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THE DEVELOPMENT OF A FLOW-INJECTION
FLUOROIMMUNOASSAY FOR SULFAMETHAZINE

By

MARK R. JOYCE

B.Sc. (Hons) DIS

A Doctoral Thesis submitted in partial fulfilment of the requirements for the
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DOCTOR OF PHILOSOPHY


Of

Loughborough University

1999

Supervisor:

Professor James N. Miller
Department Of Chemistry
Loughborough University
Loughborough
Leicestershire
UK

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This thesis is dedicated to my late farther, Maurice Kenneth Joyce and mother, Maureen Helen Joyce.

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Aims

The aim of this work was to develop farm and lab based screening assays for sulfamethazine – a sulfonamide antibiotic which is widely used in animal husbandry. The work was carried out as an EPSRC CASE project in conjunction with the Bedford based diagnostics company, Enviromed.

The project consisted of a dual approach to the monitoring of sulfamethazine residues. The first approach, conducted by my colleague Jill Hancox, at Enviromed, was to develop a test kit for on-farm monitoring of the drug, using pass-fail 'test strip' type technology; c.f. pregnancy test kit. The second approach was to develop a lab based assay for subsequent screening and confirmation of positive samples. The use of flow-injection immunoassay, a relatively new technique, was the assay format chosen for the lab based assay. The assay had to be capable of detecting the drug reproducibly at its maximum residual limit of 100 mg/kg in kidney tissue. The development of the flow-injection assay is the subject of this thesis.

The overall aim was to carry out the project with substantial technology crossover, between academia and industry, to produce a patentable detection system.

Scope And Outline Of The Thesis

The scope of the thesis is to give the reader an insight into the process of veterinary drug residue monitoring and the associated chemistry of developing a new drug residue screening/confirmation method.

The thesis is split into six chapters. Chapter one gives the reader a general introduction to the area of veterinary drug residue monitoring and covers the analytical methods and strategies currently in use. Sulfonamide antibiotics are covered in moderate detail as an understanding of basic structure-activity relationships is required in later chapters.

Chapter two covers the discipline of immunoassay and equips the reader with all the technical knowledge needed to appreciate the assay development chapters. Classical immunoassay techniques are reviewed and more recent flow-injection methods are introduced.

Chapter three describes the basic methods used which are common to all the following experimental chapters. Details of instrumentation, apparatus and reagents used are presented. Supplier lists are left to the appendices.

The experimental work was carried out in two key phases. Phase one was the organic synthesis of a fluorescently labelled analogue of the target analyte and this work, along with discussion, is presented in chapter four.

Phase two, covered in chapter five, was the assay development and optimisation using the analyte-dye conjugate synthesised in chapter four. The methods and data are presented as concisely as possible and the discussion and conclusions are left until the final chapter.

References are listed at the end of the thesis for clarity.

Abstract

Sulfonamide antibiotics have a broad spectrum of antibacterial activity and are frequently incorporated in feeds as a practical method for the prevention or treatment of a variety of so-called "confinement diseases" in animals, mainly pigs. They are also used at sub-therapeutic levels as growth promoters. Sulfamethazine has historically been the drug of choice for farmers because of its low cost and proven efficacy. However, there are concerns because of the possible presence of residues of the drug in meat.

The well established screening methods for the detection of sulfamethazine in porcine samples, enzyme linked immunosorbant assays (ELISA's), have a major drawback in that they commonly utilise micro titre plates which are both labour intensive and time consuming in operation.

Recently, high throughput flow-injection immunoassay techniques have emerged as an alternative to the ELISA format. The attractiveness of this type of assay is enhanced with the use of fluorescent dyes for detection, as background interference from the sample matrix is reduced.

This thesis describes the development of a flow-injection fluoroimmunoassay for sulfamethazine using thiophilic gel as the solid-phase immunoreactor. The methods for synthesising a fluorescently labelled analogue of sulfamethazine are presented along with the details of the assay optimisation. The assay developed was capable of detecting sulfamethazine at levels below its maximum residual limit (0.1 $\mu\text{g/g}$) with good reproducibility. CVs of 5 % were possible, even in the absence of automatic sample handling.

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List Of Abbreviations

Ab	Antibody
BB	Binding Buffer
BSA	Bovine Serum Albumin
CDR	Complementary-Determining Regions
CFA	Complete Freund's Adjuvant
CRM	Certified Reference Material
DAD	Diode Array Detection
DELFA	Dissociation-Enhanced Lanthanide Fluorescence Immunoassay
DMF	Dimethylformamide
DMSO	Dimethylsulfoxide
EB	Elution Buffer
EI	Electron-Impact
EIA	Enzyme Immunoassay
ELISA	Enzyme Linked Immunosorbant Assay
ESP	Electrospray
FAB	Fast Atom Bombardment
F _{ab}	Antigen Binding Region of the Antibody
F _c	Crystallisable Region of the Antibody
FIA	Flow-Injection Analysis
FIA	Fluoroimmunoassay
FIIA	Flow-Injection Immunoassay
FITC	Fluorescein Isothiocyanate
FT-IR	Fourier Transform Infrared
GC	Gas Chromatography
HAT	Hypoxanthine, Aminopterin, and Thymidine
HPLC	High Performance Liquid Chromatography
HPTLC	High Performance Thin Layer Chromatography
HRP	Horseradish Peroxidase
IFMA	Immunofluorimetric Assay
IRMA	Immunoradiometric Assay
LC	Liquid Chromatography
LOD	Limit Of Detection
MRL	Maximum Residual Limit
MS	Mass Spectrometry
N ⁴ -AcSMT	N ⁴ -Acetylsulfamethazine
NIR	Near Infrared
NMR	Nuclear Magnetic Resonance
PB	Particle Beam

PBS	Phosphate Buffered Saline
PEG	Polyethylene Glycol
RIA	Radioimmunoassay
SFC	Supercritical Fluid Chromatography
SIM	Selected Ion Monitoring
SMT	Sulfamethazine
SPA	Scintillation Proximity Assay
SpA	Staphylococcal Protein A
T-GEL	Thiophilic Gel
TLC	Thin Layer Chromatography
TSP	Thermospray
USP	United States Pharmacology
UV-VIS	Ultraviolet-Visible

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Chapter 1

The Analysis Of Veterinary Drugs And Their Residues In Edible Products

1. Introduction

In modern agricultural practice, veterinary drugs are being used on a large scale. The majority of these drugs are administered as feed additives or via drinking water in order to prevent the outbreak of diseases or to improve the growth of the animals. Besides that, therapeutic drugs are given in the case of disease, for drying-off purposes, or for the prevention of losses during transportation.

Unlike the situation with human drugs or veterinary drugs for pets, the use of veterinary drugs for food-producing animals such as poultry, lactating cows and swine can effect the public health and the international trade of food products because of the presence of residues of the drug, or of its metabolites, in edible products (milk, eggs, body tissue after slaughter). Depending upon the time-span between the administration of the drug and the collection of the animal product (withdrawal period), drug-related residues might be present in these products.

Other factors that determine the occurrence of residues are the route of administration, contamination of feeds or water, the physicochemical properties and metabolism of the drug, and the physical condition of the animal. The amount of drug residue that can be regarded as negligible should be based on toxicological considerations. In practice, unfortunately such internationally harmonised maximum residual limits (MRLs) often do not exist. Instead, in many cases the limits of detection of the available analytical methods determine whether residue levels are considered violative or not. For those classes of drugs that are suspected carcinogens or mutagens, no residues should be detectable. Maximum residual levels for various veterinary drugs have been established in the European Union. Table 1.1 gives the MRLs for some veterinary drugs and their residues in different kinds of matrices for food producing animals.

Obviously, the availability of sensitive and accurate analytical methods to monitor animal products for the presence of residues of veterinary drugs is

essential. In addition, pharmacokinetic and metabolism studies, which indicate the time-course of drug depletion and the presence of relevant metabolites, are of the utmost importance for the establishment of a government residue policy.

Compound	MRL ($\mu\text{g/g}$)	Matrix ^a	Species ^b	Remarks
Ampicillin	0.05	M, L, K, F	n.s.	
	0.004	Mi	B, O	
Carazolol	0.05	L, K	n.s.	
	0.005	M, F	n.s.	
Chloramphenicol	0.01	M, L, K, F	n.s.	No MRL for eggs and milk
Closantel	1.00	M, L		
	3.00	K, F		
	1.50	M, L		
	5.00	K		
	2.00	F		
Enrofloxacin	0.03	M, L, K	B, P, Ch	
Oxytetracycline	0.60	K		
	0.30	L		
	0.20	E		
	0.10	M, Mi		
	0.01	F		
Ivermectin	0.10	L	B	
	0.04	F	B	
	0.015	L	O	
	0.02	F	P, Eq	
Sulfonamides	0.10	M, Mi, L, K, F	n.s.	

^a M = Muscle; Mi = milk; L = liver; K = kidney; E = eggs; F = fat

^b B = Bovine; O = ovine; P = porcine; Ch = poultry; Eq = equine;

n.s. = not significant

Table 1.1 Established maximum residue levels (MRLs) within the European Union.

Only a few years ago, the meat and milk control of residues of veterinary drugs in most countries was based almost exclusively on microbiological methods. For eggs, sensitive and selective methods were completely absent. The microbial methods allowed one to detect a broad range of antimicrobials, but with strongly varying limits of detection (LOD) typically ranging from 2 $\mu\text{g/kg}$ to 10 mg/kg . Extensively used groups such as the sulfonamides, the

suspected mutagens or carcinogens of the nitrofurans, nitroimidazole and quinoxalin classes, the toxic antimicrobial chloramphenicol, tranquillisers and the anti parasitic drugs could not be determined with adequate sensitivity.

Recently, several sensitive, accurate and automatable non-microbiological analytical methods for the determination of residues of relevant classes of veterinary drugs in milk, eggs and animal tissues have become available. These methods are suitable for routine monitoring and surveillance programmes and for pharmacokinetic experiments, and allow the determination of relevant metabolites. Suitable spectrometric confirmation methods, which are useful for unambiguous identification, are also increasingly being developed.

1.1 Analytical Strategies In Regulatory Control

In essence, there are two types of regulatory residue programmes; (1) programmes where the animal or the product is held up pending the result of the analysis and (2) control programmes that are used to monitor the residue status of food of animal origin, without rejection of the specific product. In both cases, suspected samples should be efficiently separated from the bulk of negatives. The latter category can then be released while the, often few, positive samples can be examined further to establish whether the product contains violative residue levels. In its simplest form, a control programme therefore exists of a single analytical method that enables one to screen large numbers of samples for the presence of a variety of residues, and simultaneously to identify and quantify the residues that have been found. Unfortunately, such ideal methods are not encountered in the real world. Therefore, in practice a control programme is often divided into a screening phase and a confirmation phase, which each use appropriate analytical methods. A screening method should allow the detection of all suspect samples, preferably using a simple, routinely applicable procedure. A confirmation method should unequivocally establish the identity of the residue. During the regulatory control of non-prohibited drug residues, reliable quantification has to be carried out at an appropriate stage. Quantification

should enable one to reliably establish whether the residue concentration exceeds the maximum residual limit (MRL).

When developing or selecting analytical procedures for residue control programmes, one has to take into account a number of aspects, some of which are governed by external – e.g. political or organisational factors. Some of these are summarised in Table 1.2.

Available laboratory facilities (peripheral or central laboratory)	- personnel
Purpose and scope of control	- equipment
	- surveillance
	- multi-residue screening
Number of samples per day	
Available methodology	- development
	- implementation
Available finances	- use of MS
	- development
International agreements	- methods
	- products
Maximum residual levels	- banned drugs
	- changed with time

Table 1.2 Factors influencing the set-up of an analytical strategy.

1.2 Sulfonamide Antibiotics

1.2.1 Introduction

The discovery of the antibacterial activity of sulfonamides in the 1930's marked the beginning of the era of modern antibacterial drug research. Subsequent observation of variations in the activities of these compounds by structural changes brought into sharp focus the power of molecular modification in drug development. The elucidation of the relationship between sulfanilamide and *p*-aminobenzoic acid provided one long sought-after mechanistic basis for a biochemical approach to chemotherapy. Much of the subsequent work in chemotherapy, and drug research in general, has been based on the consideration of these facts.

The availability of a variety of sulfonamides with widely differing absorption and excretion rates has greatly increased their value in therapeutics. This, coupled with their ease of administration, wide spectrum of antimicrobial activity, non-interference with host defence mechanisms and relative freedom from the problem of superinfection, are responsible for their wide use in clinical practice six decades after their introduction.

1.2.2 Development Of Sulfonamides

The synthesis of Prontosil¹, the epoch-making report of its marked antibacterial activity in infected mice², followed by the suggestion that the activity may be due to sulfanilamide³, formed *in vivo*, and finally the isolation of sulfanilamide from the urine of patients⁴ under treatment with Prontosil started intense activity in this field.

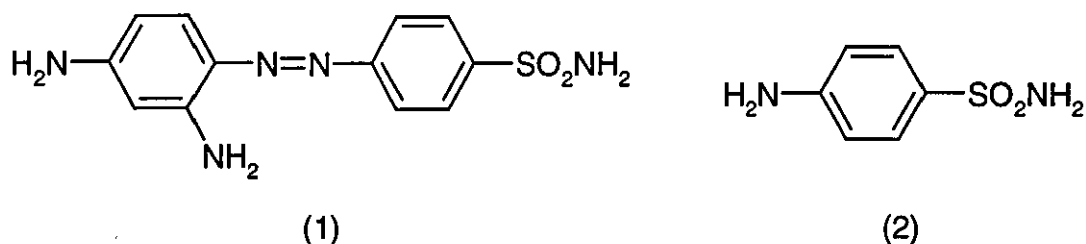


Figure 1.1 Structures of (1) prontosil and (2) sulfanilamide.

Numerous derivatives of sulfanilamide were soon synthesised to improve upon its activity and tested against bacterial, protozoal and viral infections. Sulfapyridine reported in 1938, was one of the earliest new sulfonamides to be used in clinical practice for treatment of pneumonia and remained the drug of choice until it was replaced by sulfathiazole, which possessed a higher therapeutic index. Sulfathiazole was in turn replaced by sulfadiazine, which has retained a pre-eminent position among the sulfonamides ever since. Two methylated derivatives of sulfadiazine, sulfamerazine and sulfamethazine were introduced into therapy later.

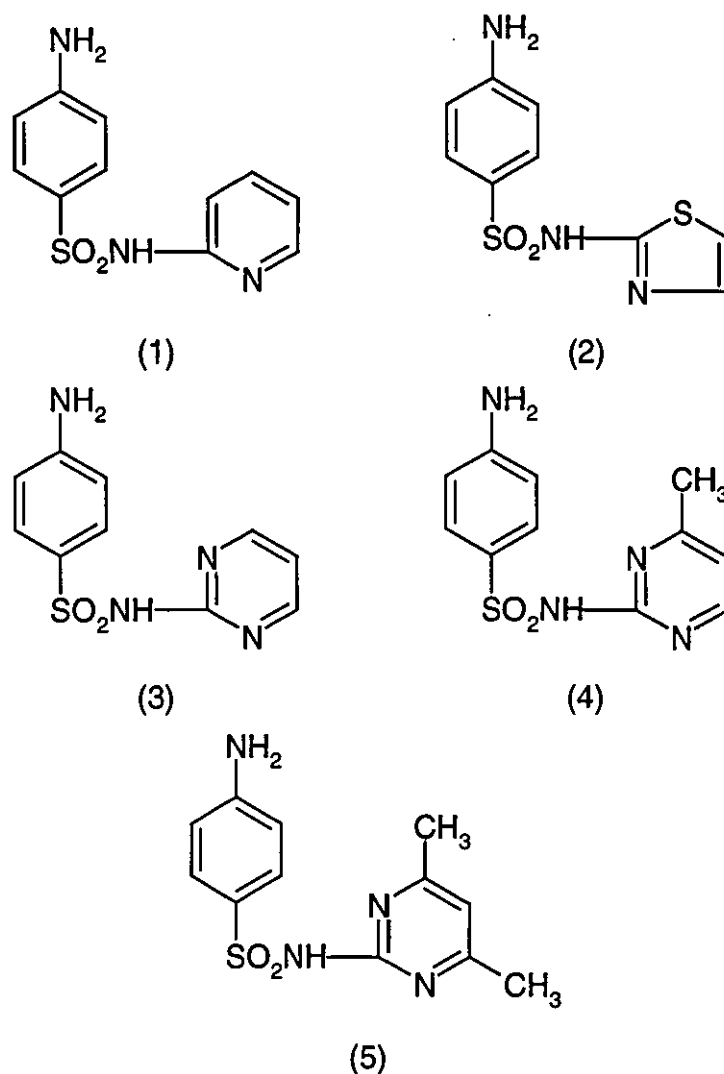


Figure 1.2 Structures of (1) sulfapyridine; (2) sulfathiazole; (3) sulfadiazine; (4) sulfamerazine; (5) sulfamethazine.

1.2.3 Mode Of Action

Sulfonamides are one of the few groups of drugs whose mode of action is known at the enzyme level. Though some of the finer points about the nature of the enzymic antagonism have yet to be settled, the theory that sulfonamides inhibit the enzymes involved in the condensation of 2-amino-4-oxo-6-hydroxymethyldihydropteridine pyrophosphate with *p*-aminobenzoic acid is unlikely to be seriously questioned.

It is not intended to discuss the effects of modifications of various structural features of sulfanilamide on its pattern of activity, other than for the sulfanilamide part, the following features are more or less inviolate for

compounds showing good activity: a) the 4-amino group, b) the benzene ring with 1,4-disubstitution, c) the singly substituted SO_2NH_2 group.

1.2.4 Toxicity

A large number of adverse reactions to sulfonamides involving almost every system of the body have been reported in the literature. Some of these are trivial and can be easily managed. Others, however, may be serious and even fatal, thus necessitating close surveillance and avoidance of indiscriminate use of these drugs.

The most serious side effects of sulfonamides are their actions on the bone marrow. Various types of blood dyscrasias have been reported, including leukopenia, agranulocytosis, aplastic anaemia, thrombocytopenia and pancytopenia. All these are serious complications and need close surveillance.

1.3 Drug Residue Screening

In this review, a screening method is defined as the first procedure that is applied to sample analyses, the purpose being to establish the presence or absence of residues of veterinary drugs. This procedure should be as simple as is possible. Still, it may be rather complex, due to, e.g. the properties of the drugs of interest or the desired limit of detection. In certain cases, the initial screening of samples will provide (semi) quantitative and quantitative information.

There are two main options when carrying out a screening programme: (1) to use multi residue methods aimed at the determination of groups of drugs having similar characteristics^{5,6} and (2) to use single residue methods applicable to one specific drug. These options are discussed below.

1.3.1 Multi-Residue Screening Methods

Veterinary drugs show a large variation in molecular structure and, consequently, in physicochemical properties and biological activity. Because the aim of the control of residues is to prevent residues in food exerting an undesirable effect on humans, it would be elegant to use this biological effect

as a detection principle in the screening procedure. Obviously, a prerequisite is that the effect is rapid, reproducible, and can be detected with great sensitivity. Generally, with the undesired effects of veterinary drug residues, one can differentiate between long-term toxic effects (carcinogenicity, mutagenicity), antimicrobial effects, allergenic effects, and technological effects. In principle, the MRLs for the drug are based on the absence of these effects.

In reality, only the antimicrobial activity can be measured quickly, simply and with high sensitivity and can therefore be used as a direct detection principle. The technological effect of veterinary drugs in milk (influence on starter cultures) primarily is an antimicrobial problem and can therefore be tested on the basis of antimicrobial activity⁷. As a consequence, all drugs that do not possess antimicrobial activity should be screened on the basis of other types of physiological reactivity, e.g. using immunochemical or receptor assays, or on the basis of their physicochemical properties.

1.3.1.1 Microbiological Multi-Methods

An analytical chemist dealing with the development of state-of-the art residue methods will probably consider the microbiological inhibition assays used for the detection of antimicrobials inadequate because they are neither very reproducible nor highly selective, have a strongly varying sensitivity for the various drugs, do not detect inactive metabolites and are rather slow (from 3 to 16 h). The opinion of a residue control officer may, on the other hand, be that the inhibition assays are excellent screening methods because: (1) they are not too selective and therefore able to simultaneously detect many drugs; (2) are often more sensitive than any other method; (3) are simple to perform (4) do not require sophisticated equipment, and (5) are completed within a day. Actually, both opinions are correct and numerous modifications of inhibition assays have, therefore, been tested to either enhance the selectivity or broaden the scope.

Methods using *B. stearothermophilus* var. *calidoactis* as a test organism, which are often used in milk control, are extremely sensitive for penicillins

(with LOD, about 5 µg/l). However, they generally are more than 100-fold less sensitive for other commonly used antibacterial agents such as macrolides, sulfonamides, tetracyclines and chloramphenicol^{7,8}. In view of this, it is even questionable whether these methods can be considered as true multi-methods. A number of rapid on-the-farm tests to check whether milk is contaminated with, mainly, penicillins are commercially available^{9,10}.

Most of the microbiological tests in meat control use muscle or kidney as target tissue. The obvious advantage of analysing muscle is that this is the edible part of the animal for which MRLs have primarily been established. A disadvantage is that a variety of microorganisms have been used to meet the MRLs for the commonly used antimicrobials, as is the case with the so-called four-plate test which initially was proposed as a routine screening method in the EC. Test systems using kidney as indicator tissue for muscle have the advantage of better sensitivity, because with most antimicrobial residues the highest free drug concentrations are found in the kidney and renal pelvis. Therefore, fewer microorganisms will be required than when testing muscle. However, there are also drawbacks such as the different ratios of residue levels in kidney and muscle of diseased animals.

1.3.1.2 Microbial Receptor-Assay Multi-Method

A multi-residue method for antimicrobials in milk had been developed which is based on the binding reaction between functional groups of the drug and receptor sites on added microbial cells (Charm-Test II,¹¹). Cells from two different organisms provide the binding sites for seven families of drugs: β -lactams, tetracyclines, macrolides, sulfonamides, aminoglycosides, novobiocin and chloramphenicol. The test employs ¹⁴C- and ³H-labelled drugs to compete for binding sites. The reported limits of detection range from below 5 µg/l for sulfonamides, β -lactams and chloramphenicol, to 10 µg/l for tetracyclines and 30 µg/l for aminoglycosides. The test can be regarded as a rather complex and sensitive screening method complementary to the microbial control methods. It can also be used as a confirmatory test for samples that have been found positive with a microbiological screening method. Compared with physicochemical screening methods for the seven drug families mentioned

above, the Charm-Test II is simple, rapid, sensitive and inexpensive. However, reported false positive results and limits of detection which are above save or maximum residue levels, indicate the necessity to confirm presumptive Charm II test results¹².

1.3.1.3 Physicochemical Multi-Methods

As has been stated above, screening procedures should be simple, but biological matrices are rather complex and different sample treatment procedures are necessary to prepare a suitable final extract. Automation is therefore highly desirable. For TLC analyses, no automated on-line sample preparation techniques for biological samples are operational. However, it is worth noting that several methods exist for the screening of sulfamethazine using TLC, with detection limits ranging from 100 ppb to 1 ppm^{13,14,15,16,17}. A field portable TLC plate reader has also been developed¹⁸. For GC, the use of a continuous flow system containing a liquid membrane, which is directly coupled to the GC part of the system has been described for the determination of amines in urine¹⁹. Another promising approach is the on-line trace enrichment of analytes from aqueous samples or extracts, using a short LC-type pre-column packed with e.g. C₁₈-bonded silica or a polymeric sorbent, and coupled on-line with a capillary GC via a retention gap²⁰. An excellent review has been published detailing HPLC methods for the determination of sulfonamides in tissue, milk and eggs²¹. Nowadays, commercially available equipment allows the fully automated solid phase extraction, either off-line or coupled on-line to an LC instrument^{22,23,24}. This method has been applied to the analysis of sulfamethazine in ovine plasma²⁵ and for the determination of 14 other veterinary drug residues in meat²⁶. The direct injection of biological fluids onto a column-switching (CS) LC system is also possible. Main applications of column-switching techniques to drug analysis have been reviewed²⁷. Today, the development of intelligent and versatile autosamplers allows one to perform essential steps such as homogenisation and liquid-liquid extraction in a fully automated mode. This is particularly useful where a derivatisation step has to be included.

The selectivity of a multi-residue screening method can be defined as its potential to discriminate between the analytes under investigation and other substances that are present in the sample, mainly matrix components. The maximum number of peaks that can be resolved in a typical TLC, LC or capillary GC run (about 20, 50 and 200 respectively²⁸), is distinctly lower than the number of residues of veterinary drugs and of matrix interferences that can be present. In other words, additional selectivity has to be introduced into the assay. If the analytes can be selectively detected because they show long wavelength (above about 350 nm) UV-VIS absorption or display native fluorescence, as do some sulfonamides²⁹, one can achieve low limits of detection (<10 µg/kg) while using simple clean-up procedures. The highly desirable use of more selective sample pre-treatment will be discussed below.

