

**IMMUNOASSAYS FOR THE DETECTION OF ILLICIT  
DRUGS AND ORGANOPHOSPHATE PESTICIDES**

**A thesis submitted for the degree of Ph.D.**

**By**

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Dublin 9,  
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**Under the supervision of Professor Richard O'Kennedy**

**This thesis is dedicated to Mum, and to Dad who is no longer  
with us and to my family.**

**“The great tragedy of science: the slaying of a beautiful hypothesis  
by an ugly fact ”**

*Tomas Huxley*

## Declaration

I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of Doctor of Philosophy, is entirely my own work, and has not been taken from the work of others, save and to the extent that such work is cited and acknowledged within the text of my results.

Signed Paul Pellon

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## Abbreviations

Ab	antibody
Abs	absorbance
AChE	acetylcholinesterase
Ach	acetylcholine
Ag	antigen
BCA	bicinchoninic acid
BIA	biomolecular interaction analysis
bp	base pairs
BSA	bovine serum albumin
bsAb	bispecific antibody
Cam	chloramphenicol
cDNA	complementary DNA
CDR	complementarity determining regions of antibody
CE	capillary electrophoresis
cfu	colony forming units
CM	carboxymethylated
conc (c)	concentration
CPM	chloropyrifos
DNA	deoxyribonucleic acid
dNTP	deoxynucleotidyl triphosphates
EBV	Epstein-Barr virus
EDC	N-ethyl-N'-(dimethylampropyl) carbodiimide
EDTA	ethylenediaminetetra-acetic acid
ELISA	enzyme-linked immunosorbent assay
EMIT	enzyme multiplied immunoassay technique
ESTs	expressed sequence tags
Fab	binding region of antibody above the hinge region
Fc	constant region of antibody molecule
FCA	Freunds's Complete Adjuvant
FIA	flow injection analysis
FN	fentrothion
Fv	variable binding region of antibody
HAT	Hypoxanthine aminopterin thymidine

HT	Hypoxanthine thymidine
HBS	Hepes buffered saline
HPLC	High Performance Liquid Chromatography
HPA	hydrophobic
IDA	iminodiacetic acid
IgG	immunoglobulin class G
IgA	immunoglobulin class A
IgD	immunoglobulin class D
IgE	immunoglobulin class E
IgM	immunoglobulin class M
IMAC	immobilised metal affinity chromatography
IR	infra-red
Ka	association affinity constant
<i>ka</i>	association rate constant
Kd	dissociation affinity constant
<i>kd</i>	dissociation rate constant
Kn	Kanamycin
L1	lipophilic
LED	light emitting diode
Ln	natural logarithm
LOD	limit of detection
Log	logarithmic
M	morphine
6-MAM	6-monoacetylmorphine
MAb	monoclonal antibody
MDH	morphine dehydrogenase
M3G	morphine-3-glucuronide
M3G-BSA	morphine-3-glucuronide-bovine serum albumin
M3G-OVA	morphine-3-glucuronide-ovalbumin
M3G-THY	morphine-3-glucuronide-thyroglobulin
M6G	morphine-3-glucuronide
MHC	Major histocompatibility complex
MRL	maximum residue level
mRNA	messenger RNA

<i>MTL</i>	mass transport limitation
MW	molecular weight
n	refractive index
NHS	N-hydroxysuccinimide
NMR	nuclear magnetic resonance
N	normorphine
NTA	nitrotriacetic acid
7-OHC	7-hydroxycoumarin
OD	optical density
OP	organophosphate
OVA	Ovalbumin
PAGE	Polyacrylamide gel electrophoresis
PBS	phosphate buffer saline
PDA	photodiode array
PEG	polyethylene glycol
pH	log of the hydrogen ion concentration
PIRM	primiphos-methyl
ppb	parts per billion
R	regression coefficient
Req	equilibrium binding response
RI	refractive index
RIA	radioimmunoassay
Rmax	maximum binding response
RT	room temperature
RU	response units
scFv	single chain Fv antibody derivative
SDS	sodium dodecyl sulphate
SIM	selected ion monitoring
SPR	surface plasmon resonance
TE	transverse electric
TED	tris (carboxymethyl) ethylenediamine
Tet	Tetracycline
TCR	T cell receptor
THY	Thyroglobulin

TIR	total internal reflection
TLC	thin layer chromatography
TM	transverse magnetic
TPB	phosphonic acid
UV	ultraviolet
VH	variable region of heavy chain
VL	variable region of light chain

## Units

$\mu\text{g}$	microgram
(k)Da	(kilo) Daltons
$\mu\text{l}$	microlitre
$\mu\text{M}$	micromoles
$^{\circ}\text{C}$	degrees Celcius
AU	arbitrary units
cm	centimetres
g	grams
h	hours
kg	kilogram
l	litre
m	metre
M	molar
mg	milligram
mm	minute
ml	millilitre
mm	millimetres
nM	nanomolar
mol	molar
pg	picograms
rpm	revolutions per minute
RU	response units
sec, s	seconds
v/v	volume per unit volume
w/v	weight per unit volume

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## **Abstract**

The research presented in this thesis describes the production and application of antibodies against morphine-3-glucuronide and parathion, an organophosphate pesticide

A variety of morphine-3-glucuronide-protein conjugates were produced and used to generate and characterise polyclonal antibodies. These polyclonal antibodies were used in the development of immunoassays in a competitive ELISA format and on the BIAcore (a surface plasmon resonance based optical biosensor capable of monitoring biomolecular interactions in 'real-time')

Combinatorial single chain Fv (scFv) antibody phage display libraries were also generated to morphine-3-glucuronide. Splenic mRNA from mice pre-immunised with a morphine-3-glucuronide-BSA conjugate was used in the amplification of antibody genes followed by cloning into vectors from a well-established phage display system. Two positive clones were isolated in the first two rounds of panning. One clone (E3) was selected and re-cloned into a plasmid for soluble scFv antibody expression. The soluble scFv antibody was purified and used in the development of a competitive ELISA-based assay. Further analysis of the E3 clone was carried out in the development of an inhibition BIAcore assay for morphine-3-glucuronide in biological matrices. Affinity determinations of the scFv antibody to morphine-3-glucuronide were carried out using 'real-time' biomolecular interaction analysis.

Polyclonal antibodies to the organophosphate pesticide, parathion, were also produced and characterised. They were used to generate competitive ELISA and BIAcore-based inhibition immunoassays. However, when analysed on the BIAcore, the parathion polyclonal antibody displayed no inhibition.

# **Chapter 1**

## **Introduction**

## **1 1 The Immune System**

The mammalian immune system has evolved a diverse range of defence mechanisms to identify and remove foreign materials such as viruses, bacteria, fungi, parasites and foreign cells. The immune system is broken up into two mechanisms of defence, innate immunity (non-adaptive immunity) and adaptive immunity. Innate immunity acts as the first line of defence against infectious agents. This type of immunity consists of a series of physical and physiological barriers. Some of the physiological factors include pH, temperature and the presence of degradative chemical substances which protect the body. Cell-mediated non-specific response to foreign cells is also part of this type of immunity and includes such internal systems as phagocytosis by macrophages and cell lysis by natural killer (NK) cells. Phagocytic cells are derived from bone marrow stem cells and their function is to engulf foreign particles present in the body, internalise them and destroy them. As the innate immune response is non-specific these cells are located in areas of the body most likely to encounter foreign bodies. Natural killer cells have the ability to recognise surface changes in cells, which are infected. They bind to the infected cells and destroy them. The body has also developed other external barriers such as the skin, which prevents the penetration of infectious agents into the body and the mucous membranes that prove effective against the majority of infectious agents (Roitt *et al*, 1998)

The adaptive immune response is divided into the humoral and cell-mediated immune response. The adaptive immune system produces a specific reaction to the infectious agent and has the ability to produce memory cells to prevent future infection (Roitt *et al*, 1998)

### **1 1 1 The Lymphoid system**

The cells involved in the immune response are organised into tissues and organs in order to perform their function. These are collectively referred to as the lymphoid system. This system consists of primary and secondary lymphoid organs (Male *et al*, 1996). The primary lymphoid organs comprise of the thymus and bone marrow

which are responsible for the antigen-independent differentiation of progenitor cells from the bone marrow into T-lymphocytes and the development of antibody-producing B-lymphocytes, respectively. There are various classes of T-cell, which possess the ability to recognise foreign antigens present in the organism, and so these cells are considered part of the adaptive immune response. The primary lymphoid organs gain their repertoire of specific antigenic receptors in order to cope with the various antigens an individual would come in contact with throughout life. Another important feature of the primary lymphoid system is that lymphocytes gain the ability to recognise the difference between self and foreign antigens.

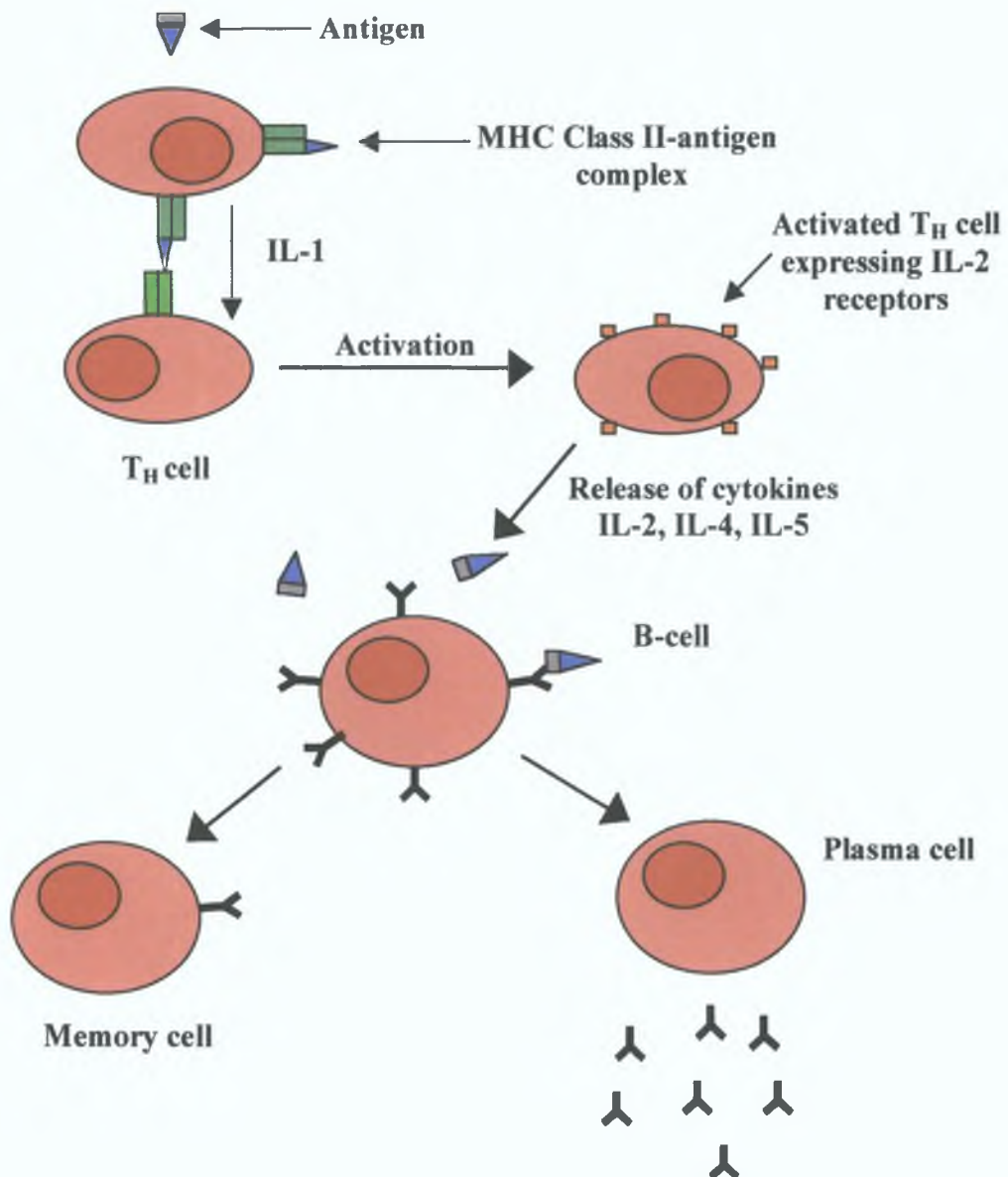
Secondary lymphoid organs include the lymph nodes, spleen, and mucous-associated lymphoid tissues (MALT), including the tonsils and Peyer's patches of the gut. The lymph nodes are scattered throughout the body and are connected to each other and the spleen by a network of lymphatic vessels. These organs create the environment in which lymphocytes can interact with one another at high concentrations to produce T-cell-dependent B-cell activation (Male *et al*, 1996).

### **1.1.2 The Humoral immune response**

The humoral immune response is mediated by antibodies produced by B-lymphocytes (Roitt *et al*, 1998). For the generation of a strong immune response, the interaction between B- and T-cells is of critical importance for the production of antigen-specific antibodies (Figure 1.1). There are two types of T-cells, T Helper ( $T_H$ ) and T cytotoxic ( $T_C$ ) cells. There are two subsets in the  $T_H$  cells,  $T_{H1}$  and  $T_{H2}$ , which are primarily different due to the cytokines they produce.  $T_{H1}$  cells produce interferon gamma ( $IFN-\gamma$ ) and tumour necrosis factor ( $TNF-\beta$ ) which are mainly responsible for the activation of T cytotoxic cells.  $T_{H2}$  cells mainly secrete interleukins and their main function is the activation of B-cells.  $T_C$  cells are responsible for destroying cells of the body displaying antigen on the surface. Following exposure to an antigen, macrophages engulf, process and present the antigen on the surface of the cell in conjunction with MHC (Major Histocompatibility Complex) Class II. These cells are known as antigen presenting cells (APC). T helper ( $T_H$ ) cells recognise antigen in

the form of MHC class II-antigen complex via their T-cell receptors. The T-cell receptor is a heterodimer comprised of  $\alpha$ - and  $\beta$ -chains which consist of variable and constant regions, which form the binding site. Contact between the T-cell and the antigen presenting cell activates the T-cell, which proliferates and forms a clone of  $T_H$  cells specific to the antigen resulting in the release of interleukin-1 (IL-1). The  $T_H$  cell then selectively stimulates B cells that have already encountered the particular antigen to proliferate by the release of IL-2. In similar fashion, antigens are also processed by B-cells. When antigens bind to antigen receptors on a B-cell, the B-cell internalises the antigen and presents the antigen fragment bound to MHC class II on the surface of the cell. However, in this case, the processing and presentation of antigen on the surface of B-cells is specific to a particular antigen.  $T_H$  cells receptors bind to the antigen-MHC complex on the surface of the B-cells, causing the B-cells to synthesise a receptor for IL-4 which is synthesised by the activated  $T_H$  cells. This leads to the production of IL-5 by B-cells which results in B-cell differentiation into memory and plasma cells. Plasma cells secrete antibody at an approximate rate of 2000 per second for the 4 to 5 day lifetime of the cells. These specific antibodies help eliminate the foreign invader. Memory B-cells, express membrane bound antibody and remain in the body for long periods of time primed for secondary exposure to the antigen (Roitt *et al*, 1998).

Once the humoral immune response has been activated, centroblasts now undergo a process known as affinity maturation, which involves somatic hypermutation of the amino acid sequence of the variable heavy and light chains. This produces high affinity selected antibodies and occurs late in the primary response to a T-dependent antigen. Most of the mutations which occur produce lower affinity antibodies. Selection of the higher-affinity antibodies for proliferation is explained as higher-affinity antibodies bind more efficiently to antigen complexes on the surface of the follicular dendritic cells. The binding interaction inhibits the apoptosis of these high-affinity binding antibodies. However, B-cells producing antibodies with lower affinities, which do not bind to the antigen-complexes, undergo apoptosis. This process ensures that only higher affinity antibodies are selected.



*Figure 1.1. Diagrammatic representation of the interaction between an antigen presenting T-cell and B-cell leading to T- and B-cell activation in a primary response. The antigen is initially phagocytosed into an antigen presenting cell and processed and presented on the surface of the cell as MHC class II-antigen complex. T<sub>H</sub> cells bind to this complex through the T-cell receptor resulting in proliferation and differentiation. Expression of cytokine receptors by the B-cell and production of cytokines by the T<sub>H</sub> cell drives the proliferation of the B-cells. As B-cells can also bind and process antigen, they are also stimulated when T<sub>H</sub> cells interact with the displayed fragment. B-cells differentiate into plasma cells with the ability to produce large quantities of antibody and memory cells, which circulate the body in order to elicit a strong antibody response to subsequent secondary infections.*

Another important part of the B-cell differentiation process is class switching. Class switching occurs during the differentiation process and is triggered to form different isotypes as a result of the type of cytokine present. B-cells expressing IgM/D antibody on the surface of the cell may switch to the synthesis of IgG, IgA or IgE molecules within this process. Once this has occurred, subsequent rounds of antigenic challenge will produce a much stronger immune response involving the higher affinity memory B-cells which are principally of an IgG class (Roitt *et al*, 1998)

## 1.2 Antibody Structure

Antibodies or immunoglobulins are a group of glycoproteins present in the serum of all mammals. Rodney Porter (Porter *et al*, 1966) in 1966 proposed the basic four polypeptide chain model for antibodies. The structure of antibodies varies depending on their isotype, however, antibody structure is generally represented by the IgG antibody as shown in Figure 1.2.

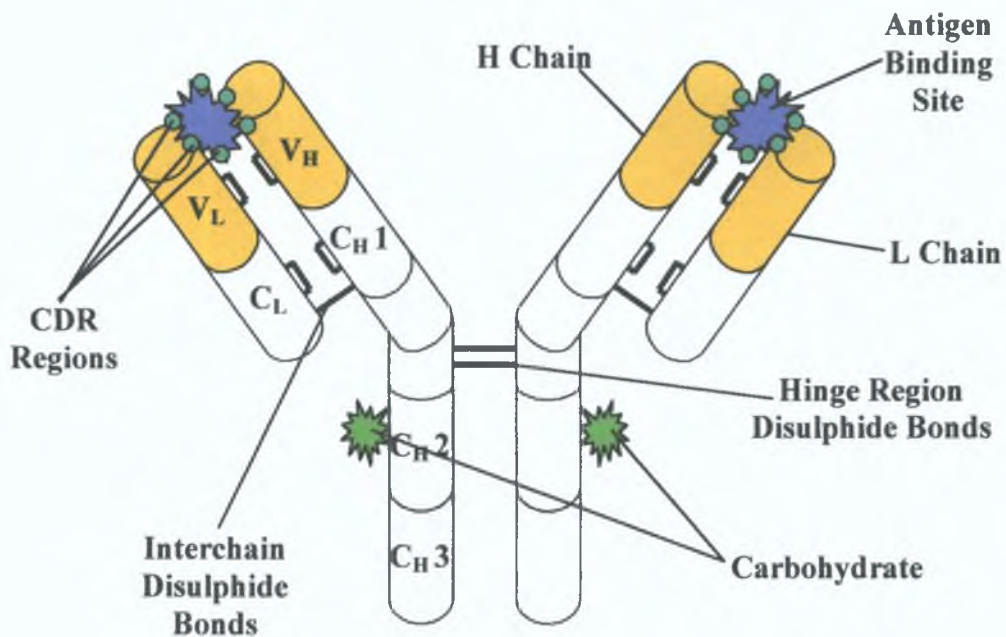
The antibody is composed of four polypeptide chains, with two identical heavy chains (H) and two identical light chains (L) (called  $\kappa$  (kappa) or  $\lambda$  (lambda)) (Hannigan, 2000). The light chain has a molecular weight of approximately 25 kDa, whereas the heavy chain has a molecular weight of approximately 50 kDa. The two heavy chains are held together by interchain disulphide bonds while various antibody chains are held together by intrachain disulphide bonds. The amino terminal end of the antibody is characterised by variability (V) of the sequences in both the heavy (H) and light (L) chains which are known as the  $V_H$  and  $V_L$  regions. The remainder of the molecule is constructed of constant regions ( $C_H$  and  $C_L$ ). These constant regions are broken up into three distinct regions:  $C_{H1}$ ,  $C_{H2}$  and  $C_{H3}$ . A hinge region is also present, which is found between the  $C_{H1}$  and  $C_{H2}$  domains, allowing conformational changes to occur in the antibody resulting in flexibility of between 60-180°. This flexibility allows the two binding sites to work independently from one another. The binding site of an antibody to an antigen is located within the variable region of the antibody. The properties of antigenic recognition, specificity and affinity are

comprised of approximately 110 amino acid residues located in the variable region. These are referred to as hypervariable regions forming a region complementary in structure to the antigen epitope. Hypervariable regions are also referred to as complementarity determining regions (CDRs) and the intervening peptide segments which are approximately 10 amino acid residues long are known as framework regions (FR). The CDR regions in both light and heavy chain V regions are subdivided into three CDRs (CDR1, CDR2, CDR3). The light and heavy chain CDRs form the six hypervariable loop structures producing a three-dimensional structure, which acts as the antigen-binding pocket.

There are five distinct classes of antibody, IgA, IgM, IgD, IgE and IgG which have been determined by the difference in the structure of their heavy chains. IgA represents 15-20% of total antibody concentration and may be present as a monomer or a dimer, and is found predominantly in seromucous secretions such as saliva, milk and tracheobronchial secretions. IgM accounts for approximately 10% of total antibody concentration and has a pentameric structure and is predominantly the early antibody seen in the immune response to primary infections. IgD accounts for less than 1% of total antibody present but is known to be present in large quantities on the membranes of many B-cells, and may have a role in lymphocyte differentiation. IgE is found in trace amounts in the body and plays a role in immunity to helminthic parasites. Hypersensitivity conditions such as hay fever is also associated with this antibody.

Finally, IgG is the major antibody present and accounts for 70-75% of the total antibody concentration. It is divided into four subclasses called IgG<sub>1</sub>, IgG<sub>2</sub>, IgG<sub>3</sub>, and IgG<sub>4</sub> which are based on the type of heavy chain present (Roitt *et al*, 1998).





*Figure 1.2. Diagrammatic representation of an immunoglobulin molecule. The amino terminal end of the antibody is characterised by variability (V) of the sequences in both the heavy (H) and light (L) chains, which are known as the V<sub>H</sub> and V<sub>L</sub> regions, respectively. The remainder of the molecule is constructed of constant regions (C<sub>H</sub> and C<sub>L</sub>). These constant regions are broken up into three distinct regions: C<sub>H</sub>1, C<sub>H</sub>2 and C<sub>H</sub>3. There is also a hinge region which is found between the C<sub>H</sub>1 and C<sub>H</sub>2 domains which allows conformational changes to occur in the antibody resulting in the flexibility of between 60-180°. The CDR regions confer the specificity of the antibody to a particular antigen and this is known as the antigen binding site. The two heavy chains are held together by interchain disulphide bonds while various antibody chains are held together by intrachain disulphide bonds.*

### 1.3 Genetics of antibody diversity

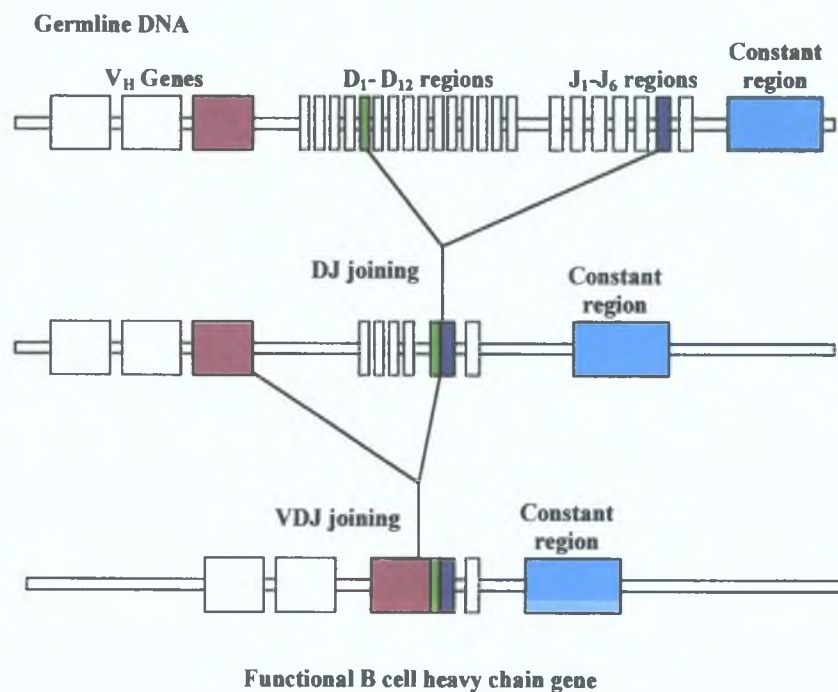
Antibodies are extremely diverse and possess the characteristic to generate numerous varied structures possessing the ability to bind to antigen. As antibodies are composed of heavy and light chains and since any light chain may combine with any heavy chain, the number of possible variant sites is a product of the number of heavy and light chains. This ability is located in the CDR regions as they are directly responsible for antigen binding. The body produces more different forms of antibody than the number of genes in the genome. This diversity is generated by the gene organisation.

Heavy and light chain variable regions of an antibody encode for antibody diversity and this is as a direct result of the genetic nature of the antibody light and heavy chain germline genes (Tizard, 1995). Variable light chain regions are composed of V (variable) and J (joining) loci, whilst heavy chain gene sequences are composed of V (variable), D (diversity) and J (joining) loci. The recombination between these three regions directly contributes to antibody variability. As a result of B-cell maturation, recombination of the various VDJ sequences on the heavy chain and the VJ sequences on the light chain takes place resulting in the formation of a functional gene. During recombination, the D region is placed adjacent to the V region through a process known as looping-out, in which the intervening heptameric and nonameric complementary sequences forming stem-loop structures are removed (Figure 1.3) (Roitt *et al.*, 1998). This process is random, with the possibility of any region recombining with another.

Further diversity may be achieved by the insertion of small sets of nucleotides between the VJ and DJ sequences resulting in changed sequences. Somatic mutation also causes single base changes allowing additional diversity (Benjamin *et al.*, 1996). The main function of this type is to increase the binding affinity of the antibody to their antigen. This allows the immune system to improve the affinity of its antibodies after the initial immunisation. Junctional site diversity may also occur during the recombination of various VJ and VDJ sequences as a result of the imprecise joining.

ends of various sequences and this changes the amino acid sequence which directly alters the binding site of the antibody.

Class switching is determined as a result of antigenic stimulation of B-cells. Constant H regions are found downstream of the variable region genes, in the specific order of  $\mu$ ,  $\delta$ ,  $\gamma$ ,  $\epsilon$ , and  $\alpha$ . Each of these constant gene sequences, except for the  $\delta$  gene, has at the 5' end a repeating base sequence called the switch region. At this region recombination of any VDJ sequences with  $C_H$  regions may occur. Depending on the individual environmental conditions to which the B-cell is exposed will determine the type of antibody produced by the cell. The heavy and light chain sequences are then translated and assembled into an intact antibody molecule (Roitt *et al.*, 1998).



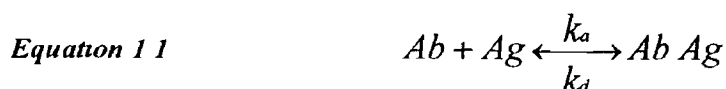
**Figure 1.3.** Diagrammatic representation of heavy chain gene recombination. There are two separate processes involved in the recombination of the gene. Heavy chain gene segments are composed of multiple VD and J regions and a constant region, which encodes for the class of the antibody. The first recombination step is the combining of the D and J segments present on the germline DNA which occurs through looping of intervening DNA by recombinase enzymes. This introduces further variability at the point of cleavage. The second step combines the V region with the DJ sequence resulting in the fixing of specificity of the antibody. This results in the formation of a functional B-cell variable heavy chain gene.

#### 1 4 Antibody affinity

The generation of an immune response to a particular antigen results in the production of antibodies. The interaction formed when an antibody comes in contact with its specific antigen is determined by a combination of different physical forces, hydrophobic interactions, hydrogen bonds, electrostatic binding and van der Waals forces (Tijssen, 1985)

Hydrophobic interactions involve the association of non-polar residues in aqueous environments. Electrostatic binding is the result of attraction between oppositely charged groups on the two interactants, the effects of which may be greatly enhanced due to the removal of water molecules as a result of hydrophobic interactions. Hydrogen bonds are also formed when hydrogen atoms are shared between two other atoms. Van der Waals forces are as a result of the interactions between the electron clouds of adjacent non-polar groups on the neighbouring molecules. This results in the induction of oscillating dipoles between the two molecules producing a net attractive force. The strength of these forces are only effective over a small distance, resulting in the epitope on the antigen and the binding site on the antibody becoming the defining factor for the strength of the interaction.

The term affinity is defined as the sum of the attractive and repulsive forces for a single antibody-antigen bond and may be described in terms of reaction kinetics. At equilibrium, the formation of an antibody-antigen complex may be expressed as



Where Ab is antibody and Ag is antigen and Ab Ag is the antibody-antigen complex and  $k_a$  and  $k_d$  are the association and dissociation rate constants, respectively

$$\text{Equation 1 2} \quad K_A = \frac{k_a}{k_d} = \frac{[\text{Ab Ag}]}{[\text{Ab}][\text{Ag}]}$$

The affinity of the antibody for the antigen is given by the equilibrium association constant  $K_A$

Equation 1.3

$$K_D = \frac{1}{K_A} = \frac{k_d}{k_a} = \frac{[Ab][Ag]}{[Ab Ag]}$$

The affinity of the antibody-antigen interaction may also be stated as the equilibrium dissociation constant,  $K_D$ , which is a reciprocal of the  $K_A$  value

## 1.5 Monoclonal antibody technology

Monoclonal antibody technology was first reported by Kohler and Milstein, (1975). Before this, all antibodies produced were of a polyclonal nature. Although polyclonal antibodies were useful in many areas, their heterogeneous nature also limited their application. However, the homologous nature of monoclonal antibodies allows the generation of antibodies with differing characteristics such as higher specificity and affinity.

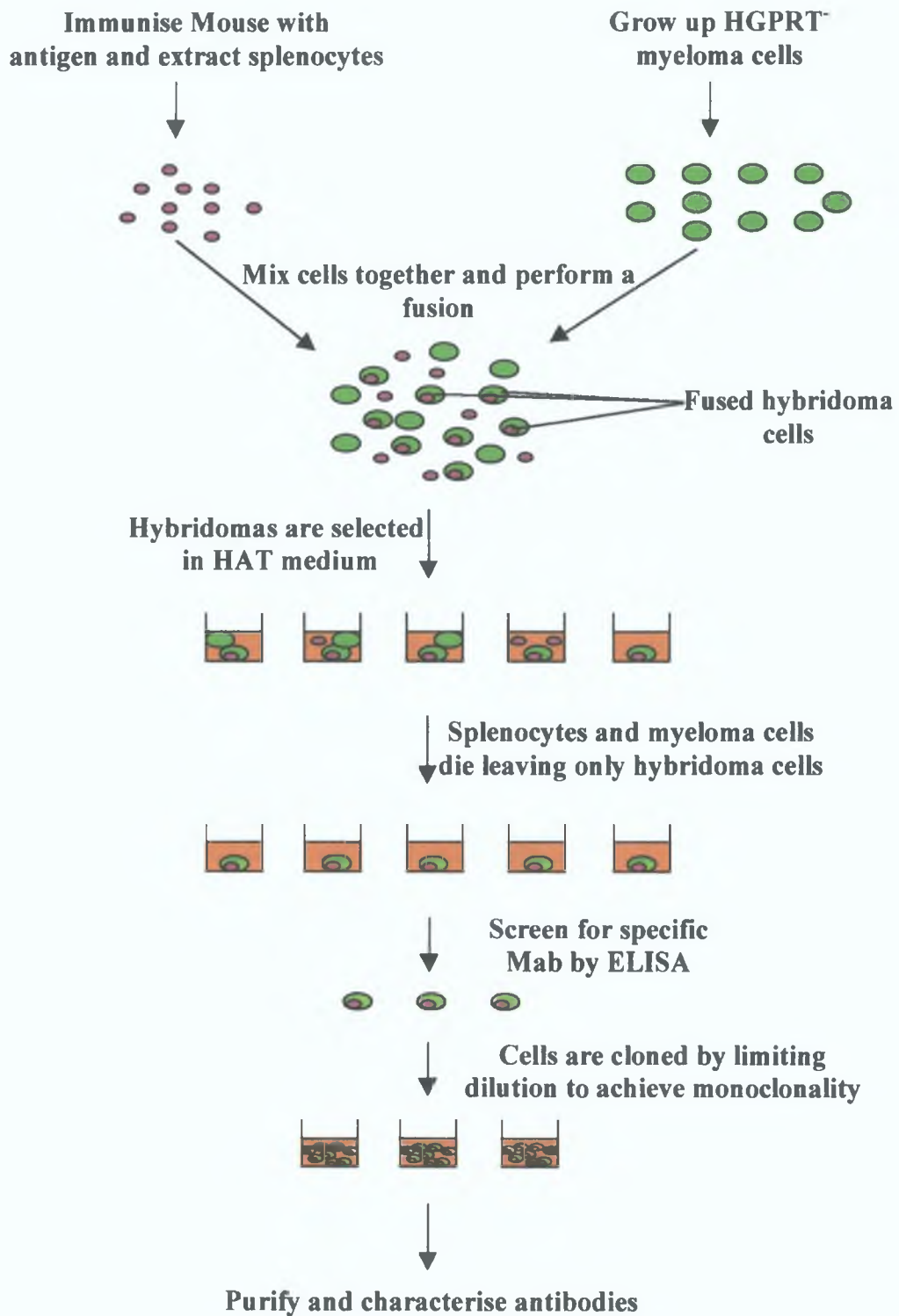
For the production of monoclonal antibodies (Figure 1.4) by somatic cell fusion, an initial immunisation of the host animal with the antigen is required. This primes the animal for the production of specific antibodies to the antigen of interest. Production of antibody to the specific antigen is monitored closely until the titre is sufficient to perform a fusion. The spleen is removed from the animal and the splenocytes harvested in order to perform the procedure. The other fusion partner in this procedure is a mouse myeloma cell line (i.e. X63-Ag8.653 and Sp2/0-ag14). Myeloma cells are tumorigenic B-lymphocytes, which may be cultured *in vitro*. These cells lack the enzyme called hypoxanthine guanine phosphoribosyl transferase (HGPRT) and are unable to proliferate in the presence of HAT (Hypoxanthine, Aminopterin and Thymidine) medium. Aminopterin blocks the *de novo* biosynthesis of purines and pyrimidines needed for the production of DNA. One of the characteristics of HGPRT cells is that they cannot utilise the salvage pathways for DNA synthesis as they do not possess the required enzyme systems. However, splenocytes carry the required enzymes to produce DNA through the salvage pathway but are unable to proliferate outside the body and die off naturally. Fused cells, however, will proliferate in HAT medium as the characteristic to use the salvage pathway to produce DNA will come from the spleen cell and the

characteristic to be immortal comes from the myeloma cell, resulting in an immortal hybridoma cell capable of antibody production

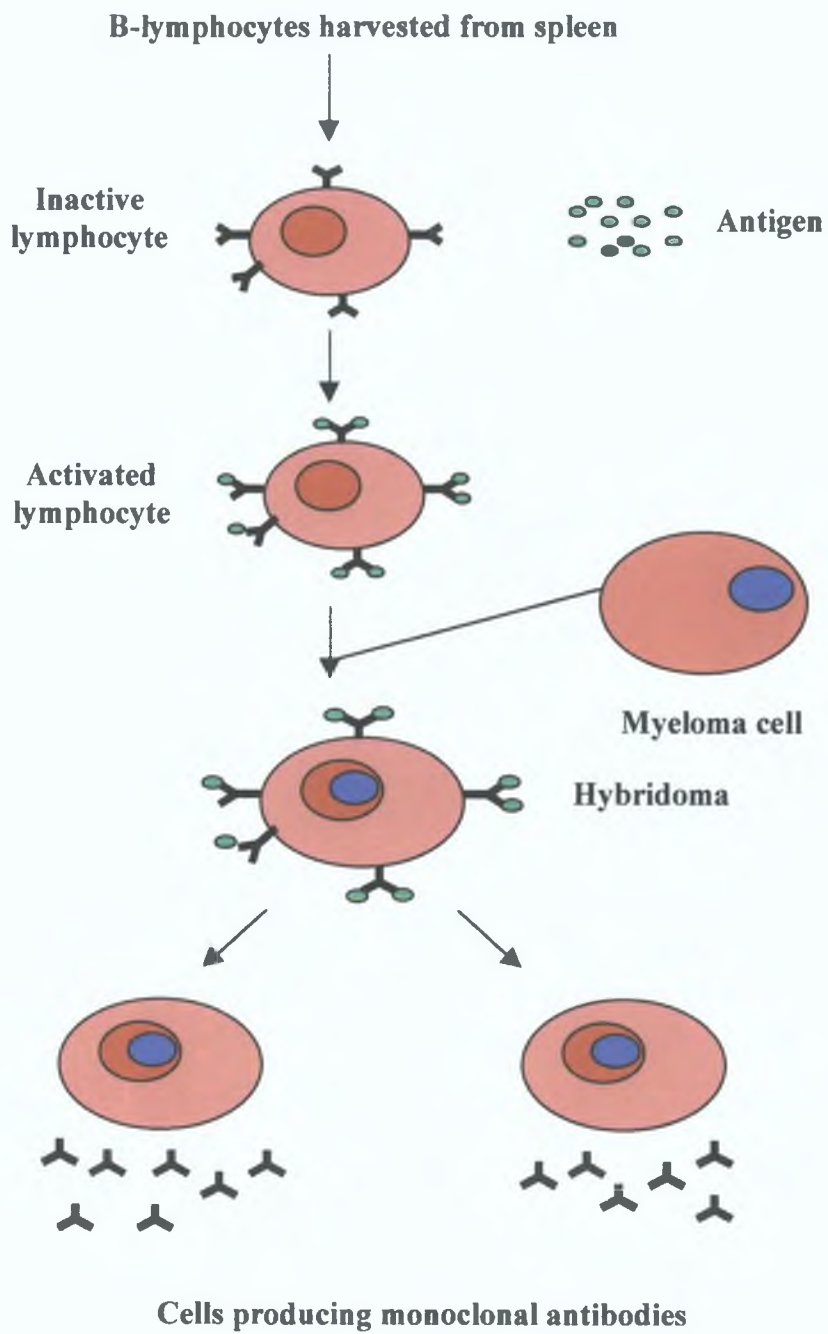
Splenocytes from the immunised animal are fused with myeloma cells by the addition of polyethylene glycol (PEG), which promotes membrane bridging, cell fusion and transfer of nuclei (Hurrell, 1985) Once hybridomas have been selected, using HAT medium, the culture supernatants are screened for hybridomas producing specific antibodies to the antigen Eventually, hybridomas from a single cell are produced using a procedure known as cloning by limiting dilution where cultures are recloned until they eventually come from a single cell (Goding, 1996)

Monoclonal antibodies may also be produced by *in vitro* immunisation (Borrebaeck *et al*, 1983) For the production of monoclonal antibodies using this method (Figure 1 5), spleen cells are harvested from naive mice and incubated with the antigen of interest for a period of 5-9 days, before the somatic cell fusion procedure Antibodies produced using *in vitro* immunisation are as a result of a primary response and are generally IgM antibody molecules with low quantities of IgGs The production of IgM antibodies is a disadvantage of this system as in general these antibodies have a lower affinity than IgG antibodies However, this system reduces the immunisation time required for *in vivo* immunisation and also the amount of antigen required for the procedure Bonwick *et al* (1996) has demonstrated the ability of this method to produce antibodies by generating antibodies to flucofluron and sulcofluron However, the lack of papers in the literature suggests that there has been very little success in the development of assays using antibodies produced by *in vitro* technology

The use of monoclonal antibodies as a therapeutic agent in humans, has been severely limited due to the fact that most of the monoclonal antibodies produced having been of murine origin The use of these antibodies in humans elicits an anti-murine antibody response, limiting the number of times the antibody may be used in any single individual Production of human monoclonal antibodies is limited with respect to the fact that human immunisation is difficult and relies on accidental immunisation through disease or infection There has also been little success in attempts to produce human monoclonal antibodies using hybridoma technology as there are few suitable



*Figure 1.4. Principle of monoclonal antibody production.*



*Figure 1.5. Production of monoclonal antibodies by in vitro immunisation.*



myeloma fusion partners of human origin. However, using the Epstein-Barr virus transformation (James and Bell, 1987) of human lymphocytes, there has been some success (Burton, 1991). This procedure can produce cell lines, which are unstable or produce low levels of antibody. However, this is still desirable as human antibodies are being produced and can be used in the treatment of humans (Sims *et al*, 1993). Protein engineering techniques have also been developed as alternatives in the production of human monoclonal antibodies (Co and Queen, 1991, Hurlle and Gross, 1994).

### **1.5.1 Humanised/Chimeric antibodies**

Since the advent of monoclonal antibody technology by Kohler and Milstein, this technique has been used in a wide variety of processes. Although murine monoclonal antibodies have been widely applied in clinical diagnostics, there has been limited success in the application of these antibodies to human therapy (Clark, 2000). Therapeutic use of murine monoclonal antibodies was found to have two major problems. Firstly, murine monoclonal antibodies did not trigger the appropriate human effector systems (Clark *et al*, 1983). Secondly, even when murine antibodies did work *in vivo*, the patient's immune system would recognise these antibodies as foreign and elicit a human anti-murine-antibody (HAMA) immune response, cutting short the time in which the antibodies had to work (Schroff, *et al*, 1985). The HAMA response may be potentially reduced by production of mouse-human chimeric antibodies. The development of chimeric mouse-human antibodies provides a double benefit of reduced immunogenicity and the ability to recruit human Fc effector functions. Such antibodies are produced by combining the entire variable (V) or binding domain of a mouse antibody with a human antibody constant (C) or Fc domain (Co and Queen, 1991). However, in some cases such as the murine antibody OKT3 used in the prevention of organ rejection, much of the antibody response is directed against the V region rather than the C region (Jaffers *et al*, 1986). This result suggests that chimeric antibodies that possess a murine V region may still result in an immunogenic effect. To inhibit this immunogenic response, V-region

humanisation was required. This involved changing some of the FR residues of the V-region on the murine antibody to sequences more similar to human sequences, while also retaining the ability to bind to antigen (Riechmann *et al* , 1988)

Two further strategies have been developed for the production of 'fully human antibodies'. Firstly, recombinant antibody technology may be used as human antibody V-regions can be selected from recombinant phage display libraries by affinity selection strategies (Winter *et al* , 1994). Secondly, the creation of transgenic mice whereby the mice immunoglobulin genes have been replaced with human immunoglobulin genes which allow the production of human antibodies upon immunisation (Mendez *et al* , 1997)

One further technique developed involves reconstituting severe combined immunodeficient (SCID) mice with human peripheral blood lymphocytes, followed by the immunisation of these animals with antigen and rescuing the immune B-cells by fusion with myeloma cells. However, antibodies produced by this procedure are found to contain unique idiotypes which may induce an immunogenic response (Clark, 2000)

Humanised antibodies have been produced for the clinical treatment of patients with cancer. One such example is described by Nakamura *et al* (2000) as they used a mouse-human chimeric monoclonal antibody (Mab) KM966, which was specific for the cell-surface tumour antigen ganglioside GM2, to produce a humanised antibody. This antibody was produced to improve the antibodies potential in clinical settings by reducing the antibodies immunogenicity. The humanised antibody developed KM8969 was found to inhibit the growth of GM2 positive tumour cells due to apoptosis induction. Further clinical studies carried out by Parajuli *et al* (2001) demonstrated that both the humanised antibody (KM8969) and the mouse-chimeric antibody (KM966) promoted the lysis of lung cancer cells by blood mononuclear cells (MNC) of lung cancer patients.

## 1 5 2 Applications of monoclonal antibodies

Monoclonal antibodies have been used for diagnostic and therapeutic applications (Voigt and Zintl, 1999) since their discovery. Detection systems for a variety of target molecules ranging from pesticides to illicit drug residues have also been developed with the use of monoclonal antibodies (Fitzpatrick *et al* , 2000)

The use of monoclonal antibodies as therapeutic agents is also gaining in importance in the treatment of a number of conditions such as cancer, cardiovascular diseases and viral infections. Monoclonal antibodies were initially thought to be of great importance for the treatment of cancer and a twofold strategy for treatment was envisaged. Initially, monoclonal antibodies locate and bind to tumour-specific antigens and this is followed by the triggering of a lethal attack against neoplastic cells. In recent years much of the work has been carried out by academic researches (Funaro *et al* , 2000) as much of the early expectations about the applications of monoclonal antibodies did not unfold.

One area for the use of monoclonal antibodies for therapeutic application is B-cell lymphomas which are suited to therapy using monoclonal antibodies due to the accessibility of malignant cells in the blood, spleen, bone marrow and lymph nodes. Production of monoclonal antibodies to the surface expressed CD20 on malignant human B-cells may be used for anti-tumour use by altering the normal signal pathways (Maloney *et al* , 1996, Shan *et al* , 1998)

Agonistic monoclonal antibodies have also been studied which possess the ability to mimic natural ligands and may also be useful in the treatment of solid tumours by targeting antigens which are involved in the signalling pathways (Gibbs, 2000). The HER2 signalling pathway is one of the best studied and it has been found that an anti-HER2 monoclonal antibody caused changes in the signal pathways within the cell resulting in an inhibition of this type of breast cancer cell (Pietras *et al* , 1999)

The use of monoclonal antibodies for the production of bispecific antibodies are continuously under investigation, enabling the potential signalling ability of these antibodies to recruit specific effector molecules to tumour cells (Hudson, 1999, Segal *et al* , 1999)

Shu *et al* (2001) recently produced a monoclonal antibody for the development of a direct ELISA to differentiate between infection and vaccination of the Japanese encephalitis (JE) virus. Monoclonal antibodies have also been produced against *Salmonella enteritidis* lipopolysaccharide (LPS) (Iankov *et al*, 2001) for the differentiation of various strains of the virus.

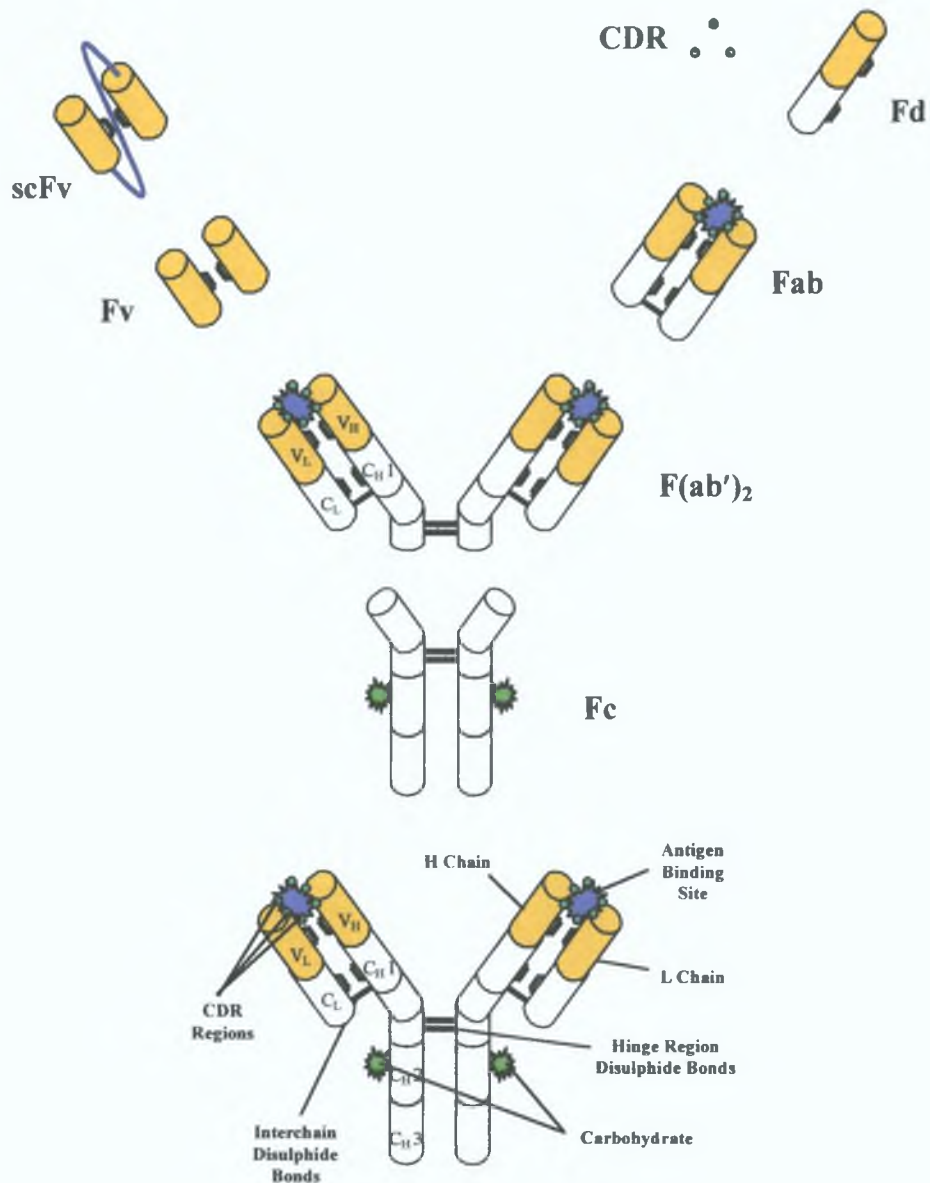
## 1.6 Antibody Engineering

Antibody engineering can be defined as the construction of designer antibodies and is one of the fastest growing fields in molecular biology. Over the last fifteen years, genetic and molecular techniques have been used as a means of generating monoclonal antibodies and antibody fragments (Smith, 1985). Recombinant antibody display technology is utilised in the generation of antibody fragments from several species including human, rabbit, mouse and sheep. This has resulted in the engineering of antibody fragments (Figure 1.6), which include the antigen-binding (Fab) and single chain variable fragment's (scFv). This truncated Fab fragment consists of one  $V_L$  domain and one  $V_H$  domain. The Fv fragment is the smallest part of the antibody required for antigen binding. The Fv fragment can be very unstable for use in therapeutics or immunoanalysis because it lacks the inter-chain disulphide bond, which is present in the Fab fragment (Killard *et al*, 1995). Protein engineering has allowed for the development of a more stable Fv fragment. A synthetic peptide linker (or in some cases the insertion of cysteine residues to form a disulphide bridge) can be incorporated into the Fv fragment to produce a more stable single chain Fv (scFv) fragment. A single chain Fv fragment is composed of a heavy chain variable region joined to a light chain variable region by a flexible 15 amino-acid linker. Smaller antibody fragments may also be engineered capable of binding to antigen. These include the Fd region which consists of one  $V_H$ , one  $C_{H1}$  domain from the heavy chain, and the complementary determining region (CDR), which is the smallest antibody fragment with the ability to bind to antigen.

The DNA with the genes required to code for antibody fragments is available from a number of sources, which include the genetic material from non-immunised or

immunised animals or humans and genes from a repertoire of naive, semi-synthetic or synthetic genes (Ditzel *et al*, 1993) Messenger RNA may also be used from hybridomas expressing specific functional antibodies to a specific antigen and used to provide the raw material for cloning the antibody variable gene segments (Winter and Milstein, 1991)

Due to the advent of antibody phage display technology, isolation of antibodies with higher affinities has become possible while simultaneously reducing the size of the antibody (De Haard *et al*, 1998)



*Figure 1.6. Illustration of an antibody and antibody fragments. The fragments illustrated above may be produced by genetic or enzymatic and chemical manipulation. The whole antibody may be initially broken up into either Fab (antigen-binding fragment) or F(ab')<sub>2</sub> (two antigen-binding fragments linked) and Fc (crystalline fragments) regions. Fab fragments may be further broken up into Fv (variable fragments), scFv (single chain Fv) and individual CDR regions which are all capable of antigen-binding.*

## 1 6 1 Combinatorial Phage Display Libraries

All the techniques used so far have relied on the use of spleen cells or hybridomas as the starting material. This changed with the development of a technique known as Combinatorial Phage Display (Barbas *et al.*, 1991). This system allowed the possibility of producing recombinant monoclonal antibodies from cloning light and heavy chain antibody genes, and expressing them in *E. coli* using phage vectors.

Huse *et al.* (1989) have used this phage display system to express a combinatorial library of Fab fragments of a mouse antibody repertoire in *Escherichia coli*. They used the Lamda ( $\lambda$ ) phage system to express their libraries. Initially using genes amplified by PCR, the heavy and light chains were cloned into  $\lambda$ Hc2 and  $\lambda$ Lc1 vectors, respectively. The oligonucleotide sequences used in the construction of these vectors contained segments for the construction, expression, and secretion of Fab fragments. The heavy and light chains were randomly combined at the two *EcoRI* sites, which were present in each vector. However, results proved that this system was capable of producing only relatively small libraries ( $10^6$ ). This resulted in work being carried out on systems based on filamentous phage display to try to improve the size of the libraries.

However, work continued using the  $\lambda$  phage system (Caton and Koprowski, 1990, Persson *et al.*, 1991). Caton and Koprowski produced a combinatorial library to Influenza-virus hemagglutinin which was generated by using mRNA obtained from an immunised donor mouse. A combinatorial library which contained  $2.5 \times 10^7$  phage particles was generated. From this, ten strong positive phage were identified and re-infected into bacteria which was co-infected by helper phage to excise a plasmid containing the heavy and light chain expression sequences. It was found by taking supernatants from these bacteria that there were Fab fragments present that were HA-specific. From this, results showed that the isolated antibodies had similar characteristics in relation to the immune status of the donor mouse. This result was significant with respect to assessing the human immune responses to different pathogenic agents, as libraries may be generated from human peripheral blood lymphocytes.

Persson *et al* (1991) also used this  $\lambda$  phage display to produce monoclonal antibodies against tetanus toxoid. He produced a combinatorial library which contained  $1 \times 10^5$  members from untreated cells and  $6 \times 10^6$  from stimulated cells. From these experiments, results showed that the libraries generated were too small ( $10^6$ ). Caton also found this, indicating the need for different techniques to produce a library with a sufficient yield. One such technique was the use of filamentous phage systems. These systems allowed foreign peptide sequences to be expressed on the surface of an infectious phage. There are a number of advantages in using this method, the first one being that a large number of phage can be screened at the same time. The second advantage was that since each phage encodes a random sequence expressed on its surface, if a phage is recovered with a low yield, amplification of phage is possible by re-infection in bacteria.

It was not until 1990 when Scott and Smith (Scott and Smith, 1990) used filamentous bacteriophage vectors into which foreign antigenic determinants were cloned into the phage gene III and shown as part of the gene III protein (pIII) which was placed at the tip of the virion. Gene III is the part of the phage which encodes the coat protein pIII which is located at one end of the virion. The carboxyl-terminal is half buried in the virion and takes part in morphogenesis while the amino-terminal half of pIII binds to the F pilus during infection. Smith, (1985) showed that it was possible to insert foreign sequences between the two domains without effecting pIII's function. Results showed that the foreign sequences were expressed on the surface of the phage. Scott and Smith produced a library, which expressed six random residue sequences on the pIII fusion protein. Once expressed on the surface of phage, it became possible to select specific phage with the required characteristics.

Devlin *et al* (1990) constructed a larger library of phage which expressed 15 random residue peptide sequences as the gene III fusion proteins. He constructed a new vector, by introducing enzyme sites into gene III and by placing a  $\beta$ -lactamase gene in the polylinker 3' of the lac promoter to produce the M13LP67 vector. This allowed the production of a mature pIII protein with the predicted sequence.



McCafferty *et al* , (1990) was the first to clone and express an antibody Fv domain in filamentous phage using V<sub>H</sub> and V<sub>L</sub> fragment genes from a murine anti-lysozyme antibody

Filamentous phage displaying systems for the selection and screening of antibodies were found to be easier to work with, and future work concentrated on developing these systems

## 1 6 2 Construction of combinatorial phage display libraries

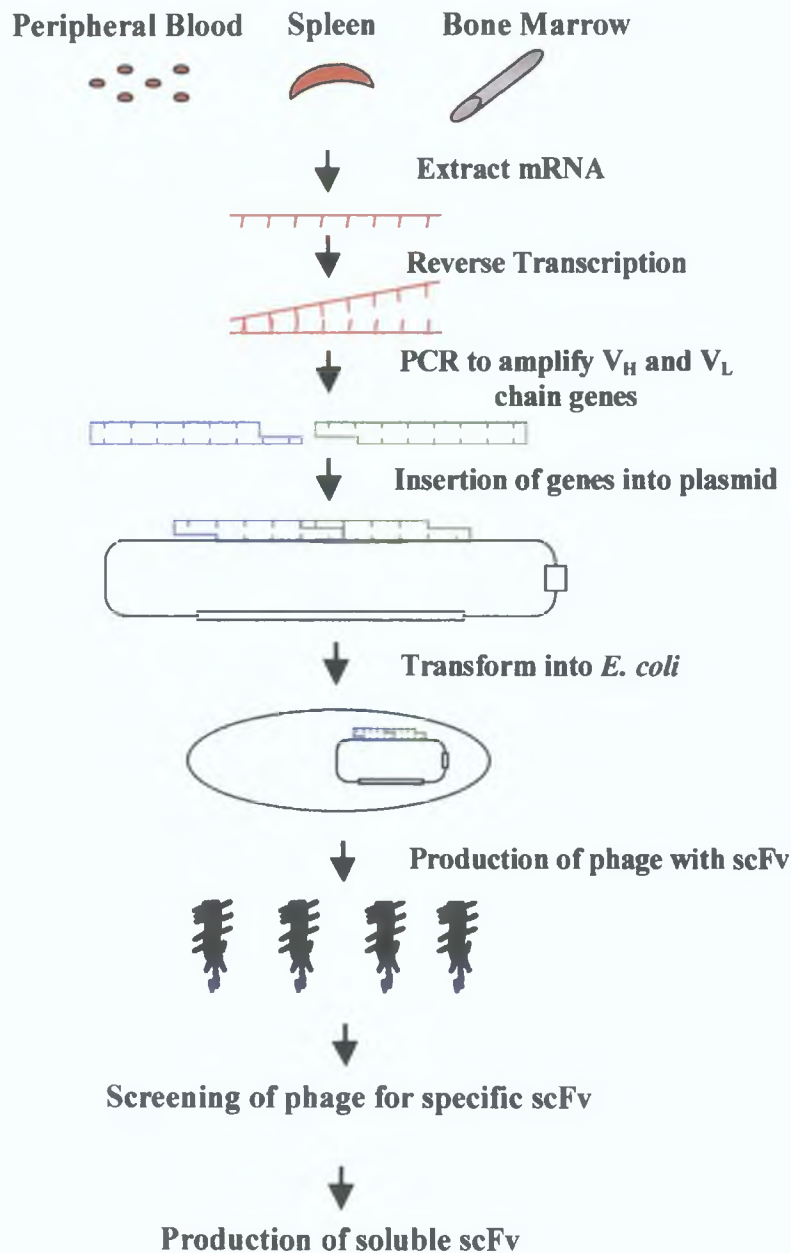
The continued progress in the use and application of recombinant DNA technology has enabled the possibility of producing specific antibodies by use of phage displaying recombinant antibody libraries

The first step in the production of a recombinant antibody library (Figure 1 7) using phage display is the isolation of mRNA from sources such as peripheral blood, spleen cells or bone marrow The isolated mRNA should contain the full repertoire of antigen specificities and may be used in the production of complementary DNA (cDNA) using reverse transcription The technology behind the use of phage display as a tool to produce antibodies is the use of this cDNA in the polymerase chain reaction (PCR) Utilising suitable primers, PCR may be applied for the amplification of heavy and light chain genes for antibody production These amplified gene fragments are then joined together and cloned into a suitable phage expression vector Once this occurs the antibody may be displayed on the surface of the phage allowing the antibody on the surface to be treated exactly like an antibody for selection and affinity studies (Hoogenboom *et al* , 1998)

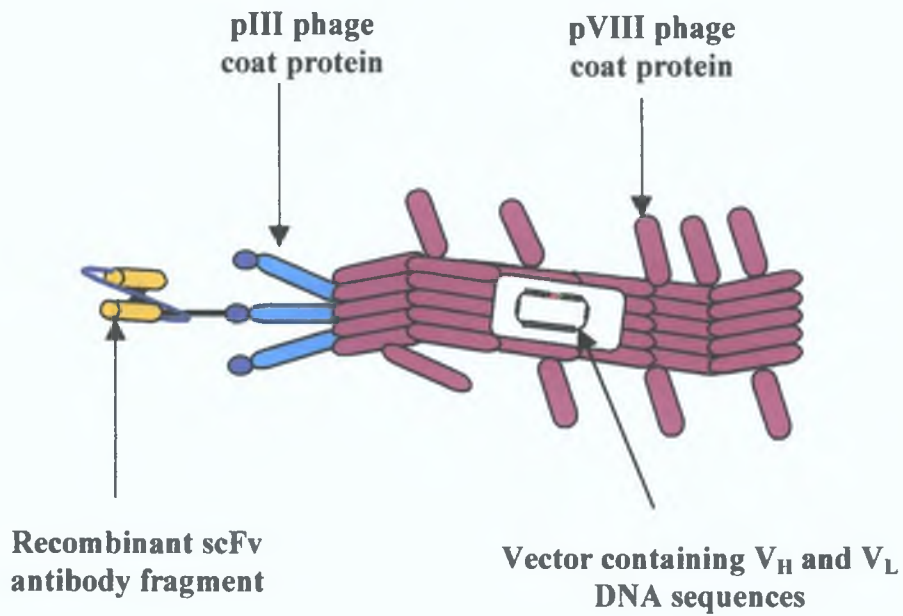
Other characteristics encoded on the phage vector include, an antibiotic resistance marker, the phage coat protein, an origin of replication, a promoter, and a tag to aid in scFv purification

The *lacZ* promoter is present to produce strain-dependent *lac* promoter expression Under control of the *lacZ* promoter expression of the vector can be induced using isopropyl- $\beta$ -D-galactopyranoside (IPTG) and suppressed in the presence of glucose Helper phage is used for the packaging of phagemid DNA into a virion This allows

the expression of recombinant antibodies on the surface of the phage. Screening for specific antibodies is carried out by propagation of *E. coli* resulting in the excretion of virions into the supernatant. The antibody fragments are usually linked to one of two phage coat proteins (pIII or pVIII) on the surface of phage particles (Figure 1.8). Following the isolation of the specific antibodies required, the DNA from these clones are sub-cloned into plasmids with the characteristic for soluble expression of antibody.



*Figure 1.7. Diagrammatic illustration of a generic model for the production of a combinatorial antibody phage display library. Messenger RNA was initially isolated from a suitable source as listed above. The mRNA undergoes reverse transcription and PCR amplification of antibody heavy and light chain genes using families of variable 5' primers and constant 3' primers. Light and heavy chain genes are joined together and ligated into a plasmid followed by transformation into *E. coli*. Phage expression is brought about by infection with helper phage, and affinity selection takes place against the desired antigen immobilised to a solid support. When specific clones have been isolated, soluble antibody may be expressed by religating specific light and heavy chain genes into a vector for soluble expression.*



*Figure 1.8. Diagram of filamentous phage expressing scFv on the surface of the phage as a fusion with the pIII phage coat protein. Light and heavy chain genes are present in the vector contained within the phage and the phage is ready for infection into *E.coli*.*

### 1 6 3 Selection and screening of specific phage antibodies

Selection processes that remove clones producing specific antibodies are of vital importance in obtaining antibodies that are required. The most popular selection methods include affinity selection (also known as biopanning) of phage displaying antibodies by use of immobilising antigen onto solid supports and columns or alternatively, the use of the BIAcore biosensor for 'real-time' analysis of antibody binding to antigen immobilised onto the surface of a chip followed by selection of the specific antibodies.

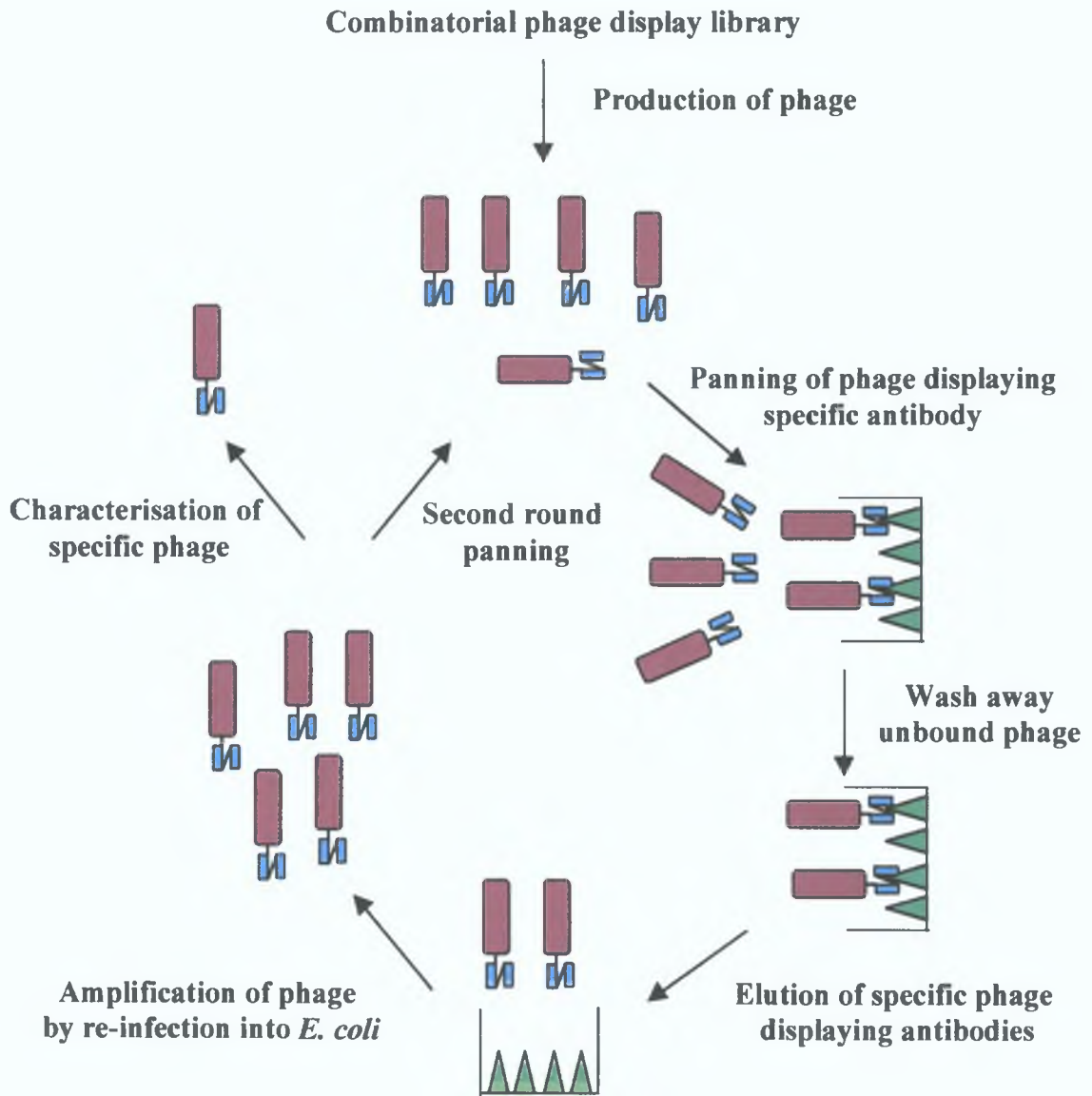
The biopanning process (Figure 1 9) for specific antibodies from combinatorial phage display libraries may be achieved by multiple rounds of phage binding to antigen, followed by washing to remove non-specific phage and elution to retrieve specific phage. This may be achieved with the use of immunoassay plates or immunotubes to which hapten-protein-conjugates are absorbed onto the surface or use of the BIAcore biosensor with conjugate immobilised onto a chip (Clackson *et al*, 1991, Marks *et al*, 1991, Griffiths *et al*, 1994, Malmborg *et al*, 1996). The higher the ratio of hapten bound to the protein, the higher the chance of phage antibodies binding to the hapten rather than the conjugate. However, conjugates which possess a high hapten to protein ratio may cause difficulties. As many haptens are hydrophobic, the more haptens present on the protein will result in insoluble complexes, which will inhibit the absorption of the conjugate onto the surface of the immunoplate or immunotube. Haptens covalently immobilised on the surface of immunoplates may also be used in the selection process for specific antibodies. This will decrease the possibility of selection of non-specific antibodies as the carrier-protein is no longer present in the selection process. However, immobilisation may depend on the availability of the functional groups on the hapten and is not suitable for all haptens.

Following selection of recombinant antibodies using the biopanning process, it may be necessary to screen large numbers of clones for selection of antibodies with the required characteristics. This is achieved with the use of a simple ELISA (Marks *et al*, 1991) with coated antigen on the surface. The polyclonal phage mixture is added to the wells of the plate and the binding allowed to come to equilibrium, followed by

the use of enzyme-labelled secondary antibodies and chromogenic substrate for detection

The problems associated with using a selection system such as the biopanning process and the screening assays are that these systems are required to reach a state of equilibrium. Using these procedures results in the selection of high affinity interactions over lower affinity interactions (Kretzchmar *et al*, 1995) which may not be desirable in some assay configurations such as the BIAcore biosensor. The BIAcore has the ability to perform 'real-time' analysis of biomolecular interactions but requires antibodies with relatively high dissociation rates for the repeated regeneration of the sensor chip surface. However, due to the nature of the ELISA screening technique, the antibodies isolated have low dissociation rates and would require more severe regeneration conditions to dissociate the antibody-antigen complex. This problem may be solved using the BIAcore biosensor for selection of positive antibody displaying phage with different affinities (Malmberg *et al*, 1996). This development has become very important as the use of antibody and antibody fragments on biosensors is becoming more prevalent (Turner, 1997). Antibodies selected using the BIAcore biosensor possess the characteristics for use on this sensor and so may be applied to the development of BIAcore biosensor assays.

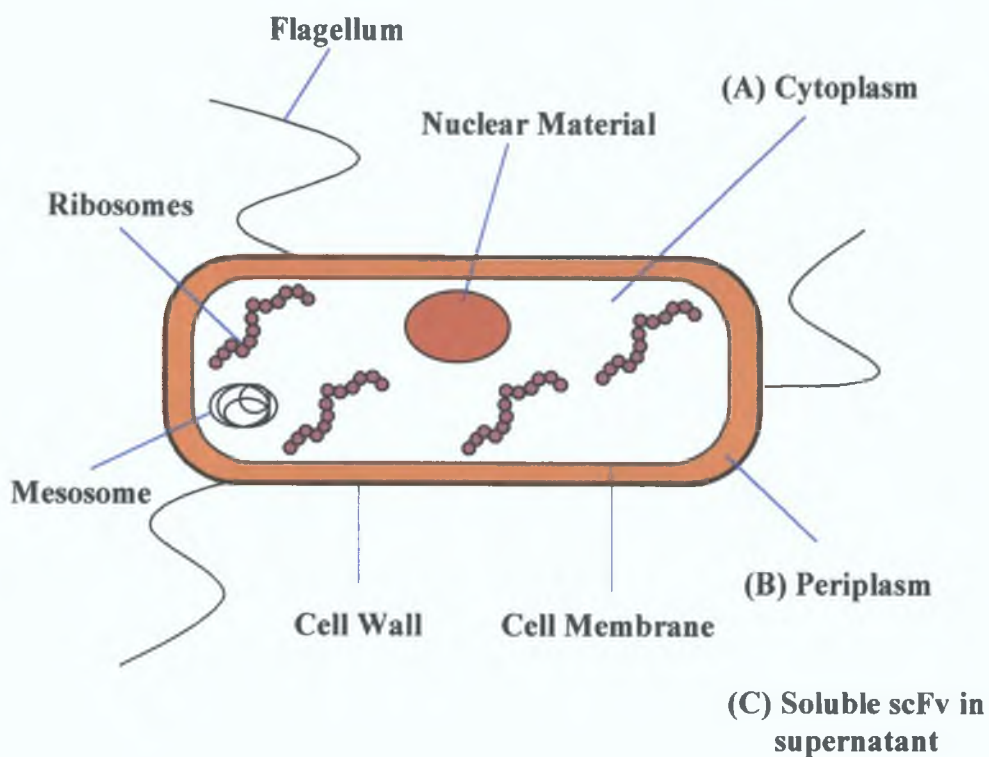
By using the BIAcore biosensor for screening, it is possible to monitor the entire binding pattern of an antibody mixture. Unpurified antibody mixtures such as supernatant may be directly injected over a surface with the antigen of interest immobilised and antibodies may be screened on the basis of their dissociation rate constants as described by Marks *et al* (1992). In this paper, antibody fragments were screened using the BIAcore biosensor and results showed the selected antibodies exhibited affinities improved by 300 times compared to conventional selection techniques.



*Figure 1.9. Illustration of affinity-selection panning process. Phage libraries are subjected to several rounds of panning which include the capture of phage by binding to antigen on a solid support. Specifically bound phage is then eluted from the antigen and used for re-infection into *E. coli* for screening of further rounds of panning.*

#### 1.6.4. Expression of recombinant scFv antibodies

Bacterial expression systems are a popular method for the production of all functional antibody fragments (Fv, Fab, F(ab')<sub>2</sub>, and scFv). Bacterial fermentation is both rapid and inexpensive, and genetic manipulation of bacteria can lead to high level production making it a perfect expression system for scFv antibodies. Unlike complex whole antibodies, which require post-translational modifications like glycosylation, scFv's can be produced in bacteria as fully functional antigen binding molecules. *E. coli* is the preferred bacterial expression system due to its ease of manipulation. Figure 1.10 shows the structure of the bacterium and the different spaces within it where scFv antibodies can be produced.



*Figure 1.10. Diagrammatic representation of an E.coli bacterium illustrating the various locations of expression of scFv antibodies. (A) Insoluble inclusion bodies (containing antibody) in the cytoplasm. (B) Soluble expression of antibody in the periplasm. (C) Soluble expression of antibody in the supernatant.*



ScFv's may be expressed without a signal sequence to specifically direct it to the periplasm resulting in the formation of insoluble inclusion bodies in the cytoplasm followed by a refolding procedure (Kipriyanov *et al*, 1997, Cho *et al*, 2000) This refolding step may be problematic and may be avoided by manipulating the *E coli* so that it mimics the secretory pathway in eucaryotic cells using a specific signal sequence that directs the scFv to the periplasmic space This signal sequence is cleaved during transport through the membrane allowing the correct folding of the antibody for expression

### **1 6 5 Purification of recombinant scFv antibodies**

Several methods for scFv purification are available These include antigen affinity chromatography (Owen *et al*, 1992, Casey *et al*, 1995, Cho *et al*, 2000), protein A chromatography (Mahler *et al*, 1997), cation exchange chromatography (Kretzschmar *et al*, 1996), size exclusion chromatography (Kretzschmar *et al*, 1996), human constant light chain tagging (Ridder *et al*, 1995, Longstaff *et al*, 1998) and immobilised metal affinity chromatography (IMAC) (Kipriyanov *et al*, 1997, Freyre *et al*, 2000)

IMAC has gained attention due to its many advantages over the more traditional antigen-affinity chromatography including higher ligand stability, higher protein loading, milder elution conditions, lower cost and complete recovery of ligand following regeneration (Arnold, 1991) IMAC involves the insertion of genes coding for a histidine (His) tag, containing 4-6 histidine residues, into the scFv sequence Insertion at the N or C terminus ensures that the antigen binding site is not effected (Casey *et al*, 1995) The column consists of transition metals such as Ni<sup>2+</sup>, Co<sup>2+</sup>, Zn<sup>2+</sup> and Cu<sup>2+</sup>, chelated by nitrotriacetic acid (NTA), iminodiacetic acid (IDA) or tris (carboxymethyl) ethylenediamine (TED) (Arnold, 1991, Muller *et al*, 1998) His tagged proteins bind to a metal-chelate solid support, while impurities are eluted Specifically bound protein may be eluted using low pH, which confers a positive charge on the His residues so they are incapable of binding metal ions, or by addition of imidazole, which competes with His for metal binding The advantage of the latter

system being that it does not denature the protein (Janknecht *et al*, 1991) Janknecht *et al* (1991) has used this system to purify human serum response factor to greater than 95% purity Subsequently, Casey *et al* (1995) has utilised Cu<sup>2+</sup>-IDA chelated IMAC to purify a His tagged protein to 90% purity, with higher yield than that of antigen affinity chromatography (10mg/l as opposed to 2.2mg/l) Kipriyanov *et al* (1997) have shown that IMAC can also be used to successfully purify scFv's from *E coli* periplasmic extracts

### **1.6.6 Applications of recombinant scFv antibodies to proteomics**

Proteomics may be defined as the study of protein properties such as expression levels, post-translational modification, interactions etc with respect to obtaining a global, integrated view of disease processes, cellular processes and networks at the protein level (Blackstock and Weir, 1999) The use of recombinant antibody phage displaying libraries for the production of recombinant antibodies are becoming increasingly important in the field of proteomics This is mainly due to recent advances, which include the development of larger combinatorial antibody phage displaying libraries which contain high-affinity binders to target antigens These larger libraries and new advances in screening technologies which use high-density antibody arrays to identify differentially expressed proteins, may be used in proteomic research (Holt *et al*, 2000)

The selection procedures used in the application of antibody phage display technology to the study of proteomics are of great importance There are three main selection strategies and these are, selection on complex antigen, conventional but highly parallel selection and use of a library of antibodies selected against a library of antigens (Holt *et al*, 2000)

The first strategy, selection on complex antigen simultaneously selects many antibodies against antigens such as cells, cellular extracts or tissue sections The characteristics of this selection method would indicate the selection of large numbers of specific antibodies, however, due to the inherent biases in phage selection, a small number of antibodies are selected toward the most abundant proteins present

(Hoogenboom *et al*, 1999) The next selection strategy concentrates on selecting antibodies against individual proteins, expressed cDNAs or peptides, performed in parallel This method has the potential for high-throughput, however, it needs the use of robotics which are expensive and problems exist when selected antibodies against protein fragments may not recognise the parent protein The third strategy for selection is to select a library of antibodies against a library of antigens allowing the production of vast quantities of antibody-antigen binding pairs (Fields and Song, 1989, Visintin *et al*, 1999)

The ProAb™ approach (Pennington and Dunn, 2001), applying phage antibodies to functional genomics is also being utilised This approach takes interesting pieces of DNA from databases of ESTs ('expressed sequence tags') and translates them into proteins Peptides are synthesised chemically to represent the protein sequence and used as antigens to which phage antibodies are selected The antibodies which are selected using this approach can then be used as detection reagents in immunocytochemistry, thereby revealing the distribution and abundance of the gene product in normal and diseased tissues

The ProxiMol™ selection technique (Pennington and Dunn, 2001) is also used in proteomics Within this method, catalysed reporter deposition (Bobrow *et al*, 1989,1992) has been utilised to allow the isolation of phage antibodies binding in close proximity to a biotinylated guide molecule (Osborne *et al*, 1998) When utilising phage display technology to screen for antibodies to a cell surface antigen, a guide molecule, such as a biotinylated target ligand, or a HRP-conjugated antibody, is added to intact cells, together with phage from a  $>10^{11}$  human antibody library Following this, biotin-tyramine reagent is added resulting in bound phage being labelled with biotin These biotin labelled phage antibodies are then recovered using streptavidin-coated magnetic beads

Both the ProAb™ strategy and ProxiMol™ method may be applied to work in conjunction with one another as the selected phage antibodies from the ProAb™ strategy can be applied as guide molecules in the ProxiMol™ method selections

Screening of positive antibodies is of vital importance in proteomics Traditional ELISA techniques have proved adequate for such a purpose However, such a

technique only allows a small percentage of the positive clones to be screened. Other methods have been developed for screening. One method of particular importance is the use of antibody arrays which have the ability to increase throughput up to several hundred times (Ekins *et al*, 1990). Antibody arrays for high-throughput screening of antibody-antigen interactions have been developed by De Wilde *et al* (2000). This technique uses high robotic picking and high density gridding of bacteria containing antibody genes followed by filter-based enzyme-linked immunosorbent assay (ELISA) screening to identify clones that express binding antibody fragments. This method had the ability to screen up to 18,342 different antibody clones at a time against 15 different antigens. This method proves that new and improved screening methods are essential for the continued progress of phage display in the study of proteomics.

#### **1.6.7 Applications of recombinant scFv antibody fragments for use in residue analysis**

Over recent years, information on recombinant DNA techniques, combined with immunoglobulin structure and function, have led to the development of recombinant single chain variable fragment (scFv) antibody technology. These antibodies have been produced in bacteria (Krebber *et al*, 1997). Such recombinant antibodies may be used for the analysis and detection of various antigens. There are many advantages in using recombinant technology compared to traditional detection methods such as the ability to modify properties through mutagenesis, speed of production of antibodies and the possibility of generating rare and novel functionalities. Selection from phage antibody libraries provides a new tool for the isolation of novel self antigens, such as disease-associated antigens (Hoogenboom *et al*, 1998). This application has been used for probing lymphocyte cell surfaces, yielding antibodies to known antigens and a number of promising uncloned new cell-type specific antigens (De Kruijff *et al*, 1995). Recombinant antibodies with a specific range of affinities are required for small antigens, however, although the literature

may suggest that obtaining such antibodies through recombinant phage display technologies is straightforward, this is not the case

Many of the detection methods using recombinant antibody technology to date have been ELISA based. Garrett *et al* (1997) produced a scFv, specific for the organophosphate pesticide parathion using DNA obtained from a hybridoma cell line secreting an anti-parathion monoclonal antibody (Mab). An ELISA was developed and the limits of detection for the scFv and Mab were 2.3 and 1.6 ng/well respectively.

Further work was carried out on the detection of organophosphate pesticides by Alcocer *et al* (2000) when a comparison was carried out between polyclonal, monoclonal and recombinant antibodies to chlorpyrifos-ethyl. Polyclonal antiserum and two murine monoclonal antibodies recognising chlorpyrifos-ethyl were produced. The hybridoma cell lines DNA was used in the production of scFv's. The two scFv's limit of detection in ELISA were either similar or improved compared to the monoclonal antibody. Cross reactivity studies were also carried out which showed all the antibodies to be specific to the chlorinated aromatic ring.

Functional scFvs have also been produced against the herbicides paraquat (Graham *et al*, 1995), atrazine (Byrne *et al*, 1996) and triazine (Kramer and Hock, 1996).

Single chain Fv fragments have also been used in the detection of potato viruses. Leafroll luteovirus (Harper *et al*, 1997) was detected using a scFv with alkaline phosphatase attached. Alkaline phosphatase removes the need for the use of a secondary enzyme-labelled antibody and decreases the time needed for the assay.

Potato virus Y potyvirus (Boonham *et al*, 1998) was detected using scFv antibodies in ELISA at concentrations of 50 ng/ml in plant sap. Results indicated that the scFv antibodies displayed similar detection characteristics to commercially available monoclonal antibodies.

Detection of environmental toxins has become increasingly important, and scFv antibodies are now being used for their detection. They have been produced against the mycotoxins zearalenone (Yuan *et al*, 1997) and aflatoxin B<sub>1</sub> (Daly *et al*, 2001). ScFv antibodies produced against aflatoxin B<sub>1</sub> were applied for use in the BIAcore biosensor and could detect levels of aflatoxin B<sub>1</sub> as low as 3 ng/ml. M<sup>C</sup>Elhiney *et al*

(2000) also developed an ELISA for the detection of the cyanobacterial hepatotoxin microcystin-LR. An scFv antibody isolated from a naive human phage display library was used in the assay. The most sensitive antibody clone isolated, detected free microcystin-LR with an IC<sub>50</sub> value of 4 μM.

These case studies of detection systems using antibody phage display technology highlight the potential of the technology for the selection of antigen specific antibodies with detection characteristics equivalent or better than traditional technologies.

## **1.7 Immunoassays**

### **1.7.1 Design of immunoassays**

This section focuses on the design of enzyme-based immunoassays. The first assay formats of interest are non-competitive, solid-phase enzyme immunoassays. These assays may be sub-divided according to the immunoreactant immobilised on the solid phase. One commonly used method is the immobilisation of antigen on solid phase for detection of antibodies with the use of enzyme labelled anti-antibody molecules. The possibility of immobilising antigen followed by detection with labelled specific antibody is also a commonly used method. This method yields higher specificity, but suffers from high background values. Another general approach associated with this type of immunoassay is the detection of antigens with the use of an antibody sandwich assay. In this assay format primary antibody is immobilised on solid phase followed by the addition of antigen, which results in specific antibody capturing the relative antigen. This is followed by the addition of a second antigen-specific enzyme-labelled antibody resulting in the formation of a sandwich assay. The final method in this class of assay is known as the class-capture technique useful in the diagnosis of infectious agents. This method works on the principal that infections inducing a secondary immune response can be detected by monitoring the different antibodies produced at each stage of infection.

Non-competitive homogenous (homogeneous assays do not require the separation of free and antibody-bound fractions of the analyte) enzyme immunoassays are further types of immunoassays which are performed without the involvement and attachment to a solid phase and they do not require the separation of free and bound label. This method of analysis may be achieved by labelling two monoclonal antibodies directed against different epitopes of the antigen with two enzymes selected so that one produces the substrate for the other.

Competitive homogenous enzyme immunoassays have several general formats. The detection of hapten is carried out with the use of enzyme-hapten conjugates used as antigen. The activity of the conjugated enzyme is modulated by the reaction of hapten-specific antibodies with the haptened enzyme. The presence of competing free hapten will decrease this modulation resulting in a detectable difference. Other methods within this group can include the use of substrate conjugated to hapten instead of enzyme. The antigen-specific antibody reacting with the antigen-substrate conjugate then prevents the substrate being acted upon by the enzyme. In the presence of free antigen, the reaction of antibody with substrate-bound antigen is prevented.

Finally, competitive, solid-phase enzyme immunoassays are the last group of interest. Within this assay format the antibody is immobilised on the solid phase and the antigen is labelled with the enzyme. Binding of antigen-enzyme conjugate to immobilised antibody is inhibited by the addition of free antigen, which results in the production of a standard curve for the detection of hapten (Tijssen 1985).

### **1.7.2 Immunoassays-experimental**

The increased need for systems with the ability to monitor, detect and quantify various molecules has led to the growth of immunoassays. Immunoassays (Price and Newman, 1997) are bioanalytical methods in which the detection and quantification of a molecule is dependent on the binding of the molecule to a specific antibody.

Enzyme-Linked Immunosorbent Assay (ELISA) (Gosling, 1990, Crowther, 1995) is the most commonly used technique for measuring hapten-antibody interactions and is

a solid-phase heterogeneous technique used for the detection of a wide range of analytes. ELISA may have a number of formats. However, two assay formats, indirect ELISA and competitive ELISA, were primarily used in the development of assays in relation to the work in this thesis.

A schematic representation of a competitive ELISA technique is shown in Figure 1.11. As direct immobilisation of small haptens onto an immunoplate may prove difficult, the hapten is coupled to protein through various coupling chemistries and added to the wells of a 96 well immunoplate, which is known as coating. Proteins bind primarily to the surface of the wells of  $\gamma$ -irradiated 96-well immunoplates by hydrophobic interactions. After a suitable incubation period, the wells are washed to remove any loosely bound conjugate. A suitable blocking solution (i.e. 2% Marvel) is then added to the wells to 'block' any unreacted sites on the plate that may result in non-specific binding of either primary or secondary antibodies. Solutions are then prepared with a constant amount of antibody present with varying concentrations of free hapten. After washing the blocked immunoplate, these antibody-hapten mixtures are added to the wells of the plate and incubated. During this incubation period, competition occurs between the hapten bound on the surface of the well and free hapten in solution for the antibody. Again following washing, a suitable secondary antibody (e.g. horseradish peroxidase-labelled goat anti-rabbit antibody) is added to the wells of the plate followed by chromogenic substrate for detection purposes. The intensity of the colour produced by the enzyme-substrate reaction is monitored spectrophotometrically at the appropriate wavelength. With this type of assay, the colour intensity is inversely proportional to the amount of free hapten in solution (i.e. the higher the concentration of free hapten the weaker the colour intensity). The results from such assays may be used to construct standard curves, from which the concentration of hapten in unknown solutions may be determined.

Figure 1.12 represents the format for an indirect ELISA. This type of ELISA is mainly used for the determination of antibody titres in purified and unpurified samples. The format of this ELISA is similar to the format of the competitive ELISA. However, dilutions of primary antibody are added to the wells of the immunoplate instead of the antibody-hapten mixtures.



When analysing both indirect and competitive ELISA formats, it is evident that the sensitivity of both assays depends solely on the affinity of the antibody for the hapten

The BIAcore biosensor is also used in the development of inhibitive immunoassays in this thesis. As discussed in section 4.1.1.1, the BIAcore works on the principle of surface plasmon resonance and may be used to develop immunoassays to various metabolites. With this system, either hapten or antibody may be immobilised onto the surface of the chip. Immobilisation of haptens may prove difficult depending on the functional groups available. This is why most assays developed immobilise protein-hapten conjugates onto the surface. Standards of free haptens are prepared and premixed with antibody and after a suitable incubation time, passed over the surface of the chip with protein-hapten conjugate immobilised. The haptens in solution inhibit the binding of antibody to the surface and the amount of antibody that binds to the surface is inversely proportional to the amount of free haptens in solution. The results of this immunoassay may be used in the construction of a standard curve and unknown concentrations determined from such a curve.

### **1.7.3 Immunoassay optimisation**

Assay design requires the selection of reagents and a protocol whereby the main objective of the assay, which is the most accurate measurement of analyte concentration, is achieved. There is little doubt that the ability of an assay to detect haptens is directly related to the affinity and selectivity of the antibody for the particular haptens. When analysing a competitive assay, the main function of enzyme conjugates and free haptens is to compete for a limited number of binding sites present on the specific antibodies, according to a reversible antibody-hapten equilibrium competition with an antibody-enzyme conjugate. Lower affinity of the antibody for the competitor, compared with the haptens, will shift the equilibrium conditions in favour of the formation of an antibody-hapten complex. Association of the antibody with the labelled-antigen is then inhibited, hence the structure of the labelled competitor has a key role to play in the sensitivity of the immunoassay.

Dose-response curves are a common feature of such assay systems. As fixed amounts of antibody and enzyme conjugates are present, the enzyme activity determined by absorption readings is related to the hapten concentration by a dose response curve. When response is plotted against the logarithm of the hapten concentration, the curve produced is of a sigmoidal nature with a relatively linear portion near the point of half inhibition, which is known as the  $IC_{50}$ . The change in the absorbance reading in this curve correlate to the hapten concentrations used in the assay.

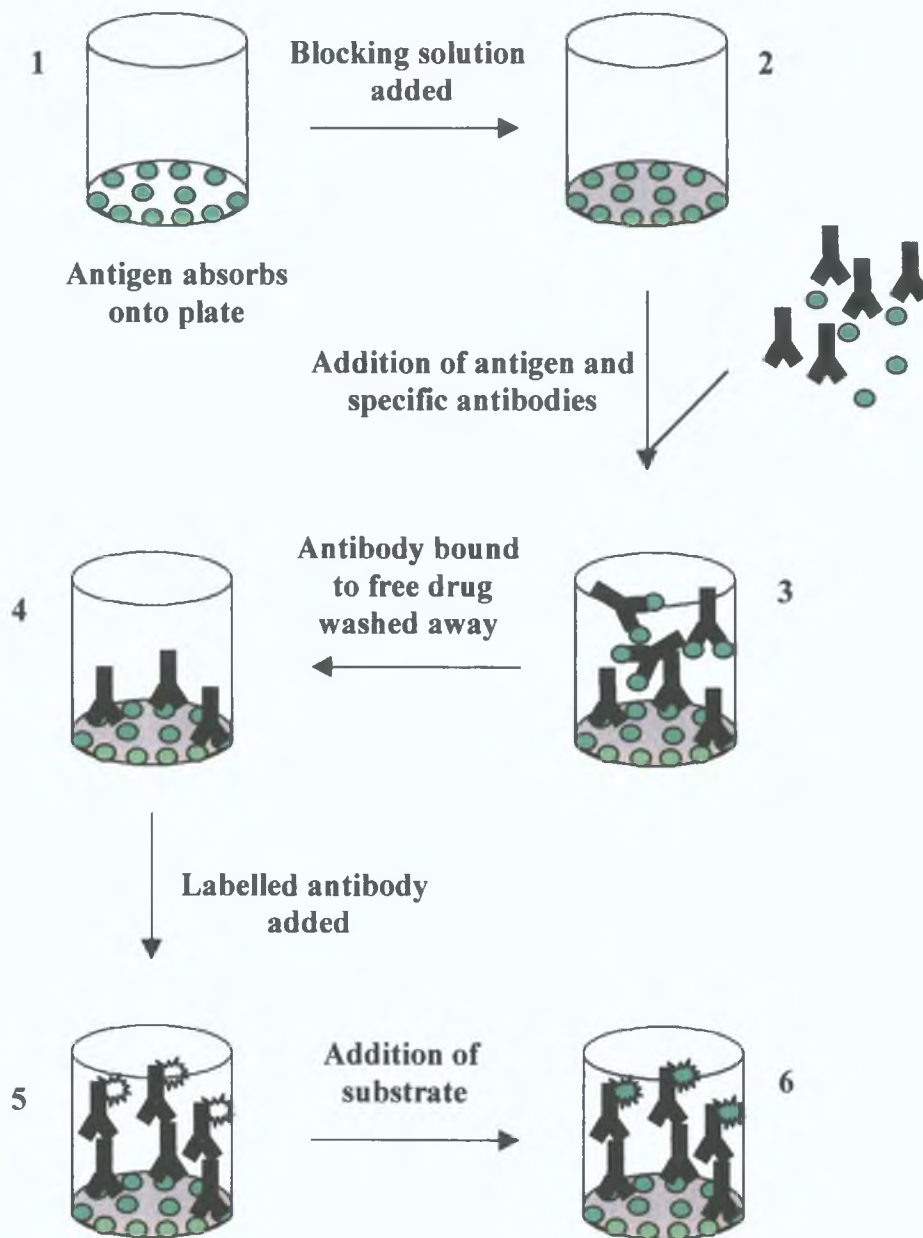
Characterisation of these assays requires the determination of the limit of detection and the limit of quantification. The limit of detection (LOD) is defined as the smallest concentration of hapten which results in a change in signal enabling accurate differentiation between it and the signal from the zero concentration of hapten. Although there are several methods of determining sensitivity, the one most favoured for use is selection of the dose which inhibits 10% of the binding of the antibody with the enzyme label at 90%. However, the limit of quantification is the level above which the quantitative results can be obtained with a reasonable degree of accuracy and precision. In the case of many assays with sigmoidal curves, the accuracy of the assay decreases as the curve approaches the asymptotes at both the higher concentrations of free hapten and the lower concentrations.

Assays require high levels of precision and reproducibility. The precision of an assay is defined as the way in which replicate analysis of sample agree with one another. The reproducibility of the assay may be defined as the assays ability to provide the same result when used on different days or by different users. With respect to a normal sigmoidal curve we find that the variability is not uniform and that the errors increase as the ranges move to the asymptotes of the curve at the highest and lowest concentrations of sample. In the case of many assays the highest precision is obtained for concentrations close to the  $IC_{50}$ .

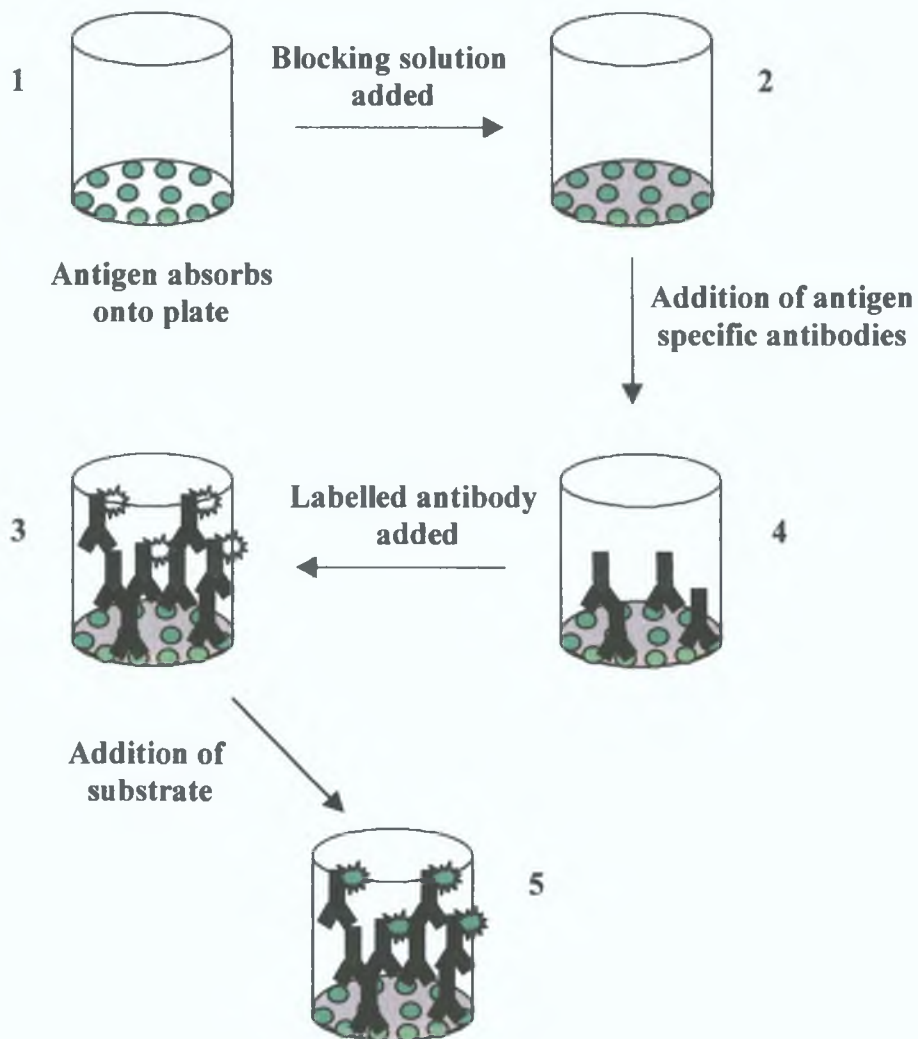
The accuracy of an assay is defined as the closeness of agreement of the measured test results with the expected true reference value. Depending on the type of assay required low accuracy values may be acceptable if the assay is a confirmatory assay.

However, if the assays function is to determine exact concentration of hapten high accuracy values are required

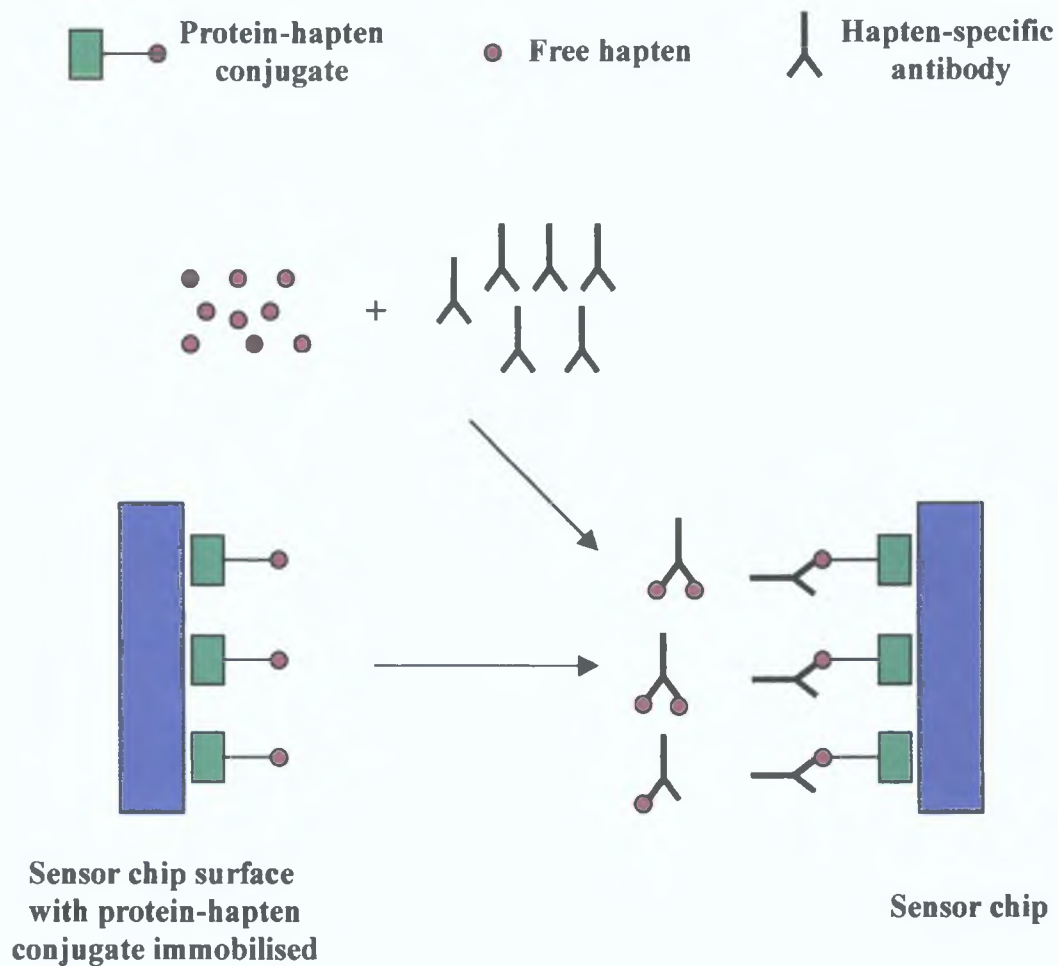
The specificity of the assay is the ability of the assay to produce a signal representing a measurement of the hapten being analysed. Quantitative determinations of hapten concentration are reliable only if there is no cross-reactivity. Cross reactivity with other haptens will produce false results within the assay. However, various antigens may possess similar epitopes resulting in the production of antibodies with cross-reactivity to these antigens. This results in the need to fully understand and determine the antibodies cross-reactivity.



*Figure 1.11. Schematic diagram of a typical competitive ELISA. (1) Wells of an immunoplate are coated with a protein-hapten conjugate. (2) Wells are blocked with a suitable blocking solution (e.g. 2% marvel). (3) The antibody-hapten solutions are then added to the wells of the plate and allowed to incubate. (4) Wells are washed to remove unbound antibody and hapten, the bound antibody remains on the surface of the well. (5) A species specific secondary enzyme-labelled antibody is added to the wells and allowed to incubate. (6) With the addition of substrate, in the presence of the enzyme on the secondary antibody, a colour is produced and detected spectrophotometrically at the appropriate wavelength.*



*Figure 1.12. Schematic diagram of a typical indirect ELISA mainly used for the determination of purified and unpurified antibody titre. (1) Wells of an immunoplate are coated with a protein-hapten conjugate. (2) Wells are blocked with a suitable blocking solution (e.g. 2% marvel). (3) Dilutions of antibody are prepared and added to the wells of the plate. (4) A species specific secondary enzyme-labelled antibody is added to the wells and allowed to incubate. (5) With the addition of substrate, in the presence of the enzyme on the secondary antibody, a colour is produced and detected spectrophotometrically at the appropriate wavelength.*



**Figure 1.13.** Schematic representation of the BIAcore inhibitive immunoassay format for quantitative determination of hapten. Standards of free haptens were prepared and premixed with antibody and after a suitable incubation time, passed over the surface of the chip with protein-hapten conjugate immobilised. The excess antibody in the mixtures binds to the sensor chip surface and the amount of antibody, which binds, is inversely proportional to the amount of free haptens in solution.

## **1 8 Aims of Research**

The aim of this project was to develop antibodies to morphine-3-glucuronide and parathion for use in the development of competitive ELISA immunoassays and rapid and sensitive BIAcore inhibition immunoassays for the analysis of these compounds

Chapter 3 describes the production and characterisation of drug-protein conjugates to morphine-3-glucuronide used for the production of two polyclonal antibodies to morphine-3-glucuronide in rabbits. Both affinity purified polyclonal antibodies were used to develop competitive ELISA immunoassays for morphine-3-glucuronide in urine

In chapter 4, the polyclonal antibodies were used in the development of BIAcore-based inhibition immunoassays for the detection of morphine-3-glucuronide in urine. Studies were carried out on the suitability of these antibodies for application on the BIAcore biosensor and results displayed good reproducibility with low CVs

Chapter 5 describes the development of combinatorial phage display libraries using the Krebber system for the production of single chain Fv (scFvs) antibodies that detect morphine-3-glucuronide. Competitive ELISA and BIAcore inhibition immunoassays were developed for the detection of morphine-3-glucuronide using an affinity selected scFv antibody

Chapter 6 describes the derivatisation of parathion to amino-parathion and following characterisation, was used in the production and characterisation of drug-protein conjugates to the organophosphate pesticide parathion. The drug-protein conjugates were then used for the production of a polyclonal antibody to parathion, its purification and use in a competitive ELISA immunoassay. Studies were also carried out on the applicability of this antibody for the development of a BIAcore-based immunoassay

## **Chapter 2**

### **Materials & Methods**



## 2 1 Materials

All standard laboratory reagents were purchased from Sigma Chemical Co , Poole, Dorset, England unless stated otherwise

Reagent	Supplier
Acetic acid Hydrochloric acid Sodium chloride Tween 20 Parathion	Riedel de-Haen AG, Wunstorfer, Strabe 40, D-30926, Hannover, Germany
Bacteriological Agar Tryptone Yeast Extract PBS tablets	Oxoid, Basingstoke, Hampshire, RG24 8PW, England
Bicinchoninic acid assay (BCA) kit	Pierce and Warriner (UK) Ltd , Chester, England
DNA ligase	Boehringer-Mannheim Ltd , East Sussex, England
CM-Dextran	Fluka Chemicals, Gillingham, Dorset, England
Hepes Trizol	Gibro BRL, Renfew Rd, Paisley PA49RF, Scotland
HPLC-grade solvents	Lab-Scan, Stillorgan, Co Dublin, Ireland
Hydrogen peroxide	BDH Chemicals Ltd , Poole, Dorset, England
PCR Optimizer Kit ProBond™ Resin	Invitrogen, 9704-CH-Groningen, Netherlands
PCR Primers	Sigma-Genosys Ltd, Cambridge CB2, England
SfiI enzyme	New England Biolabs, Hitchin, Herts , England
VCSM13 Helper Phage	Stratagene, North Torrey Pines Rd La Jolla, USA
Wizard Plus Mini-prep kit and PCR prep DNA purification kit PCR-related Reverse-Transcription System	Promega Corporation, 2800 Wood Hollow Rd, Madison, WI 53711-5399, USA

## 2 2 Equipment

Equipment	Supplier
Atto dual minislabs AE-6450 Atto AE-6100	Atto Corp, Bunhyo-Kui, Tokyo 113, Japan
BIAcore 1000™ BIAcore 3000™ CM5 chips	Pharmacia Biosensor AB, Uppsala, Sweden
Eppendorf tubes Sterile universal containers	Sarstedt, Wexford, Ireland
Heraeus Christ Labofuge 6000 Biofuge A Microcentrifuge	Heraeus Instruments Inc, 111-a Corporate Bolevard, South Plainfield, New Jersey, USA
Titretek Twinreader Plus	Medical Supply Company, Damastown, Mulhuddart, Dublin 12, Ireland
Image Master VDS	Pharmacia Biotech, San Francisco, CA , USA
IR Spectrophotometer	The Nicolet Instrument Corp , Madison, USA
NMR Spectrometer, 400MHz AC400	Brucker, Coventry, CV4 9GH, England
NUNC MaxiSorb plates	NUNC, Kamstrup DK, Roskilde, Denmark
3015 pH meter	Jenway Ltd , Essex, England
Orbital incubator	Gallenkamp, Leicester, England
RM6 Lauda waterbath T-Gradient BIOMETRA-PCR Millipore Filtration Apparatus	AGB Scientific Ltd , Dublin Industrial Estate, Glasnevin, Dublin 9, Ireland
SB1 Blood tube rotator	Stuart Scientific, London, England
Sorvall RC-5B refrigerated centrifuge	Du Pont instruments, Newtown, Connecticut, USA
Sterile cell culture lab-wear	Brownes, Foxrock, Dublin, Ireland
Stuart Platform Shaker STR6	Lennox, Naas Rd , Dublin, Ireland
Ultrafiltration cell 8400	Amicon Inc Beverly, Massachusetts 01915, USA
UV-160A spectrophotometer	Shimadzu Corp , Kyoto, Japan
UVP ImageStore 7500 gel documentation system	Ultra Violet Products, Upland, CA , USA

## 2 3 Methods

### 2 3 1 General formulations

#### 2 3 1 1 Culture media formulations

2 x Tryptone and yeast extract (TY) medium	Tryptone	16 g/l
	Yeast Extract	10 g/l
	NaCl	5 g/l
Luria broth (LB) medium	Tryptone	16 g/l
	Yeast Extract	10 g/l
	NaCl	5 g/l
	Bacteriological agar	15 g/l
Non-expression medium (NE)	Tryptone	16 g/l
	Yeast Extract	10 g/l
	NaCl	5 g/l
	Glucose	1%, v/v
	Chloramphenicol	25 µg/ml
Low expression medium (LE)	Tryptone	16 g/l
	Yeast Extract	10 g/l
	NaCl	5 g/l
	Glucose	1%, v/v
	Chloramphenicol	25 µg/ml
	IPTG	0.5 mM
Expression Medium (EM) for soluble scFv antibodies	2 x TY medium	
	Chloramphenicol	25 µg/ml
Super optimal catabolites (SOC) medium	Tryptone	20 g/l
	Yeast Extract	5 g/l
	NaCl	0.5 g/l
	KCl	2.5 mM
	MgCl <sub>2</sub>	20 mM
	Glucose	20 mM
	pH	7.0
Tryptone and yeast extract (TYE) medium	2 x TY medium	
	Bacteriological agar	15 g/l

### **2 3 1 2 *Buffer formulations***

#### **Phosphate buffered saline (PBS)**

One tablet (Oxoid) was dissolved per 100 mls of distilled water according to manufacturer's instructions. When dissolved, the tablets prepare Dulbecco's A PBS which contains 0.15 M NaCl, 2.5 mM potassium chloride, 10 mM disodium hydrogen phosphate and 18 mM sodium dihydrogen phosphate, pH 7.4. This buffer will be referred to throughout as PBS.

#### **Tris buffered saline (TBS)**

Tris buffered saline containing 1 mM CaCl<sub>2</sub>, 0.05 M Tris, 0.15 M NaCl, pH 7.4, was prepared as the dilution buffer for the monoclonal anti-FLAG antibody for western blotting and ELISA analysis of soluble scFv's.

#### **Tris-acetic acid-EDTA buffer (TAE)**

A stock solution of 50 X TAE buffer was prepared in a final volume of 1 l by dissolving 242 g Tris, and addition of 57.1 ml glacial acetic acid followed by 100 ml of 0.5 M EDTA, pH 8.0. All gels were run in 1 X TAE.

#### **Hepes Buffered Saline (HBS)**

Hepes buffered saline (BIACORE running buffer) containing 50 mM NaCl, 10 mM HEPES, 3.4 mM EDTA and 0.05% (v/v) Tween-20 was prepared by dissolving 8.76 g of NaCl, 2.56 g of HEPES, 1.27 g of EDTA and 500 µl of Tween 20 in 800 ml of distilled water. The pH of the solution was then adjusted to pH 7.4, by the addition of 2 M NaOH. The final volume was then made up to 1,000 ml in a volumetric flask. The solution was filtered through a 0.2 µm filter and degassed prior to use.

## **2 3 2 Production of drug-protein conjugates**

### **2 3 2 1 *Production of protein conjugates to morphine-3-glucuronide***

A 50 mg/ml solution of morphine-3-glucuronide (M3G) was prepared in 50 mM HCl and made up to a final volume of 5 ml with 0.2 M borate buffer, pH 8.5. Concentrations of EDC (N-ethyl-N-(dimethyl-aminopropyl) carbodiimide hydrochloride) and NHS (N-hydroxysuccinimide) were then added to give a final molarity of 0.4 and 0.1 M, respectively, in the solution and incubated at room temperature without agitation for 10 min. Protein (BSA, OVA, THY) was prepared at a molar ratio of 1:100 to M3G in 5 ml of 0.2 M borate buffer, pH 8.5, and added dropwise to the M3G solution. This solution was then incubated at room temperature for 2 h with stirring. The solution was dialysed against 50 volumes of PBS overnight at 4°C.

### **2 3 2 2 *Production of parathion conjugates***

Production of parathion conjugates was performed according to Ercegovich *et al* (1981).

#### **2 3 2 2 1 *Preparation of reduced parathion***

Parathion (150 mg) was dissolved in 6 ml of diethyl ether and extracted with cold 1% (w/v) sodium carbonate ( $\text{Na}_2\text{CO}_3$ ) in distilled water (4 x 50 ml) to remove any phenol impurities. 5 ml of 9.1% acetic acid-concentrated HCl and 0.5 g of zinc powder were added to the phenol free ether solution. The yellow reaction mixture was stirred under reflux for 45 min. The solids were then filtered from the colourless solution and subsequently washed with 10 ml of carbon tetrachloride ( $\text{CCl}_4$ ). The combined organic phase was then washed with 5 ml of distilled water and dried over 0.5 g of anhydrous sodium sulphate. The solvent was subsequently removed under reduced

pressure Characterisation of the product was carried out by NMR and IR spectroscopy

#### **2 3 2 2 2 *Preparation of parathion conjugates***

Reduced parathion (10 mg) was dissolved in 5 ml of distilled water containing 2 mM HCl The solution was cooled on an ice bath, and a chilled 0.1 M sodium nitrite ( $\text{NaNO}_2$ ) solution was added dropwise until a positive starch-iodide paper test was observed The mixture was stirred for 30 min and the excess nitrous acid was decomposed with urea The reaction mixture was added to 50 mg of OVA dissolved in 10 ml of 0.2 M borate buffer, pH 9, and stirred in an ice bath for 2 h A bright orange colour was then observed The conjugation mixture was dialysed against 2 changes of 5 L of distilled water daily for 5 days at 4°C The dialysed product was then stored at -20°C The same procedure was carried out for the production of BSA, THY and dextran conjugates

### **2.3.3 Characterisation of protein-drug conjugates**

#### **2.3.3.1 *Ultraviolet spectroscopy***

Conjugates were prepared at a concentration of 1 mg/ml in PBS, and their UV spectra analysed from 200-400 nm. UV spectra of similar concentrations of control protein were also prepared and recorded. Comparison of the two spectra gave putative confirmation of the conjugation of parathion to protein carrier molecules.

#### **2.3.3.2 *Immunisation***

Mice were immunised as described in section 2.3.5.3 with preparations of M3G-protein conjugates as described in section 2.3.2.1. Blood was taken from the mice as described in section 2.3.6.2 after 10 days and analysed for production of antibodies to the specific drug as described in section 2.3.8.1.

### **2 3 4 Production of immunoaffinity matrices**

An immunoaffinity matrix was prepared by the chemical coupling of parathion-BSA to sepharose-4B. The immunoaffinity matrix was then used for the affinity-purification of polyclonal antibodies to parathion from ammonium sulphate precipitated-rabbit immunoglobulin fractions.

#### **2 3 4 1 Preparation of parathion-BSA Sepharose column**

Parathion-BSA was coupled to CNBr-activated sepharose 4B according to the manufacturer's instructions. 1.5 g of CNBr-activated sepharose 4B was weighed out and swollen in 250 ml of 1 mM HCl for 1 h, giving approximately 5 ml of wet gel. The gel was sucked dry on a Buckner funnel with a filter of pore size 0.2  $\mu\text{m}$ , and washed twice with 100 ml of PBS. 50 mg of parathion-BSA was dissolved in 10 ml of coupling buffer (0.1 M  $\text{NaHCO}_3$ , pH 8.3, with 0.5 M NaCl). The coupling solution was then added dropwise to the gel, under gentle rotation, and the mixture rotated overnight at 4°C. The gel was then washed successively with 50 ml each of, 0.1 M sodium bicarbonate, pH 8.5, PBS and PBS containing 1.0 M NaCl to remove any non-chemically bound parathion-BSA. The gel was then mixed with 10 ml of 1.0 M ethanolamine for 2 h under gentle rotation to block the excess active amine groups. The gel was then washed with 0.1 M acetate buffer, pH 4.0, containing 0.5 M NaCl and with coupling buffer, and stored in PBS with azide (0.05%, v/v) at 4°C.



## **2 3 5 Antibody production**

### ***2 3 5 1 Licensing for drug and animal use***

The possession and use of morphine-3-glucuronide for analytical purposes is licensed by the Department of Health. All processes involving the use of live animals is also licensed. Extreme care was taken to minimise stress to the animals involved.

### ***2 3 5 2 Immunisation procedure for the production of rabbit antiserum to morphine-3-glucuronide and parathion***

A 1 mg/ml solution of conjugate was prepared (M3G-BSA, parathion-OVA) in PBS and added to an equal volume of Freund's Complete adjuvant. This solution was then vortexed until an emulsion was present. New Zealand White female rabbits were injected subcutaneously at a number of sites with 1 ml of the emulsion. The immunisation was repeated 21 days later with Freund's Incomplete adjuvant. Bleeds were taken from the marginal ear vein of the animals between 10 and 15 days after immunisation, and the cycle of reboosting and bleeding procedure was repeated until an adequate titre of no less than 1/200,000 was obtained. Once a sufficient titre was obtained the animals were sacrificed with an overdose of anaesthetic and the serum removed as described in section 2 3 6 1.

### ***2 3 5 3 Immunisation procedure of BALB/c mice for the production of single chain Fv antibodies to morphine-3-glucuronide***

A 1 mg/ml solution of M3G-BSA was prepared in PBS and added to an equal volume of Freund's Complete adjuvant. This solution was vortexed until an emulsion was present. 5-10 week old BALB/c mice were immunised subcutaneously with a total volume of 250  $\mu$ l over several sites. Further intraperitoneal immunisations were carried out after 21 days using Freund's Incomplete adjuvant. The mice were bled 7 days later and the antibody titre against the specific drug determined. The reboosting

and bleeding procedure was repeated until an adequate titre of no less than 1/1,000 was obtained. The animals were then immunised intravenously through the tail vein with 250  $\mu$ l of 1 mg/ml M3G-BSA in PBS 3-4 days prior to sacrifice. This was followed by sacrifice by cervical dislocation and removal of the spleen.

## **2 3 6 Preparation of serum**

### **2 3 6 1 *Preparation of rabbit serum***

1 ml blood samples from the marginal ear vein were collected for estimation of specific antibody titre in rabbit serum. Blood was allowed to clot for 2 h at room temperature, followed by overnight at 4°C, thus allowing the clot to tighten. It was then centrifuged at 4,000 rpm for 20 min and serum removed and stored at -20°C. For collection of polyclonal antisera, the rabbit was anaesthetised and sacrificed by cardiac puncture and blood removed from the heart with a syringe. The whole blood was treated as described above.

### **2 3 6 2 *Preparation of mouse serum for estimation of titre***

10-20 µl of blood was removed from the tail of the mouse for estimation of specific antibody titre in the sample of blood. Blood was allowed to clot at room temperature for 30 min, followed by centrifugation at 14,000 rpm for 2 min. The serum was removed and stored at -20°C until further analysis.

## **2 3 7 Antibody purification and characterisation**

### **2 3 7 1 *Purification of polyclonal antibody from whole serum***

Purification of polyclonal antibody from rabbit serum was initially carried out by precipitation with saturated ammonium sulphate. This was followed by affinity purification using a protein G column or immunoaffinity matrices consisting of a parathion-BSA sepharose column.

### **2 3 7 2 *Saturated ammonium sulphate precipitation***

The ammonium sulphate precipitation of antibody from rabbit serum was carried out according to the method of Hudson & Hay, (1980). Saturated ammonium sulphate (SAS) was prepared by dissolving 500 g of ammonium sulphate in 500 ml distilled water at 50°C, leaving the solution overnight at 4°C and adjusting the pH to 7.2. 10 ml of cold saturated ammonium sulphate (100%, w/v) was added dropwise to an equal volume of rabbit serum on ice with stirring. The mixture was allowed to stir at room temperature for 30 min, followed by centrifugation at 3,000 rpm for 20 min. The supernatant was discarded and the precipitate washed twice in 10 ml of 45% (w/v) ammonium sulphate. The washed pellet was dissolved in 5 ml of PBS and dialysed in 5 L of PBS overnight at 4°C to remove residual ammonium sulphate.

### **2 3 7 3 *Protein G affinity chromatography***

A 2 ml protein G-sepharose column was poured and equilibrated with 50 ml of PBS, pH 7.4, containing 0.3 M NaCl (running buffer). 2 ml of the dialysed ammonium sulphate rabbit immunoglobulin fraction was applied to the column and the flow rate adjusted to 1 ml/min. The sample was then re-applied to the column twice followed by washing the column with 20 ml of PBS, pH 7.4, containing 0.5 M NaCl and 0.05% (v/v) Tween (wash buffer). Affinity captured-antibody was eluted with 0.1 M glycine/HCl, pH 2.2. 1 ml fractions were collected and immediately neutralised to

pH 7.0 by addition of 100  $\mu$ l of 1.5 M Tris/HCl, pH 8.7, to each fraction, in order to prevent denaturation of antibody. The optical density of each fraction was recorded at 280 nm, and those fractions containing protein were pooled and dialysed overnight in PBS at 4°C, and sodium azide was added to 0.05% (v/v). The sample was aliquotted (0.5 ml fractions) and stored at -20°C.

#### ***2.3.7.4 Affinity purification of polyclonal anti-parathion antibodies using a parathion-BSA sepharose column***

Purification of the anti-parathion polyclonal antibody was carried out as described in section 2.3.7.3, except that a 5 ml parathion-BSA sepharose column was used.

#### ***2.3.7.5 Bicinchoninic acid (BCA) protein assay***

Standard protein solutions with concentrations between 0-2 mg/ml were prepared in PBS using either bovine serum albumin (BSA) or for immunoglobulin concentration determination IgG. 10  $\mu$ l of protein sample or standard was placed in wells of a 96 well microtitre plate. 190  $\mu$ l of BCA working reagent (50 parts reagent A to 1 part reagent B) was added. The plate was incubated at 37°C for 30 min after gentle mixing. The absorbance of the wells was determined at 562 nm on a Titretrek Twinreader Plus plate reader. A standard curve of known protein concentration versus absorbance was constructed from which unknown sample protein concentrations were determined.

#### ***2.3.7.6 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)***

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed to assess antibody purity. The compositions of gels, electrophoresis buffer and sample buffer are shown in Table 2.1. Samples and protein markers were prepared in sample loading buffer (4.1, sample buffer) and boiled for 5 min. Samples were run at 50 mA per gel on an Atto AE-6450 minigel gel until samples had reached

the base of the stacking gel, and then at 20 mA per gel until the dye had reached the base of the resolving gel. Coomassie blue was used to stain the gel or the gel was used for western blotting.

### **2.3.7.7. Coomassie blue staining for SDS-PAGE gel**

Coomassie blue staining solution (0.2% (w/v) Coomassie blue R250 in 30:10:60 (v/v/v) methanol:acetic acid:water) was prepared and gels were stained for 30 min and destained overnight at 4°C (in destaining solution 10:7:5:3 (v/v/v) methanol:acetic acid:water).

### **2.3.7.8. Western blotting of scFv antibodies**

Proteins were transferred from acrylamide gels to nitrocellulose using a BioRad wet blotter for 90 minutes at 72 V, in electrophoresis buffer containing 20% (v/v) methanol. Blocking of the membrane was carried out with blocking solution (5% (w/v) non-fat milk powder, 0.01% (v/v) antifoam A, 0.02% (v/v) sodium azide in Tris Buffered Saline (TBS) (50 mM Tris-HCl, 150 mM NaCl, pH 7.4) for 2 h at room temperature or overnight at 4°C. Monoclonal anti-FLAG antibody (1/500 dilution) was added in blocking solution containing 1 mM calcium chloride (CaCl<sub>2</sub>) for 1.5 h at room temperature. This was followed by washing the nitrocellulose three times in TBS containing 1 mM CaCl<sub>2</sub> for 10 min each time. Alkaline phosphatase-labelled anti-mouse IgG prepared in 5% (w/v) fat free milk powder in TBS containing 1 mM calcium chloride was added to the nitrocellulose filter for 1.5 h shaking at room temperature. Final washing consisting of four 10 min washings in TBS containing 1 mM CaCl<sub>2</sub>. The western blot was developed with one-step 5-bromo-4-chloro-3'-indolyphosphate/nitro blue tetrazolium chloride (BCIP/NBT) reagent (Promega). Colour development was stopped by the addition of 2 mM EDTA in PBS.

**Table 2 1** Composition of stacking gel, resolving gel, electrophoresis buffer and sample loading buffer for SDS-PAGE

Stacking gel	<p>5% (w/v) acrylamide  0 13% (w/v) bis-acrylamide  125 mM Tris (pH 6 8)  0 1% (w/v) SDS  0 15% (w/v) ammonium persulphate  0 25% (v/v) TEMED</p>
Resolving gel	<p>10% (w/v) acrylamide  0 27% (w/v) bis-acrylamide  375 mM Tris (pH 6 8)  0 1% (w/v) SDS  0 08% (w/v) ammonium persulphate  0 08% (v/v) TEMED</p>
Electrophoresis buffer	<p>25 mM Tris (pH 8 8)  192 mM glycine  0 1% (w/v) SDS</p>
Sample loading buffer	<p>60 mM Tris (pH 6 8)  25% (v/v) glycerol  2% (w/v) SDS  14 4 mM 2-mercaptoethanol  0 1% (w/v) bromophenol blue</p>

### **2 3 7 9 *Agarose gel electrophoresis***

Samples from 10 to 20  $\mu\text{l}$  were prepared in 6 X loading buffer (0.25% (w/v) bromophenol blue, 40% (w/v) sucrose). Samples were loaded into the wells of agarose gels, typically between 0.8 and 1.2%. Gels were prepared using TAE and Type 1-A agarose. Ethidium bromide was added to gels at 0.5  $\mu\text{g/ml}$ . Samples were electrophoresed at 80 V per gel for 2 h.



## **2 3 8 General enzyme immunoassay protocol using polyclonal antibodies**

### **2 3 8 1 *Non-competitive ELISA for determination of polyclonal antibody titre and optimal concentration of protein conjugates***

Ranges of protein drug conjugates of appropriate type (M3G-THY, M3G-OVA, parathion-BSA, parathion-OVA, cephalixin-BSA) were coated onto microtitre (Nunc) plates and incubated overnight at 4°C. Plates were washed three times with PBS containing 0.05% (v/v) Tween (PBST) and three times with PBS. The plates were then blocked with 2% (w/v) marvel in PBS for 1 h at 37°C. Serial dilutions of polyclonal antibody in PBS were added to the wells of each conjugate concentration on the plate and allowed to bind at 37°C for 1 h. This was followed by addition of a 1 in 5000 dilution in PBS of HRP-labelled goat anti-rabbit antibody and incubation for 1 h at 37°C. Plates were again washed and chromogenic substrate (0.4 mg/ml *o*-phenylenediamine (*o*-PD), in 0.05 M phosphate citrate buffer, pH 5.0, and 0.4 mg/ml of urea hydrogen peroxidase) was added and incubated for 30 min at 37°C. Absorbance was read at 492 nm.

### **2 3 8 2 *Competitive ELISA for polyclonal antibody***

Competitive immunoassay was performed using this polyclonal antibody as described in section 2.3.8.1. However, standards of free analyte were mixed with antibody and added to the wells following coating and blocking.

## **2 3 9 Production of murine scFv antibody libraries to morphine-3-glucuronide**

### **2 3 9 1 *Isolation of total RNA from the spleen of BALB/c mice***

The spleen was removed using aseptic technique. The weight of the spleen was recorded and 1 ml of Trizol Reagent was added per 40 mg of spleen present. This solution was then homogenised and incubated at room temperature for 5 min. The mixture was centrifuged at 14,000 rpm for 15 min. The upper aqueous phase (represents approx 40% of total volume) was removed and precipitation of the RNA was carried out by adding 0.5 ml isopropanol/ml of trizol. The solution was incubated for 10 min at room temperature, followed by centrifugation at 14,000 rpm for 10 min. Removal of excess salt from the mixture is essential as this will interfere with cDNA synthesis and PCR. The mixture was washed with 1 ml of EtOH/ml of trizol reagent. The pellet was dislodged by inverting the eppendorf tubes several times. Following centrifugation at 10,000 rpm for 5 min, the EtOH was removed and the pellet allowed to dry at 37°C for 10 min. The final pellet was dissolved in 30 µl of ultrapure water at 4°C overnight.

### **2 3 9 2 *Reverse transcription of mouse spleen mRNA***

Reverse transcription was carried out by using a Promega PCR-related Reverse Transcription System. cDNA was synthesised by using random hexamer primers (Promega). A mixture of MgCl<sub>2</sub>, 10X buffer, dNTP (dATP, dCTP, dGTP, dTTP mix) and ultrapure water was prepared. The required volume of enzyme was added followed by the appropriate concentration of mRNA. The reaction was incubated at room temperature for 10 min, 42°C for 2 h and then finally stored at -20°C.

**2 3 9 2 1. Reverse transcription components for cDNA synthesis**

<b><u>Component</u></b>	<b><u>Stock concentration</u></b>	<b><u>Concentration in 20µl</u></b>
MgCl <sub>2</sub>	25 mM	5 mM
10X Buffer	10 X	1 X
dNTP mix	10 mM	1 mM
RNase Inhibitor	40 U/µl	1 U/rxn
Random Primer	0.5 µg/µl	0.2 µg/rxn
AMV RT	25 U/µl	15 U/rxn
Ultrapure water		to 20 µl including RNA
RNA		7-10 µg/rxn

### 2 3 9 3 *Amplification of antibody light and heavy chain genes using polymerase chain reaction*

#### 2 3 9 3 1 *PCR Primers*

The listed PCR primers were obtained from Sigma-Genosys Ltd. The primers were used for assembling mouse scFv fragments which are compatible with the pAK vector system as performed by Krebber *et al*, 1997

#### Variable light chain back primers

- LB1 5'gccatggcggactacaaaGAYATCCAGCTGACTCAGCC3'
- LB2 5'gccatggcggactacaaaGAYATTGTTCTCWCCCAGTC3'
- LB3 5'gccatggcggactacaaaGAYATTGTGMTMACTCAGTC3'
- LB4 5'gccatggcggactacaaaGAYATTGTGYTRACACAGTC3'
- LB5 5'gccatggcggactacaaaGAYATTGTRATGACMCAGTC3'
- LB6 5'gccatggcggactacaaaGAYATTMAGATRAMCCAGTC3'
- LB7 5'gccatggcggactacaaaGAYATTCAGATGAYDCAGTC3'
- LB8 5'gccatggcggactacaaaGAYATYCAGATGACACAGAC3'
- LB9 5'gccatggcggactacaaaGAYATTGTTCTCAWCCAGTC3'
- LB10 5'gccatggcggactacaaaGAYATTGWGCTSAACCAATC3'
- LB11 5'gccatggcggactacaaaGAYATTSTRATGACCCARTC3'
- LB12 5'gccatggcggactacaaaGAYRTTKTGATGACCCARAC3'
- LB13 5'gccatggcggactacaaaGAYATTGTGATGACBCAGKC3'
- LB14 5'gccatggcggactacaaaGAYATTGTGATAACYCAGGA3'
- LB15 5'gccatggcggactacaaaGAYATTGTGATGACCCAGWT3'
- LB16 5'gccatggcggactacaaaGAYATTGTGATGACACAACC3'
- LB17 5'gccatggcggactacaaaGAYATTTTGCTGACTCAGTC3'
- LBλ 5'gccatggcggactacaaaGATGCTGTTGTGACTCAGGAATC3'

### Variable light chain primers for

- LF1 5'ggagccgccgcc(agaaccaccacc)<sub>2</sub>ACGTTTGATTTCAGCTTGG3'  
LF2 5'ggagccgccgcc(agaaccaccacc)<sub>2</sub>ACGTTTTATTTCAGCTTGG3'  
LF4 5'ggagccgccgcc(agaaccaccacc)<sub>2</sub>ACGTTTTATTCCAACCTTGG3'  
LF5 5'ggagccgccgcc(agaaccaccacc)<sub>2</sub>ACGTTTCAGCTCCAGCTTGG3'  
LFλ 5'ggagccgccgcc(agaaccaccacc)<sub>2</sub>ACCTAGGACAGTCAGTTTGG3'

### Variable heavy chain back primers

- HB1 5'ggcggcggcggctccggtggtggtgatccGAKGTRMAGCTTCAGGAGTTC3'  
HB2 5'ggcggcggcggctccggtggtggtgatccGAGGTBCAGCTBCAGCAGTC3'  
HB3 5'ggcggcggcggctccggtggtggtgatccCAGGTGCAGCTGAAGSASTC3'  
HB4 5'ggcggcggcggctccggtggtggtgatccGAGGTCCARCTGCAACARTC3'  
HB5 5'ggcggcggcggctccggtggtggtgatccCAGGTYCAGCTBCAGCARTC3'  
HB6 5'ggcggcggcggctccggtggtggtgatccCAGGTYCARCTGCAGCAGTC3'  
HB7 5'ggcggcggcggctccggtggtggtgatccCAGGTCCACGTGAAGCAGTC3'  
HB8 5'ggcggcggcggctccggtggtggtgatccGAGGTGAASSTGGTGGAAATC3'  
HB9 5'ggcggcggcggctccggtggtggtgatccGAVGTGAWGYTGGTGGAGTC3'  
HB10 5'ggcggcggcggctccggtggtggtgatccGAGGTGCAGSKGGTGGAGTC3'  
HB11 5'ggcggcggcggctccggtggtggtgatccGAKGTGCAMCTGGTGGAGTC3'  
HB12 5'ggcggcggcggctccggtggtggtgatccGAGGTGAAGCTGATGGARTC3'  
HB13 5'ggcggcggcggctccggtggtggtgatccGAGGTGCARCTTGTTGAGTC3'  
HB14 5'ggcggcggcggctccggtggtggtgatccGARGTRAAGCTTCTCGAGTC3'  
HB15 5'ggcggcggcggctccggtggtggtgatccGAAGTGAARSTTGAGGAGTC3'  
HB16 5'ggcggcggcggctccggtggtggtgatccCAGGTTACTCTRAAAGWGTSTG3'  
HB17 5'ggcggcggcggctccggtggtggtgatccCAGGTCCAACCTYCAGCARCC3'  
HB18 5'ggcggcggcggctccggtggtggtgatccGATGTGAACTTGGAAGTGTC3'  
HB19 5'ggcggcggcggctccggtggtggtgatccGAGGTGAAGGTCATCGAGTC3'

**Variable heavy chain for primers**

HF1 5'ggaattcggcccccgagggcCGAGGAAACGGTGACCGTGGT3'

HF2 5'ggaattcggcccccgagggcCGAGGAGACTGTGAGAGTGGT3'

HF3 5'ggaattcggcccccgagggcCGCAGAGACAGTGACCAGAGT3'

HF4 5'ggaattcggcccccgagggcCGAGGAGACGGTGACTGAGGT3'

### **2 3 9 3 2** *Components of PCR reaction for amplification of antibody light and heavy chain genes*

The concentration of stock primers varied. However, 0.1 nmol concentration of the forward and reverse primers were used per reaction.

<u>Component</u>	<u>Stock concentration</u>	<u>Concentration in 50 <math>\mu</math>l</u>
Forward Primers	varied	0.1 nmol each primer/rxn
Reverse Primers	varied	0.1 nmol each primer/rxn
10X Buffer	10 X	1 X
MgCl <sub>2</sub>	25 mM	1.0 mM
dNTP	20 mM	0.4 mM
Ultrapure water		to 50 $\mu$ l including <i>Taq</i> and DNA
cDNA		6-8 $\mu$ g of cDNA per 50 $\mu$ l rxn
<i>Taq</i> polymerase	5 U/ $\mu$ l	5 U/rxn (multiplex)

### **2 3 9 3 3** *PCR conditions for amplification of antibody light and heavy chain genes*

Typical thermal cycling conditions were as follows:

94°C X 5 min 'Hot-Start'

94°C X 1 min

63°C X 30 sec

58°C X 50 sec

72°C X 1 min

repeat X 7 cycles

94°C X 1 min

63°C X 30 sec

72°C X 1 min

repeat X 24 cycles

72°C X 10 min

All ramping rates were at 4°C/sec

#### **2 3 9 4 *Purification of PCR reaction products***

PCR purification was performed using the Wizard PCR prep DNA purification kit (Promega). An Atto AE-6100 gel electrophoresis system was used. The PCR product was run on a 0.5% (w/v) low melt agarose gel containing 0.5 µg/ml ethidium bromide for 2 h at 50 V until the 400 base pair (bp) fragment was separated from the unincorporated primer. The 400 bp fragment was cut from the gel with a sterile scalpel and the DNA purified from the low melt agarose as follows. The sample was incubated at 70°C in a waterbath/heating block causing the agarose to melt within 5 min. 1 ml of resin was immediately added and mixed thoroughly by pipetting for 15 sec. For each PCR preparation a plunger from a 5 ml syringe was attached to a Wizard Minicolumn and the resin was slowly pushed through. The column was then washed with 2 ml of 80% (v/v) isopropanol. The column was centrifuged for 2 min at 14,000 rpm to remove any residual buffers. The minicolumn was placed in a new 1.5 ml eppendorf tube and 20 µl of autoclaved ultrapure water was applied to the minicolumn and allowed to incubate for 1 min. The column was centrifuged at 14,000 rpm for 20 sec to elute the DNA fragment and stored at -20°C until required.

#### **2 3 9 5 *Quantification of purified PCR products***

After purification the fragments were re-electrophoresed on an agarose gel with a quantitative Molecular Weight Marker (Promega, 100 bp Molecular Weight Markers), as the concentration must be determined prior to splice by overlap extension PCR (SOE-PCR). The 500 bp fragment of this marker was used as a



reference for densitometric quantitation as 5 µl contains 150 ng of the 500 bp fragment and 50 ng of the other molecular weight fragments

### **2 3 9 6 Components of Splice by overlap extension (SOE) PCR**

Gel purified light and heavy chains were joined and amplified together to produce a 800 bp fragment using SOE PCR

#### **2 3 9 6 1 PCR Primers**

##### **SOE Primers**

Single chain back 5'ttactcgcggcccagccggccatggcggactaccccG3'

Single chain forward 5'ggaattcggcccccgag3'

#### **2 3 9 6 2 SOE PCR components for amplification of joined antibody light and heavy chain genes**

<b><u>Component</u></b>	<b><u>Stock concentration</u></b>	<b><u>Concentration in 50 µl</u></b>
10 X buffer	10 X	1 X
MgCl <sub>2</sub>	25 mM	1 0 mM
dNTP	20 mM	0 4 mM
Ultrapure water	to 50 µl including <i>Taq</i> , DNA and scfor, scback	
VH	as detemined	10 ng/rxn
VL	as detemined	10 ng/rxn
scfor	varied	0 05 nmol /rxn
scback	varied	0 05 nmol/rxn
<i>Taq</i> polymerase	5 U/µl	5 U/rxn

**2 3 9 6 3 SOE PCR conditions for amplification of joined antibody light and heavy chain genes**

92°C X 1 min

45°C X 50 min

72°C X 1 min

repeat X 5 cycles

Add 0.05 mM scfor and scback primers

5 U *Taq* Polymerase/rxn

92°C X 1 min

68°C X 30 sec

72°C X 1 min

repeat X 25 cycles

All ramping rates were at 4°C/sec

**2 3 9 7 Preparation and purification of pAK100 vector using Wizard miniprep system**

A single colony of *E coli* XL1-Blue containing pAK100 (all vectors were kindly donated by Andreas Pluckthun, University of Zurich, Switzerland) was picked off an agarose plate and grown overnight at 37°C with vigorous shaking in 5 ml of 2 x TY supplemented with 30 µg/ml tetracycline. Purification of the plasmid was carried out using the Wizard miniprep system as per the manufacturer's instructions. The culture was centrifuged at 4°C at 7,000 rpm for 5 min. The supernatant was discarded and the cells resuspended in 250 µl of cell resuspension solution (50 mM Tris-HCl, pH 7.5, 10 mM EDTA, 100 µg/ml RNase A). 250 µl of cell lysis solution (0.2 M NaOH, 1% (w/v) SDS) was added and the mixture inverted three times. A clear lysate was produced from this procedure. 10 µl of alkaline phosphatase was added and the mixture inverted four times. The mixture was then incubated at room temperature for

5 min 350 µl of neutralisation solution (4.09 M guanidine hydrochloride, 0.759 M potassium acetate, 2.12 M glacial acetic acid, pH 4.2) was added, followed by inverting the mixture four times and centrifuging at 14,000 rpm for 10 min. The clear lysate was transferred to a spin column and centrifuged at 14,000 rpm for 1 min at room temperature. Supernatant from the column was discarded and the column was washed with 750 µl of column wash solution (6 mM potassium acetate, 10 mM Tris-HCl, pH 7.5, 60% (v/v) ethanol) by centrifuging at 14,000 rpm for 1 min at room temperature. The column wash step was repeated using 250 µl of column wash solution and centrifuging at 14,000 rpm for 2 min. 50 µl of autoclaved ultrapure water was placed in the column and allowed to incubate for 1 min. Elution of the column was carried out by centrifugation at room temperature at 14,000 rpm for 1 min. The purified vector was stored at -20°C in ultrapure water.

### ***2.3.9.8 Digestion of pAK100 and antibody light and heavy chain genes SOE PCR products***

Digestion of pAK100 and SOE products was performed using *SfiI* restriction enzyme. pAK100 requires digestion with *SfiI* restriction enzyme prior to ligation with light and heavy chain genes. Antibody light and heavy chain gene SOE products are also digested with *SfiI* as 4 bp are spliced off from either end.

#### ***2.3.9.8.1. Components for digestion of pAK100 and antibody light and heavy chain genes SOE PCR products***

1.5 µl reaction buffer

0.2 µl BSA

2 U *SfiI*

30-50 ng/rxn VH/VL or 50-100 ng/rxn pAK100

Sterile ultrapure water was added to a final volume of 10 µl

The mixture was incubated at 50°C overnight. Purification of fragments was carried out using a Promega purification kit as described previously in section 2.3.9.4.

### **2.3.9.9 Ligation of antibody light and heavy chain genes into the pAK100 vector**

Gel purified VL/VH genes were ligated into the pAK100 plasmid vector using the following conditions:

60 ng pAK100 vector

40 ng of gel purified SOE light and heavy chain genes

10 U ligase (Promega)

2 µl of 5X reaction buffer

Sterile ultrapure water was added to give a final volume of 10 µl

The concentrations of SOE VL/VH and pAK100 digests were added at a ratio of 1:5:1. The reaction mixture was incubated at 15°C overnight and then transformed into *E. coli* XL1-Blue supercompetent cells.

### **2.3.9.10 Transformation of *E. coli* XL1-Blue supercompetent cells with pAK100 vector containing light and heavy chain genes and measurement of transformation efficiencies**

Supercompetent *E. coli* XL1-Blue (Stratagene) cells were thawed gently on ice. Cells were gently mixed by hand and aliquotted into two 20 ml sterile tubes (one tube was for the experimental transformation and the other tube was for the control transformation). 17 µl of β-mercaptoethanol was added to each of the two aliquots of bacteria, giving a final concentration of 25 mM in each tube. The contents of the tubes were stirred gently and incubated on ice for 10 min, swirling gently at 2 min intervals. 0.1-50 ng of vector, containing light and heavy chain genes was added to one of the tubes and swirled gently. 1 µl of pUC18 control vector was added to the second tube and also swirled. Both tubes were then incubated on ice for 30 min.