Selectivity can be introduced by chemical transformation of drug(s) of interest into compounds that possess favourable detection characteristics. In GC, pre-column derivatisation with a reagent that enables trace-level determination with nitrogen-phosphorous detection (NPD) or ECD detectors for sensitive nitrogen-phosphorous, and halogen or other heteroatom detection is quite common³⁰. Two methods for the determination of sulfamethazine by GC involving methylation prior to analysis have been reported^{31,32}. With TLC, spraying of the plate with a suitable reagent prior to development is used for many drugs. Fluorescamine is a commonly used derivatisation agent for sulfonamides as it reacts with the primary amine of the sulfanilamide part of the molecule and yields a fluorescent product which is easily quantified^{13,14,15,16}. With both GC and TLC, such derivatisation procedures are performed off-line and are therefore not very attractive because the automation potential is low and side products are often formed which may interfere in the determination of the analytes(s) of interest. The same is true for off-line and is true to a lesser extent (no automation problem) for on-line pre-column LC derivatisation. General strategies and selection of derivatisation reactions for LC and capillary electrophoresis are reviewed in reference³³.

Post-column LC derivatisation has a number of advantages. For example, (1) the LC separation is not influenced, (2) the procedure is on-line and can,

therefore, easily be automated, and (3) the reaction can be incomplete as long as it is reproducible. However, there are also disadvantages because the LC eluent is often not the ideal reaction medium, some additional band broadening may be introduced, and an additional pulseless pump is (often) required for reagent introduction.

Various post-column reactions for veterinary drug analysis have been reported^{34,35}. For sulfonamides, these include utilisation of the fluorescent reagent *o*-phthaldialdehyde³⁶, and dimethylaminobenzadehyde (DMAB) which is detected at 450 nm³⁷. Pre-column methodologies for the derivatisation of sulfamethazine commonly utilise the fluorescamine reaction^{38,39,40}. It is worth noting that several methods for the liquid chromatographic determination of sulfonamides exist which do not involve either pre- or post-column derivatisation of the analyte(s). These methods frequently involve extensive sample clean-up and involve UV detection at 265 nm^{41,42,43,44,45,46,47}.

1.3.2 Single Residue Screening Methods

The above discussion on multi-residue screening methods shows that, in relatively simply equipped laboratories, only microbiological methods can be used for routine application. However, to quote two examples, the extensively used antimicrobial chloramphenicol and the sulfonamides cannot be detected with sufficient sensitivity using such methods. This has prompted the development of simple specific tests for these compound classes, which should be used in addition to the microbiological methods suited for other microbials.

Immunoassays are widely used in therapeutic drug monitoring for humans⁴⁸. The antibody-antigen interaction is highly selective and theoretically enables analytical procedures to be carried out with little or no sample treatment. In simple terms, the assay is performed by bringing the antibody into contact with the analyte and adding an amount of radio-, enzyme, or fluorescently labelled analyte, which competes with the non-labelled analyte for the available binding sites. The amount of labelled analyte is then determined directly or after the addition of suitable substrate that is transformed into a selectively detectable

product. The principles and practice of immunoassay will be discussed in detail in chapter two.

A large number of enzyme-linked immunosorbant assays (ELISA) have been reported for the detection of sulfamethazine in a variety of matrices^{49,50,51,52,53,54,55,56,57,58,59}. Enzyme labels include horseradish peroxidase (HRP), and less commonly, β -galactosidase. Chromogenic substrates commonly used are tetramethylbenzidine (TMB), and 2,2'-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid (ABTS).

A number of ELISA assays for sulfamethazine are commercially available. There are at least four kits on the market for detection covering a variety of matrices; e.g. urine, milk and plasma. Two of these are classical ELISA methods requiring microtitre plates and a spectroscopic reader^{60,61}. The remaining two tests are in the form of a card or cup, and require no further instrumentation^{62,63}.

All these tests are intended for pre-slaughter or pre-milk delivery screening of animals and milk. The limits of detection range from 10 $\mu\text{g/l}$ in milk to 100 $\mu\text{g/l}$ in plasma and 400 $\mu\text{g/l}$ in urine. The latter two limits are supposed to be sufficiently low to ensure that animals negative in the test will not contain violative levels ($>100 \mu\text{g/kg}$) of sulfamethazine in meat. In all instances, only minimal sample handling is required.

Immunochemical tests have been developed for a number of other important veterinary drugs. These include chloramphenicol⁶⁴, the benzimidazoles⁶⁵, the ionophoric coccidiostat monensin⁶⁶, the cephalosporin cephalixin⁶⁷, and the antibiotic colistin⁶⁸. A direct (on-farm) enzymatic assay for β -lactams in milk is commercially available⁶⁹.

The development of biological sensors as selective and simple instruments for the on-site control of drug residues in edible products is an interesting

challenge for analytical chemists. One such study has been reported for the development of a penicillin sensor based on immobilised penicillinase⁷⁰.

1.4 Drug Residue Confirmation

If a screening test indicates the presence of a violative concentration of a drug residue, the next steps are to establish whether the amount of residue exceeds the MRL and to confirm the identity of the residue.

1.4.1 Non-Spectrometric Methods

The identity of antimicrobials found positive in a microbiological screening test is generally confirmed by means of high-voltage electrophoresis. Although this approach combines efficient separation with microbiological detection using different microorganisms, unfortunately neither quantitative nor direct structural information is provided. The combination of liquid-liquid extraction, HPLC and microbiological detection (bioautography) has been proposed as an alternative confirmation technique for antimicrobials^{71,72}. For penicillins, false positive samples can be identified by repeating the test in the presence of a β -lactamase. If the inhibition zone disappears in the presence of β -lactamase, this proves that a penicillin is present. Another indirect confirmation method, which uses a receptor assay (Charm-test), has been discussed above and has been used for the confirmation of sulfonamide residues⁷³. For a growing number of antimicrobials, chromatographic methods are being developed for quantitation and confirmation.

1.4.2 Spectrometric Methods

Confirmation methods should preferably provide direct structural information, which is usually obtained by means of one of two spectroscopic techniques; diode array detection (DAD) or mass spectrometric (MS) detection. In addition, increasing attention is being paid to fourier transform infrared spectroscopy (FT-IR) and nuclear magnetic resonance (NMR) detection.

Nowadays, FT-IR can be coupled on-line with capillary GC. However, it is still mostly used as a standalone technique. That is, identification and quantification are achieved by fraction collection of eluting LC peaks or the

scraping off of TLC spots. The removal of highly interfering/absorbing solvents before FT-IR detection either by deposition or coupling LC and FT-IR on-line via a suitable interface enhances the potential of the technique for trace-level analysis. The use of FT-IR coupled with capillary supercritical fluid chromatography for the confirmation of sulfonamides has been reported^{74,75}.

DAD is frequently used routinely for detection in LC; it mainly provides information on the basis of spectral match of the UV spectrum recorded for the peak of interest and that of the analyte standard, and of the purity of the peak⁶⁴. DAD can be used successfully for confirmation purposes when the sample clean-up has efficiently removed interfering UV-VIS absorbing compounds. Horie *et al.*⁷⁶ have used this approach for the analysis of sulfamethazine and its metabolite N⁴-acetylsulfamethazine (N⁴-AcSMT). The reported limit of detection was 0.02 µg/ml.

There is general agreement about the fact that MS detection provides more structural information at low analyte levels than any other analytical technique. The mass spectrum contains information regarding the mass of parent and fragment ions along with their relative abundance^{77,78}. Selectivity is best when full spectral scans can be acquired to compare sample and standard peaks. MS detection is generally combined with capillary GC separation, which means that in many cases that the analytes of interest have to be derivatised before analysis. This approach has been applied to the GC-MS analysis of sulfamethazine^{79,80,81,82,83,84,85,86} – chemical ionisation is the MS mode most commonly employed. The most frequently used derivatisation of sulfamethazine involves formation of the methyl derivative. Limits of detection range from 0.01 ppm to 0.1 ppm using this method.

In practice, the confirmation procedure is often divided into two steps. Firstly, the sample is analysed by means of a well-established LC method. The amount of analyte is quantified and the fraction of the LC eluate containing the analyte is collected. Next, this fraction is processed to make it suitable for direct injection into a mass spectrometer or for GC-MS. Advantages of this approach over direct GC-MS analysis are: (a) extra clean-up through LC

separation; (b) reliable quantitative information; (c) tentative identification by means of e.g. DAD detection; (d) use of the expensive and vulnerable mass spectrometer in a limited number of cases only i.e. when the MRL is exceeded.

In the past few years, on-line LC-MS has become a popular technique. This is especially due to the improved performance of, e.g. the thermospray (TSP), particle beam (PB), and electrospray (ESP) interfaces. Multi-residue analysis with microbore LC-ESP-MS has the advantage that no post-column splitting of the eluent is required and all of the analyte is transferred to the ESP interface⁸⁷. There are several reports of this technique being applied to the confirmation of sulfamethazine residues^{88,89,90,91}. Today, tandem mass spectrometry, i.e. MS-MS, is also being used for drug residue confirmation. Applications have been reported for the residue analysis of sulfonamides⁹², β -lactams⁸⁷, betamethasone and clenbuterol⁹³. Tandem MS approaches generally provide sub ng/g detection limits, more or less independent of the biological matrix that is analysed.

Recent developments in on-line coupled LC-GC, especially normal phase LC-GC, open the possibility to achieve highly selective and fully automated determination of analytes in, preferably, non-aqueous samples or sample extracts. Until now, most LC-GC and related applications are in the area of environmental and food analysis²⁰.

1.5 Validation Of Results

In official residue control, regulatory action can only be taken after unequivocal identification of contaminated products. The control system should also be able to effectively identify suspected samples in a large population. This implies that the analytical results should be accurate and precise, i.e. agree with the actual situation. This can only be achieved when (1) adequate analytical methods are available, and (2) trained personnel under quality assurance conditions carry out the work.

1.5.1 Adequate Analytical Methods

Whether an analytical method is adequate or not depends upon the purpose for which it is going to be used. A screening method should allow the detection of all the suspected samples, using a relatively simple, routinely applicable procedure. A quantitative method should allow the user to reliably establish whether the residue level exceeds the MRL. Finally, a confirmatory method should give unequivocal evidence on the identity of the residue. Until recently an analytical method was believed to be adequate only after it had successfully been tested in a full collaborative study. Because of the tremendous cost of such studies, the time necessary to complete a test, the rapid progress made in method development and the large number of compounds for which new methods are required, this view has now changed. Within the EC, a group of experts has defined a number of criteria that have to be met by so-called reference methods for drug residue control^{94,95}. These EC reference methods are to be used in case of an international dispute. The criteria include general demands on precision, limit of detection, limit of determination, accuracy, testing for interferences, calibration curves and the relationship between the established MRL and the limits of identification and determination. Besides, a number of identification criteria for chromatographic (TLC, LC, GC), immunochemical, and spectrometric (MS, DAD, IR) techniques have been laid down. These criteria are very useful to establish whether a method has a sound basis and they provide guidelines for the analyst developing non-reference methods. With regard to international regulatory control, the "criteria approach" should result in a set of equivalent reference methods producing comparable results for each (group of) veterinary drug(s), rather than having one method laid down in detail. In 1992 the commission of the EC has issued the first version of a summarising booklet describing EC criteria and candidate reference methods⁹⁶.

1.5.2 Quality Assurance

Working under quality assurance conditions in essence means that there is a guarantee that the analytical method is carried out according to the procedure laid down, and that any deviations are registered and approved of by responsible staff. Actually, no statement is made regarding the quality of the

method, but rather regarding the conditions under which it is carried out. Certification of analytical methods, or even laboratories, by (inter)national accrediting agencies will become highly important in the future European open-market situation. A method will only be certified if a number of internal or external quality assurance measures have been included in the procedure. A number of these are described in Ref.⁶⁴ quoted above for the immunochemical, LC and GC-MS analysis of chloramphenicol residues in meat. Briefly, these measures comprise: (1) inclusion of recovery samples with each series; (2) inclusion of blank samples; (3) inclusion of known (internal) or "blind" (external) samples containing naturally incurred residues (control or reference material); (4) prevention of contamination; (5) determination in duplicate; (6) establishing criteria on the maximum allowable deviation of individual results from the mean recovery, the precision and the mean or certified value of a control/reference material (RM); (7) involvement in quality-control collaborative studies.

With regard to the last aspect, it is clear that there is a need for (inter)national quality-control studies, even if one uses provisionally certified control materials containing one or more drugs. Such studies should aim at the comparison of results obtained by different laboratories using different methods⁹⁷. In addition, certified reference materials (CRM) are needed such as those provided by United States Pharmacology (USP) and the EC, to test the accuracy of the total analytical methods. Biological reference materials are still very scarce and are currently only available for a number of hormones and chloramphenicol. Therefore other quality-control evaluation programmes have been initiated by individual countries to assess the quality of drug residue monitoring. One such programme is the Food Analysis Performance Scheme that has been set up by the United Kingdom Ministry of Agriculture Food and Fisheries and which evaluates samples containing oxytetracycline, sulfamethazine and chloramphenicol⁹⁸.

In summary, it is only the combination of high-quality analytical methods and laboratory quality assurance procedures that can safeguard the quality of the food by identifying contaminated products and prevent false positive results.

Chapter 2

Immunoassays

2. Introduction

Immunoassay is now the fastest-growing analytical technology in use for detection and quantification of biomolecules. Along with the various chromatographic techniques, immunoassay is probably the most commonly used technology for the analysis of biomolecules; each technique, of course, has its individual merits. Thus although chromatographic techniques can detect and quantify a family of compounds, the immunoassay is generally intended for the specific determination of a single molecular species. It may also be argued that chromatographic techniques are capable of greater discrimination between chemical structures, and they are often used as the analytical tool that precedes the development of an immunoassay for a new diagnostic marker. A review of the technology available shows that immunoassays can be developed that use less complex equipment, allowing them to be used in a wider range of testing environments. Thus, immunological and chromatographic assays should be considered as complementary techniques.

Ultimately, all developments in immunoassay stem from the report of the first immunoassay by Yalow and Berson in 1959⁹⁹. Their immunoassay was for insulin, employed a radiolabelled form of the hormone, and was able to detect some 10 to 40 pg of insulin. This radioimmunoassay (RIA) doubtless appeared as an esoteric research tool in 1959, but such assays were rapidly transformed into routine quantitative methods for many analytes and because such RIAs provided superior sensitivity, greater specificity and higher throughput than traditional methods of analysis, they gained rapid and widespread acceptance among clinical chemists.

In contrast, food scientists were slow to adopt and adapt such assays. Publications in this field were slow to appear, the total reaching only seven by 1973¹⁰⁰. Several reasons can be suggested for this slow transfer of technology:

(a) conservatism among food analysts;

- (b) high overall initial cost because of the need for special equipment, special facilities for the handling and disposal of radioisotopes, and training of personnel; and in particular:
- (c) difficulties in dealing with solid samples;
- (d) recognition of lay concern, not to say hysteria, at the thought of radioactivity in close proximity to human food.

The means to circumvent the last two objections, and thus potentially the first objection also, was provided in 1971 with simultaneous reports of a major development^{101,102}. It was demonstrated that radiolabels could be replaced with enzyme labels and thus the radioisotopes and their attendant problems could be dispensed with once the enzyme immunoassay (EIA) had been developed.

2.1 The Antigen-Antibody Reaction

An immunoassay is a quantitative technique that depends on the reaction between the molecule of interest, 'the antigen', and a complementary molecule, 'the antibody'. The antigen is so called because it is a molecule capable of eliciting an immune response when injected into an animal (in which it is treated as a foreign species). The nature of the immune response and the production of antibodies are discussed in section 2.3.

The antigen can be a small molecule (hapten), such as sulfamethazine (M_r 278), or it can be a large protein such as albumin (M_r 66,000). However, there is only one portion of the antigen that binds to the antibody; the *epitope* of the antigen binds with the *paratope* of the antibody. An antigen may contain several epitopes, but only be capable of binding one antibody molecule. The remaining surface of the molecule being unavailable for binding because of steric hindrance of the first antibody molecule; thus a small molecule may consist of a series of overlapping epitopes, although only one can function at a time. On the other hand, a large biomolecule will contain many epitopes and indeed copies of the same epitope. Furthermore a large biomolecule will have many independent epitopes; i.e. it is capable of binding several antibody molecules at the same time.

2.2 Structure Of Immunoglobulins

Most immunoglobulins are found in the fraction of serum known the gamma globulin fraction (on the basis of their electrophoretic mobility). All immunoglobulin molecules are heterodimers with a similar architecture consisting of four polypeptide chains linked by disulphide bridges: two identical large or heavy (H) chains of about 450-600 amino acid residues and two small or light (L) chains of about 220 residues. Sequence analysis of H and L chains and X-ray crystallography shows that each chain consists of homologous segments of about 110 amino acids that form independently folded domains. H and L chains all contain a variable region and a constant region, which are encoded by separate gene segments. The amino-terminal domain of both H and L chains differs in antibodies of different specificity and are called variable (V) regions. Within each V region, three segments exhibit hypervariability and constitute the complementary-determining regions (CDR) of the immunoglobulin. The six CDRs form hypervariable loops at the ends of the two variable domains, which are labelled L1, L2 and L3 (L chain) and H1, H2 and H3 (H chain), respectively.

The remaining domains in each chain are invariant and are called constant (C) regions. In the H chain of IgG, there are three constant domains called C_H^1 , C_H^2 and C_H^3 . Between the C_H^1 and C_H^2 domains there is an additional segment termed the hinge which contains the inter-heavy chain disulphide bridges and which offers a certain amount of flexibility on the molecule. The L chains contain a single constant domain called C_L .

All immunoglobulins can be cleaved at the middle of their H chains by various proteases. Papain cleaves γ -chains at the N-terminal side of the disulphide bridges that keep the H-chains together, thereby generating two F_{ab} (fragment antigen binding) fragments and one F_c (fragment crystallisable) fragment (Figure 2.2). Each F_{ab} fragment contains one of two identical combining sites of the immunoglobulin.

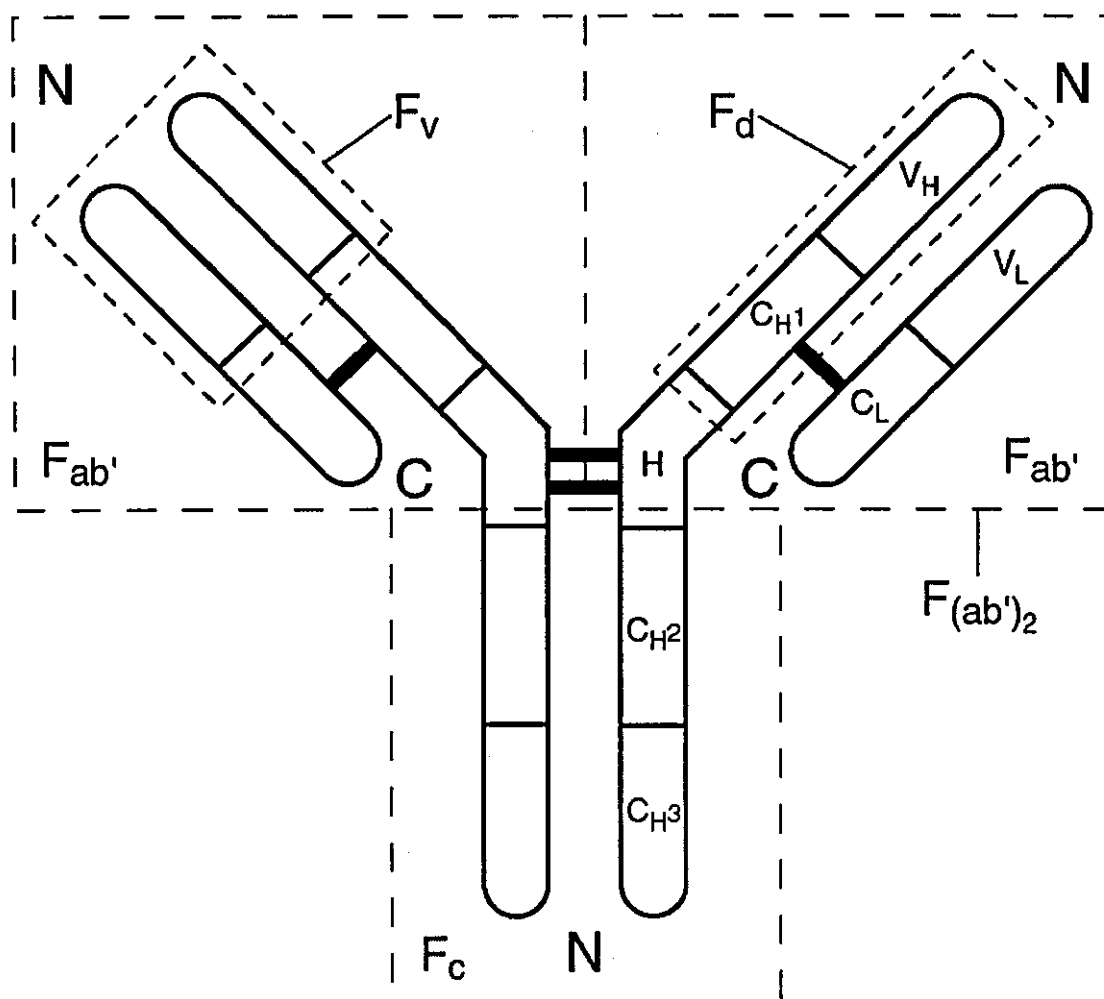


Figure 2.1 Schematic representation of an antibody molecule (human subclass IgG) showing carboxyl (C) and amino (N) termini.

Pepsin cleaves γ -chains at the C-terminal side of the disulphide bridges and generates a single bivalent $F(ab')_2$ fragment containing two combining sites, as well as several smaller fragments of the C_H^2 and C_H^3 domains.

2.3 Production And Purification Of Antibodies

In the immune system, a single B lymphocyte produces a single type of antibody molecule. In a typical immune response to an antigen, e.g. a foreign protein, many B-lymphocytes produce many different antibody molecules all directed to different parts of the antigen. These areas of recognition on the antigen are known as epitopes. Such an antibody population is, thus, considered polyclonal. Monoclonal antibodies, on the other hand, are specific for one epitope and are produced by cell fusion techniques.

2.3.1 Polyclonal Antibodies

Polyclonal antibodies are useful reagents for a variety of immunochemical applications including immunohistochemistry, western blots and many immunoassays. Polyclonal antibodies offer the advantage of a simpler method of production with the disadvantage of the heterogeneous and often crossreactive binding properties of the antisera.

Animal	Serum volume (ml)	Comments
Rabbits	500	Good choice for polyclonal antibody Production even with limited antigen.
Mice	2	Low serum volume. Many inbred strains available.
Rats	20	Some inbred strains available.
Hamsters	20	Good choice for polyclonal antibody production when antigen is limited.
Guinea Pigs	30	Hard to bleed.
Chickens	50	Good for highly conserved mammalian species.
Goats	litres	High serum volume. Multiple bleeds with additional antigen boosts.

Table 2.1 Polyclonal antisera production.

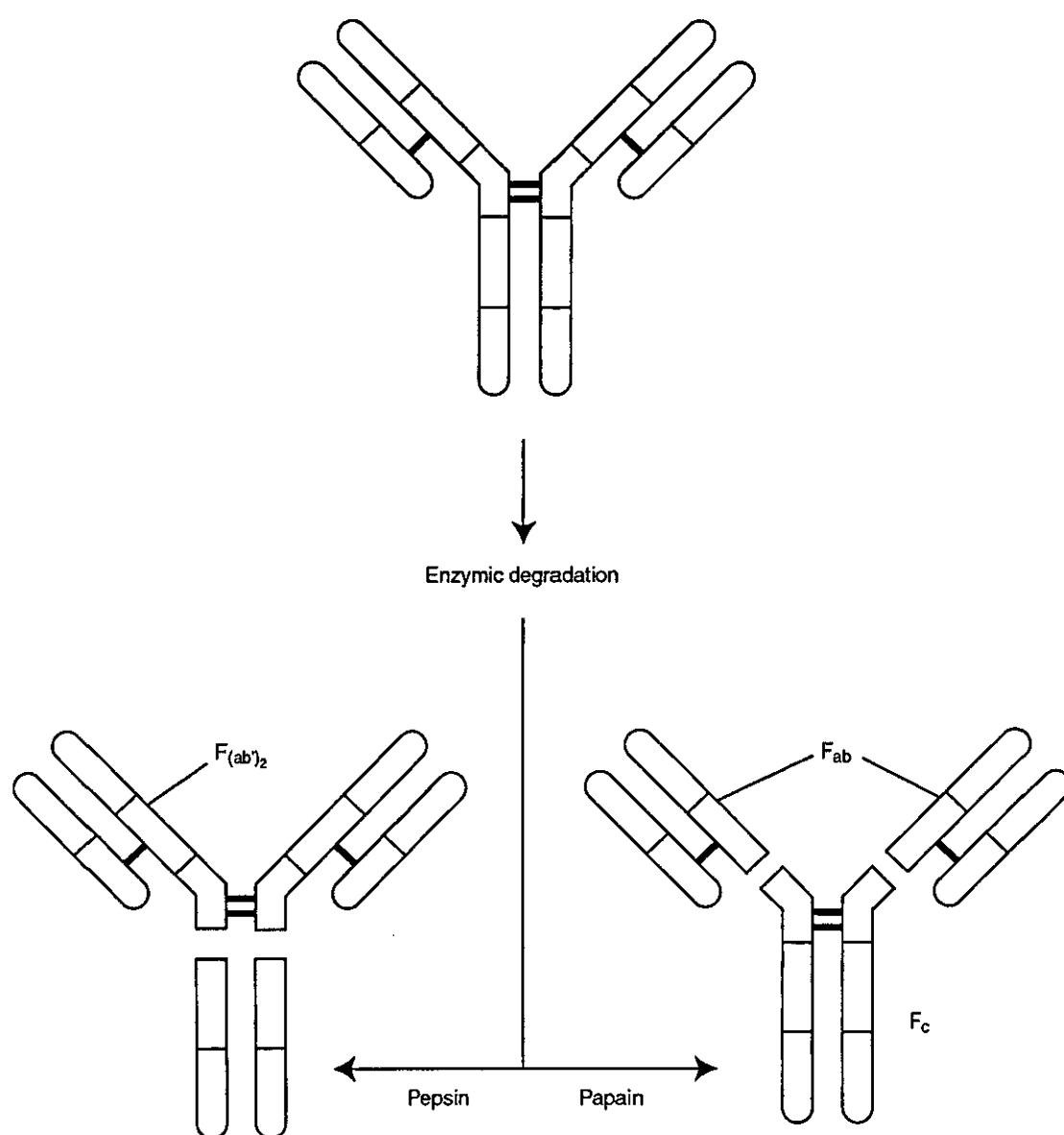


Figure 2.2 Generation of antibody fragments.