Cells were heat-pulsed for 45 sec at 42°C followed by incubation on ice for 2 min. 0.9 ml of SOC medium, preincubated at 37°C, was then added and the tubes incubated at 37°C for 1 h with shaking at 225-250 rpm. pAk100 transformants were plated out on LB agar supplemented with 25 µg/ml chloramphenicol and 1% (v/v) glucose. Control transformants were plated out on LB agar supplemented with 20 µg/ml of ampicillin. Both sets of plates were allowed to grow overnight at 37°C. pAK100 transformed colonies were scraped off the plates and used as library stocks. These stocks were suspended in 15% (v/v) glycerol and stored at -80°C.

## **2 3 10 Production of recombinant scFv antibodies to morphine-3-glucuronide**

### **2 3 10 1 Preparation of phagemid particles**

50 ml of NE medium was inoculated with  $10^9$  cells from the library glycerol stocks. The culture was grown in a shaking incubator at 250 rpm at 37°C until the optical density at 550 had reached 0.5 AU.  $10^{11}$  VCSM13 helper phage (Stratagene) and 25 µl of 1 M IPTG (isopropyl-β-D-thiogalactopyranoside) were added and after incubation at 37°C for 15 min without agitation, the culture was diluted with 100 ml of LE medium. The culture was shaken (250 rpm) for 2 h at 26°C followed by addition of 30 µg/ml of kanamycin. The culture was then allowed to continue shaking at 26°C for a further 8 h. Phage particle precipitation was then carried out using PEG/NaCl.

### **2 3 10 2 PEG/NaCl precipitation of recombinant scFv antibodies**

150 ml of the *E coli* XL1-Blue culture containing phage particles was transferred to a Sorvall RC-5B centrifuge tube and centrifuged at 10,000 rpm for 10 min at 4°C. The supernatant was collected and one fifth the volume of PEG/NaCl (20% (w/v) polyethylene glycol 6000, 2.5 M NaCl) was added, followed by incubation for 1 h at 4°C. The culture was centrifuged at 10,000 rpm for 30 min and the pellet resuspended in 40 ml of 4% (w/v) PEG/NaCl (4% (w/v) polyethylene glycol 6000, 2.5 M NaCl). Following mixing, it was incubated at 4°C for 20 min. After incubation, the mixture was centrifuged at 10,000 rpm for 20 min. The supernatant was removed and the pellet re-centrifuged at 7,000 rpm for 10 min at 4°C to allow removal of any remaining supernatant. The pellet was resuspended in 2 ml of sterile PBS. This was followed by micro-centrifugation at 4,000 rpm for 10 min at 4°C to remove any bacterial debris. The phage supernatant was collected and stored (short term at 4°C, long term at -80°C in 15% (v/v) glycerol solution).

### **2 3 10 3 *Titre of phage particles***

4 ml of 2 x TY supplemented with 30 µg/ml tetracycline was inoculated with a single colony from an *E coli* XL1-Blue stock plate and grown overnight at 37°C. The culture was then diluted 1/100 in 100 ml of 2 x TY supplemented with 30 µg/ml tetracycline, and allowed to grow at 37°C with shaking (250 rpm) until the optical density at 550 had reached 0.5 AU. After incubation for 15 min at 37°C without agitation, serial dilutions of phage particles ( $10^1$ - $10^9$ ) were prepared using the *E coli* XL1-Blue exponentially growing bacterial culture (180 µl *E coli* XL1-Blue with 20 µl phage). The culture was incubated for 30 min at 37°C without agitation. 100 µl of each dilution was plated on TYE agar containing 25 µg/ml chloramphenicol and 1% (v/v) glucose. Colonies were allowed to grow overnight at 37°C. The colony forming units were counted in order to calculate the titre of the phage particles.

### **2 3 10 4 *Affinity selection of scFv antibodies by panning***

An immunotube (NUNC) was coated with 100 µg/ml of conjugate in PBS and incubated overnight at 4°C. The tube was washed five times with PBS containing 0.05% (v/v) Tween (PBST) followed by five washes with PBS. 5% (w/v in PBS) marvel (blocking solution) was added to the immunotube and allowed to incubate for 1 h at 37°C. After washing the immunotube (as before), 4 ml of PBS containing 2% (w/v) marvel and  $10^{11}$  of phagemid particles was added and incubated for 2 h at room temperature with gentle shaking. This was followed by washing the immunotube to remove any non-specifically bound phage. Specifically bound phage particles were eluted from the immunotube by addition of 800 µl of 0.1 M glycine/HCl, pH 2.2, for 10 min with shaking. Eluted phage particles were then neutralised using 48 µl of 2 M Tris. Determining the titre of this phage was carried out as described in section 2 3 10 3.

### **2 3 10 5 *Preparation of library stocks from panning***

4 ml of 2 x TY supplemented with 30 µg/ml tetracycline was inoculated with a single colony from an *E coli* XL1-Blue stock plate and grown overnight at 37°C. The culture was then diluted 1/100 in 2 x TY supplemented with 30 µg/ml tetracycline, and allowed to grow at 37°C with shaking (250 rpm) until the optical density at 550 had reached 0.5 AU. After allowing the culture to incubate without agitation for 15 min at 37°C, 1-2 ml of eluted phage from panning was then re-infected into this *E coli* XL1-Blue culture. This culture was again incubated for 15 min at 37°C without agitation, followed by centrifugation at 4,000 rpm for 10 min at room temperature. The supernatant was discarded and the pellet resuspended in 500 µl of 2 x TY medium. The culture was plated on NE medium and grown overnight at 37°C. This was followed by scraping the colonies off the plates (using a glass spreader) and resuspending in 2 x TY medium. The culture was centrifuged at 4,000 rpm for 10 min, and the pellet resuspended in 3 ml of 2 x TY medium containing 15% (v/v) glycerol. This stock was then stored at -80°C until required for further use.

### **2 3 10 6 *Preparation of clones for phage ELISA***

Single colonies were selected from plates and used to inoculate individual wells of a 96 well cell culture plate containing 200 µl 2 x TY containing 1% (v/v) glucose, 25 µg/ml chloramphenicol and 30 µg/ml tetracycline. The plate was shaken at 150 rpm overnight at 37°C and used as a master plate to inoculate an identical plate. 30 µl of 60% (v/v) glycerol was added to each well of the master plate and this was stored at -20°C. The second plate was grown at 37°C for 6-8 h with shaking at 150 rpm in 200 µl 2 x TY containing 1% (v/v) glucose, 25 µg/ml chloramphenicol and 30 µg/ml tetracycline. After allowing the plate to incubate for 15 min at 37°C without agitation, 25 µl of 2 x TY containing 1% (v/v) glucose, 25 µg/ml chloramphenicol, 1.5 mM IPTG and  $5 \times 10^9$  VCSM13 helper phage per ml was added to each well, and incubated at 37°C for 15 min followed by shaking at 150 rpm for 2 h at 26°C. Plates



were then centrifuged at 4,000 rpm for 10 min. The supernatant was discarded, and the pellets resuspended in 200  $\mu$ l 2 x TY containing 1% (v/v) glucose, 25  $\mu$ g/ml chloramphenicol, 1.5 mM IPTG and 30  $\mu$ g/ml kanamycin. The plate was incubated overnight at 26°C and centrifuged at 4,000 rpm for 10 min. 75  $\mu$ l of supernatant was used for analysis of phage by ELISA.

### **2.3.10.7 Phage ELISA**

A microtitre plate (Nunc) was coated with 100  $\mu$ l of 100  $\mu$ g/ml of M3G-THY or M3G-OVA and incubated overnight at 4°C. Plates were washed three times with PBST, three times with PBS, and blocked with 100  $\mu$ l of 2% (w/v) marvel in PBS for 1 h at 37°C. 75  $\mu$ l of supernatant and 25  $\mu$ l of 4% (w/v) marvel in PBS were added to each well for 2 h at room temperature. The plates were re-washed as before, and a 1 in 500 dilution of anti-M13 rabbit antibody was added followed by incubation for 1 h at 37°C. This was followed by addition of a 1 in 5000 dilution of HRP-labelled goat anti-rabbit antibody and incubation for 1 h at 37°C. Following washing, substrate was added as described in section 2.3.8.1 and incubated for 30 min at 37°C. Substrate reactions were stopped by addition of 25  $\mu$ l of 2 M H<sub>2</sub>SO<sub>4</sub> per well. Absorbance was read at 492 nm.

### **2.3.10.8 Preparation of phage particles for non-competitive and competitive phage ELISA**

5 ml of NE medium was inoculated with 5  $\mu$ l of positive clone from the master plate. This culture was allowed to grow overnight at 37°C in an incubator at 250 rpm. The culture was then diluted 1/100 in 5 ml of NE medium, and allowed to grow at 37°C with shaking (250 rpm) until the optical density at 550 nm had reached 0.5 AU. 2.5 ml of NE medium supplemented with  $5 \times 10^9$  VCSM13 helper phage and 1.5 mM IPTG was added and the culture incubated at 26°C overnight. The culture was then centrifuged at 4,000 rpm for 10 min and the supernatant removed.

### **2 3 10 8 1 *Non-competitive phage ELISA for determination of scFv antibody titre***

Protein drug conjugates of appropriate type (M3G-THY, M3G-OVA) were coated onto a microtitre (Nunc) plate at a concentration of 100 µg/ml and incubated overnight at 4°C. Plates were washed three times with PBST, three times with PBS and blocked with 100 µl of 2% (w/v) marvel in PBS for 1 h at 37°C. Dilutions of phage-scFv antibody in PBS were added to the wells of the plate and allowed to bind at 37°C for 1.5 h. Again the plates were washed as before and a 1 in 500 dilution of anti-M13 rabbit antibody was added, followed by incubation for 1 h at 37°C. This was followed by addition of a 1 in 5000 dilution of HRP-labelled goat anti-rabbit antibody and incubation for 1 h at 37°C. Following washing, substrate was added as described in section 2 3 8 1 and incubated for 30 min at 37°C. Substrate reactions were stopped by addition of 25 µl of 2 M H<sub>2</sub>SO<sub>4</sub> per well. Absorbance was read at 492 nm.

### **2 3 10 8 2 *Competitive phage ELISA for scFv antibody clones***

Competitive immunoassay was performed using culture supernatant as described in section 2 3 10 8 1. However, standards of free analyte were mixed with antibody and added to the wells following coating and blocking.

### **2 3 10 9 *Isolation of light and heavy chain genes from pAK100 vector from positive clones***

Positive clones were identified from the phage ELISA and 10 µl of each clone was taken from the master plate and inoculated into 10 ml of NE medium and grown overnight at 37°C. Glycerol was added to 5 ml of this culture to give a final concentration of 15% (v/v) glycerol and stored at -80°C as stock cultures. The remaining 5 ml containing the pAK100 vector with light and heavy chain genes was purified with the Wizard miniprep system as described in section 2 3 9 7. Purified pAK100 vector was digested with *Sfi*I digestion enzyme as described in section

2 3 9 8, and the product was run on a 0.5% (w/v) low melt agarose gel, containing 0.5 µg/ml ethidium bromide, for 2 h at 50 V until the 800 base pair (bp) fragment containing the light and heavy chain genes is well separated from the digested pAK100 vector. The 800 bp fragment was cut from the gel with a sterile scalpel and the DNA purified from the low melt agarose as described in section 2 3 9 4. The 800 bp fragment containing the light and heavy chain genes was ready for ligation into the pAK400 vector.

### ***2 3 10 10 Ligation of positive light and heavy chain genes into pAK400 for soluble expression of scFv antibodies***

Ligation of antibody light and heavy chain genes into pAK400 was carried out using the same protocol as described in section 2 3 9 9 for ligation with pAK100.

### ***2 3 10 11 Preparation of calcium chloride-competent JM83 bacterial cells***

A single colony of JM83 (kindly donated by the Biotechnology Department technical staff in DCU) was inoculated into a 5 ml culture of NE medium containing 25 µg/ml of streptomycin-sulphate, and grown overnight at 37°C with shaking (250 rpm). The culture was then diluted 1/100 in 100 ml of NE medium containing 25 µg/ml of streptomycin-sulphate, and allowed to grow at 37°C with shaking (250 rpm) until the optical density at 550 nm had reached 0.5 AU. The culture was then cooled on ice for 15 min followed by centrifugation at 2,000 rpm for 20 min at 4°C. Supernatant was decanted and the pellet resuspended in 20 ml of cold 100 mM MgCl<sub>2</sub>. Cells were collected by centrifugation at 2,000 rpm for 20 min at 4°C, and resuspended in 20 ml of cold 50 mM CaCl<sub>2</sub>. The cells were allowed to incubate on ice for 30 min and again centrifuged at 2,000 rpm for 20 min at 4°C. The supernatant was discarded and the pellet resuspended in 2 ml of 50 mM CaCl<sub>2</sub>. The culture was stored at -80°C until further use.

### **2 3 10 12 Transformation of JM83 calcium chloride competent cells with pAK400 vector**

JM83 calcium chloride competent cells were transformed with pAK400 as followed

JM83 culture	200 µl
pAK400	3 µl

The mixture was allowed to sit on ice for 1 h followed by incubation for 2 min at 42°C. The mixture was immediately placed back on ice. 0.7 ml of NE medium was added and incubated for 1 h at 37°C with gentle shaking. The culture was plated on NE agar and grown overnight at 37°C. Single colonies were inoculated in 5 ml cultures of NE medium containing 25 µg/ml chloramphenicol and grown overnight at 37°C, followed by addition of glycerol to give a final concentration of 15% (v/v). This stock was then stored at -80°C until required for further use.

### **2 3 10 13 Soluble expression of scFv in pAK400**

For soluble expression, 20 to 200 ml expression medium (EM) (2 x TY containing 25 µg/ml chloramphenicol) was inoculated with 200 µl to 2 ml of preculture (JM83 harbouring the expression vector for morphine-3-glucuronide), and allowed to grow at 37°C with shaking (250 rpm) until the optical density at 600 nm had reached 0.5 AU. 1 mM IPTG/ml was added and the culture allowed grow for 4 h at 24°C. The culture was centrifuged at 4,000 rpm for 10 min and the supernatant containing soluble scFv antibodies stored at 4°C.

### **2 3 10 14 Concentration of culture supernatant**

100 ml of supernatant containing soluble scFv antibodies from the relevant cell line was collected as described in section 2 3 10 13. Sodium azide was added to a final concentration of 0.05% (w/v) to the supernatants, which were then stored at 4°C until

required. The supernatant was concentrated 10-fold to a final volume of 10 ml, using a stirred ultrafiltration cell with a 76 mm diaflo ultrafilter membrane, which had a molecular weight cut-off of 10,000 daltons. The resulting concentrate was stored at 4°C until required.

### **2.3.10.15 Purification of soluble scFv antibodies using ProBond™ Resin**

A 5 ml ProBond™ Resin column was poured and equilibrated with 50 ml of 20 mM phosphate buffer, pH 7.8, containing 500 mM NaCl (running buffer). 20 ml of concentrated antibody supernatant was added to the column and the flow rate adjusted to 1 ml/min. The sample which ran through was then re-applied to the column three times followed by washing the column with 20 ml of running buffer. Affinity-captured scFv antibody was eluted with running buffer containing 300 mM imidazole. 5 ml fractions were collected. The optical density of each fraction was recorded at 280 nm, and those fractions containing protein were pooled and dialysed overnight at 4°C against 5 L PBS, and sodium azide was added to a final concentration of 0.05% (v/v). The sample was aliquotted (0.5 ml fractions) and stored at -20°C. The ProBond™ Resin column was stripped and recharged using the following procedure:

The column was washed as follows:

Twice with 8 ml 50 mM EDTA to strip away nickel ions,

Once with 16 ml 0.5 M NaOH to denature and dilute protein bound to the column,

Once with 16 ml sterile distilled water.

The column was recharged with 16 ml NiCl<sub>2</sub> (5 mg/ml) and washed with 16 ml distilled water. Finally the charged column was stored in 20% (v/v) ethanol until use.

## **2 3 10 16 General enzyme immunoassay protocol using soluble scFv antibodies**

### **2 3 10 16 1 *Non-competitive ELISA for determination of soluble scFv antibody titre***

Protein drug conjugates of appropriate type (M3G-THY, M3G-OVA) were coated onto a microtitre (Nunc) plate at a concentration of 100 µg/ml and incubated overnight at 4°C. Plates were washed three times with PBST, three times with PBS and blocked with 100 µl of 2% (w/v) marvel in PBS for 1 h at 37°C. Dilutions of soluble scFv antibody in PBS were added to the wells of the plate and allowed to bind at 37°C for 1.5 h. The plates were washed, as before, and a 1 in 400 dilution of anti-FLAG rabbit antibody was added, followed by incubation for 1 h at 37°C. This was followed by addition of a 1 in 5000 dilution of HRP-labelled goat anti-rabbit antibody and incubation for 1 h at 37°C. Following washing, substrate was added as described in section 2.3.8.1 and incubated for 30 min at 37°C. Substrate reactions were stopped by addition of 25 µl of 2 M H<sub>2</sub>SO<sub>4</sub> per well. Absorbance was read at 492 nm.

### **2 3 10 16.2. *Competitive ELISA for soluble scFv antibody clones***

Competitive immunoassay was performed using the supernatant as described in section 2.3.10.16.1. However, standards of free analyte were mixed with antibody and added to the wells following coating and blocking.

## **2 3 11. BIAcore studies**

CM5 research grade sensor chips were used in all experiments. Running buffer for all BIAcore experiments was HBS buffer, pH 7.4, containing 10 mM HEPES, 150 mM NaCl, 3.4 mM EDTA, and 0.05% (v/v) Tween 20. The running buffer was filtered (pore size of 0.22  $\mu\text{m}$ ) and degassed using a millipore filtration apparatus (millipore sintered glass filtration unit) immediately before use.

### **2 3 11 1 *Preconcentration studies***

For all investigations, proteins were immobilised on the sensor surface by means of N-hydroxysuccinimide esterification. The standard conditions used lead to activation of 30-40% of the carboxyl groups on the dextran. Primary amine groups present allow covalent attachment of biomolecules. It is necessary to carry out an initial "preconcentration" step, resulting from electrostatic binding of protonated amine groups on the biological component to negatively-charged carboxyl groups on the chip surface, to take place.

Preconcentration for the native form of a protein can be facilitated by adjusting the pH below the isoelectric point (pI). However, modification via conjugation of drug molecules to proteins often radically alters the pI. Therefore, when dealing with modified proteins, the correct pH for preconcentration has to be determined experimentally. Protein solutions were prepared in 10 mM sodium acetate at a range of different pHs. These solutions were passed over an underivatized chip surface, and the degree of electrostatic binding was monitored. The highest pH at which satisfactory preconcentration was observed was chosen as the pH for immobilisation, since using a buffer below pH 4.0, inhibits the activity of N-hydroxysuccinimide esterification.

### **2 3 11 2 *Immobilisation of interactants***

The carboxymethylated dextran matrix was activated by mixing equal volumes of 100 mM NHS (N-hydroxysuccinimide) and 400 mM EDC (N-ethyl-N-(dimethyl-

aminopropyl) carbodiimide hydrochloride), and injecting the mixture over the sensor chip surface for 7 min at a flowrate of 5  $\mu$ l min<sup>-1</sup>. The conjugate or analyte to be immobilised was diluted in 10 mM sodium acetate at the appropriate pH, and at a typical concentration of 50-200  $\mu$ g/ml. This solution was then injected over the derivatised chip surface for 20 min. Unreacted NHS groups were capped, and non-covalently bound protein removed, by injection of 1 M ethanolamine hydrochloride, pH 8.5, for 7 min.

### **2.3.11.3 *Direct immobilisation of parathion onto chip surface***

Immobilisation of parathion directly onto the chip surface was performed outside the BIAcore instrument. The chip was first allowed to equilibrate to room temperature. The chip was primed for direct immobilisation by addition of 40  $\mu$ l of HBS buffer to the sensor chip well and allowing the chip to incubate for 5 min at room temperature. This solution was then removed using lint-free adsorbent paper, taking great care to ensure the paper did not touch the dextran matrix. Equal volumes of 0.4 M EDC and 0.1 M NHS were then mixed, and 40  $\mu$ l of the resulting solution, containing 0.05 M NHS and 0.2 M EDC in ultrapure water, was added to the chip well and incubated for 15 min at room temperature. The solution of EDC/NHS was then removed using adsorbent paper and parathion, at a concentration of 100  $\mu$ g/ml in 10 mM sodium acetate buffer, pH 5.5, was added to the chip well and allowed to incubate for 20 min. The chip was then blocked by adding 40  $\mu$ l of 1.0 M ethanolamine, pH 8.5, to the chip well and incubating for 20 min. The chip was then washed extensively with distilled water and dried over nitrogen. The chip was stored in HBS buffer at 4°C or in a desiccator at room temperature until required.

### **2.3.11.4 *Regeneration studies***

To assess the stability of the immobilised drug-protein conjugate surfaces, a known concentration of antibody was passed over the chip surface, and the surface regenerated by passing over various concentrations of NaOH and HCl ranging from



1-100 mM or 1 M ethanolamine, pH 13.6. This cycle of binding and regeneration was usually completed for greater than 50 cycles, and the binding signal measured to assess the stability and suitability of the immobilised surface for assay purposes.

#### **2.3.11.5 *Universal standard curve***

Conjugates were immobilised as described in section 2.3.11.2. Serial dilutions of purified antibody were passed over the surface of the chip under conditions of mass transport limitation (MTL). The rate of antibody binding ( $dR/dT$ ) was calculated from the slope of the binding curve, and plotted against the logarithm of the reciprocal of the antibody dilution factor used. The concentration of antibody (nM) in both solutions was calculated from the curve and algorithm.

#### **2.3.11.6 *Non-specific binding studies***

Purified polyclonal and scFv antibody solutions at the requisite dilution were passed over a dextran surface and a surface with the protein of interest immobilised on the dextran matrix. Non-specific binding to either dextran or immobilised protein surface was titred out by the addition of either molecule to the antibody solution, causing these non-specific antibodies to bind to either protein or dextran in solution.

#### **2.3.11.7 *BIAcore inhibitive immunoassay***

Standards of free analyte (morphine-3-glucuronide, parathion, and cephalexin) were prepared at varying concentration ranges. All further additions of reagents and incubation steps were automated. Each sample was incubated with an equal volume of antibody, and allowed to equilibrate for a specific time interval (10 min) when automated, or manually for 2 h at room temperature, and then passed over the sensor surface. The equilibrated mixtures were passed sequentially, in random order, over the chip surface, and the chip surface regenerated between cycles by pulses of the appropriate regeneration solution for each antibody type. A calibration curve was

constructed by plotting the change in response (RU) for each standard against the log of concentration, and sample concentrations were determined from this curve

The intra-day variability of the assay was investigated by running a set of standards across the linear range three times in one day, and determining the coefficient of variation (CV) between the calculated concentrations for each

The inter-day variability of the assay was assessed by running standards across the linear range on three different days, and determining the CV's between concentrations for the standards from each of the three standard curves

#### **2.3.11.8 *Solution affinity analysis using BIAcore***

Drug-protein conjugates were immobilised using conventional EDC/NHS coupling chemistry. Purified anti-M3G scFv antibodies of known concentration were passed over the immobilised surface, and a calibration curve was constructed of mass bound measured in terms of response units, versus antibody concentration (nM). Varying concentrations of M3G were incubated with a known concentration of scFv antibody and allowed to reach equilibrium overnight. The samples were then passed over the immobilised surface and the binding response calculated. The amount of free antibody in the equilibrium mixtures was calculated using the response values measured from the constructed calibration curve. Free antibody concentration (nM) was then plotted against drug concentration (nM), and using the solution phase interaction models in BIAevaluation 3.1 software, the overall affinity constant could be determined.

## **Chapter 3**

# **Production and Characterisation of Polyclonal Antibodies to Morphine-3-glucuronide**

### 3 1 Introduction

#### 3 1 1 The Opiates

Opium is a naturally occurring substance, which can be obtained from the opium poppy, *Papaver somniferum* (Julian *et al* , 1981) The name opium is derived from the Greek name for the dried latex (juice) extracted from the unripe capsules of the opium poppy The main active ingredient of opium is morphine, which possesses analgesic properties Opioids are compounds that have morphine-like pharmacological activity but are not necessarily structurally related (Jaffe *et al* , 1990)

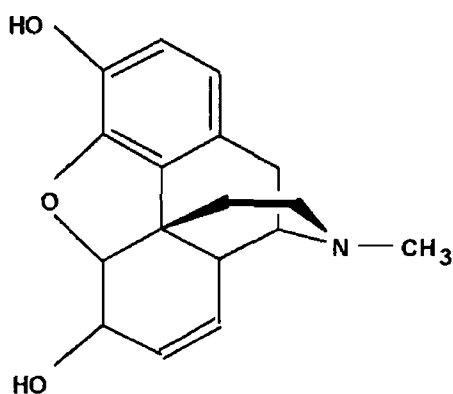
Opiates are those drugs that are structurally related to morphine (either natural or synthetic) and which exert actions upon the body similar to those induced by morphine They are used in the relief of pain, and possess a wide variety of pharmacological effects and differing potency for the production of analgesia, sedation, respiratory depression, decreased gut motility and cough suppression (Jaffe *et al* , 1990) The earliest writings of the effects of opium are dated back to 300 B C However, some references to opiates date back as far as 3000 B C The medicinal use of opium to relieve pain is well known and was extensively used for this and recreational purposes in Europe throughout the sixteenth and seventeenth centuries

Opium contains at least 20 different compounds and in the early 1800's Serturmer, a German pharmacist isolated morphine, which is the main active ingredient in opium Opium and morphine were sometimes referred to as 'GOM', "God's Own Medicine" and were used for a wide variety of problems i.e. alcoholism, asthma, dysentery and were also used to treat such problems as the simple cough Opium contains over 20 alkaloids, of which the most abundant is morphine (10-15%) followed by codeine (1-3%), thebaine (1-2%), noscapine (4-8%) and papaverine (1-3%) (Perrine *et al* , 1996) However, only morphine and codeine have analgesic properties Thebaine has a chemical structure quite similar to morphine, however, unlike morphine, it exhibits no analgesic effects and it causes 'strychnine-like' convulsions By the early part of the twentieth century, 1 in 400 Americans was addicted to morphine or opium

derivatives. For this reason, there was concern about the possible harmful effects and the addictive nature of both morphine and other opiates. This concern sparked the government into action and in 1914 the Harrison Narcotic Act was passed. The conditions of this act made the use of opiate drugs illegal except for medical applications.

### 3.1.2 Morphine, Heroin and Morphine-3-Glucuronide

As already stated, morphine (Figure 3.1) is the main active ingredient of opium associated with pain relief. It was first isolated in pure form in the early 19<sup>th</sup> century by Serturmer (Perrine *et al.*, 1996). Once isolated, morphine became a widely used drug in the United States as it became freely available and accessible from both drug and general stores. Morphine became commonly used for both medicinal and recreational purposes, until 1914 when the Harrison Narcotic Act was passed (Julian *et al.*, 1981) making non-medical uses of morphine illegal.

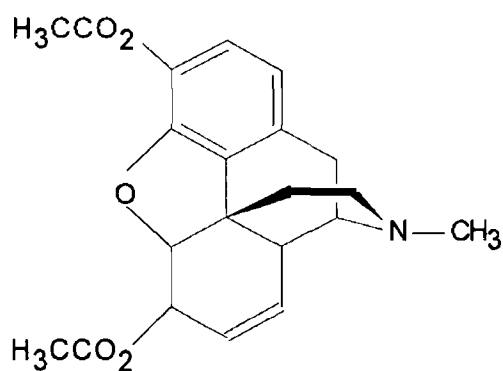


*Figure 3.1 Structure of morphine.*

Morphine is still used today in modern medical practice. It is a potent opioid analgesic used traditionally for the short-term treatment of surgical pain and for the long-term treatment of moderate-to-severe cancer pain. Euphoria is experienced when morphine is administered. This is an important component of its analgesic

effect as anxiety and agitation are greatly reduced, which are associated with a painful illness. It can be administered intravenously or orally. Oral administration is much less potent because of rapid metabolism in the intestinal wall and liver. This resulted in intravenous administration becoming the preferred route of entry as its effects began within an hour (Rang *et al*, 1995). The most important effects of morphine are on the central nervous system and the gastrointestinal tract, although other systems have been affected to a lesser extent. Constipation can be caused by reduced gastrointestinal motility and is a common side effect of morphine, which can be very severe. Respiratory depression, suppression of cough, nausea and vomiting and histamine release, causing bronchoconstriction and hypotension are also lesser pharmacological effects of morphine (Rang *et al*, 1995).

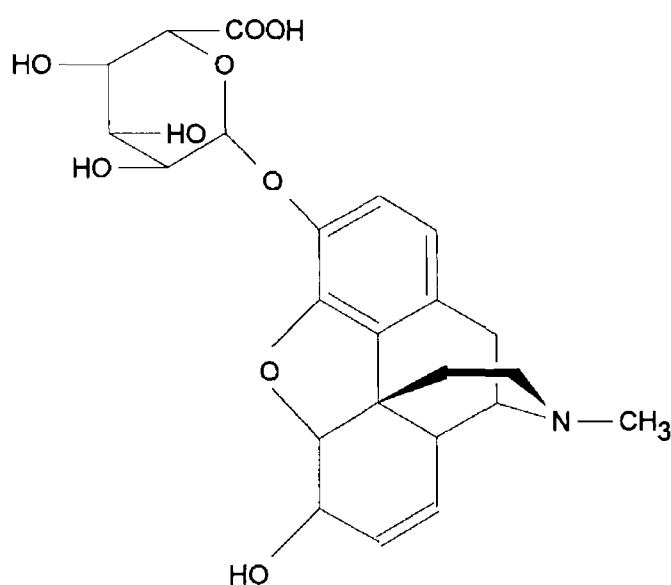
Heinrich Dreser and Felix Hoffman of the Friedrich Bayer laboratories first introduced heroin (Figure 3.2) in the late 1890's. Heroin is not a naturally occurring opiate. It is a semi-synthetic opioid. Both Dreser and Hoffman believed that by forming the acetyl ester of a naturally occurring substance (i.e. morphine), the pharmacological profile would be increased. Acetylation was carried out twice on morphine to produce heroin (diacetylmorphine). Heroin is just over 3 times more potent than morphine as an analgesic i.e. it takes three milligrams of heroin to produce the same analgesic effects as 10 milligrams of morphine. Absorption occurs more rapidly from the gastrointestinal tract and enters the brain faster than morphine. Although most of heroin's effects are due to it being metabolised into morphine, it is more lipophilic and reaches the brain faster (Braithwaite *et al*, 1995).



*Figure 3.2 Structure of heroin*

This difference provides greater euphoric effects than morphine. The majority of scientific studies have been carried out on morphine, due to heroin's rapid transformation into morphine once present in the body. Once this occurs the pharmacological sequence is the same for both.

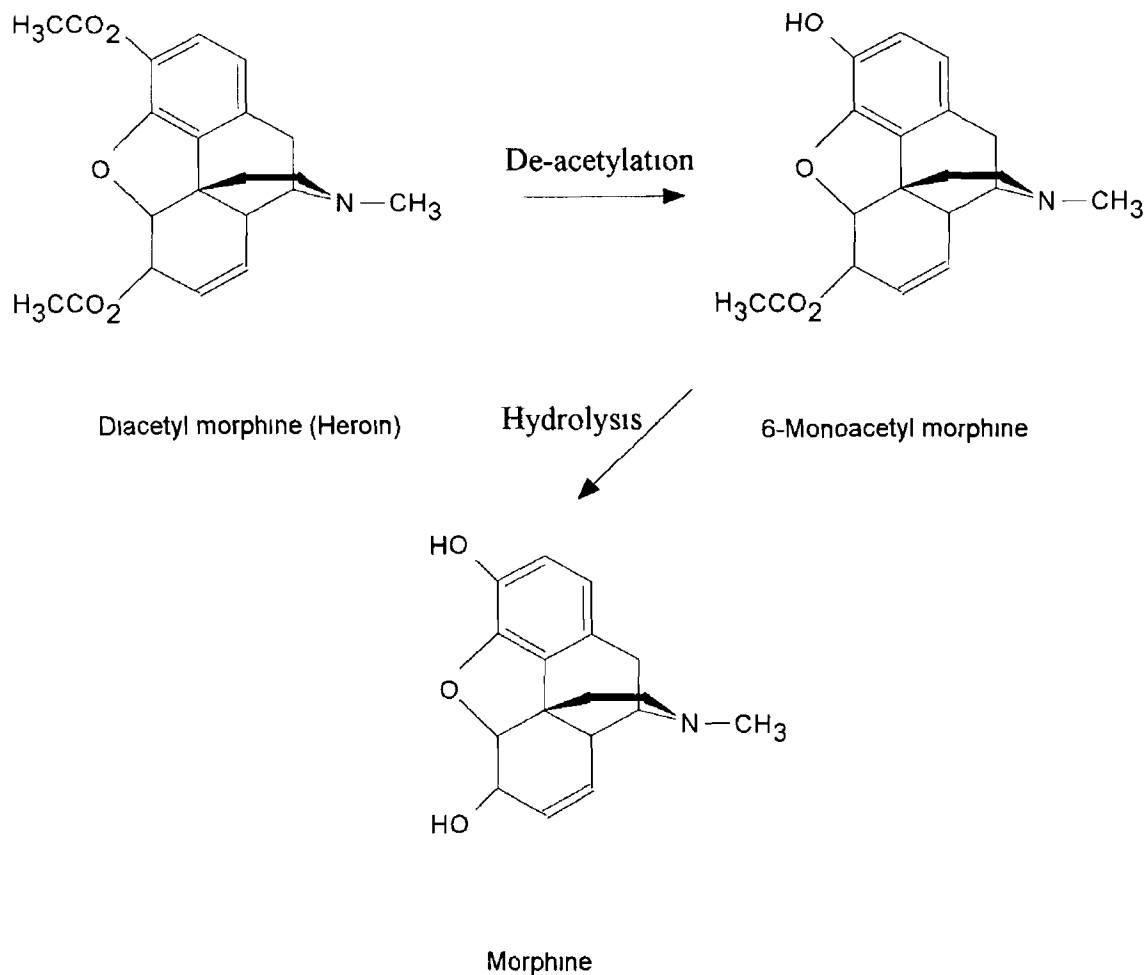
Morphine-3-glucuronide (Figure 3.3) is the main metabolite formed in the body for both morphine and heroin (Figure 3.4) (Moffat *et al.*, 1986, Coyle *et al.*, 1987, Gough *et al.*, 1991). This characteristic of morphine-3-glucuronide is of great significance for the indirect determination of heroin or morphine usage.



*Figure 3.3 Structure of morphine-3-glucuronide.*

### 3.1.3 Metabolism

Heroin is rapidly hydrolysed to 6-monoacetylmorphine (6-MAM) which, is hydrolysed to morphine following intravenous administration into humans (Goodman & Gilman, 1991). Results have shown this reaction to occur as rapidly as 5 min and high levels are found in the heart, forebrain, lungs and liver. However, once heroin is metabolised into morphine it follows the same path of metabolism as morphine in the system.

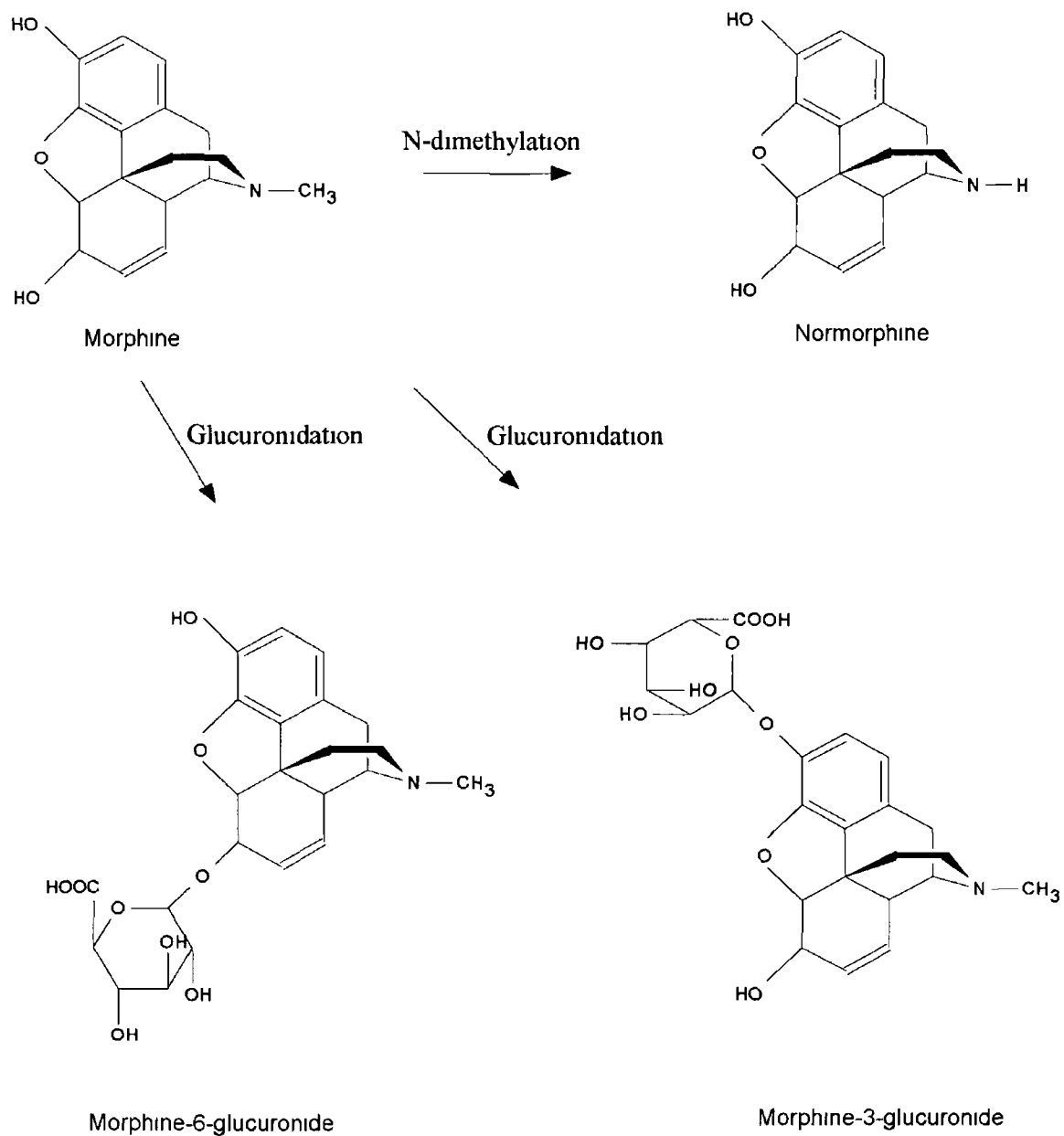


**Figure 3 4 Metabolic pathway of heroin** Once it is metabolised to morphine, it follows the same pathway as in Figure 3 5

Morphine's main effects are exerted primarily on the central nervous system, the eye, and the gastrointestinal tract. In the case of continuous morphine use or poisoning, the common symptoms associated are sedation, decreased respiration rate, severe constipation and pinpoint pupils (Rang *et al*, 1995). On administration morphine is quickly absorbed from the gastrointestinal tract. However, due to the high first-pass elimination on passage through the liver, only 40% of the drug remains present (Stanski *et al*, 1978, Sarve *et al*, 1981, Sawe *et al*, 1986). Morphine is metabolised



(Figure 3 5 ) by conjugation with glucuronic acid at both the 3-phenolic and 6-alcoholic positions for excretion purposes



*Figure 3 5 Metabolic pathway of morphine showing the production of the main metabolites, morphine-3-glucuronide, morphine-6-glucuronide and normorphine.*

These metabolites are known as morphine-3-glucuronide (M3G) (60%) and morphine-6-glucuronide (M6G) (10%) (Yoshimura *et al*, 1969, Oguri *et al*, 1970, Yeh *et al*, 1979, Gong *et al*, 1991, Faura *et al*, 1996)) Normorphine (N), normorphine-glucuronide and morphine-3,6-diglucuronide are also formed in small quantities (Boerner and Delle, 1975, Yeh, 1975) Glucuronidation mainly occurs in the liver while the intestine and kidneys also possess the same ability to a lesser extent (Brunk *et al*, 1974, Boerner *et al*, 1975) Codeine is also metabolised in minor quantities M6G possesses significant biological activity, the most significant being its greater analgesic potency than morphine (Pasternak *et al*, 1987, Abbott *et al*, 1988, Osborne *et al*, 1988, Frances *et al*, 1992, Stain *et al*, 1995) This discovery suggested the metabolites themselves perhaps contained the majority of the analgesic properties rather than morphine Results also showed M3G to have no analgesic effects (Shimomura *et al*, 1971) Smith *et al* (1990) suggested that rather than acting as an agonist, M3G is, in fact, an antagonist and that it may be involved in the development of tolerance to and dependence on morphine

### 3 1 4 Detection of Opiates

The Misuse of Drugs Act (1971) renders the possession and use of opiates illegal for non-medical purposes. This creates a need for sensitive methods of detection and identification of individual opiates. Problems can arise in the detection of individual opiates as a number possess very similar characteristics and follow the same metabolic pathways. Detection methods for opiates have traditionally been based on thin-layer chromatography (TLC), gas chromatography (GC), high performance liquid chromatography (HPLC) and capillary electrophoresis (CE).

Low *et al* (1995) developed an assay system using HPLC for detection of opiates and heroin in urine. A 200 x 2 mm ID, 3 µm silica column with dichloromethane-pentane-diethylamine-methanol mobile phase was used. The detection limits were found to be quite low and ranged from 4 to 20 ng/ml.

Gerostamoulos *et al* (1995) also used HPLC for detection of morphine and its metabolites. Reverse phase ion-pair chromatography was used to achieve separation with a C<sub>18</sub> bonded column. Acetonitrile, lauryl sulphate and sodium dihydrogen orthophosphate buffer at low pH was used as the mobile phase and electrochemical detection (ECD) in conjunction with ultraviolet (UV) spectrophotometric detection was used for quantification of the drugs. This assay had a detection range of between 10 and 5,000 ng/ml.

Zheng *et al* (1998) reported on a high-performance liquid chromatography tandem mass spectrometry-mass spectrometry (LC-MS-MS) assay for the analysis of morphine, morphine glucuronides and normorphine in plasma samples from rats. Extraction of the analytes was carried out by using C<sub>2</sub> solid-phase extraction cartridges. Recoveries were found to range from 100% for morphine, 84% for morphine-3-glucuronide, 64% for morphine-6-glucuronide and 88% for normorphine. The limits of detection were 3.8 ng/ml for morphine, 5.5 ng/ml for morphine-3-glucuronide, 12 ng/ml for morphine-6-glucuronide and 5.0 ng/ml for normorphine.

Beike *et al* (1999) developed an immunoaffinity-based extraction method for the determination of morphine and its glucuronides in human blood. Specific antisera against morphine, morphine-3-glucuronide and morphine-6-glucuronide were

coupled to 1,1-carbonyldiimidazole-activated tris-acrylgel and used for immunoaffinity extraction of morphine and its glucuronides from blood. The resulting extracts were analysed by HPLC with native fluorescence detection. Mean recoveries from spiked blood were 71, 76 and 88% for morphine, morphine-3-glucuronide and morphine-6-glucuronide, respectively, and the limit of detection was 3 ng/g blood and the limit of quantitation was 10 ng/g blood for all three analytes.

Meng *et al* (2000) developed a method of analysis with increased sensitivity and specificity for morphine and its metabolites by using a cleaner extraction method. The extraction method involves both the hydrophobic isolation on a carbon cartridge and ion-exchange isolation on ion-exchange resin. The combination of these two steps results in the successful purification of these drugs from human plasma with maximum removal of interfering substances compared with a conventional C<sub>18</sub> cartridge. The analytes were quantified by HPLC on a reverse-phase C<sub>18</sub> column employing a mobile phase consisting of 25% (v/v) acetonitrile in 0.05 M phosphate buffer, and 2.5 mM sodium dodecyl sulphate as the pairing ion with a combination of electrical and fluorometric detection. Recoveries for morphine, morphine-3-glucuronide, morphine-6-glucuronide and hydromorphone after the SPE procedure were 86±7.1, 82±6.9, 79±6.0 and 85±6.0%, respectively. Limits of detection for this method are 0.1 ng/ml for morphine and 0.18 ng/ml for morphine-3-glucuronide and morphine-6-glucuronide and limits of quantitation are 0.25 ng/ml for morphine and 0.45 ng/ml for morphine-3-glucuronide and morphine-6-glucuronide.

As described above there are a large number of HPLC techniques for the detection of opiates in biological fluids. However, HPLC techniques are not extensively used in routine clinical laboratories for either initial screening or for the confirmation of samples found positive by other detection techniques (Braithwaite *et al*, 1995). The lack of application of HPLC in clinical settings is due to the expense of the technique, the extraction requirement, the relatively slow analysis time and the expertise required for operation.

The combination of gas chromatography with mass spectrometry can also be used in the identification of individual opiates. Selected ion monitoring (SIM) provides sufficient sensitivity for the confirmation of immunoassay results down to the

threshold concentrations of 300 µg/l (Braithwaite *et al* , 1995) Mule *et al* (1988) and Cone *et al* (1990) both used this configuration in the identification of 6-monoacetylmorphine in urine as direct evidence of heroin usage

Naidong *et al* (1999) developed a simultaneous assay for morphine, morphine-3-glucuronide and morphine-6-glucuronide in human plasma using normal-phase liquid chromatography-tandem mass spectrometry with a silica column and an aqueous organic mobile phase. Detection ranges were 0.5-50 ng/ml for morphine, 1.0-100 ng/ml for morphine-6-glucuronide and 10-1000 ng/ml for morphine-3-glucuronide

In the past, the GC-MS systems were slow and expensive to operate, however, recent developments in the production of bench-top systems has left such systems within the budget of many smaller laboratories

However, these analytical methods require many clean up steps, are very time consuming and may be expensive. Over the years, antibody-based methods have been developed as favourable alternatives for either the identification or quantification of opiates. Immunoassays have been developed and are sold commercially. One well known system is the enzyme multiplied immunoassay technique (EMIT) (Gough, 1991, McCleave, 1992, Wu *et al* , 1993). In this system the activity of the conjugated enzyme is modulated by their interaction of the drug-specific antibody with the haptenated enzyme. The presence of free drug causes a decrease in the modulation, which indicates the presence of an illicit drug.

Spector *et al* (1971) developed a radioimmunoassay (RIA) for the quantification of morphine using anti-morphine antibodies. The sensitivity of the assay in serum was found to be between 50 to 100 pg of morphine, which provides very good detectability. Affinity-purified morphine antibody was used to develop a solid phase radioimmunoassay by Steiner *et al* (1978) which produced a sensitive assay with detection limits for morphine of 500 ng/l of serum. Radioimmunoassays possess a number of advantages and disadvantages. There is minimal cross reactivity to morphine metabolites or closely related opiates when using RIAs. This technique is extremely sensitive and provides positive results for use of heroin, morphine or codeine (as codeine is metabolised into morphine). As codeine is available for general medical use, these assays may not be used as preliminary screening assays for

opiate abuse, they are more applicable to pharmacokinetic studies of morphine (Braithwaite *et al* , 1995)

An enzyme-linked immunosorbent assay (ELISA) was developed by Usagawa *et al* (1993) to detect morphine using a morphine-specific monoclonal antibody. Concentrations as low as 100 pg/ml of morphine were detected using this assay. This assay showed the ability of the morphine monoclonal antibody to detect very low levels of morphine confirming the usefulness of such assays for the detection of opiates.

In 1995 Holt *et al* developed an amperometric system for the detection of opiates. This system used two microbial enzymes, acetylmorphine carboxyesterase (heroin esterase) and morphine dehydrogenase (MDH). They were incorporated into an amperometric assay for morphine and heroin using phenazine methosulphate as a mediator. The limits of detection for morphine were found to be 6.8 µg/ml. Holt *et al* (1996) also developed a bioluminescence assay for heroin and its metabolites. This involved using heroin esterase, morphine dehydrogenase, and the bacterial luciferase. Detection levels were found to be between 89 ng/ml of heroin and 2.0 ng/ml of morphine.

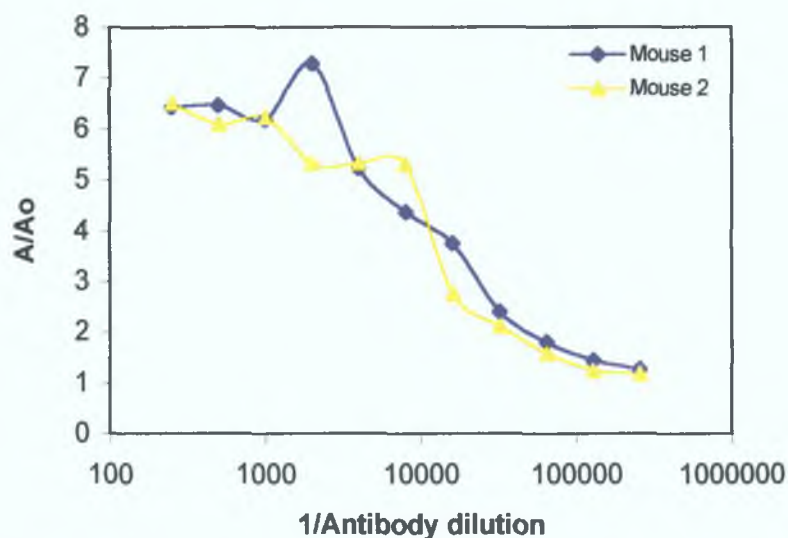
The development of enzyme-linked immunosorbent assays for the detection of morphine-3-glucuronide (M3G) (the main metabolite of heroin and morphine) is described in this chapter as very little work was reported in the literature for detection of M3G using such assays. Two polyclonal antibodies were produced and used in the development of model ELISA assays to measure M3G in PBS. Analytical studies were carried out on the precision and reproducibility of the assays. The immunoassays were subsequently used in the analysis of free M3G in urine.

## 3.2 Results

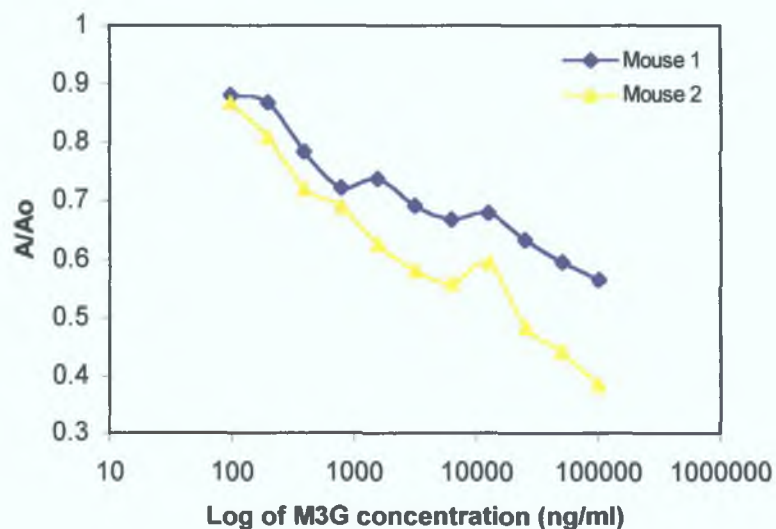
### 3.2.1 Production and characterisation of morphine-3-glucuronide protein conjugates

Conjugates to M3G were prepared using carbodiimide coupling chemistry as described in section 2.3.2.1. Characterisation of conjugates was indirectly carried out by immunisation followed by the characterisation of the specific antibody produced in serum. *BALB/c* mice were immunised (section 2.3.3.2) with hapten-protein-conjugates to elicit an immune response followed by removal of blood 10 days after immunisation. Detection of specific antibody in serum was carried out by use of ELISA (Figure 1.12) as described in section 2.3.8.1. Initially, M3G-BSA was coated on a microtitre plate followed by the addition of serial dilutions of serum ranging from 1/250 to 1/256,000 taken from two mice immunised with M3G-BSA to monitor specific antibody produced. The absorbance readings obtained at each antibody dilution ( $A$ ) was divided by the absorbance reading determined in the presence of zero antibody ( $A_0$ ), to give a normalised reading. Results indicated that antibodies were produced to the hapten-protein-conjugate as shown in Figure 3.6. However, a competition assay was carried out using these antibodies to determine the specificity of the antibodies to M3G and not the carrier-protein.

A competition ELISA was carried out as shown in Figure 1.11 and described in section 2.3.8.2. Standards of free M3G were prepared in PBS containing 0.5 mM HCl between the ranges of 97 and 100,000 ng/ml and added to the wells of the plate with a 1:1 ratio of antibody at a 1/8,000 dilution (antibody dilution factor determined from Figure 3.6). The absorbance readings at each antigen concentration ( $A$ ) was divided by the absorbance reading determined in the presence of zero antigen concentration ( $A_0$ ), to give a normalised reading. Results indicated that as the concentration of M3G increased in solution, the absorbance readings decreased confirming that free drug displaces antibody (Figure 3.7). The presence of specific antibodies to M3G in serum indicated the successful coupling of M3G to protein. Further M3G conjugates were produced and characterised using the same method.



*Figure 3.6. Analysis of serum from mice immunised with M3G-BSA conjugate by ELISA. Serum was taken 10 days after immunisation from two mice immunised with M3G-BSA. Serial dilutions of serum from 1/250 to 1/256,000 were added to wells coated with M3G-BSA. A high response was found to the M3G-BSA conjugate. The optimal antibody dilution for a competitive ELISA taken as 50% of the maximum absorbance was found to be 1/8000.*



*Figure 3.7. Results from a competitive ELISA using serum taken from mice immunised with M3G-BSA. Standards of free M3G were prepared and added in a ratio of 1:1 with a 1/8000 dilution of antibody to the wells of a microtitre plate coated with M3G-BSA. As free drug concentration increases, the absorbance signal decreases indicating the displacement of antibody by M3G and confirming the presence of antibodies specific to M3G.*



### **3 2 2 Production and characterisation of polyclonal anti-morphine-3-glucuronide antibodies**

Polyclonal antibodies to M3G were produced, purified by saturated ammonium sulphate precipitation and protein G affinity chromatography. The antibodies purity was assessed with the use of SDS-PAGE gel electrophoresis, and the working dilution of the antibodies for competitive assay analysis was optimised.

#### **3 2 2 1 Determination of titre**

New Zealand White female rabbits were immunised with hapten-protein-conjugates to M3G as described in section 2 3 5 2. Analysis of the antibody titre was carried out using the ELISA as described in section 2 3 8 1. Serial dilutions of serum samples from both rabbits were prepared at ranges between 1/250 and 1/256,000 and these added to a microtitre plate coated with 100 µg/ml of M3G-BSA, followed by labelled anti-rabbit antibody and subsequently, chromogenic substrate. The resulting titres were greater than 1/256,000 following immunisation and are shown in Figure 3 8 for both rabbit-1 (R-1) and rabbit-2 (R-2) antibodies.

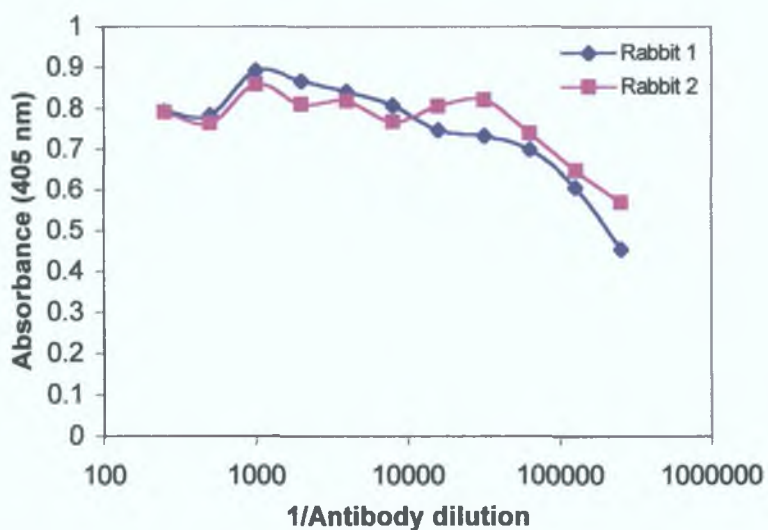
#### **3 2 2 2 Purification of polyclonal antiserum**

The serum was prepared as described in section 2 3 6 1. Initial purification was carried out on the rabbit serum by saturated ammonium sulphate precipitation, as described in section 2 3 7 2. Protein G affinity chromatography (section 2 3 7 3) was then carried out to further purify the antibodies. Partially purified saturated ammonium sulphate precipitated-antibody was passed through the column allowing IgG to bind to protein G. After extensive washing of the column to remove non-specifically bound antibodies the M3G antibodies were eluted with glycine buffer, pH 2.2 (Figure 3 9). Eluted fractions were collected and protein concentration monitored by spectrophotometric analysis with absorbance readings at 280 nm. Results indicated that the majority of antibody eluted within the first six to seven fractions.

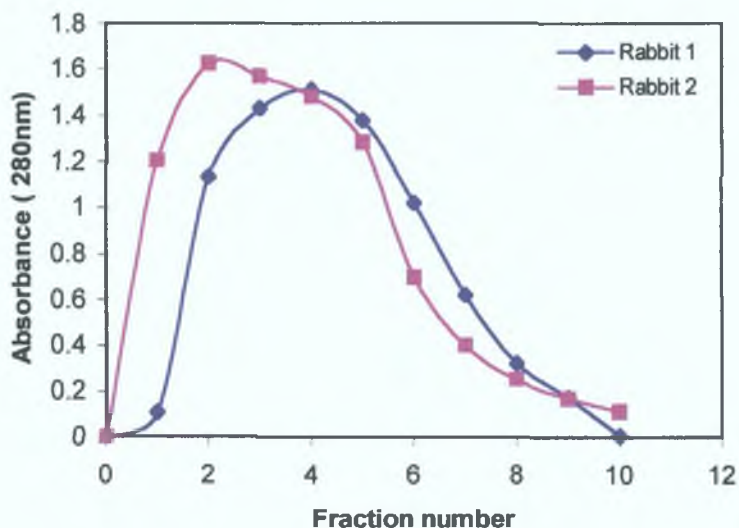
These fractions were pooled and dialysed into PBS buffer. The titre of the affinity-purified antibody was determined using an ELISA as described in section 2.3.8.1 and showed a specific antibody titre of greater than 1/500,000 for R-1 and 1/1,000,000 for R-2.

### **3.2.2.3 Characterisation of purified antibody by SDS-PAGE**

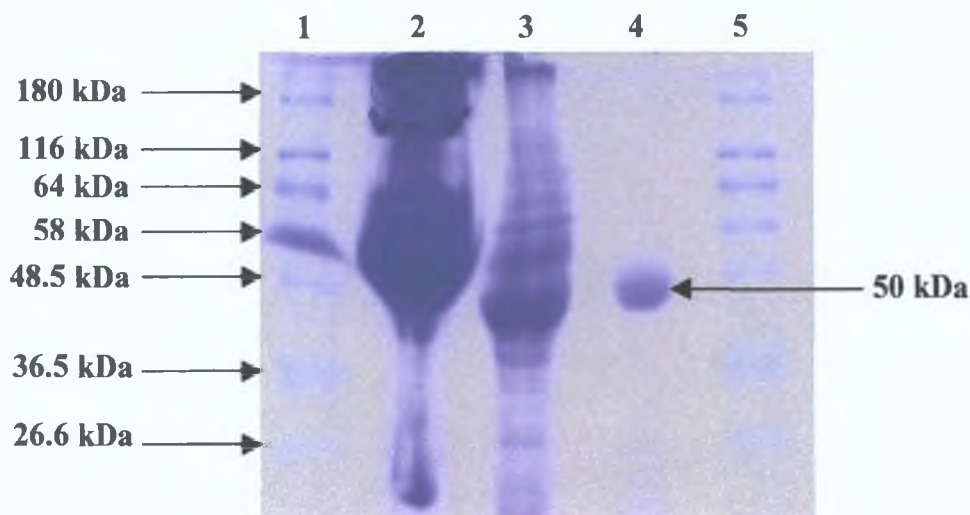
The purity of both R-1 and R-2 antibodies were analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) as described in section 2.3.7.6 and the gels are shown in Figures 3.10 and 3.11, respectively. SDS-PAGE is a technique which separates proteins on the basis of their molecular weight. The samples and molecular weight markers were boiled with mercaptoethanol causing the polypeptide chains of the immunoglobulin to separate. The gel pictures show the affinity-purified anti-M3G antibodies (lane 4 on both Figures 3.10 and 3.11) with a molecular weight band at approximately 50 kDa which represents the heavy chain of the immunoglobulins.



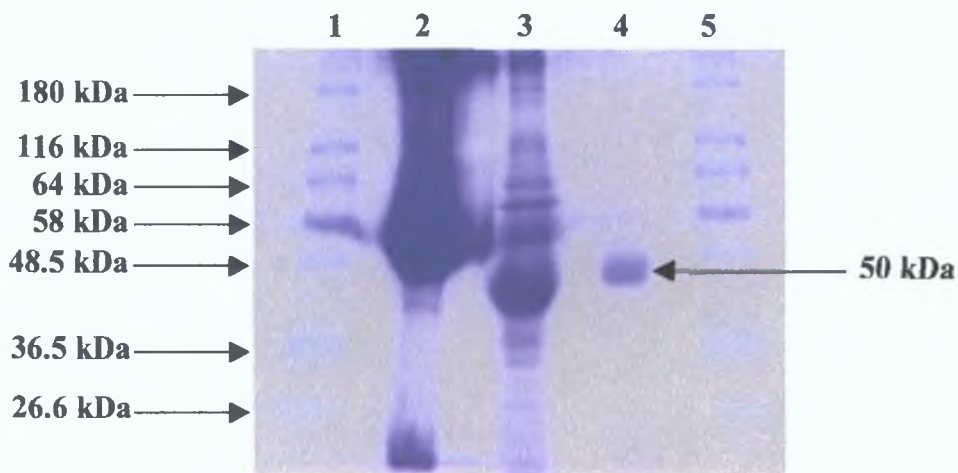
*Figure 3.8. Plot showing the antibody titres of R-1 and R-2 serum. Serial dilutions of serum were added to wells coated with M3G-BSA. At a dilution of 1/256,000 a high response from both serum samples is present.*



*Figure 3.9. A typical elution profile for polyclonal anti-M3G R-1 and R-2 antibodies from a protein-G-sepharose column. Saturated ammonium sulphate purified antibody was passed down the column. The column was washed and the bound antibody eluted with 0.1 M glycine/HCl, pH 2.2. 0.5 ml fractions were collected, and the protein concentration was measured by absorbance at 280 nm. The majority of antibodies for both purification's eluted during the first seven fractions.*



*Figure 3.10. SDS-PAGE gel of R-1 affinity-purified anti-M3G antibody on a protein-G-sepharose column. Lanes 1 and 5 contain molecular weight markers ranging from 26.6 to 180 kDa. Lane 2 represents unpurified R-1 anti-M3G antibody rabbit serum. Lane 3 represents rabbit antiserum purified by saturated ammonium sulphate precipitation followed by lane 4 representing affinity-purified saturated ammonium sulphate precipitated antibody on a protein-G-sepharose column.*



*Figure 3.11. SDS-PAGE gel of R-2 affinity-purified anti-M3G antibody on a protein-G-sepharose column. Lanes 1 and 5 contain molecular weight markers ranging from 26.6 to 180 kDa. Lane 2 represents unpurified R-2 anti-M3G antibody rabbit serum. Lane 3 represents rabbit antiserum purified by saturated ammonium sulphate precipitation followed by lane 4 representing affinity-purified saturated ammonium sulphate precipitated antibody on a protein-G-sepharose column.*

### **3 2 3 Development of a competitive enzyme-linked immunosorbent assay (ELISA) for morphine-3-glucuronide**

Competitive ELISA's were developed for the detection of free M3G in solution using the affinity-purified polyclonal antibodies

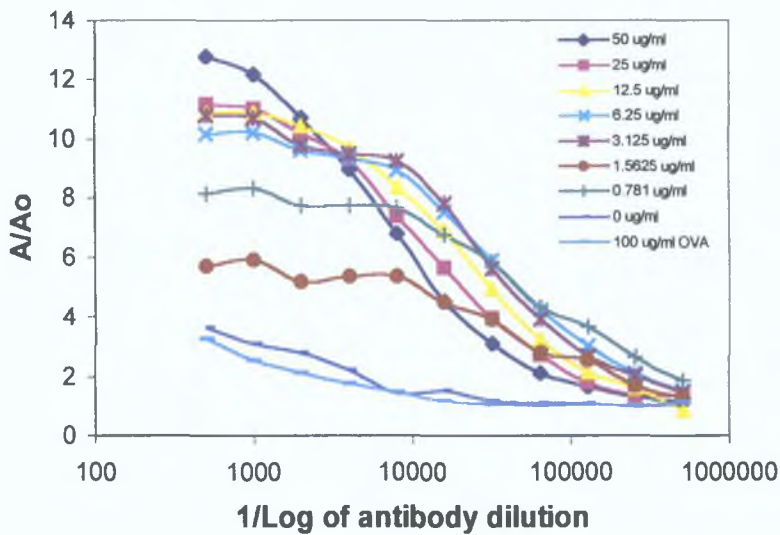
#### **3 2 3 1 Checkerboard ELISA for determination of optimal loading ratios of conjugate and optimal antibody dilution for R-1 and R-2 anti-morphine-3-glucuronide polyclonal antibodies**

The working dilution of these antibodies was determined using the format already discussed in section 2 3 8 1 M3G-OVA was coated on the wells of a microtitre plate at varying concentrations from 50 to 1 56  $\mu\text{g/ml}$  Dilutions of M3G affinity-purified antibody were prepared at ranges from 1/500 to 1/512,000 for R1 and 1/500 to 1/100,000 for R2 and each dilution of antibody was added to each concentration of conjugate on the microtitre plate The results were plotted for R-1 antibody as shown in Figure 3 12 The optimal conjugate loading ratio for R-1 was found to be 12 5  $\mu\text{g/ml}$  with an antibody dilution determined to be 1/50,000 Results were also plotted for R-2 antibody as shown in Figure 3 13 The optimal conjugate loading ratio and dilution for R-2 were found to be 1 56  $\mu\text{g/ml}$  and 1/50,000, respectively

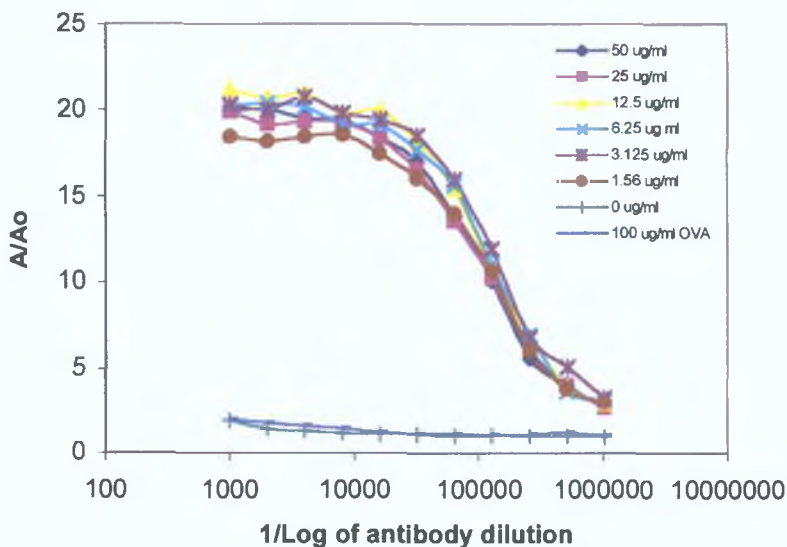
#### **3 2 3 2 Linear range of detection of morphine-3-glucuronide in ELISA for R-1 and R-2 polyclonal antibodies**

The immunoassay format for the detection of M3G as described in section 2 3 8 2 was used to determine the optimal range of detection of free M3G Microtitre plates were coated with 12 5  $\mu\text{g/ml}$  and 1 56  $\mu\text{g/ml}$  of M3G-OVA for R-1 and R-2 antibodies, respectively, for the determination of the detection ranges of each antibody to M3G Standards were prepared of M3G in PBS containing 0 5 mM HCl ranging from 4 to 500,000 pg/ml and these were added to the plate at a 1 1 ratio with R-1 and R-2 antibodies (1/50,000 dilution) After incubation, enzyme-labelled anti-

rabbit Ig was added, followed by chromogenic substrate. The linear range of detection for R-1 and R-2 antibodies were found to be between 244 and 250,000 pg/ml and 122 and 31,250 pg/ml, respectively.



*Figure 3.12. Checkerboard ELISA carried out to determine the optimal dilution of antibody and concentration of coating conjugate for R-1 polyclonal antibody. The optimal conjugate loading density was determined as 12.5  $\mu\text{g/ml}$  and the optimal dilution of antibody for use in a competitive assay was 1/50,000.*



*Figure 3.13. Checkerboard ELISA carried out to determine the optimal dilution of antibody and concentration of coating conjugate for R-2 polyclonal antibody. The optimal conjugate loading density was determined as 1.56  $\mu\text{g/ml}$  and the optimal dilution of antibody for use in a competitive assay was 1/50,000.*

### **3 2 3 3 Intra-day assay variability studies of R-1 and R-2 anti-morphine-3-glucuronide polyclonal antibodies**

To carry out intra-day assay variability studies, five sets of standards ranging from 4 to 500,000 pg/ml were prepared for each antibody and assayed on the same day and their means plotted. The intra-day assay variability study for R-1, shown in Figure 3 14, had a linear range of detection between 244 and 250,000 pg/ml. The  $R^2$  value for this range was 0.99. The coefficients of variation (CV's) were determined to assess the precision of the analytical method, expressing standard deviation as a percent function of the mean. These ranged from 1.29% to 4.68% as shown in Table 3 1. The intra-day assay variability study for R-2 shown in Figure 3 15 had a linear range of detection between 62 and 31,250 pg/ml. The  $R^2$  value for this range was 0.99. The CV's were determined and ranged from 1.81% to 6.13% as shown in Table 3 2. The percentage accuracy values obtained for both R-1 and R-2 ELISA intra-day assay studies were found to be variable and are listed in Tables 3 1 and 3 2.

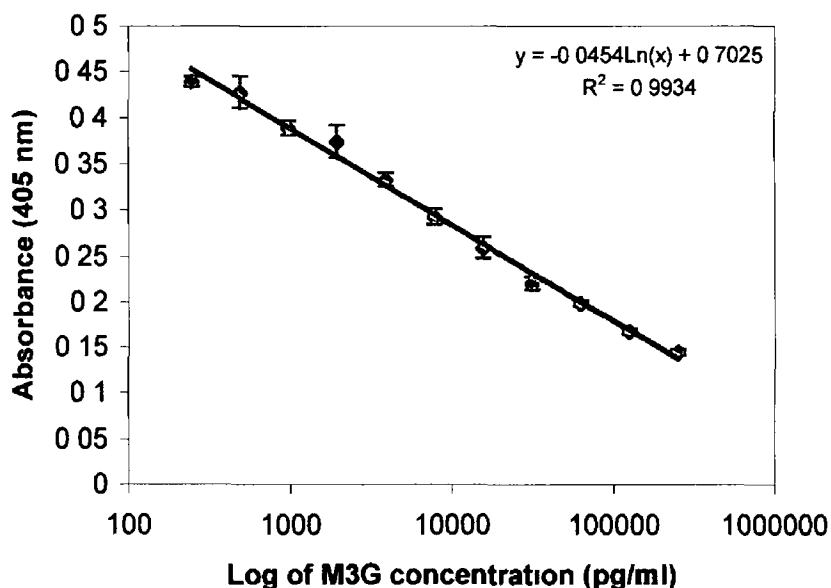
### **3 2 3 4 Inter-day assay variability studies of R-1 and R-2 anti-morphine-3-glucuronide polyclonal antibodies**

To carry out inter-day variability studies, five sets of standards ranging from 4 to 500,000 pg/ml were prepared for each antibody and assayed five times over five days. The inter-day assay variability study for R-1 had a linear range of detection between 244 and 250,000 pg/ml as shown in Figure 3 16. The  $R^2$  value for this range was 0.99. The CV's were determined and ranged from 1.63% to 6.86% as shown in Table 3 3. The inter-day assay variability study for R-2 had a linear range of detection between 61 and 31,250 pg/ml as shown in Figure 3 17. The  $R^2$  value for this range was 0.99. The CV's were determined and ranged from 3.06% to 11.72% as shown in Table 3 4. The percentage accuracy values obtained for both R-1 and R-2 ELISA inter-day assay studies were found to be variable and are listed in Tables 3 3 and 3 4.



**Table 3 1 Intra-day assay variation for R-1 ELISA assay Five sets of eleven standards were analysed on the same day**

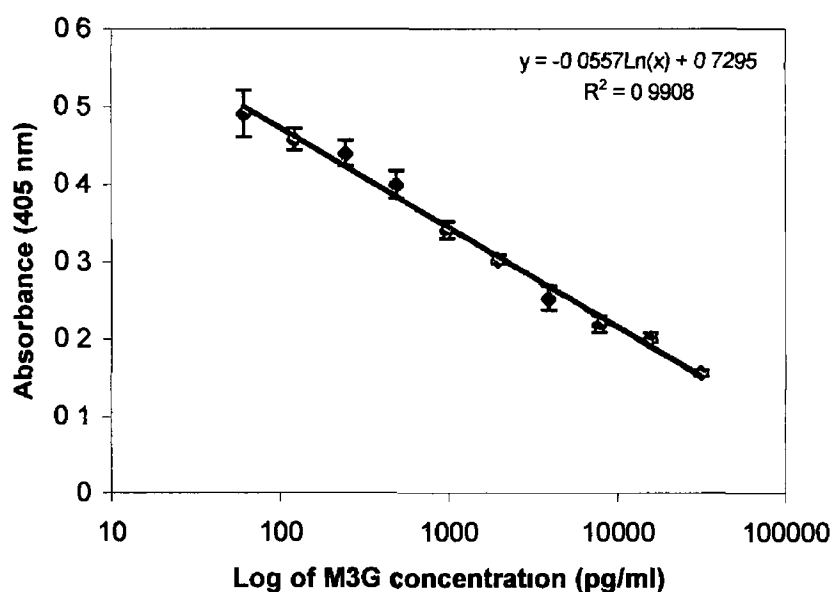
M3G Concentration, (pg/ml)	Calculated mean $\pm$ S D , Abs	Coefficients of variation (CV's), %	% Accuracy
250000	0 145 $\pm$ 0 003	2 22	13 80
125000	0 167 $\pm$ 0 003	1 92	6 08
62500	0 199 $\pm$ 0 002	1 33	4 80
31250	0 221 $\pm$ 0 007	3 26	29 16
15625	0 259 $\pm$ 0 011	4 46	11 80
7812 5	0 292 $\pm$ 0 008	2 90	8 15
3906 25	0 333 $\pm$ 0 007	2 27	12 32
1953 1	0 374 $\pm$ 0 017	4 68	28 93
976 5	0 389 $\pm$ 0 008	2 06	2 15
488 2	0 427 $\pm$ 0 017	4 11	11 52
244	0 439 $\pm$ 0 005	1 29	35 90



**Figure 3.14. Intra-day competitive ELISA assay of R-1 antibody for determination of the optimal range of detection of free M3G using an antibody dilution of 1/50,000 M3G-OVA was coated at a concentration of 12.5  $\mu$ g/ml. The absorbance at 405 nm is inversely proportional to the amount of free drug in solution. The linear range of detection was found to be between 488 and 250,000 pg/ml.**

**Table 3 2 Intra-day assay variation for R-2 ELISA assay Five sets of nine standards were analysed on the same day**

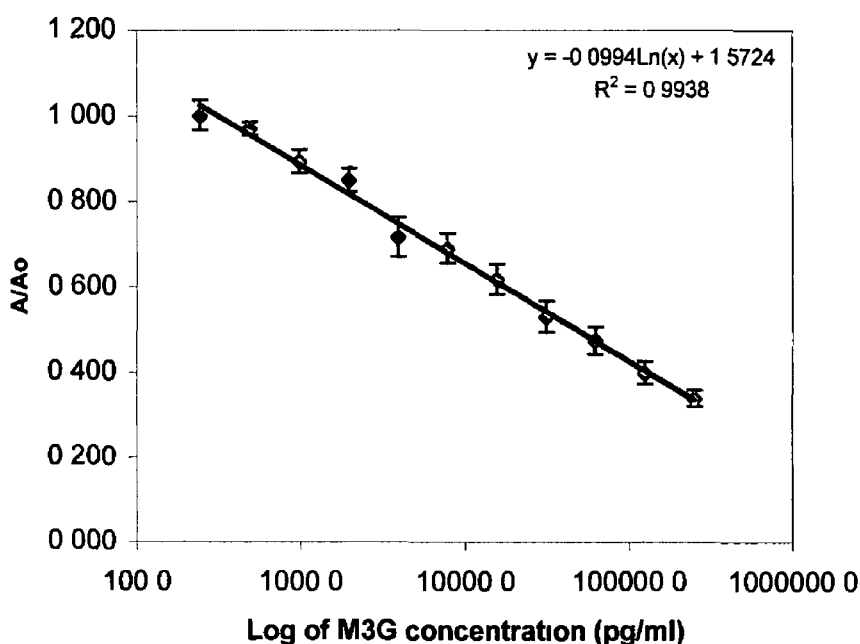
M3G Concentration, (pg/ml)	Calculated mean $\pm$ S.D., Abs	Coefficients of variation (CV's), %	% Accuracy
31250	0.157 $\pm$ 0.0041	2.64	6.90
15625	0.203 $\pm$ 0.0055	2.71	18.47
7812.5	0.219 $\pm$ 0.0104	4.74	22.34
3906.25	0.253 $\pm$ 0.0155	6.13	32.87
1953.1	0.304 $\pm$ 0.0055	1.81	6.39
976.5	0.341 $\pm$ 0.0109	3.21	9.51
488.2	0.399 $\pm$ 0.0180	4.53	22.60
244	0.440 $\pm$ 0.0166	3.77	25.89
122	0.458 $\pm$ 0.0140	3.05	7.27
61.5	0.491 $\pm$ 0.0299	6.09	16.73



**Figure 3 15 Intra-day competitive ELISA assay of R-2 antibody for determination of the optimal range of detection of free M3G using an antibody dilution of 1/50,000 M3G-OVA was coated at a concentration of 1.56  $\mu$ g/ml. The linear range of detection was found to be between 61 and 31,250 pg/ml**

**Table 3 3 Inter-day assay variation for R-1 ELISA assay Five sets of ten standards were analysed over five different days**

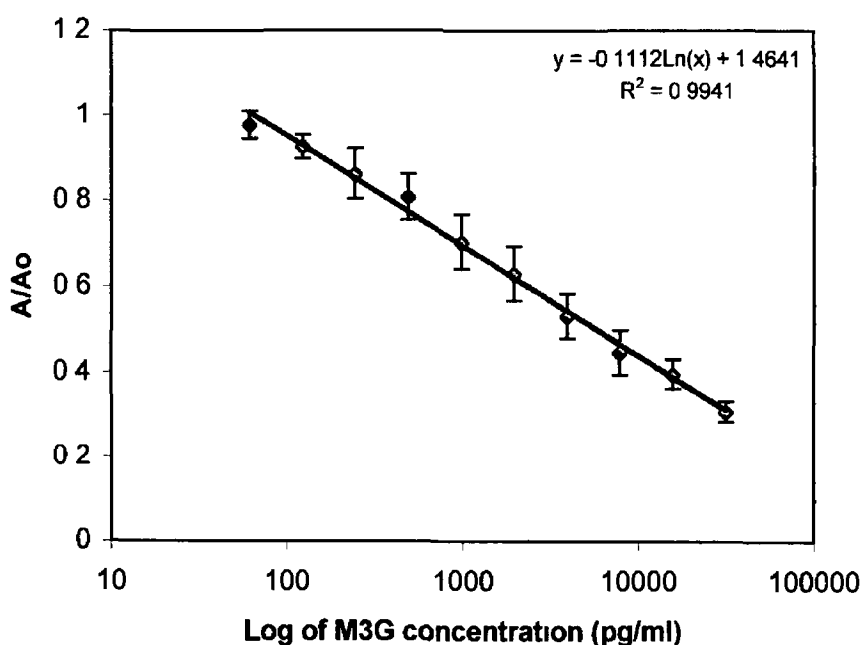
M3G Concentration, (pg/ml)	Calculated mean $\pm$ S D , A/A <sub>0</sub>	Coefficients of variation (CV's), %	% Accuracy
250000	0.341 $\pm$ 0.019	5.51	4.00
125000	0.401 $\pm$ 0.027	6.75	4.98
62500	0.467 $\pm$ 0.031	6.49	8.08
31250	0.524 $\pm$ 0.036	6.86	21.83
15625	0.587 $\pm$ 0.036	5.89	29.28
7812.5	0.655 $\pm$ 0.035	5.07	30.45
3906.25	0.733 $\pm$ 0.046	6.45	19.04
1953.1	0.753 $\pm$ 0.028	3.27	94.66
976.5	0.882 $\pm$ 0.028	3.08	6.29
488.2	0.922 $\pm$ 0.016	1.63	42.27
244	0.979 $\pm$ 0.035	3.46	60.24



**Figure 3 16. Inter-day competitive ELISA assay of R-1 antibody for determination of the optimal range of detection of free M3G using an antibody dilution of 1/50,000 M3G-OVA was coated at a concentration of 12.5  $\mu$ g/ml. The linear range of detection was found to be between 244 and 250,000 pg/ml**

**Table 3 4 Inter-day assay variation for R-2 ELISA assay Five sets of nine standards were analysed over five different days**

M3G Concentration, (pg/ml)	Calculated mean $\pm$ S D , $A/A_0$	Coefficients of variation (CV's), %	% Accuracy
31250	0 308 $\pm$ 0 024	7 99	4 79
15625	0 396 $\pm$ 0 034	8 57	5 01
7812 5	0 446 $\pm$ 0 052	11 72	21 17
3906 25	0 532 $\pm$ 0 052	9 87	11 83
1953 1	0 631 $\pm$ 0 063	10 07	8 19
976 5	0 704 $\pm$ 0 063	8 98	4 73
488 2	0 809 $\pm$ 0 053	6 57	25 84
244	0 863 $\pm$ 0 058	6 80	8 75
122	0 926 $\pm$ 0 028	3 06	3 55
61	0 977 $\pm$ 0 032	3 32	30 91



**Figure 3 17 Inter-day competitive ELISA assay of R-2 antibody for determination of the optimal range of detection of free M3G using an antibody dilution of 1/50,000. M3G-OVA was coated at a concentration of 1 56  $\mu$ g/ml. The linear range of detection was found to be between 61 and 31,250 pg/ml.**

### **3 2 4 Urine analysis of anti-morphine-3-glucuronide antibodies**

The quantitative determination of M3G in urine samples was also investigated. The ionic composition of urine can vary considerable from sample to sample. To compensate for the potential of great inter-individual variability in the salt composition of urine, the antibody sample was prepared in PBS of twice the normal ionic strength (2 x PBS). Known samples of M3G ranging from 10 to 50,000 pg/ml were spiked into control urine and assayed for the presence of free drug with both R-1 and R-2 polyclonal antibodies.

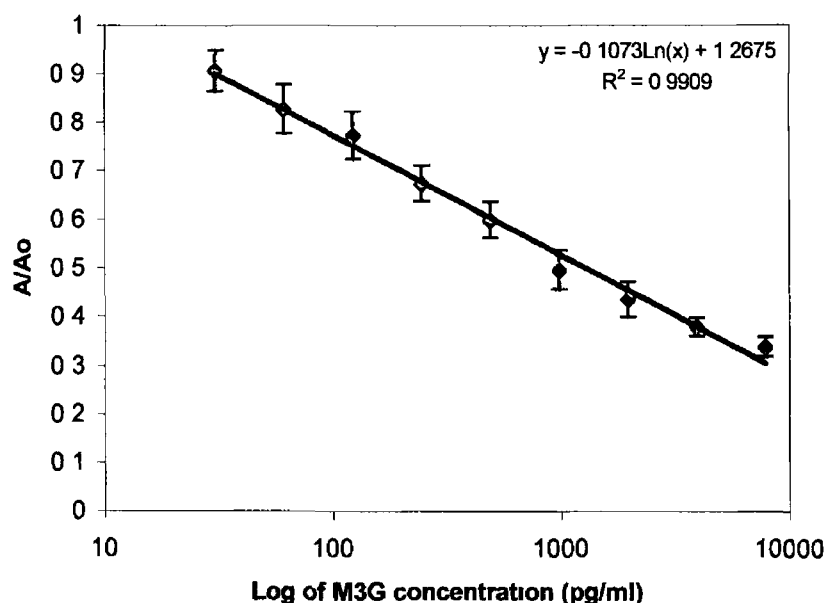
#### **3 2 4 1 Intra-day assay variability studies of R-1 and R-2 anti-morphine-3-glucuronide polyclonal antibodies in urine**

To carry out intra-day assay variability studies, five sets of standards ranging from 10 to 50,000 pg/ml were prepared in urine for each antibody and assayed on the same day and their means plotted. The intra-day assay variability study for R-1 had a linear range of detection between 30 and 7,812 pg/ml. The  $R^2$  value for this range was 0.99. The CV's were determined and ranged from 1.76% to 8.47% as shown in Table 3.5. The intra-day assay variability study for R-2 had a linear range of detection between 30 and 1,950 pg/ml. The  $R^2$  value for this range was 0.98. The CV's were determined and ranged from 0.00% to 3.77% as shown in Table 3.6. The percentage accuracy values obtained for both R-1 and R-2 ELISA intra-day assay studies in urine were found to be variable and are listed in Tables 3.5 and 3.6.

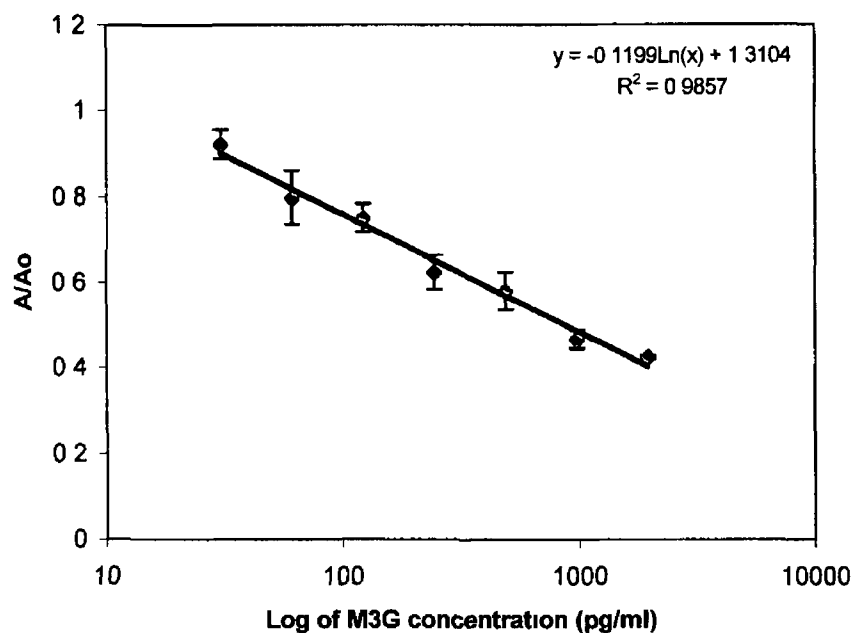
#### **3 2 4 2 Inter-day assay variability studies of R-1 and R-2 anti-morphine-3-glucuronide antibodies in urine**

To carry out inter-day variability studies, five sets of standards ranging from 10 to 50,000 pg/ml were prepared for each antibody and assayed five times over five days. The inter-day assay variability study for R-1 had a linear range of detection between 30 and 7,812 pg/ml as shown in Figure 3.18. The  $R^2$  value for this range was 0.99.

The CV's were determined and ranged from 4.71% to 8.31% as shown in Table 3.7. The inter-day assay variability study for R-2 had a linear range of detection between 30 and 1,953 pg/ml as shown in Figure 3.19. The  $R^2$  value for this range was 0.98. The CV's were determined and ranged from 1.04% to 7.77% as shown in Table 3.8. The percentage accuracy values obtained for both R-1 and R-2 ELISA inter-day assay studies in urine were found to be variable and are listed in Tables 3.7 and 3.8.



**Figure 3.18.** Inter-day competitive ELISA assay of R-1 antibody for determination of the optimal range of detection of free M3G in urine using an antibody dilution of 1/50,000. M3G-OVA was coated at a concentration of 12.5 µg/ml. The linear range of detection was found to be between 30 and 7,812 pg/ml.



**Figure 3 19.** *Inter-day competitive ELISA assay of R-2 antibody for determination of the optimal range of detection of free M3G in urine using an antibody dilution of 1/50,000 M3G-OVA was coated at a concentration of 1.56 µg/ml. The linear range of detection was found to be between 30 and 1,950 pg/ml.*

*Table 3 5 Intra-day variation for R-1 ELISA assay in urine. Five sets of nine standards were analysed on the same day*

<b>M3G Concentration, (pg/ml)</b>	<b>Calculated mean ± S.D., Abs</b>	<b>Coefficients of variation (CV's), %</b>	<b>% Accuracy</b>
7812	0 101 ± 0 008	8 47	26 48
3906	0 115 ± 0 002	2 28	2 58
1953	0 132 ± 0 002	1 91	18 12
976	0 150 ± 0 002	1 76	39 24
488	0 182 ± 0 014	7 80	8 60
244	0 203 ± 0 011	5 68	16 80
122	0 234 ± 0 017	7 52	5 81
61	0 257 ± 0 006	2 34	4 24
30	0 284 ± 0 010	3 80	12 00

*Table 3 6. Intra-day assay variation for R-2 ELISA assay in urine. Five sets of seven standards were analysed on the same day*

<b>M3G Concentration, (pg/ml)</b>	<b>Calculated mean ± S D , Abs</b>	<b>Coefficients of variation (CV's), %</b>	<b>% Accuracy</b>
1953	0 066 ± 0 0041	3 15	21 77
976	0 072 ± 0 0055	0 00	14 65
488	0 088 ± 0 0104	3 00	0 001
244	0 097 ± 0 0155	2 73	25 40
122	0 115 ± 0 0055	3 61	1 12
61	0 123 ± 0 0109	2 60	30 63
30	0 147 ± 0 0180	3 77	23 66



*Table 3 7 Inter-day assay variation for R-1 ELISA assay in urine. Five sets of nine standards were analysed over five different days*

<b>M3G Concentration, (pg/ml)</b>	<b>Calculated mean <math>\pm</math> S D , <math>A/A_0</math></b>	<b>Coefficients of variation (CV's), %</b>	<b>% Accuracy</b>
7812	0 338 $\pm$ 0 020	6 01	53 55
3906	0 380 $\pm$ 0 018	4 76	0 00
1953	0 435 $\pm$ 0 036	8 31	19 86
976	0 496 $\pm$ 0 040	8 16	35 86
488	0 598 $\pm$ 0 036	6 14	4 91
244	0 673 $\pm$ 0 036	5 35	4 09
122	0 772 $\pm$ 0 049	6 35	16 98
61	0 826 $\pm$ 0 050	6 13	0 00
30	0 9 6 $\pm$ 0 042	4 71	0 03

*Table 3 8 Inter-day variation for R-2 ELISA assay in urine. Five sets of seven standards were analysed over five different days*

<b>M3G Concentration, (pg/ml)</b>	<b>Calculated mean <math>\pm</math> S D , <math>A/A_0</math></b>	<b>Coefficients of variation (CV's), %</b>	<b>% Accuracy</b>
1953	0 422 $\pm$ 0 004	1 04	15 46
976	0 465 $\pm$ 0 021	4 61	18 13
488	0 578 $\pm$ 0 044	7 70	7 99
244	0 622 $\pm$ 0 040	6 50	27 45
122	0 749 $\pm$ 0 032	4 35	11 47
61	0 796 $\pm$ 0 061	7 77	18 03
30	0 920 $\pm$ 0 034	3 73	13 53

### 3 2 5 Cross reactivity studies of R-1 and R-2 polyclonal antibodies

The specificity of the polyclonal antibodies to morphine-3-glucuronide is critical for the performance of the immunoassay. Cross reactivity studies were carried out with a number of available drugs for this purpose using the ELISA developed as described in section 2 3 8 2. Microtitre plates were coated with 12.5 µg/ml of M3G-OVA for R-1 antibody and 1.56 µg/ml of M3G-OVA for R-2 antibody and blocked with 2% (w/v) marvel. Standards of morphine, codeine, nor-codeine and 6-monoacetylmorphine (6-MAM) were prepared and added to the microtitre plate at a 1:1 ratio with antibody at a dilution of 1/50,000. Table 3 9 shows the percentage inhibition of each compound.

*Table 3 9 Cross reactivity studies of affinity-purified anti-morphine-3-glucuronide polyclonal antibodies with structurally related molecules*

<b>Compound</b>	<b>Inhibition expressed as a percentage of the slope of R-1</b>	<b>Inhibition expressed as a percentage of the slope of R-2</b>
Morphine-3-glucuronide	100%	100%
Morphine	83%	42%
Codeine	92%	97%
Nor-codeine	0.8%	56%
6-monoacetylmorphine	97%	98%

### 3 3 Discussion

Polyclonal antibody production is the main focus of the work described in this chapter. Production of polyclonal antibodies begins with the preparation of a suitable antigen. Small haptens such as M3G (461.5 Da) are not immunogenic, and so require conjugation to a larger carrier-protein, such as BSA, OVA or THY (Hermanson, 1996). The coupling chemistry used to prepare such immunogenic hapten-carrier protein molecules is an important consideration for the production of antibodies with the correct specificity. Many methodologies for conjugation exist and vary depending on the functional groups which are available on both the antigen and the carrier molecule. Production of conjugates should also take into consideration the orientation of the antigen to produce optimal presentation to the immune system. When considering conjugation of morphine-3-glucuronide, the structure was analysed and a carboxyl group was located on the glucuronide. This carboxyl group may be used to conjugate morphine-3-glucuronide to amine groups present on proteins utilising carbodiimide coupling chemistry. This coupling process allows carbodiimide to react with carboxylic groups available on the M3G molecule at the glucuronide site. This forms a highly active *O*-acylsourea intermediate which reacts with the amine present on the carrier protein of interest to form an amide bond allowing the release of the EDC mediator.

Spectrophotometric analysis of conjugates could not be carried out due to both M3G and protein exhibiting the same characteristics, as both compounds show the presence of shoulders between 200 nm and 280 nm. This presented a problem for characterisation of conjugates by UV/visible spectroscopy. However, characterisation of conjugates was carried out by immunising *BALB/c* mice and monitoring of specific antibody produced. Initially, conjugates were thoroughly dialysed to remove small molecular weight components. Dialysis would remove any unbound M3G as the molecular cut off weight for the dialysis tubing was 10,000 daltons allowing M3G bound to protein to remain. If the conjugation reaction had failed to couple protein and M3G, M3G would be removed from the mixture. Immunisation of such a conjugate would only produce specific antibody to protein.

However, once coupling had occurred, immunisation of *BALB/c* mice would produce specific antibody to both protein and M3G. 10 days after immunisation to allow production of antibody, serum was taken from the mice and prepared as described in section 2.3.6.2. The same conjugate was used for both immunisation and screening. This specific antibody was monitored by ELISA as described in section 2.3.8.1. A titre of the antibody present in serum was carried out against the M3G-BSA conjugate and found to titre out at 1 in 200,000. The dilution of antibody was determined for a competitive assay, allowing the antibody to be the limiting factor in the assay by picking a dilution of antibody on the linear part of the curve (Figure 3.6). This resulted in the use of a 1 in 8,000 dilution of antibody. Competition assays were carried out to determine the antibody specificity to M3G. These competitive assays confirmed that specific antibody to M3G was present in serum. Figure 3.7 shows that the antibodies can detect free drug in a range of 100 to 100,000 ng/ml. This confirmed that M3G was coupled to BSA. Further conjugates were produced to OVA and THY and characterised in the same manner.

Polyclonal antibodies to morphine-3-glucuronide were then produced by immunising New Zealand White rabbits with the EDC/NHS coupled M3G-BSA conjugate. Once a titre of over 1/256,000 (Figure 3.8) from serum was obtained, the animal was sacrificed and the serum removed.

Initial purification of the antibodies from both animals was performed by saturated ammonium sulphate precipitation. Affinity purification of anti-M3G polyclonal antibodies was then carried out, employing high pH elution of the antibody from the protein G affinity column. Figure 3.9 illustrates the elution profile for both antibodies on a protein-G column. Characterisation of the purity of the antibodies was carried out by SDS-PAGE gel (Figures 3.10 and 3.11).

Both purified polyclonal anti-M3G antibodies were produced for the detection of the drug M3G in a competitive immunoassay format in which free drug in solution and surface bound M3G-OVA conjugate compete with one another to bind to antibody.

For this type of competitive assay to function effectively, a number of parameters must be optimised. The limits of detection in competitive ELISA's are a function of the antibody affinity, and the equilibrium between both the free and immobilised conjugate. As a result, too high a concentration of coating conjugate will shift the binding equilibrium in favour of binding to the ELISA plate and cause reduced sensitivity to free antigen in solution. Similarly, the concentration of antibody used must be the limiting factor. If this were not the case, antibody would be capable of binding to free drug and bound conjugate in excess, resulting in a signal that could not be measured. The optimal antibody concentration was determined by titres of the two polyclonal antibodies and taking between 50-70% of the maximum binding. Therefore, it is essential to optimise both the loading concentration of solid-phase conjugate and the optimal dilution of antibody.

Checkerboard ELISA's were carried out for both polyclonal antibodies for the determination of the optimal loading conjugate concentration and the optimal antibody dilution. Conjugate concentrations for R-1 and R-2 were found to be optimal at 12.5 (Figure 3.12) and 1.56  $\mu\text{g/ml}$  (Figure 3.13), respectively. These concentrations were chosen as increasing concentrations did not give an increase in signal and gave the widest linear detection range over the range of antibody dilutions used.

The antibody dilution used for both R-1 and R-2 polyclonal antibodies was 1/50,000 (Figures 3.12 and 3.13) resulting in these dilutions of antibodies being the limiting factor in the assay. These working dilutions for both R-1 and R-2 antibodies were picked from the linear portion of the antibody dilution curve, where minimal changes in antibody concentration give the greatest change in absorbance readings. Antibody dilutions chosen from the asymptotes of the antibody dilution curve show minimal changes in response to changes in antibody concentration and may not be used. Controls were also carried out by adding antibody to wells coated with OVA. Results reflect that there is negligible specific antibodies present to the screening conjugate protein.

A model competitive assay was developed for these antibodies in PBS where standards of M3G ranging from 4 to 500,000 pg/ml were prepared and mixed with purified polyclonal antibody at dilutions of 1/50,000. Both antibodies showed competition within these ranges. R-1 showed a range of detection between 244 to 250,000 pg/ml (Figure 3.14) and R-2 showed a range of detection between 61 to 31,250 pg/ml (Figure 3.15). Both assays displayed excellent linearity and both antibodies show very low limits of detection. Intra-day and inter-day assay variability statistics were then analysed in an attempt to look at the reproducibility of the assay. Intra-day and inter-day assay coefficients of variation (CV's) for R-1 were found to be between 1.29 to 4.68% and 1.63 to 6.86%, respectively, for 5 replicates (Tables 3.1 and 3.3). Intra-day assay CV's for R-2 were found to be between 1.81 to 6.13% for 5 replicates, however, inter-day assay CV's for R-2 were found to be higher and ranged between 3.06 to 11.72% (Tables 3.2 and 3.4). These results indicate that using R-1 anti-M3G antibody gives an accurate, specific and reproducible assay. The low nature of the CV's for R-1 shows that the assay may be used to detect real samples. However, the CV's for the R-2 assay were higher indicating that the assay may not provide as accurate a result as the R-1 antibody, however the R-2 antibody was still suitable for this assay format. Studies carried out on the percentage accuracy levels for both R-1 and R-2 intra- and inter-day assays were found to be relatively high resulting in the assay becoming more applicable as a confirmatory assay for the use of illicit drugs.

Having shown that both polyclonal antibodies were suitable for use in an assay of this type, the aim was to develop a method of detection of M3G in complex biological matrices. The matrix of study in this chapter is urine.

As already discussed in section 3.1.3 the main metabolites of heroin and morphine are known as morphine-3-glucuronide (M3G) (60%) and morphine-6-glucuronide (M6G) (10%) (Yoshimura *et al*, 1969, Oguri *et al*, 1970, Yeh *et al*, 1979). This characteristic of morphine-3-glucuronide is significant as detection of this compound directly relates to heroin or morphine usage. The development of an immunoassay

for the detection of M3G in urine can be applied to relate back to use of heroin or morphine due to both of these molecules producing M3G as one of their metabolites

A competitive assay in urine was developed for both anti-M3G antibodies to investigate the ability of the antibodies to detect real samples. Standards of M3G ranging from 10 to 50,000 pg/ml were prepared in urine and mixed with purified polyclonal antibody. Both antibodies showed competition within these ranges. R-1 showed a range of detection between 30 to 7,812 pg/ml and R-2 showed a range of detection between 30 to 1,953 pg/ml. These results when compared to the assays developed in PBS are favourable as the detection limits for both antibodies have decreased when using urine as the biological matrix. R-1 antibody has lowered its detection range by three orders of magnitude, from a lower detection limit of 244 pg/ml in PBS to 30 pg/ml in urine. R-2 has also lowered its detection limits from 122 pg/ml in PBS to 30 pg/ml in urine. This is a very favourable result as assays with the ability to detect low quantities of the drug are desirable. However, the advantage of lower detection limits for these assays also produces a disadvantage as the overall range of detection of the drug decreases. Intra-day and inter-day assay variability statistics were again analysed in an attempt to look at the reproducibility of the assays in urine. Intra-day and inter-day CV's for R-1 were found to be between 1.76 to 8.47% and 4.71 to 8.31% respectively for 5 replicates (Tables 3.5 and 3.7). Intra-day CV's for R-2 were found to be between 0.00 to 3.77% for 5 replicates, however, inter-day CV's for R-2 were found to be higher and ranged between 1.04 to 7.77% (Tables 3.6 and 3.8). The low nature of the CV's demonstrates the applicability of the assay to be used in the analysis of samples. Further studies were carried out on the percentage accuracy levels for both R-1 and R-2 intra- and inter-day assays in urine and results produced relatively high values which correlated to the results found when the assays were performed in PBS resulting in these assays becoming more applicable for use as a confirmatory test for drug abuse.

Cross reactivity is defined as a measure of the antibody response to structurally similar molecules. One of the initial steps in designing an immunoassay is the

assessment of reactivity toward structurally similar molecules (Wild, 1994) Serial dilutions of the cross reactive analyte of interest are prepared with similar dilutions of the specific antigen and a competitive ELISA carried out as described in section 2.3.8.1 Table 3.9 shows the percentage inhibition of each compound using the slope of the line, if 100% inhibition equals the slope of the line for the M3G immunoassay Competitive assays were carried out with a range of metabolites of heroin Morphine, codeine, 6-monoacetylmorphine and nor-codeine standards were prepared and added onto a microtitre plate with a mixture of both R-1 and R-2 antibodies at dilutions of 1/50,000 Results for both R-1 and R-2 were comparative except for their recognition of morphine and nor-codeine R-1 antibody shows twice as high percentage recognition for morphine as R-2 antibody and while R-1 antibody shows a negligible response to nor-codeine, R-2 antibody shows 56% cross reactivity The cross-reactivity of the affinity-purified polyclonal antibodies demonstrates an almost identical cross-reactivity to codeine and 6-monoacetylmorphine These results show that both antibodies possess high cross reactivity with the metabolites of heroin However, as M3G is the main metabolite the assay may still be used for real sample analysis

Overall, the competitive immunoassay for quantitative determination of M3G in urine compares well with the traditional analytical methods The assay developed in our lab has extremely low levels of detection While many of the ELISA based assays have concentrated on the detection of heroin and morphine, many of the traditional analytical tools have been used for the development of assays to heroin's metabolites

The assays developed in our lab to M3G compare favourable to assays in the literature Of the analytical techniques discussed in section 3.1.4, the HPLC method developed by Meng *et al* (2000) has a lower limit of detection As very little work has been carried out in the development of ELISA assays to M3G, morphine ELISA assays are used as a comparison of limits of detection of this family of molecules Radioimmunoassays developed by Spector *et al* (1971) and Steiner *et al* (1978) have limits of detection of 50 pg/ml and 500 pg/ml, respectively, for morphine In comparison to the detection



limits of these assays, the assay developed in our lab compares very well. Our assay is also a much simpler assay, with lower costs and does not require a radiation suite, as in the case of these assays. One ELISA assay developed for detection of morphine by Usagawa *et al* (1993), however, in comparison to the assays developed in our lab has proved to possess limits of detection as low as 100 pg/ml and thus possesses similar detection limits compared to our assays. With respect to the detection of opiates using HPLC, GC or MS methods as described in section 3.1.4, even though these methods can detect low concentrations of drug, these methods require many clean up steps and are expensive resulting in the need for alternative methods of detection.

## **Chapter 4**

### **Development of a BIAcore-based Inhibition Immunoassay for the Detection of Morphine-3-glucuronide in Biological Matrices**

## 4 1 Introduction

### 4 1 1 Biosensors

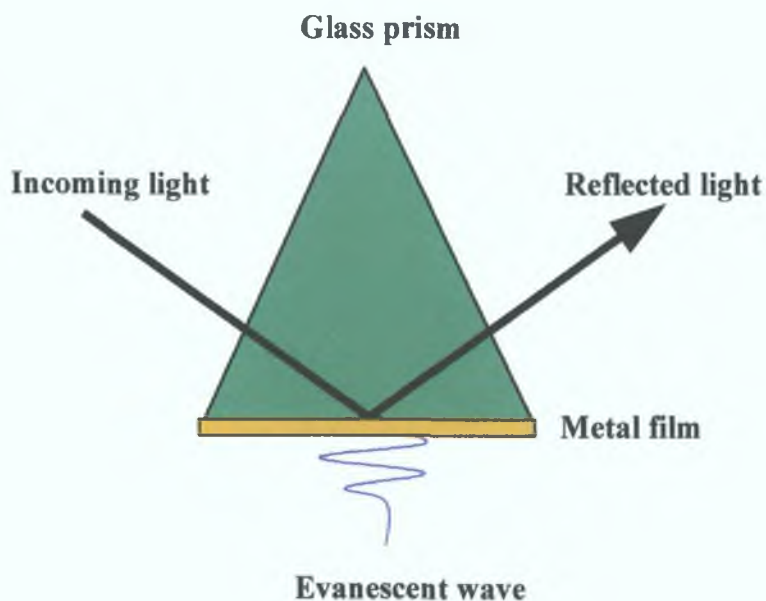
The development of rapid analytical devices such as biosensors for the detection of compounds has grown enormously over the past few decades (Pancrazio *et al* , 1999, Rogers, 2000) Biosensors are devices incorporating a biological sensing element coupled to a variety of transducing mechanisms (i.e. electrochemical, optical-electronic) When biological molecules interact specifically and reversibly, a change occurs in one or more physio-chemical parameters associated with the interaction This change causes the production of ions, electrons, gases, heat, mass or light These quantities are then converted by transducers into electrical signals and displayed in a suitable form The transducer translates the response of the biological component into a readable electronic signal (Rogers, 2000) The use of biosensors has ranged from environmental monitoring (Dennison and Turner, 1995), medical applications (Connolly, 1995) to the detection of coumarins (Keating *et al* , 1999) and mycotoxins (Daly *et al* , 2000)

The biological component of a biosensor should possess a high degree of specificity and stability, should not contaminate the sample and should retain biological activity when immobilised (Hall, 1990) The specificity of the biosensor is completely dependent on the properties of the biological component as the biological component must recognise the analyte for there to be an interaction Biosensors may be divided into two categories, the catalytic sensor and the affinity sensor The catalytic sensor works on the principle that the molecules bind and alter the analyte in some way The affinity-based sensors actually monitor the binding between the two molecules The use of biomolecules and biological systems in biosensors was reviewed by McCormack *et al* (1998), and may be divided into the following groups: enzymes, antibodies, whole cells, receptors, tissues, nucleic acids, lectins and antigens Recently, several commercial biosensors have been developed using surface plasmon resonance (SPR) technology as their detection method In particular, the BIAcore

SPR-based biosensor has become a very important tool in the detection of a number of analytes.

#### 4.1.1.1. Surface plasmon resonance (SPR) biosensors

Several biosensors have been developed based on the phenomenon of surface plasmon resonance. This phenomenon allows the detection of biomolecular interactions in 'real-time'. The principle behind SPR is described in the context of BIAcore for convenience. At an interface between two media of different refractive index (e.g. glass and water), light coming from the side of the higher refractive index is partly reflected and refracted. Above a certain critical angle of incidence, the light is totally internally reflected and no light is refracted across the interface between the two surfaces of different refractive index.

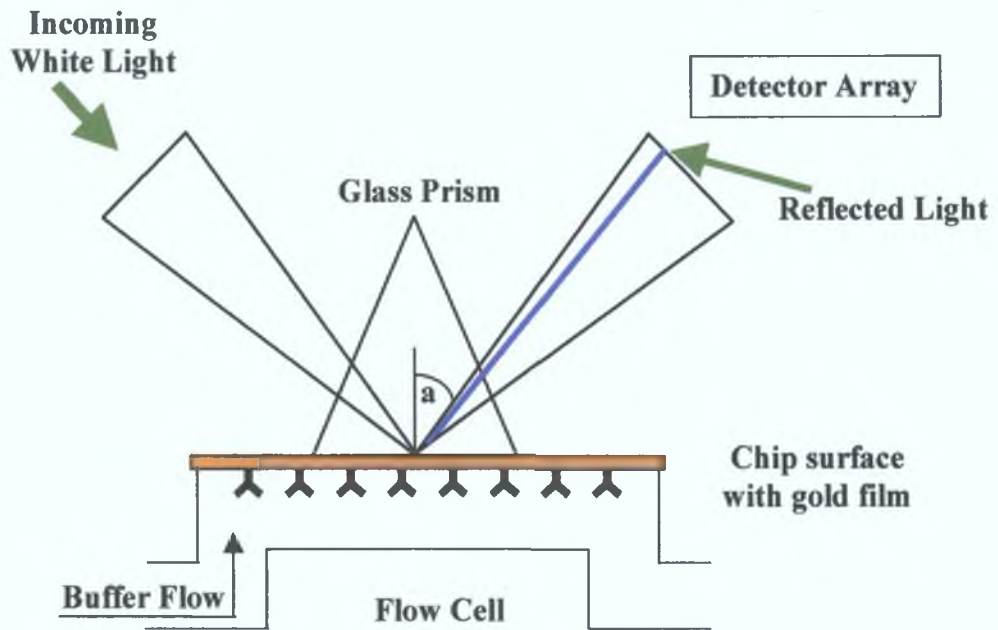


*Figure 4.1. Under conditions of total internal reflection at a metal-coated interface, an evanescent wave propagates into the medium of lower refractive index on the non-illuminated side.*

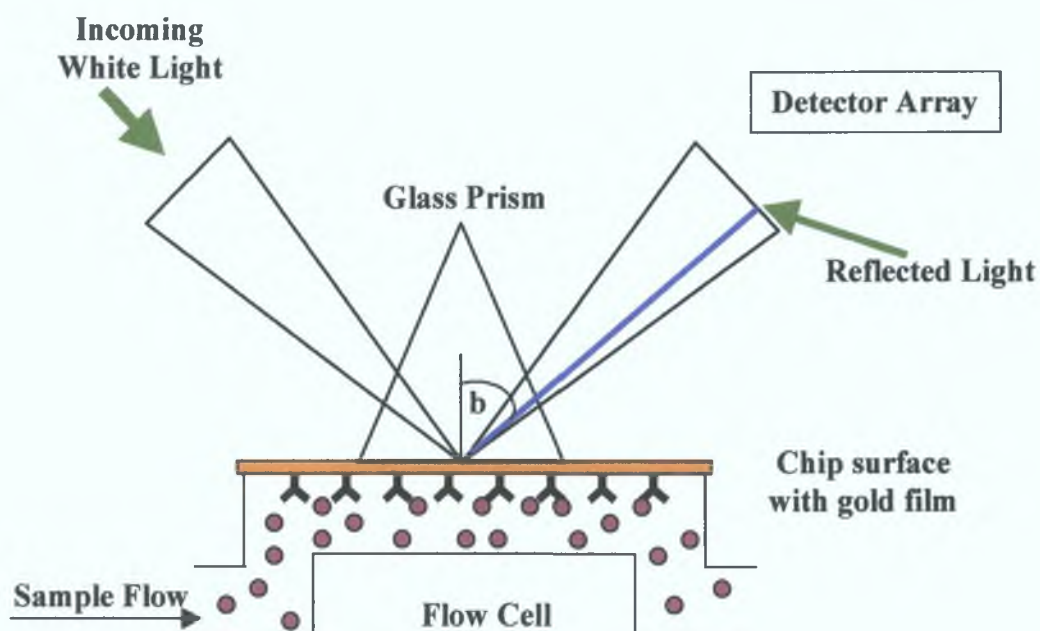
Under total internal reflection conditions (TIR), an electromagnetic field component called the evanescent wave penetrates into the medium of lower refractive index a short distance in the order of one wavelength (Figure 4 1) As the evanescent wave moves further away from the interface into the lower dense medium, the wave decays exponentially If the interface between the media is coated with a thin layer of metal (in the case of BIAcore, this metal layer is gold), containing electron clouds at the surface and the passage of the evanescent wave through this metal layer causes the plasmons to resonate which results in a quantum mechanical wave known as a surface plasmon Some of the energy of the reflected light (incident light) is taken up by the surface plasmon wave, resulting in a dip in the intensity of reflected light at a certain angle being observed (Panayotou *et al*, 1993) The incident light angle at which this dip is observed is known as the SPR angle The SPR angle is dependent on a number of factors These factors include the properties of the metal film (e.g thickness, uniformity and composition), the wavelength and polarisation of incident light and the refractive index of the media on either side of the metal film In real-time BIA, the properties of the metal film, the wavelength and refractive index of the denser medium are kept constant The SPR signal can be used to monitor the refractive index of the aqueous layer immediately adjacent to the gold metal layer The light source in the BIAcore instrument is a high-efficient light emitting diode with a wavelength in the near infra-red region This light is focused on an interface consisting of glass and gold on the sensor chip in a wedge shaped beam, producing a fixed range of incident angles (Figure 4 2) A two-dimensional diode array is used to monitor the reflected light Changes in the refractive index are a direct result of changes in the mass or concentration on the surface of the chip and this characteristic of SPR has been used to monitor biological interactions (Figure 4 3) The change in reflected light is interpolated as a sensorgram (Figure 4 4)

At the heart of the BIAcore instrument, as with the majority of SPR-based commercially available instruments, is the sensor chip as shown in Figure 4 5 The BIAcore sensor chip consists of a glass slide with a thin layer of gold deposited on one side Gold was chosen as it possesses the characteristics of chemical inertness

and good SPR response. This gold layer was in turn covered with a covalently bound carboxymethylated dextran matrix attached by a hydroxyalkyl thiol linker layer. The matrix allows the covalent immobilisation of analytes onto the surface of the chip and increases sensitivity by increasing the binding capacity of the surface.

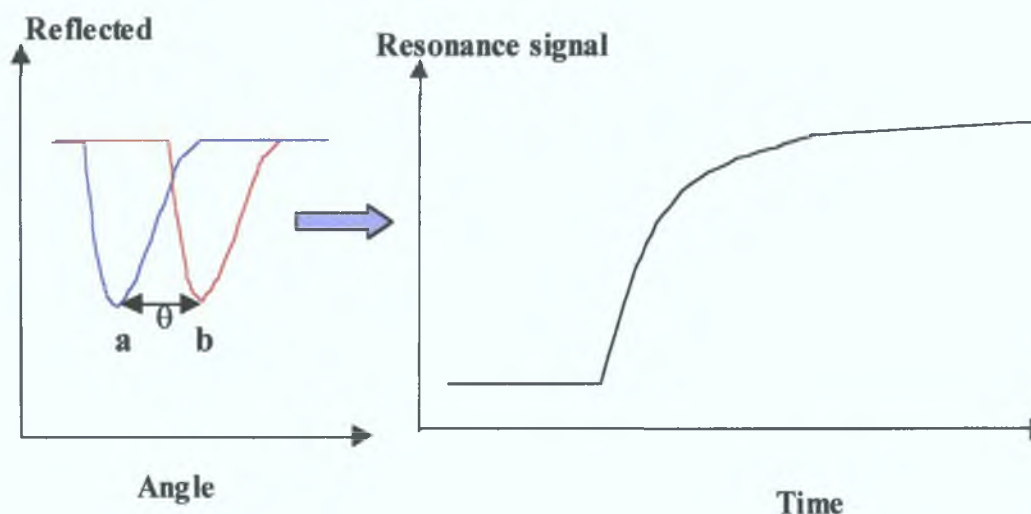


*Figure 4.2. Shows the operation of surface plasmon resonance (SPR) in the BIAcore biosensor instrument. Antibody (Y) is immobilised onto the surface of the chip using conventional EDC/NHS chemistry. Light from a high intensity light emitting diode (LED) is focused onto the gold chip surface by means of a prism under conditions of total internal reflection. A two-dimensional photo-diode array was used to measure the reflected light. Under conditions of total internal reflection at a metal-coated interface, an evanescent wave propagates into the medium of lower refractive index on the non-illuminated side. This leads to a dip in the intensity of reflected light at a particular angle known as the SPR angle (SPR angle = angle a) and this is shown as a blue line in the Figure above. This SPR angle is sensitive to a variety of factors such as the refractive index at the gold film side of the interface and any biospecific interactions.*



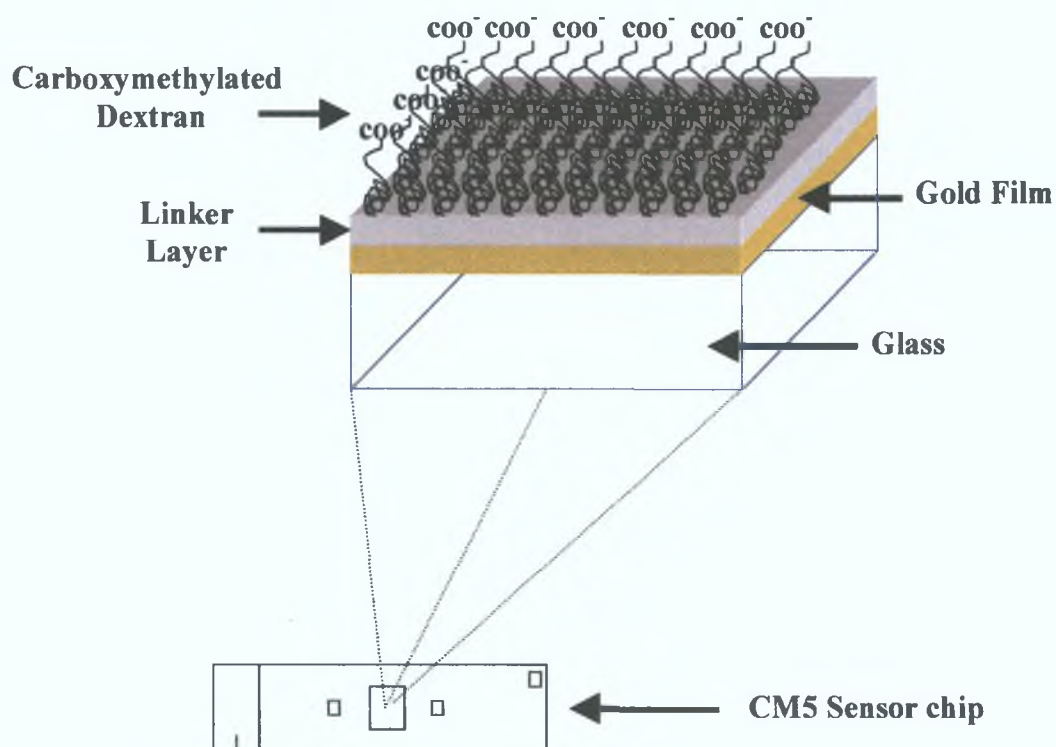
*Figure 4.3. Shows the injection of antigen (\*) over a chip surface immobilised with antibody (Y). The specific interaction of antigen binding to the antibody causes an increase in the mass bound at the sensor chip surface resulting in changes in the refractive index. This change in the refractive index is responsible for a shift in the resonant angle of the reflected light as the angle changes from a in Figure 4.2 to b above. As changes in the resonant angle are a direct result of changes in the mass or concentration on the surface of the chip, the mass of analyte binding to the chip surface may be determined.*



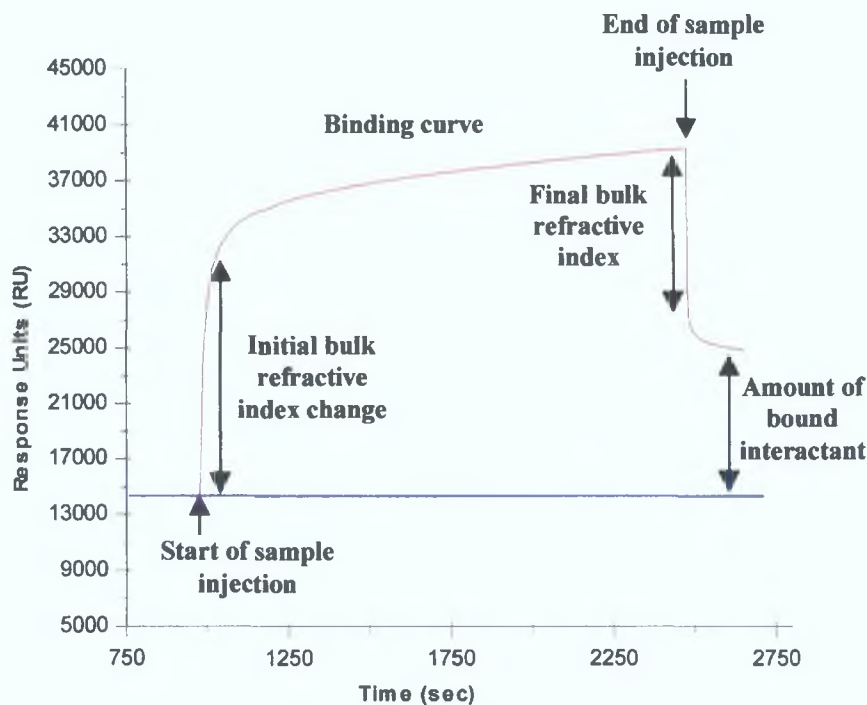


*Figure 4.4. The SPR angle may be seen as a dip in the intensity of the reflected light. As an antibody:antigen interaction occurs, changes in the resonant angle ( $\theta$ ) are monitored continuously and displayed as a sensorgram. Biomolecular interactions at the chip surface causes a mass change and an increase in the SPR angle which is seen as a gradual increase in the signal of the sensorgram. The signal is interpolated into response units (RU) by the instrument software. An increase of 1000 RU as a result of a biomolecular interaction corresponds to approximately 1 ng/mm<sup>2</sup> of protein present on the chip surface.*

The matrix also provides a hydrophilic environment with very low non-specific binding. The matrix forms one wall of a micro-flow cell where interactions are monitored. The carboxymethylated side of the chip comes in contact with the solution of interest, while the gold side of the chip is illuminated from the other side, through the glass. SPR is generated through the interaction of the light energy with the gold film and this is used to monitor concentrations of analyte on the surface of the chip. There are currently a wide variety of different sensor chips available for the BIAcore instrument for varying purposes. However, all chips use the same optical principle.



*Figure 4.5. Diagram representing the surface of the BIAcore carboxymethylated 5 (CM5) sensor chip. The surface of the chip consists of three layers: glass, a thin gold film and a carboxymethylated dextran layer. The carboxymethylated dextran matrix is bonded to the gold film through an inert linker layer. This matrix allows the covalent immobilisation of analytes onto the surface of the chip.*



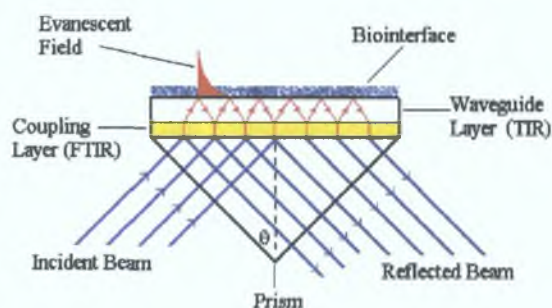
*Figure 4.6. Schematic sensorgram illustrating the binding of analyte to immobilised ligand. Before the start of the injection of sample the initial steady baseline is due to the flow of running buffer over the chip surface. As the sample is injected over, the initial bulk refractive index changes causing a sharp increase in the signal. This is followed by a steady increase in signal over the course of the injection as binding of the two interactants is allowed to occur. At the end of the sample injection a sharp decline or final bulk refractive index change in the signal is observed as the running buffer is again passed over the surface which is a result of a change in the refractive index of the buffer. The difference in the recorded response units (RU) between the signal after the injection and the initial baseline response units corresponds to the amount of interactant that remains bound to the sensor chip surface.*

A typical sensorgram for binding of analyte to immobilised ligand is shown in Figure 4.6. Running buffer flowing over the surface at a fixed volume per min produces the initial level baseline at the sensor surface. A sharp rise in signal is then observed at the precise moment of the injection of sample over the surface. This is due to a difference in the bulk refractive index between the running buffer and the sample buffer. The binding can be seen as an initial jump or initial bulk refractive index change in the signal followed by a steady increase in signal over the course of the injection. This is because large quantities of analyte bind followed by the sensor surface slowly becoming saturated resulting in the steady signal. At the end of the sample injection, a sharp decrease in the signal is observed as running buffer of lower refractive index is again allowed to pass over the surface. The difference in the recorded response units (RU) between the signal after the injection and the initial baseline response units corresponds to the amount of interactant that remains bound to the sensor chip surface. A change in response unit signal of 1000 RU corresponds to a change in surface concentration on the sensor chip of about  $1 \text{ ng/mm}^2$  (Stenberg *et al.*, 1991).

The BIAcore biosensor has a number of major advantages compared with conventional detection techniques. The absence of a need for labelling is of major significance as it minimises interference with the binding interaction being studied and also eliminates expensive and time-consuming purifications in many situations. Real-time analysis of interactants is also one of the main advantages of the BIAcore instrument as interactions may now be monitored as they occur providing valuable diagnostic information as well as kinetic data. Further advantages include the ability of the chip to be reused a significantly large (up to 1000 times) number of times and also the ability of the instrument to allow rapid and automated analysis. The BIAcore instrument is provided as a complete system, which includes processing unit, sensor chip, controlling computer and the appropriate software for data collection and analysis.

#### 4.1.1.2. IAsys biosensor

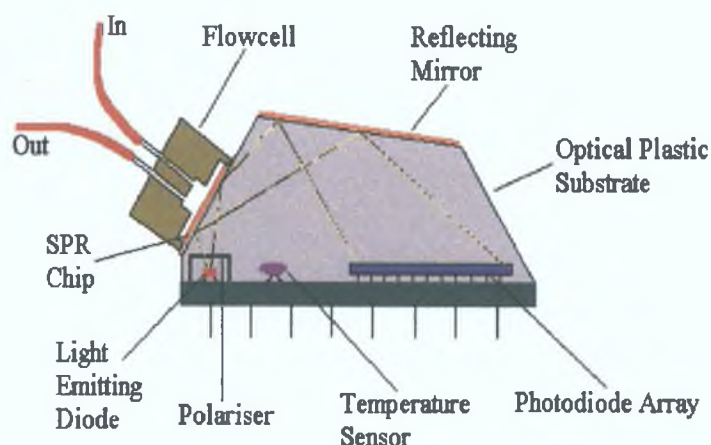
The IAsys series of optical biosensors are analytical instruments that utilise advanced resonant mirror optical biosensor technology and apply it to the recognition of biomolecules. A dielectric layer of high refractive index is used instead of the gold sensing layer in BIAcore (Cush *et al.*, 1993). The sensor consists of a glass prism coated with a thin layer of silica and high refractive index resonant layer (e.g. titanium), which is in contact with the sample solution (Figure 4.7). Polarised laser light is directed at the prism and under conditions of total internal reflection illuminates the underside of the sensor surface at angles greater than the critical angle. At one angle which is known as the resonant angle, a component of light can couple through the low refractive index layer and propagate along the high refractive index layer before being coupled back into the prism. During resonance a phase shift occurs between the reflected electric (TE) and magnetic (TM) modes resulting in a phase shift in the measured response which is observed as constructive interference and can be measured in 'real-time'. The resonant angle at which coupling occurs is essentially dependent on the refractive index at the surface of the sensor. As a result, changes in the refractive index or mass will change the resonant angle corresponding to signal increases as mass increases and signal decreases as mass decreases.



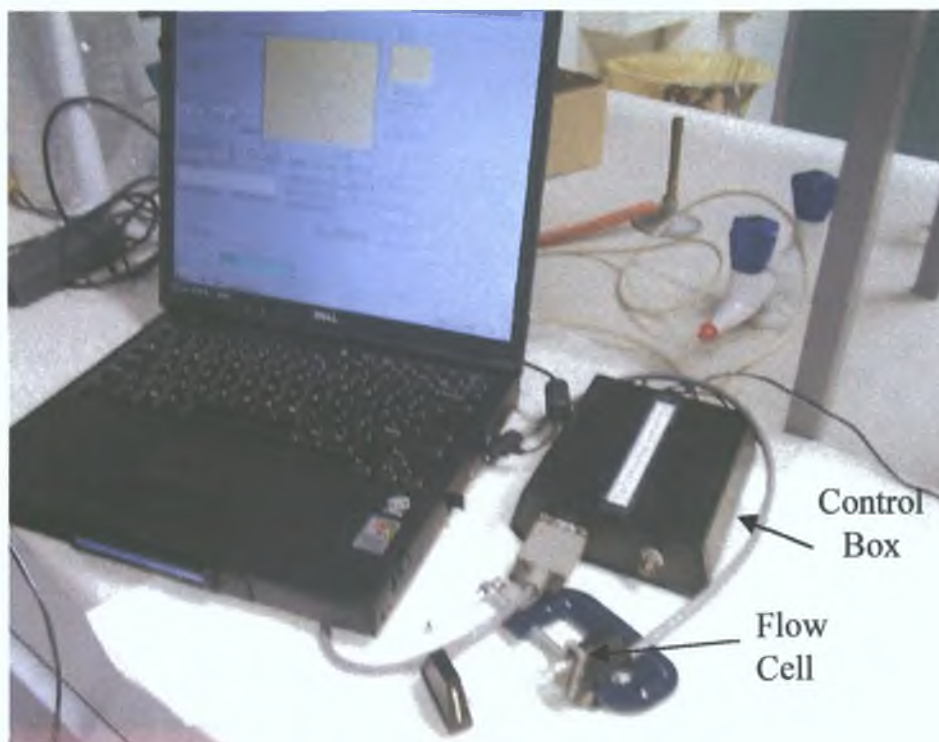
*Figure 4.7. Illustration of the resonant mirror configuration. Incident light is passed through a prism and undergoes total internal reflection at the boundary with the low refractive index layer and generates an evanescent field. The resonant angle at which coupling occurs is essentially dependent on the refractive index at the surface of the sensor. Binding interactions can be monitored using this instrument as shifts in reflected light are related to mass change on the sensor chip surface.*

#### 4.1.1.3. Miniature TI-SPR sensor

The miniature TI-SPR device was first released in 1996 by Texas Instruments, and consists of a light emitting diode (LED), polarizer, thermistor allowing correction due to temperature changes and two 128 silicon photo diode arrays (Figure 4.8). These components are mounted on a single platform using conventional semi-conductor-based opto-electronic manufacturing techniques (Melendez *et al.*, 1996). The platform is encapsulated in an epoxy resin moulding structure in the form of the Kretschmann geometry prism. The width of light produced by the light emitting diode is controlled by the polarizer and reduces the emission of transverse electric radiation. Under conditions of total internal reflection the wedged shaped beam is directed onto a linear photo diode array by a mirror. An SPR-induced minimum is determined by processing the signal from the photo diode array in 'real-time' using dedicated signal software (Kukanskis *et al.*, 1999). Temperature fluctuations can also be corrected during analysis as there is a built in temperature sensor in the device. This system is available in a hand-held format and is known as the Spreeta™ device which possesses a control box housing the components of the instrument, which may be attached to a laptop to produce a portable device (Figure 4.9).



*Figure 4.8. Cross-section of miniature TI-SPR instrument. The instrument consists of an electro-optical component mounted on a single platform encapsulated in an optically transparent plastic. Changes in mass are related to changes in the resonant angle and this change is measured by the photodiode array.*



*Figure 4.9. Schematic of TI-SPR device fitted with flow cell. A control box interfaces with the device and this may be installed on a personal computer. SPR-MINI MS-Windows software can be used for data acquisition and processing to produce a display via a virtual control panel as shown on the laptop.*

#### **4.1.2. Applications of SPR technology**

With the birth of SPR technology and the ability to monitor interactions between biomolecules in ‘real-time’ the applications involving SPR technology has grown quickly over recent years. The characteristic of label-free detection of binding interactions between molecules is also considered to be of great significance in the future development of assays as previous labelling procedures have shown to affect the binding activities of antibodies (Høyer-Hansen *et al.*, 2000). Applications of SPR biosensing systems have been used by Quinn *et al.* (1997) for the detection of blood group antigens in whole blood using antibodies. Assays have also been developed using the SPR-based BIAcore biosensor, for detection of aflatoxin B<sub>1</sub> (Daly *et al.*,

2000) These applications show that SPR technology is a powerful technique in the study of both surface properties and surface interactions. SPR technology has the potential to measure a number of factors ranging from rates of adsorption, total amounts of immobilised material, degree of desorption of material from the surface, relative binding affinities, equilibrium constants, association of analyte on the surface and dissociation of analyte off the surface. These can all be measured quickly and in 'real-time'.

#### **4.1.2.1 Application of SPR technology for the measurement of small molecular weight analytes and characterisation of target molecules**

The most obvious problem with SPR technology is the analysis of small molecules. As changes in the refractive index are mass dependent, the ability of the sensor to detect low molecular weight compounds is compromised. This problem however may be overcome by linking small molecules to larger carrier molecules. When developing BIAcore-based assays for detection of these small molecular weight molecules, the format used most frequently is an inhibition assay. In this assay format, the immobilisation of the analyte is performed by immobilisation of a carrier molecule with the analyte of interest attached. Standards of free analyte mixed with specific antibody are then passed over the surface. Excess antibody is allowed to bind to the surface and the amount of antibody bound is inversely proportional to the amount of free analyte in solution.

Daly *et al* (2000) developed an inhibition assay for the detection of aflatoxin B<sub>1</sub> using carrier proteins (BSA) as the ligand for immobilisation onto the surface of the chip. Polyclonal antibodies were produced to aflatoxin B<sub>1</sub> and found to be regenerable using an organic solution consisting of 1 M ethanolamine with 20% (v/v) acetonitrile, pH 12.0. The polyclonal antibodies were added to standards of free aflatoxin and passed over the surface immobilised with aflatoxin B<sub>1</sub>-BSA. The remaining free antibody bound to the surface of the chip, providing a signal inversely proportional to the amount of free aflatoxin in solution.



Keating *et al* (1999) also developed an inhibition assay for the determination of total-7-hydroxycomarin (7-OHC) in human serum samples using the BIAcore biosensor. A 7-OHC-BSA conjugate was produced and immobilised onto the chip surface. Standards of free 7-OHC were premixed with a polyclonal anti-7-OHC antibody and injected over the surface. As in the previous assay, excess antibody bound to the immobilised conjugate, generating a binding response that was inversely proportional to the amount of 7-OHC in solution. The assay had a range of detection for 7-OHC of between 0.5 to 80 µg/ml.

The BIAcore biosensor has been used to develop a number of different assays to various analytes. Hains and Patel, (1995) developed an inhibition BIAcore assay for the detection of food borne pathogens *Salmonella* and *Listeria*. Polyclonal antibodies to bacterial cell wall antigens were added to cultures of food samples and filtered to remove all bacteria. The filtrate was passed over a chip surface with anti-Fab antibody immobilised, producing a signal when free antibody bound to the surface.

Further developments in SPR technology have been made with the development of the BIAcore 3000 instrument. This instrument has increased sensitivity, reduced flow cell size compared to the BIAcore 2000 and possesses on-line reference curve subtraction, enabling better detection of small molecular weight analytes (Morton & Myska, 1998).

Markgren *et al* (1998, 2000) monitored the binding of an inhibitor to HIV-1 proteinase and showed how the BIAcore instruments may be used in the selection of binders. HIV-1 was immobilised onto the surface of a chip and inhibitors were detectable by analysis of their association and dissociation rates. Malmquist, (1999) demonstrated the ability to monitor the weak-affinity interaction of lactose binding to an immobilised antibody. Nieba *et al* (1996) developed a method utilising competition assays on BIAcore for measuring the true affinities of binding interactions. Kampranis *et al* (1999) also studied the binding kinetics between wild-type and mutant DNA gyrases to novobiocin. Results from the SPR analysis allowed the identification of residues responsible for binding of novobiocin to the DNA gyrases.

This ability of the BIAcore biosensor instruments to monitor the binding interactions of small molecules will have a significant impact on the pharmaceutical industry. Previous characterisation and development of a small molecule drug was estimated to take 5-7 years with a cost of \$500 million dollars. However, the technology is now available for the identification of potential new drug molecules more rapidly and with less expense. The lack of labelling requirements and the ability of the BIAcore instruments to use very low quantities of sample enables the BIAcore to be used as a secondary screening tool. BIAcore has the ability to provide binding information not available from normal screening methods as the interaction may be monitored in 'real-time' while also providing both affinities and binding kinetics (Markgren *et al*, 2000). Small molecule detection may also be useful in pharmacological studies. An important example of this is the ability to screen drug candidates. These may be screened for binding to carrier plasma proteins and lipoproteins as well as for membrane permeability. Thus, information may be acquired on the oral adsorption to, and transfer across, the gastrointestinal tract and exchange across the blood/organ barrier of these molecules (Rich & Myszka, 2000).

#### **4 1 2 2 Membrane surface applications**

As advances are made in SPR technology, new areas of analysis become possible. One such area of analysis is the monitoring of protein interactions with lipid surfaces and membrane-associated proteins. Such analysis is possible using the newly developed HPA (hydrophobic) and L1 (lipophilic) sensor chips produced by BIAcore to create stable membrane surfaces within the flow cell. The HPA chip possesses a self-assembly monolayer of alkylthiols covalently linked to the gold surface. It is then possible to fuse liposomes to this surface to form a hybrid lipid monolayer (Evans & MacKensie, 1999). This monolayer may then be used to monitor interactions. The L1 chip differs as it contains an alkyl chain immobilised to the dextran matrix. In this case, liposomes are captured on the surface, forming a stable lipid bilayer which mimics the biological membranes (Rich & Myszka, 2000).

Further advantages of using lipid surfaces on such chips may be seen in the ability to specifically orientate the proteins within the membrane and also multiple interactions may be monitored due to the fluidity of the membrane resulting in protein diffusion through the membrane (Celia *et al*, 1999) Celia *et al* (1999) observed the binding of soluble T-cell receptors (TCRs) to major histocompatibility complex (MHC) molecules attached to the membrane Danelian *et al* (1999) described a new method to estimate the fraction of drug absorbed in the human intestine based on sensor technology Liposomes were immobilised onto the surface of the chip and the interaction between drugs and liposomes were monitored directly using SPR technology In all 27 drugs were studied and results correlated with previous studies carried out on fractions of drugs absorbed by humans

#### **4 1 2 3. Proteomics**

Proteomics is an area of research devoted to determine the function of the encoded protein repertoire to find out how it regulates the behaviour of individual cells and, ultimately, the whole animal throughout its development both in health and disease In its infancy, proteomics was the word used to describe technology that allowed large scale protein separation and mass identification In a paper by Celis *et al* (1989) the expression of proteins was analysed at the same time from human cells in order to study cell proliferation and differentiation This study, to provide an overview of a number of proteins expressed at one time when a cell is carrying out a particular function, is recognised today as proteomics However, recently proteomics has divided into three areas, protein profiling, interaction analysis and structural genomics Characteristics of the BIAcore instrument are real-time monitoring, an auto injection system and the ability to analyse large numbers of samples while using small amounts of material These provide a platform for analysis in the area of proteomics (Natsume, 2000) Generally 2D gel electrophoresis is used to separate and analyse 5,000 proteins simultaneously Identification of the proteins is carried out by cutting the proteins from the gel followed by digestion with trypsin into fragments The fragments are then identified by comparing them with databases of

protein or DNA sequences with the use of mass spectrometry. However, this process is very time consuming and this is why new quicker methods of identification are being developed. Sonksen *et al* (1998) developed a method, which combined BIA technology with Mass spectrometry. Affinity bound molecules were recovered from the surface of the BIAcore chip in a few microliters, ready for MS analysis. BIAcore provides information on the concentration of protein bound to the surface of the chip while MS reveals the identity of the compound by molecular mass determination. Calculation of the total surface molar concentration of affinity-bound molecules was possible by combining the information provided by the two instruments.

#### **4 1 3 BIAcore as a tool in antibody engineering**

Antibodies with the desired affinities and specificity's for use in biomedical or industrial applications are required. Hybridoma technology has been used for this purpose for more than a decade. However, recent developments in genetics have produced an explosion in the development of genetically engineered antibodies (Parmley & Smith, 1988, McCafferty *et al*, 1990). With this new technology, screening of combinatorial phage display libraries prompted the development of novel systems capable of selecting antibodies of the desired specificities. The BIAcore biosensor provides a simple and rapid approach for screening of recombinant antibody fragments, kinetic selection of phage displayed antibodies, characterisation and epitope mapping of monoclonal antibodies and their fragments (Malmborg & Borrebaeck, 1995).

In the past, ELISA has been the most common method used for screening recombinant antibodies. However, due to the nature of the ELISA method being based on equilibrium, antibodies with high "off-rates" may be lost. This problem may be solved with the used of BIAcore as this instrument enables the evaluation of the entire binding pattern. An example of this was published by Marks *et al* (1992) where bacterial cultures were screened for positive antibodies by direct injection into the biosensor without any previous purification. Clones were selected with up to 300 times improved affinities using this method. Malmborg *et al* (1996) also showed

that it is possible to increase specific phage titres by 10-57% by using elution and re-screening of eluted phage selection procedures on BIAcore

The BIAcore instrument may also be used to further characterise already screened clones. Deng *et al* (1994) reported that low affinity anti-carbohydrate antibody clones when subjected to random mutagenesis resulted in clones with higher affinities being obtained. In this case the BIAcore was used to characterise these clones and it was found that the increased affinity was mainly due to an increased association rate constant.

BIAcore may also be used to perform epitope mapping allowing the complete characterisation of interactions by studying the stoichiometry and kinetic characteristics of antibodies. Fagerstam *et al* (1990) characterised epitopes on recombinant HIV-1 core protein p24 for 29 mouse monoclonal antibodies by fixing antibody on the surface with capture antibody followed by injection of antigen. This was followed by injection of a further antibody to analyse if each antibody bound to a different epitope on the antigen.

Finally, SPR technology may be used to determine the active concentration of single chain antibody concentrations in crude periplasmic fractions (Kazemier *et al*, 1996). This allows screening of antibodies with respect to their affinities, as the concentration of active antibody is required for this purpose.

The development of BIAcore-based immunoassays for the detection of M3G as a model system in PBS and in biological matrices is investigated in this chapter. Two polyclonal antibodies were developed and used for an inhibition-based BIAcore immunoassay application. The ability of the model assays to measure free drug concentrations was studied. Subsequently, the assays were developed and used in the measurement of M3G in urine samples. The assays were also assessed with respect to accuracy and precision.