Polyclonal antisera can be made in a variety of animals. Immunisation of different species of animals provides the possibility of generating antibodies with different reactivity profiles based on the differences in B cell repertoires in the different species. Genetically in-bred strains of animals are available for rodent and rabbit species that can provide a potentially more predictable immune response to a given antigen. The choice of carrier protein, dose and route of immunisation and choice of adjuvant all need to be considered for effective polyclonal antibody production.

One procedure for the production of polyclonal antisera in goats involves injection by either intramuscular or subcutaneous routes of 25-200 μg of antigen emulsified in complete Freund's adjuvant (CFA) on day 0 and day 30. This is followed by another injection of antigen on days 60 and 90. At 9 to 11 days after the last injection, the animal is bled and the serum titre determined for the antigen in question. Additional boosts can be made on a monthly basis. Antiserum is collected by plasmapheresis 9 to 11 days after the boost.

2.3.2 Monoclonal Antibodies

For the production of monoclonal antibodies in, for example, mice, the animal must first be immunised with the antigen of interest to provoke its immune system into producing antibodies to the antigen. The mouse spleen is then removed. This contains a high concentration of B-lymphocytes (splenocytes) which can produce the desired antibody. The spleen cells are then fused with a suitable non-antibody producing myeloma cell with polyethylene glycol (PEG). This induces the membranes of both cells to merge. Following fusion, what remains is mixed cell population of hybridomas and unfused splenocytes and myelomas. The splenocytes die off after a short period of growth. Unfused myeloma cells are removed using a selective growth medium called HAT (hypoxanthine, aminopterin, and thymidine). What remains is a population of fused hybridoma cells.

The fusion mixture can be conveniently divided into many culture wells. The fused hybridomas are then allowed to grow. Periodically, supernatant from the wells can be analysed for the presence of antibody to the desired antigen

using a suitable assay system, usually an enzyme immunoassay. Positive wells can then be further divided and eventually some wells will contain clones of hybridomas derived from a single parent cell, which produces a single antibody type. Large-scale propagation can take place *in vitro* or *in vivo*. This process is shown schematically in Figure 2.3.

2.4 Classification Of Immunoassays

Immunoassays can be classified according to a range of criteria including sample type, nature of analyte, assay conditions etc., (Gosling¹⁰³). The majority of approaches have been described for the quantification of antigens, although most are equally applicable in the case of assays for large molecules, for antibodies.

The three major criteria for classification that have the greatest influence on the performance that can be expected of an assay with regard to precision and sensitivity are: (1) the use of a limited or excess reagent format; (2) the use of a homogeneous or heterogeneous format and (3) the use of a label or unlabelled assay format and then the choice of label. The accuracy of an assay will primarily, but not solely, depend on the characteristics of the complementary molecule (i.e. antibody in the case of an assay for antigen). The influences of factors other than the antibody are listed in Table 2.2.

2.4.1 Limited Reagent

In this assay format for determining the amount of antigen present, a limited amount of antibody is used (the so called 'limited reagent') which is insufficient to bind all of the antigen. A fixed amount of labelled antigen competes with the unlabelled antigen (from the sample) for the limited number of antibody binding sites. The concentration of unlabelled antigen can be determined from the proportion of labelled antigen that is bound to the antibody (or remains free). This principle is illustrated in Figure 2.4.

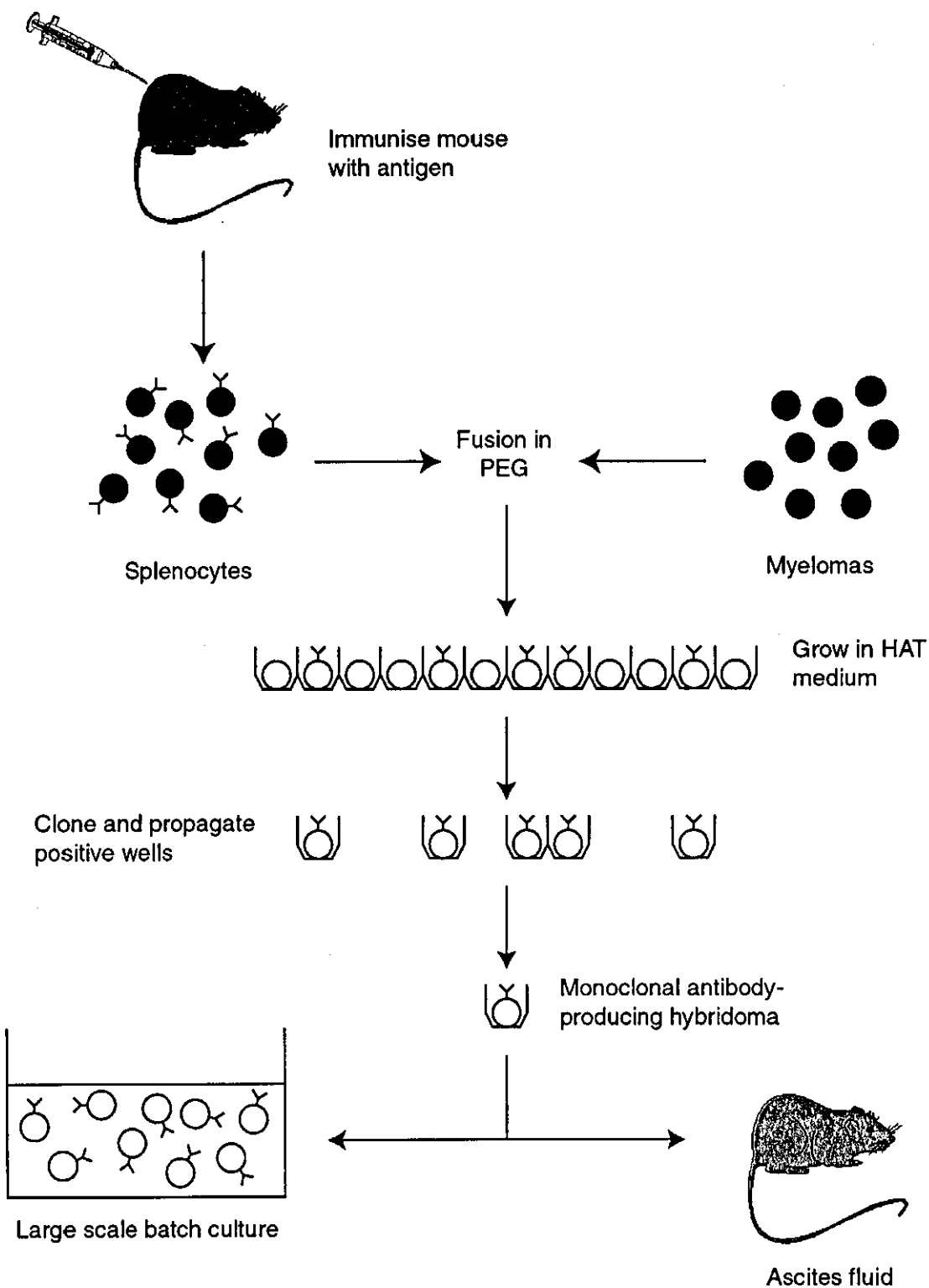


Figure 2.3 Production of monoclonal antibodies from the mouse.

Sample	
Nature of antigen	Size
	Polarity
Matrix	Viscosity
	Presence of binding proteins
	Presence of unusual antibodies
	Presence of cross-reacting species (e.g. metabolites)
	Interference with label
Reagent	Immunoassay buffer pH, ionic strength
	Polymer enhancers
	Detergents
	Preservatives
Reaction environment	Temperature
	Mixing
	Sequence and speed of addition of reagents

Table 2.2 Factors that influence the design and performance of an immunoassay.

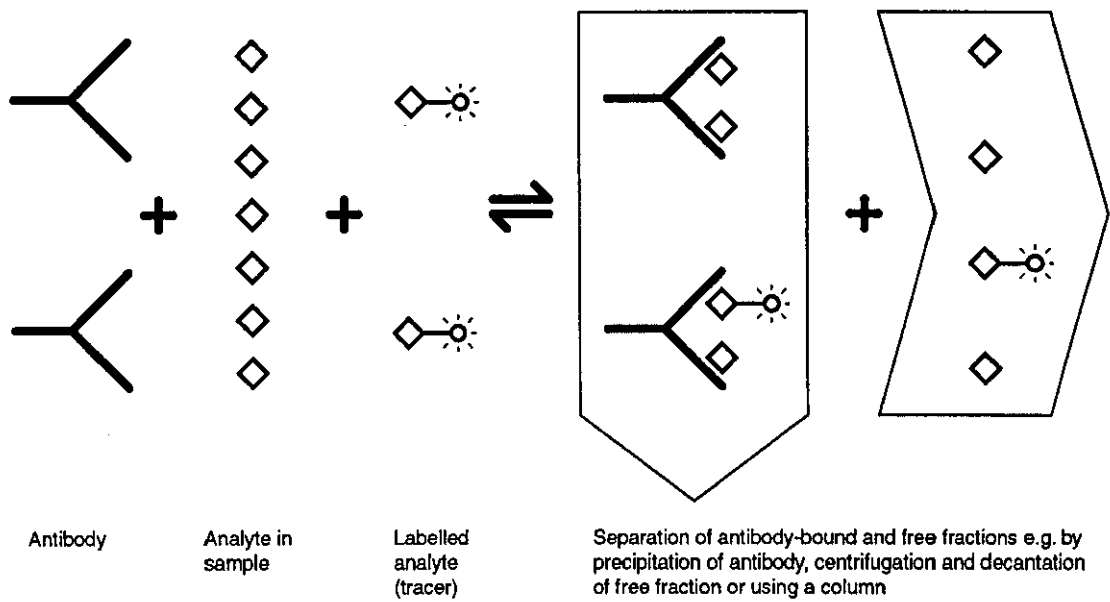


Figure 2.4 Limited reagent (competitive) immunoassay.

Clearly it is important that the amounts of labelled antigen and antibody are kept constant, so that the amount of bound (or free) labelled antigen can be compared with a series of calibrators to obtain a quantitative result.

2.4.2 Excess Reagent

In this assay format, antigen binds to an excess of antibody; a variety of approaches have been developed to detect the bound antigen. The most common is the two-site immunometric assay (the so-called 'sandwich' assay); in this approach, the first antibody in excess is coupled to a solid phase. The bound antigen is then detected with a second antibody labelled in a way that aids detection (e.g. by use of an enzyme, fluorophore etc.). In this instance, the amount of labelled antibody captured on the solid phase (i.e. forming the sandwich) is directly proportional to the amount of antigen in the sample. This is illustrated in Figure 2.5. Another approach to the reagent excess assay involves the separation of the antibody-bound labelled analyte and free antibody using an antigen-bound solid phase. This is illustrated in Figure 2.6.

The delineation into 'limited' or 'excess' reagent assay has important practical consequences. First, the response in a limited reagent assay is highly dependant on the amount of labelled antigen and antibody present, as well as sample antigen concentration; therefore sample and reagent metering have an important bearing on assay precision. Second, and of more positive benefit, is the fact that a situation of antigen excess never arises because, as the amount of sample antigen increases, the amount of bound labelled antigen diminishes towards zero. Third, in a limited reagent competitive assay the equilibrium constant is of vital importance because the sensitivity of the assay is determined by its value.

Conversely, in a situation where there is an 'excess' of reagent antibody, metering of reagent is less critical. However, the situation of antigen excess has finite limits and, when it occurs, leads to a 'hook effect'. When antibody reagents are added simultaneously; above a certain sample concentration the response is no longer proportional to concentration and indeed falls, with the

consequence that a particular response may be given for two different sample concentrations.

2.4.3 *Heterogeneous And Homogeneous Immunoassays*

The use of either competitive or immunometric assays requires differentiation of bound from free label. This can be achieved either: (1) by separating bound from free label using a means of removing the antibody (heterogeneous assay) or (2) modulation of the signal of the label when antigen is bound to antibody compared to when it is free (homogeneous assay).

2.4.3.1 Heterogeneous

There are many ways of separating bound from free label. These can briefly be described in terms of (1) precipitation of antibody; (2) coupling of antibody to a solid phase; and (3) chromatographic techniques. In each case, the performance is governed by (1) the completeness of the separation; (2) the efficiency of the washing to remove any unbound label; and (3) the level of non-specific binding.

2.4.3.2 Homogeneous

The need to separate free from bound label constitutes another analytical step (two more steps if you include washing), which can influence method precision. Furthermore, it has proved a challenge to automation of immunoassays and is probably one of the main stimuli for what has been a burgeoning development in homogeneous immunoassays over the past two decades.

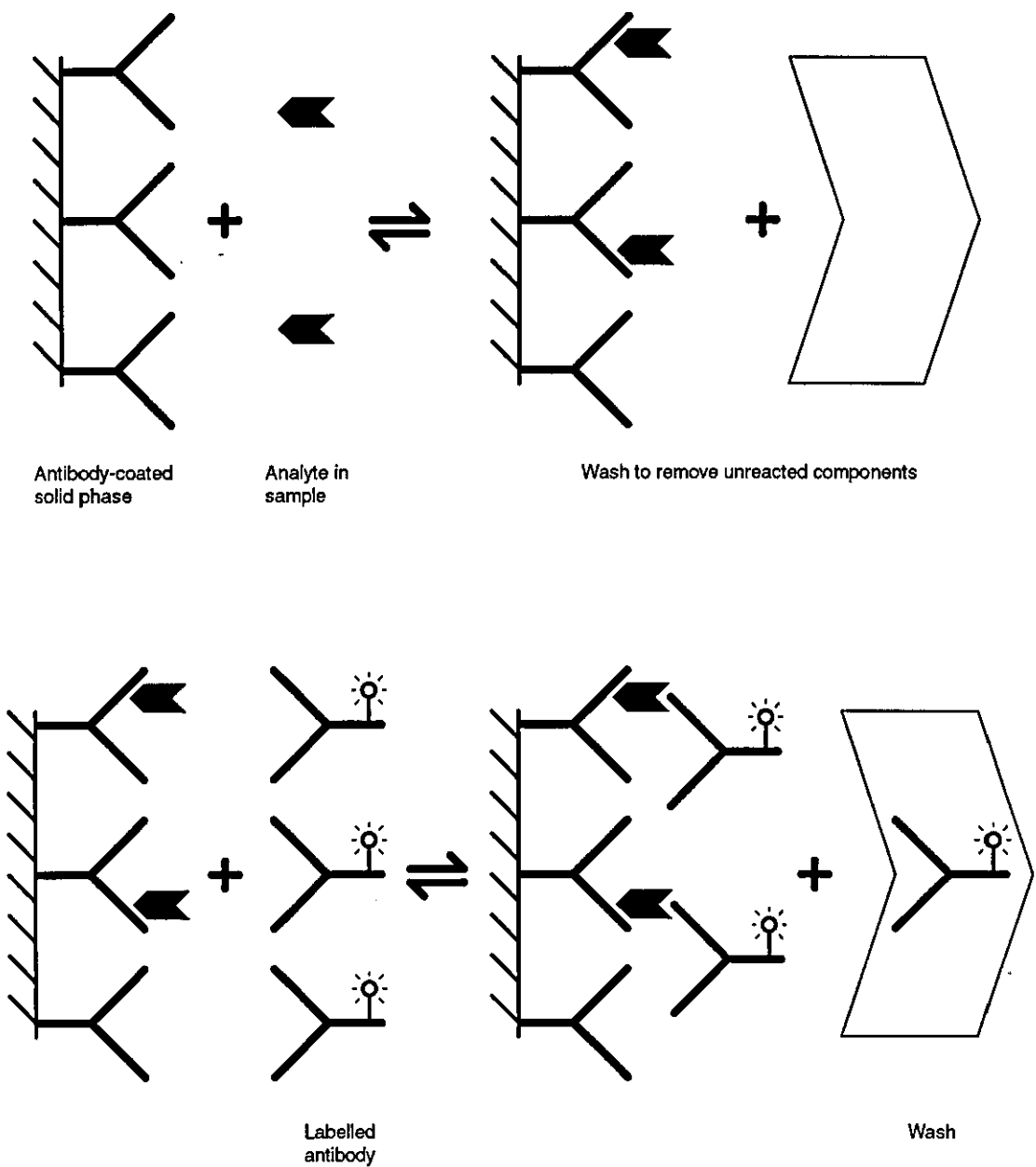


Figure 2.5 Two-site immunometric assay ('sandwich' assay).

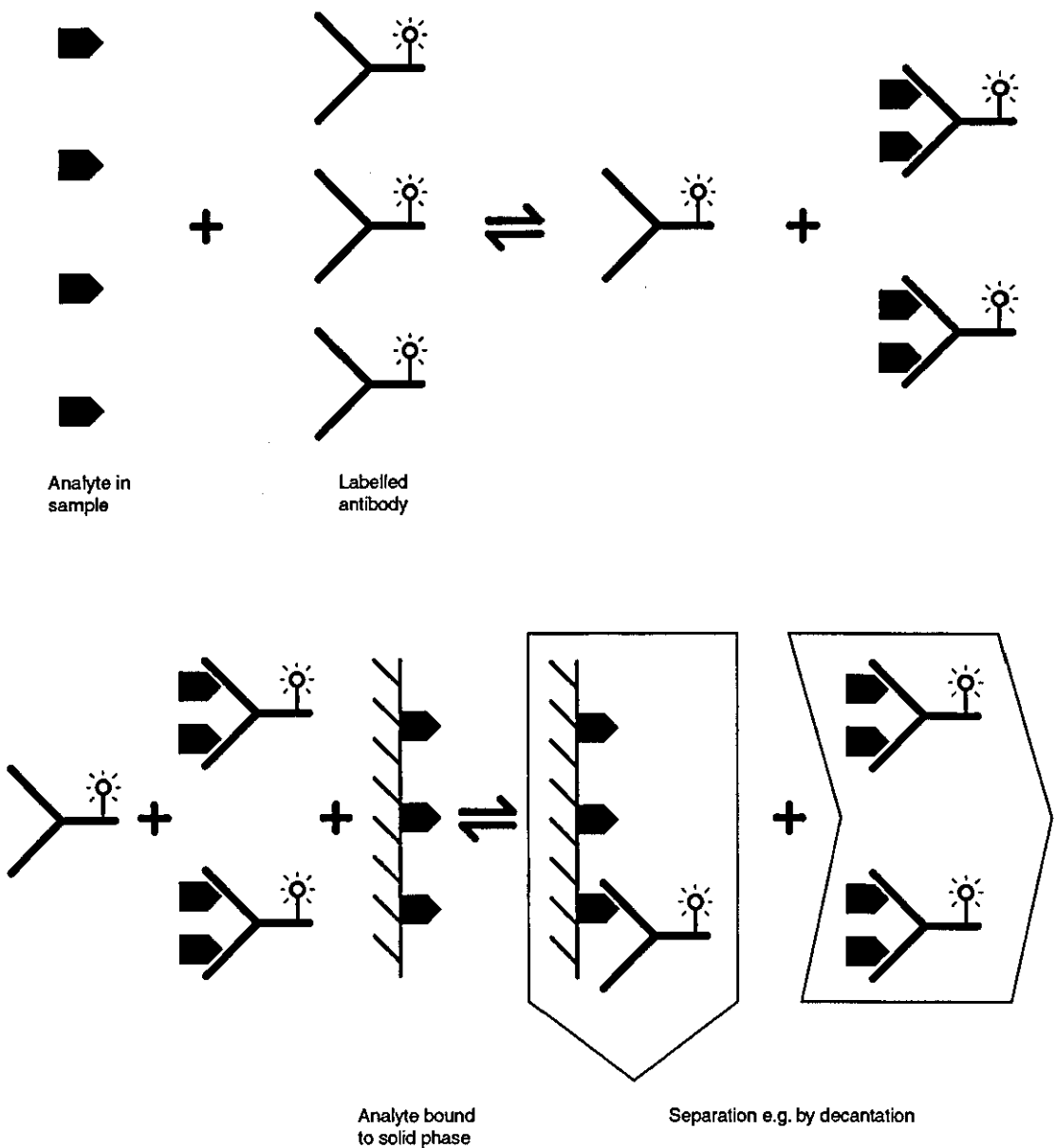


Figure 2.6 Antibody excess immunometric assay.

2.5 Methods Of Detection In Immunoassay

Quantification of the antigen-antibody reaction can be by visual assessment or using a variety of instrumentation. The choice of immunoassay format (limited or excess reagent), label and instrumentation can all influence the characteristics of the assay with respect to detection limits, precision and analytical range¹⁰⁴. A summary of the choice of labels and instrumentation used are given in Table 2.3 and Table 2.4. The most commonly utilised immunoassay detection techniques (radio, enzyme, electrochemical and fluorescence labelling) are overviewed later in this section.

Radioisotope, e.g. ^{125}I , ^{14}C
Enzyme, e.g. alkaline phosphatase,
Enzyme cofactor, e.g. flavine adenine dinucleotide
Enzyme substrate, e.g. galactosyl umbelliferone
Fluorophore, e.g. fluorescein
Luminescent species, e.g. acridinium ester, luminol
Particle, e.g. latex, carbon sol
Metal ion, e.g. Au^{3+}
Ionophore, e.g. valinomycin

Table 2.3 Choice of labels in immunoassay.

Colorimetry	Sensors
Fluorimetry	Potentiometric
Phosphorescence	Amperometric
Luminescence	Piezoelectric
Turbidimetry	Surface plasmon resonance
Nephelometry	Ellipsometry
Photon correlation	Total internal reflection
Photothermal	
Isotope counting	
Neutron activation	
Atomic absorption	

Table 2.4 Methods of detection used in immunoassay.

2.5.1 Immunoassays Without Labels

In this type of assay, antigen molecules react with antibody molecules to form an immunoaggregate that can be detected by its turbidity. In its simplest form, it is only applicable to larger molecules. Effectively, the assay can be considered as a direct modulation homogeneous immunoassay, with the antibody acting as its own label. Greater sensitivity can be achieved by further labelling antibody with a particle.

2.5.2 Radio-Immunoassay

Although most current developments have been in the area of nonisotopic immunoassays, methods based on radiolabels still make up a significant portion of the routine immunoassays currently in use. The techniques that are included in this group include the competitive binding RIA, the immunoradiometric assay (IRMA), and the scintillation proximity assay (SPA).

The first labels used were the radioisotopes, ^{131}I and then ^{125}I , which were used to label the protein and polypeptide hormones. As the technique was extended to the measurement of steroids and drugs, which could not be iodinated directly, tritium- (^3H) and ^{14}C -labelled antigens were used in their place. The use of radioisotopes as labels has a number of advantages over other labelling techniques used in immunoassay. These are shown in Table 2.5.

Small size	Small size means minimal disruption to the biological properties of the molecule they are attached to.
High sensitivity and selectivity	In the range of 10^{-12} to 10^{-17} mol.
Wide applicability	Radioimmunoassays have been developed for a wide range of compounds, for example, steroid hormones ¹⁰⁵ and drugs ¹⁰⁶ .

Table 2.5 Advantages of radioisotopes as labels in immunoassay.

Radioimmunoassays do, however, suffer from several disadvantages. Radioisotopically labelled antigens do have limited stability. Whereas ^{125}I -labelled compounds have short shelf lives, mainly due to the half-life of the isotope (60 days), antigens labelled with ^3H or ^{14}C are often chemically

unstable and also need replacing at intervals of 6-13 months. The cost of radiolabelled antigens is therefore relatively high and the capital cost of radioactivity counting instruments as well as of scintillation cocktails should also be borne in mind. Although biological samples do not interfere with the radioactive measurements, the signal cannot be modified and therefore a separation phase is required. Automation of RIAs has therefore presented some difficulties. A number of alternatives to radioactive labels have been investigated, and the most commonly employed are discussed in the following sections.