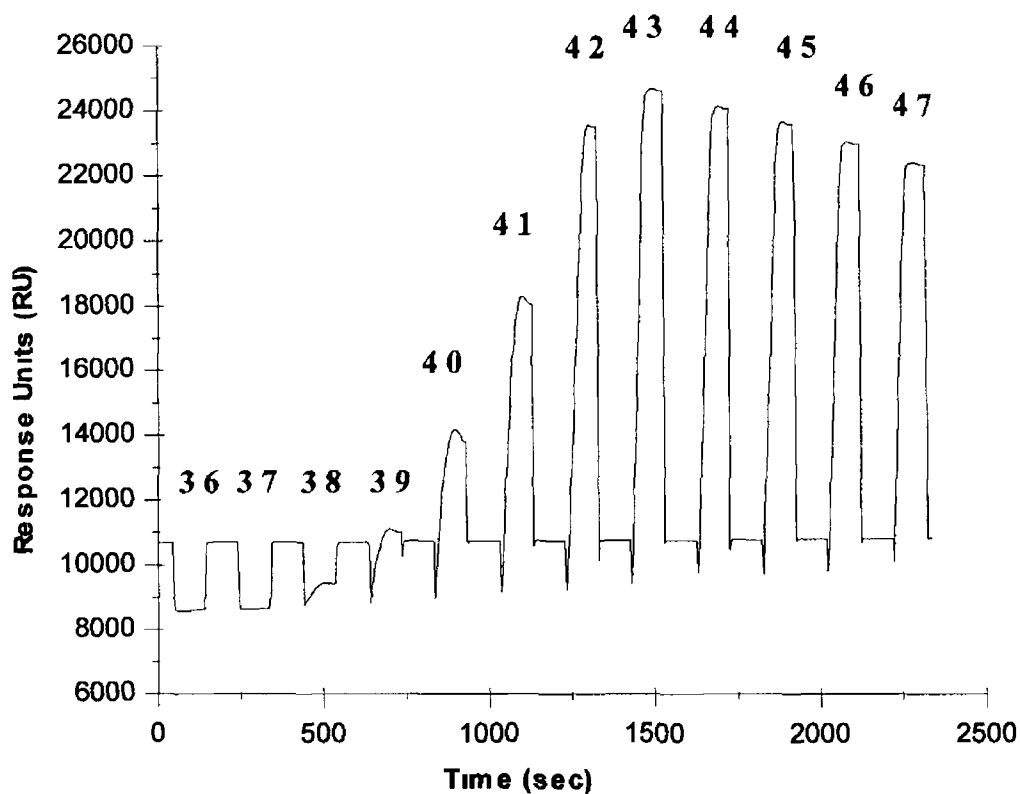
## **4 2 Results**

### **4 2.1 Preconcentration of morphine-3-glucuronide-ovalbumin conjugate**

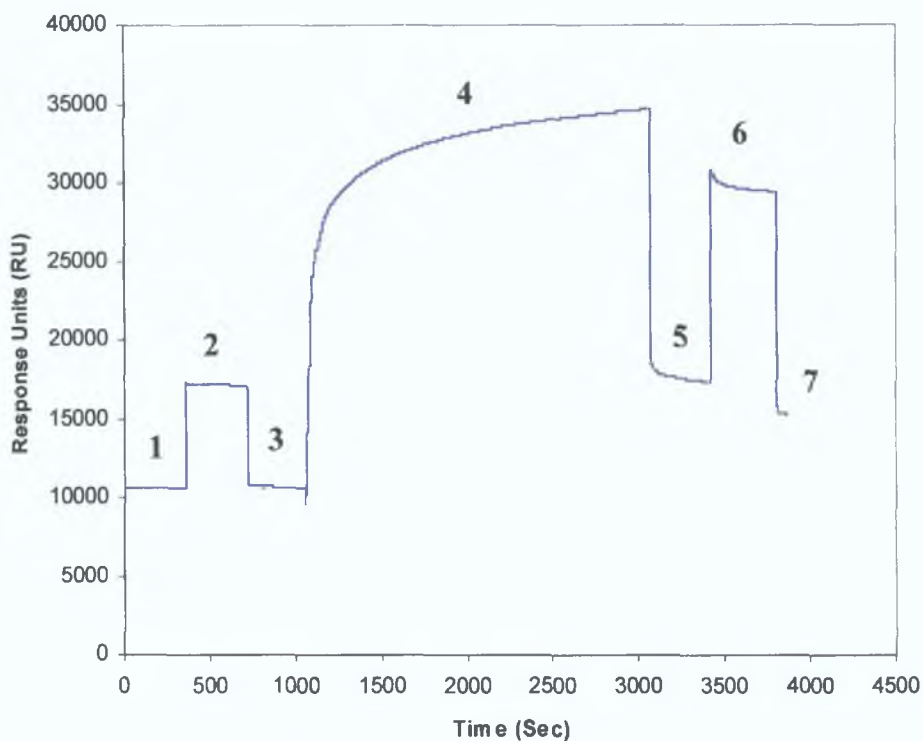
For the development of a model inhibition BIAcore assay to M3G in PBS, the first step of such an assay was the immobilisation of morphine-3-glucuronide-ovalbumin (M3G-OVA) onto the surface of a CM5 sensor chip. The immobilisation chemistry used was EDC-mediated NHS esterification as discussed in section 2 3 11 2. For the successful immobilisation of M3G-OVA onto the surface of the sensor chip, it is essential to maximise the interaction between the carboxymethylated dextran surface and the conjugate of interest. To achieve maximum binding, a preconcentration study of M3G-OVA was carried out as described in section 2 3 11 1. 100 µg/ml of M3G-OVA was prepared in 10 mM sodium acetate buffer. This solution was aliquotted and each aliquot adjusted to the required pH by addition of 10% acetic acid. These solutions were then injected over an underivatized chip surface. Figure 4 10 shows the differing degree of binding of conjugate to the surface at pHs ranging from 3 6 to 4 7. The optimal pH determined from this graph for immobilisation of M3G-OVA is 4 3. All subsequent immobilisations of M3G-OVA were carried out by preparing the conjugate at this pH.

### **4 2.2 Immobilisation of morphine-3-glucuronide-ovalbumin conjugate**

The immobilisation of conjugates onto the surface of a CM5 chip was carried out as described in section 2 3 11 2. The chip surface was activated by passing over a solution of EDC/NHS followed by the M3G-OVA conjugate, prepared in 10 mM sodium acetate, pH 4 3. The unreacted sites were then capped by passing over a solution of 1 M ethanolamine, pH 8 5. Figure 4 11 shows a typical sensorgram for the immobilisation of M3G-OVA onto the surface of a chip. The total amount of protein bound to the surface of the chip after immobilisation was found to be approximately 6,500 response units (RU).



**Figure 4 10** *Preconcentration of M3G-OVA conjugate onto the surface of a CM-dextran chip* Solutions of 100  $\mu\text{g/ml}$  of M3G-OVA in 10 mM acetate buffer at a range of pH from 3.6 to 4.7 were passed over the same unactivated chip surface at 2  $\mu\text{l/min}$  for 2 min. Electrostatic attraction between the negatively charged dextran layer and the positively charged protein is enhanced in the presence of the acetate buffer. A measurement of preconcentration was taken 5 sec before the end of the injection. As the injection finishes the protein is dissociated from the surface of the chip by the ionic strength of the HBS buffer (150 mM NaCl) passing over the surface. The optimal pH for immobilisation was determined to be pH 4.3. All subsequent immobilisations of M3G-OVA were carried out using 100  $\mu\text{g/ml}$  of M3G-OVA prepared in 10 mM sodium acetate buffer, pH 4.3



**Figure 4.11.** Typical sensorgram for the immobilisation of M3G-OVA onto the surface of a sensor chip. (1) HBS buffer is passed over the sensor chip. (2) A solution of 0.05 M NHS and 0.2 M EDC is passed over the sensor chip surface resulting in activation of the carboxymethylated dextran on the chip surface. (3) Again HBS buffer is passed over the surface and the baseline returns to normal with a small increase in response units which can be attributed to the binding of NHS-esters to the surface. (4) A solution of 100 µg/ml of M3G-OVA prepared in 10 mM acetate buffer, pH 4.3, was passed over the surface for 20 min. (5) Shows the amount of bound protein to the surface of the chip and this is recorded. (6) Deactivation of the surface NHS-esters was carried out with 1 M ethanolamine hydrochloride (pH 8.5) which removes weakly bound protein. (7) The value recorded here is the amount of protein bound to the surface of the chip and this figure is presented as response units (RU). 6,500 RU bound to the surface of the chip.



### **4 2 3 Development of a model inhibition assay for morphine-3-glucuronide in PBS**

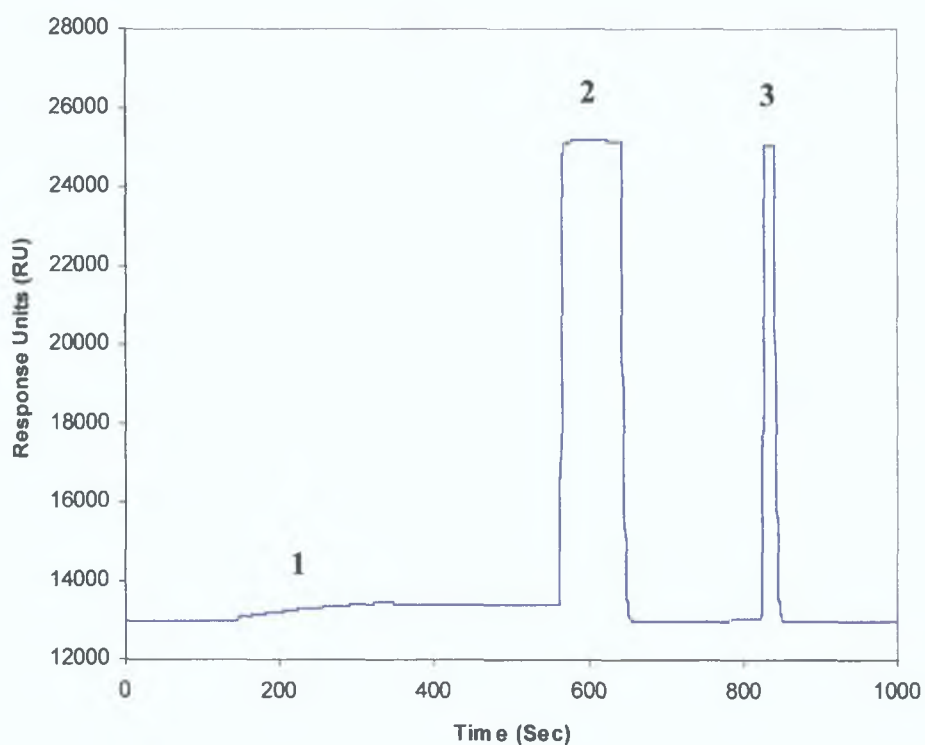
For the successful development of an inhibition BIAcore assay to M3G, a number of parameters must be optimised. Regeneration of the surface is essential for the surfaces repeated use in assay formats. Also removal of non-specific interactions is important.

1/500 dilutions of both R-1 and R-2 antibodies were found to produce a binding response of antibody of approximately 450 RU and 400 RU, respectively. Regeneration conditions for both R-1 and R-2 polyclonal antibodies were optimised. R-1 antibody required two pulses for 2 min followed by a 1 min pulse of 1 M ethanolamine, pH 13.6. R-2 antibody required three 1 min pulses in the order of 40 mM HCl, 40 mM NaOH followed by 40 mM NaOH. Figure 4.12 shows a typical sensorgram for the binding and regeneration of R-1 polyclonal antibody to M3G-OVA immobilised onto the surface of the chip.

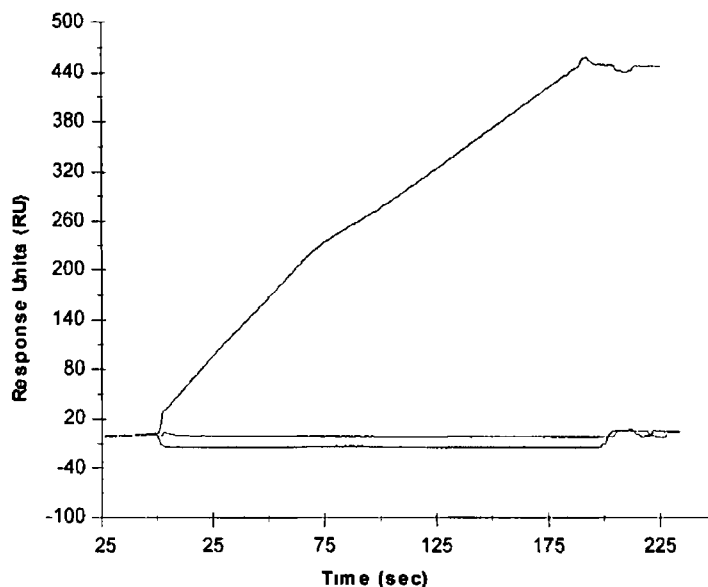
Non-specific interactions between both anti-M3G R-1 and R-2 polyclonal antibodies were analysed for both the carrier protein used to prepare the conjugate and the dextran matrix on the surface of the sensor chip. Binding of the polyclonal antibodies to either OVA or dextran would result in high background signals and have a negative effect on the sensitivity of the assay. Polyclonal R-1 and R-2 antibodies were passed over both immobilised M3G-OVA, OVA and dextran surfaces at dilutions of 1/500. Figures 4.13 and 4.14 show the binding of R-1 and R-2 polyclonal antibodies, respectively, to these surfaces. Binding of both R-1 and R-2 antibodies to the OVA and dextran surfaces were found to be negligible as less than 10 RU bound compared with approximately 400 RU binding to the conjugate immobilised surface for each antibody.

Once the regeneration conditions for both antibodies were optimised, the antibody solutions were repeatedly injected and the surface regenerated in order to conduct regeneration studies. These studies demonstrated that for the R-1 antibody, it was possible to regenerate the surface up to approximately 60 times (Figure 4.15). After 58 cycles of binding and regeneration, the response after the injection of the last

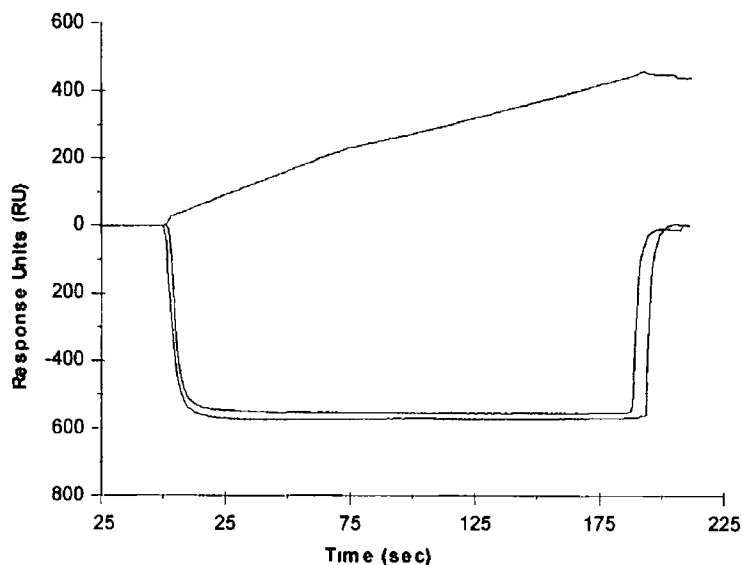
binding pulse was 377.6 RU compared with the first binding pulse of 451.8 RU. This resulted in a 16% decrease in antibody binding capacity over this number of regenerations. However, between cycles 1 and 10 there is a 13.4% drop. After the first 10 cycles, the binding capacity of the remaining regeneration cycles drops by only 2.6%. Consequently, assays performed using such conjugate surfaces were exposed to at least 10 regeneration cycles prior to sample analysis. Studies performed on the R-2 antibody demonstrated that it was possible to regenerate the surface up to approximately 50 times (Figure 4.16). After 51 cycles of binding and regeneration, the response after the injection of the last binding pulse was 365.7 RU compared with the first binding pulse of 422.8 RU. The ligand binding capacity was shown to decrease by approximately 13.5% over the course of the regeneration cycles.



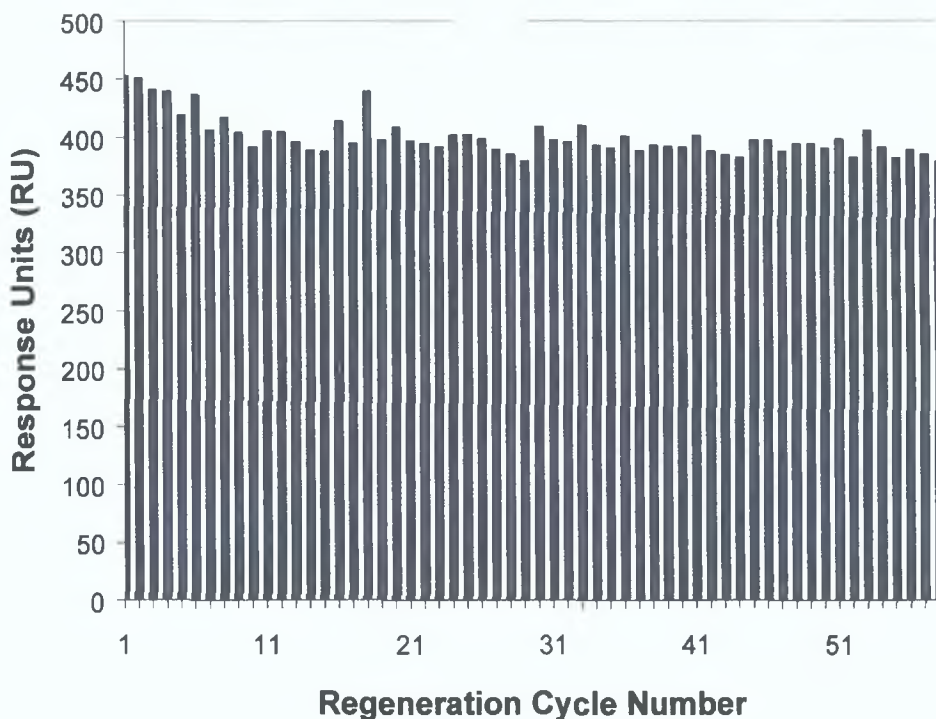
*Figure 4.12. Typical sensorgram for the binding and regeneration of R-1 polyclonal antibody to M3G-OVA immobilised onto the surface of the chip. (1) A 1/500 dilution of antibody was passed over the surface of the chip with M3G-OVA immobilised with approximately 400 response units of antibody binding. The surface was regenerated by passing 1 M ethanolamine, pH 13.6, over the surface with an initial 2 min (2) pulse followed by a 1 min pulse of the same solution (3).*



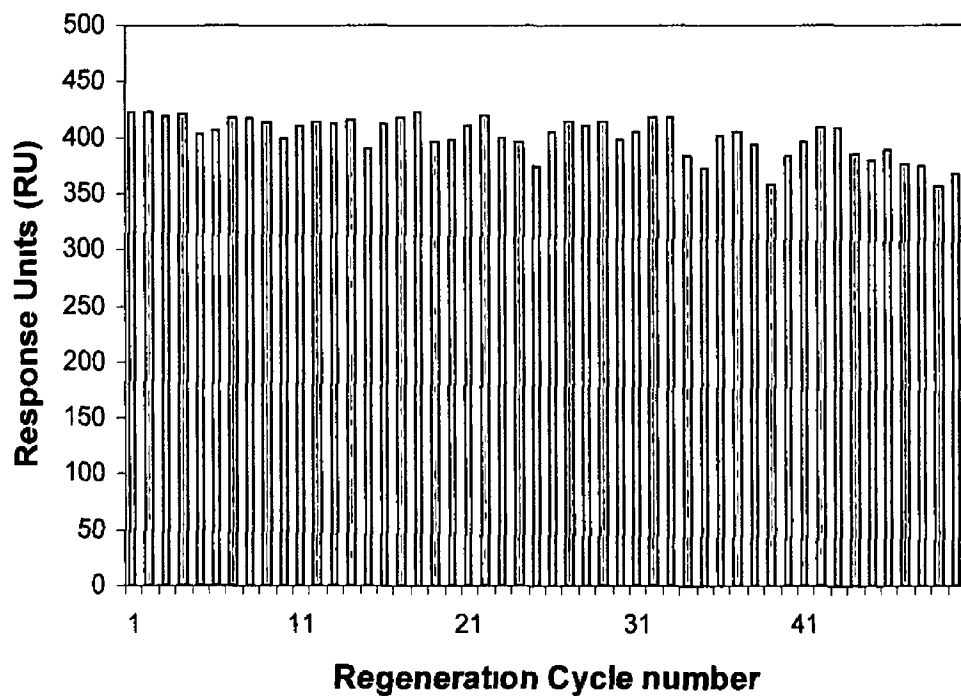
**Figure 4 13** Overlay plot demonstrating the binding of affinity-purified R-1 polyclonal antibody to immobilised M3G-OVA (—) and OVA (---) surfaces and also over a blank dextran surface (·-·-) There was negligible binding to the control OVA and dextran surfaces However, approximately 450 response units of R-1 antibody bound to the immobilised M3G-OVA surface indicating the antibody is specific to the M3G portion of the conjugate.



**Figure 4 14** Overlay plot demonstrating the binding of affinity-purified R-2 polyclonal antibody to immobilised M3G-OVA (—) and OVA (---) surfaces and also over a blank dextran surface (·-·-) This resulted in negligible binding to the control OVA and dextran surfaces. However, approximately 400 response units of R-2 antibody bound to the immobilised M3G-OVA surface indicating the antibody is specific to the M3G portion of the conjugate.



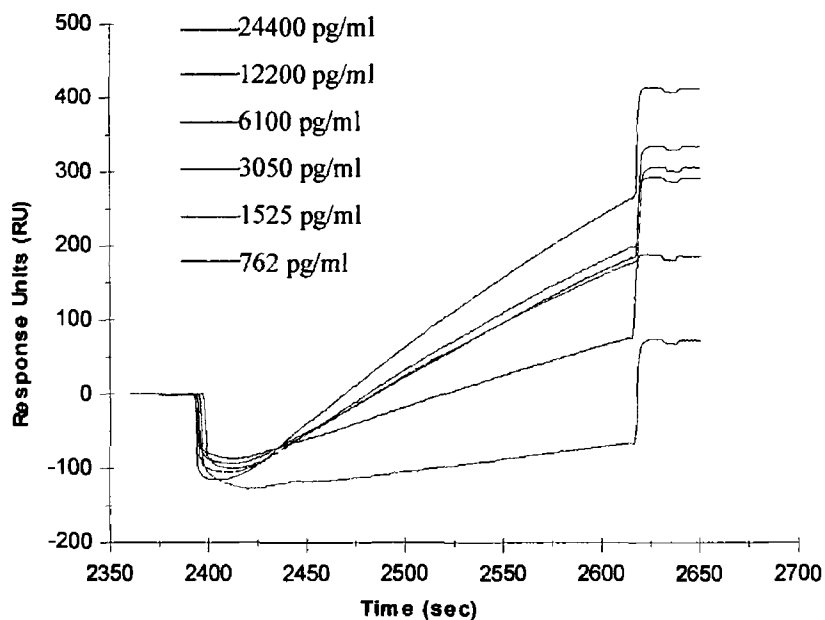
*Figure 4.15. Typical regeneration profile for approximately 60 cycles of a 4 min binding pulse of affinity-purified R-1 polyclonal antibody to the surface of a chip immobilised with M3G-OVA. A 1/500 dilution of polyclonal antibody was used and the surface regenerated with one 2 min pulse followed by a 1 min pulse of 1 M ethanolamine, pH 13.6. After 58 cycles of binding and regeneration, the response after the injection of the last binding pulse was 377.6 RU compared with the first binding pulse of 451.8 RU. The ligand binding capacity was shown to decrease by approximately 16% over the course of the regeneration cycles. However, between cycles 1 and 10 there is a 13.4% drop. After the first 10 cycles, the binding capacity of the remaining regeneration cycles drops to 2.6%. When performing assays the first 10 regenerations were disregarded.*



*Figure 4.16. Typical regeneration profile for approximately 50 cycles of a 4 min binding pulse of affinity-purified R-2 polyclonal antibody to the surface of a chip immobilised with M3G-OVA. Polyclonal antibody at a 1/500 dilution was used and the surface regenerated with three 1 min pulses in the order of 40 mM HCl, 40 mM NaOH followed by 40 mM NaOH. After 51 cycles of binding and regeneration, the response after the injection of the last binding pulse was 365.7 RU compared with the first binding pulse of 422.8 RU. The ligand binding capacity was shown to decrease by approximately 13.5% over the course of the regeneration cycles. As can be seen there is some fluctuation in the binding of antibody to the surface of the chip which may be dependent on the regeneration solutions used.*

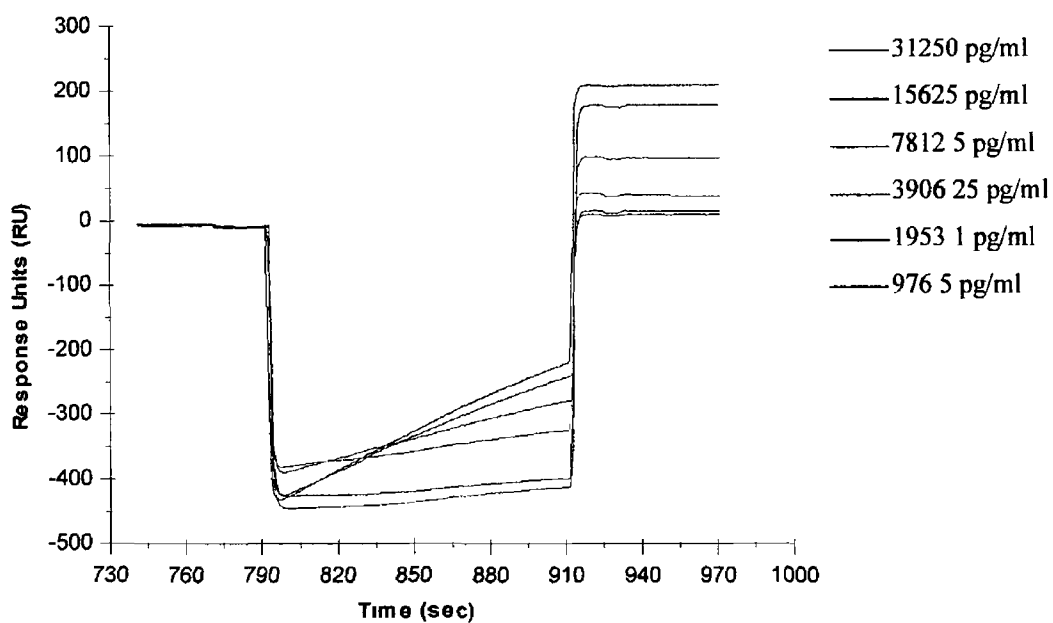
#### **4.2.3.1 Determination of the range of detection of morphine-3-glucuronide in PBS in a BIAcore inhibition assay for R-1 and R-2 anti-morphine-3-glucuronide polyclonal antibodies**

For the determination of the range of detection of M3G for a BIAcore inhibition assay, a range of standards of M3G were prepared in PBS ranging from 11 to 48,800 pg/ml for R-1 and from 122 to 250,000 pg/ml for R-2 antibody. The affinity purified polyclonal antibodies to M3G were mixed with the corresponding concentration of free M3G using the BIAcore autosampler, and allowed to equilibrate for 10 min. The samples were then passed over the surface of the sensor chip immobilised with M3G-OVA in random order, followed by regeneration of the surface using the appropriate regeneration solution for each antibody (R-1 antibody: two pulses for 2 min and 1 min of 1 M ethanolamine, pH 13.6, R-2 antibody: three 1 min pulses in the order of 40 mM HCl, 40 mM NaOH followed by 40 mM NaOH). Typical antibody binding responses for each mixture of antibody and drug concentration is shown in Figures 4.17 and 4.18 for R-1 and R-2 antibodies, respectively. Figure 4.17 and 4.18 also show that as the free drug concentration increases in solution the binding response of antibody decreases. Calibration curves for both R-1 and R-2 antibodies were constructed by plotting the change in response for each standard of free drug against the concentration of M3G. The range of detection for R-1 and R-2 antibodies were found to be between 762 and 24,400 pg/ml (Figure 4.19) and 976 and 31,250 pg/ml (Figure 4.20), respectively.



**Figure 4 17.** An overlay plot for a typical set of binding curves taken from one of the replicates in an inhibition assay. M3G standards ranging from 762 to 24,400 pg/ml of free drug were mixed with R-1 polyclonal antibody and passed over an M3G-OVA immobilised surface. The measured binding response was used to construct the calibration curves for the intra-day assay variability studies, and used to calculate the normalised binding response in conjunction with 2 other normalised binding response curves for the inter-day assay variability studies





**Figure 4 18.** An overlay plot for a typical set of binding curves taken from one of the replicates in an inhibition assay. M3G standards ranging from 976 to 31,250 pg/ml of free drug were mixed with R-2 polyclonal antibody and passed over an M3G-OVA immobilised surface. The measured binding response was used to construct the calibration curves for the intra-day assay variability studies.

#### **4.2.3.2 Intra-day assay variability studies of R-1 and R-2 anti-morphine-3-glucuronide polyclonal antibodies**

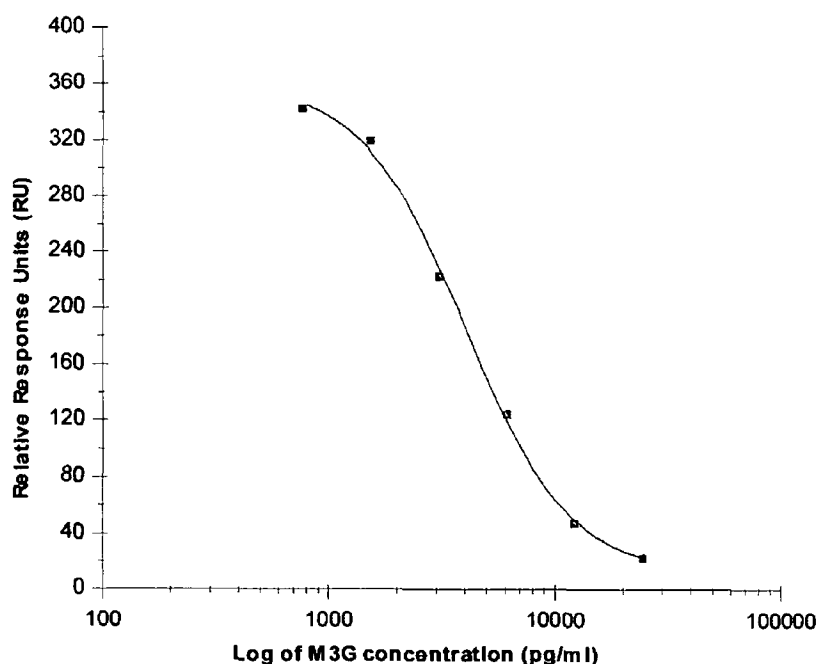
To carry out intra-day assay variability studies, three sets of a range of standards of M3G were prepared in PBS ranging from 11 to 48,800 pg/ml for R-1 and from 122 to 250,000 pg/ml for R-2 antibody and assayed on the same day and their means plotted. The intra-day assay variability study for R-1 shown in Figure 4.19 had a range of detection between 762 and 24,400 pg/ml. The coefficients of variation (CV's) were determined to assess the precision of the analytical method, expressing standard deviation as a percent function of the mean. These ranged from 3.29% to 15.04% as shown in Table 4.1. The intra-day assay variability study for R-2 antibody shown in Figure 4.20 had a range of detection between 976 and 31,250 pg/ml. The CV's were determined and ranged from 2.07% to 7.09% as shown in Table 4.2.

#### **4.2.3.3. Inter-day assay variability studies of R-1 and R-2 anti-morphine-3-glucuronide polyclonal antibodies**

To carry out inter-day variability studies, three sets of standards of M3G were prepared in PBS ranging from 11 to 48,800 pg/ml for R-1 and from 122 to 250,000 pg/ml for R-2 antibody and assayed three times over three days. The binding response at each M3G concentration ( $R_{AG}$ ) was divided by the antibody binding response determined in the presence of zero M3G concentration ( $R_0$ ) to give a normalised binding response, which allowed inter-day variability comparison of the assay to be made. The inter-day assay variability study for R-1, carried out over six concentrations, had a range of detection between 762 and 24,400 pg/ml as shown in Figure 4.21. The CV's were determined and ranged from 1.31% to 11.98% as shown in Table 4.3. The inter-day assay variability study for R-2 had a range of detection between 976 and 31,250 pg/ml as shown in Figure 4.22. The CV's were determined and ranged from 4.06% to 8.19% as shown in Table 4.4.

**Table 4 1** *Intra-day assay CV's for R-1 antibody BIAcore inhibition assay Three sets of six standards ranging from 762 to 24,400 pg/ml were analysed on the same day and the CV's calculated.*

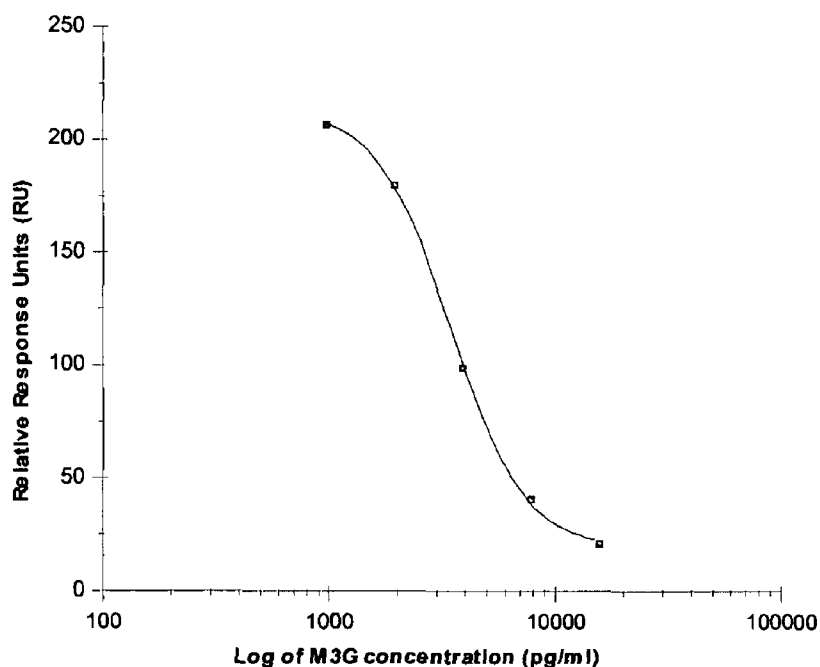
<b>M3G Concentration, (pg/ml)</b>	<b>Calculated mean <math>\pm</math> S.D , RU</b>	<b>Coefficients of variation (CV's), %</b>
24400	23 43 $\pm$ 3 52	15 04
12200	48 23 $\pm$ 3 55	7 37
6100	125 16 $\pm$ 4 11	3 29
3050	223 25 $\pm$ 9 40	4 21
1525	319 86 $\pm$ 29 63	9 26
762	343 16 $\pm$ 24 12	7 03



**Figure 4 19** *Intra-day assay curve for M3G using the R-1 polyclonal antibody on an M3G-OVA immobilised chip surface The residual plot shows the excellent correlation of the data set to the four-parameter model fit. The calibration curve was constructed using BIAevaluation 3 1 software package. The results shown are the average of triplicate results and the range of detection of free M3G was found to be between 762 and 24,400 pg/ml.*

**Table 4 2** *Intra-day assay CV's for R-2 antibody BLAcore inhibition assay Three sets of six standards ranging from 976.5 to 31,250 pg/ml were analysed on the same day and the CV's calculated.*

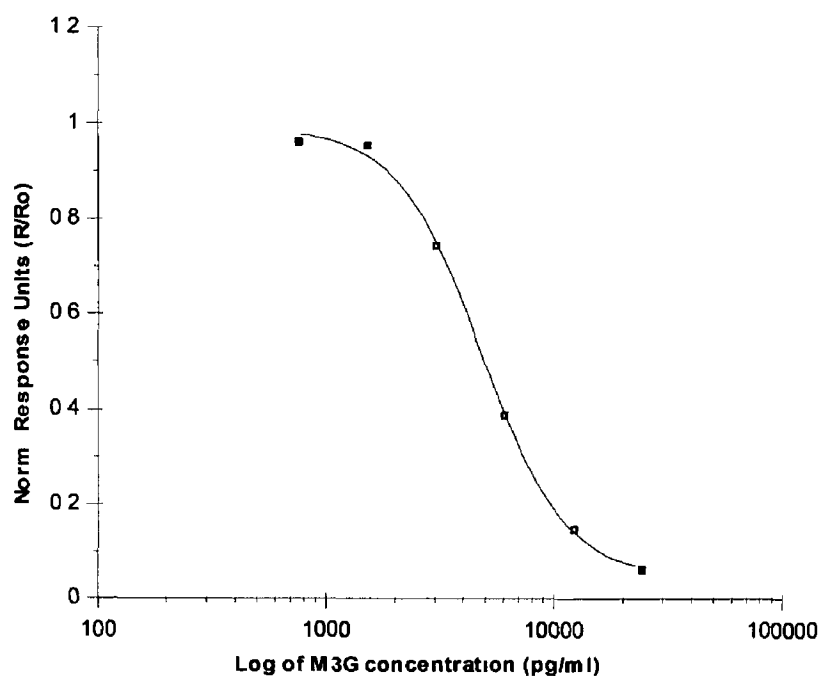
<b>M3G Concentration, (pg/ml)</b>	<b>Calculated mean <math>\pm</math> S D , <i>RU</i></b>	<b>Coefficients of variation (CV's), %</b>
31250	15 90 $\pm$ 1 126	7 09
15625	21 36 $\pm$ 1 159	5 42
7812 5	41 20 $\pm$ 0 854	2 07
3906 25	98 86 $\pm$ 4 521	4 57
1953 1	179 46 $\pm$ 6 782	3 78
976 5	206 46 $\pm$ 7 454	3 61



**Figure 4 20** *Intra-day assay curve for M3G using the R-2 polyclonal antibody on an M3G-OVA immobilised chip surface The residual plot shows the excellent correlation of the data set to the four-parameter model fit. The results shown are the average of triplicate results and the range of detection of free M3G was found to be between 976.5 and 31,250 pg/ml*

**Table 4 3 Inter-day assay CV's for R-1 antibody BLAcore inhibition assay** Three sets of six standards ranging from 762 to 24,400 pg/ml were analysed over three different days and the CV's calculated.

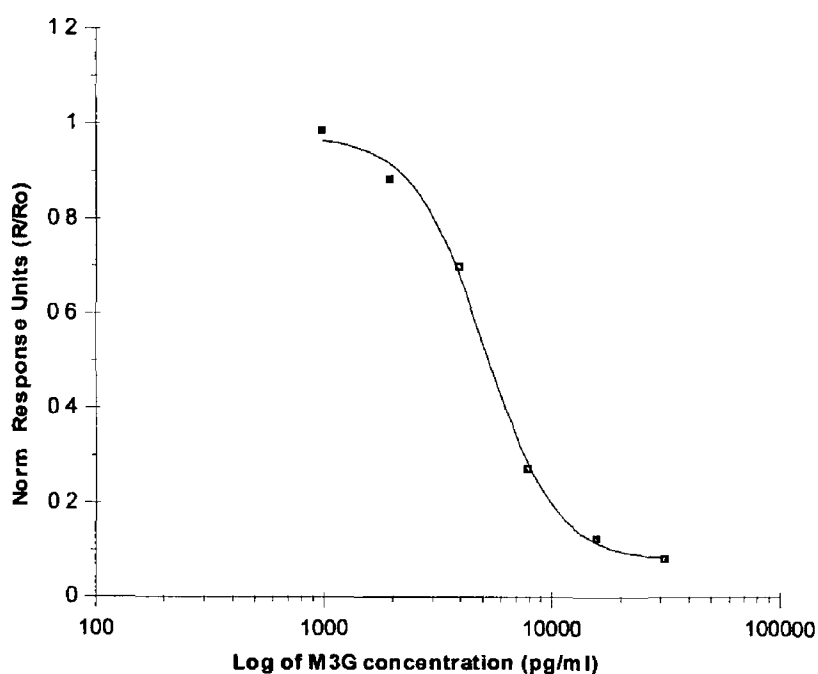
M3G Concentration, (pg/ml)	Calculated mean $\pm$ S D., $R/R_0$	Coefficients of variation (CV's), %
24400	0.063 $\pm$ 0.007	11.98
12200	0.148 $\pm$ 0.014	9.85
6100	0.390 $\pm$ 0.036	9.37
3050	0.744 $\pm$ 0.065	8.83
1525	0.954 $\pm$ 0.078	8.26
762	0.963 $\pm$ 0.012	1.31



**Figure 4 21 Inter-day assay curve for M3G using the R-1 polyclonal antibody on an M3G-OVA immobilised chip surface.** The results shown are the average of triplicate results and the range of detection of free M3G was found to be between 762 and 24,400 pg/ml. The binding response at each M3G concentration ( $R_{AC}$ ) was divided by the antibody binding response determined in the presence of zero M3G concentration ( $R_0$ ) to give a normalised binding response ( $R/R_0$ )

**Table 4 4 Inter-day assay CV's for R-2 antibody BLAcore inhibition assay** Three sets of six standards ranging from 976 5 to 31,250 pg/ml were analysed in triplicate for three assays over three different days and the CV's calculated from these assays

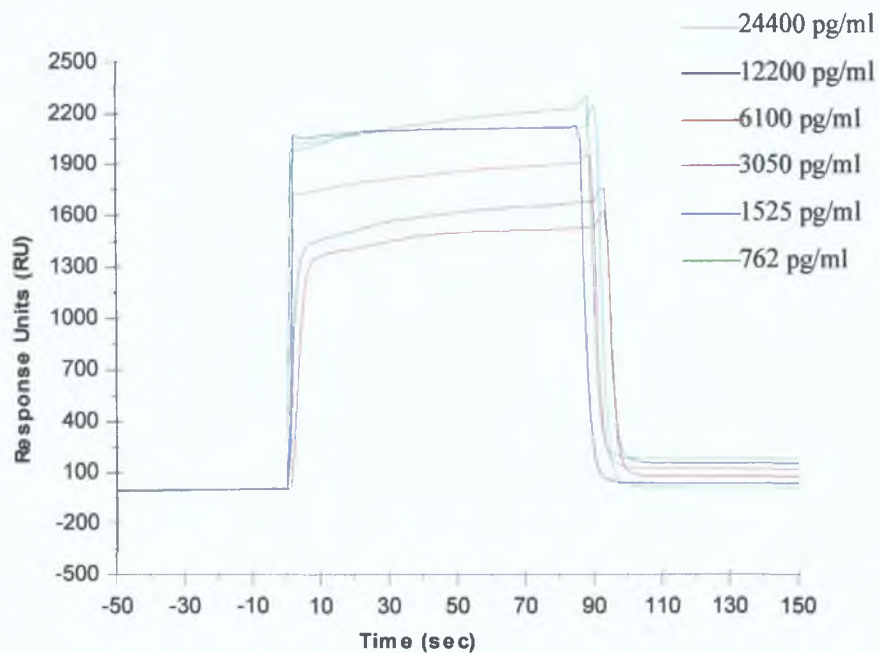
M3G Concentration, (pg/ml)	Calculated mean $\pm$ S.D., $R/R_0$	Coefficients of variation (CV's), %
31250	0 084 $\pm$ 0 003	4 06
15625	0 126 $\pm$ 0 008	6 84
7812 5	0 274 $\pm$ 0 011	4 28
3906 25	0 701 $\pm$ 0 057	8 19
1953 1	0 886 $\pm$ 0 022	5 24
976 5	0 987 $\pm$ 0 053	5 45



**Figure 4 22 Inter-day assay curve for M3G using the R-2 polyclonal antibody on an M3G-OVA immobilised chip surface.** The residual plot shows the excellent correlation of the data set to the four-parameter model fit. The results shown are the average of triplicate results and the range of detection of free M3G was found to be between 976 5 and 31,250 pg/ml. The binding response at each M3G concentration ( $R_{AG}$ ) was divided by the antibody binding response determined in the presence of zero M3G concentration ( $R_0$ ) to give a normalised binding response ( $R/R_0$ )

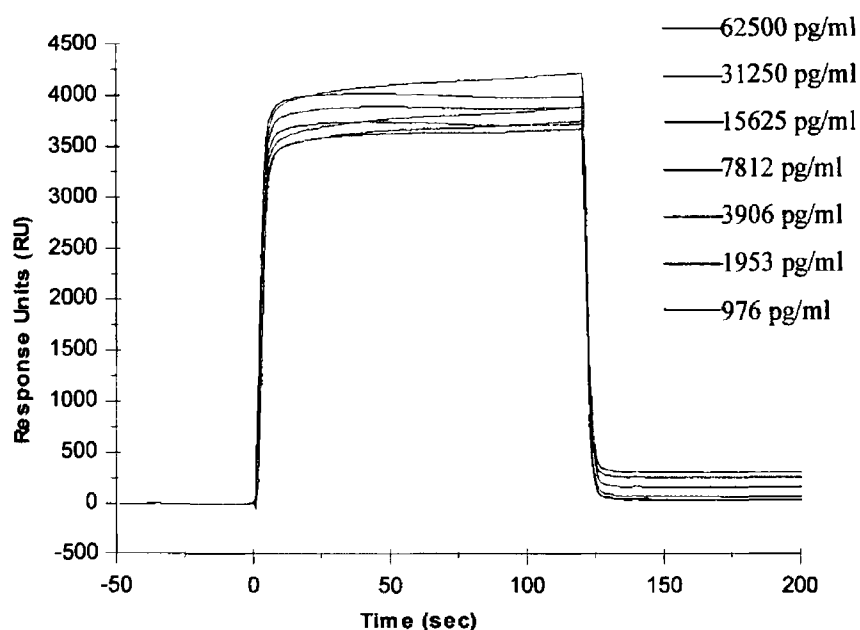
#### **4 2 4 Urine analysis of anti-morphine-3-glucuronide antibodies**

The quantitative determination of M3G in urine samples was also investigated. The ionic composition of urine can vary considerable from sample to sample. To compensate for the potential wide inter-individual variability in the salt composition of urine, the antibody sample was prepared in PBS of twice the normal ionic strength (2 x PBS). Known samples of M3G ranging from 11 to 48,800 pg/ml for R-1 and from 122 to 250,000 pg/ml for R-2 antibody were spiked into control urine and assayed for the presence of free drug with both antibodies. Figure 4 23 and 4 24 show that as the free drug concentration increases in solution the binding response of antibody decreases. Calibration curves for both R-1 and R-2 antibodies were constructed by plotting the change in response for each standard of free drug against the concentration of M3G. The range of detection in urine for R-1 and R-2 antibodies were found to be between 762 and 24,400 pg/ml (Figure 4 25) and 976 and 62,500 pg/ml (Figure 4 26), respectively.



*Figure 4.23. An overlay plot for a typical set of binding curves in a BIAcore inhibition assay developed in urine. M3G standards containing 762 to 24,400 pg/ml of free drug were mixed with R-1 polyclonal antibody and passed over an M3G-OVA immobilised surface. The measured binding response was used to construct the calibration curves for the intra-day assay variability studies, and used to calculate the normalised binding response in conjunction with 2 other normalised binding response curves for the inter-day assay variability comparison.*





**Figure 4 24** *An overlay plot for a typical set of binding curves in a BIAcore inhibition assay developed in urine. M3G standards containing 976 to 62,500 pg/ml of free drug were mixed with R-2 polyclonal antibody and passed over an M3G-OVA immobilised surface. The measured binding response was used to construct the calibration curves for the intra-day assay variability studies, and used to calculate the normalised binding response in conjunction with 2 other normalised binding response curves from assays for the inter-day assay variability comparison.*

#### **4 2 4 1 Intra-day assay variability studies of R-1 and R-2 anti-morphine-3-glucuronide polyclonal antibodies in urine**

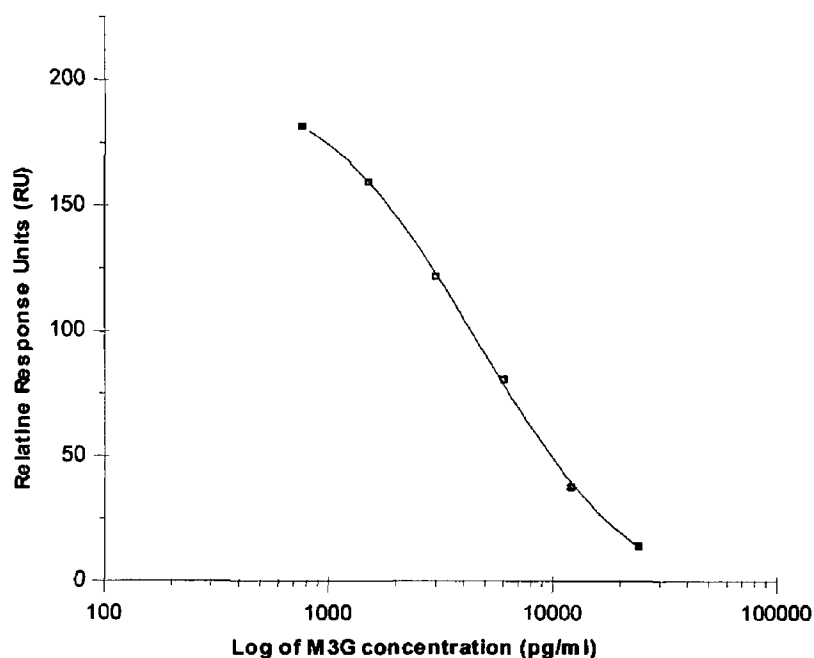
To carry out intra-day assay variability studies, three sets of standards of M3G were prepared ranging from 11 to 48,800 pg/ml for R-1 and from 122 to 250,000 pg/ml for R-2 antibody in urine and assayed on the same day and their means plotted. The intra-day assay variability study for R-1 had a range of detection between 762 and 24,400 pg/ml (Figure 4 25). The CV's were determined and ranged from 2.07% to 8.70% as shown in Table 4 5. The intra-day assay variability study for R-2 had a range of detection between 976 and 62,500 pg/ml (Figure 4 26). The CV's were determined and ranged from 0.44% to 1.92% as shown in Table 4 6.

#### **4 2 4 2 Inter-day assay variability studies of R-1 and R-2 anti-morphine-3-glucuronide antibodies in urine**

To carry out inter-day variability studies, three sets of standards of M3G were prepared in PBS ranging from 11 to 48,800 pg/ml for R-1 and from 122 to 250,000 pg/ml for R-2 antibody and assayed three times over three days. The inter-day assay variability study for R-1 had a range of detection between 762 and 24,400 pg/ml as shown in Figure 4 27. The CV's were determined and ranged from 2.95% to 10.80% as shown in Table 4 7. The inter-day assay variability study for R-2 had a range of detection between 976 and 62,500 pg/ml as shown in Figure 4 28. The CV's were determined and ranged from 1.48% to 11.24% as shown in Table 4 8.

**Table 4 5** *Intra-day assay CV's for R-1 antibody BIAcore inhibition assay in urine. Three sets of six standards ranging from 762 to 24,400 pg/ml were analysed on the same day and the CV's calculated.*

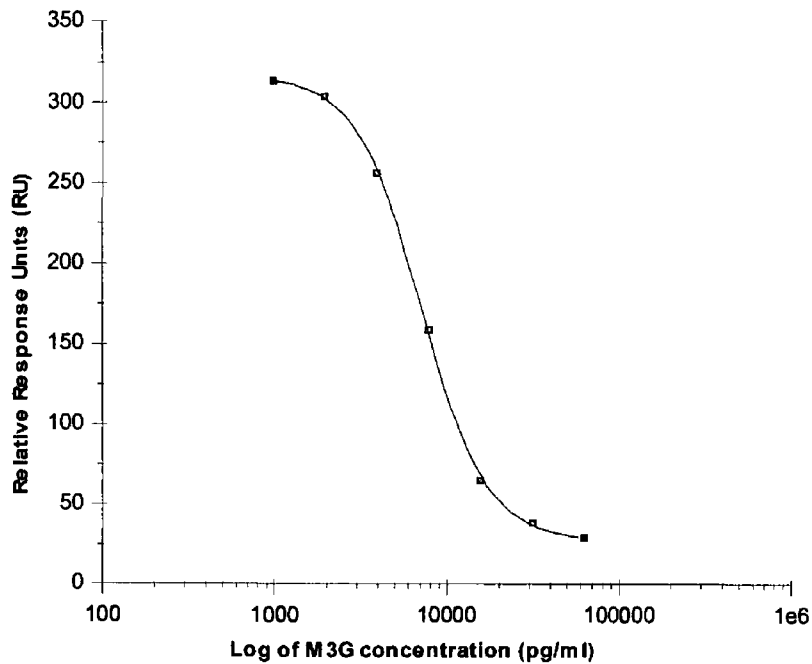
<b>M3G Concentration, (pg/ml)</b>	<b>Calculated mean <math>\pm</math> S D , <i>RU</i></b>	<b>Coefficients of variation (CV's), %</b>
24400	14 66 $\pm$ 0 40	2 76
12200	37 83 $\pm$ 3 29	8 70
6100	81 33 $\pm$ 1 77	2 19
3050	122 30 $\pm$ 2 80	2 29
1525	159 56 $\pm$ 7 44	4 66
762	181 66 $\pm$ 3 76	2 07



**Figure 4 25** *Intra-day assay curve for M3G in urine using the R-1 polyclonal antibody on an M3G-OVA immobilised chip surface. The results shown are the average of triplicate results and the range of detection of free M3G was found to be between 762 and 24,400 pg/ml*

**Table 4 6. Intra-day assay CV's for R-2 antibody BIAcore inhibition assay in urine. Three sets of seven standards ranging from 976.5 to 62,500 pg/ml were analysed on the same day and the CV's calculated.**

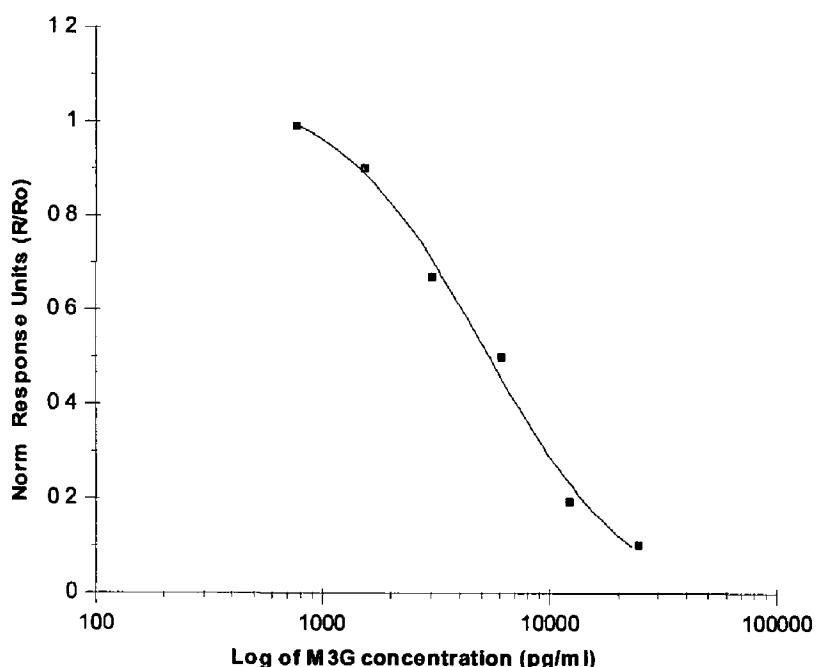
<b>M3G Concentration, (pg/ml)</b>	<b>Calculated mean <math>\pm</math> S D , <i>RU</i></b>	<b>Coefficients of variation (CV's), %</b>
62500	30 40 $\pm$ 0 400	1 32
31250	39 76 $\pm$ 0 763	1 92
15625	66 13 $\pm$ 0 550	0 83
7812 5	159 40 $\pm$ 0 800	0 50
3906 25	256 50 $\pm$ 1 135	0 44
1953 1	304 36 $\pm$ 3 842	1 26
976 5	313 70 $\pm$ 3 019	0 96



**Figure 4 26. Intra-day assay curve for M3G in urine using the R-2 polyclonal antibody on an M3G-OVA immobilised chip surface. The results shown are the average of triplicate results and the range of detection of free M3G was found to be between 976 5 and 62,500 pg/ml.**

**Table 4 7 Inter-day assay CV's for R-1 antibody BIAcore inhibition assay in urine. Three sets of six standards ranging from 762 to 24,400 pg/ml were analysed over three different days and the CV's calculated.**

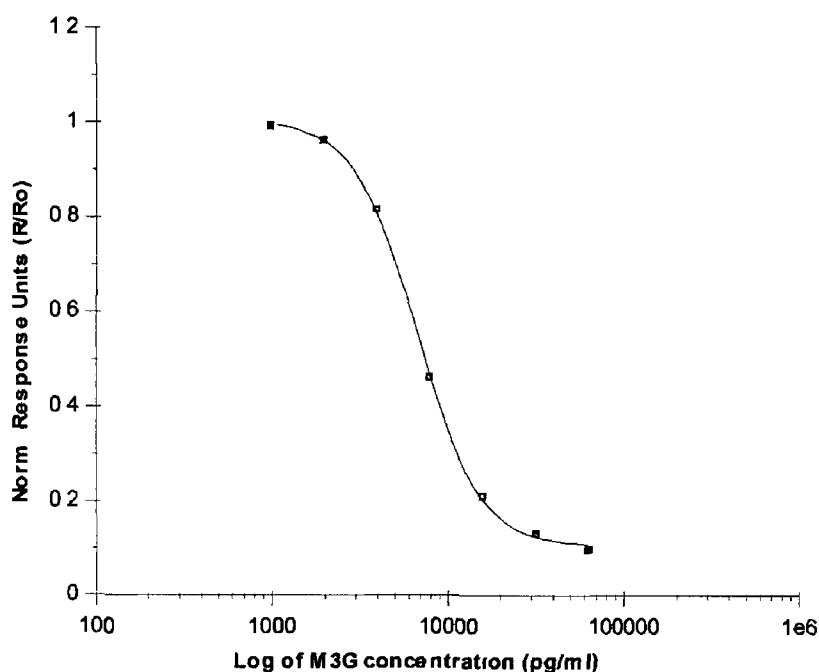
<b>M3G Concentration, (pg/ml)</b>	<b>Calculated mean <math>\pm</math> S.D , <math>R/R_0</math></b>	<b>Coefficients of variation (CV's), %</b>
24400	0.104 $\pm$ 0.011	10.80
12200	0.196 $\pm$ 0.013	6.90
6100	0.501 $\pm$ 0.014	2.95
3050	0.671 $\pm$ 0.029	4.46
1525	0.903 $\pm$ 0.096	10.63
762	0.993 $\pm$ 0.064	6.51



**Figure 4 27 Inter-day assay curve for M3G in urine using the R-1 polyclonal antibody on an M3G-OVA immobilised chip surface. The results shown are the average of triplicate results and the range of detection of free M3G was found to be between 762 and 24,400 pg/ml.**

**Table 4 8. Inter-day assay CV's for R-2 antibody BIAcore inhibition assay in urine. Three sets of seven standards ranging from 976.5 to 62,500 pg/ml were analysed over three different days and the CV's calculated.**

<b>M3G Concentration, (pg/ml)</b>	<b>Calculated mean <math>\pm</math> S D , <math>R/R_0</math></b>	<b>Coefficients of variation (CV's), %</b>
62500	0.101 $\pm$ 0.007	7.82
31250	0.134 $\pm$ 0.008	6.09
15625	0.213 $\pm$ 0.005	2.63
7812.5	0.465 $\pm$ 0.052	11.24
3906.25	0.818 $\pm$ 0.025	3.10
1953.1	0.963 $\pm$ 0.019	2.07
976.5	0.995 $\pm$ 0.014	1.48



**Figure 4 28. Inter-day assay curve for M3G in urine using the R-2 polyclonal antibody on an M3G-OVA immobilised chip surface. The results shown are the average of triplicate results and the range of detection of free M3G was found to be between 976.5 and 62,500 pg/ml.**

### 4.3 Discussion

For the successful development of an inhibition BIAcore assay to M3G, a number of parameters were optimised. The first step in setting up such an immunoassay was the immobilisation of M3G-OVA onto the surface of a CM5 sensor chip. Immobilisation of conjugates to the surface of the chip at sufficiently high yields to carry out routine sample analysis may prove difficult when using EDC/NHS chemistry, as many hapten-protein conjugates are characterised by very acidic isoelectric points. It is essential to maximise the interaction between the dextran surface of the chip and the conjugate of interest in order to immobilise as much conjugate as possible onto the surface of the chip. At pH values greater than 7, the surface of the chip has a net negative charge. Proteins prepared in low ionic strength buffers, which result in the pH of the proteins being below their isoelectric point (pI), cause the proteins to be electrostatically attracted to this surface. By preparing proteins in varying pHs of this buffer, the attraction between each protein solution and the surface of the chip may be monitored and the pH which provides optimal binding of conjugate to the surface of the chip may be determined. This preconcentration step is an effective way to determine the solutions required for optimal immobilisation of conjugate onto the surface of the chip. Figure 1.10 shows the optimal pH for immobilisation of M3G-OVA was found to be pH 4.3.

The immobilisation of conjugates onto the surface of a CM5 chip (section 2.3.11.2) requires a number of steps. The carboxyl groups in the hydrogel of the chip are activated by employing EDC/NHS chemistry. The water soluble EDC transforms surface carboxyl groups into active ester functional groups in the presence of NHS. These surface NHS esters are then available to react with a suitable amine group. Conjugates prepared in low ionic solutions optimised from the preconcentration step are passed over the surface and binding allowed to occur. Immobilisation of M3G-OVA resulted in approximately 6,500 RU binding to the surface of the chip as shown in Figure 4.11 corresponding to a protein concentration on the chip of approximately  $6.5 \text{ ng/mm}^2$ .

Non-specific binding analysis of the antibodies was carried out by passing both R-1 and R-2 antibodies over an OVA immobilised surface and an underivatized dextran surface. For both R-1 (Figure 4 13) and R-2 (Figure 4 14) antibodies the non-specific interactions were found to be negligible. This result shows that the antibodies are specific for M3G rather than the carrier molecule of the conjugate.

An important consideration when using BIAcore as an immunoassay tool is the ability of the antibodies to be regenerated from the surface of the chip. The regeneration of the immunosensor surface is of critical importance for the development of an assay. The use of hapten-protein conjugates immobilised on the surface of the chip can prove difficult when trying to regenerate a surface capable of high affinity antibody antigen interactions. This may result in the need for harsh regeneration solutions to regenerate the surface, which can denature the immobilised conjugate. The binding-capacity of both antibodies to conjugate immobilised surfaces were determined by a series of binding and regeneration sequences to assess how many regenerations were possible before the binding-capacity falls below the desired performance parameters (< 20%) (Wong *et al* , 1997). Ideally, it is preferable to be able to perform multiple binding and regenerations of antibody on the surface of one derivatised chip. Regeneration conditions for both R-1 and R-2 polyclonal antibodies were optimised. R-1 antibody required two pulses for 2 min followed by a 1 min pulse of 1 M ethanolamine, pH 13.6. R-2 antibody required three 1 min pulses in the order of 40 mM HCl, 40 mM NaOH followed by 40 mM NaOH. Results from surface regeneration studies showed that R-1 antibody was regenerable approximately 60 times (Figure 4 15) with a loss of 16% antibody binding capacity over this number of regeneration's. However, between cycles 1 and 10 there was a 13.4% drop. After the first 10 cycles, the binding capacity of the remaining regeneration cycles drops by only 2.6%. Consequently, assays performed using such conjugate surfaces were exposed to at least 10 regeneration cycles prior to sample analysis to minimise loss in antibody binding capacity. Studies performed on the R-2 antibody demonstrated that it was possible to regenerate the surface approximately 50



times (Figure 4 16) resulting in a 13 5% decrease in antibody binding capacity over this number of regeneration's

These results are favourable for the development of BIAcore inhibition assays as both antibodies can be regenerated a sufficient number of times without the binding-capacity falling below the desired performance parameters This enables the development of an inhibition assay

A model inhibition assay was then developed as described in section 2 3 11 7 for M3G in PBS for both R-1 and R-2 polyclonal antibodies Standards of M3G ranging from 11 to 48,800 pg/ml for R-1 and from 122 to 250,000 pg/ml for R-2 antibody were mixed with R-1 and R-2 affinity-purified antibodies at dilutions of 1/500 These mixtures were allowed to incubate for 10 min followed by injecting them over the M3G-OVA immobilised surface for 4 min The antibody binding response to the surface is inversely proportional to the concentration of free drug in solution These binding responses were plotted in Figure 4 19 for R-1 and Figure 4 20 for R-2 antibodies and showed that the detection range of R-1 antibody for M3G was between 762 and 24,400 pg/ml and R-2 antibody was between 976 and 31,250 pg/ml

Both assays displayed low limits of detection Intra-day and inter-day assay variability statistics were then analysed in an attempt to look at the reproducibility of the assay Intra-day analysis was compared by taking the mean of each antibody binding response at each concentration of free M3G for an assay on one day and plotting this response on a standard curve against the concentration of M3G The binding response at each antigen concentration ( $R_{AG}$ ) were divided by the antibody binding response determined in the presence of zero antigen concentration ( $R_0$ ), to give the normalised binding response used for the determination of inter-day assay analysis Inter-day assay precision utilised the mean from each of the three days as described by Wong *et al* (1997) Intra-day and inter-day assay coefficients of variation (CV's) for R-1 were found to be between 3 29 to 15 04% and 1 13 to 11 98%, respectively, for 3 replicates (Tables 4 1 and 4 3) Intra-day assay and inter-day assay

CV's for R-2 were found to be between 2.07 to 7.09% and 4.06 to 8.19% for 3 replicates (Tables 4.2 and 4.4). These results indicate that using R-1 and R-2 anti-M3G antibodies gives accurate, specific and reproducible assays.

Both R-1 and R-2 antibodies demonstrate low detection limits of M3G in the BIAcore biosensor format. However, the R-1 antibody shows relatively high CV's in both the intra-day and inter-day assays as they reach 15%. R-2 antibody shows CV variations do not reach higher than 8%.

Having completed the analysis of both antibodies for the development of BIAcore inhibition assays in PBS, results showed both antibodies were suitable for application on this biosensor. The antibodies were found to have negligible non-specific interactions with both the carrier protein and the dextran surface. Both R-1 and R-2 antibodies were regenerable from the surface of the chip approximately 60 and 50 times, respectively. This demonstrated the stability of the conjugate on the surface and the applicability of these antibodies to such an assay system. With all these considerations, and the fact that both antibodies were applicable to this system producing excellent results, the aim was to develop a method for the detection of M3G in biological matrices.

An inhibition assay was then developed for detection of M3G in urine as described in section 2.3.11.7. Considerable variations in the ionic composition of urine are evident from different samples. To compensate for the variation in urine salt composition, the antibodies were prepared in 2 x PBS. R-1 antibody showed a range of detection between 762 to 24,400 pg/ml and R-2 showed a range of detection between 976.5 to 62,500 pg/ml. Intra-day and inter-day assay coefficients of variation (CV's) for R-1 were found to be between 2.07 to 8.70% and 2.95 to 10.80%, respectively, for 3 replicates (Tables 4.5 and 4.7). Intra-day assay and inter-day assay CV's for R-2 were found to be between 0.44 to 1.92% and 1.48 to 11.24% for 3 replicates (Tables 4.6 and 4.8). The low nature of the CV's indicates the reproducibility of these assays when used in sample analysis for the detection of M3G. These low CV's may be attributed

to the use of 2 x PBS as the diluent for the antibodies in these assays as without the stabilising affect of this buffer the pH and ionic strength of urine may have interfered with the antibody-antigen interaction

BIAcore technology may be used as an alternative for traditional analytical detection systems such as HPLC, CE and ELISA techniques. In this case, the BIAcore assay developed in our lab compares favourably with other methods of detection for M3G. Of the analytical techniques discussed in section 3.1.4, only the HPLC method developed by Meng *et al* (2000) has a lower limit of detection than our assay. Also the majority of the methods discussed involve some type of sample clean up. When comparing the M3G assays developed in this chapter to previous assays developed using the BIAcore biosensor, results are favourable as both assays developed were capable of detecting M3G at pg concentrations. BIAcore inhibition assays developed by Daly *et al* (2000) and Keating *et al* (1999) to the mycotoxin aflatoxin B<sub>1</sub> and 7-hydroxycomarin, respectively, possess limits of detection for aflatoxin B<sub>1</sub> of 3 ng/ml and 50 ng/ml of 7-hydroxycomarin.

Further comparison of other assays developed include radioimmunoassays developed by Spector *et al* (1971) and Steiner *et al* (1978) which have limits of detection of 50 pg/ml and 500 pg/ml, respectively, for morphine. One ELISA assay developed for detection of morphine by Usagawa *et al* (1993) has very low limits of detection down as low as 100 pg/ml. These assays are more sensitive, however, for screening and confirmatory analysis, the assay discussed in this chapter is suitable to such applications.

The purpose of opiate analysis in biological samples is mainly carried out to monitor therapeutic levels in patients, drug concentrations in human and animal pharmacokinetic studies, for the investigation of opiate abuse for drug control as well as to characterise causes of intoxication or death in cases of clinical, pathological or forensic interest (Rashid *et al*, 1998). As opioids are metabolised into morphine and then morphine-3-glucuronide and various other pharmacologically active compounds, there is a need for

sensitive and specific methods of detection of free and glucuronidated forms (Wernly *et al*, 1993)

The relationship between heroin and other CNS depressants resulting in death by overdose is of prime importance and has several implications for research purposes. At present, very little information is available on blood morphine levels in living, intoxicated heroin users. Methods for the determination of opiates in living and fatal overdose cases are desirable as they would contribute to our understanding of opiate related fatalities (Darke and Zador, 1996)

Opiates are widely used clinically in the relief of moderate to severe pain in advanced cancer patients. Detection systems may also be used for the clinical study of opiate concentrations in blood and urine in the determination of dose requirements for patients in severe pain as monitoring of pain relief may be correlated to specific opiate concentrations (Sawe, 1986)

Detection of opiate abuse in rehabilitation centres, industry (includes pre-employment testing and for-cause testing), correctional departments and emergency rooms is also of prime importance. The testing options for opiates include screening only and screening and confirmation of specific opiates or any opiate found. Due to the fact that some legally available opiates (codeine) result in false positives, the cut-off concentrations were raised (cut-off concentration for morphine and codeine were raised from 300 to 2000 ng/ml) in 1997 in an attempt to prevent these false positives. The detection range of the assays developed in this chapter are lower than the cut-off concentrations, however, they may be used to determine positive results by dilution of samples to within the detection range of the assay (Welsh, 1998)

## **Chapter 5**

### **Production of Murine Recombinant Antibody Libraries for Selection of Anti-Morphine-3-glucuronide Single Chain Fv (scFv) Antibodies for Applications to Immunoassays**

## 5 1 Introduction

### 5 1 1 The Krebber system for the production of single chain Fv (scFv) antibodies

The main requirement of recombinant antibody technology, using hybridomas or immune repertoires as the source of DNA, is the reliable cloning of functional light and heavy chain genes. For this purpose, Krebber developed a standard phage display system optimised for robustness, vector stability, tight control of scFv-gIII expression, primer usage for PCR amplification of variable region genes, scFv assembly strategy and subsequent directional cloning using a single rare cutting restriction enzyme. The Krebber (Krebber *et al*, 1997) system allows the rapid harvesting of antigen binding scFvs derived from spleen cell repertoires of mice immunised with morphine-3-glucuronide conjugates.

A single phagemid vector system used to efficiently express intact scFv fragments on the surface of phage was produced by Krebber. The vector is known as pAK100 (Figure 5 1 A) and contains a tetracycline (tet) resistance gene, which was picked to allow monitoring of digestion by gel electrophoresis and origin of replication for *E coli* and a chloramphenicol (cam) gene used in the selection of scFv fragments. It also contains an origin of phage replication and has directional cloning sites for PCR-amplified SOE products. The SOE-PCR product is ligated down stream of the single pelB leader sequences for efficient *E coli* periplasmic transport allowing a low level of scFv-gIII expression upon induction by IPTG. The SOE-PCR product is ligated to the C terminal to the gIII gene sequence where, upon expression, it forms a fusion protein. The *lac* repressor is present to produce strain-dependent *lac* promoter expression. The phagemid is now ready to produce phage particles with the help of helper phage. For soluble expression of scFv fragments the pAK400 (Figure 5 1 B) vector is used as the gIII gene has been removed with the addition of a much stronger Shine-Dalgarno sequence (SDT7g10) to enhance protein expression in the JM83 expression host.