2.5.3 Enzyme Immunoassay

In 1971, enzymes were first introduced as alternatives to radioisotopes in immunoassays¹⁰², and they have since become the most versatile and popular class of labelling substance for nonisotopic immunoassays. Enzyme-immunoassays have been the primary subject of quite a large number of books (Tijssen¹⁰⁷, 1985; Kemeny and Challcombe¹⁰⁸, 1988; Ternynck and Avrameas¹⁰⁹, 1988; Wreghitt and Morgan-Capner¹¹⁰, 1990; Crowther¹¹¹, 1995) and reviews (Oellerich¹¹², 1984; Ishikawa¹¹³, 1987; Ishikawa *et al.*¹¹⁴, 1989; Ngo¹¹⁵, 1991), and an important concern in many others.

Enzymes are biological catalysts; by lowering the activation energy they accelerate chemical reactions without themselves being changed in the process. Almost all enzymes are high molecular mass proteins or glycoproteins and the part of the enzyme that interacts with the reactants is called the active site. Like all true catalysts, enzymes do not affect the state of equilibrium of a reaction; they just permit equilibrium to be attained more rapidly.

The use of enzyme-labelled reagents for immunoassays has provided the most convenient means by which the sensitivity and specificity of RIA can be more generally applied. The reagents used are those commonly employed in general laboratories and are thus not associated with special hazards: the enzymes used for labelling are cheap and may be stored for in excess of one year at 4 °C, or at room temperature when freeze-dried. Similarly, the

equipment required for enzyme immunoassay (EIA) is common to the majority of the laboratories and requires only the availability of accurate pipettes and dispensers, and a colourimeter or spectrophotometer. End-point determination, i.e. the quantification of colour or fluorescence, need not be rate limiting. Microtitre plate readers enable automatic optical density determination at approximately one per second and microprocessor interfacing is common for data reduction.

Enzyme immunoassays have been extensively used to monitor drugs given to food producing animals. Several papers for the detection of drug residues (including sulfamethazine) in swine and other food producing animals were reviewed in section 1.3.2.

2.5.4 Electrochemical Immunoassay

Electrochemistry offers a convenient route to the detection of a wide range of organic and inorganic species. It offers substantially simplified transduction mechanisms that rely on the direct intrinsic redox behaviour either of an analyte species or of some reporter molecule. Instrumentation can also be considerably simpler than, say, for spectrophotometric analysis, with the measurement of either a simple current or a voltage change. Potentially, a direct, analyte-responsive probe can be made that demands the minimum in sample preparation and is capable of operating in optically opaque media, readily miniaturisable and ultimately amenable to mass fabrication. Although these are the proposed advantages of biosensors generally¹¹⁶, they have actually been realised in only a few cases.

Applications of electrochemical immunosensors to environmental monitoring have been reviewed in ref.¹¹⁷. Compounds for which electrochemical immunoassays have been developed include phenytoin¹¹⁸, the anti-asthmatic theophylline¹¹⁹ and the pesticide 2,4-dichlorophenoxyacetic acid¹²⁰. Ivnitski *et al.* have reported the development of a one-step, separation free amperometric enzyme immunosensor¹²¹. A competitive, separation flow-injection immunoassay has been developed by Stiene *et al.*¹²² to detect African Swine Fever Virus. The detection was based on the competition

between a peroxidase labelled monoclonal antibody and the specific pig polyclonals for the biotinylated virus protein.

2.5.5 Fluoroimmunoassay

Antibodies labelled with fluorophores have been used since 1941 in histological immunofixation techniques¹²³, but immunoassay applications have taken longer to develop. Reports by O'Donnell *et al.*¹²⁴ and Bright¹²⁵ outline the usefulness of fluorophores as labels in immunoassay. Over the past 15 years, the introduction of fluorescent probes into heterogeneous immunoassay systems has provided some alternative methods for analytes such as drugs or proteins that are present in relatively high concentrations in biological fluids. Detection limits for these methods can be high because of background fluorescence and quenching problems, however, novel approaches to assay design and the use of labels which fluoresce in the near-infrared region (700-1000 nm) provide a route to assays with excellent sensitivity (10^{-12} M is easily possible using laser diode excitation).

The use of time-resolved fluorescence techniques using europium chelates and specialised counting equipment has provided methods that are viable alternatives to existing RIA and IRMA methods. This system has been commercialised under the acronym DELFIA (dissociation-enhanced lanthanide fluorescence immunoassay). DELFIA type assays have found widespread application in analysis of foods. Recent methods include assays for androstenedione¹²⁶, clenbuterol¹²⁷, aflatoxin B1¹²⁸ and medroxyprogesterone¹²⁹. Detection limits as low as 10 pg/ml have been reported¹²⁷. Kakabakos *et al.* have reported the development of an assay which can determine four compounds (lutropin, follitropin, choriogonadotropin and prolactin) simultaneously using specially distinct fluorescent areas quantified by laser-excited solid-phase time-resolved fluorimetry¹³⁰.

Polarisation fluoroimmunoassays have also gained in popularity due to ease of automation and the availability of commercial analysers such as the Abbott TDx^{131,132,133}. Plowman *et al.*¹³⁴ have reported an evanescent

fluoroimmunosensor which can detect femtomolar concentrations of clinical markers such as human chorionic gonadatropin (hCG) and creatine kinase.

Liposomes have been used as signal amplifiers in fluoroimmunoassay^{135,136,137,138}. They carry the components for detection, such as fluorophores. Liposomes can be disrupted by complete lysis and in this way, considerable signal amplification can be achieved.

2.6 Flow-Injection Analysis

The seeds for the development of flow-injection analysis were present by the end of 1959. The concept of a sample being injected into a continuously flowing stream with continuous recording downstream is inherent in the basic concepts of gas chromatography as developed by James and Martin in 1952¹³⁹. The concept of injecting the sample into the stream with a hypodermic was already established by 1959¹⁴⁰. The development in 1957 by Skeggs¹⁴¹ of an automated technique for analysis of discrete samples by use of segmented continuously flowing streams was a key development in the early history of flow-injection analysis.

In 1974, Stewart *et al.* first described the automated analysis of discrete samples with unsegmented continuously flowing streams¹⁴². By 1975 there was a considerable increase in the reports on flow-injection analysis. Ruzicka and Hansen published the first in their classic series of papers on flow-injection analysis¹⁴³. In this paper they described their concepts of unsegmented continuous flow analysers as an adjunct to the segmented continuously flowing systems. In the same year Beecher *et al.* published the first detailed description of their automated system for the analysis of discrete enzyme samples by use of unsegmented continuously flowing streams¹⁴⁴.

There have been numerous reviews published since this early work^{145,146,147,148,149,150,151}. Of particular interest is a review published in 1995 by Lopez-Fernandez *et al.* on the assessment of the quality of flow-injection methods used in food analysis¹⁵². This review evaluates over 200 reported flow-injection methods for food analysis in terms of accuracy, applicability,

precision, selectivity, sensitivity, determination range and sample throughput. A quality scale is proposed to enable the most appropriate method for a given type of food analysis, to be selected easily by the analyst.

A basic flow-injection system consists of a two-channel pump, an injection valve, a reactor and a detector (Figure 2.7). The reagent is added continuously to the carrier stream (top) which allows the injected sample zone to be merged with it (centre). The resulting reaction product forms a concentration gradient corresponding to the concentration of analyte throughout the entire sample zone length. Typical flow rates are 1 ml/min, sample volume 50 μ l, residence time 30 s and a sampling frequency of 2 samples per minute.

It is not intended to give a detailed description of all the technical factors affecting sample dispersion in a flow-injection system, as these have been covered elsewhere¹⁵³. Briefly, they are:

Factor	Ref.
Flow rate	154,155
Tube length	154,155,156
Sample volume	154,155,155
Reactors	157,158,159
Temperature	160,161,162
Multiline system	163,164,165,166

Table 2.6 Instrumental factors affecting performance in flow-injection systems.

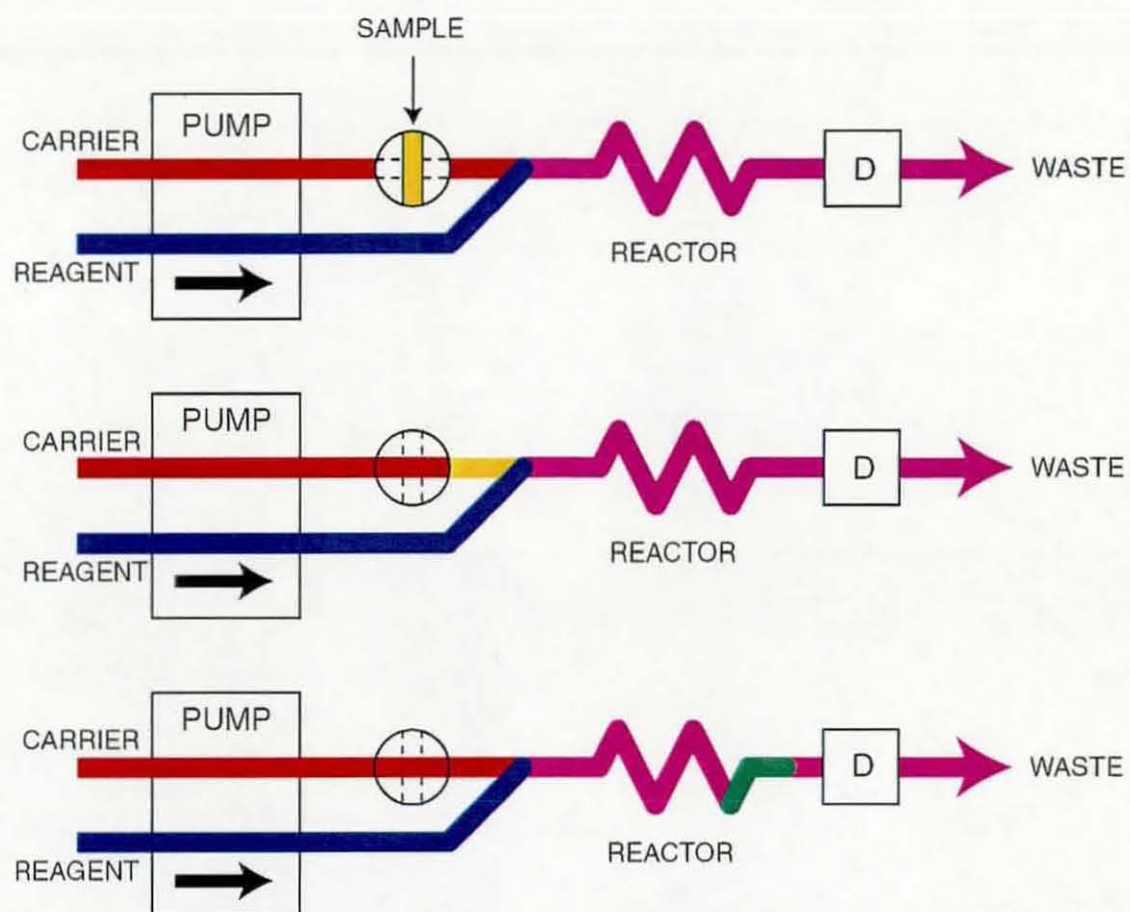


Figure 2.7 A simple two channel flow-injection system.

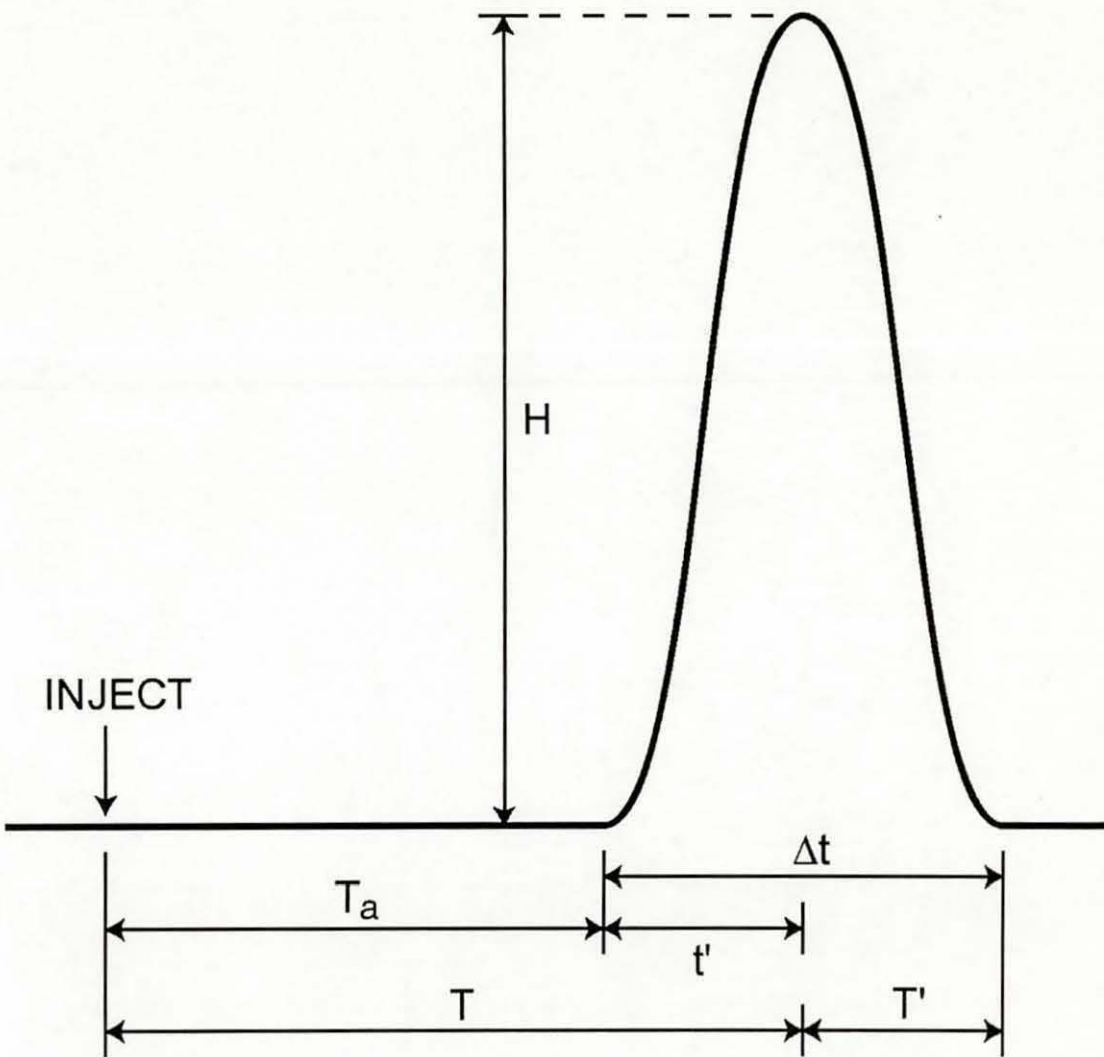


Figure 2.8 FIA signal.

The resulting detector signal can be described as in Table 2.7.

<i>H</i>	Peak height (related to concentration of sample)
<i>A</i>	Peak area (related to concentration of sample)
<i>T</i>	Residence time. The time elapsed from injection to maximum signal.
<i>T_a</i>	Travel time. The period elapsed between injection and start of the signal ($t' = T - T_a$)
<i>T'</i>	Return time. The period between the appearance of maximum signal and the return to the baseline
Δt	The interval between the start of the signal and its return to the baseline

Table 2.7 Essential features of a flow-injection detector response.

2.6.1 Affinity Chromatography

Affinity chromatography is a type of adsorption chromatography in which the molecule to be purified is specifically and reversibly adsorbed by a complementary binding ligand immobilised onto an insoluble support matrix. The immobilised ligand retains its specific binding affinity for the substance of interest and it is available for selective desorption of the bound substances. After washing away the non-interacting material, the desired molecule is eluted by adjusting the elution conditions, e.g. by changing the pH or ionic strength. In heterogeneous flow-injection immunoassays, affinity chromatography is used in the form of a solid phase reactor to separate antibody-analyte complexes from unbound analyte. Immobilised protein A and thiophilic gels are commonly used in the affinity chromatography of antibodies. The application of affinity chromatography in flow-injection immunoassay is discussed below.

2.6.2 Flow-Injection Immunoassay

The wide success of flow-injection analysis is due to (1) its extremely high flexibility in adapting most chemical and biochemical reaction procedures; (2) its compatibility with virtually any detection method; and (3) its reliability in low volume, rapid experiments. It is not surprising that the advantages inherent to FIA also have led to a large number of applications in the field of life sciences¹⁶⁷. The most common biochemical reactions monitored by FIA are enzymatic assays, where the selective reagent is either in solution or immobilised in open tubular or packed reactors¹⁶⁸. However, the concept of an antibody-supported flow-injection system has received relatively little attention. Only recently has the advantage of the specificity inherent to most immune reactions been exploited in FIA procedures. Some examples are given in Table 2.8. There have been several reviews published on the subject of flow-injection immunoassay^{169,170,171}.

Analyte	Detection method	Ref.
Atrazine	Enzyme	172
α -Fetoprotein, Insulin and 17- α -Hydroxyprogesterone	Enzyme/chemiluminescence	173
Theophylline	Enzyme/electrochemical	174
α -(Difluoromethyl)ornithine	Enzyme/fluorescence	175
Serum IgG	Enzyme/fluorescence	176
Testosterone	Fluorescence	177
Mouse IgG1	Fluorescence	178
Albumin	Fluorescence	179
Theophylline	Fluorescence	180
Mouse antiovine IgG	HRP/Chemiluminescence	181
Polychlorinated biphenyls	HRP/Electrochemical	182
Atrazine	HRP/H ₂ O ₂	183
Theophylline	Liposomes	137, 184
Human IgG	Precipitation	185

Table 2.8 Examples of flow-injection immunoassays.

Both homogeneous and heterogeneous flow-injection immunoassays (FIAs) have been developed. The simplest FIA would be of the homogeneous format. Here, samples and reagents are mixed simply by the merging of two streams or by injection of the sample into a flowing reagent stream.

Although homogeneous assays are very interesting, heterogeneous formats are more straightforward to develop and offer many advantages. These assays are inherently more sensitive, especially for large proteins, they are less prone to interferences and are extremely flexible as to the choice of detection principle and solid phase. Figure 2.9 shows the instrumentation set-up for a simple dual stream FIA system. There are five basic parts:

- A pump – to propel buffer and reagents through the system.
- An injection valve – consisting of a loop, enabling a fixed volume of a sample to be injected into the flowing stream.
- A switching valve – used to control, which buffer flows through the immunoreactor.

- An immunoreactor – The reaction area of the system.
- A detector – For example in the case of fluorescence detection, this would be a flow cell in a fluorimeter.

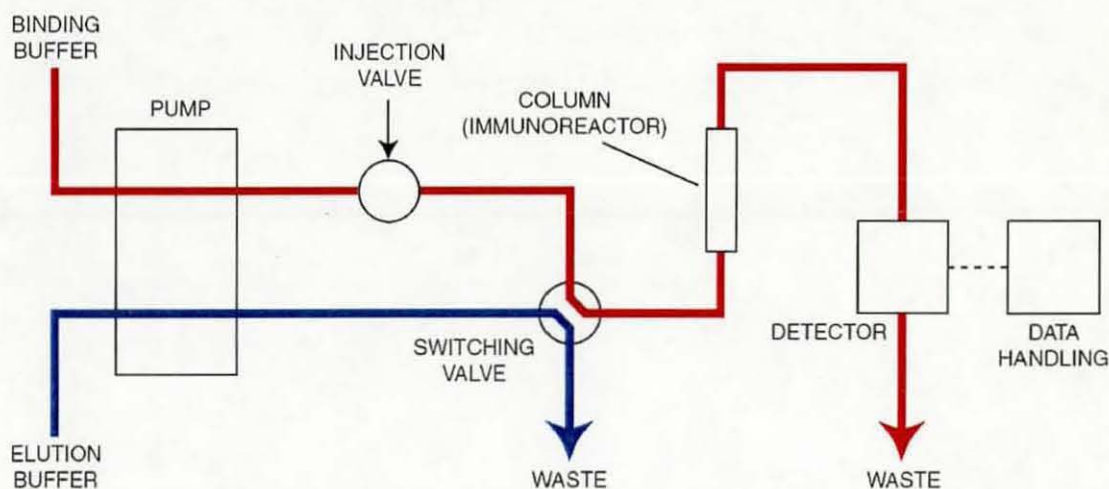


Figure 2.9 Schematic diagram of a dual stream FIA system.

2.6.2.1 Solid Phases In Flow-Injection Immunoassay

The immunoreactor can contain any one of a number of rigid supports to couple the antibody or ligand binder. Some examples are given in Table 2.9.

Support Material	Ref.
Sepharose	186
Non porous silica	186,187
Trisacryl	188
Divinylbenzene	189
Polymeric beads	190
Controlled pore glass	191

Table 2.9 Examples of solid phases used in FIIA.

In the heterogeneous FIIA system shown in Figure 2.9, the principle requirements of the immunoreactor are that it has the ability to bind and elute antibodies repeatedly, with little or no degradation of the solid phase. Two supports with this specification are staphylococcal protein A (SpA) and thiophilic gel (t-gel). These are discussed in more detail in the following sections.

2.6.2.1.1 Protein A

Staphylococcal protein A (SpA) is a bacterial protein, which is produced by *Staphylococcus aureus* (*S. aureus*). SpA specifically binds the F_c region of IgG, from a variety of species, with varying affinity and can be effectively immobilised on a variety of solid supports.

Species of polyclonal IgG	Protein A binding
Human	Strong
Mouse	Strong
Rabbit	Strong
Guinea pig	Strong
Pig	Strong
Dog	Strong
Goat	Weak
Sheep	Weak
Cow	Weak
Horse	Weak
Rat	Weak
Chicken	No binding

Table 2.10 Binding affinity of IgG from different species to SpA.

Palmer *et al.* have reviewed the use of protein A in FIAs¹⁹². The same group have also developed a FIA for testosterone using a Texas Red labelled derivative of the analyte¹⁷⁷. Evans has developed a flow-injection immunoassay for phenytoin¹⁵³ using SpA immobilised on a porous divinylbenzene polymer matrix, marketed under the name POROS. POROS is mechanically rigid and high flow rates are possible due to the nature of the pores within each polymer bead. Binding of antibody molecules to SpA is achieved at neutral pH (7.4) and elution is facilitated at acidic pH (2.5).

2.6.2.1.2 Thiophilic Gel

Many of the flow-injection immunoassays to date have used protein A as the solid phase for binding and isolation of antibodies. However, as elution of the bound antibody-analyte complex is achieved using a pH change, this could be detrimental for fluoroimmunoassays where a pH sensitive fluorophore is used

e.g. fluorescein. As a result, a new class of adsorbents, thiophilic gels (t-gel) have found application in this area.

Thiophilic interaction chromatography was discovered by chance when it was observed that a divinylsulfone-activated gel, coupled to mercaptoethanol (the t-gel) bound protein from human serum albumin in a salt dependent manner¹⁹³. When other low molecular weight thiols were substituted for mercaptoethanol, there was a similar pattern of protein binding, but when ethanolamine was coupled to the activated gel, or the activated groups reacted with hydroxyls, no protein binding was observed. To emphasise the importance of the sulfone and thioether sulfur atoms, this interaction was termed "thiophilic".

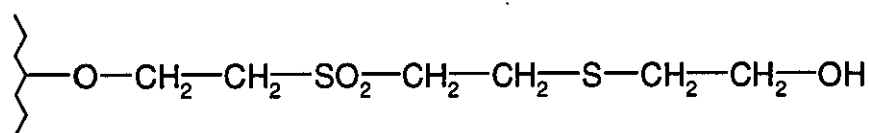


Figure 2.10 Structure of t-gel.

Thiophilic chromatography differs from hydrophobic chromatography in that different proteins are bound to the gels; for example hydrophobic adsorbents predominantly bind albumin from serum, while thiophilic adsorbents bind immunoglobulins¹⁹³. Thiophilic interactions are independent of temperature^{194,195,196} while hydrophobic interactions decrease with decreasing temperature. Both types of interactions are salt-dependant. NaCl tends to decrease thiophilic interactions while strengthening hydrophobic interactions¹⁹⁷.

The mechanism of protein binding has been postulated to be through the formation of an electron donor-acceptor complex between adjacent electron-rich and deficient regions situated within a hydrophobic cavity on the protein, and the electron-donating thioether and electron-accepting sulfone of the ligand¹⁹⁸.

Thiophilic gels have previously been used to purify antibodies^{199,200} until their additional use in flow-injection immunoassay was realised. Palmer and Miller have demonstrated their use in flow-injection immunoassay^{179,201}. The group have developed flow-injection immunoassays for albumin and testosterone using a small column containing t-gel as the immunoreactor.

Chapter 3

Instrumentation, Apparatus And Reagents

3. Introduction

This chapter describes the instrumentation, apparatus and reagents and basic methods used throughout the work.

3.1 Instrumentation

3.1.1 *Perkin-Elmer LS 50 B Fluorescence Spectrometer*

The excitation source is a special xenon flash tube that produces an intense, short duration pulse of radiation over the spectral range of the instrument. A small festoon lamp close to the excitation source maintains an even triggering of the xenon flash tube. The path of the radiation is shown in Figure 3.1. Energy from the source is focused by the ellipsoidal mirror M(E)5 and reflected by the toroidal mirror onto the entrance slit of the excitation monochromator. The monochromator consists of the entrance slit, a 1440 lines per millimetre grating, a spherical mirror and an exit slit. A narrow wavelength band emerges from the exit slit, with the centre wavelength being determined by the setting of the grating, the angle of which, is controlled by a stepper motor. The majority of the excitation beam is transmitted to the sample area via the focusing toroidal mirror M(T)1, a small portion is reflected by the beam splitter onto the reference photomultiplier. To correct the response of the reference photomultiplier, a rhodamine correction curve is stored within the instrument. Rhodamine dye absorbs energy from 230 to 630 nm and fluoresces at about 650 nm with nearly constant quantum efficiency.

Energy emitted by the sample is focused by the toroidal mirror M(T)1' onto the entrance slit of the emission monochromator. The monochromator consists of the entrance slit, a spherical mirror M(S)3', a 1200 lines per millimetre grating and the exit slit. A narrow wavelength band emerges from the exit slit, with the centre wavelength being determined by the setting of the grating. The excitation and emission monochromators can be scanned over their ranges independently, synchronously or driven to selected points in their ranges.

The LS 50 B was fitted with a red sensitive photomultiplier tube (R928) supplied by Hamamatsu. The instrument was allowed to warm up for 20 minutes prior to use, unless otherwise indicated.

3.1.2 Hitachi F4500 Fluorescence Spectrometer

The Hitachi instrument is fitted with a continuous xenon source and this is the principle difference between it and the LS 50 B described above. The Hitachi instrument is also more modern and interfaced to a personal computer running a Windows™ operating system. This improves data collection, processing and storage. The Hitachi F4500 performance specifications are listed in Table 3.1.

Sensitivity	S/N 100 or higher for Raman spectrum of water at Ex wavelength 350 nm, slit width 5 nm.
Minimum sample volume	0.6 ml.
Photometric system	Monochromatic light monitoring ratio calculation.
Light source	150 W Xe lamp.
Monochromator	Aberration – corrected concave diffraction grating: 900 l/mm. Blazed bandwidth: Ex 300 nm, Em 400 nm.
Measuring wavelength range	200 – 730 nm and 0 nm.
Slit width	Ex: 1.0, 2.5, 10 nm. Em: 1.0, 2.5, 10, 20 nm.
Resolution	1.0 nm.
Wavelength accuracy	Within ± 2 nm.
Wavelength scan speed	15, 60, 240, 1200, 2400, 12000, 30000nm/min.

Table 3.1 Performance specifications for Hitachi F4500 fluorimeter.

3.1.3 UV/VIS Spectrometer

All UV-VIS spectra were recorded on a Pye-Unicam PU8600 single beam spectrometer. The diluent buffers were used as the reference.

3.1.4 pH Measurement

All pH measurements were made using a Philips PW9420 meter, calibrated with appropriate standards before each measurement.

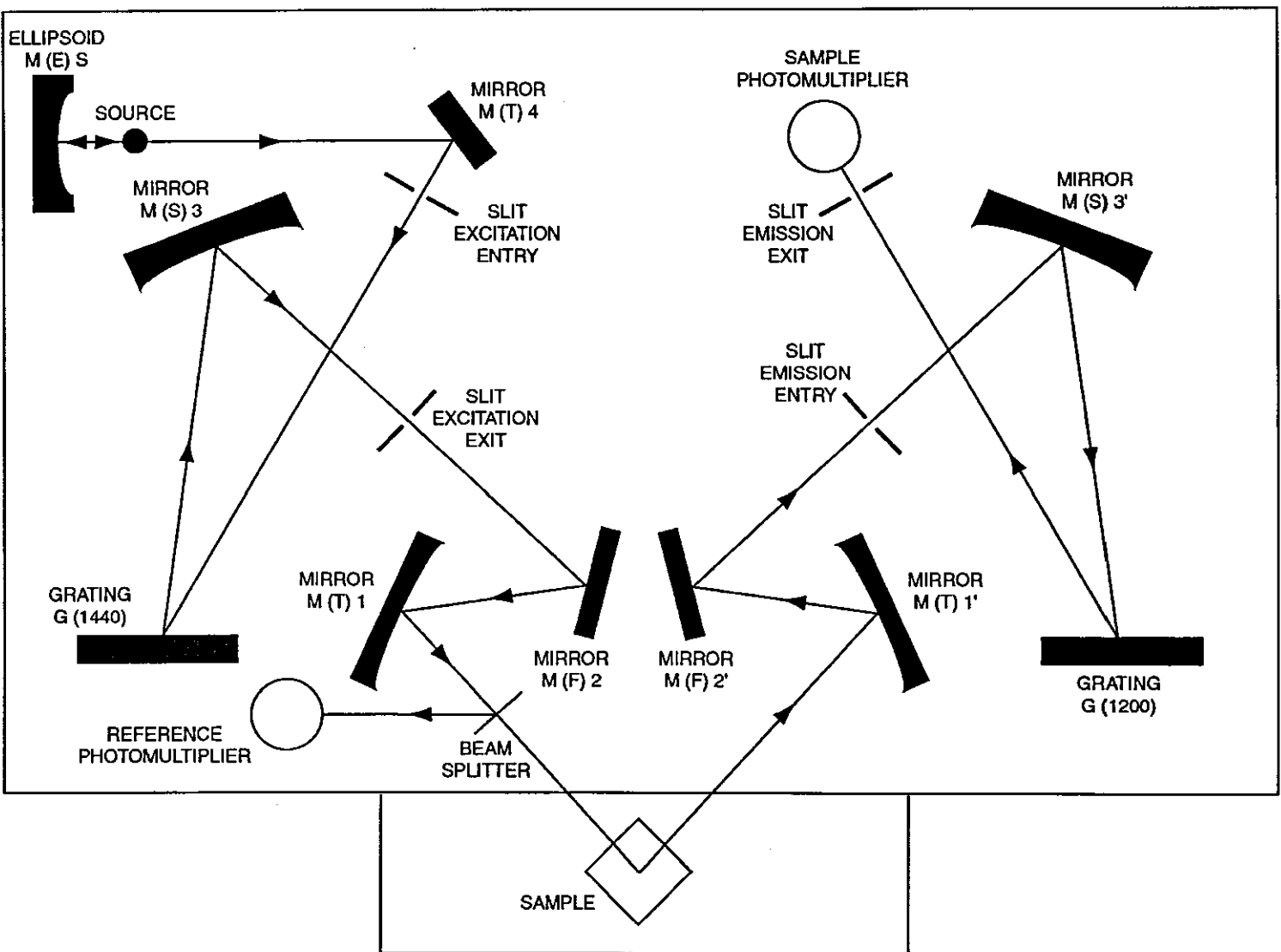


Figure 3.1 The LS 50 B optical diagram.

3.2 Apparatus

3.2.1 Automatic Pipettes

Automatic pipettes were supplied by Gilson. Pipettes were routinely calibrated and clean tips were used for each dispensation.

3.2.2 Flow-Injection Equipment

A schematic diagram of the flow-injection system used is shown in Figure 2.7, Chapter 2.

3.2.3 Peristaltic Pump

A Minipuls 3 peristaltic pump from Gilson was employed to propel the buffer through the flow-injection system. The pump head consists of ten rollers and was capable of propelling up to four channels of buffer via the peristaltic pump tubing, which was made of PVC (Anachem). Different flow rates could be achieved by either altering the rotational speed of the pump head or the diameter of the PVC pump tubing.

3.2.4 Flow Rate Calculations

The buffer reservoir was charged with deionised water and pumped through the system. The system effluent was collected for one minute in a pre-weighed glass vial. After this time, the sample vial was re-weighed and the flow-rate calculated using a density of 1.0 g/cm^3 for water.

3.2.5 Transmission Tubing

0.8 mm ID Teflon transmission tubing (Anachem) was used throughout.

3.2.6 Manual Injection Valve

The injection valve used was a low pressure Rheodyne 5020 Teflon rotary valve. All parts of the valve, which are in contact with the eluent, are of Teflon construction, the remainder are stainless steel and propylene. The valve has six ports, two ports to form the loop, one port for injecting the sample, one for excess of injected sample and the last two for inlet and outlet of the eluent towards the detector. A $25 \mu\text{l}$ injection loop was used throughout.

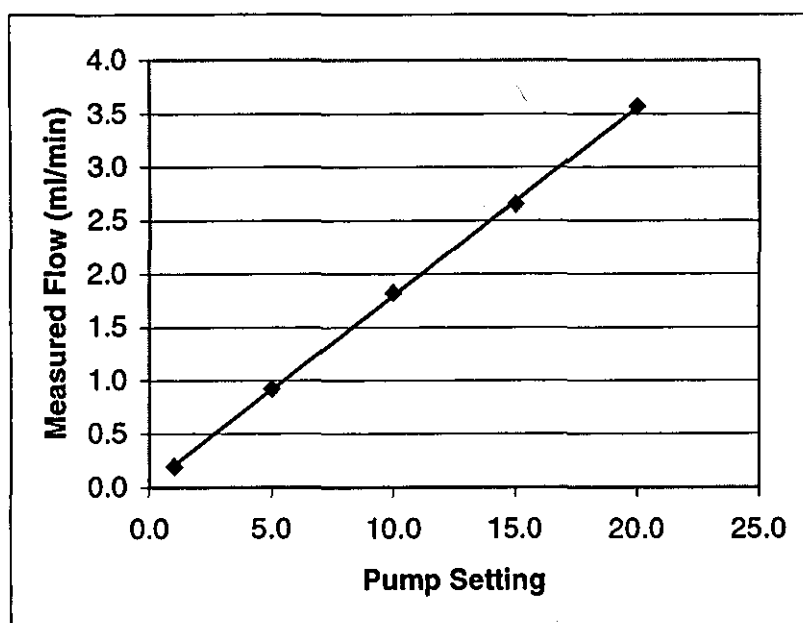


Figure 3.2 Calibration graph for Minipuls 3 pump.

3.2.7 Manual Switching Valve

The manual switching valve incorporated in the manifold was a two way valve with four ports and key allowing flow between two ports at 90° (Omnifit).

3.2.8 Column Equipment

The borosilicate columns used for packing the t-gel were supplied by Omnifit with the dimensions 100 mm length, 6.6 mm ID. Each column is sealed with adjustable end pieces containing porous polyethylene frits (pore size 25 μ m). The adjustable end pieces could be screwed closer together or further apart to vary the bed volume of the column.

3.2.9 Flow Cell

The flow cell used in the LS 50 B fluorimeter was supplied by Hellma, and has a volume of 100 μ l.

3.2.10 Packing Of Size Exclusion Gel Column

Sephadex G-25 gel (Pharmacia) was swollen in 0.9% w/v sodium chloride overnight. 1 g of gel swelled to approximately 5 ml. A PD-10 column was filled with 10 ml of pre-swollen gel using a pasteur pipette. The column was then equilibrated by pumping through with appropriate buffer before use.

3.2.11 Packing The Solid Phase Immunoreactor

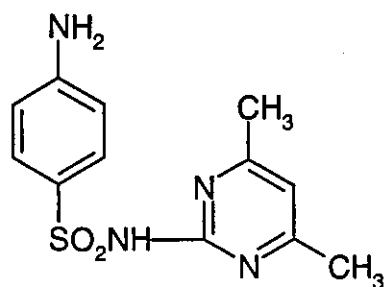
Thiophilic gel (pre-swollen by the manufacturer) was carefully pipetted into the glass column using a pasteur pipette. The adjustable column end pieces were then gently pushed down onto the gel and fixed in place. The column bed volume could be altered by varying the amount of gel and the distance between the column end pieces. Typically, approximately 1 ml of gel slurry was required to produce a bed volume of 3 mm length by 6 mm ID.

The packed column was equilibrated with appropriate buffer before use and the performance was qualitatively checked by varying the flow rate of buffer through the column. An over packed column would only allow low flow rates. A similar procedure was used to pack columns with POROS Protein A.

3.3 Reagents

All reagents used were analytical grade or above, unless otherwise stated. A full list of chemicals and suppliers is given the appendices.

3.3.1 Sulfamethazine



Sulfamethazine, 99%, M_r 278.33 was supplied by Sigma. The compound was stored desiccated in the dark at 4 °C. Care was taken to allow the sample to rise to room temperature before opening as sulfamethazine is sensitive to moisture.

3.3.2 Solvents

All solvents used were HPLC grade and were supplied by Fisher. Anhydrous solvents were supplied in sure seal bottles by Aldrich.

3.3.3 Deionised Water

Deionised water was triply distilled by reverse osmosis and supplied via an Elga Maxima Ultrapure system. The measured resistance of the water was never less than 18 M Ω .

3.3.4 Buffer Solutions

The following buffer solutions were made up as required by dissolving the necessary quantity of reagent in deionised water. The pH of each was adjusted using NaOH or HCl as appropriate. The buffers were stored at 4 °C and sodium azide (0.1% w/v) was added as preservative. The buffers were allowed to equilibrate to room temperature before use.

3.3.4.1 Phosphate Buffered Saline (PBS) pH 7.4

Sodium chloride	8.0 g/l (0.137 M)
Potassium dihydrogen phosphate	0.2 g/l (0.00147 M)
Disodium hydrogen phosphate	2.9 g/l (0.020 M)

3.3.4.2 T-Gel Binding Buffer (BB) pH 8.0

Potassium sulphate	87.14 g/l (0.5 M)
Disodium hydrogen phosphate	13.41 g/l (0.095 M)
Sodium dihydrogen phosphate	7.8 g/l (0.050 M)

3.3.4.3 T-Gel Elution Buffer (EB) pH 8.0

Disodium hydrogen phosphate	13.41 g/l (0.095 M)
Sodium dihydrogen phosphate	7.8 g/l (0.050 M)

3.3.4.4 Protein A Binding Buffer pH 7.4

Sodium chloride	8.0 g/l (0.137 M)
Potassium dihydrogen phosphate	0.2 g/l (0.00147 M)
Disodium hydrogen phosphate	2.9 g/l (0.020 M)

3.3.4.5 Protein A Elution Buffer pH 2.5

Citric acid	21.0 g/l (0.1 M)
Sodium chloride	29.26 g/l (0.5 M)

3.3.5 Thiophilic Gel

Thiophilic gel was purchased from the Sigma Chemical Company.

3.3.6 POROS Protein A

POROS IIA (20 μm particle size) was supplied by Perceptive Biosystems.

3.3.7 Antisera

Anti-sulfamethazine antisera was supplied by Enviromed Plc. The antibody was raised in sheep using ovalbumin as the carrier protein. The pooled serum was then purified by ammonium sulfate precipitation according to. The final concentration of IgG, by electrophoresis, was 20 mg/ml.

Chapter 4

Labelling Strategies For Sulfamethazine

4. Introduction

There are two principle labelling formats used in immunoassay, as described in section 2.4. One can label the antigen, as in the case of a limited reagent (competitive) immunoassay (section 2.4.1), or the antibody, as in the case of the two-site immunometric assay (section 2.4.2). There are advantages and disadvantages to choosing either format. Essentially, as in this case, the decision may depend on the availability of the various reagents needed to develop the assay and on commercial patent pressures. In order to develop a sandwich type immunoassay, two different antibodies with affinities for different epitopes on the same analyte molecule are required. It is then relatively simple to label the antibody chosen to produce the analytical signal. Indeed, sandwich immunoassays for SMT have already been heavily patented. A reagent limited assay, on the other hand, requires only one type of antibody, but it is then necessary to produce a labelled version of the analyte. This can involve significant amounts of synthetic organic chemistry when a small molecule, such as sulfamethazine, is to be labelled. There are no reported patents for a reagent limited immunoassay for SMT.

For commercial reasons, the competitive assay format was chosen. This chapter introduces the area of fluorogenic labelling and the strategy for producing a fluorescently labelled version of sulfamethazine.

4.1 Fluorescent Labels

The choice of label in fluoroimmunoassay (FIA) is critical and must satisfy several requirements. A fluorescence label should possess stability; have high molar absorptivity and quantum yield; absorb and fluoresce at appropriate wavelengths; and fulfil specific structural criteria. The labels most frequently used are listed in Table 4.1.

The first requirement implies both short- and long-term stability and consists of achieving stable fluorescence reading upon irradiation with light of an appropriate wavelength. Long term stability is more difficult to define, but

implies that fluorescence characteristics of the label are constant during long-term storage, i.e., several years.

The second condition is desirable in order to have the maximum absorption of light on a molar basis and a significant portion of this absorbed light re-emitted in the form of fluorescence without appreciable losses due to radiationless decay processes. Fluorescence from the label should occur in a region of the spectrum where photomultiplier phototubes are sensitive.

The last condition necessitates that the label not interfere with the antigen-antibody reaction. Conjugation may or may not significantly perturb the electronic structure of a label and cause shifts in absorption and fluorescence maxima. Molar absorptivity and/or quantum yield may also be affected by the conjugation.

Label	Excitation max. (nm)	Emission max. (nm)	Decay time (ns)	Quantum yield
Fluorescein	492	520	4.5	0.85
Rhodamine B-Isothiocyanate	550	585	3.0	0.7
Lissamine-rhodamine B-sulphonyl chloride	530, 565	595	1.0	-
Umbelliferones	380	450	-	-
Dansyl Chloride	340	480-520	14	0.3
Anillo-naphthaline sulphonic acid (ANS)	385	471	16.0	0.8
Fluorescamine	394	475	7.0	0.1
Lucifer Yellow	430	540	-	-
Porphyrins	400-410	619-633	-	-
Chlorophylls	430-453	648-669	-	-
Phycobiliprotein	550-620	580-660	-	0.5-0.98
Erythrosin	492	517	10 ⁸	0.01

Table 4.1 Properties of fluorescent probes used in FIA and IFMA.

4.1.1 Principles Of Fluorescence

Fluorescent molecules can absorb energy in the form of radiation and emit this as photons (Figure 4.1). Absorbed light excites the electron field of the molecule from its ground state singlet (S_0) to a higher state (S_1 , S_2 etc.).

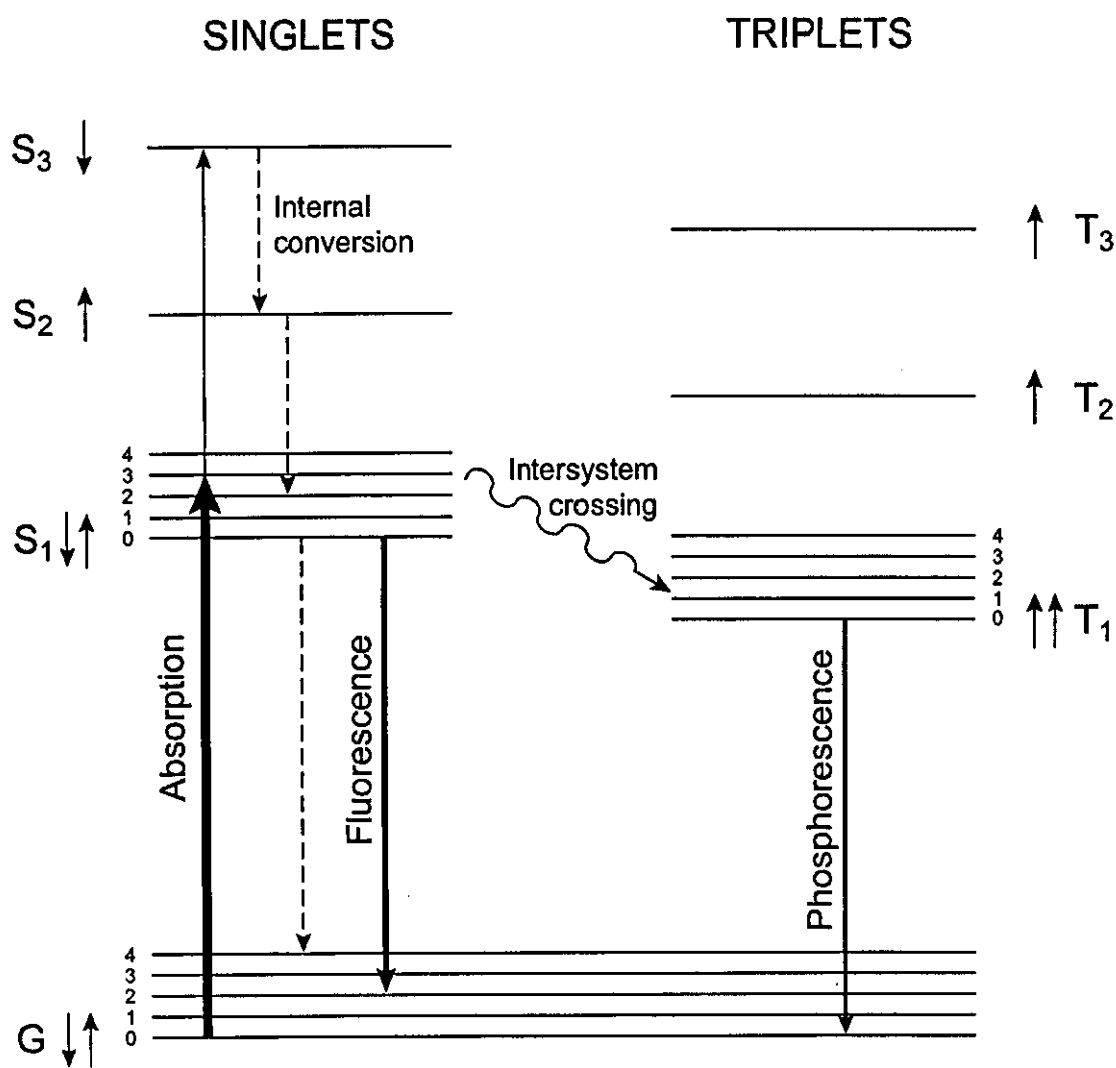


Figure 4.1 Principle of fluorescence and phosphorescence.

Energy may be lost from non-radiative conversion (for example, as heat), by radiative transition to the ground state (fluorescence) or through a semi-stable triplet state (phosphorescence).

Some rare earth ions, particularly of europium (Eu^{3+}) and terbium (Tb^{3+}) form highly luminescent chelates with suitable organic ligands. In this case, light absorption by the ligand is followed by energy transfer from the excited singlet state through its triplet state to the resonance energy levels of the lanthanide ion.

The wavelength of emitted light is always longer than that of the excited energy because of energy losses before emission. This wavelength difference (the "Stokes shift") is generally small – in the region of 30-50 nm for fluorescent organic molecules – but is greater for phosphorescent molecules (e.g. 150 nm for erythrosin) and much greater for luminescent lanthanide chelates (over 200 nm for europium and samarium). The decay times for phosphorescence (10^{-4} to 1 s) and lanthanide chelate luminescence (10^{-5} to 10^{-2} s) are much longer than for fluorescent organic molecules (10^{-9} to 10^{-8} s) and this factor has permitted the development of time-resolved fluorescence techniques.

4.1.2 Visible Labels

The most commonly used fluorogenic label, which fluoresces in the visible region (400-700 nm), is fluorescein, the luminescent power of which was demonstrated in 1867 by Goppelsroder²⁰². He suggested that 10 kg of fluorescein be thrown into the river Danube. Three days later, the characteristic green fluorescence of fluorescein was detected in the waters of the Rhine, demonstrating that these two great rivers were linked.

Fluorescein is a pH sensitive member of the xanthene dye family and has absorption and emission maxima at 495 nm and 525 nm respectively. Fluorescein is available in two isomeric forms and these can either be reactive or unreactive depending on which substituents are present in the molecule.

Figure 4.2 shows the structure of the most commonly used reactive fluorescein derivative, fluorescein isothiocyanate (FITC). The diagram shows the extended electron conjugation possible at alkaline pH, leading to enhanced fluorescence and explains why fluorescein is non-fluorescent in strongly acidic solutions.

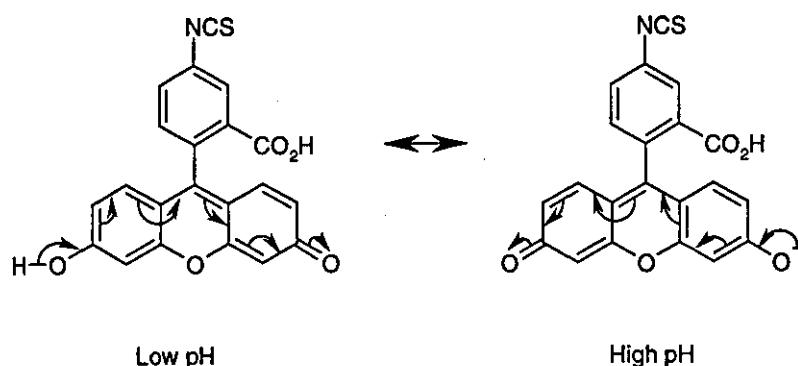


Figure 4.2 Fluorescein isothiocyanate.