An outline of the Krebber system is shown in Figure 5.2. The first step in the production of a recombinant library using this system was to isolate mRNA from murine splenocytes either pre-immunised with an antigen or from an unimmunised mouse. The mRNA isolated should theoretically contain the full repertoire of antigen specificities. Complementary DNA (cDNA) may then be synthesised from the mRNA using random hexamer primers which complement the 5' end of the mRNA and using reverse transcriptase to convert mRNA into its cDNA strand.

This cDNA is then used as a template for the PCR amplification of light and heavy chain genes using a primer mix specific for this amplification followed by thermal cycling to amplify these fragments. The PCR primers are so designed that they also introduce restriction sites at the ends of the amplified genes for ligation into the pAK100 vector. The amplified light and heavy chain genes are then joined together by splice by overlap extension (SOE) PCR and this product is purified and digested along with the pAK100 vector with the SfiI restriction enzyme (Figure 5.3). The digested scFv genes are then ligated into the digested vector and transformed into *E. coli*.

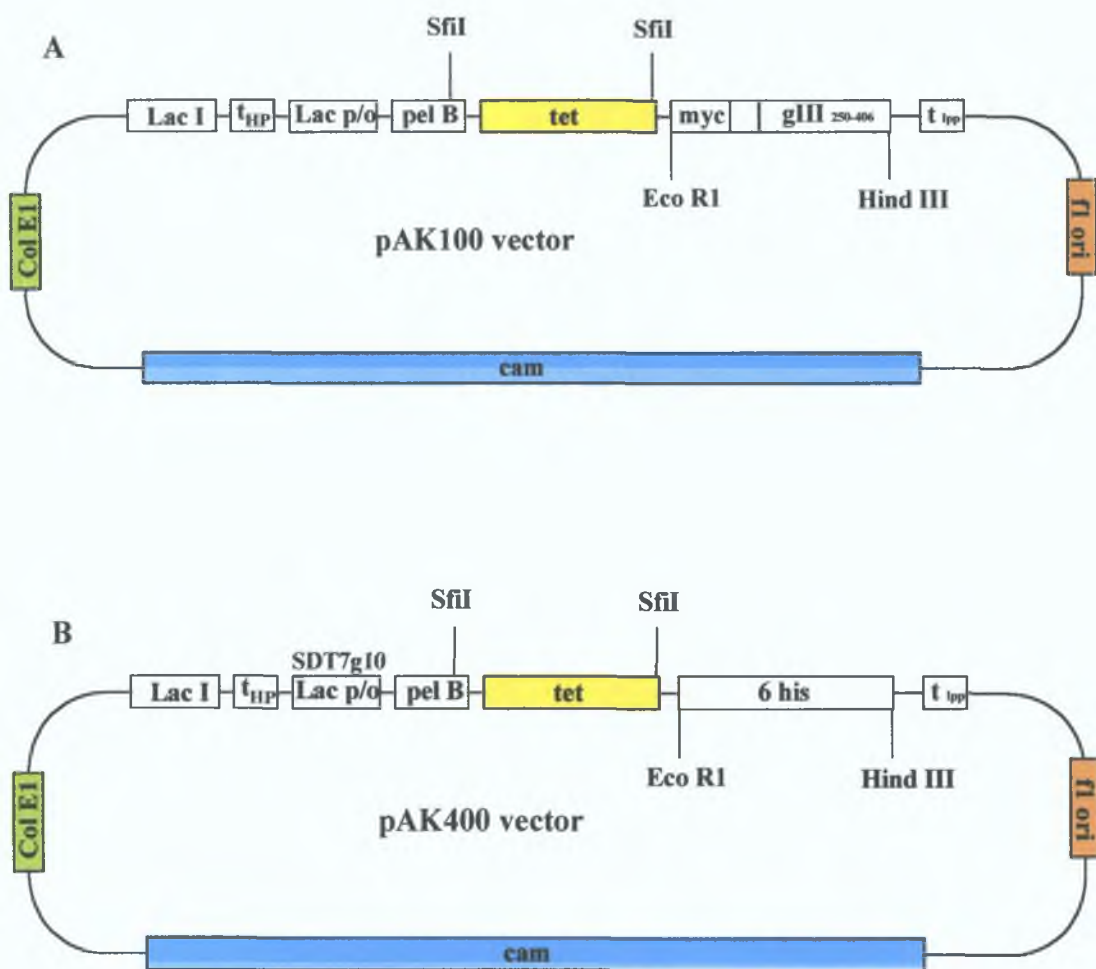
Helper phage is used to package phagemid DNA into a virion. This results in the expression of recombinant antibodies on the surface of the phage. Screening for specific scFvs is carried out by propagation of *E. coli* resulting in the excretion of virions into the supernatant. After separation of phage from cells by centrifugation, the phage are added to wells of a microtitre plate coated with the antigen of interest, resulting in binding of specific scFv's. After washing to remove non-specific scFv's, specific fragments are removed from the plate and isolated.

Production of soluble scFv's is carried out by digestion of the specific DNA from positive clones and religation into the pAK400 vector (with the gIII gene removed to inhibit phage production) containing a much stronger Shine-Dalgarno sequence for increased expression. This vector is transformed into JM83 cells for soluble expression.

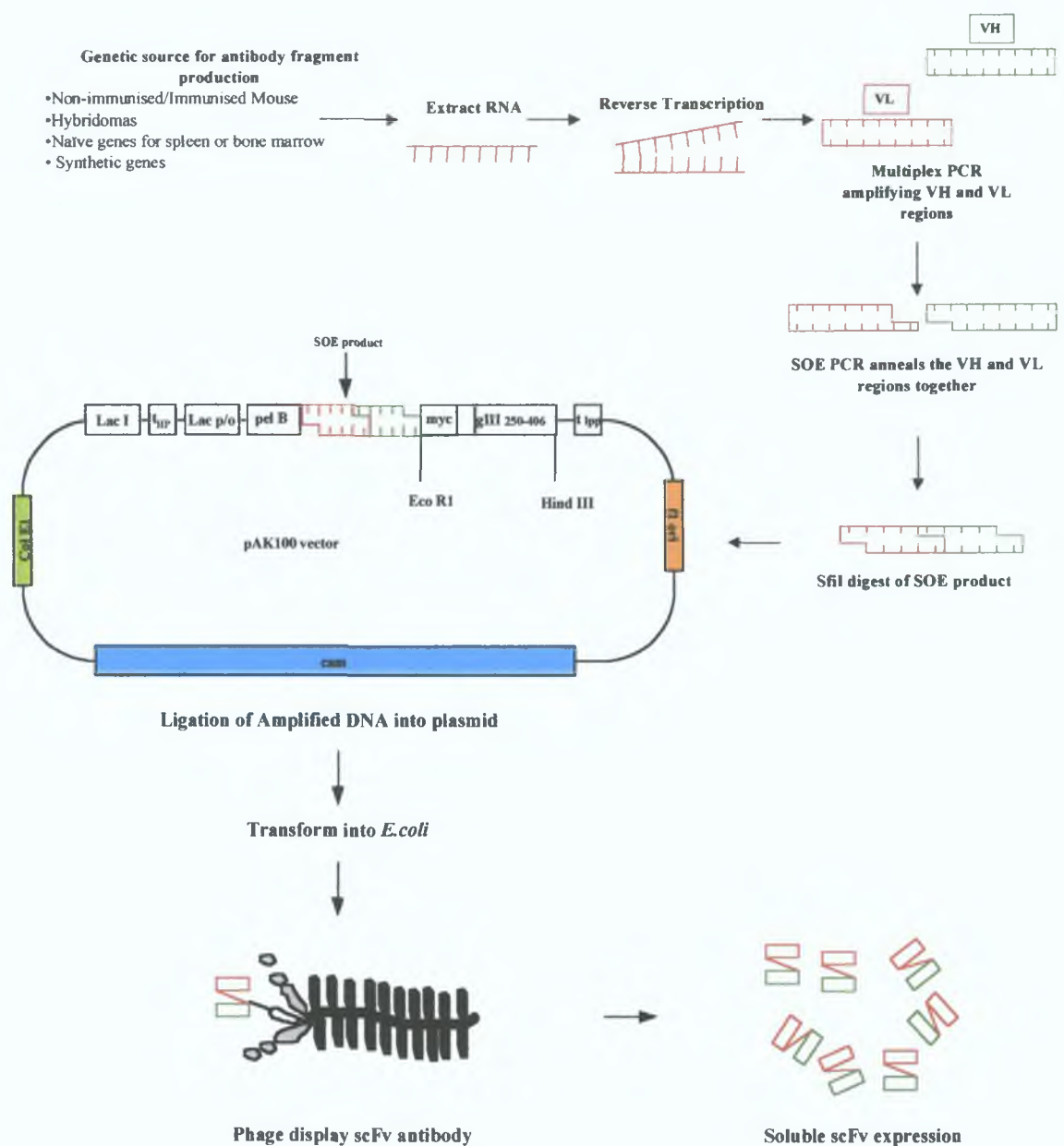
As discussed in the paper produced by Krebber *et al* (1997), their system contains a number of improved features compared to other systems. The Krebber system

contains more diverse primers enabling the amplification of more light and heavy chain genes. The system also contains a FLAG peptide for easier purification and splice by overlap extension (SOE) PCR for efficient assembly of scFv fragments. Further features include using only one restriction enzyme for digestion of SOE products and vectors and insertion of an amber codon allowing switching between expression of scFv attached to phage and soluble scFv by changing the expression host.

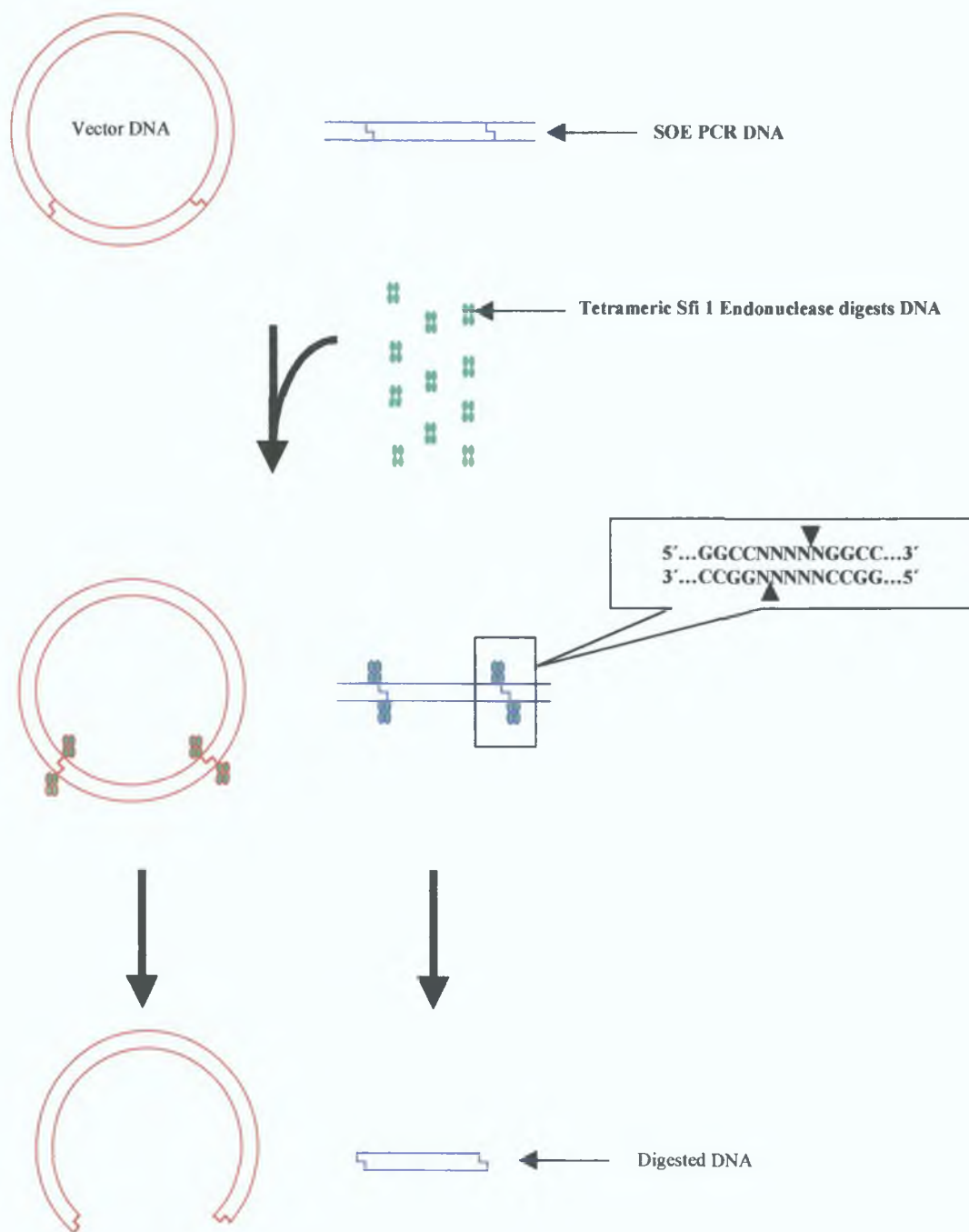




**Figure 5.1.** (A) The pAK100 phagemid vector. Replication in *E. coli* is via the *Col E1* ori. Resistance is via the *cam* (chloramphenicol) and *tet* (tetracycline) resistance genes. For phage packaging, pAK100 carries the intrinsic region from *f1* phage. Directional insertion of light and heavy chain genes ligated to produce SOE product is via the *SfiI* restriction enzyme sites, which are downstream from the *Lac* promoter/operator and the *pelB* leader sequence. Downstream of the SOE insertion position is the *gIII*, for phage surface expression of scFv fragments. (B) The pAK400 vector is used for soluble expression of scFv fragments, there are two main differences from the pAK100 vector. The *gIII* gene is replaced with a 6 His tag for IMAC purification and a much stronger Shine-Dalgarno sequence for enhancement of protein expression.



**Figure 5.2.** Schematic diagram illustrating the production of antibody fragments using the Krebber system. Antibody mRNA is isolated from a suitable source as listed above. The mRNA undergoes reverse transcription and PCR amplification using families of variable 5' primers and constant 3' primers, encoding a restriction site for cutting with *Sfi*I. Amplified light and heavy chain genes are joined together by SOE-PCR. Vector and PCR product are digested, ligated and transformed in *E. coli*. Phage expression is brought about by infection with helper phage, and affinity selection takes place against the desired antigen immobilised to a solid support. When specific clones have been isolated, soluble antibody may be expressed by religating specific light and heavy chain genes into the pAK400 vector (with *gIII* gene removed).



**Figure 5.3.** Diagrammatic representation of SfiI restriction digestion of amplified scFv fragment and pAK100 vector. SfiI is the only restriction enzyme used in this system for antibody cloning.

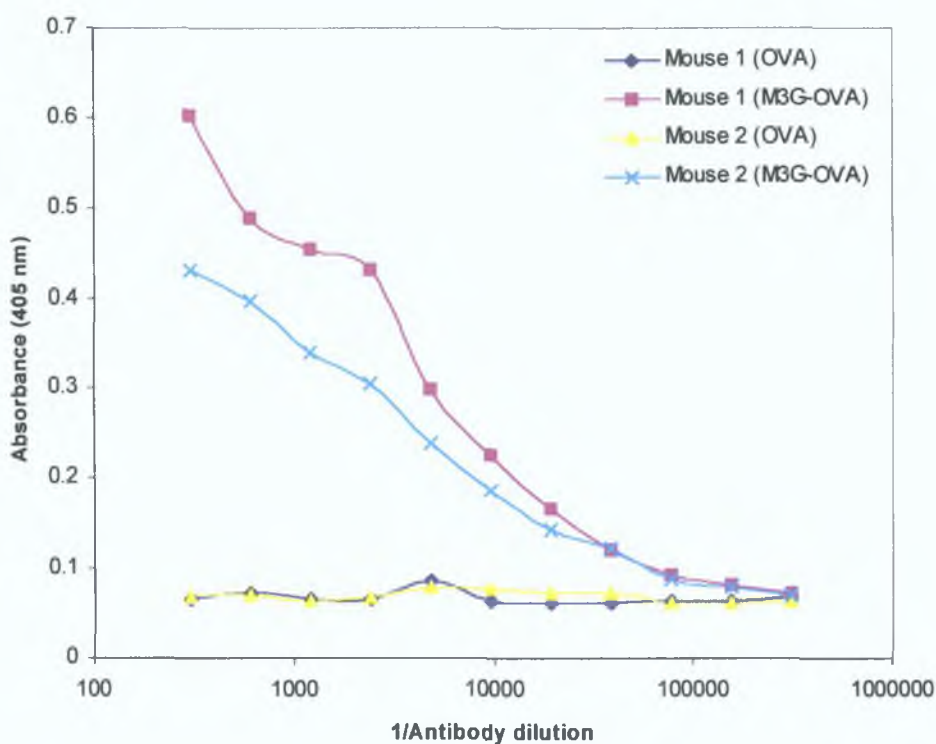
## **5 2 Results**

### **5 2 1 Production of murine scFv antibody libraries to morphine-3-glucuronide**

#### **5 2 1 1 Immunisation of mice with morphine-3-glucuronide-bovine serum albumin conjugate**

Balb/c mice were immunised with a carbodiimide-coupled bovine serum albumin conjugate to M3G as described in section 2 3 5 2. Immunisations were carried out by subcutaneous and intraperitoneal injections of conjugate in Freund's adjuvant and the antibody concentrations monitored by taking blood samples to estimate the titre of specific antibody. Analysis of the titre was carried out using the ELISA as described in section 2 3 8 1.

Serial dilutions of mouse serum from 1/300 to 1/300,000 were prepared and added to wells of a microtitre plate coated with M3G-OVA conjugate, followed by labelled anti-rabbit antibody and chromogenic substrate. Serum from both mice gave a very high response to M3G-OVA and both antibodies titred out at about 1/300,000 (Figure 5 4). On control wells coated with OVA, there was a negligible response indicating the antibodies are specific to the M3G portion of the conjugate. These serum titrations indicated a sufficient response from the host to isolate splenic mRNA as described in section 2 3 9 1. The concentrations of isolated mRNA taken from mice are listed in Table 5 1.



*Figure 5.4. Overlay plot showing the antibody titres of Mouse-1 and Mouse-2 serum before sacrifice. Serial dilutions of serum were added to wells coated with M3G-OVA. Both mice immunised with M3G-BSA gave similar titres of greater than 1/150,000 to the M3G-OVA conjugate and showed minimal non-specific interactions with OVA.*

Mouse Number	mRNA Concentration ( $\mu\text{g}/\mu\text{l}$ )
1.1.	7.34
1.2.	8.52
1.3.	10.88
2.1.	6.76
2.2.	7.71
2.3.	11.43

*Table 5.1. Showing the concentration of isolated mRNA from 2 mice immunised with M3G-BSA. Spleen cells from each mouse were aliquotted into three samples and the concentration of mRNA of each aliquot determined.*

### **5 2 1 2 Selection of primers for amplification of light and heavy chain genes**

The set of mouse primers (section 2 3 9 3 1) described in this chapter for the amplification of mouse light and heavy chain genes have been optimised to incorporate all mouse  $V_H$ ,  $V_\lambda$  and  $V_\kappa$  sequences collected by the Kabat data base (Kabat *et al*, 1991) and combine extended primer sets described by Kettleborough *et al* (1993), Ørum *et al* (1993) and Zhou *et al* (1994)

### **5 2 1 3 Amplification of V region genes and assembly into the scFv format**

Reverse transcription to synthesise cDNA was initially carried out on mRNA isolated from mice spleen cells immunised with M3G-BSA. Using the standard protocol for the amplification of heavy and light chain genes as described in section 2 3 9 3 3 primers amplified a single band of approximately 400 bp for both light and heavy chain genes using this cDNA. To amplify light chain genes the  $V_L$  back primer mix was paired with five  $V_L$  forward primers and to amplify heavy chain genes the  $V_H$  back mix was paired with four  $V_H$  forward primers. In all cases, the first PCR amplification yielded sufficient amounts of products for cloning, with a sharp band produced at the predicted band of 375-402 bp (Figure 5 5) for  $V_L$  or 386-440 bp (Figure 5 6) for  $V_H$ . These fragments were purified from the gel as described in section 2 3 9 4 and their concentration determined (Table 5 2)

### **5 2 1 4 Splice by overlap extension (SOE) PCR**

All amplified  $V_L$  and  $V_H$  domains after purification were linked together by SOE-PCR to produce a combined molecular weight fragment of approximately 800 bp (Figure 5 7)

### **5 2 1 5 Digestion of pAK100 vector and SOE-PCR products formed by linking antibody light and heavy chain genes**

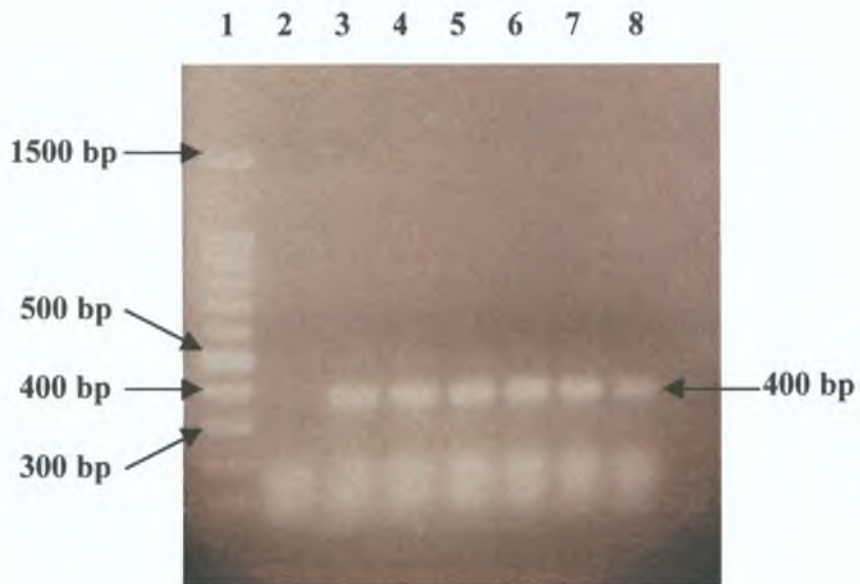
The pAK100 vector and SOE-PCR products formed by linking antibody light and heavy chain genes were digested with SfiI restriction enzyme as shown in Figure 5 7 for digestion of SOE-product and Figure 5 8 for digestion of pAK100 and described for both in section 2 3 9 8. Figure 5 7 shows the optimisation of concentrations required of SfiI restriction enzyme for the digestion of SOE-PCR product resulting in 20 units of restriction enzyme being required to digest the fragment fully. Figure 5 8 identifies two main bands labelled A and B. A represents cut pAK100 vector ready for ligation with cut SOE-PCR product while B represents the tetracycline gene fragment cut from the vector. Band A was purified from the gel as described in section 2 3 9 4 and the concentration determined (Table 5 2).

### **5 2 1 6 Ligation of antibody light and heavy chain genes into the pAK100 vector**

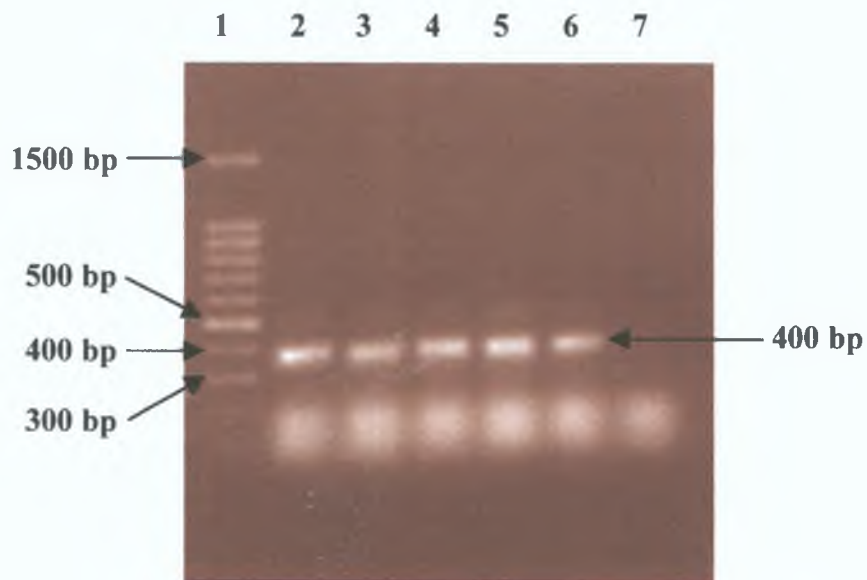
After purification, the SOE-PCR fragment was ligated into the pAK100 vector at a molar ratio of vector to insert of 1 5 1 as described in section 2 3 9 9.

### **5 2 1 7 Transformation of *E coli* XL1-Blue supercompetent cells with pAK100 vector containing light and heavy chain genes**

Supercompetent *E coli* XL1-Blue cells, with a transformation efficiency of  $1.0 \times 10^9$  cfu/ $\mu$ g of DNA, were used for the production of a recombinant antibody library to M3G using the procedure described in section 2 3 9 10. Ligation reactions of pAK100 vector and SOE-PCR products were transformed resulting in the production of a M3G recombinant library consisting of approximately  $5 \times 10^3$  transformants.

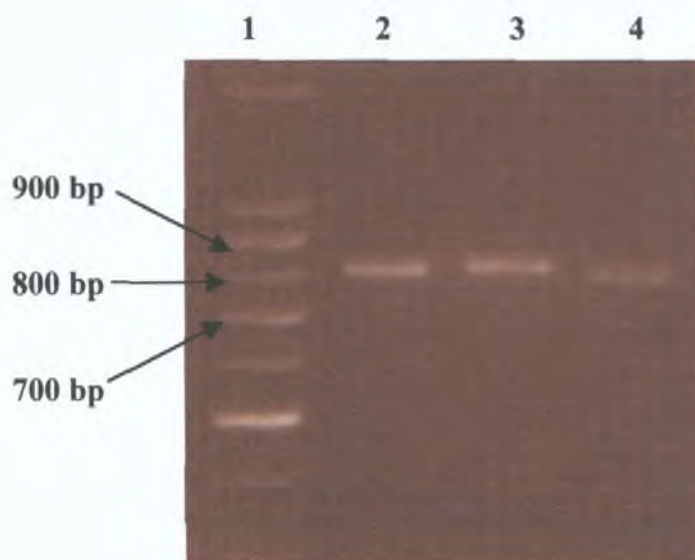


*Figure 5.5. Gel showing the amplification of murine light chain genes from mouse 1.1 immunised with M3G-BSA. Lanes: (1) 100 bp molecular weight makers. (2) Negative control for PCR without any cDNA. (3-8) Amplified 400 bp fragment using primer combinations listed in section 2.3.9.3.1. under conditions described in section 2.3.9.3.3.*

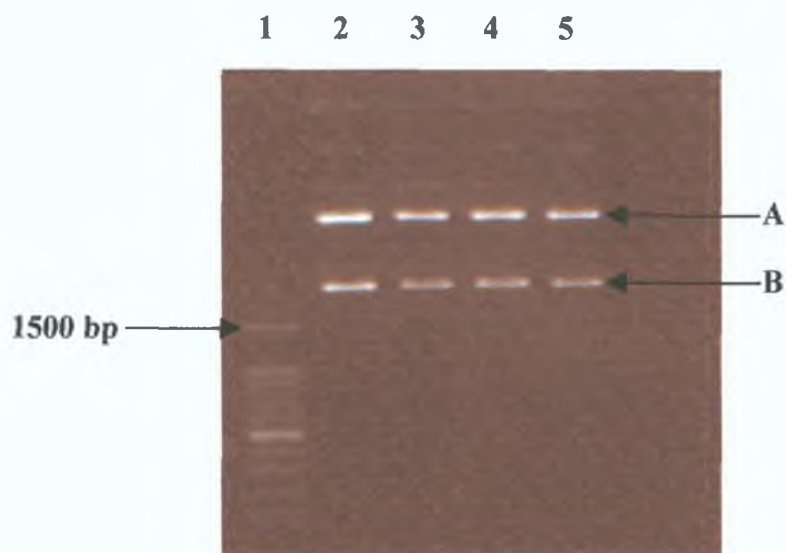


*Figure 5.6. Gel showing the amplification of murine heavy chain genes from mouse 1.1 immunised with M3G-BSA. Lanes: (1) 100 bp molecular weight makers. (2-6) Amplified 400 bp fragment using primer combinations listed in section 2.3.9.3.1. under conditions described in section 2.3.9.3.3. (8) Negative control for PCR without any cDNA.*





**Figure 5.7.** Gel showing undigested SOE-PCR product and optimisation of double digestion of SOE-PCR product using *SfiI* restriction enzyme. Lanes: (1) 100 bp molecular weight markers. (2) Shows amplified  $V_L$  and  $V_H$  domains linked by SOE-PCR. (3) SOE-PCR product digested using 10 units of *SfiI* restriction enzyme. (4) Digested SOE-PCR using 20 units of *SfiI* restriction enzyme.



**Figure 5.8.** Restriction digestion of pAK100 vector. Lanes: (1) 100 bp molecular weight markers. (2-5) Gel showing the digestion of pAK100 vector with *SfiI* restriction enzyme. (A) Represents digested pAK100 plasmid with *SfiI* restriction enzyme. (B) Represents digested insert from plasmid after restriction digest proving the digestion of the pAK100 plasmid.

*Table 5 2 Showing the concentration of DNA of the components for library production*

<b>Samples</b>	<b>Concentration of DNA (ng/μl)</b>
M3G Heavy chain Mouse No 1 1	37 7
M3G Heavy chain Mouse No 2 1	39 7
M3G Light chain Mouse No 1 1	25 6
M3G Light chain Mouse No 2 1	17 2
Splice by Overlap extension (SOE)	7 5
Digested SOE	4 9
PAK 100 Vector	16 3
PAK 400 Vector	18 5

## **5.2.2 Production of single chain Fv antibodies to morphine-3-glucuronide**

### **5.2.2.1 First round selection and screening of functional scFv sequences to morphine-3-glucuronide from morphine-3-glucuronide recombinant antibody library using phage ELISA**

After transformation of the ligation reaction into supercompetent *E. coli* XL1-Blue cells, production of phagemid particles from the M3G recombinant antibody library was carried out as described in section 2.3.10.1. This resulted in a phage titre (section 2.3.10.3) of approximately  $4 \times 10^{10}$  phage particles being produced. These phage particles were then used in the first round affinity selection of scFv antibodies by panning with M3G-OVA as described in section 2.3.10.4. After panning and elution from the immunotube a titration was carried out on the remaining phage resulting in a  $5.1 \times 10^6$  phage particle count. Bacterial phage stock cultures were produced as described in section 2.3.10.5 after the first round of panning.

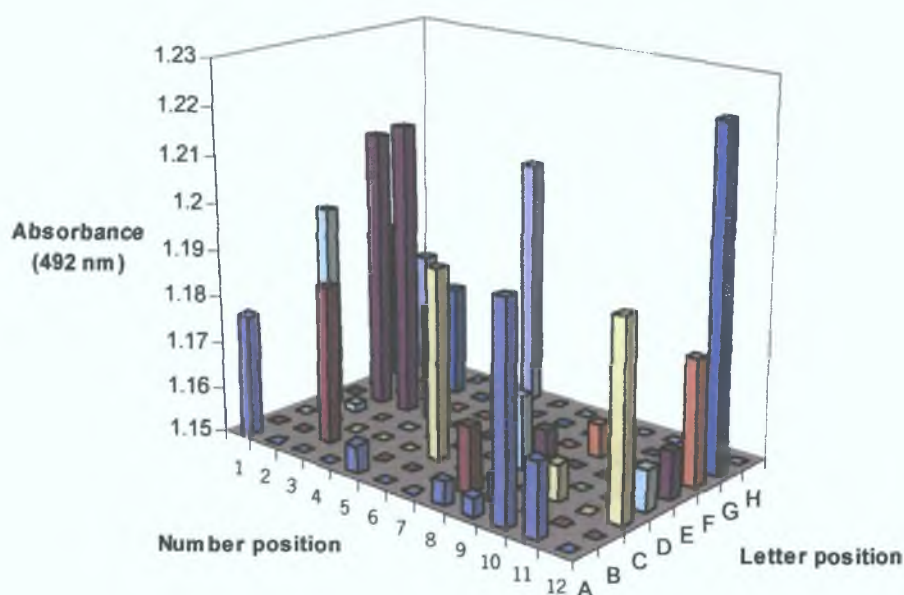
95 individual clones (Table 5.3 and Figure 5.9) from the M3G recombinant antibody library, following the first round of affinity selection, were grown separately and infected by helper phage as described in section 2.3.10.6. The recombinant scFvs, displayed on the surface of filamentous phage, were screened on a plate coated with M3G-thyroglobulin (THY) for antigen binding in a typical phage ELISA as described in section 2.3.10.7. Results from the phage ELISA showed that all clones analysed possessed affinity to the conjugate producing absorbance readings of 1 or higher. Six positive clones, B8, C6, E2, E3, G3 and H5 were selected on the bases of the strength of their absorbance readings for characterisation with respect to their affinity for free drug. However, all the clones showing high absorbance values may be positive clones, six were chosen as a model system.

#### **5 2 2 2 Analysis of supernatant of anti-morphine-3-glucuronide clones for the presence of specific scFv**

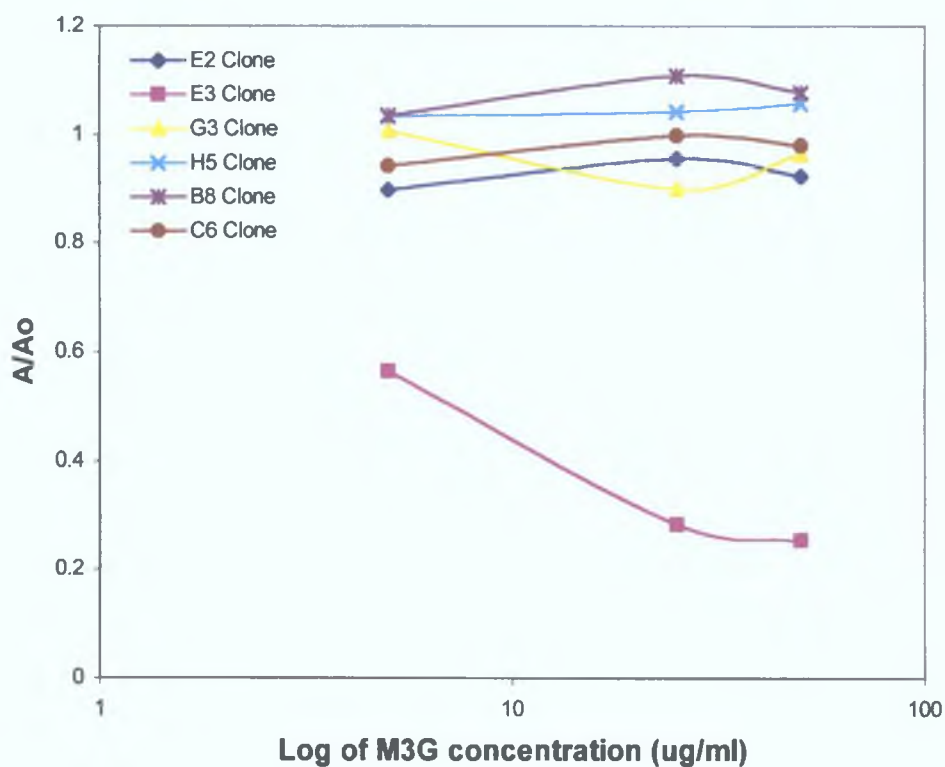
Competitive phage ELISA's were carried out for the six clones selected as described in section 2 3 10 8 2 Standards of free drug were prepared at drug concentrations of 50, 25 and 5  $\mu\text{g/ml}$  and added to neat supernatant at a ratio of 1 1 The mixtures were then added to the wells of a microtitre plate coated with 100  $\mu\text{g/ml}$  of M3G-THY Results indicated that the E3 clone was the only clone to exhibit affinity for free M3G (Figure 5 10) as absorbance readings decreased as drug concentrations increased

**Table 5.3. Phage ELISA data of 95 randomly selected clones after the first round of panning of the M3G library screened against M3G-OVA. Six scFv antibody clones (bold) were picked and analysed for their ability to recognise free M3G.**

	1	2	3	4	5	6	7	8	9	10	11	12
A	1.176	1.146	1.150	1.093	1.156	1.102	0.369	1.155	1.154	1.196	1.166	1.143
B	1.141	1.093	1.184	1.099	1.097	1.131	1.118	<b>1.164</b>	1.156	1.111	1.120	1.124
C	1.119	1.107	1.098	1.126	1.104	<b>1.191</b>	1.080	1.120	1.127	1.158	1.113	1.192
D	1.194	1.152	1.102	1.094	1.057	1.119	0.850	1.166	1.086	1.089	1.082	1.159
E	1.112	<b>1.210</b>	<b>1.213</b>	1.175	1.103	1.092	1.150	1.156	1.094	1.092	1.037	1.160
F	1.090	1.095	1.122	1.138	1.111	1.156	1.147	1.122	1.157	1.108	1.122	1.177
G	1.185	1.138	<b>1.174</b>	1.132	1.148	1.147	1.083	1.130	1.125	1.115	1.151	1.223
H	1.176	1.104	1.142	1.170	<b>1.203</b>	1.076	1.113	1.070	1.133	1.146	1.109	0.276



**Figure 5.9. Graphical representation of absorbance data from ELISA carried out on 95 clones after the first round of panning. B8, C6, E2, E3, G3 and H5 scFv antibody clones were picked in order to study their ability to bind to free M3G.**



*Figure 5.10. Competition enzyme immunoassay carried out for free M3G at drug concentrations of 50, 25 and 5  $\mu\text{g/ml}$  on supernatant of six scFv antibody clones picked from the first round of panning. As in the cases of B8, C6, E2, G3 and H5 scFv antibody clones, no competition was noted. However, the E3 clone shows competition for free M3G.*

### **5 2 2 3 Second round selection and screening of functional scFv sequences to morphine-3-glucuronide from morphine-3-glucuronide recombinant antibody library using phage ELISA**

Production of phagemid particles from the M3G recombinant antibody library first round panning stocks was carried out as described in section 2 3 10 1 This resulted in a phage titre (section 2 3 10 3 ) of approximately  $6.2 \times 10^{11}$  phage particles being produced M3G-THY conjugate was used as the coating conjugate for the second round panning procedure as described in section 2 3 10 4 After panning and elution from the immunotube a titration was carried out on the remaining phage resulting in a  $6.0 \times 10^8$  phage particles count Bacterial phage stock cultures were produced after the second round of panning as described in section 2 3 10 5

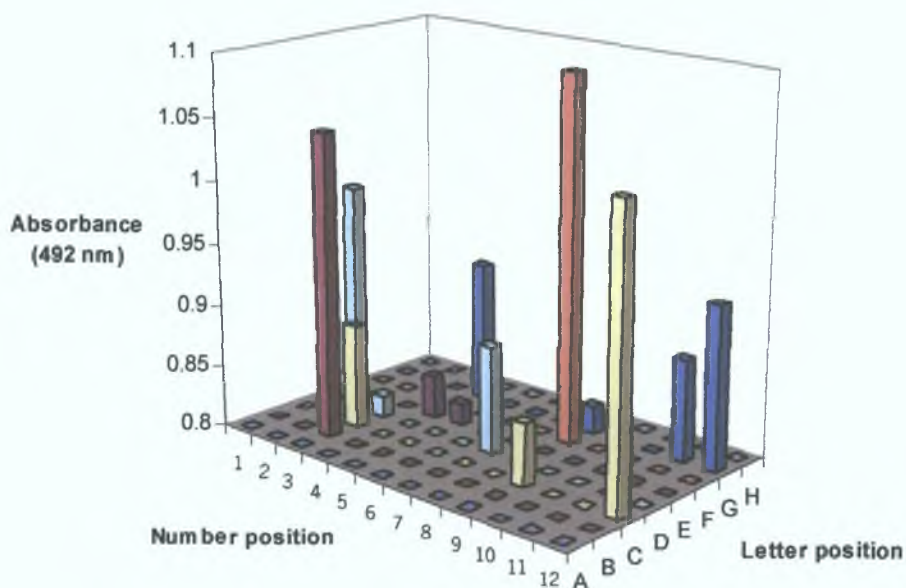
Again as for the first round of panning, 95 individual clones following the second round of affinity selection, were grown separately and infected by helper phage as described in section 2 3 10 6 The recombinant scFvs, were screened on a plate coated with M3G-OVA for antigen binding in a typical phage ELISA as described in section 2 3 10 7 Results from the phage ELISA are shown in Table 5 4 and Figure 5 11 showing absorbance readings and a graphical representation of the higher absorbance readings, respectively Six positive clones, B3, C12, D2, F8, G4 and G12 were selected on the bases of the strength of their absorbance readings for characterisation with respect to their affinity for free drug

### **5 2 2 4 Analysis of supernatant of anti-morphine-3-glucuronide clones for the presence of specific scFv after second round of panning**

Competitive phage ELISA's were carried out for the six clones selected as described in section 2 3 10 8 2 Standards of free drug were prepared at drug concentrations of 50  $\mu\text{g/ml}$ , 25  $\mu\text{g/ml}$  and 5  $\mu\text{g/ml}$  and added to neat supernatant at a ratio of 1:1 The mixtures were then added to the wells of a microtitre plate coated with 100  $\mu\text{g/ml}$  of M3G-OVA Results indicated that the G12 clone was the only clone to exhibit affinity for free M3G (Figure 5 12)

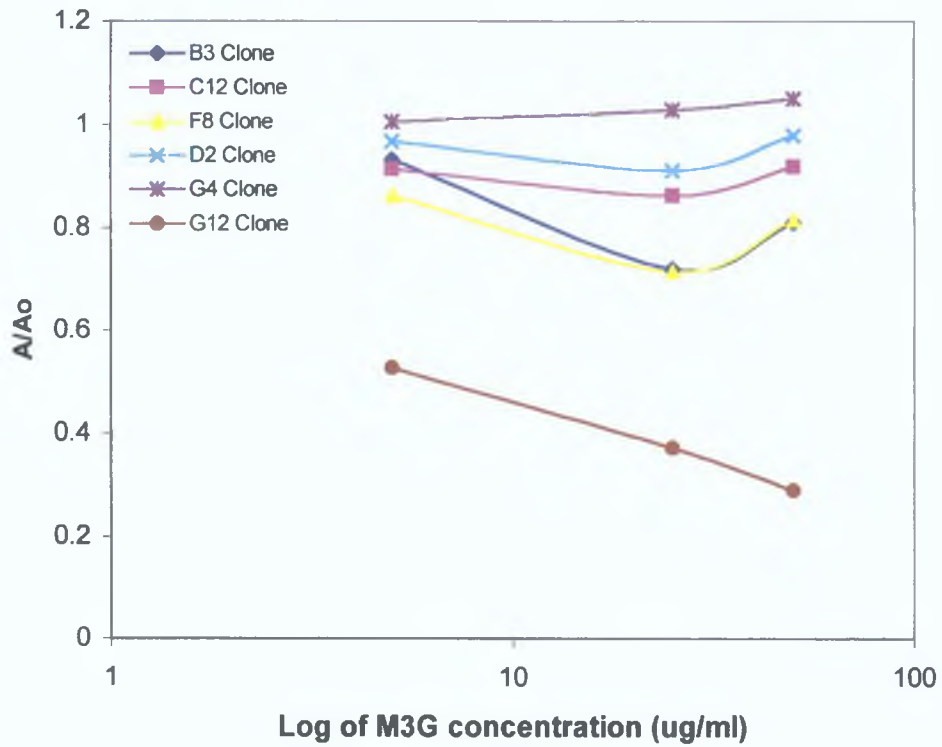
**Table 5.4. Phage ELISA data of 95 randomly selected clones after the second round of panning of the M3G library screened against M3G-THY. Six scFv antibody clones (bold) were picked and analysed for their ability to recognise free M3G.**

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.492	0.657	0.664	0.634	0.625	0.654	0.565	0.652	0.702	0.568	0.571	0.591
B	0.637	0.592	<b>1.043</b>	0.630	0.660	0.709	0.673	0.532	0.669	0.645	0.762	0.687
C	0.654	0.592	0.884	0.681	0.514	0.767	0.609	0.690	0.850	0.746	0.670	<b>1.035</b>
D	0.564	<b>0.984</b>	0.818	0.558	0.695	0.730	0.887	0.602	0.721	0.693	0.768	0.634
E	0.462	0.526	0.669	0.834	0.818	0.629	0.677	0.616	0.746	0.646	0.699	0.670
F	0.582	0.626	0.597	0.756	0.675	0.578	0.498	<b>1.092</b>	0.740	0.678	0.651	0.660
G	0.520	0.760	0.602	<b>0.913</b>	0.724	0.636	0.606	0.822	0.639	0.698	0.882	<b>0.931</b>
H	0.570	0.765	0.610	0.529	0.589	0.662	0.595	0.715	0.690	0.700	0.713	0.198



**Figure 5.11. Graphical representation of absorbance data from ELISA carried out on 95 scFv antibody clones after the first round of panning. B3, C12, D2, F8, G4 and G12 clones were picked in order to study their ability to bind to free M3G.**

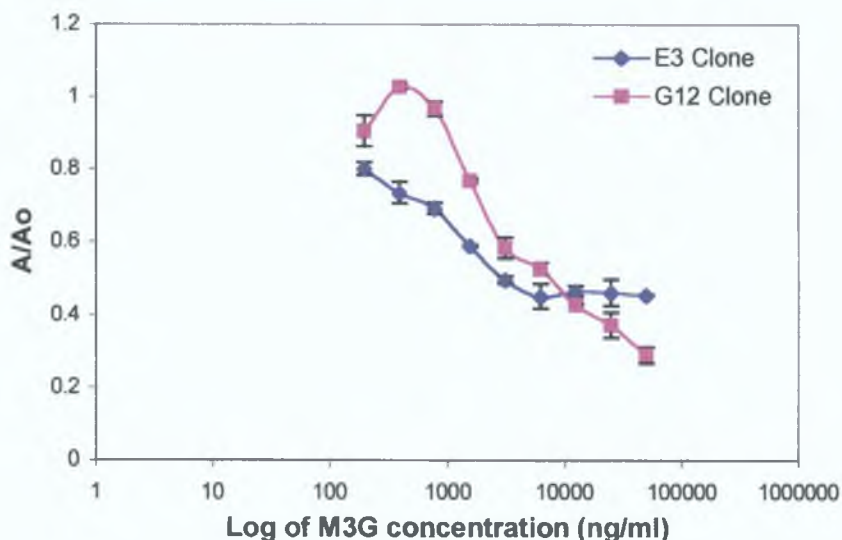




*Figure 5.12. Competition enzyme immunoassay carried out for free M3G at drug concentrations of 50, 25 and 5 µg/ml on supernatant of six scFv antibody clones picked from the second round of panning. As in the cases of B3, C12, D2, F8 and G4 clones, no competition was noted. However, the G12 clone clearly shows competition for free M3G.*

### 5.2.2.5. Broad range competition phage ELISA for positive clones

To further characterise the positive clones screened from the first two rounds of panning and to confirm the ability of the scFv antibody clones to recognise free M3G over a broader range of drug concentrations, competitive phage ELISA's were carried out on both E3 and G12 clones. Standards of free drug were prepared at drug concentrations ranging from 195 to 50,000 ng/ml and added to neat supernatant at a ratio of 1:1. The mixtures were then added to the wells of a microtitre plate coated with 100  $\mu$ g/ml of M3G-THY for the E3 clone and 100  $\mu$ g/ml of M3G-OVA for the G12 clone. Results indicated that both clones had the ability to recognise free M3G over a broad range (Figure 5.13). Further analysis was carried out on the E3 clone for the development of a model system as the E3 clone produced the most accurate linear detection range and seems to have the ability to detect lower concentrations of M3G.



*Figure 5.13. Competition phage ELISA carried out on supernatant of positive scFv antibody clones screened from round one and round two panning. Standards of free M3G between 195 and 50,000 ng/ml were prepared and added to the wells of a microtitre plate coated with conjugate (M3G-THY for the E3 clone and M3G-OVA for the G12 clone) in a ratio of 1:1 with neat supernatant. As free drug concentration increases, the absorbance signal decreases indicating the presence of antibodies specific to M3G.*

### **5 2 2 6 Digestion of E3 scFv antibody light and heavy chain genes and pAK400 vector for production of soluble E3 scFv antibody**

E3 clone antibody light and heavy chain genes were digested with SfiI restriction enzyme as shown in Figure 5 14 There are two main bands present labelled A and B A represents cut pAK100 vector while B represents the fragment cut from the vector containing E3 light and heavy chain genes

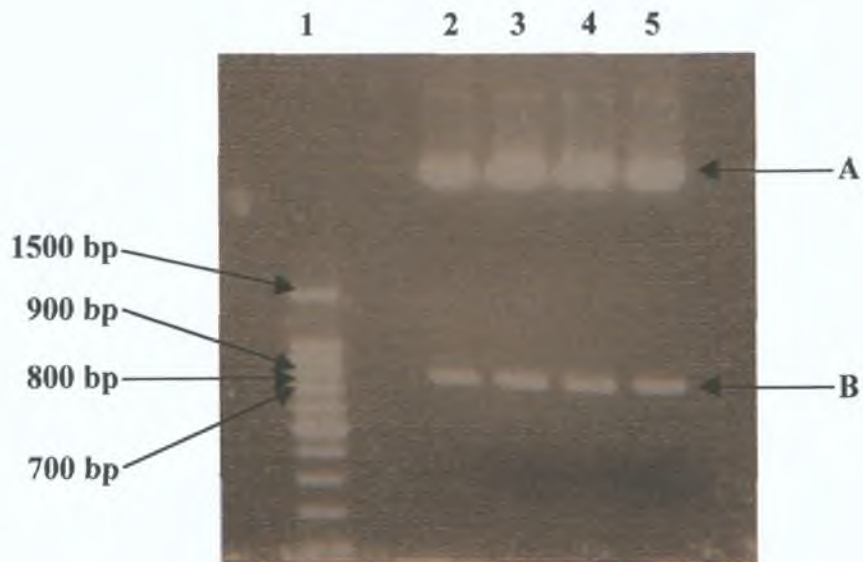
The pAK400 vector is used for the production of soluble scFv fragments Digestion of the pAK400 vector is shown in Figure 5 15 showing two main bands present labelled B and C B represents the digested pAK400 plasmid while C represents digested insert from plasmid after restriction digest as described in section 2 3 9 8

The band represented by B was gel-purified as described in section 2 3 9 4 and the concentration determined (Table 5 2) followed by ligation with E3 clone light and heavy chain genes

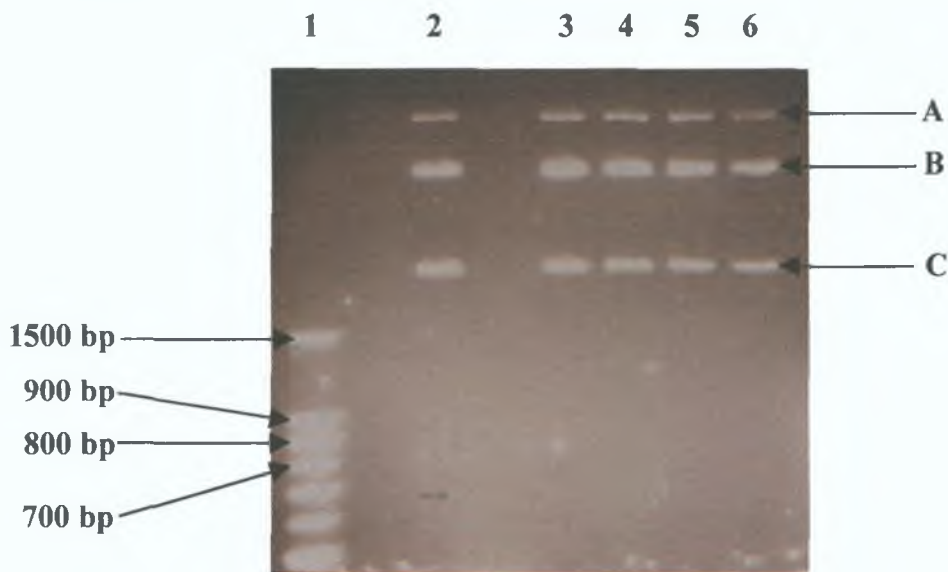
Ligation reactions of pAK400 vector and E3 light and heavy chain genes were transformed into calcium chloride JM83 competent cells as described in section 2 3 10 12 resulting in the production of E3 clones capable of producing soluble scFv antibodies

### **5 2 2 7 Purification and characterisation of E3 soluble scFv antibodies**

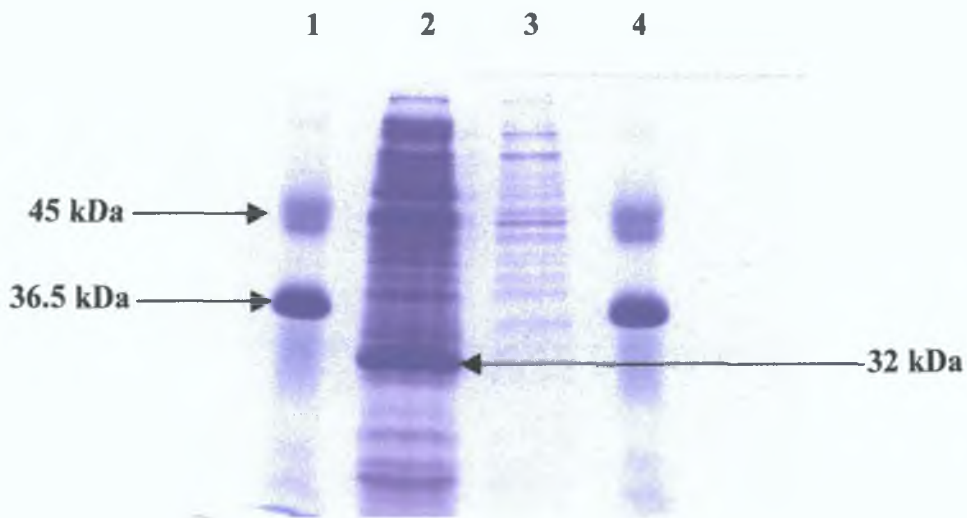
Soluble E3 scFv antibodies were produced in supernatant as described in section 2 3 10 13 and purified using Probond™ resin as described in section 2 3 10 15 The purified E3 scFv antibody was characterised using both SDS-PAGE gel (section 2 3 7 6 ) and western blot analysis (section 2 3 7 8 ) as can be seen in Figures 5 16 and 5 17, respectively SDS-PAGE gel shows the presence of a band at the molecular weight of 32 kDa while Western blotting confirms that the band at 32 kDa is in fact scFv antibody as the detection antibody used was an anti-FLAG antibody capable of recognising the 6 His tag present on the scFv antibody



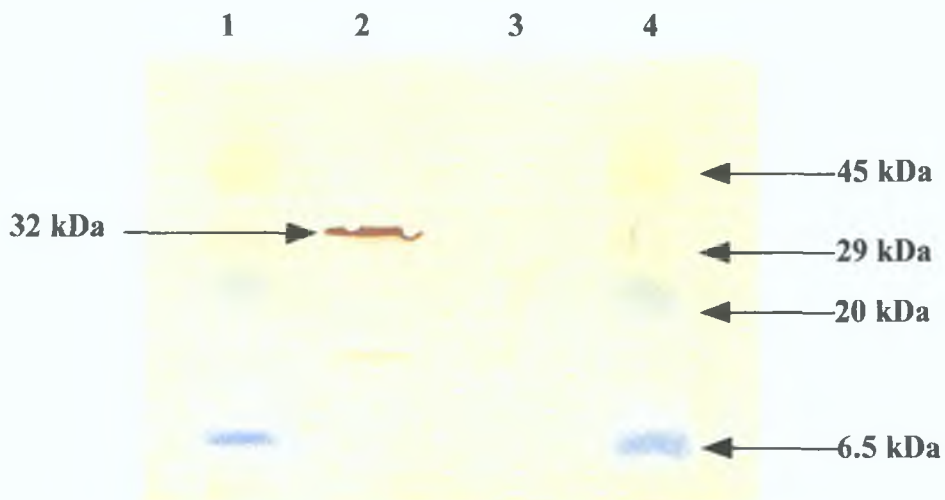
**Figure 5.14.** Restriction digestion of pAK100 vector containing E3 clone genes. Lanes: (1) 100 bp molecular weight markers. (2-5) Gel showing the digestion of pAK100 vector with *SfiI* restriction enzyme. (A) Represents digested pAK100 plasmid. (B) Represents digested insert from plasmid after restriction digest containing light and heavy chain genes for E3 clone.



**Figure 5.15.** Restriction digestion of pAK400 vector. Lanes: (1) 100 bp molecular weight markers. (2-6) Gel showing the digestion of pAK400 vector with *SfiI* restriction enzyme. (A) Represents undigested pAK400 plasmid (B) Represents digested pAK400 plasmid. (C) Represents digested insert from plasmid after restriction digest.



**Figure 5.16.** SDS-PAGE gel analysis of purified E3 scFv antibody. Lanes 1 & 4 contain prestained molecular weight markers ranging from 26 kDa to 116 kDa. Lane 2 represents concentrated purified E3 scFv antibody showing a strong molecular weight band of 32 kDa relative to the 36.5 kDa marker (Lactic Dehydrogenase). Lane 3 represents supernatant from E3 clone.



**Figure 5.17.** Western blot analysis of purified E3 soluble scFv antibody. Lanes 1 & 4 contain prestained molecular weight markers ranging from 6.5 kDa to 45 kDa. Column 2 represents concentrated purified E3 scFv antibody showing a specific molecular weight of 32 kDa. Lane 3 represents supernatant from E3 clone. However, the supernatant was dilute and showed no band at 32 kDa.

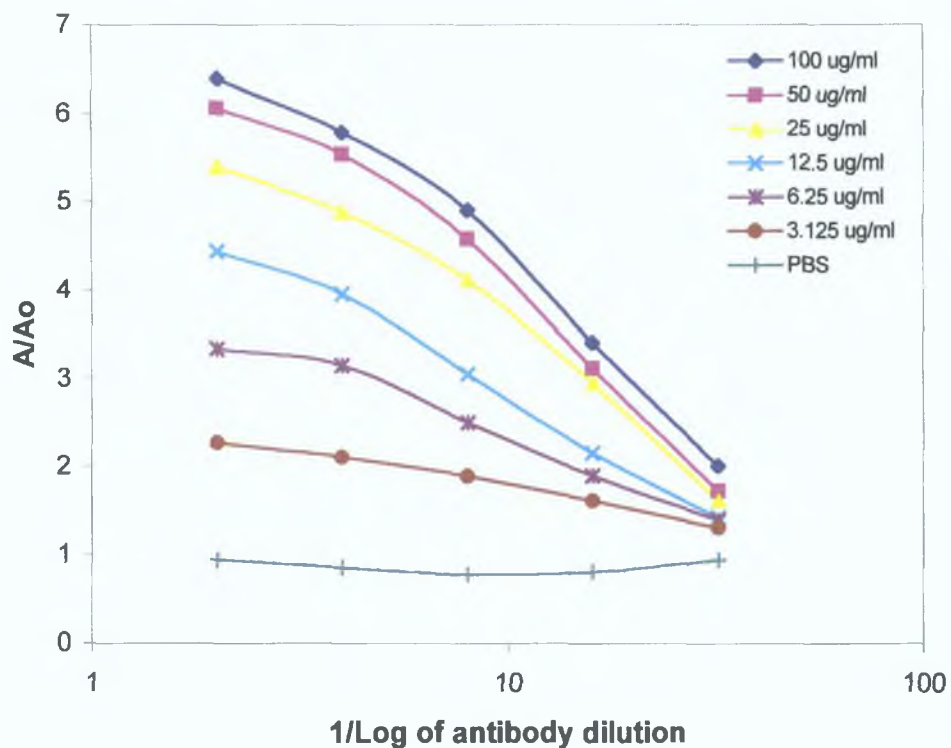
### **5 2 3 Development of a competitive enzyme-linked immunosorbent assay (ELISA) for morphine-3-glucuronide**

#### **5 2 3 1 Checkerboard ELISA for determination of optimal loading ratio of conjugate and optimal E3 scFv antibody dilution**

The working dilution of these antibodies was determined using the format already discussed in section 2 3 10 16 1 M3G-THY was coated on the wells of a microtitre plate at varying concentrations from 3 125 to 100  $\mu\text{g/ml}$ , and blocked with 2% (w/v) marvel Dilutions of purified E3 scFv antibody were prepared at ranges from 1/2 (488  $\mu\text{g/ml}$ ) to 1/32 (30 5  $\mu\text{g/ml}$ ) and each dilution of antibody was added to each concentration of conjugate on the microtitre plate The results were plotted for the E3 scFv antibody as shown in Figure 5 18 The optimal conjugate loading ratio was found to be 50  $\mu\text{g/ml}$  with the optimal antibody dilution determined to be 1/10

#### **5 2 3 2 Intra- and inter-day assay variability studies of E3 scFv antibody**

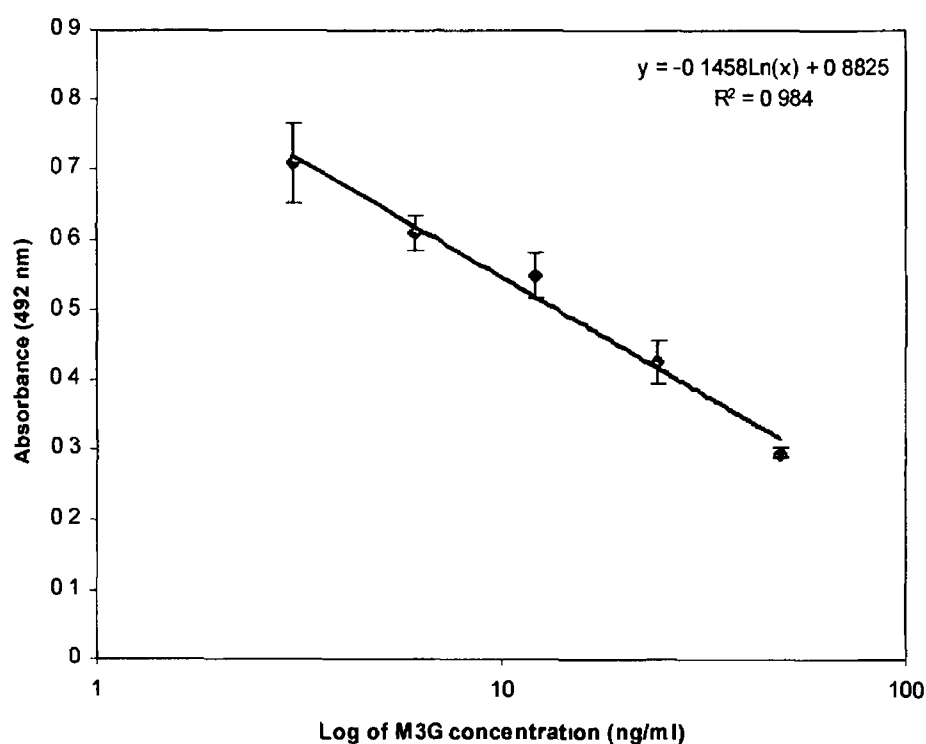
To carry out intra-day assay variability studies, three sets of standards were prepared for the antibody and assayed on the same day and their means plotted The intra-day assay variability study for E3 antibody shown in Figure 5 19 had a linear range of detection between 3 05 and 48 8 ng/ml The  $R^2$  value for this range was 0 98 The coefficients of variation (CV's) were determined to assess the precision of the analytical method, expressing standard deviation as a percent function of the mean These ranged from 2 64% to 8 03% as shown in Table 5 5 The inter-day assay variability study for E3 scFv antibody also had a linear range of detection between 3 05 and 48 8 ng/ml as shown in Figure 5 20 The  $R^2$  value for this range was 0 98 The CV's were determined and ranged from 2 52% to 12 55% as shown in Table 5 6 Studies on the % accuracy level values showed relatively high values for the inter-assay variability studies (Table 5 6)



*Figure 5.18. Checkerboard ELISA carried out to determine the optimal dilution of antibody and concentration of coating conjugate for E-3 scFv antibody. The optimal conjugate loading density was determined as 50  $\mu\text{g/ml}$  and the optimal dilution of antibody for use in a competitive assay was 1/10.*

**Table 5 5** Intra-day assay variation for E-3 scFv antibody ELISA assay Three sets of five standards ranging from 3 05 to 48 8 ng/ml were analysed on the same day

M3G Concentration, (ng/ml)	Calculated mean $\pm$ S.D , Abs	Coefficients of variation (CV's), %	% Accuracy
48 8	0 296 $\pm$ 0 007	2 64	14 42
24 4	0 425 $\pm$ 0 031	7 38	5 57
12 2	0 549 $\pm$ 0 031	5 79	19 27
6 1	0 609 $\pm$ 0 024	3 94	6 88
3 05	0 709 $\pm$ 0 056	8 03	7 54

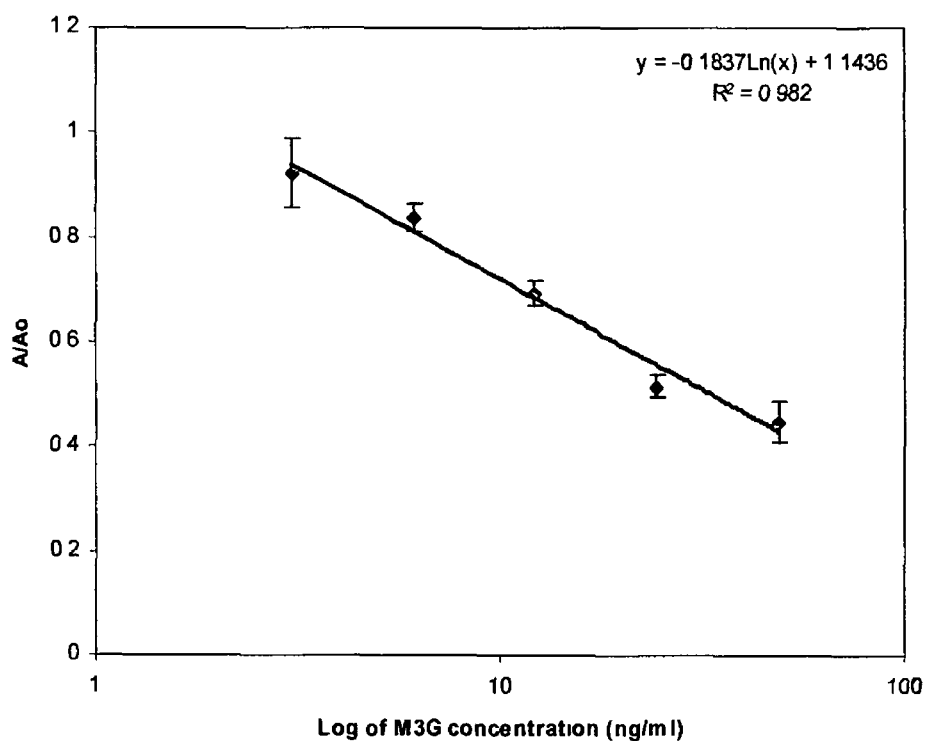


**Figure 5 19** Intra-day competitive ELISA assay of E-3 scFv antibody for determination of the optimal range of detection of free M3G using an antibody dilution of 1/10 M3G-THY was coated at a concentration of 50  $\mu$ g/ml. The linear range of detection was found to be between 3 05 and 48 8 ng/ml.



**Table 5.6. Inter-day assay variation for E-3 scFv antibody ELISA assay** To carry out triplicate analysis, three sets of five standards ranging from 3.05 to 48.8 ng/ml were analysed over three different days

M3G Concentration, (ng/ml)	Calculated mean $\pm$ S D , A/A <sub>0</sub>	Coefficients of variation (CV's), %	% Accuracy
48.8	0.578 $\pm$ 0.072	12.55	55.46
24.4	0.658 $\pm$ 0.058	8.83	42.37
12.2	0.780 $\pm$ 0.026	3.40	40.68
6.1	0.854 $\pm$ 0.022	2.63	20.81
3.05	0.954 $\pm$ 0.024	2.52	8.19



**Figure 5.20. Inter-day competitive ELISA assay of E-3 scFv antibody for determination of the optimal range of detection of free M3G using an antibody dilution of 1/10** M3G-THY was coated at a concentration of 50  $\mu$ g/ml. The linear range of detection was found to be between 3.05 and 48.8  $\mu$ g/ml

*Table 5 7 Cross reactivity studies of purified anti-morphine-3-glucuronide E-3 scFv antibody with structurally related molecules*

<b>Compound</b>	<b>Inhibition expressed as 100% inhibition of E-3 antibody</b>
Morphine-3-glucuronide	100%
Morphine	23 9%
Codeine	28 4%
Nor-codeine	6 5%
6-monoacetylmorphine	15 7%

#### **5 2 4 Cross reactivity studies of E3 scFv antibody**

The specificity of the E3 scFv antibody to morphine-3-glucuronide is critical for the performance of the immunoassay. Cross reactivity studies were carried out with a number of available drugs for this purpose using the ELISA developed as described in section 2 3 10 16 2. Microtitre plates were coated with 50 µg/ml of M3G-THY and blocked with 2% (w/v) marvel. Standards of morphine, codeine, nor-codeine and 6-monoacetylmorphine (6-MAM) were prepared and added to the microtitre plate at a 1:1 ratio with antibody at a dilution of 1/10. Table 5 7 shows the percentage inhibition of each compound indicating the E3 scFv cross reacts with morphine, codeine and 6-monoacetylmorphine.

## **5 2 5 Analysis of E3 scFv on the BIAcore biosensor**

### **5 2 5 1 Preconcentration and immobilisation of morphine-3-glucuronide-thyroglobulin conjugate**

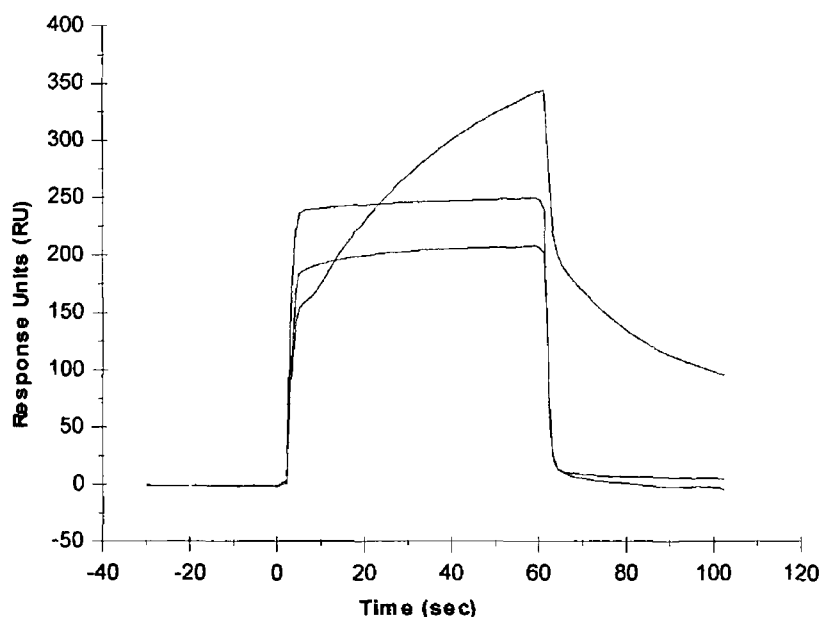
For the development of a model inhibition BIAcore assay to M3G in PBS, the first step of such an assay was the immobilisation of morphine-3-glucuronide-thyroglobulin (M3G-THY) onto the surface of a CM5 sensor chip. The immobilisation chemistry used was EDC-mediated NHS esterification as discussed in section 2.3.11.2. To achieve maximum binding, a preconcentration study of M3G-THY was carried out as described in section 2.3.11.1 and shown in Figure 4.10. 100 µg/ml of M3G-THY was prepared in 10 mM sodium acetate buffer at a range of pH's from 3.7 to 4.7. These solutions were then injected over an underivatized chip surface and the optimal pH determined for immobilisation of M3G-THY was 4.3. All subsequent immobilisation's of M3G-THY were carried out by preparing the conjugate at this pH.