The isothiocyanate group (NCS) reacts with primary amines under basic conditions to yield a covalent thiourea linkage. Fluorescein is also available itself with a primary amine moiety. The compound, so called fluoresceinamine, is reactive towards carboxyl containing compounds.

Other commonly used visible fluorophores include tetramethylrhodamine, lissamine rhodamine, Texas Red and cyanine 3.18. Wessendorf *et al.* have compared these fluorophores with fluorescein for use in fluorescence microscopy²⁰³.

4.1.3 Near-Infrared Labels

The far visible and the near-infrared (NIR) spectral regions (600 - 1000 nm) are areas of low interference, where only a few classes of molecules exhibit significant absorption or fluorescence²⁰⁴. These features of the NIR spectral region make it ideal for using fluorogenic labels as background fluorescence from matrix components is minimal. Unfortunately, until recently, the NIR region has been less accessible to inexpensive instrumentation. However, this

situation has changed rapidly since the introduction of semiconductor based detectors (e.g. photodiodes) and semiconductor lasers or laser diodes.

There is an abundant variety of polymethine dye compounds known to fluoresce in this wavelength region, whose absorption maxima coincide with the output wavelengths from the commercially available laser diodes mentioned above. Waggoner *et al.* have commercialised the cyanine dyes under the trademark 'Cy'. These labels are sulfoindocyanine compounds and possess sulfonate groups to increase aqueous solubility^{205,206}. Figure 4.3 shows the bis-form of Cy5, complete with succinimidyl ester groups that are reactive towards amines. The label absorbs at 650 nm and fluoresces at 670 nm. Mank *et al.* have used similar five carbon cyanine dyes for precolumn derivatisation in the chromatography of amines and thiols^{207,208}. A review published in 1996 by the same author covers diode laser-based detection in liquid chromatography and capillary electrophoresis²⁰⁹.

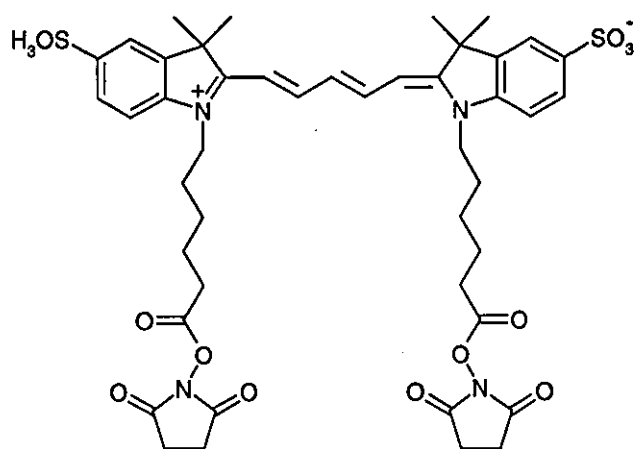


Figure 4.3 Cy5 succinimidyl ester (Cy5.18.OSucc).

The maximum absorption and emission wavelengths of cyanine dyes are determined mainly by the length of the cyanine chain - the longer the chain, the longer the wavelength. Each CH-CH group increases the absorption maximum by approximately 110 nm. However, chemical and photostability decrease as the methine chain increases. Substituents on the aromatic or heterocyclic rings also affect the maximum absorption or emission wavelengths, although less significantly.

Using these basic rules, synthesis of dyes containing seven carbon atoms between the heterocyclic rings, which fluoresce around 800 nm, is possible. A large number of publications exist, outlining the synthesis of these long wavelength fluorophores^{210,211,212,213,214}. Figure 4.4 shows a heptamethine cyanine dye with an isothiocyanate reactive group. This dye has been used to label goat anti-human antibody (GAHG) in a solid-phase immunoassay for human immunoglobulin (HulgG)²¹⁵. Other applications of these heptamethine labels include DNA sequencing²¹⁶ and chromatographic determination of human serum albumin²¹⁷.

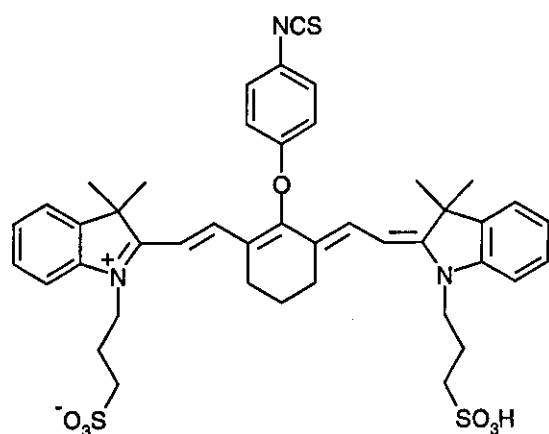


Figure 4.4 A heptamethine cyanine dye with an isothiocyanate reactive group.

An important point to note, is that the majority of the reported applications mentioned above involve fluorescent labelling of large protein molecules or immunoglobulins. There are very few examples in the literature of fluoroimmunoassays in which, small hapten molecules have been labelled.

4.2 Structure Of Sulfamethazine

To label sulfamethazine (SMT) in such a way that its antibody binding properties are not affected, one must have detailed information about the structure of both SMT and the antibody to be used. The antibodies used in this work were produced by the central veterinary laboratory in Cambridge and supplied by Enviromed Plc. As SMT is a hapten, i.e. too small to elicit an immune response by itself, it was first converted into a succinyl derivative and

then coupled to ovalbumin (Figure 4.5) to furnish the immunogen (Figure 4.6). Studies have shown that the elbow shaped region, in SMT, around the sulfone group, along with the unique heterocyclic aromatic ring are key to its recognition using an antibody raised in this way. Figure 4.7 shows the three-dimensional structure of sulfamethazine as determined by Basak *et al.*²¹⁸.

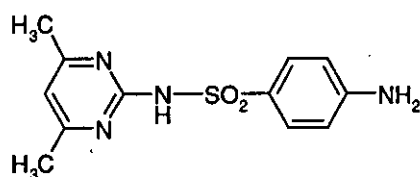


Figure 4.5 Two-dimensional structure of sulfamethazine.

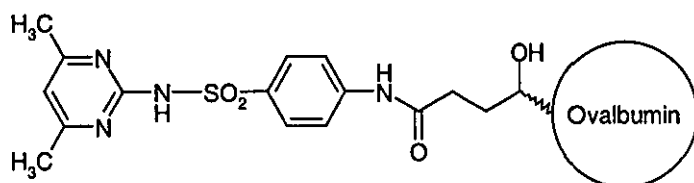


Figure 4.6 SMT-Ovalbumin immunogen.

It follows that, to label SMT without perturbing its antibody binding properties, we must do so selectively at the primary amine group. Another important consideration is whether to label SMT directly, or to put a spacer group in between the drug and the label. Both of these approaches are covered in the following sections. All of the prepared conjugates were tested in the same way. The method is described in section 4.5.

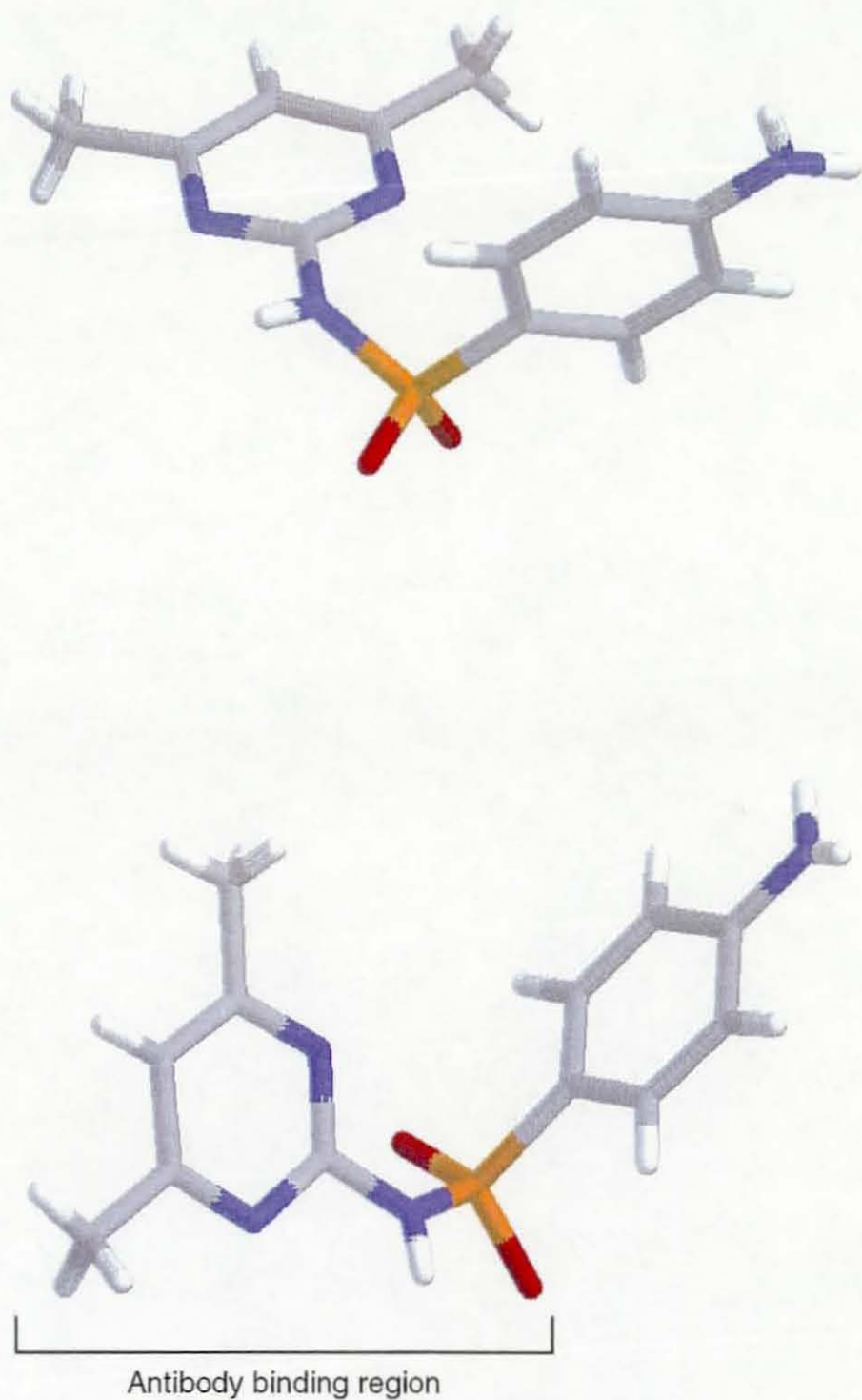


Figure 4.7 Three-dimensional structure of sulfamethazine showing the antibody binding region.

4.3 Indirect Labelling

4.3.1 Synthesis Of Succinyl Sulfamethazine

A solution of sulfamethazine (3.03 g, 10 mmol) and succinic anhydride (1.51 g, 15 mmol) in ethanol (30 ml) was refluxed for 90 minutes. The solution was then left to cool overnight, after which time, white crystals had formed. The ethanol was decanted off and the solid was refluxed in a mixture of water and ethanol (1:1.5) for 10 minutes. The solution was then cooled, filtered and the resulting crystals were washed three times with the water/ethanol mixture. The crystals were then dried in a vacuum oven at 45 °C.

Full analytical data for this compound can be found in ref²²¹.

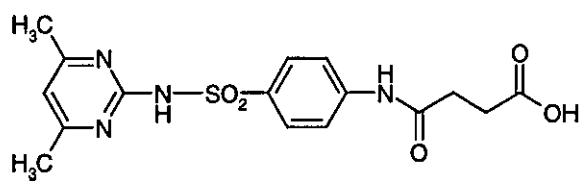


Figure 4.8 Succinyl sulfamethazine (SSMT).

4.3.2 Preparation Of A Succinyl Sulfamethazine-Biotin-LC-Hydrazide Conjugate

In an attempt to ensure that no biotin was left unreacted, an excess of succinyl sulfamethazine was reacted with Biotin-LC-hydrazide as follows:

25 μ l of EDC (200 mM in 0.1 M MES buffer, pH 5.5) was added to 1 ml of SSMT (0.5 mM in 0.1 M MES buffer, pH 5.5) and stirred for 30 minutes at room temperature. 25 μ l of biotin-LC-hydrazide (2 mM in DMSO) was then added and the resulting mixture was then left to stir overnight.

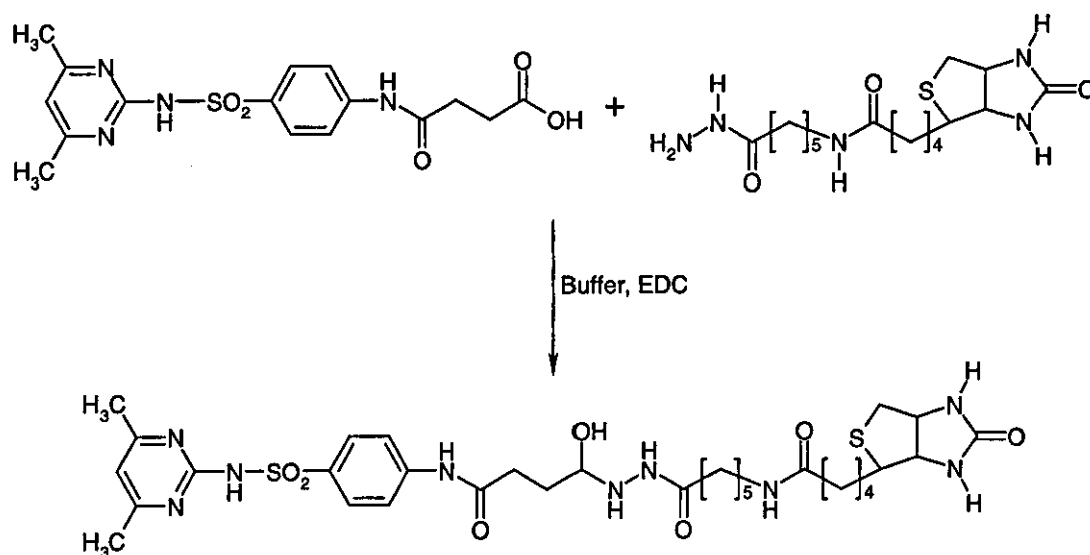


Figure 4.9 Preparation of succinyl sulfamethazine-biotin-LC-hydrazide conjugate.

4.3.3 Preparation Of A BODIPY FL Labelled Succinyl Sulfamethazine-Biotin-LC-Hydrazide Conjugate

0.5 ml of BODIPY FL labelled avidin (0.031 mM in buffer) was added to 1.05 ml of the biotin solution (0.045 mM) prepared in 4.3.2. An excess of biotinylated SSMT over avidin was used to ensure that no unconjugated label was left at the end of the reaction. The reaction was stirred gently for 12 hours after which time it was passed down a 10 cm Sephadex G-10 size exclusion column in order to separate the conjugate fraction from the unreacted starting materials and by-products. The column effluent was monitored using the LS 50B fluorimeter. The detector settings were: λ_{Ex} 500 nm, λ_{Em} 515 nm with excitation and emission monochromator slits both set at 10 nm. The fractions containing the conjugate were then pooled and concentrated prior to testing.

4.3.4 Preparation Of A FITC-BSA-SMT Conjugate

Bovine serum albumin (20 mg, 298 μmol) and *p*-benzoquinone (200 μl , 149 μmol) was dissolved in PBS buffer (0.01 M, pH 6) and stirred for 2 hours. The solution was dialysed against saline (0.9 % w/v, 5 l) for 2.5 hours. The dialysed protein solution was added to a solution of sulfamethazine (20 mg in 1.6 ml PBS buffer : 0.4 ml DMF) and the pH adjusted to 8 by the addition of carbonate buffer (0.1 M, pH 9.6). The solution was incubated at 4 °C for 36

hours after which time the pH was raised to 9.3 using a saturated sodium carbonate solution.

Fluorescein isothiocyanate (5 mg, 1.26 μmol) was added and the solution was incubated for 1 hour, with stirring every 10 minutes. Lysine (1 M, 400 μl) was added and the solution was stirred for a further 2 hours. The conjugate was isolated using gel permeation chromatography: sephadex G-25, eluted with PBS buffer. The column effluent was monitored using a fluorescence detector set at λ_{Ex} 495 nm, λ_{Em} 525 nm.

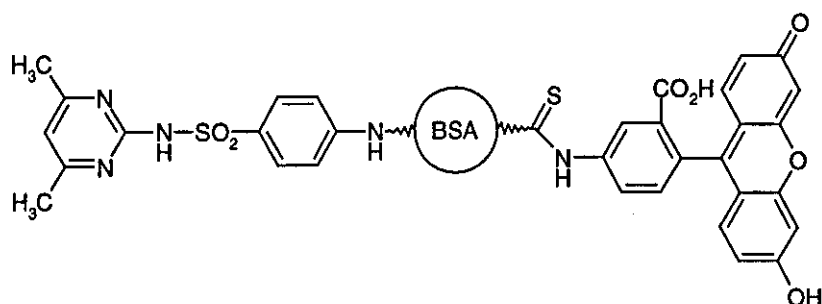


Figure 4.10 FITC-BSA-SMT conjugate.

4.3.5 Preparation Of A Cy5-HRP-SMT Conjugate²¹⁹

A solution of sulfamethazine-horseradish peroxidase (10 ml, 0.95 μM , pH 5) was added to Cy5.18.OSucc (3.7 mg, 3.95 μmol) and stirred for 4 hours at room temperature in the dark. After this time, the reaction mixture was passed down a sephadex G-25 column (eluted with PBS, pH 7.2). The HRP positive fractions (using 4-chloronaphthol/hydrogen peroxide indicator) were collected and pooled. The concentrations of Cy5 and HRP were determined spectrophotometrically and the ratio of Cy5:HRP:SMT was 1:1:2.

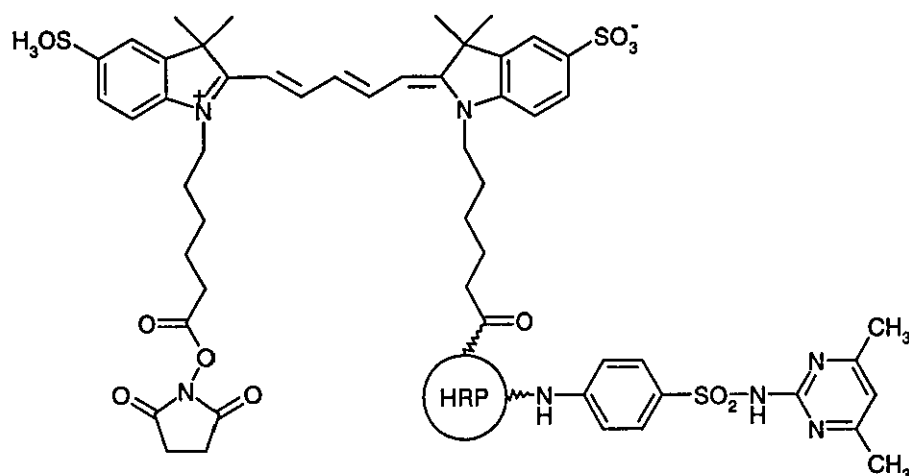


Figure 4.11 Cy5-HRP-SMT conjugate.

4.4 Direct Labelling

4.4.1 Preparation Of A Cy5-SMT Conjugate²¹⁹

Sulfamethazine (10 mg, 36 μmol), triethylamine hydrochloride (68 mg, 0.36 mmol) and Cy5.18.OSucc (16.8 mg, 17.95 μmol) were dissolved in methanol (0.5 ml) and stirred under nitrogen for 16 hours. After this time, the reaction mixture was evaporated to dryness using nitrogen. The residue was then redissolved in 0.1 ml of methanol and applied to a preparative TLC plate which was eluted with 79:20:1 ethyl acetate:methanol:acetic acid. The band at $R_f = 0.25$ was scraped off and extracted into methanol.

$\text{C}_{49}\text{H}_{59}\text{O}_{11}\text{N}_6\text{S}_3$; yield = 14 %; $\lambda_{\text{max}} = 653 \text{ nm}$; $\epsilon = 51,000 \text{ M}^{-1}\text{cm}^{-1}$.

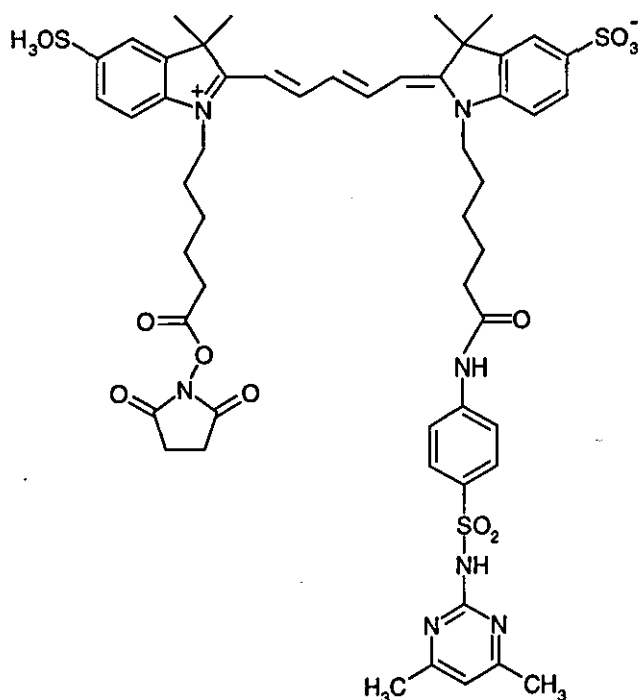


Figure 4.12 Cy5-SMT conjugate.

4.4.2 Preparation Of A Cy7-SMT Conjugate²¹⁹

A solution of 4-(2,3,3-trimethyl-3H-indolio)butanesulfonate (0.80 g, 2.71 mmol), 2-formyl-6-[(E)-1-hydroxymethylidene]-1-cyclohexenyl-4-(4-[(4,6-dimethyl-2-pyrimidinyl)amino)sulfonyl]anilo)-4-oxobutanoate (0.69 g, 1.35 mmol) in benzene/butan-1-ol (30 ml: 70 ml) was refluxed using a Dean-Stark condenser at 95 °C for 12 hours. The solvents were removed *in vacuo* and the resulting solid was washed with diethyl ether. The washed product was passed down an LH-20 gel chromatography column (eluting 75 % chloroform/25 % methanol, 3.5 ml/min, then 1.0 ml/min) and the green fractions collected.

$C_{55}H_{63}O_{10}N_6S_3$; TLC (75 % chloroform/25 % methanol): $R_f = 0.23$; yield = 75 % (crude), $\lambda_{max} = 782 \text{ nm}$; $\epsilon = 61,000 \text{ M}^{-1}\text{cm}^{-1}$.

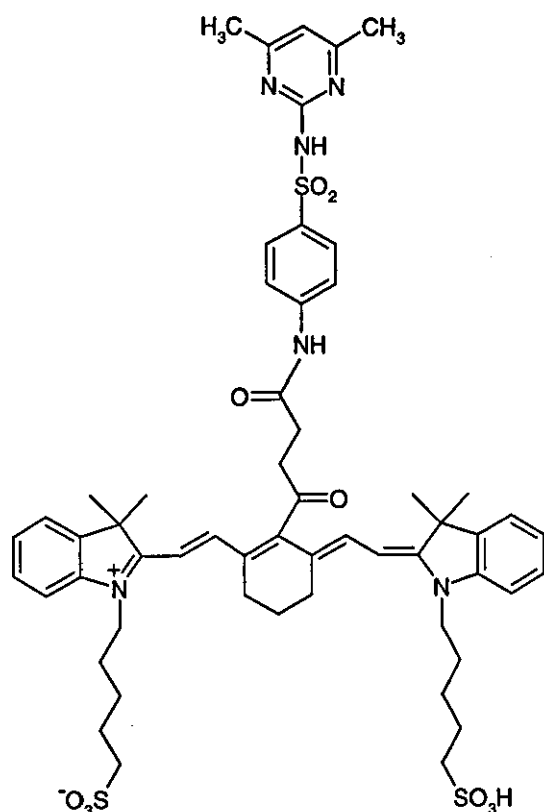
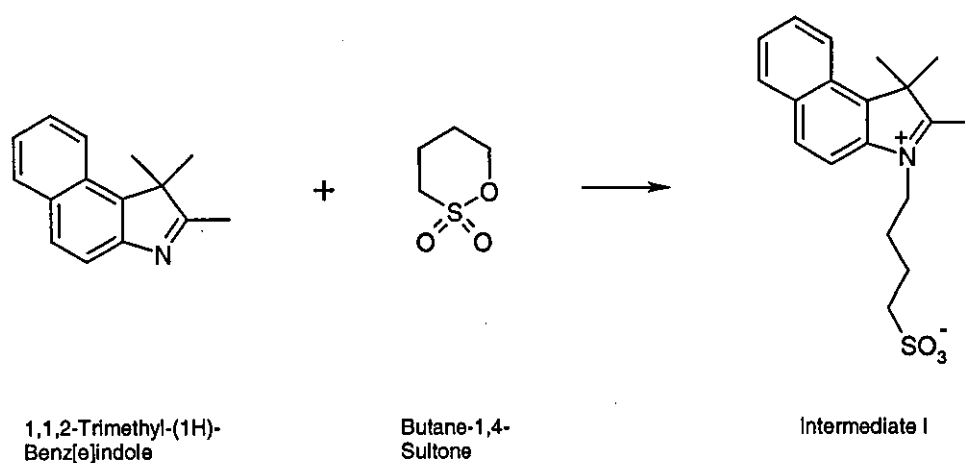


Figure 4.13 Cy7-SMT conjugate.

4.4.3 Preparation Of Cy5.4a-1A

An attempt was made to synthesise the near infrared fluorophore, Cy5.4a-1A, first synthesised by Mank et al.²⁰⁸.

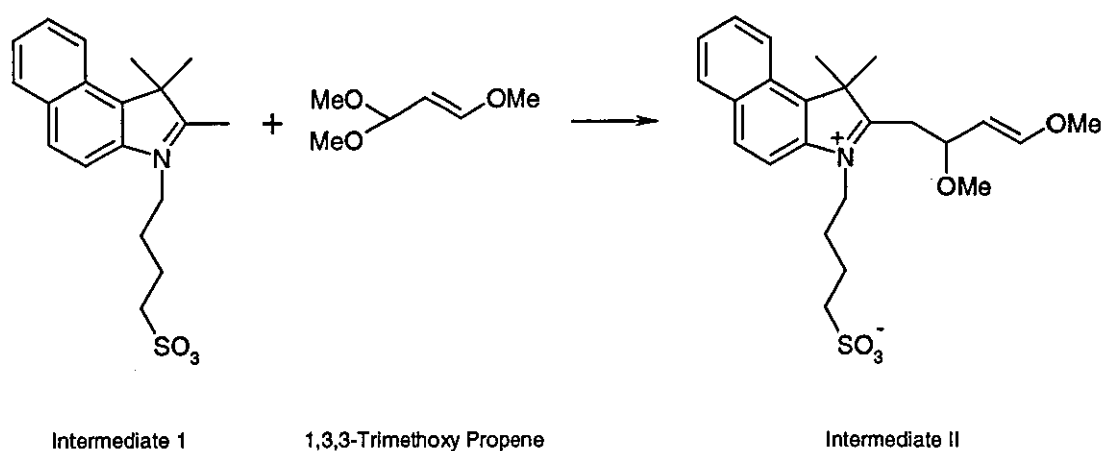
4.4.3.1 Intermediate I



To a 100 ml 2 neck RB flask was added 5.0 g (0.024 mols) of 1,1,2-trimethyl-(1H)-benz[e]indole and 7.3 ml (0.072 mols) butane-1,4-sultone, followed by

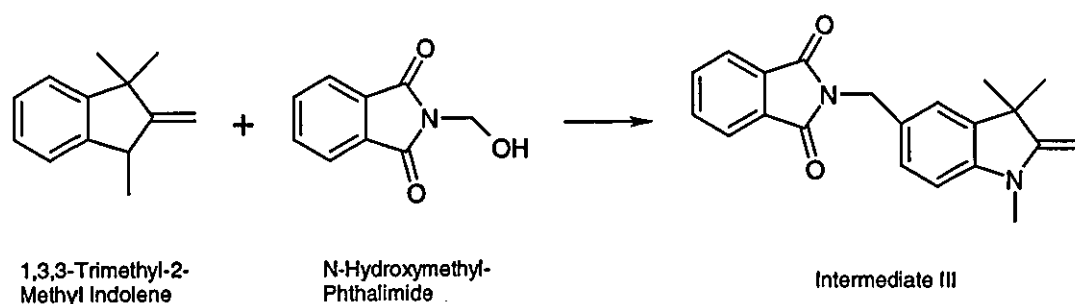
50 ml of anhydrous 1,2-dichlorobenzene. A thermometer and septum's were fitted and the mixture was heated for 12 hours at 120 °C under nitrogen. The mixture was then allowed to cool to room temperature during which time a precipitate formed. Excess dichlorobenzene was decanted off and small amount of acetone was added to the suspension of crude product. The mixture was then filtered and the precipitate was dissolved in a minimum of hot methanol. The solution was then allowed to cool and acetone was added to precipitate the product. This process was repeated three times until a single spot was obtained by TLC.

4.4.3.2 Intermediate II



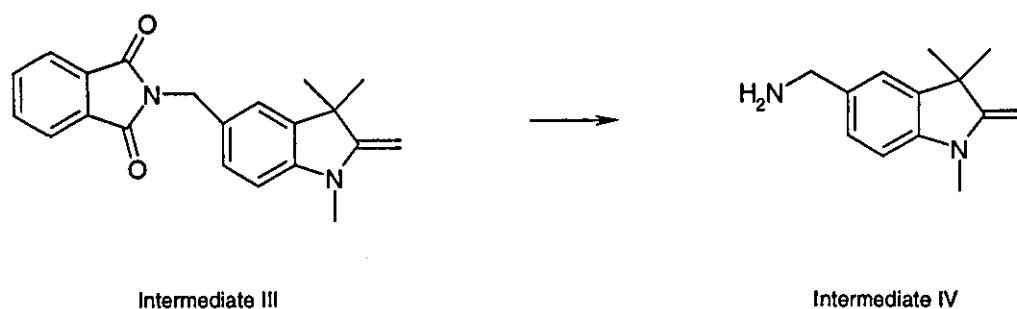
69 mg (0.2 mmol) of intermediate I and 119 mg (0.9 mmol) of 1,3,3-trimethoxypropene were transferred into a 1 ml vial, to which was added 1 ml of concentrated acetic acid and a small magnetic stirrer bar. The mixture was then stirred for 30 minutes at room temperature. The resulting yellow-brown mixture was then diluted with 10 ml of anhydrous diethyl ether and cooled in an ice bath. A yellow-green gum separated. The supernatant was decanted off and the gum was dissolved in 5 ml of methanol, followed by fast addition of 25 ml of cold diethyl ether. The resulting green precipitate was collected by suction filtration and used without further purification. The reaction was repeated a further four times in order to obtain enough product for the next step.

4.4.3.3 Intermediate III



5.08 ml (0.029 mol) of 1,3,3-trimethyl-2-methyleneindolene was added to a stirred solution of 50 ml concentrated sulphuric acid, followed by 5.11 g (0.029 mol) of N-hydroxymethylphthalimide portion wise over 45 minutes. The mixture was stirred at room temperature for 70 hours after which time the solution was poured onto ice water and basified with ammonia to yield a yellow solid. The crude product was then recrystallised with dichloromethane-ethanol to give a yellow solid which slowly turned red if left standing in air at room temperature. The purified product was stored under nitrogen at 0 °C.

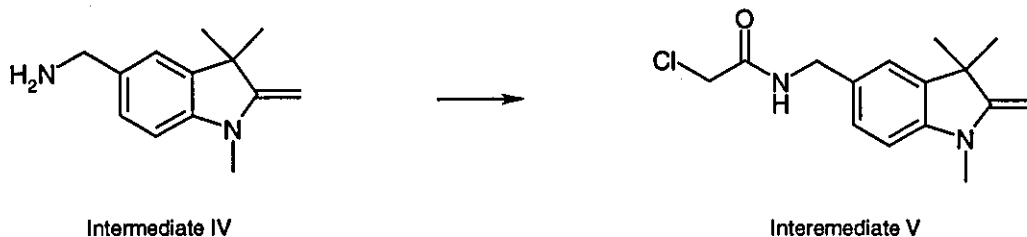
4.4.3.4 Intermediate IV



2.0 g (6.02 mmol) of intermediate III, 50 ml of concentrated HCl and a few anti-bumping granules were placed in a 100 ml RB flask. The mixture was then refluxed for 10 hours after which time it was allowed to cool to room temperature. Further cooling in ice caused the precipitation of phthalic acid which was removed by reduced pressure filtration. The filtrate was then basified with ammonia and salt added. The product was then extracted with 3 x 25 ml diethyl ether. The ether was removed using a rotary evaporator to furnish a yellow oil which if left standing in air, rapidly turned red. Owing to the

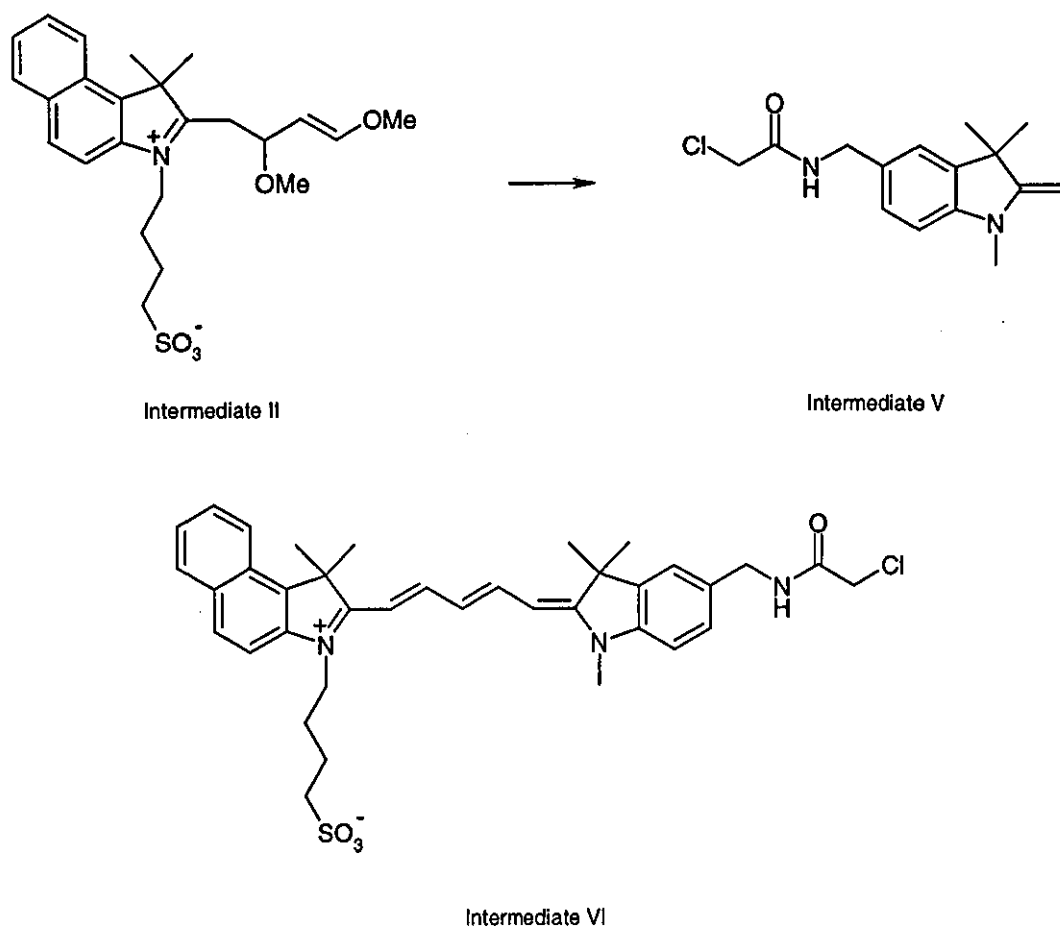
unstable nature of the product, no attempt was made to ascertain its purity and the compound was used without further purification.

4.4.3.5 Intermediate V



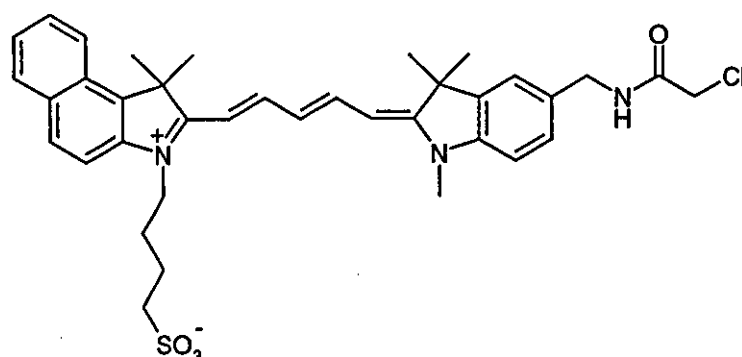
To a 100 ml beaker was added: 0.2 ml (1.48 mmol) of triethylamine, 0.3 g (1.48 mmol) of intermediate IV and 50 ml dichloromethane, all of which was then stirred. To a 100 ml 2 neck RB flask was added 0.17 g (1.48 mmol) chloroacetyl chloride and 20 ml of dichloromethane. A stirrer bar, septum's and thermometer were fitted and the mixture cooled to 0 °C under nitrogen. The solution of intermediate IV was then added to the cooled solution of chloroacetyl chloride maintaining the temperature at 0 °C. After 1.5 hours, the solution was diluted by adding 200 ml of dichloromethane, followed by washing twice with 1 M HCl. The acidic aqueous layer was separated and neutralised with 2M NaOH. The product was then isolated by extraction with 5 x 25 ml ether. After removal of the ether, the product was used immediately (if left standing, the product rapidly turned red in air).

4.4.3.6 Intermediate VI

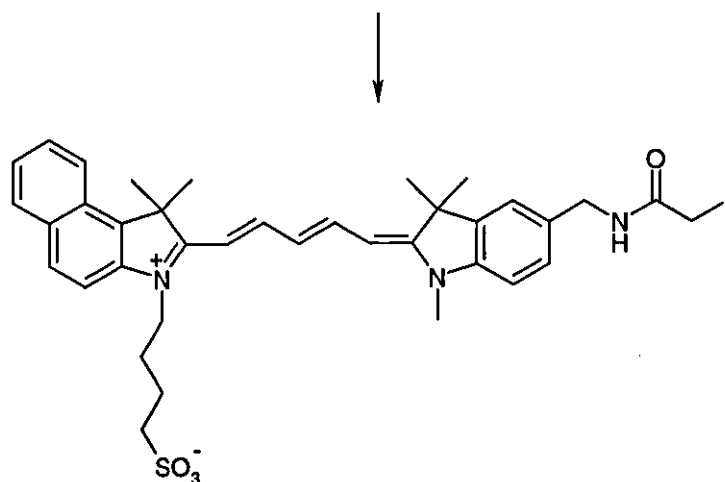


67 mg (0.15 mmol) of intermediate II and 42 mg (0.15 mmol) of intermediate V were transferred into a small vial and just enough methanol was added to dissolve both compounds. The mixture was then stirred for 24 hours, followed by cooling in ice. The resulting product was then isolated by decantation of the methanol followed by washing with diethyl ether.

4.4.3.7 Cy5.4a-IA



Intermediate VI



Cy5.4a-IA

4.4.4 Preparation Of A FITC-SMT Conjugate

Sulfamethazine (10 mg, 36 μmol) was dissolved in a mixture of methanol (0.5 ml), triethylamine hydrochloride (49 mg, 0.36 mmol) and FITC (7 mg, 18 μmol) and stirred at room temperature for 16 hours. Purification of the FITC-SMT conjugate was by preparative TLC (80:19:1 ethyl acetate:methanol:acetic acid). The band at $R_f = 0.65$ was scraped off the plate and extracted into methanol.

$\text{C}_{33}\text{H}_{27}\text{N}_5\text{O}_7\text{S}_2$; yield = 42 %; $\lambda_{\text{max}} = 493 \text{ nm}$; $\epsilon = 30,000 \text{ M}^{-1}\text{cm}^{-1}$; FAB Mass Spec.: No molecular ion seen, fragments at 651, 593, 407, 273.

NMR data for this compound can be found in ref.²²¹.

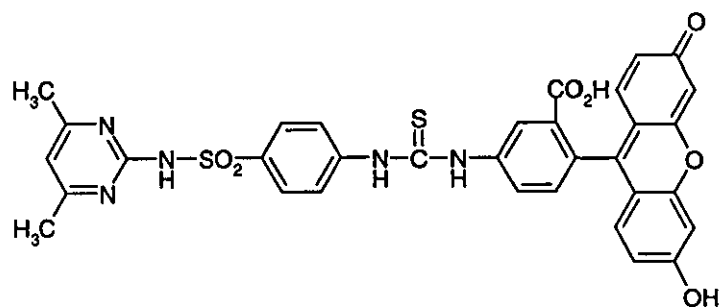


Figure 4.14 FITC-SMT conjugate.

4.5 Conjugate Testing

A general method was applied to all of the prepared conjugates to test their viability as a tracer in a flow-injection fluoroimmunoassay. The first step was to determine the spectral characteristics of the conjugate by measuring its UV-VIS and fluorescence spectra. Fluorescence spectra were measured by filling the flow cell with conjugate. This minimised wastage and gave a clearer indication as to the concentration of conjugate which would be needed to give a good detector response.

The next step was to test the fluorescent conjugate in a flowing system. This was done using the set-up shown in Figure 2.9, initially without the immunoreactor in place. The conjugate was injected into the binding buffer stream (flowing at 1.5 ml/min) which carried the sample to the detector. The signal size and shape was noted. Next, the sample was injected with the immunoreactor in place. After the first, unbound peak had returned to the baseline, the flow was switched to elution buffer. This was the first test for non-specific binding of the conjugate to the immunoreactor. A peak at this stage would indicate that the conjugate was binding non-specifically to the column. The flow was then switched back to binding buffer followed by an injection of 10 % (w/v) CHAPS (a zwitterionic detergent) to try and wash any bound conjugate off the column. A peak at this stage would also indicate non-specific binding of the conjugate to the immunoreactor.

If the conjugate did not bind non-specifically to the column then a simple immunoassay was tried. This consisted of incubating the conjugate (50 μ l) with 50 μ l of binding buffer and 50 μ l 1:10 sulfamethazine antibody for 5 minutes.

The incubation mixture was then loaded into the 25 μ l injection loop and injected into the binding buffer stream which carried the sample to the immunoreactor and subsequently to the detector. After the excess, unbound conjugate had passed through the detector, the flow was switched to elution buffer. A peak at this stage was characteristic of elution of antibody-bound conjugate i.e. the analytical signal.

4.6 Results And Discussion

4.6.1 Indirect Labelling

Indirect labelling, i.e. placing a spacer group between the SMT molecule and the label is attractive for three reasons. Firstly, initial experiments by colleagues at Enviromed had suggested that the primary amine group present in SMT was not as reactive as previously thought, due to the electron withdrawing effect of the *para*-sulfone group. This made direct labelling of SMT, with small quantities of expensive reactive fluorophores, problematical and costly. The second attractive reason for using indirect labelling is that steric hindrance between the analyte-label conjugate and antibody is reduced, and therefore the chance of problems with antibody recognition and binding are less. Finally, it was thought the spacer group would reduce the chance of the label properties; either absorption and emission wavelengths or molar absorptivity, being altered upon conjugation.

4.6.1.1 Synthesis Of Succinyl Sulfamethazine

Any derivatisation of sulfamethazine to furnish a group more reactive than the primary amine, had to be simple, relatively cheap and furnish a product with high yield, which needed little or no further purification. One reaction which was known to work well, involved succinylation of SMT to produce a carboxylic acid derivative. A large number of labels are available for carboxylic acids.

The reaction produced a white crystalline solid in high yield with melting point and spectral data consistent with the literature. The sample was stored desiccated in the dark at 4 °C.

4.6.1.2 Biotinylation Of Sulfamethazine

One method for labelling the succinylated sulfamethazine involved biotinylation. Biotinylation relies on the specific reaction between avidin (a high molecular weight protein) and biotin, which form a strong complex. A large number of biotin derivatives are available which react with a variety of functional groups, including carboxylic acids. Equally important, a large number of avidin substrates, labelled with various fluorophores, are commercially available.

The strategy was to first label succinyl sulfamethazine with biotin-LC-hydrazide, so chosen because of the long chain (LC) between the biotin moiety and the hydrazide reactive group. The next step was then to react the biotinylated SSMT with avidin, pre-labelled with the fluorescent dye BODIPY FL.

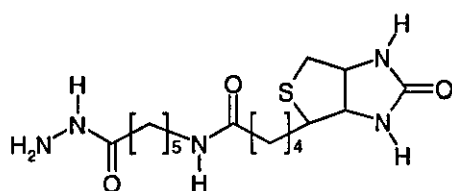


Figure 4.15 Biotin-LC-hydrazide.

The isolated conjugate was tested according to the method in section 4.5. Unfortunately, it appeared that the biotinylation strategy had been unsuccessful. The principle reason for this was the very low final concentration of conjugate. Even when neat conjugate was injected into the flow-injection system, it was impossible to detect a signal.

The limiting step in the biotinylation reaction was the very poor solubility of succinyl sulfamethazine in aqueous media. The procedure was repeated using dry DMF as the derivatisation solvent in the reaction between succinyl sulfamethazine and biotin-LC-hydrazide; the DMF being removed before the reaction with labelled avidin. The limiting factor then became the cost of the biotin reagent itself, meaning concentrations barely higher than in aqueous media were feasible.

4.6.1.3 Preparation Of A FITC-SMT-BSA Conjugate

It is well known that large protein molecules are easier to label with fluorescent tags than small hapten molecules. Colleagues at Enviromed had previously prepared SMT-HRP conjugates for use in conventional ELISA assays using TMB as the chromogenic substrate. It was also known that SMT had been effectively conjugated to ovalbumin in order to produce the SMT immunogen (Figure 4.6). The strategy was therefore to first produce a conjugate of SMT and a suitable protein, and then to label the protein with a fluorescent dye.

Bovine serum albumin was chosen as it is widely available and cheap. More importantly, work had already been done in our laboratory regarding labelling BSA for use in flow-injection immunoassay.

Once the reaction mixture had been applied to the gel permeation column and elution started, two strongly coloured yellow bands could be observed migrating down the column. The first band was assumed to be conjugate band to its more rapid migration. The second band moved only very slowly down the column and was therefore assumed to be unconjugated FITC.

The pooled conjugate fraction was tested for antibody binding activity as described in section 4.5. Although the conjugate exhibited little non-specific binding, it showed no antibody binding activity in the flowing system. This was surprising as the conjugate was also tested by colleagues in a conventional ELISA assay at Enviromed and showed a good level of activity.

It is still unclear why the conjugate did not work in the flowing system. With hindsight it would have probably been more profitable to repeat exactly the ovalbumin-SMT conjugation reaction used to produce the immunogen and then label that product.

4.6.1.4 Preparation Of A Cy5-HRP-SMT Conjugate

A Cy5-HRP-SMT conjugate was supplied by Enviromed and tested according to the method outlined in section 4.5. Like the visible SMT-protein conjugate described above, the Cy5 conjugate also showed no antibody binding activity

in the flowing system. The reasons for this are unclear as this conjugate too showed activity in a standard ELISA assay conducted at Enviromed.

4.6.2 Direct Labelling

All of the indirect labelling methods described above utilise proteins as the linker group. The advantage of this is that proteins have many possible binding sites and are thus, easy to conjugate to small molecules. One drawback of this technique, however, is that any one conjugation reaction can yield products, conjugated to different extents, even when reaction conditions are carefully controlled. The other major problem is that proteins must be stored at 4 °C, otherwise their shelf lives are dramatically reduced. This is a particularly important consideration if one is to produce a robust, commercial assay.

Direct labelling involves reacting sulfamethazine with a reactive fluorescent dye to produce a chemically stable, and fully characterisable conjugate. This was attempted using both visible and near-infrared fluorophores.

4.6.2.1 Preparation Of Cy5- And Cy7-SMT Conjugates

The cyanine dyes are attractive for a number of reasons, as outlined in section 4.1.3. They fluoresce in the NIR region of the electromagnetic spectrum and thus can be excited by small, inexpensive laser diodes. However at the time this project was instigated, cyanine reactive dyes were only commercially available through one company, Biological Detection Systems (BDS) who were subsequently taken over by Amersham International. The dyes were therefore very expensive to buy (\approx £500 per 5 mg). The labels were originally produced to label large protein molecules for visualisation in DNA sequencing techniques. Using these fluorophores to label small hapten molecules would therefore present a difficult problem.

It was decided that colleagues at Enviromed would synthesise these labels in house as the methods had already been published. An attempt was made to use these synthesised dyes to directly label SMT. Neither of the conjugates showed antibody binding activity when tested.

The Cy7 conjugate was especially problematical due to it being extremely light sensitive – a solution of the dye in buffer (or methanol) would visually discolour if left on the bench for more than two hours, producing non-fluorescent decomposition products. The conjugate also bound non-specifically to the t-gel column. This could be as a result of unreacted dye being present in the conjugate mix.

4.6.2.2 Preparation of Cy5.4a-IA

An attempt was made to synthesise Cy5.4a-IA. Unfortunately this proved problematical because two of the intermediate compounds, IV and V were highly unstable oils, which rapidly decomposed despite efforts to use them immediately in the next synthetic step. Synthesis of the final product, the iodoacetamide derivative of intermediate VI was therefore impossible.