### **5 2 5 2 Development of a model inhibition assay for morphine-3-glucuronide in PBS**

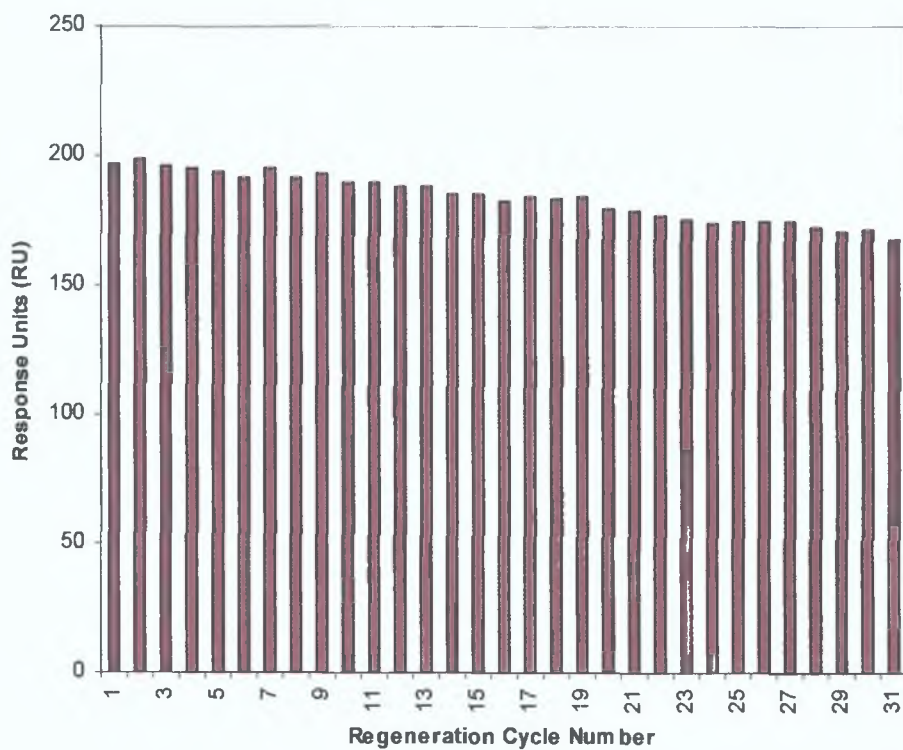
For the successful development of an inhibition BIAcore assay to M3G, regeneration of the surface is essential for the surfaces repeated use in assay formats. Also removal of non-specific interactions is important. A 1/2 dilution of E3 scFv antibody was found to produce a binding response of approximately 200 RU. Regeneration conditions for the E3 scFv antibody were optimised and found to require a 1 min pulse of 10 mM NaOH.

Non-specific interactions of the E3 scFv antibody for both the carrier protein used to prepare the conjugate and the dextran matrix on the surface of the sensor chip were analysed and found to be negligible. The E3 scFv antibody was passed over both immobilised M3G-THY, THY and dextran surfaces at dilutions of 1/2. Figure 5.21 illustrates the binding of E3 scFv antibody to these surfaces. Once the regeneration

conditions for both antibodies were optimised, surface regeneration studies were conducted by a series of antibody binding and regeneration pulses. These studies demonstrated that for the E3 scFv antibody, it was possible to regenerate the surface up to approximately 30 times (Figure 5 22) resulting in a 15% decrease in antibody binding capacity over this number of regenerations.



**Figure 5 21** Shows overlay plot demonstrating the binding of affinity purified E-3 scFv antibody to immobilised M3G-THY (---) and THY (—) surfaces and also over a blank dextran surface (· · ·). There was negligible binding to the control THY and dextran surfaces. However, approximately 200 response units of E-3 antibody bound to the immobilised M3G-THY surface indicating the antibody is specific to the M3G portion of the conjugate.



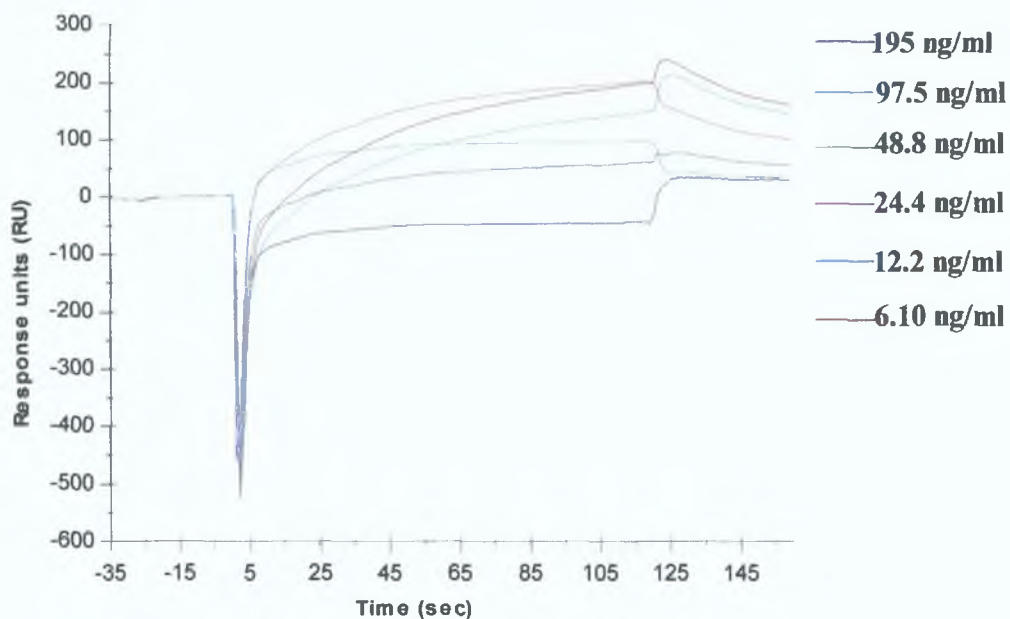
*Figure 5.22. Typical regeneration profile for approximately 30 cycles of a 2 min binding pulse of purified E-3 antibody to the surface of a chip immobilised with M3G-THY. A 1/2 dilution of antibody was used and the surface regenerated with a 1 min pulse of 10 mM NaOH. After 30 cycles of binding and regeneration, the response after the injection of the last binding pulse was 167.7 RU compared with the first binding pulse of 197.2 RU. The ligand binding capacity was shown to decrease by 14.9% over the course of the regeneration cycles.*

### **5.2.5.3 Determination of the range of detection of morphine-3-glucuronide in PBS in a BIAcore inhibition assay using E3 anti-morphine-3-glucuronide scFv antibodies**

For the determination of the range of detection of M3G for a BIAcore inhibition assay, a range of standards of M3G were prepared in PBS ranging from 1.5 to 3,125 ng/ml for E3 scFv antibody. The purified antibody to M3G was mixed with the corresponding concentration of free M3G, and allowed to equilibrate for 1 h. The samples were then passed over the surface of the sensor chip immobilised with M3G-THY in random order, followed by regeneration of the surface using a 1 min pulse of 10 mM NaOH. A typical antibody binding response for each mixture of antibody and drug concentration is shown in Figure 5.23. A calibration curve was constructed by plotting the change in response for each standard of free drug against the concentration of M3G. The range of detection for the E3 scFv antibody was found to be between 6.10 and 195 ng/ml (Figure 5.24).

### **5.2.5.4 Intra- and inter-day assay variability studies of E3 scFv antibody**

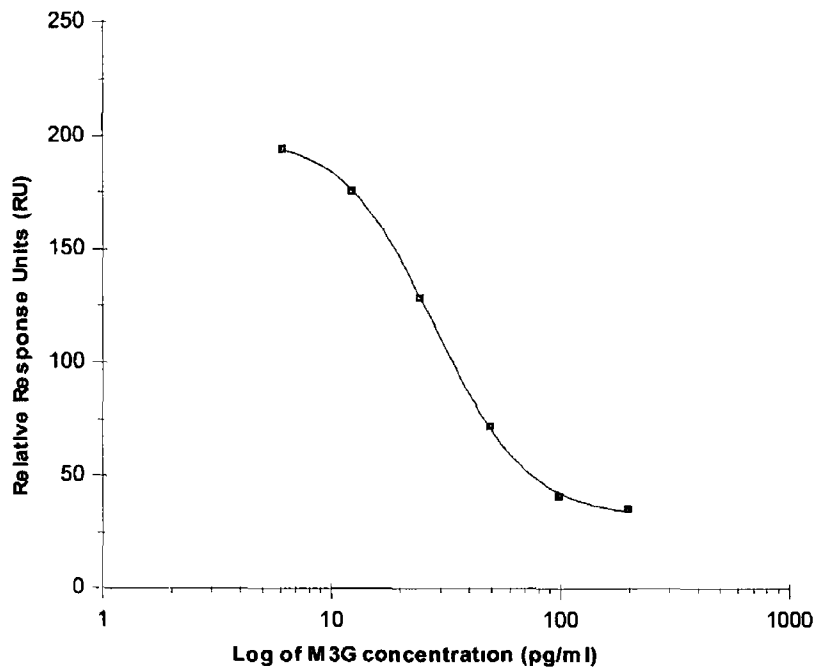
To carry out intra-day assay variability studies, three sets of standards were prepared for the antibody and assayed on the same day and their means plotted. The intra-day assay variability study for E3 shown in Figure 5.24 had a range of detection between 6.10 and 195 ng/ml. The CV's were determined and ranged from 0.62% to 1.55% as shown in Table 5.8. The inter-day assay variability study carried out over three separate days for the E3 scFv antibody had a range of detection between 3.05 and 195 ng/ml as shown in Figure 5.25 and CV's ranged from 0.96% to 6.36% as shown in Table 5.9.



*Figure 5.23. An overlay plot for a typical set of binding curves in an inhibition assay. M3G standards ranging from 6.10 to 195 ng/ml of free drug were mixed with E-3 scFv antibody and passed over an M3G-THY-immobilised surface. The measured binding response was used to construct the calibration curves for the intra-day assay variability studies, and used to calculate the normalised binding response in conjunction with 2 other normalised binding response curves for the inter-day assay variability studies.*

**Table 5 8. Intra-day assay CV's for E-3 scFv antibody BIAcore inhibition assay** Three sets of six standards ranging from 6 10 to 195 ng/ml were analysed on the same day and the CV's calculated.

<b>M3G Concentration, (ng/ml)</b>	<b>Calculated mean <math>\pm</math> S D , RU</b>	<b>Coefficients of variation (CV's), %</b>
195	35 83 $\pm$ 0 404	1 13
97 5	41 10 $\pm$ 0 458	1 11
48 8	72 16 $\pm$ 0 929	1 29
24 4	128 90 $\pm$ 0 793	0 62
12 2	175 80 $\pm$ 2 535	1 44
6 10	194 26 $\pm$ 3 008	1 55

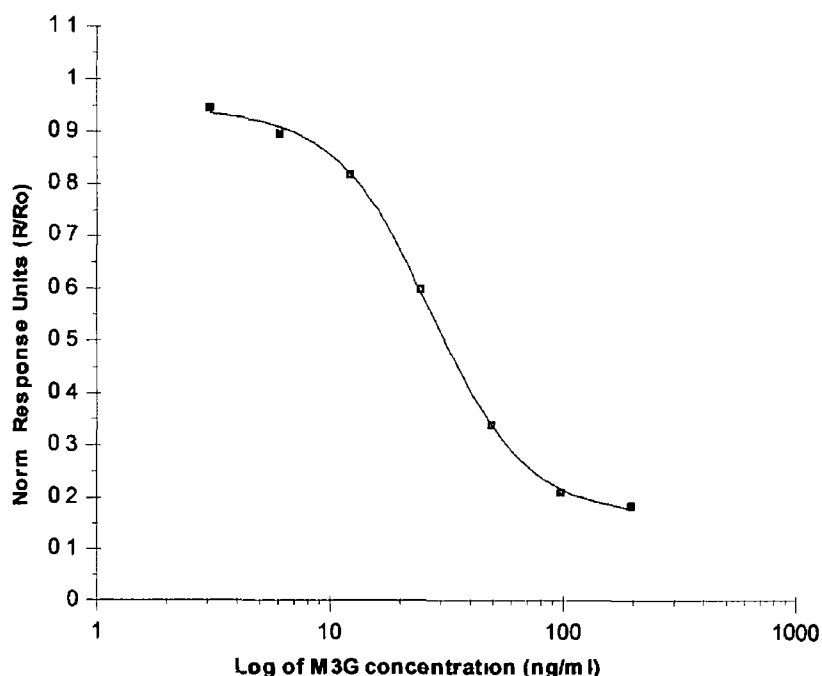


**Figure 5 24 Intra-day assay curve for M3G using the E-3 scFv antibody on an M3G-THY immobilised chip surface** The residual plot shows the excellent correlation of the data set to the four-parameter model fit. The results shown are the average of triplicate results and the range of detection of free M3G was found to be between 6 10 and 195 ng/ml



**Table 5 9 Inter-day assay CV's for E-3 scFv antibody BIAcore inhibition assay** Three sets of seven standards ranging from 3 05 to 195 ng/ml were analysed over three different days and the CV's calculated.

<b>M3G Concentration, (ng/ml)</b>	<b>Calculated mean <math>\pm</math> S.D , <math>R/R_0</math></b>	<b>Coefficients of variation (CV's), %</b>
195	0 186 $\pm$ 0 008	4 56
97 5	0 212 $\pm$ 0 011	5 51
48 8	0 342 $\pm$ 0 016	4 77
24 4	0 600 $\pm$ 0 038	6 36
12 2	0 820 $\pm$ 0 040	4 94
6 10	0 898 $\pm$ 0 051	5 72
3 05	0 945 $\pm$ 0 009	0 96



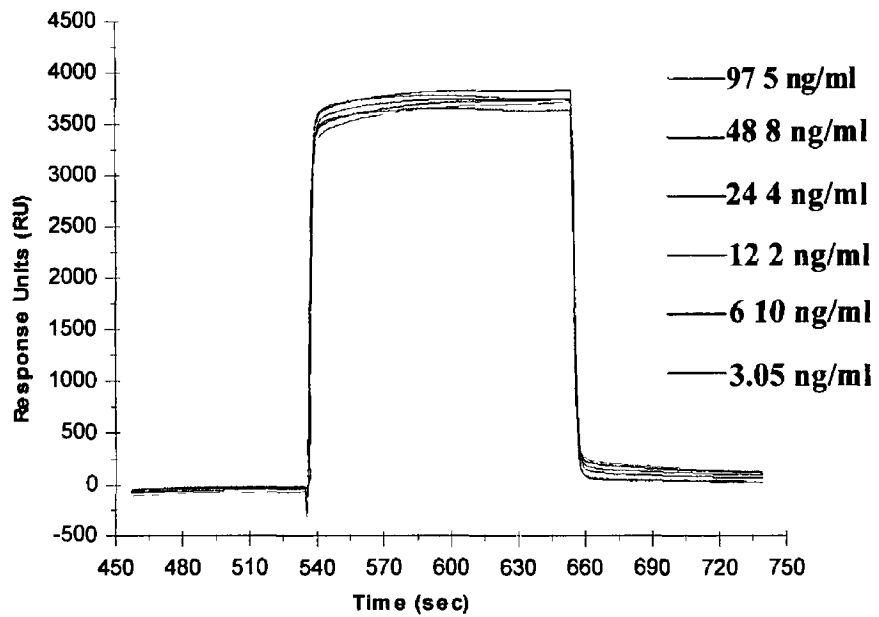
**Figure 5 25 Inter-day assay curve for M3G using the E-3 scFv antibody on an M3G-THY-immobilised chip surface.** The results shown are the average of triplicate results and the range of detection of free M3G was found to be between 3 05 and 195 ng/ml. The binding response at each M3G concentration ( $R_{AG}$ ) was divided by the antibody binding response determined in the presence of zero M3G concentration ( $R_0$ ) to give a normalised binding response ( $R/R_0$ )

## **5 2 6 Urine analysis of anti-morphine-3-glucuronide E3 scFv antibody**

The quantitative determination of M3G in urine samples was also investigated. Known samples of M3G ranging from 1.5 to 3,125 ng/ml were spiked into control urine and assayed for the presence of free drug using the E3 scFv antibody. Figure 5 26 shows that as the free drug concentration increases in solution the binding response of antibody decreases. A calibration curve was constructed by plotting the change in response for each standard of free drug against the concentration of M3G. The range of detection in urine was found to be between 3.05 and 97 ng/ml (Figure 5 27).

### **5 2 6.1. Intra- and inter-day assay variability studies of E3 scFv antibody in urine**

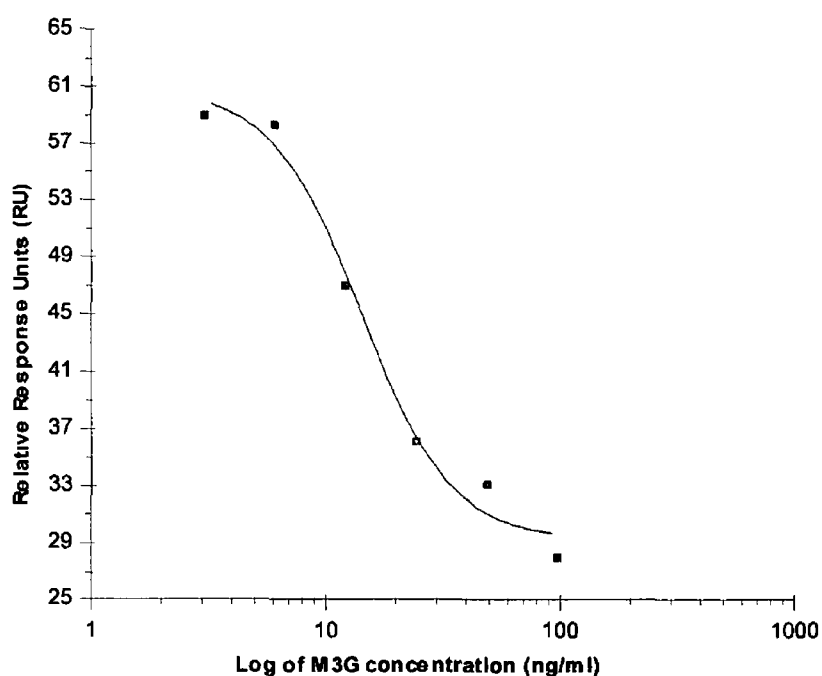
The intra-day assay variability study for E3 shown in Figure 5 27 had a range of detection between 3.05 and 97.5 ng/ml. The CV's were determined and ranged from 0.39% to 5.35% as shown in Table 5 10. The inter-day assay variability study carried out for the E3 scFv antibody had a range of detection between 3.05 and 97.5 ng/ml as shown in Figure 5 28 and CV's ranged from 0.48% to 18.38% as shown in Table 5 11.



*Figure 5.26. An overlay plot for a typical set of binding curves in an inhibition assay in urine. M3G standards ranging from 3.05 to 97.5 ng/ml of free drug were mixed with E-3 scFv antibody and passed over an M3G-THY-immobilised surface. The measured binding response was used to construct the calibration curves for the intra-day assay variability studies, and used to calculate the normalised binding response in conjunction with 2 other normalised binding response curves for the inter-day assay variability studies.*

**Table 5 10 Intra-day assay CV's for E-3 scFv antibody BIAcore inhibition assay in urine. Three sets of six standards ranging from 3 05 to 97 5 ng/ml were analysed on the same day and the CV's calculated.**

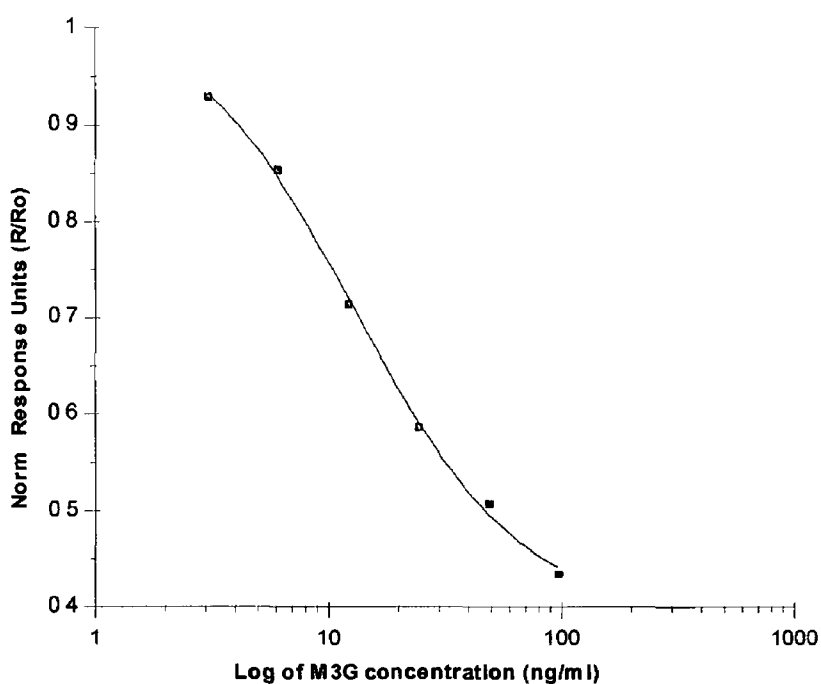
<b>M3G Concentration, (ng/ml)</b>	<b>Calculated mean <math>\pm</math> S D , RU</b>	<b>Coefficients of variation (CV's), %</b>
97 5	28 13 $\pm$ 1 504	5 35
48 8	33 20 $\pm$ 0 529	1 59
24 4	36 20 $\pm$ 0 141	0 39
12 2	47 06 $\pm$ 0 513	1 09
6 10	58 40 $\pm$ 0 435	0 75
3 05	59 06 $\pm$ 1 274	2 16



**Figure 5 27 Intra-day assay curve for M3G in urine using the E-3 scFv antibody on an M3G-THY-immobilised chip surface. The results shown are the average of triplicate results and the range of detection of free M3G was found to be between 3 05 and 97 5 ng/ml.**

**Table 5.11** *Inter-day assay CV's for E-3 scFv antibody BIAcore inhibition assay in urine. Three sets of six standards ranging from 3.05 to 97.5 ng/ml were analysed over three different days and the CV's calculated.*

<b>M3G Concentration, (ng/ml)</b>	<b>Calculated mean <math>\pm</math> S.D , <math>R/R_0</math></b>	<b>Coefficients of variation (CV's), %</b>
97.5	0.435 $\pm$ 0.002	0.59
48.8	0.508 $\pm$ 0.093	18.38
24.4	0.587 $\pm$ 0.052	8.98
12.2	0.714 $\pm$ 0.068	9.58
6.10	0.854 $\pm$ 0.004	0.48
3.05	0.929 $\pm$ 0.007	0.77



**Figure 5.28** *Inter-day assay curve for M3G in urine using the E-3 scFv antibody on an M3G-THY-immobilised chip surface. The results shown are the average of triplicate results and the range of detection of free M3G was found to be between 3.05 and 97.5 ng/ml. The binding response at each M3G concentration ( $R_{AC}$ ) was divided by the antibody binding response determined in the presence of zero M3G concentration ( $R_0$ ) to give a normalised binding response ( $R/R_0$ ).*

### 5 2 7 Solution Phase Steady State Affinity determinations

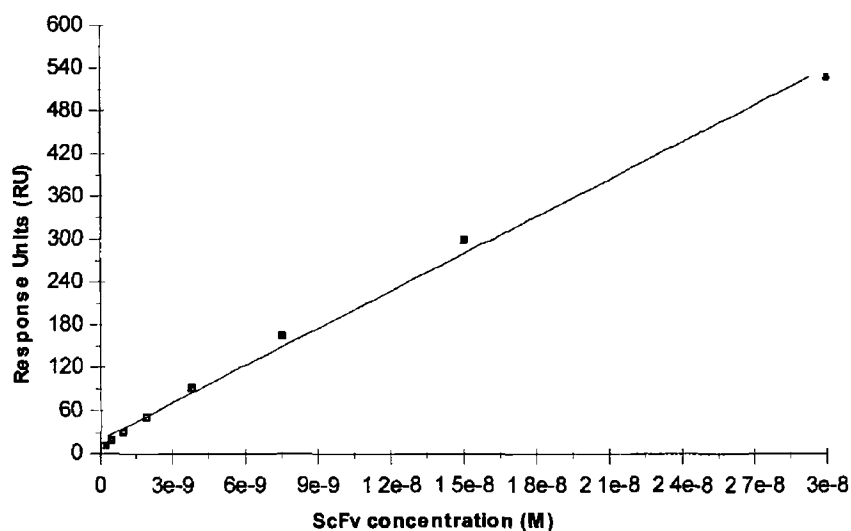
The use of BIAcore technology has been used to determine the dissociation constant of the E3 scFv antibody by using 'real-time' biomolecular interaction. A known concentration of anti-M3G scFv antibody was serially doubly diluted in PBS buffer and used to construct a calibration curve of free antibody concentration versus response as described in section 2 3 11 8 and shown in Figure 5 29. A known concentration of antibody was then mixed with known concentrations of M3G and allowed to reach equilibrium. Each antibody drug mixture was passed over the surface of a chip immobilised with M3G-THY and assayed for 'free-unliganded' anti-M3G scFv antibodies. The concentration of free antibody at equilibrium was determined by reference to the standard curve.

$$B_{free} = \frac{B - A - K_d}{2} + \sqrt{\frac{(A + B + K_d)^2}{4} - AB}$$

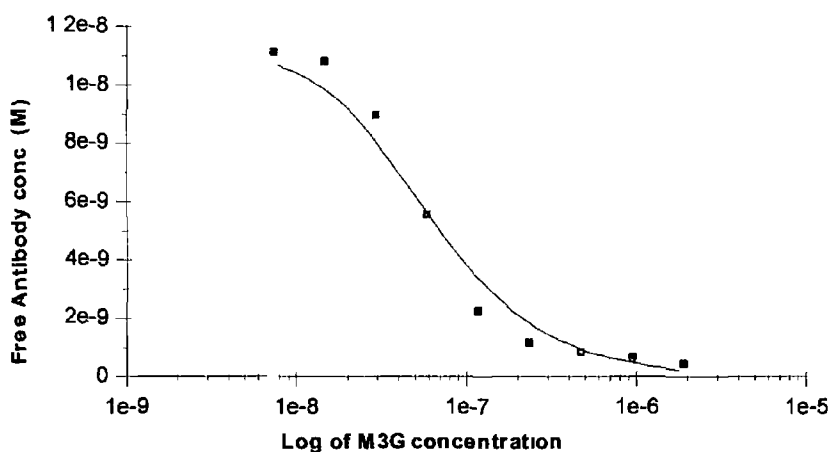
Where  $B_{free}$  is the free concentration of anti-M3G scFv  
 $A$  is the total concentration of M3G  
 $B$  is the total anti-M3G scFv concentration  
 $K_d$  is the equilibrium dissociation constant

*Equation 5 1 Shows the model for solution phase affinity*

The equilibrium dissociation constant was calculated by constructing a plot of free anti-M3G scFv antibodies against free M3G concentration as shown in Figure 5 30. Equation 5 1 is used as the model for the solution phase affinity constant as the model calculates monovalently bound antibody. For scFv analysis, this is ideal as the scFv possess one antigen binding site. The equilibrium dissociation constant for the E3 scFv antibody was found to be  $K_D = 2.13 \times 10^{-8}$  M.



**Figure 5 29** Serial doubling dilutions of anti-morphine-3-glucuronide antibodies of known concentration (M) were passed sequentially over an M3G-THY-coated sensor chip surface. A calibration plot was constructed of anti-M3G scFv antibodies (M) versus response measured (RU) The calibration plot was then used to calibrate the concentration of free antibody at equilibrium, results shown are the average of triplicate measurements.



**Figure 5 30** Determination of overall solution equilibrium constant for E3 scFv clone and M3G on an M3G-THY-coated chip surface. M3G concentrations were plotted against free M3G scFv antibody concentration, determined by reference to a calibration plot of anti-M3G antibodies. A 1:1 interaction model was used to describe the interaction (equation 5 1), and fitted to the data set using BIAevaluation software, deriving an equilibrium dissociation constant of  $K_D = 2.13 \times 10^{-8}$  M, for the interaction between E3 clone and M3G

### 5.3 Discussion

The mouse antibody titres produced by immunisation with M3G-BSA were extremely high. Both mice produced titres of approximately 1/300,000. Titrations of 1/1000 are adequate for scFv production. However, higher titres are preferable. This indicated a high concentration of B-cells producing specific antibody. Splenic mRNA was isolated from these spleen cells and used in the production of a recombinant antibody library. The isolated mRNA was used in the production of cDNA followed by the amplification of the light and heavy chain genes.

In many cases, primer sets have been too restricted to amplify either particular light or heavy chain genes. However, the set of primers produced by Krebber *et al* (1997) and used in this chapter have been extended and optimised. They incorporate all mouse  $V_H$ ,  $V_\lambda$  and  $V_\kappa$  sequences collected by the Kabat data base (Kabat *et al*, 1991) and combine extended primer sets described by Kettleborough *et al* (1993), Ørum *et al* (1993) and Zhou *et al* (1994). The PCR for the production of light and heavy chain genes was optimised and the conditions used are described in section 2.3.9.3.3. Bands of light (375-402 bp) and heavy (386-440 bp) chain gene amplification can be seen in Figures 5.5 and 5.6, respectively. Results show that the bands for both light and heavy chain genes are very clear with minimal production of non-specific bands. This is a significant result as production of non-specific bands would lower the concentration and diversity of the specific bands and decrease the overall diversity of the recombinant library produced using the PCR products.

Purification of amplified light and heavy chain genes was performed as described in section 2.3.9.4. The light and heavy chain genes were then assembled together by splice by overlap extension (SOE) PCR (Figure 5.7) (Horton *et al*, 1989). To avoid the occurrence of incorrect overlaps during assembly PCR, the four (Gly<sub>4</sub>Ser) repeats in the single chain linker region were encoded by different codons (Krebber *et al*, 1997). ScFv fragments may have a tendency to dimerize or aggregate. In order to reduce the occurrence of this, the linker between the  $V_L$  and  $V_H$  regions is 20 amino-



acids in length rather than the 15 amino acids long variant which is frequently used (Krebber *et al*, 1997) The SOE-PCR product was purified and digested along with pAK100 vector This was followed by ligation of digested SOE-PCR product with digested pAK100 as described in section 2.3.9.9 Attempts to clone digested heavy and light chain SOE-PCR products into the digested pAK100 vector produced low yields of transformants ( $5 \times 10^3$ ) The failure to produce high numbers of transformants may be due to the inefficiency of the SOE-PCR and pAK100 to digest or to ligate together properly Inadequate overhangs can reduce the efficiency of restriction digestion However, due to the pre-immunised nature of the splenic cells, which contain highly specific mRNA for production of antibodies to M3G, low levels of efficiency may in fact produce specific scFv antibodies

Selection of scFv antibodies to M3G was carried out by rounds of affinity selection panning to enrich for antibodies recognising the M3G conjugates The first round of panning was carried out by using M3G-OVA as the screening conjugate while M3G-THY was used for the subsequent round of panning in order to inhibit the possibility of screening for scFv's specific to the carrier protein Results from the first round of panning showed 100% of clones selected showing recognition for the M3G-THY conjugate which would seem to suggest a non-specific or high back round as the cause for such a result However, due to the DNA for library production originating from pre-immunised splenic mRNA, results were assumed to be accurate as this would suggest the amplified light and heavy chain genes were of a highly specific nature After the second round of panning, results also showed high levels of response to the conjugate for the clones analysed

Screening of positive scFv producing clones was performed by growing individual colonies separately and infecting them with helper phage as described in section 2.3.10.6 The recombinant scFvs, displayed on the surface of the filamentous phage, were tested for antigen binding in a typical phage ELISA This would simultaneously show the recognition of scFv for antigen, while also demonstrating extracellular expression if culture supernatant were used However, clones may prove negative in

phage ELISA when culture supernatant is analysed but may in fact be positive clones. This may result due to scFv's not being efficiently secreted due to particular protein structures that result in misfolding and intracellular aggregation (Buchner and Rudolph, 1991). This would result in positive scFv antibodies not reaching the extracellular matrix, such as the supernatant, causing the ELISA screening technique to produce a negative result.

Six clones found to be positive in phage ELISA were picked from each round of panning and further characterised by antigen competition studies to verify that the binding was antigen specific. In each round of panning one clone was found to be antigen specific and these clones were E3 (Figure 5.10) from round one and G12 (Figure 5.12) from round two. At this point the E3 clone was picked for further characterisation and analysis as a soluble scFv in order to develop a model system for M3G detection.

As the E3 clone possesses antigen-specific characteristics as shown by phage display, the next step was to produce a soluble scFv antibody. This was carried out by digestion of the pAK100 vector harbouring the light and heavy chain genes of the E3 clone and sub-cloning into the pAK400 vector for soluble expression in a JM83 cell line. Expression medium devoid of glucose was used to increase expression levels of recombinant protein (De Bellis and Schwartz, 1990). The pAK400 vector also contains a much stronger Shine-Dalgarno sequence (Krebber *et al*, 1997), resulting in a further significant enhancement of protein expression. However, the increase in expression levels did not occur for the E3 clone as can be seen in Figure 5.18 illustrating a titration of purified and concentrated scFv in which the scFv titres out at 1/32. However, enough antibody was produced to carry out further analysis.

A ProBond<sup>TM</sup> resin column was used in the purification of scFv antibodies as described in section 2.3.10.15. ScFv fragments produced in this library contain a six histidine tag allowing purification by IMAC (Lindner *et al*, 1992). However, using this column was not successful as can be seen in Figure 5.16, as the concentrated

sample of scFv contains a number of non-specific bands. This problem has been encountered before and may be due to the protein of interest being present in small fractions only. This results in several contaminating proteins binding to the column and co-eluting with the specific protein (Muller *et al*, 1998). Examples of such contaminating proteins produced by *E. coli* are superoxidase dismutase, chloramphenicol acetyltransferase, cAMP receptor protein, heat-shock protein and many more as described by Wulfig *et al* (1994). Further options were analysed for the secondary purification of the scFv and using an anti-FLAG mAb immunoaffinity column was a possible option (Kalinke *et al*, 1996). However, due to the lack of availability of a good anti-FLAG antibody this option was not pursued. With regards to purification, this is an area which may need further optimisation in the future to produce a high level of scFv purity. The IMAC-purified scFv was analysed further for the purpose of developing a model system. From the SDS-PAGE gel and the Western blot analysis it is evident that scFv was purified and concentrated up into a usable concentration of 976.77 µg/ml. The non-specific bands in the SDS-PAGE gel are present but may not effect the further characterisation of the scFv.

The purified scFv was then used in the development of a competitive enzyme-linked immunosorbent assay for M3G. A checkerboard ELISA for determination of optimal loading ratios of conjugate and optimal anti-morphine-3-glucuronide scFv antibody was performed and results are shown in Figure 5.18. An M3G-THY conjugate concentration was found to be optimal at 50 µg/ml. This concentration was chosen as increasing concentrations of conjugate did not give an increase in signal and gave the widest linear detection range over the range of antibody dilutions used.

The scFv antibody dilution used for E3 scFv antibodies was 1/10 resulting in the dilution of antibody being the limiting factor in the assay. Antibody dilutions chosen from the asymptotes of the antibody dilution curve would show minimal changes in response to small changes in antibody concentration resulting in an assay with very low sensitivity levels. The working dilution for the scFv antibody was picked from

the linear portion of the dilution curve as minimal changes in antibody concentration give the greatest change in absorbance readings. Results showed minimal binding of scFv to THY indicating negligible specific antibodies present to the protein part of the conjugate. As the screening conjugate used was M3G-OVA, there should be minimal binding of scFv antibody to any other protein and this is confirmed by the low level of non-specific binding of the scFv.

A model competitive ELISA assay was developed for this antibody in PBS where standards of M3G ranging from 1.5 to 781 ng/ml were prepared and mixed with purified antibody at dilutions of 1/10. The E3 scFv antibody showed a range of detection between 3.05 and 48.8 ng/ml. The assay displayed excellent linearity with very low limits of detection. Intra-day and inter-day assay variability statistics were then analysed in an attempt to look at the reproducibility of the assay. Intra-day and inter-day assay coefficients of variation (CV's) for the E3 scFv were found to be between 2.64 to 8.03% and 2.52 to 12.55%, respectively. This result indicates that this anti-M3G antibody gives an accurate, specific and reproducible assay. The low nature of the CV's shows that the assay may be used to detect samples of M3G in urine. Studies carried out on the % accuracy levels resulted in high values particularly for the inter-day variability studies indicating the assay may only be applicable for confirmatory assay analysis. However, the main problem associated with the assay is the concentrations of antibody used to perform the assay. A 1/10 dilution of antibody is required for the assay, limiting the number of assays that can be performed due to the amount of antibody available. Further analysis of this antibody for sample analysis in ELISA was not carried out for this reason as the quantities of antibody required were too great.

Cross reactivity is defined as a measure of the antibody response to structurally similar molecules. Table 5.7 shows the inhibition expressed as 100% inhibition of the E3 scFv antibody. Competitive assays were carried out with a range of metabolites of heroin. Morphine, codeine, nor-codeine and 6-monoacetylmorphine standards were prepared and added onto a microtitre plate with a mixture of a 1/10 dilution of E3 scFv antibody. As results indicate the scFv antibody shows some cross

reactivity with morphine, codeine and 6-monoacetylmorphine with very little cross reactivity to nor-codeine. These results demonstrate that the scFv recognises an epitope common to some of the molecules related to morphine-3-glucuronide.

Having shown that the E3 scFv antibody was suitable for use in an ELISA assay of this type, the aim was to develop a similar method of detection of M3G using the BIAcore biosensor.

For the successful development of an inhibition BIAcore assay to M3G a number of parameters were optimised as discussed in chapter 4. The first step in setting up such an immunoassay was the immobilisation of M3G-THY onto the surface of a CM5 sensor chip. It is essential to maximise the interaction between the dextran surface of the chip and the conjugate of interest in order to immobilise as much conjugate as possible onto the surface of the chip, this was achieved by preparing the M3G-THY conjugate in sodium acetate buffer, pH 4.3.

Non-specific analysis was carried out by passing the scFv antibody over a THY-immobilised surface and an underivatized dextran surface. For the scFv antibody the non-specific interactions were found to be negligible. This result shows that the antibody is specific for M3G rather than the carrier molecule of the conjugate.

As discussed in chapter four an important consideration when using BIAcore as an immunoassay tool is the ability of the antibodies to be regenerated from the surface of the chip. The binding-capacity of antibody to conjugate immobilised surfaces was determined by a series of binding and regeneration sequences to assess how many regenerations were possible before the binding-capacity falls below the desired performance parameters (< 20%). Ideally, it is preferable to be able to perform multiple binding and regenerations of antibody on the surface of one derivatised chip. Regeneration conditions for the E3 scFv antibodies were optimised and found to require a 1 min pulse of 10 mM NaOH. Results from surface regeneration studies

showed that the antibody was regenerable approximately 30 times with a loss of 14.9% antibody binding capacity over this number of regeneration's

These results are favourable for the development of BIAcore inhibition assays as the E3 scFv antibody can be regenerated a sufficient number of times without the binding-capacity falling below the desired performance parameters. This enables the development of an inhibition assay. The optimised regeneration solutions required were found to be of a low concentration resulting in minimal damage being caused to the surface immobilised conjugate during the assay.

Model inhibition assays were then developed as described in section 2.3.11.7 for M3G in PBS. Standards of M3G were prepared and mixed with the E3 purified antibody at a dilution of 1/2 followed by incubation at 37°C for 1 h to allow the reactions to reach equilibrium. These mixtures were then passed over an M3G-THY immobilised surface for 2 min resulting in an antibody binding response to the surface being inversely proportional to the concentration of free drug in solution. This binding response was plotted in Figure 5.24 showing that the detection range of the E3 scFv antibody for M3G was between 6.10 and 195 ng/ml.

Studies were performed on the intra- and inter-day variability of the assay. The CV's for both were found to be low, especially in the intra variability assay where CV values were typically found to be lower than 2%. Inter variability CV's were found to be slightly higher in the order of approximately 5%, however, these are well within the desired performance parameters. Overall, these results indicate that the E3 scFv anti-M3G antibody gives accurate, specific and reproducible assays in PBS.

An inhibition assay was then developed for detection of M3G in urine as described in section 2.3.11.7. The E3 scFv antibody showed a range of detection of M3G between 3.05 and 97.5 ng/ml. Intra-day and inter-day assay coefficients of variation (CV's) for the assay were found to be between 0.39 to 5.35% and 0.48 to 18.38%, respectively. The overall results from the CV's for the assays developed in urine were found to be

low. A CV of 18.38% was seen for the 48.8 ng/ml concentration of M3G in the inter-day variability studies. However, all other points within the assay are below 10% indicating the assay may be used for sample analysis.

The assays developed in our lab to M3G using both the ELISA and BIAcore formats compare favourably to some HPLC and GC-MS assays in the literature. Of the analytical techniques discussed in section 3.1.4, assays developed by Low *et al* (1995), Zheng *et al* (1998) and Beike *et al* (1999) have detection limits down to low ng/ml levels compared to a detection limit of 3 ng/ml for the assays developed in our lab. However, assays developed by Meng *et al* (2000), Spector *et al* (1971) and Steiner *et al* (1978) have all shown to have detection limit in the low pg/ml levels resulting in these assays being much more sensitive than the assays developed in our lab. The suitability of our assay for sample analysis is still possible, as the assay range is suitable for the confirmatory cut-off concentrations for opiate abuse (i.e. morphine and codeine- 2000 ng/ml, 6-MAM- 10 ng/ml).

The final step in the analysis of the E3 scFv antibody clone was to determine the solution phase steady state affinity constant. The method employed as described in section 5.2.7 used the same principle as that employed in the method of Friguet *et al* (1985). However, the method employed in this chapter holds a number of advantages over the Friguet method. The main advantage is the short contact time of equilibrated mixtures in the flow cell and continuous flow of equilibrated mixtures resulting in a decrease in the possibility of re-equilibration and underestimation of the affinity constant. The method on BIAcore also does not require labels which may affect the calculated constant. The solution phase steady state affinity constant was calculated using equation 5.1 and determined to be  $K_D = 2.13 \times 10^{-8}$  M. The data obtained from the analysis fits the monovalent model particularly well and describes the interaction between the antibody and the conjugate coated surface accurately.

## **Chapter 6**

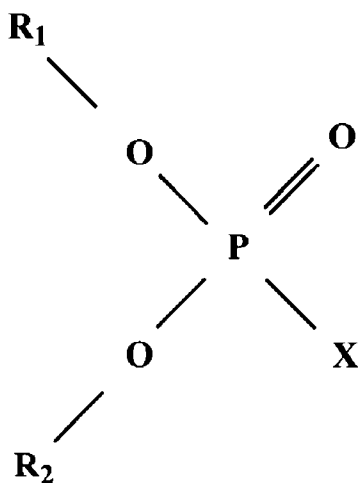
# **Production and Application of Polyclonal Antibodies to Parathion**



## 6 1 Introduction

### 6 1 1 Organophosphates

Organophosphates (OPs) are the most commonly used insecticides (Table 6 1 ) Although they were developed in the early 19<sup>th</sup> century, it was not until the early 1930's that their toxicity to insects was discovered resulting in neurological diseases (O'Brien *et al* , 1967) Structurally, organophosphates possess a central phosphorus atom and numerous side chains The generic structure of the organophosphate compounds is shown in Figure 6 1 where X is a leaving group of variable structure and R<sub>1</sub> and R<sub>2</sub> are alkoxy, amino, thioalkyl, phenyl or other substituent groups (Storm *et al* , 2000) OPs are categorised based on the nature of the atoms immediately surrounding the central phosphorus atom (Chambers, 1992)



*Figure 6 1 Generic organophosphate structure*

One characteristic of the OPs which has led to their wide usage in agriculture is that they are much less persistent in the environment compared to other insecticides OPs have been extensively used to control pests in agriculture for many years resulting in the accumulation of these pesticides over this period of time within the environment

to such an extent that OPs are now considered environmental pollutants. Public health authorities are thus extremely concerned with the levels of OPs within the food chain. This has prompted health authorities to set up acceptable Maximum Residue Levels (MRLs) which are stringently adhered to.

In recent years, OPs are being replaced in some uses with the carbamate insecticides which possess lower toxic properties towards humans and wildlife.

**Table 6 1 Organophosphates**

Acephate	Formothion
Azinphos-methyl	Isofenphos
Bensulde	Malathion
Carbophenothion	Methidathion
Chloropyrifos	Methyl-parathion
Coumaphos	Mevinphos
Demeton-S-methyl	Monocrotophos
Diazinon	Naled
Dichlorvos/DDVP	<b>Parathion</b>
Dicrotophos	Phorate
Dimethoate	Phosalone
Disulfoton	Phosmet
Endothion	Phosphamidan
Ethion	Phoxim
Fenamiphos	Propetamphos
Fenitrothion	Temephos
Fenthion	Terbufos
Fonofos	Trichlorfon

### 6.1.1.1 Mechanism of action and toxicity

Organophosphate pesticides (OPs) have the same toxic mechanism for both insects and mammals. Cytochrome P450-dependent monooxygenases have the ability to activate phosphorothionate insecticides to toxic anti-cholinesterases (Davison *et al*, 1955, Gage *et al*, 1953, Wagner *et al*, 1989). The organophosphate pesticides are converted into their oxon analogs (Figure 6.3) by oxidative desulfuration (Levi and Hodgson, 1985). This reaction requires reduced NADP and O<sub>2</sub> and is inhibited by

CO (Kulkarni and Hodgson, 1984) The majority of the oxon active metabolites are assumed to be produced in the liver, from where it gains entry into the circulatory system to find its target molecule, the enzyme acetylcholinesterase (AChE)

OP compounds cause the enzyme acetylcholinesterase to become inactive (Miles *et al*, 1998) The function of this enzyme is to increase the rate of breakdown of acetylcholine (ACh) ACh is produced in the nerve cells and allows the transfer of nerve impulses from one nerve cell to a receptor cell (i.e. muscle cell)

AChE's function is as a negative feedback control enzyme, breaking down ACh by chemical inactivation thus regulating the transfer of nerve impulses Without the breakdown of ACh by the enzyme AChE nerve impulses will carry on continuously High levels of ACh results in a number of effects in mammals such as weakness or paralysis of the muscle (Gallo *et al*, 1991) Death may occur when AChE's activity falls to about 10-20 % of normal levels of activity and this causes respiratory failure or cardiac arrest

Individual OPs possess different mechanisms of action Some function as contact poisons while others are stomach poisons Humans may come in contact with OPs in a number of ways, which include absorption through the skin, inhalation and direct ingestion However, OPs are toxic through all routes of entry Inhalation of OPs initially affects the respiratory system, and may cause bloody and runny nose, chest discomfort, coughing and wheezing Contact with skin causes sweating and involuntary muscle contractions Contact with eyes can cause effects such as pain, tears, bleeding, and blurred vision Symptoms may appear within a time period of a few minutes to twelve hours Other symptoms include nausea, vomiting, diarrhoea, headache, dizziness and confusion In severe cases the central nervous (spinal cord, brain) and the peripheral nervous systems (nerve and fibres) can be greatly affected (Smith, 1993) Symptoms include defecation, psychosis, unconsciousness, convulsions, coma and irregular hearts beats OPs do not affect reproduction with respect to normal exposure levels (Gallo *et al*, 1991, Storm *et al*, 2000) However, some organophosphates (i.e. malathion) have been found to affect pregnancies of women exposed to high levels

OPs have the ability to stay active for months after their application. However, they are much less persistent than other pesticides. This feature causes no great threat to water pollution over the long term. The main organophosphate of interest in this chapter is parathion and its mode of action is described in section 6.1.2.1.

### 6.1.1.2 Detection of organophosphate pesticides

Many of the detection methods for organophosphate pesticide analysis have been based on conventional chromatographic (HPLC, GLC) and colourimetric procedures. For example, Ding and Krull, (1984) used high-performance liquid chromatography (HPLC) with on-line photolysis ( $h\nu$ ), followed by electrochemical detection of 20 different organophosphates. Minimum quantities of malathion and parathion detectable by this method were 50 ppb and 20 ppb, respectively (enabling this method to be applied to crop extracts).

Bottomley *et al* (1984) developed a multi-residue method for determination of organophosphate pesticides in gram by GLC. Following extraction using acetone-methanol, pesticides were separated from co-extractives by a partition process with dichloromethane and chromatography on an acidic aluminium oxide column. Packed column gas-liquid chromatography with flame-photometric detection was used with minimal limits of detection of  $0.1 \text{ mg Kg}^{-1}$ .

Further methods of detection of pesticides are continuously being developed as described by Sng *et al* (1997). This paper describes the development of a solid-phase microextraction (SPME) method for the analysis of pesticides in water. The advantage of this system is the ability of the system to directly analyse chemical species in aqueous systems avoiding sample clean-up. The samples are then analysed by gas chromatography.

A method for the detection of seventeen pesticides by gas chromatography was developed by Soleas *et al* (2000). Solid-phase extraction of a 0.5 ml sample of wine was followed by direct injection of  $1 \mu\text{l}$  of the eluent onto a DB-5 MS gas chromatographic column. This was followed by mass-selective detection using one target and two qualifier ions for each pesticide. Recovery rates for all compounds

were found to be higher than 80% and the detection and quantitation limits were 2 µg/l and 10 µg/l, respectively

Tuovinen *et al* (2000) have developed a system for the detection of pesticides from liquid matrices by aspiration of ion mobility spectrometry. This technology is based on ion mobility which is proportional to the molecular weight and charge and has the ability to measure changes in the product ion as well as mobility changes of reactant ions. The detection method is based on differences in the gas phase profiles of the different compounds and results have shown the ability of this system to analyse pesticides with limits of detection ranging from ng to µg levels. However, most of these methods require many clean-up steps and are quite labour intensive.

Development of immunoassays have become a favourable alternative to these methods in relation to the cost involved, the ease of use of the method and the lack of clean-up steps required. For low molecular weight analytes such as pesticides in solution, competitive tests have been employed using limiting antibody concentrations. There are two main formats used in such assays. The first allows the antibody to be directly immobilised onto the surface of the plate and competition occurs as labelled and unlabelled analyte compete with each other for the binding sites on the antibody. The second method employs a system where a hapten-carrier conjugate is immobilised onto the surface of a plate and free and bound analyte compete for the binding site on the antibody. The amount of antibody bound to the surface of the plate is directly proportional to the amount of free analyte in solution.

Deschamps *et al* (1990) also employed polyclonal and monoclonal antibodies for the detection of the pesticide picloram. The assay developed using polyclonal antibodies had a linear range of detection from 5 to 5,000 ng/ml. Using a monoclonal antibody the range of detection was from 1 to 200 ng/ml. The results indicated that the monoclonal antibody was more sensitive, accurate and precise compared to the polyclonal antibody and only the monoclonal antibody assay was suitable for quantitative determination of picloram.

An immunoassay was developed by McAdam *et al* (1992). Initially fenitrothion (FN) was coupled to a carrier-protein for the production of monoclonal and polyclonal antibodies. These antibodies were used in the development of an ELISA

assay for fenitrothion. Assay formats using either immobilised antibody or the immobilised hapten-protein conjugate were used to determine concentrations of fenitrothion in solution resulting in assays with detection limits as low as 1 ng

Skerritt *et al* (1992) developed simple competitive ELISA's for three organophosphates, fenitrothion (FN), chlorpyrifos (CPM) and pirimiphos-methyl (PIRM). These assays had sensitivities of low parts per million ranges. No clean-up of samples was required and grain, flour and wheat were analysed for these compounds. The assays used polyclonal antibodies to PIRM and monoclonal antibodies to FN and CPM. Minimum detection limits for the pesticides were 2 ng/ml for PIRM, 30 ng/ml of FN and 10 ng/ml for CPM.

In general, anti-OP antibodies developed for detection assays are very specific towards a single analyte. Although antibody specificity is an advantageous characteristic for low level detection of trace amounts of individual OPs, there are applications in which broad-specificity antibody recognition of groups of analytes would be desirable. Such antibodies may be applied in cost effective screening programmes and for assays with the purpose of providing a positive or negative result for the presence of the analytes. Alcocer *et al* (2000) produced broad-specificity antibodies against a group of organophosphate pesticides by using phosphonic acid (TPB) as a generic hapten. Results showed that detection of a number of pesticides had a poor limit of detection using this antibody. It was possible to lower the limits of detection 20 fold by coating the OP of lower affinity (diethyl-parathion-BSA) onto the surface of the plate.

Progress is also being made in detection of pesticides in the field of immunosensors. Some of these sensors use optical systems such as surface plasmon resonance or grating couplers. Minunni and Mascini, (1993) used the BIAcore biosensor in the development of a detection method for atrazine while Bier and Schmid, (1994) developed a grating coupler immunosensor for determination of terbutryn, a triazine herbicide with detection limits of 5.05 µg/l and 3.6 µg/l, respectively.

The future in pesticide detection may lie in the new strategies in antibody production being developed. Genetically engineering antibodies with the required affinity and stability for use in specific assays is now possible as these antibodies may be tailored

for a specific characteristic. One example of the use of this technology is described in a paper by Garrett *et al* (1997) where anti-parathion scFv antibodies were produced and applied to an assay system for the detection of parathion.

## 6.1.2 Parathion

Parathion was discovered by Schrader in 1946 and since then has become one of the most widely used of all the organophosphate pesticides. Parathion is a yellow to dark brown liquid with a phenol like odour. It is used in a wide variety of crops i.e. cereals, fruit, vines, vegetables and field crops. The structure of parathion is shown in Figure 6.2 and contains the characteristic phosphorus group attached to oxygen, which is common to the majority of organophosphate pesticides. Sulphur, nitro, two ethyl groups, and a ring structure are also present in this molecule.

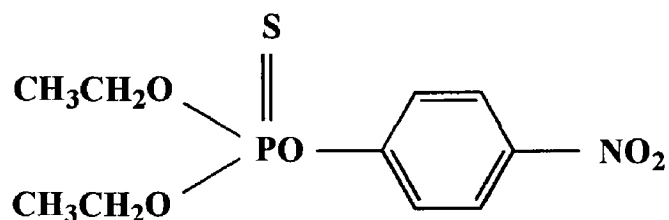


Figure 6.2 Structure of parathion

Parathion is considered to be a very toxic chemical. Exposure can occur through inhalation or through contact with the skin. Death may occur from contact with the skin even though no discomfort or irritation may be felt. Symptoms associated with exposure to this organophosphate are similar to those produced by all organophosphates and are sweating, nausea, headaches, diarrhoea, vomiting and loss of coordination (Kamrin, 1997).

### 6 1 2 1 Mechanism of action

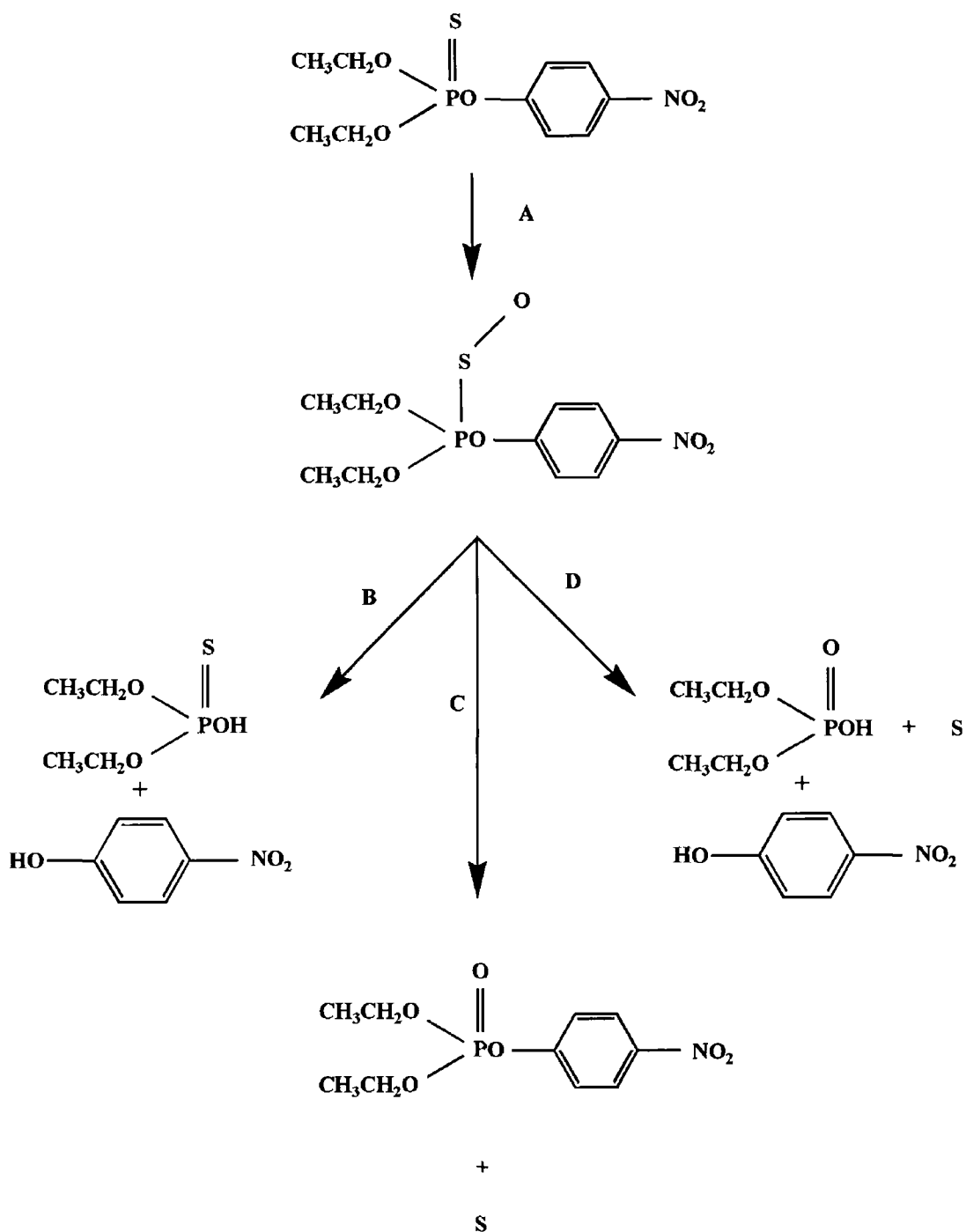
Parathion is a non-systemic insecticide and acaricide with contact action, through the stomach and also possesses some respiratory action. Parathion itself is a poor inhibitor of acetylcholinesterase, whereas its oxon analogue is a very active anticholinesterase agent. The toxicity of parathion is therefore due to its *in vivo* metabolism. Upon exposure the mammalian system metabolises parathion (Figure 6 3) to its toxic oxygen analogue, paraoxon, and to its non-toxic analogs p-nitrophenol and diethyl phosphate or diethyl thiophosphate by the cytochrome P450-dependent monooxygenase pathway (Neal *et al* , 1967, Nakatsugawa *et al* , 1968)

Most instances of death in mammals exposed to parathion are due to failure of the respiratory system. Once parathion has been metabolised into paraoxon, it begins to inhibit the activity of the acetylcholinesterase. Paraoxon is then transferred to the lungs and brain by the circulatory system. Respiratory failure occurs as AChE is inhibited as discussed in section 6 1 1 1

### 6 1 2 2 Detection of parathion

In the past, residues of parathion have been detected by gas-liquid chromatography (GLC), spectrophotometry and polarographic methods. However, over the past few decades, antibody-based methods have been developed as favourable alternatives for either the identification or quantification of pesticides. Ercegovich *et al* (1981) developed a radioimmunoassay (RIA) which provides a simpler procedure that required less clean-up steps than conventional methods as real samples may be used without the need for purification. Parathion-specific polyclonal antibodies were produced and used in the development of an RIA method by using either <sup>3</sup>H- or <sup>14</sup>C-labelled parathion as a tracer. The lowest detection levels were found to be 4 ng of parathion in model solutions and between 10-20 ng in blood plasma and lettuce without any cleanup of the sample. However, this method also has its disadvantages as it requires expensive equipment and highly trained personnel and also possesses radiation risk with long analysis times.





*Figure 63 Proposed metabolic scheme for cytochrome P450-dependent biotransformation of parathion (A) Represents reaction catalysed by cytochrome P450, while all others occur nonenzymatically (C) Represents reaction of activation to paraoxon and S (B) Detoxification to p-nitrophenol and diethyl phosphate. (D) Detoxification to p-nitrophenol and diethyl thiophosphate.*

Brimfield *et al* (1985) developed a competitive enzyme immunoassay for paraoxon, which is the principal metabolite of parathion. Monoclonal antibodies were produced to paraoxon. Significant cross reactivity was found with parathion and paraoxon with respect to these antibodies. Results from the CIEIA showed a detection linear range from 10 to 100 µg/ml with a minimum detectable concentration of 1 µg/ml for paraoxon.

Garrett *et al* (1997) described a method for the production of recombinant scFv anti-parathion antibodies. The scFv was produced from a hybridoma cell line secreting anti-parathion antibodies. In this paper the feasibility of such an antibody was tested by comparing the scFv to a monoclonal antibody. Results showed the scFv possessed similar characteristics to the monoclonal antibody when tested against parathion in food extract analysis. Both antibodies were found to be stable in the presence of 10% (v/v) methanol and the minimum limits of detection were 16 ng/ml and 23 ng/ml for the monoclonal and scFv fragment, respectively. These findings indicate the potential for the use of recombinant scFv antibodies for the detection of organophosphate compounds in food samples.

A method of analysis for detection of paraoxon, was developed by Campanella *et al* (1996) using biosensors. The study involved the comparison of two new solid state biosensors which used a graphite electrode, coated with an ion-selective polymeric membrane sensitive to pH. The principle of both combined the inhibiting action of paraoxon with the enzyme butrylcholinesterase. The first biosensor used the enzyme butrycolinesterase immobilised on a functional nylon membrane. The second biosensor format used butrycholinesterase coated on a polymeric membrane by a polyazetidine prepolymer. Results from the first biosensor analysis which used the enzyme butrycolinesterase immobilised on a functional nylon membrane, showed a minimum detection limit of 10 ng/ml for paraoxon while the second format showed a detection limit of 30 ng/ml. When also comparing analysis times the second biosensor proved a more promising format for paraoxon analysis.

Horacek *et al* (1998) used recombinant anti-parathion single chain Fv antibody (scFv) fragments to characterise antibody-parathion interactions using a piezoelectric

biosensor Parathion was chemically linked to a layer of aminothiophenol using either bovine serum albumin or dextran as spacer molecules Dissociation ( $K_d$ ) and association ( $K_a$ ) rate constant studies were then carried out Regeneration of parathion-BSA coated surfaces was not successful However, parathion-dextran surfaces were successfully regenerated Comparisons were then carried out between the anti-parathion monoclonal antibody and the scFv antibody Results showed a 50 x lower affinity of the scFv antibodies for parathion compared to the monoclonal antibodies

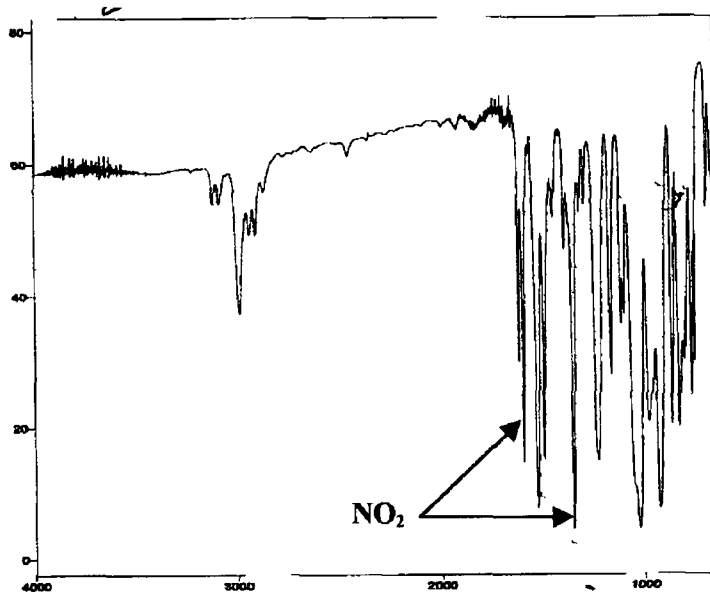
The development of ELISA-based immunoassays for the detection of parathion as a model system in PBS is investigated in this chapter Polyclonal antibodies were generated and used for the development of a competitive ELISA for parathion The ability of the model assays to measure free drug concentrations was studied and the results obtained were assessed in relation to the accuracy and the precision of the assays Further studies were carried out on the applicability of this anti-parathion antibody to biosensor analysis

## 6 2 Results

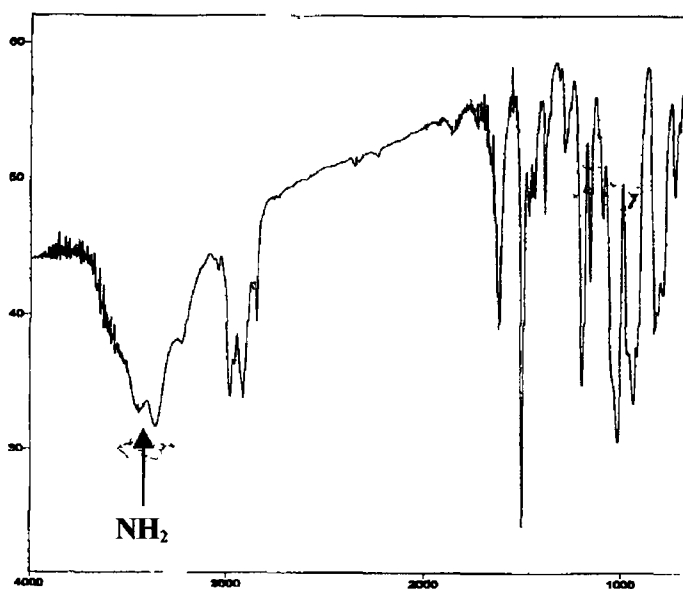
### 6 2 1 Production and characterisation of parathion protein conjugates

Conjugates to parathion were prepared according to the method of Ercegovich *et al* (1981) This required the reduction of parathion to amino-parathion as described in section 2 3 2 2 1 Parathion and amino-parathion were analysed by infra red spectrophotometric scans for identification purposes The IR spectrum of the parathion (Figure 6 4) molecule was found to possess stretches at 1350 and 1526  $\text{cm}^{-1}$  corresponding to the symmetrical and asymmetrical stretches associated with an  $-\text{NO}_2$  The IR spectrum for the amino-parathion (Figure 6 5) molecule was found to possess stretches at 3322 and 3402  $\text{cm}^{-1}$  corresponding to the  $\text{NH}_2$  asymmetrical and symmetrical stretches However, the bands at 1350 and 1526  $\text{cm}^{-1}$  were not present in the amino-parathion molecule confirming that the  $-\text{NO}_2$  group has been exchanged for an  $-\text{NH}_2$  group

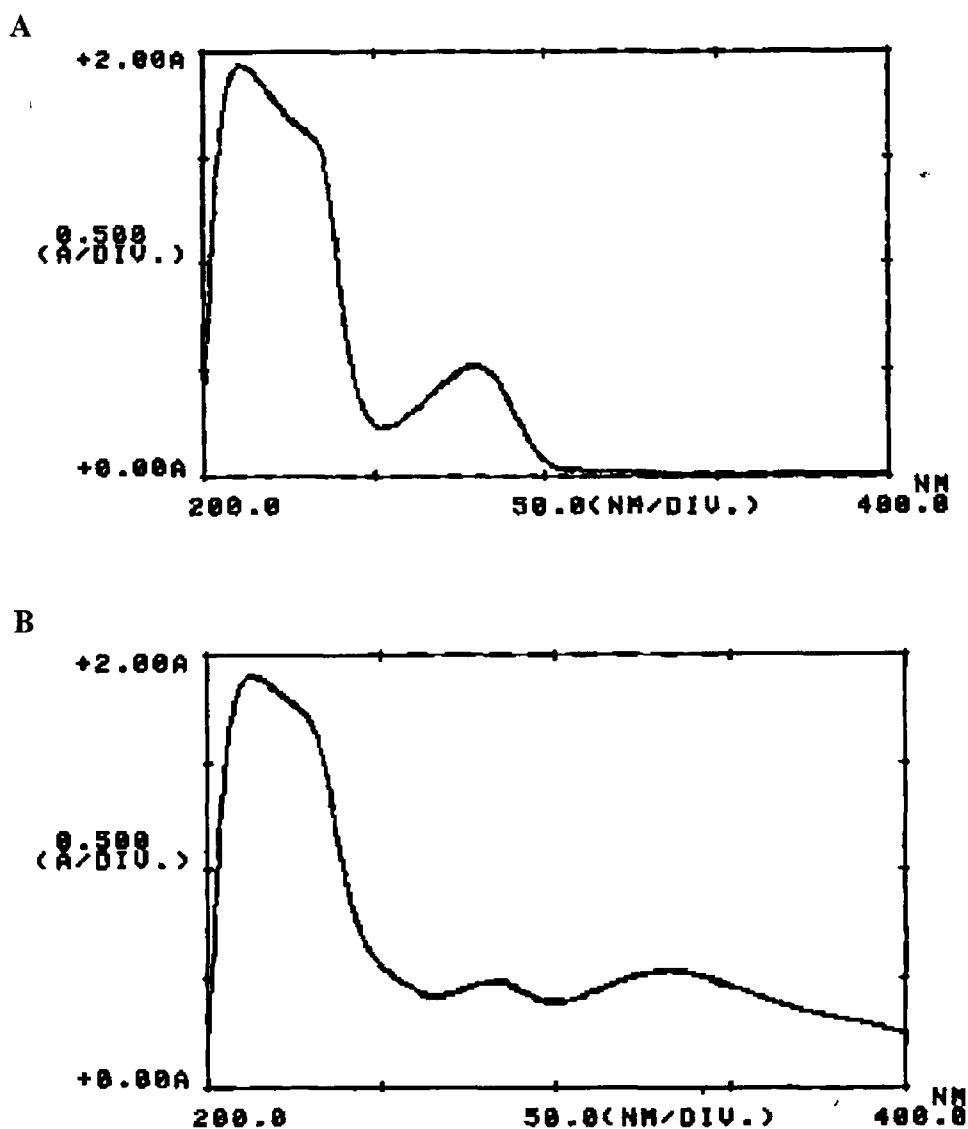
A number of amino-parathion-protein conjugates were generated for the production of polyclonal antibodies and also for the affinity purification of antibodies for assay development Amino-parathion was coupled to various proteins (Ercegovich *et al*, 1981) using diazo-coupling chemistries as described in section 2 3 2 2 2 The conjugates produced were dialysed extensively against  $\text{H}_2\text{O}$  over five days to remove any unconjugated amino-parathion In all cases, a characteristic orange colour appeared indicating the formation of a diazonium bond Characterisation of a parathion-OVA conjugate was carried out and is shown in Figure 6 6 Spectrophotometric scans were carried out on OVA before and after conjugation to parathion Figure 6 6(A) shows the OVA molecule with characteristic peaks at 260 and 280 nm However, after conjugation to parathion a third peak is present at 320 nm (Figure 6 6(B)) representing the attached parathion The resultant conjugate was used for the production of anti-parathion polyclonal antibodies



*Figure 6.4 Infrared spectrum for parathion from 600 to 4000  $\text{cm}^{-1}$ . The two strong bands, at  $\sim 1526 \text{ cm}^{-1}$  and  $1350 \text{ cm}^{-1}$  correspond to the  $\text{NO}_2$  asymmetric and symmetric stretches, respectively which is present on the parathion molecule.*



*Figure 6.5 Infrared spectrum for amino-parathion from 600 to 4000  $\text{cm}^{-1}$ . The two strong bands at  $\sim 3322$  and  $3402 \text{ cm}^{-1}$  correspond to the  $\text{N-H}_2$  asymmetric and symmetric stretches.*



*Figure 6.6. Spectrophotometric scans of ovalbumin (OVA) (A) and parathion-OVA (B) from 200 to 400 nm. A comparison of the parathion-OVA conjugate and OVA shows a distinct difference in the shoulder between 300 and 400 nm on the parathion-OVA sample, which was not present on the OVA sample. This was explained by the presence of parathion in the parathion-OVA sample, which was indicative of the linkage of parathion to OVA.*

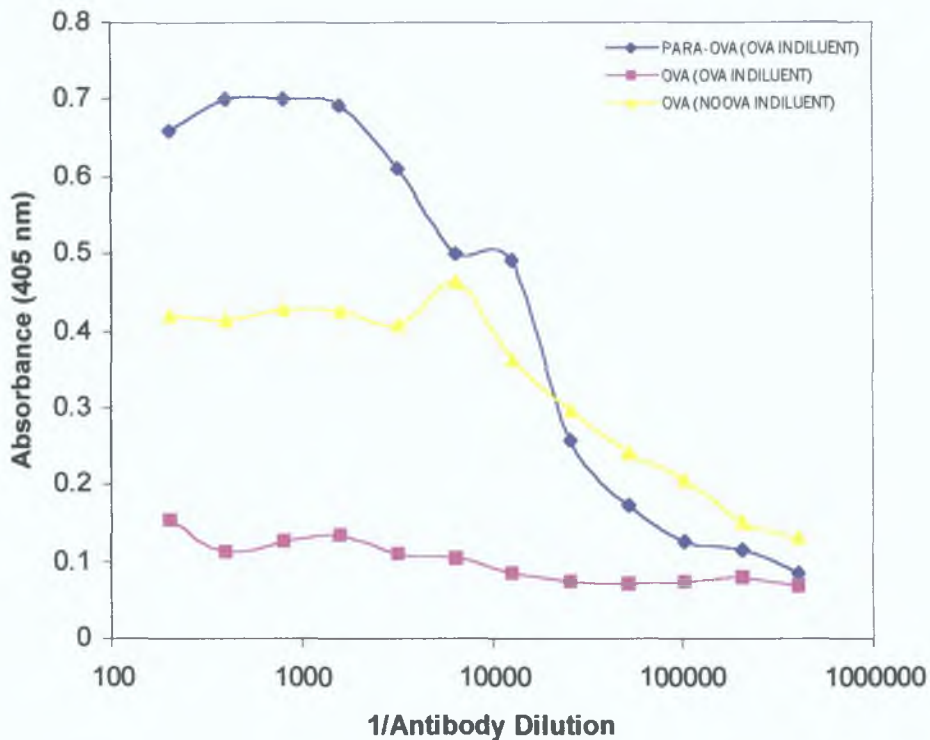
## **6 2 2 Production, purification and characterisation of polyclonal anti-parathion antibodies**

For the production of anti-parathion polyclonal antibodies, rabbits were immunised intradermally with the characterised parathion-OVA conjugate as described in section 2 3 5 2. The resultant serum was purified by saturated ammonium sulphate precipitation and affinity chromatography using a parathion-BSA-sepharose column. The antibody purity was assessed by SDS-PAGE gel electrophoresis, and the working dilution of the anti-parathion antibody for competitive assay analysis was determined.

Analysis of the antibody titre to parathion in serum was carried out using the ELISA as described in section 2 3 8 1. The results of the titre are shown in Figure 6 7 and indicate that there is a specific titre to parathion present of greater than 1/400,000 (Figure 6 7). This titre was sufficiently high to justify the sacrifice and harvesting of blood. The serum was then prepared as described in section 2 3 6 1. Initial partial purification was carried out on the rabbit serum by saturated ammonium sulphate precipitation, as described in section 2 3 7 2. A parathion-BSA-sepharose immunoaffinity matrix was constructed for the purification of the antibodies as described in section 2 3 4 1. Partially purified polyclonal antibody from the ammonium sulphate precipitation was passed through the column resulting in the binding of specific parathion antibodies. After extensive washing of the column to remove non-specifically bound antibodies, the anti-parathion antibodies were eluted with 0.1 M glycine/HCl, pH 2.2 (Figure 6 8). Eluted fractions were collected and protein concentration monitored by absorbance readings at 280 nm (Figure 6 8). The results indicated that the majority of specific antibody eluted between fractions five and fifteen. These fractions were pooled and dialysed into PBS buffer.

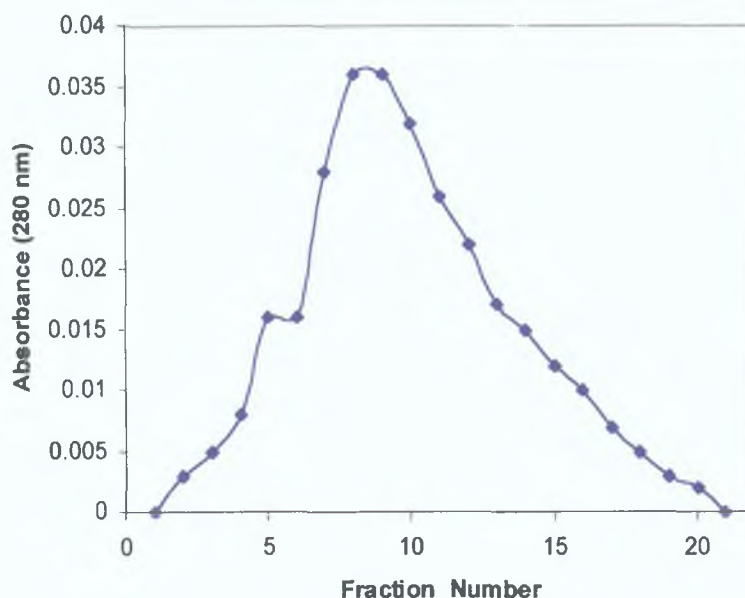
The purity of the antibody was analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) as described in section 2 3 7 6 and the gel is shown in Figure 6 9. SDS-PAGE is a technique which separates proteins on the basis of their molecular weight. The samples and molecular weight markers were boiled with

mercaptoethanol causing the polypeptide chains of the immunoglobulin to separate, producing bands at 25 and 50 kDa. The gel picture shows the affinity-purified anti-parathion antibody (lane 4) with a molecular weight band at approximately 50 kDa which represents the heavy chain of the immunoglobulin.

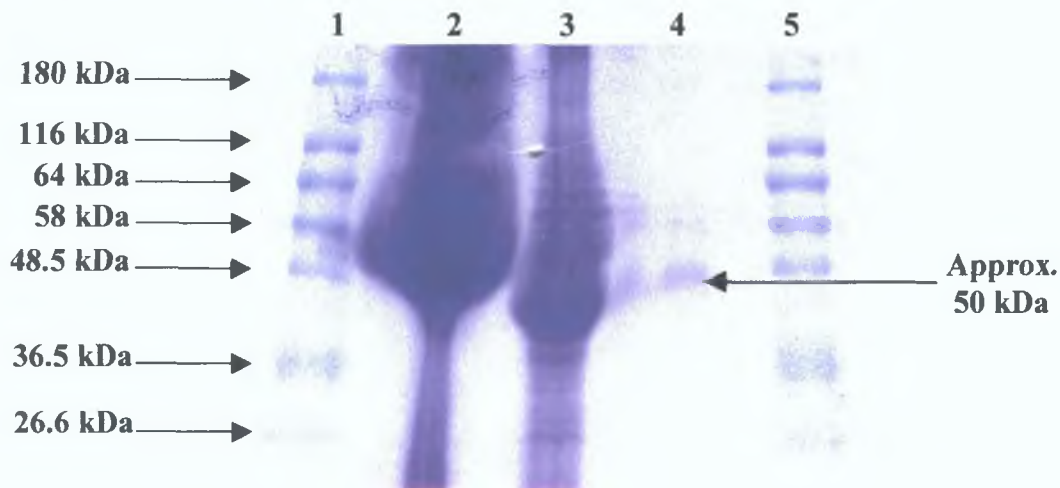


*Figure 6.7. Overlay plot showing the final titre of anti-parathion antibody in serum before sacrifice. Serial dilutions of serum from 1/200 to 1/400,000 were prepared in diluent with and without OVA and added to wells coated with parathion-OVA and OVA. Results show that OVA in diluent binds to OVA antibodies present in the sample effectively inhibiting their ability to bind to OVA coated on the wells of the plate. The antibody response titres out at approximately 1/400,000 indicating a high titre of specific antibodies present to parathion.*





*Figure 6.8. A typical elution profile for polyclonal anti-parathion antibody from a parathion-BSA-sepharose column. Saturated ammonium sulphate-partially-purified antibody was applied to the column. The column was washed and the bound antibody eluted with 0.1 M glycine/HCl, pH 2.2. 0.5 ml fractions were collected, and the protein concentration was measured by absorbance at 280 nm. As can be seen the majority of antibody eluted between fraction 5 and 15.*



*Figure 6.9. SDS-PAGE gel of affinity purified anti-parathion antibody on a parathion-BSA-sepharose column. Lanes 1 and 5 contain molecular weight markers ranging from 26.6 to 180 kDa. Lane 2 represents unpurified anti-parathion antibody rabbit serum. Lane 3 represents rabbit antiserum purified by saturated ammonium sulphate precipitation. Lane 4 represents the antibody fraction following affinity-purification on a parathion-BSA-sepharose column.*

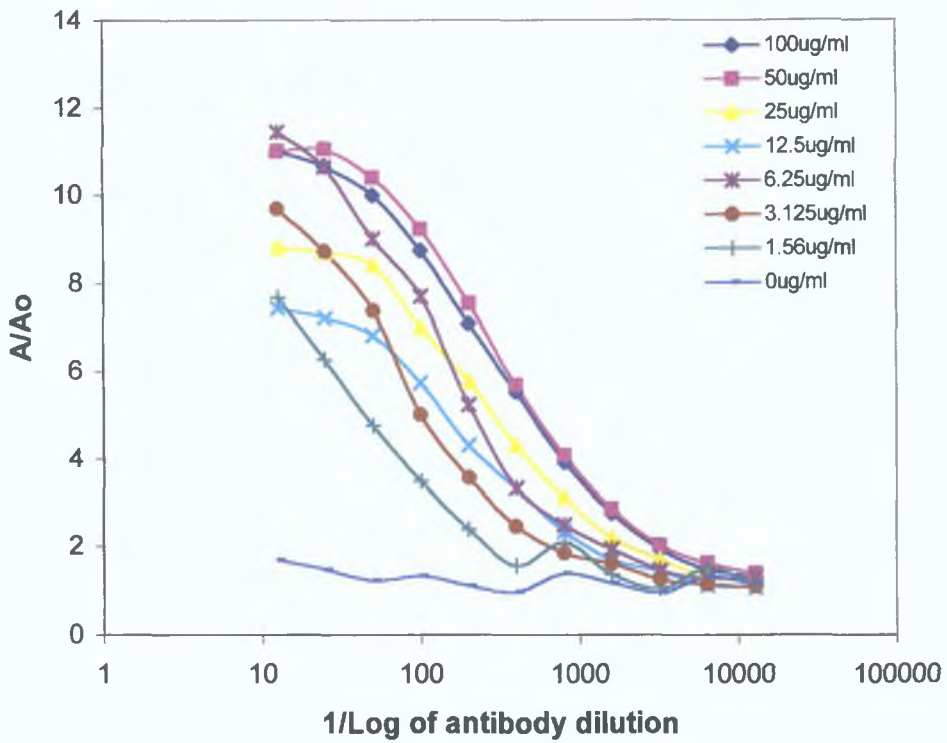
### **6 2 3 Development of a competitive enzyme-linked immunosorbent assay (ELISA) for parathion**

#### **6 2 3 1 Checkerboard ELISA for determination of optimal loading ratio of conjugate and optimal anti-parathion antibody dilution**

The working dilution of these antibodies was determined using the format already described in section 2 3 8 1 Parathion-BSA was coated on the wells of a microtitre plate at varying concentrations from 1 56 to 100  $\mu\text{g/ml}$ , and blocked with 2% (w/v) marvel Dilutions of affinity-purified anti-parathion antibody were prepared at ranges from approximately 1/12 to 1/128,000 and each dilution of antibody was added to each concentration of conjugate on the microtitre plate The results were plotted for the anti-parathion antibody as shown in Figure 6 10 The optimal conjugate loading ratio was found to be 50  $\mu\text{g/ml}$  with the optimal antibody dilution determined to be 1/500

#### **6 2 3 2 Intra- and inter-day assay variability studies of anti-parathion antibody**

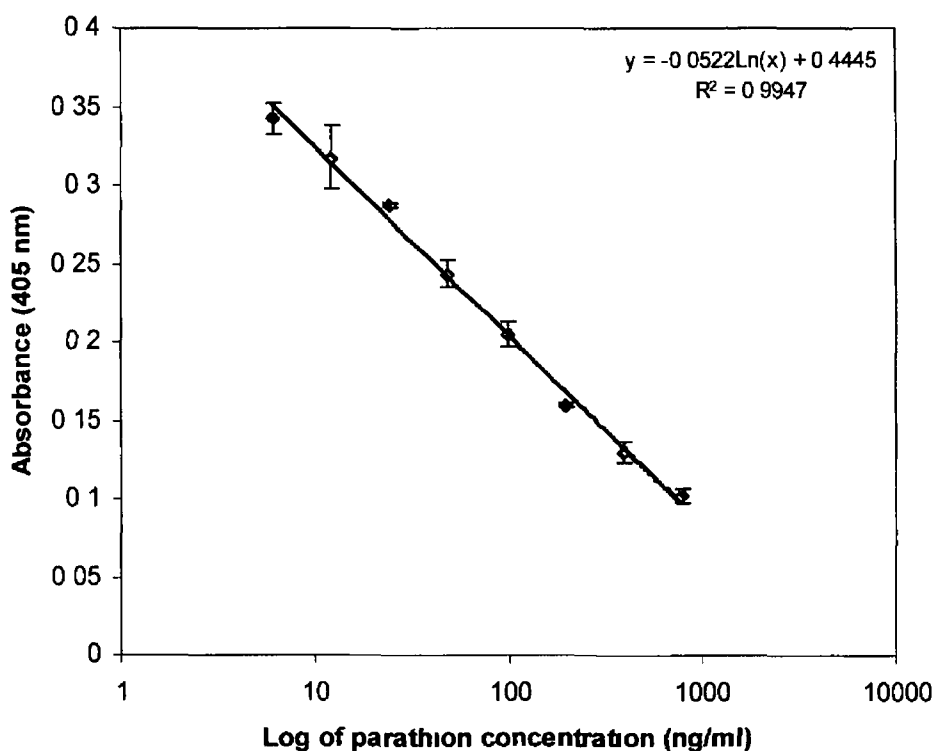
To carry out intra-day assay variability studies, five sets of standards were prepared for the antibody and assayed on the same day and the means plotted The intra-day assay variability study for the anti-parathion antibody shown in Figure 6 11 had a linear range of detection between 6 10 and 781 ng/ml The  $R^2$  value for this range was 0 99 The coefficients of variation (CV's) were determined to assess the precision of the analytical method, expressing standard deviation as a percent function of the mean These ranged from 0 20% to 2 55% as shown in Table 6 2 The inter-day assay variability study for the anti-parathion antibody also had a linear range of detection between 6 10 and 781 ng/ml as shown in Figure 6 12 The  $R^2$  value for this range was 0 99 The CV's were determined and ranged from 3 72% to 6 09%, as shown in Table 6 3 The percentage accuracy values obtained for both the intra- and inter-day assay variation were typically of a low nature demonstrating the accuracy and reproducibility of the method (Tables 6 2 and 6 3)



*Figure 6.10. Checkerboard ELISA carried out to determine the optimal dilution of antibody and concentration of coating conjugate (parathion-BSA) for anti-parathion polyclonal antibody. The optimal conjugate loading density was determined as 50  $\mu\text{g/ml}$  and the optimal dilution of antibody for use in a competitive assay was 1/500.*

**Table 6 2** *Intra-day assay variation for parathion ELISA assay Five sets of eight standards ranging from 6.10 to 781 ng/ml were analysed on the same day*

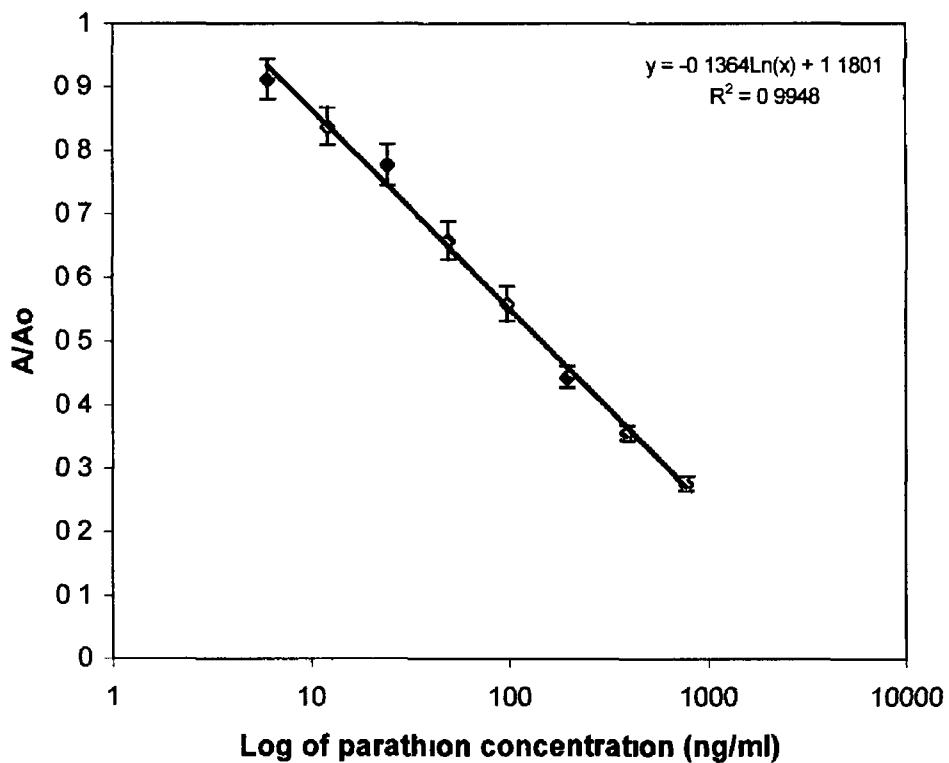
Parathion Concentration, (ng/ml)	Calculated mean $\pm$ S D , Abs	Coefficients of variation (CV's), %	% Accuracy
781	0.102 $\pm$ 0.0017	1.70	9.45
390	0.130 $\pm$ 0.0025	1.93	6.05
195	0.159 $\pm$ 0.0005	0.36	21.69
97.5	0.205 $\pm$ 0.0030	1.49	0.82
48.8	0.243 $\pm$ 0.0320	1.32	2.72
24.4	0.287 $\pm$ 0.0005	0.20	16.27
12.2	0.317 $\pm$ 0.0080	2.55	5.72
6.10	0.342 $\pm$ 0.0040	1.18	16.78



**Figure 6 11** *Intra-day competitive ELISA assay of anti-parathion antibody for determination of the optimal range of detection of free parathion using an antibody dilution of 1/500 Parathion-BSA was coated at a concentration of 50  $\mu$ g/ml. The absorbance at 405 nm is inversely proportional to the amount of free drug in solution. The linear range of detection was found to be between 6.10 and 781 ng/ml.*

**Table 6.3 Inter-day assay variation for parathion ELISA assay** Five sets of eight standards ranging from 6.10 to 781 ng/ml were analysed over three different days

Parathion Concentration, (ng/ml)	Calculated mean $\pm$ S D , A/A <sub>0</sub>	Coefficients of variation (CV's), %	% Accuracy
781	0.276 $\pm$ 0.011	4.25	3.17
390	0.356 $\pm$ 0.017	4.82	7.85
195	0.444 $\pm$ 0.027	6.09	13.15
97.5	0.558 $\pm$ 0.029	5.34	1.88
48.8	0.657 $\pm$ 0.032	4.88	5.13
24.4	0.776 $\pm$ 0.028	3.72	20.70
12.2	0.837 $\pm$ 0.031	3.77	1.39
6.10	0.911 $\pm$ 0.047	5.23	17.86



**Figure 6.12 Inter-day competitive ELISA assay of anti-parathion antibody for determination of the optimal range of detection of free parathion using an antibody dilution of 1/500** Parathion-BSA was coated at a concentration of 50  $\mu$ g/ml. The linear range of detection was found to be between 6.10 and 781 ng/ml.

#### 6 2 4 Cross reactivity studies of the anti-parathion polyclonal antibody

The specificity of the polyclonal antibodies to parathion is critical for the performance of the immunoassay. Cross reactivity studies were carried out with a number of available drugs for this purpose using the ELISA developed as described in section 2 3 8 2. Standards of parathion, methyl-parathion, carbophenothion, fenitrothion, etrimfos, mevinphos and dichlorvos were prepared and added to a microtitre plate coated with parathion-BSA at a 1:1 ratio with antibody at a dilution of 1/500. Table 6 4 shows the percentage inhibition of each compound.

*Table 6 4 Cross reactivity studies of the anti-parathion polyclonal antibody with structurally related molecules*

<b>Compound</b>	<b>Inhibition expressed as a percentage of slope of parathion antibody</b>
Parathion	100%
Methyl-Parathion	0 8%
Carbophenothion	0 015%
Fenitrothion	0 5%
Etrimfos	No cross reactivity
Mevinphos	No cross reactivity
Dichlorvos	No cross reactivity

## **6 2 5 Development of a model BIAcore inhibition assay for parathion in PBS using the anti-parathion polyclonal antibody**

### **6 2 5 1 Preconcentration and immobilisation of parathion-BSA conjugate**

The first step in the development of a model inhibition BIAcore assay to parathion was the immobilisation of parathion-BSA onto the surface of a CM5 sensor chip. The immobilisation chemistry used was EDC-mediated NHS esterification as discussed in section 2.3.11.2. To achieve maximum binding, a preconcentration study of parathion-BSA was carried out as described in section 2.3.11.1. 100 µg/ml of parathion-BSA was prepared in 10 mM sodium acetate buffer at a range of pH's from 3.7 to 4.7. These solutions were then injected over an underivatized chip surface and the optimal pH determined for immobilisation of parathion-BSA was 4.1. Parathion-BSA was subsequently prepared in this solution for all immobilisations.

An inhibition BIAcore assay to parathion was then developed using the affinity-purified anti-parathion polyclonal antibody. For this purpose a number of parameters were optimised including the conditions required for the regeneration of the antibody and also for the removal of non-specific interactions.

Non-specific interactions between the anti-parathion polyclonal antibody and for both the carrier protein used to prepare the conjugate and the dextran matrix on the surface of the sensor chip were analysed. The anti-parathion antibody was passed over immobilised parathion-BSA, BSA and dextran surfaces at a dilution of 1/5. Binding of the antibody to both control surfaces was negligible (Figure 6.13) compared with approximately 350 RU binding to the conjugate immobilised surface.

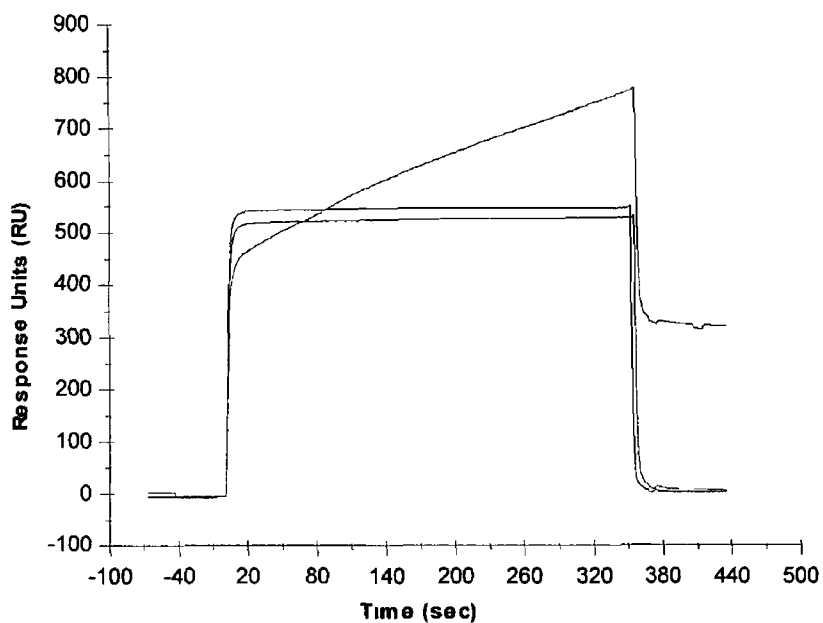
Regeneration conditions for the anti-parathion antibody required a 1 min pulse of 40 mM NaOH with 5% (v/v) acetonitrile. The antibody solution was repeatedly injected and the surface regenerated in order to conduct regeneration studies. These studies demonstrated that for the anti-parathion antibody, it was possible to regenerate the

surface up to approximately 65 times (Figure 6 14) After 65 cycles of binding and regeneration, the response after the injection of the last binding pulse was 253 1 RU compared with the first binding pulse of 256 9 RU resulting in a decrease of 1 4% in binding capacity over the course of the regenerations

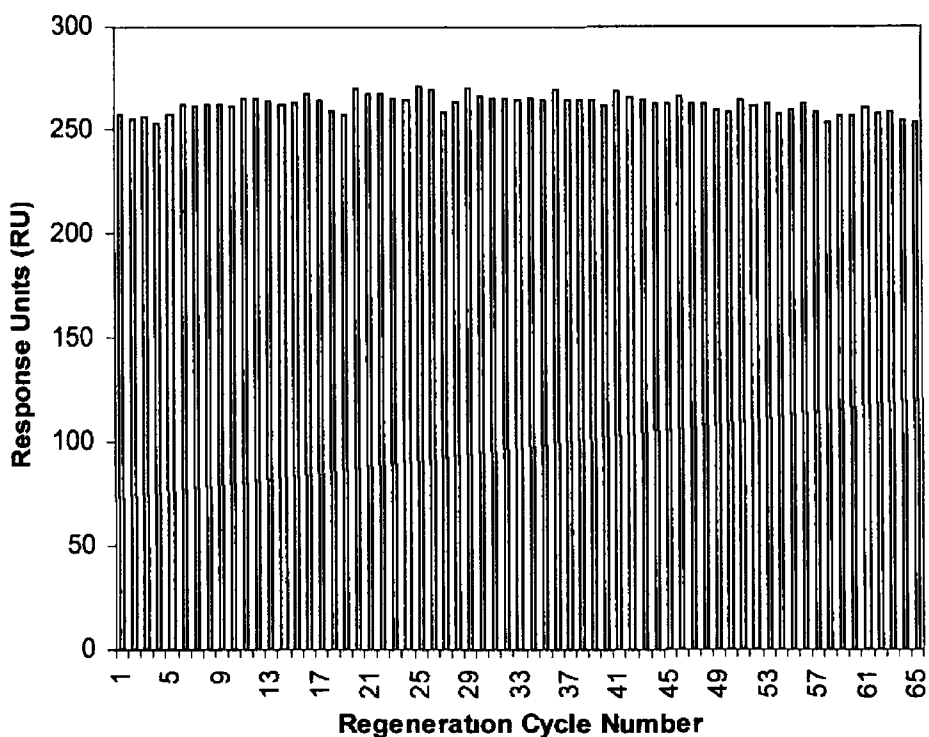
The binding capacity of surfaces directly immobilised with parathion (section 2 3 11 3 ) were also assessed using the anti-parathion antibody Again a 1/5 antibody dilution was used resulting in approximately 300 RU binding to the surface, however, to regenerate the surface a 1 min pulse of 40 mM NaOH with 20% (v/v) acetonitrile was required This surface displayed a 12% decrease in binding capacity over 40 regenerations (Figure 6 15) which is well within acceptable parameters of 20% (Wong *et al* , 1997)

A BIAcore inhibition assay was then set up on surfaces immobilised with parathion-BSA and parathion Standards of parathion were prepared in PBS with 1% (v/v) methanol ranging from 100,000 to 781 ng/ml and mixed with a 1/5 dilution of anti-parathion antibody The samples were then passed in random order over two individual surfaces, one immobilised with parathion-BSA and the other with parathion directly immobilised This was followed by regeneration of the parathion-BSA surface with a 1 min pulse of 40 mM NaOH with 5% (v/v) acetonitrile and the parathion surface with a 1 min pulse of 40 mM NaOH with 20% (v/v) acetonitrile A calibration curve was constructed for both surfaces by plotting the change in response for each standard of free drug against the concentration of parathion in solution Results from the surface immobilised with parathion-BSA (Figure 6 16), showed no inhibition of the anti-parathion antibody However, results from the surface with directly immobilised parathion (Figure 6 17) showed inhibition of antibody binding to the surface when mixed with the higher concentrations of free parathion

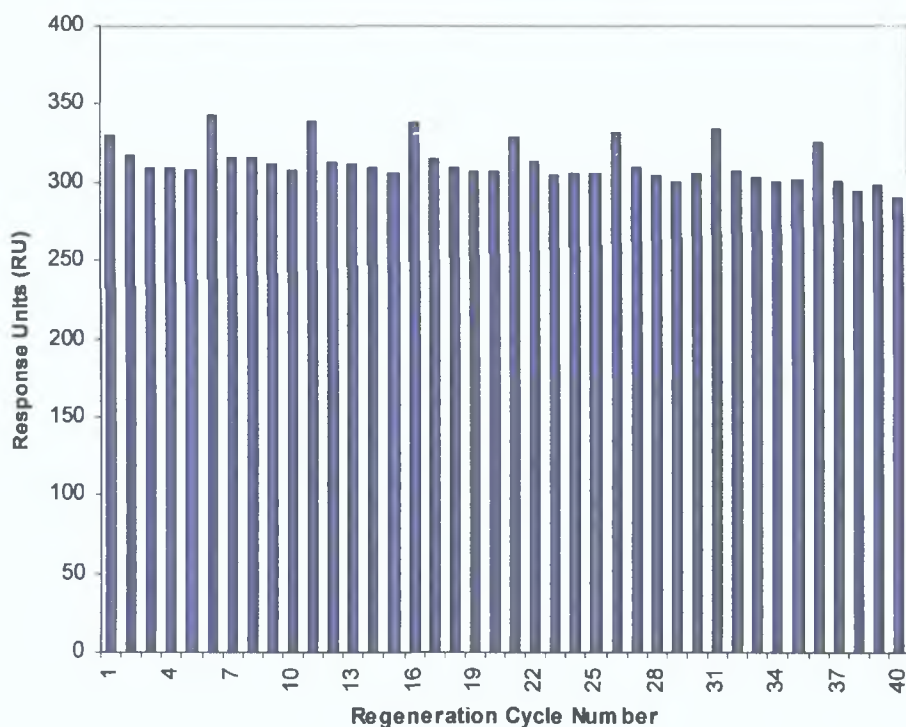




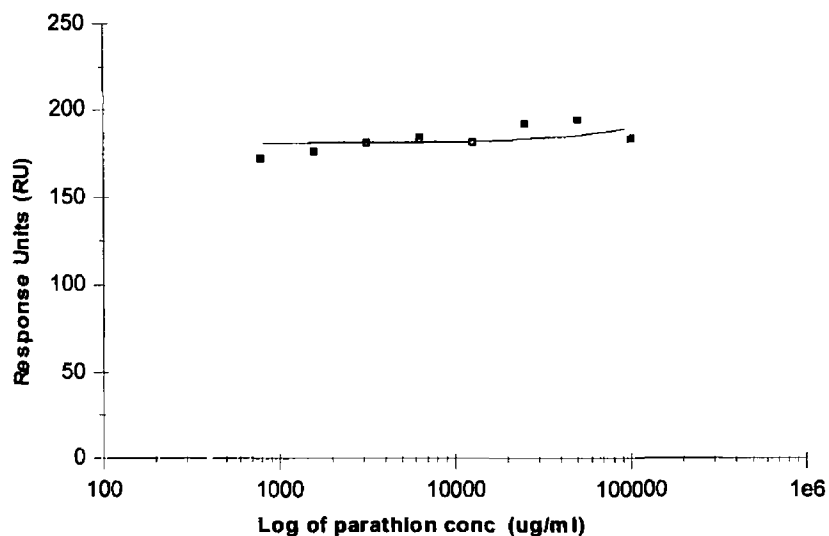
**Figure 6 13** *Overlay plot demonstrating the binding of affinity-purified anti-parathion polyclonal antibody to immobilised parathion-BSA (—) and BSA (---) surfaces and also over a blank dextran surface (· · ·). There was negligible binding to the control BSA and dextran surfaces. However, approximately 350 response units of anti-parathion antibody bound to the immobilised parathion-BSA surface indicating the antibody is specific to the parathion portion of the conjugate.*



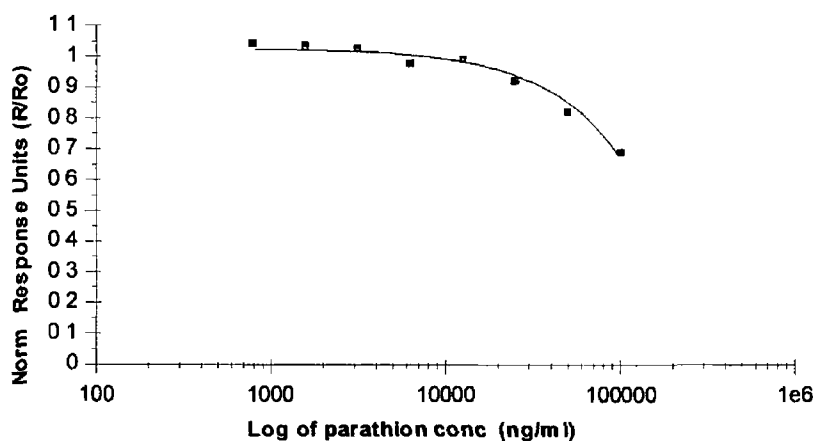
*Figure 6 14 Typical regeneration profile for approximately 65 cycles of a 5 min binding pulse of affinity-purified anti-parathion polyclonal antibody to the surface of a chip immobilised with parathion-BSA Polyclonal antibody at a 1/5 dilution was used and the surface regenerated with a 1 min pulse of 40 mM NaOH with 5% (v/v) acetonitrile. After 65 cycles of binding and regeneration, the response after the injection of the last binding pulse was 253.9 RU compared with the first binding pulse of 256.9 RU The ligand binding capacity was shown to decrease by approximately 1.4% over the course of the regeneration cycles*



*Figure 6.15. Typical regeneration profile for approximately 40 cycles of a 5 min binding pulse of affinity purified anti-parathion polyclonal antibody to the surface of a chip directly immobilised with parathion. Polyclonal antibody at a 1/5 dilution was used and the surface regenerated with a 1 min pulse of 40 mM NaOH with 20% (v/v) acetonitrile. After 40 cycles of binding and regeneration, the response after the injection of the last binding pulse was 289.7 RU compared with the first binding pulse of 329.6 RU. The ligand binding capacity was shown to decrease by approximately 12% over the course of the regeneration cycles.*



**Figure 6.16.** Assay curve for parathion using the affinity-purified anti-parathion polyclonal antibody on a parathion-BSA immobilised chip surface. The calibration curve was constructed using BIAevaluation 3.1 software package. The result shows the absence of inhibition using a surface immobilised with parathion-BSA conjugate.



**Figure 6.17** Assay curve for parathion using the affinity-purified anti-parathion polyclonal antibody on a chip surface directly immobilised with parathion. The result shows inhibition of antibody binding to the surface of the chip at the higher concentrations of free parathion.

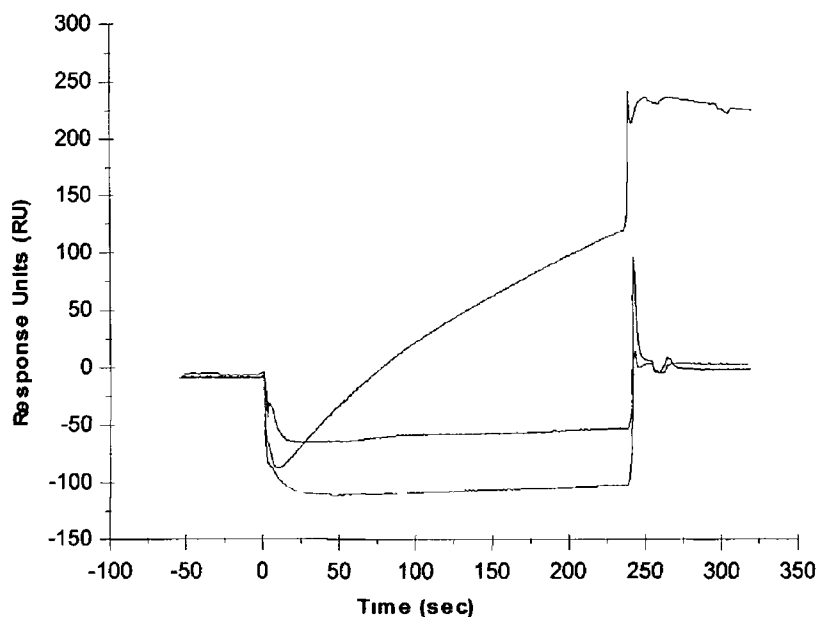
## **6 2 6 Development of a model BIAcore inhibition assay for parathion in PBS using the broad specificity antibody**

The broad specificity polyclonal antibody was supplied by the Institute of Food Research, Norwick, UK for the development of a BIAcore inhibition assay

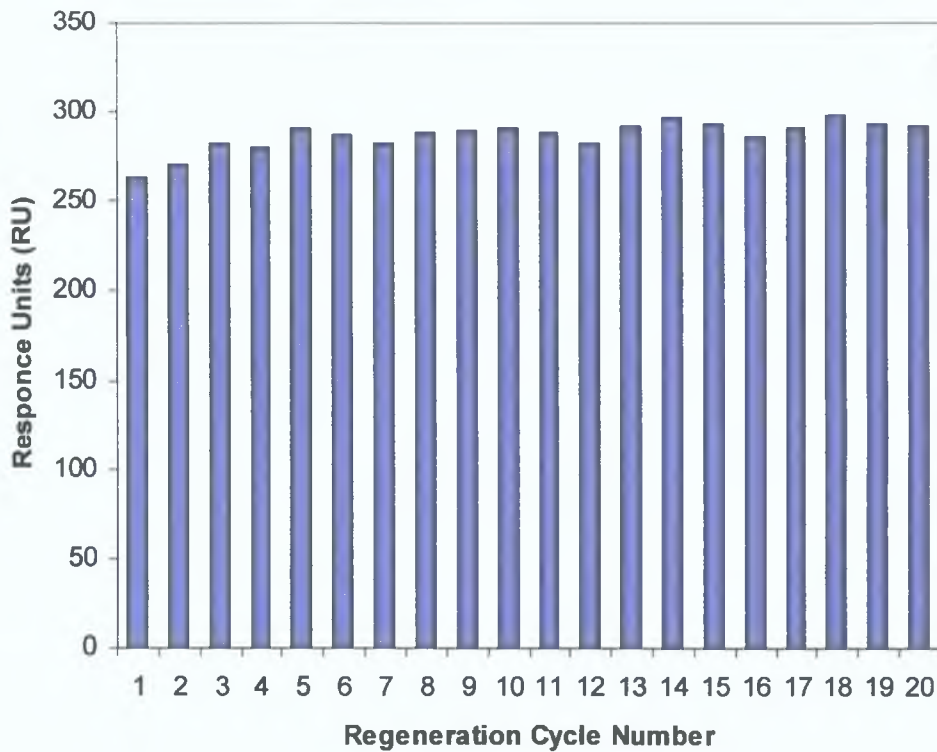
For the development of such an assay, parathion-BSA was immobilised onto the surface of the chip using already optimised parameters as described in section 6 2 5 1

Non-specific interactions between the broad specificity polyclonal antibody and for both the carrier protein (BSA) used to prepare the conjugate and the dextran matrix on the surface of the sensor chip were analysed Binding of the antibody (1/5 dilution) to both control surfaces was negligible (Figure 6 18) compared with approximately 220 RU binding to the conjugate immobilised surface

Regeneration conditions for the broad specificity antibody required two 1 min pulses in the order of 20 mM NaOH followed by 20 mM HCl The antibody solution was repeatedly injected and the surface regenerated in order to conduct regeneration studies Results demonstrated that it was possible to regenerate the surface up to approximately 20 times (Figure 6 19) After 20 cycles of binding and regeneration, results show that over the first five regeneration cycles there is a slight increase in binding of antibody However, after the first five binding regeneration cycles, binding of antibody to the surface of the chip stabilises and is found to be very reproducible



**Figure 6 18. Shows overlay plot demonstrating the binding of the affinity purified broad specific polyclonal antibody to immobilised parathion-BSA (-----) and BSA (—) surfaces and also over a blank dextran surface (.....) There was negligible binding to the control BSA and dextran surfaces However, approximately 220 response units of broad specific anti-organophosphate polyclonal antibody bound to the immobilised parathion-BSA surface indicating the antibody is specific to the parathion portion of the conjugate**



*Figure 6.19. Typical regeneration profile for approximately 20 cycles of a 5 min binding pulse of affinity-purified broad specific polyclonal antibody to the surface of a chip immobilised with parathion-BSA. Polyclonal antibody at a 1/5 dilution was used and the surface regenerated with two 1 min pulses in the order of 20 mM NaOH followed by 20 mM HCl. After 20 cycles of binding and regeneration, results show that there is a slight increase in binding of antibody over the first five regeneration cycles, resulting in the first five regenerations in any future work being disregarded. However, after the first five, binding of antibody to the surface of the chip stabilises and is found to be reproducible.*

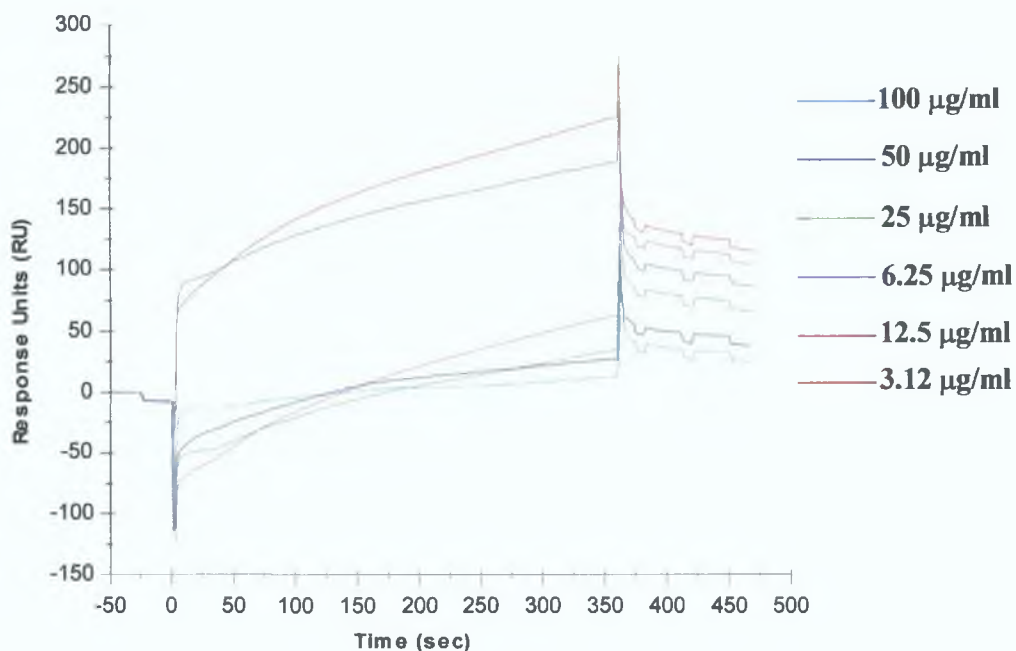
### **6 2 6 1 Determination of the range of detection of parathion in PBS in a BIAcore inhibition assay using the broad specific polyclonal antibody**

For the determination of the range of detection of parathion for a BIAcore inhibition assay, a range of standards of parathion were prepared in PBS with 1% (v/v) methanol ranging from 0.78 to 100 µg/ml for the broad specificity antibody. The antibody, prepared at a dilution of 1/5 in PBS was mixed with the corresponding concentration of free parathion. Each mixture was allowed to incubate at room temperature for ten min and then passed over the surface of a sensor chip immobilised with parathion-BSA in random order. This was followed by regeneration of the surface with two 1 min pulses in the order of 20 mM NaOH followed by 20 mM HCl. Figure 6.20 illustrates a typical antibody binding response for each concentration of parathion. From the calibration curve constructed the range of detection for the broad specificity antibody for parathion was found to be between 3.12 and 100 µg/ml (Figure 6.21).

### **6 2 6 2 Intra- and inter-day assay variability studies of broad specificity polyclonal antibody**

Three sets of standards were prepared for the broad specificity antibody to study the intra-day variability of the assay and assayed on the same day and their means plotted. The range of detection was found to be between 3.12 and 100 µg/ml (Figure 6.21). Table 6.5 shows the CV's which ranged from 1.85% to 12.24%. The inter-day assay variability study carried out over three separate days for the broad specificity polyclonal antibody, had a range of detection between 3.12 and 100 µg/ml as shown in Figure 6.22 and the CV's ranged from 2.51% to 23.99% as shown in Table 6.6.

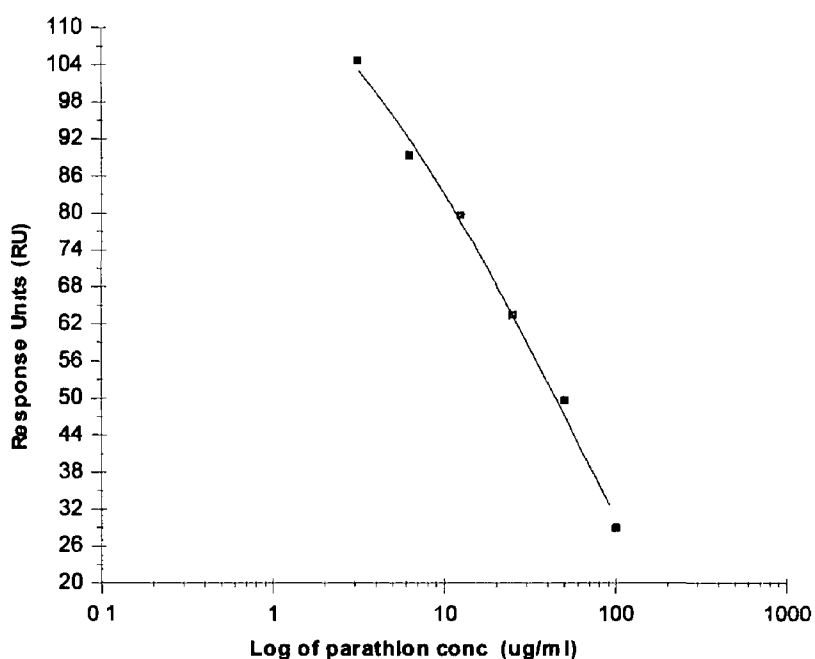




*Figure 6.20. An overlay plot for a typical set of binding curves in a parathion inhibition assay. Parathion standards ranging from 3.12 to 100 µg/ml of free organophosphate were mixed with the broad specific polyclonal antibody and passed over a parathion-BSA immobilised surface. The measured binding response was used to construct the calibration curves for the intra-day assay variability studies. This result was normalised and used in conjunction with 2 other normalised binding response curves for the inter-day assay variability studies.*

**Table 6 5** *Intra-day assay CV's for broad specificity antibody BIAcore inhibition assay*  
*Three sets of six standards ranging from 3 12 to 100 µg/ml were analysed on the same day*  
*and the CV's calculated.*

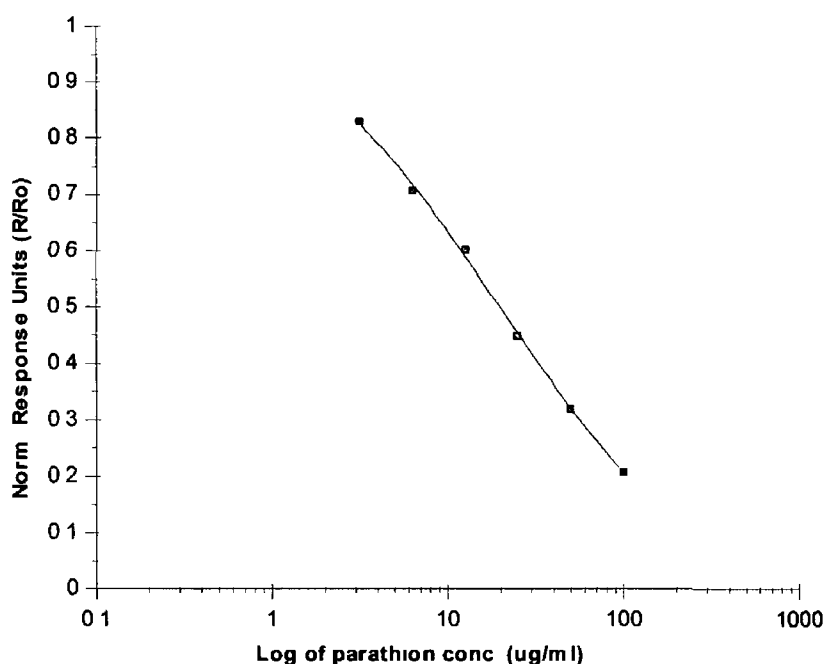
<b>Parathion Concentration, (µg/ml)</b>	<b>Calculated mean ± S D , RU</b>	<b>Coefficients of variation (CV's), %</b>
100	29 06 ± 3 55	12 24
50	49 75 ± 0 91	1 85
25	63 53 ± 3 58	5 64
12 5	79 63 ± 5 52	6 94
6 25	89 30 ± 5 95	6 67
3 12	104 73 ± 2 95	2 82



**Figure 6 21.** *Intra-day assay curve for parathion using the broad specificity polyclonal antibody on a parathion-BSA immobilised chip surface* *The residual plot shows the excellent correlation of the data set to the four-parameter model fit. The calibration curve was constructed using BIAevaluation 3 1 software package. The assay was carried out in triplicate and the mean response calculated for each concentration of free organophosphate. The range of detection of free parathion was found to be between 3 12 and 100 µg/ml*

**Table 6.6. Inter-day assay CV's for broad specificity antibody BIAcore inhibition assay**  
**Three sets of six standards ranging from 3.12 to 100 µg/ml were analysed over three**  
**different days and the CV's calculated.**

<b>Parathion Concentration, (µg/ml)</b>	<b>Calculated mean ± S D , <math>R/R_0</math></b>	<b>Coefficients of variation (CV's), %</b>
100	0.207 ± 0.032	15.81
50	0.319 ± 0.076	23.99
25	0.449 ± 0.082	18.45
12.5	0.603 ± 0.041	6.87
6.25	0.708 ± 0.017	2.51
3.12	0.830 ± 0.020	2.48



**Figure 6.22 Inter-day assay curve for parathion using the broad specificity polyclonal antibody on a parathion-BSA immobilised chip surface. The residual plot shows the excellent correlation of the data set to the four-parameter model fit. The assays were carried out in triplicate over three days and normalised and the mean calculated for each concentration of free organophosphate. The range of detection of free parathion was found to be between 3.12 and 100 µg/ml.**

### 6.3 Discussion

As discussed in chapter three, the production of a polyclonal antibody initially requires the preparation of a suitable antigen. Small haptens such as parathion, the hapten of interest in this chapter, are not immunogenic and so require conjugation to a larger molecule such as a protein. As described by Ercegovich *et al* (1981), the coupling technology for parathion to these proteins was via diazo linkage between the amino group present on the hapten and the nitrogen on imidazole and phenolic ring structures found on the protein. As parathion does not possess an amine group, the amine group was synthesised by reducing the nitro group present on the molecule to an amine group. This was carried out as described in section 2.3.2.2.1. Characterisation of this amine group and the purity of the compound was analysed by use of infra-red (IR) studies. The infra-red spectrum of a compound is the superposition of absorbance bands of specific functional groups. Thus, IR analysis of compounds may be used to confirm the presence of specific functional groups on a compound. The IR spectrum of parathion and reduced-parathion are shown in Figures 6.4 and 6.5, respectively, for comparison. As can be seen from Figure 6.5, the two strong bands at  $\sim 3322$  and  $3402\text{ cm}^{-1}$  correspond to the  $-\text{NH}_2$  asymmetric and symmetric stretches confirming the presence of an amino group on the reduced form of parathion.

The resultant amino-parathion product was ready for linking to proteins by diazonium coupling chemistry. The aromatic amine present on the parathion molecule is converted to a highly reactive and unstable diazonium under acidic conditions at  $0^\circ\text{C}$  with sodium nitrite. This diazonium ion is now ready to attack aromatic rings on residues present in proteins. The resultant diazonium bond bound amino-parathion to protein and is characterised by the formation of coloured compounds. The conjugate was characterised by spectrophotometric analysis. Figure 6.6 shows the spectra of unconjugated protein and conjugated protein for comparison between 200 and 400 nm. The presence of bands in both spectra at 260 and 280 nm represent the characteristic peaks present on proteins. However, the peak at 320 nm on the

conjugated protein represents the presence of parathion in the sample confirming the production of a parathion-protein conjugate

Polyclonal antibodies to parathion were then produced by using these conjugates for immunisation. Once an antibody titre of over 1/400,000 from serum was obtained (Figure 6.7), the host animal (rabbit) was sacrificed and the serum removed.

Initial purification of the antibodies from both animals was carried out by saturated ammonium sulphate precipitation. A parathion-BSA-sepharose column was produced and used to affinity purify specific parathion antibodies. The elution profile is shown in Figure 6.8. Checking the purity of the eluted antibody fraction was carried out by SDS-PAGE gel (Figure 6.9). This gel shows the process of purification of antibodies, starting with samples of serum followed by saturated ammonium sulphate precipitation and finally the affinity purified antibody. The affinity-purified antibody fraction was found to be pure as there were no contaminating bands present on the gel.

The purified polyclonal anti-parathion antibody was produced for the detection of the pesticide parathion in a competitive immunoassay format, in which the antibody would compete for the free analyte in solution and the surface bound parathion-BSA conjugate.

The limits of detection in competitive ELISA's are a function of the antibody affinity, and the equilibrium between both the free and immobilised conjugate. As a result, the coating conjugate concentration must be optimised as too high a concentration will shift the binding equilibrium in favour of binding to the immunoplate and cause reduced sensitivity to free antigen in solution. Similarly, the concentration of antibody used must be optimised allowing the antibody to be the limiting factor in the assay. If this were not the case, antibody would be capable of binding to free drug and bound conjugate in excess. The optimal antibody concentration was determined by taking a dilution of antibody producing a signal between 50-70% of maximum.

binding from a titre of the antibody. Antibody dilutions chosen from the asymptotes of the antibody dilution curve would show minimal changes in response to small changes in antibody concentration. Therefore, it is essential to optimise both the loading concentration of solid-phase conjugate and the optimal dilution of antibody. Results from the checkerboard ELISA (Figure 6.10) for the anti-parathion antibody gave an optimal concentration of loading conjugate of 50 µg/ml and optimum dilution of antibody of 1/500. At this dilution of antibody, minimal changes in antibody concentration give maximum changes in absorbance readings.

A competitive assay was developed for parathion in PBS using the affinity-purified antibody and optimised parameters determined from the checkerboard ELISA. Standards of parathion ranging from 0.38 to 25,000 ng/ml were prepared and mixed with purified polyclonal antibody at a dilution of 1/500. The assay showed a range of detection between 6.10 to 781 ng/ml (Figure 6.11) and displayed excellent linearity with low limits of detection. Intra-day and inter-day assay coefficients of variation (CV's) for the anti-parathion antibody were found to be between 0.20 to 2.55% and 3.72 to 6.09% respectively for 5 replicates (Tables 6.2 and 6.3). The percentage accuracy values obtained for both the intra- and inter-day assay variation were typically of a low nature demonstrating the accuracy and reproducibility of the method (Tables 6.2 and 6.3). When analysing the results produced from the assay, it is clearly evident that the antibody produces an accurate, specific and reproducible assay.

Cross reactivity is defined as a measure of the antibody response to structurally similar molecules. One of the initial steps in designing an immunoassay is the assessment of reactivity towards structurally similar molecules (Wild, 1994). Serial dilutions of the cross reactive analytes of interest are prepared with similar dilutions of the specific antigen and a competitive ELISA carried out as described in section 2.3.8.1. Table 6.4 shows the percentage inhibition of each compound using the slope of the line as a comparison. 100% inhibition equals the slope of the line for the parathion immunoassay and the slope of the line of each analyte studied is determined as a percentage of this slope. Results showed minimal cross reactivity of

the antibody with the analytes tested. The antibody mixture is clearly specific for parathion. This may be mainly due to its purification using the parathion-BSA-sepharose column.

Having shown that the anti-parathion polyclonal antibody was suitable for use in a competitive ELISA assay, the aim was to develop an inhibition assay using the BIAcore biosensor.

The first step in setting up such an immunoassay was the immobilisation of parathion-BSA and parathion onto the surface of a CM5 sensor chip. It is essential to maximise the interaction between the dextran surface of the chip and the conjugate of interest in order to immobilise as much conjugate as possible onto the surface of the chip. This was achieved by preparing the parathion-BSA conjugate in sodium acetate buffer pH 4.1. Parathion was directly immobilised onto the chip as described in section 2.3.11.3.

Non-specific analysis of the anti-parathion polyclonal antibody was carried out by passing the antibody over a BSA immobilised surface and an underivatized dextran surface. For the anti-parathion polyclonal antibody the non-specific interactions were found to be negligible (Figure 6.13).

For the successful development of an inhibition BIAcore assay, an important aspect of the biosensor assay, that must be optimised (as discussed in chapter 4), is the binding capacity of the immobilised ligand. This is not necessary in ELISA. Multiple regenerations are desirable on a single chip surface without a decrease in binding capacity of more than 20% (Wong *et al.*, 1997) to allow adequate routine sample analysis.

In this chapter, the binding capacity of surfaces immobilised with parathion-BSA and parathion were analysed. This was achieved by carrying out a series of binding and surface regenerations to assess the binding capacity deviation over these regenerations. Regeneration conditions were initially optimised for the anti-parathion

antibody and found that conjugate surfaces required a 1 min pulse of 40 mM NaOH with 5% (v/v) acetonitrile for regeneration while directly immobilised surfaces required a 1 min pulse of 40 mM NaOH with 20% (v/v) acetonitrile. Results showed that the binding capacity of the conjugate immobilised surface decreases by only 1.4% over the course of approximately 70 binding-regeneration cycles. However, results from regeneration studies from the directly immobilised surface showed a decrease in binding capacity of 12% over 40 binding-regeneration cycles. This result implies that the conjugate surface is more applicable for such an assay system than the directly immobilised surface. However, previous results (Fitzpatrick, 2001) have shown that directly immobilised surfaces are more applicable to the BIAcore biosensor. In this case, surface regeneration studies conducted demonstrated that the sensor chip surfaces with directly immobilised 4'-aminowarfarin were essentially inexhaustible with respect to antibody-binding capacity, and were regenerated up to 1000 times. This is because directly immobilised surfaces may be regenerated with harsher solutions than that used for regeneration of conjugate immobilised surfaces without resulting in denaturation of the hapten on the surface. Harsh solutions denature proteins resulting in antibody-antigen binding interactions being disrupted when using a conjugate immobilised surface.

The regeneration conditions required for the dissociation of the antibody-antigen complex on the surface of a parathion-conjugate surface were not quite as harsh as those required for regeneration of the surfaces with parathion directly immobilised. This may explain the excellent stability of the conjugate surfaces compared to the directly immobilised surfaces. Leaching of directly immobilised parathion from the surface of the chip may be occurring as a result of the harsher regeneration solutions. Leaching would cause antibody binding to decrease over a number of regenerations as the surface is slowly losing ligand, reducing the availability of binding sites on the surface. Results also show a slightly more variable binding response of antibody to the directly immobilised surface, calling into question the accuracy of the regeneration profile (Figure 6.15) for further analysis. The results demonstrate the use of hapten-protein conjugates is applicable to the development of an inhibition



immunoassay as the antibody binding capacity of the surface is extremely stable (Figure 6 14)

A BIAcore inhibition assay was then set up on surfaces immobilised with parathion-BSA and parathion. Standards of parathion were prepared in PBS/methanol ranging from 781 to 100,000 ng/ml and mixed with a 1/5 dilution of anti-parathion antibody. However, no inhibition of antibody binding to the conjugate immobilised surface was detected and inhibition of binding of antibody to the directly immobilised surface was present only at the higher concentrations of antibody. However, as shown in Figure 6 15 the antibody possesses a variable binding response to the surface and thus inhibition of antibody at the higher concentrations of parathion is not accurate and cannot be considered as a true result due to these variable binding responses.

The absence of inhibition with the use of conjugate immobilised surfaces is an interesting result. Using the same antibody in ELISA, competition occurs proving the antibodies affinity for free parathion. However, the result on BIAcore would suggest differently. At closer inspection of both techniques, there are many differences. ELISA is based on the antibody-antigen interaction reaching a state of equilibrium which allows binding of a range of antibodies with different affinities. The antibody-antigen interaction is also given a relatively long time to occur (i.e. 1.5 h). The BIAcore biosensor on the other hand is based on inhibition, the antibody-antigen complex is premixed for a period of time followed by injection over the surface of a chip immobilised with the appropriate antigen. The unbound excess antibody binds to the surface allowing the generation of a standard curve for different concentrations of free analyte. In the BIAcore system the antibody-antigen mixture does not reach equilibrium and bound antigen and free antigen do not compete with one another for binding to the antibody, so the assay is referred to as an antigen inhibition assay rather than a competitive assay.

The minimal inhibition seen in the BIAcore assay may be explained by the range of detection of free parathion using the polyclonal antibody differing from the range detected on ELISA. The detection of parathion may be of a higher range than the concentrations set up in the assay. Higher ranges of parathion were not analysed as parathion was found to precipitate out of solution at higher concentrations. An

increase in methanol concentration to combat this precipitation resulted in loss of antibody activity

The conjugate concentration on the surface of the chip may also be too high resulting in a shift in the binding equilibrium in favour of antibody binding to the surface of the chip, reducing sensitivity to free antigen in solution. To test this theory a reduction in the conjugate concentration on the surface of the chip is required, which would result in an increase in the concentration of antibody required. However, with respect to the amount of antibody available, this experiment was not feasible.

Due to the fact that the surface of the sensor chip is a hydrogel and is not the same solid state surface as in the case of the ELISA. Steric hindrance with a high dissociation rate of antibody may be a cause for the lack of displacement in the BIAcore assay (Kusterbeck *et al*, 1990). The short half-life of an antibody-antigen interaction may be characterised by a continuous association-dissociation process of the antibody-antigen complex. If the parathion antibody has a high dissociation constant, this would result in the antibody becoming free to bind to the surface of the chip with parathion-BSA immobilised, resulting in a non-measurable displacement response.

Schofield *et al* (1996) carried out a study on the affinities of antibodies produced to the influenza A virions in both ELISA and using surface plasmon resonance. No kinetic results from the SPR system for mAB HC58 were obtained although it had one of the highest affinities using the ELISA format, and was 28-fold higher than another mAB (HC10) which gave good data by SPR. With regards to this assay, the orientation of the analyte on the surface of the chip made it difficult for the antibody to access its epitope in the flow cell resulting in the lack of antibody binding. This may be indirectly applied to the anti-parathion antibody. If the opposite were the case in the anti-parathion displacement assay and the epitope present on the conjugate was placed on the surface of the chip allowing easy access for the antibody. This in conjunction with a high dissociation rate would allow the antibody to bind more freely to the immobilised antigen instead of the free antigen in solution resulting in an assay with no displacement.

A broad specificity polyclonal antibody was supplied by the Institute of Food Research, Norwick, UK with the ability to bind to a number of organophosphate pesticides including parathion (Alcocer *et al*, 2000) This antibody was used in the development of a BIAcore inhibition assay for parathion Controls were initially carried out and the antibody showed negligible binding to BSA and dextran Regeneration of the surface was achieved with two 1 min pulses in the order of 20 mM NaOH followed by 20 mM HCl 20 cycles of binding and regeneration were carried out and results showed there to be is a slight increase in binding of antibody over the first five regeneration cycles However, after the first five, binding of antibody to the surface of the chip stabilised and was found to be very reproducible An inhibition assay was then set up and the range of detection of parathion was found to be between 3.12 and 100 µg/ml The assay displayed low limits of detection Intra-day and inter-day assay variability statistics were then analysed in an attempt to look at the reproducibility of the assay The intra-assay CV's were found to be quite low However, the CV's for the inter-day assay were not found to be very reproducible with results as high as 20%

When comparing the results from the anti-parathion polyclonal and the broad specificity polyclonal antibody inhibition assays, it was found that all the parameters used were the same for both assays except for the antibody and the regeneration conditions The conjugate immobilised on the surface and the free pesticide concentrations were prepared in the same manner This proves that the lack of inhibition seen in the anti-parathion polyclonal antibody assays was not as a result of some flaw in the assay protocol or design Instead, some characteristic of the polyclonal antibody is responsible for the lack of inhibition

The toxicity of OPs is well known, nevertheless, OPs have been extensively used in the control of pests in the agricultural industry for many years This usage has resulted in the accumulation of these toxic compounds in the environment to such an extent that they are now considered an environmental pollutant Evidence would suggest that these compounds have entered the food chain, resulting in the need for

detection systems. As many traditional analytical systems have been used in the past for detection of parathion (section 6.1.2.2), recent methods have concentrated on the production of methods which are rapid, accurate, relatively inexpensive and capable of screening large numbers of samples. This need for detection systems is the main factor behind the ELISA method developed in this chapter for the analysis of parathion. The ELISA competition assay developed in this chapter for parathion illustrates the potential of using the ELISA technique compared to other detection techniques for the quantification of parathion. This assay compares favourably with various traditional analytical based detection methods for organophosphates as discussed in section 6.1.1.2. Various immunoassays developed by Ercegovich *et al* (1981) and Garrett *et al* (1997) have detection limits in the low ng levels while Brimfield *et al* (1985) possesses detection limits of low  $\mu\text{g}$  levels for parathion. When comparing these assays to the assay developed in this chapter we find that our assay has detection limits which are also in low ng levels.

Biosensors have also become very prominent in the detection of various analytes. The BIAcore biosensor was used in the development of an assay to parathion as this biosensor possesses many advantages such as 'real-time' monitoring of interactions, label free detection, reusable chip surface and the ability for rapid analysis. With all these in mind, an inhibition assay for parathion was developed using a broad specificity polyclonal antibody to organophosphates.

## **Chapter 7**

### **Overall Conclusions**

## 7.1 Overall Conclusions

The research work described in this thesis is mainly concerned with the development of polyclonal antibodies to two compounds, morphine-3-glucuronide and parathion. Following characterisation, they were applied to antibody-based immunoassay systems for the detection of these compounds. Genetic techniques were also developed for the production of single chain Fv (scFv) antibodies to morphine-3-glucuronide for development of antibody-based immunoassays.

The initial work carried out in this thesis was the production and characterisation of drug-protein conjugates of morphine-3-glucuronide. Conjugation of this hapten to the carrier protein was carried out by coupling the amine group present on the protein to a carboxyl group present on morphine-3-glucuronide. A variety of drug-protein conjugates were produced and used in the production and characterisation of polyclonal antibodies. Two polyclonal antibodies were produced and purified and used in the development of competitive ELISA immunoassays for morphine-3-glucuronide. These antibodies displayed the ability for use at high dilution factors and the ability to detect low concentrations of morphine-3-glucuronide in both model systems developed in PBS and urine.

Chapter 4 has outlined the development of BIAcore-based inhibition immunoassays for morphine-3-glucuronide. Both R1 and R2 polyclonal antibodies described in chapter 3 were used in the development of BIAcore inhibition immunoassays. A model immunoassay for detection of M3G in buffer was initially developed followed by the development of an immunoassay in urine. The biosensor-based assay used a surface with drug-protein conjugate immobilised. Studies were carried out on the binding/regeneration reproducibility of the antibodies. Both antibodies exhibited excellent binding and regeneration characteristics (Wong *et al.*, 1997) allowing the development of inhibition assays. The assays developed displayed good intra- and inter-day coefficients of variation while also exhibiting very low levels of detection for morphine-3-glucuronide. The low degree of sample preparation for BIAcore analysis highlights some of the advantages of BIAcore over some traditional detection techniques such as HPLC and GC-MS which require extensive sample

clean-up steps before analysis. As a result of this, the sample throughput of BIAcore assays is comparatively better than traditional techniques.

Results obtained from chapter 5 show the production of recombinant scFv antibodies with antigen specific characteristics using the Krebber system. Libraries produced from pre-immunised spleen mRNA do not necessarily require high transformant numbers in order to isolate positive clones. Instead, as results have shown in this chapter, the high titres of immunised mice and the resultant specific mRNA produced, allowed the screening of specific clones to M3G from a small library. Once specific clones were isolated, the scFv antibodies produced by these clones were studied for their application in an assay format. The ELISA results show that the scFv antibody produced is applicable to this assay format but large quantities of scFv antibody is required. The characterisation of the scFv antibody on the BIAcore biosensor produced very different results. With the ability of the BIAcore to carry out assays with minimal use of samples, an assay was developed for M3G. Results showed that the assays produced were repeatable and reliable, confirming the ability of the scFv antibody to be used in such an assay format both in a model system and for sample analysis.

In chapter 6, parathion was used for the production of parathion-protein conjugates. These conjugates were subsequently characterised by spectrophotometric analysis and used in the production of rabbit polyclonal antibodies. Antibodies from rabbit serum were purified and used in the development of a competitive ELISA for parathion analysis. Studies clearly demonstrated the accuracy and reproducibility of the assay. However, when the anti-parathion antibody was used in the BIAcore biosensor format, it was found not to be applicable to such a system. Inhibition assays were developed for parathion using a broad specificity antibody, indicating the possibility of development of a BIAcore parathion assay with antibodies with the required characteristics for use in this system.

To summarise, the BIAcore biosensor possesses strengths and weaknesses. The instrument was used to perform accurate and reproducible assays for morphine-3-glucuronide using both polyclonal and single chain Fv antibodies. However, the fact that the anti-parathion polyclonal antibody produced showed no inhibition on BIAcore while exhibiting competition on the ELISA format indicates that not all antibodies are applicable to such an assay format.



## **Chapter 8**

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## **Appendix A**

### **Glossary of terms and definitions commonly employed in Bioanalytical Validation procedures**

The terms listed below are commonly referred to in bioanalytical validation procedures and the criteria which they can be defined under have been extensively reviewed (Karnes *et al* , 1991, Shah *et al* , 1991, Braggio *et al* , 1996, Hubert *et al* , 1999, Currie, 1999 and Findlay *et al* , 2000)

#### **Mean**

Describes the average of replicate ( $x$ ) measurements (i.e.  $n_1+n_2+\dots+n_x/x$ )

#### **Standard Curve**

This describes the relationship between the measured analyte response (i.e. absorbance, response units) and the analyte concentration

#### **Non-specific binding**

This describes matrix effects which affect the degree of binding of the antibody antigen interaction, and can occur as a result of increased protein concentration and sample viscosity in the sample matrix (e.g. plasma), and also as result of altered ionic composition (e.g. urine) of the sample matrix. The assay design should therefore be robust enough to be able to withstand such variations in sample composition, and the degree of non-specific binding minimised prior to sample analysis (e.g. use of detergents and/or surface treatment with non-specific protein prior to sample analysis)

### **Coefficient of Variation (% C V )**

A quantitative measure of the precision of an analytical measurement expressed as a percent function of the mean value, also referred to as the Relative Standard Deviation (% R S D )

$$\% C V = [S D / \text{Mean value}] \times 100$$

### **Limit of Detection (LOD)**

Lowest concentration of analyte for which the response can be reliably distinguished from background noise

### **Normalised Response Values**

The response recorded in response units (RU) at each particular antigen concentration divided by the response recorded in the presence of zero antigen

i e

$$\text{Normalised Response} = \frac{\text{Response measured at particular antigen concentration}}{\text{Response measured at zero antigen concentration}}$$

### **Normalised Absorbance Values**

The absorbance recorded (AU) at each particular antigen concentration divided by the absorbance recorded in the presence of zero antigen

i e

$$\text{Normalised Absorbance} = \frac{\text{Absorbance measured at particular antigen concentration}}{\text{Absorbance measured at zero antigen concentration}}$$