4.6.2.3 Preparation Of A FITC-SMT Conjugate

After failing to produce a conjugate using NIR labels it was becoming increasingly important to synthesise an SMT conjugate using any available fluorophore, if for no other reason, to prove that a flow-injection fluoroimmunoassay for SMT was possible. This was, after all, the aim of the project.

Fluorescein isothiocyanate (FITC) is a very cheap, widely available visible fluorophore. Using FITC, it was possible to scale-up the organic chemistry and repeat the reaction as many times as necessary to yield enough of the product for use in further method development. In the event, the FITC-SMT conjugation reaction had a surprisingly good yield (42 %) and this could doubtless have been increased further with reaction optimisation.

The conjugate was purified, in the absence of a suitable HPLC system, using the somewhat laborious, but reliable method of preparative TLC. The isolated conjugate was dried and stored in the freezer, in the dark, where it has remained stable for over a year.

Synthesis of FITC-SMT has provided a fully characterisable organic product which has excellent long term stability. More importantly, the FITC-SMT conjugate was the only one of all the conjugates tested, which showed activity in the flowing system. The conjugate also exhibited only a very small amount of non-specific binding.

The FITC-SMT conjugate was therefore chosen for the assay development stage, covered in the following chapter.

Chapter 5

Assay Optimisation

5. Introduction

The previous chapter introduced the area of fluorogenic labelling, and outlined some of the difficulties of working with a small molecule, such as sulfamethazine. This chapter outlines the science of immunoassay design and optimisation, followed by details of the experimental work using the FITC-SMT conjugate described in the previous chapter.

5.1 Immunoassay Design And Optimisation

In order to select the optimum concentration of antibody, an experiment can be performed in which, labelled antigen is incubated (1) alone with a series of antibody dilutions and (2) with the same set of dilutions but together with a small increment of unlabelled antigen ($[\Delta Ag]$), sufficient in magnitude to cause a significant displacement of the resulting antibody dilution curve shown in Figure 5.1. A further crucial feature of the experiment is that each point on each of the antibody dilution curves is determined at least in duplicate, and preferably using 5-10 replicates. This experiment thus yields not only the two dilution curves shown in Figure 5.1, but, equally importantly, an estimate of the standard deviation of the response at each measured point along the curves. However, because the number of replicate estimations at each point is limited (thereby causing SD estimates to be statistically unreliable) it is useful to plot the measured SD values as a function of the response variable (B), permitting the fitting of a further curve – representing the response error relationship (RER) – through the points. (Figure 5.2)

Turning again to the dilution curves, it is also evident that the vertical distance between the dilution curves varies as a function of antibody concentration. This difference, representing the change in the bound labelled antigen fraction (ΔB) caused by the increment in antigen concentration ($[\Delta Ag]$), may likewise be plotted (Figure 5.3) as a function of the corresponding value of B_0 (i.e. the value of B when only labelled antigen is present), again reducing the effects of experimental errors on the individual measurements of ΔB .

We now have two curves: one relating to the SD in the measurement of B (i.e. σ_B) to B; the other the reduction in B (ΔB), caused by the presence of $[\Delta Ag]$, in the system, also as a function of B. We are thus able to calculate the quotient of $\sigma_B/\Delta B$ as a function of B. This quotient represents the estimated precision of measurement of zero dose (i.e. assay sensitivity corresponding to all values of B_0 , and implicitly to all antibody dilutions. It may likewise be plotted, either as a function of B or of antibody dilution (Figure 5.4), its minimum value corresponding to the conditions yielding maximal assay sensitivity.

The example has been described in some detail in order to illustrate a logical and scientifically sound approach to the choice of antibody concentration yielding maximal assay sensitivity. Analogous procedures can be readily devised to establish the values of other parameters, for example, the optimum labelled antigen concentration or the optimum amount of adsorbent to separate bound and free antigen fractions etc. These principles were applied to the optimisation of the immunoassay for sulfamethazine.

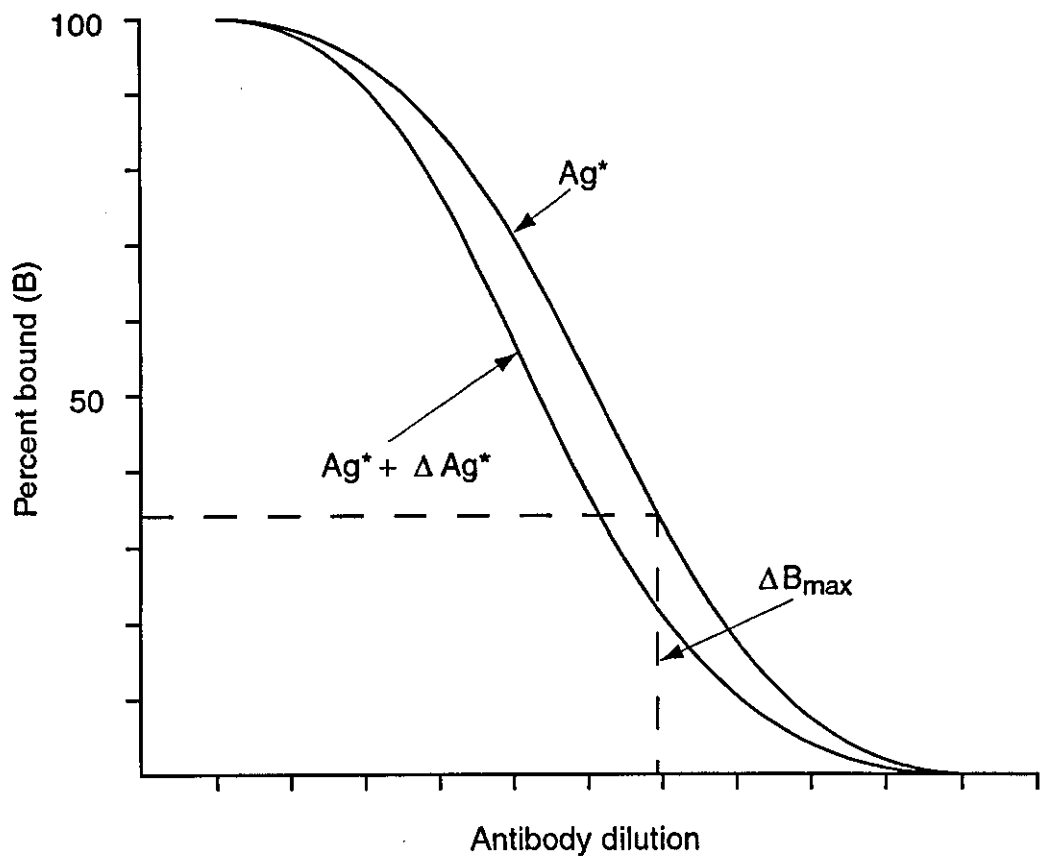


Figure 5.1 Antiserum dilution curves obtained using trace labelled antigen only (Ag^*) and labelled antigen together with a small increment of unlabelled antigen ($Ag^* + \Delta Ag$). Note that the maximum difference in the fraction of antigen bound (ΔB_{max}) is observed when Ag^* is 33% bound.

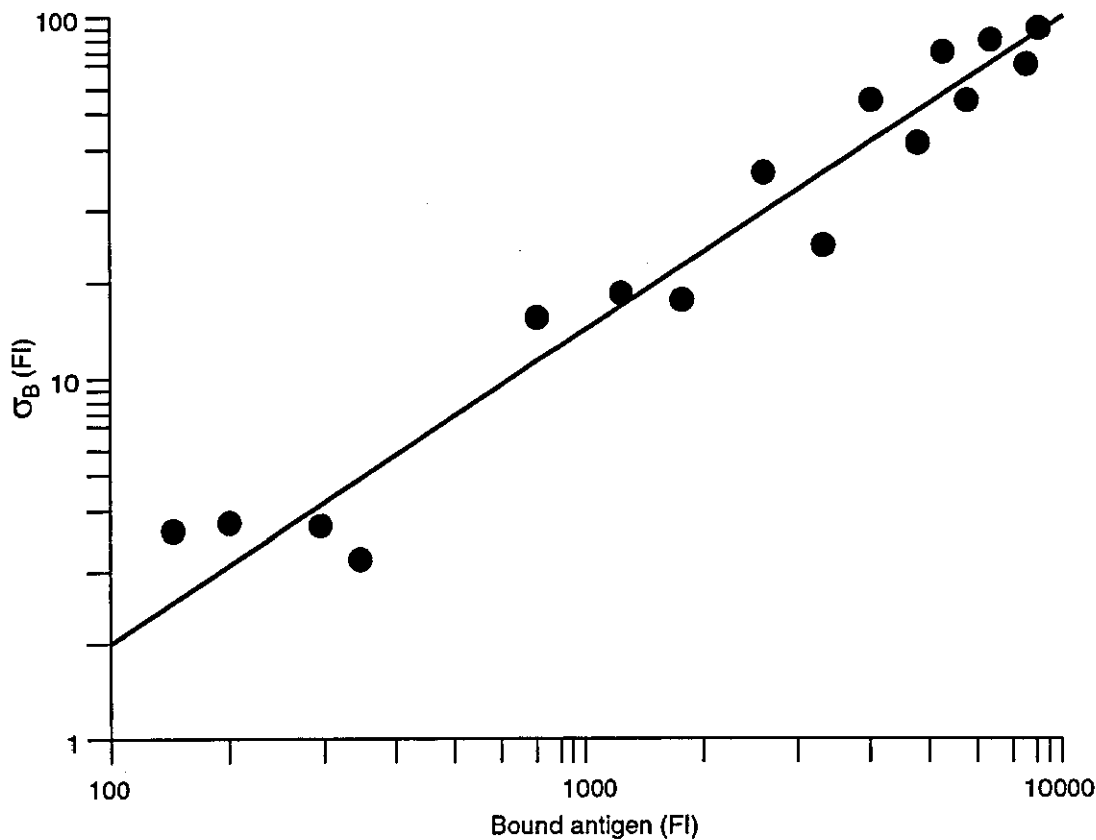


Figure 5.2 Curve representing the response error relationship (RER), i.e. the standard deviation in bound fluorescence intensity (σ_B) plotted as a function of bound fluorescence intensity.

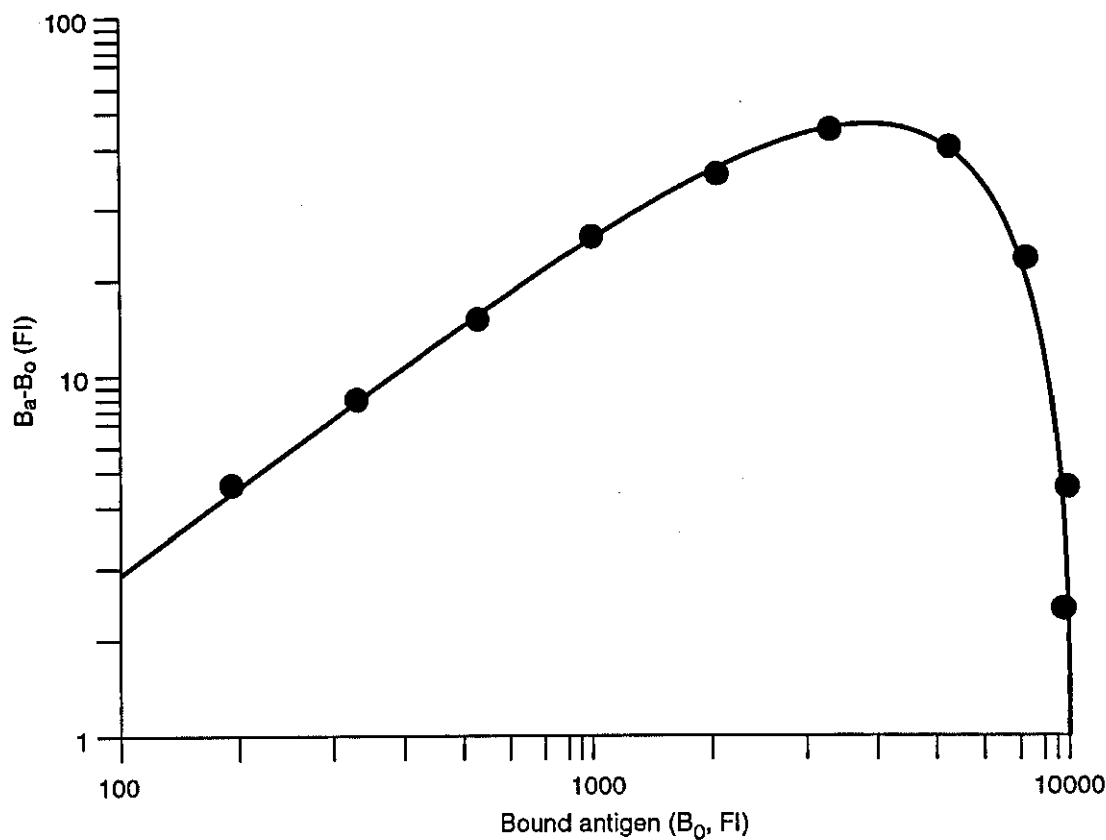


Figure 5.3 Curve representing the difference (ΔB) in fluorescence intensity in the presence (B_a) and absence (B_o) of the antigen increment (ΔA).

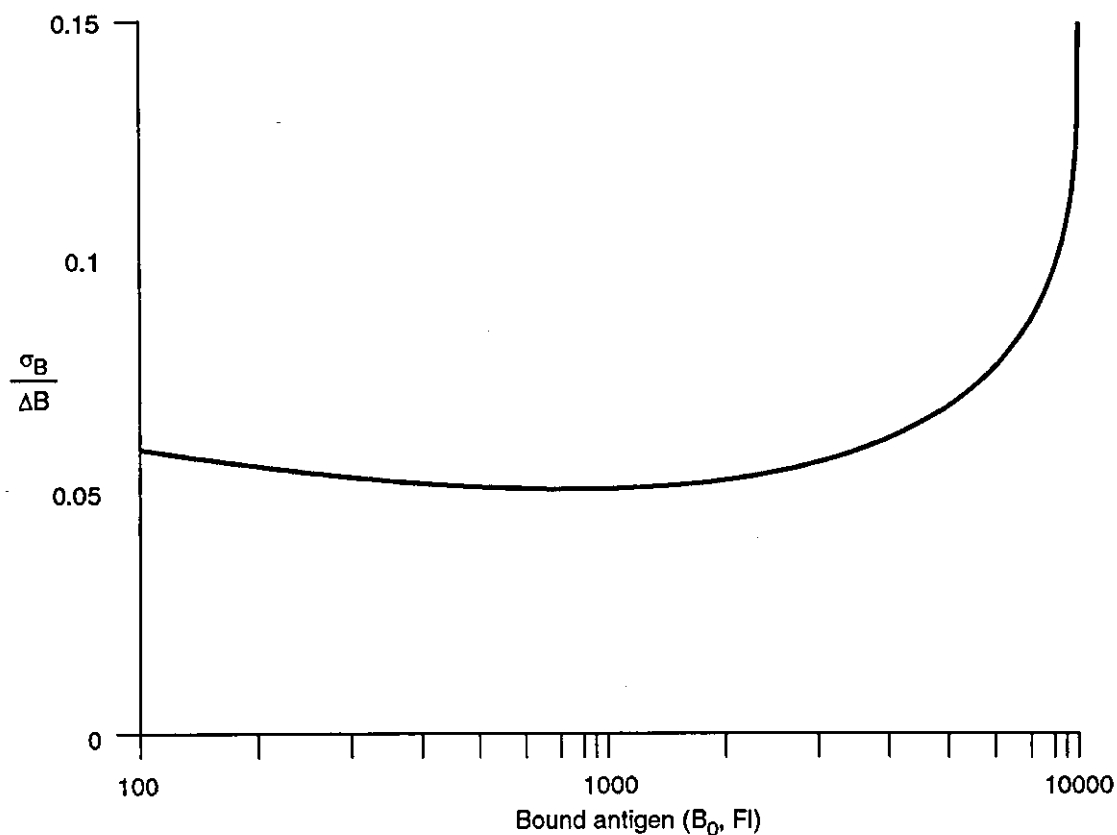


Figure 5.4 Curve representing the quotient of $\sigma_B/\Delta B$ as a function of fluorescence intensity. The value of the response variable B_0 (bound fluorescence intensity) at which $\sigma_B/\Delta B$ is minimal (about 1000 FI units) indicates the antibody dilution at which the SD in the zero-dose measurement ((σ_A) , given by $\Delta A\sigma_B/\Delta B$) is minimal, i.e. sensitivity is maximal.

5.2 Experimental Approach To Immunoassay Optimisation

Where the term conjugate is used, this refers to the FITC-SMT conjugate described in the previous chapter. All antibody concentrations are expressed in terms of dilution of the original stock antibody solution (section 3.3.7); i.e. 1:10 refers to a ten fold dilution of the original stock solution.

Where the term Ab is used, this refers to the SMT antisera supplied by Enviromed.

5.3 Antibody Binding Onto Solid Phases

The binding of the sheep anti-SMT, supplied by Enviromed, to POROS protein A and thiophilic gel was investigated. The aim of the experiments was to check the affinity of the Enviromed supplied SMT antisera for both protein A (in the form of POROS IIA) and t-gel. The method used was the same for each matrix:

- A small borosilicate glass column (10 cm x 6 mm ID) was packed with the appropriate matrix according to the method in section 3.2.11. The packed bed measured approximately 3 mm x 6 mm ID.
- The packed column was then equilibrated with appropriate binding buffer for ten minutes. The binding buffers were PBS (pH 7.4) and PBS with 0.5 M K_2SO_4 (pH 8.0) for protein A and thiophilic gel respectively.
- The flow-rate was kept constant at 1.82 ml/min throughout the experiment (pump setting 10.0).
- Initially, 25 μ l of 1:10 antisera, diluted in appropriate binding buffer (BB), was injected into the flowing stream and any unbound antibody was detected down stream using the LS 50 B fluorimeter with settings: λ_{Ex} 280 nm, λ_{Em} 335 nm (native protein fluorescence), slits Ex/Em, 10/10 nm.

- The flow was then switched to elution buffer (EB); citrate (pH 2.5) in the case of protein A and PBS without K_2SO_4 (pH 8) for t-gel. The bound antibody signal was detected as before. The antisera was diluted as necessary to give an 'in range' instrument response and the procedure repeated.
- The ratio of unbound to bound antibody was then calculated.

Figure 5.5 shows the binding profile of sheep anti-SMT on POROS protein A. The first peak is the unbound fraction and the second is the bound. Table 5.1 shows the integration.

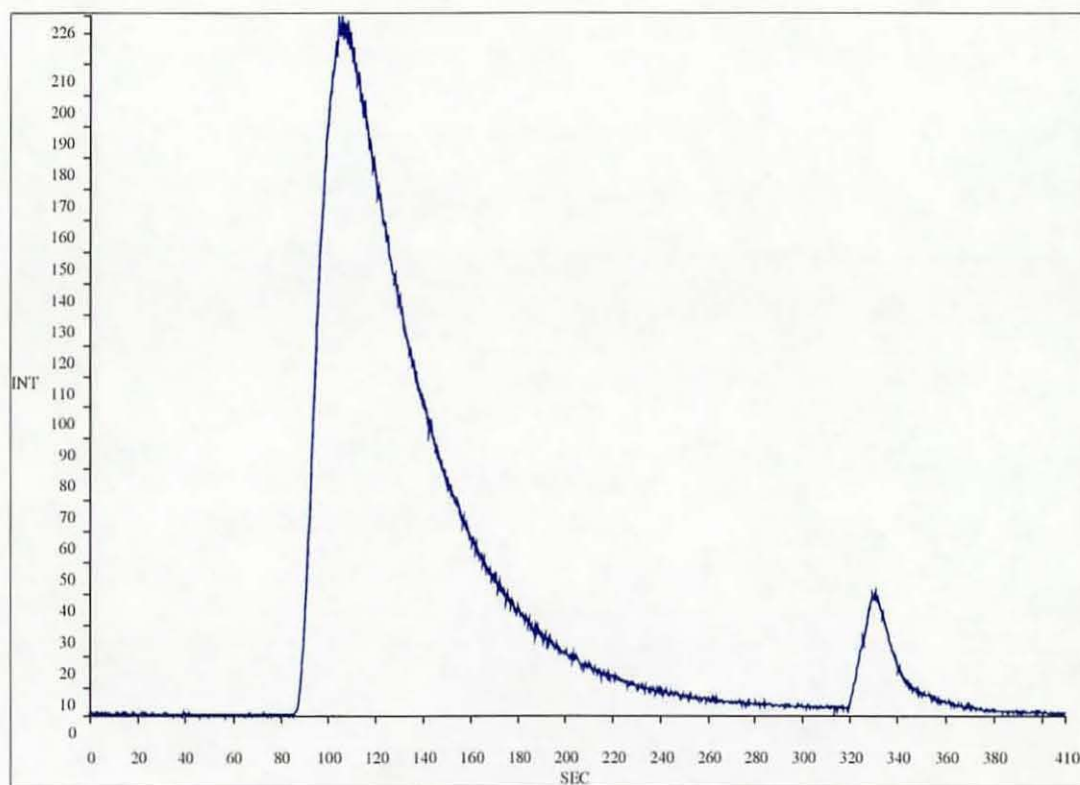


Figure 5.5 Binding profile of sheep anti-SMT on POROS protein A.

	Peak Area	% of Total
Unbound	11125	95.3
Bound	554	4.7
Total	11679	100.0

Table 5.1 Binding profile of sheep anti-SMT on POROS protein A.

5.3.1 T-Gel

Figure 5.6 shows the binding profile of sheep anti-SMT on t-gel and Table 5.2 gives the integration.

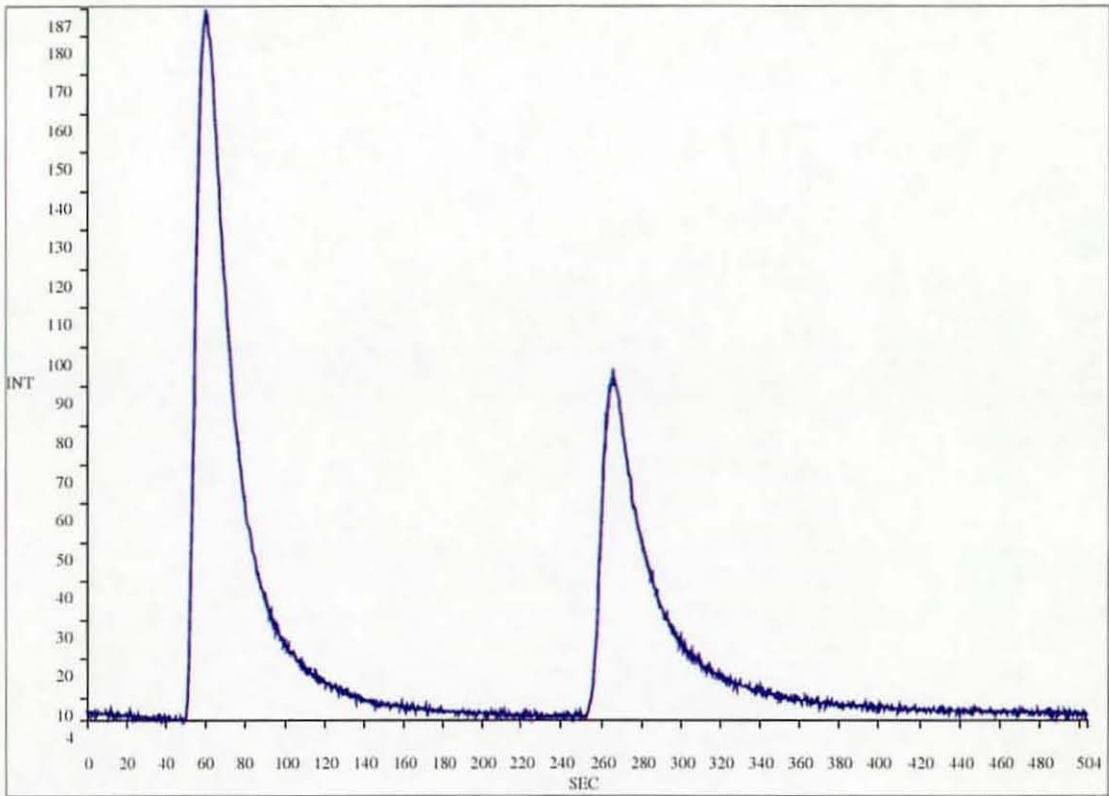


Figure 5.6 Binding profile of sheep anti-SMT on thiophilic gel.

	Peak Area	% of Total
Unbound	7117	53.7
Bound	6128	46.3
Total	13245	100.0

Table 5.2 Binding profile of sheep anti-SMT on thiophilic gel.

5.4 POROS Protein A Feasibility Experiments

An investigation was undertaken to determine whether it was feasible to use POROS for further method development despite its low affinity for sheep-raised anti-SMT.

5.4.1 Initial Investigations

A third reagent stream was added to the simple flow-injection system illustrated in Figure 2.9. The purpose of this stream was to introduce alkali to the system in order to raise the pH of the effluent stream. The resulting system is shown schematically in Figure 5.7.

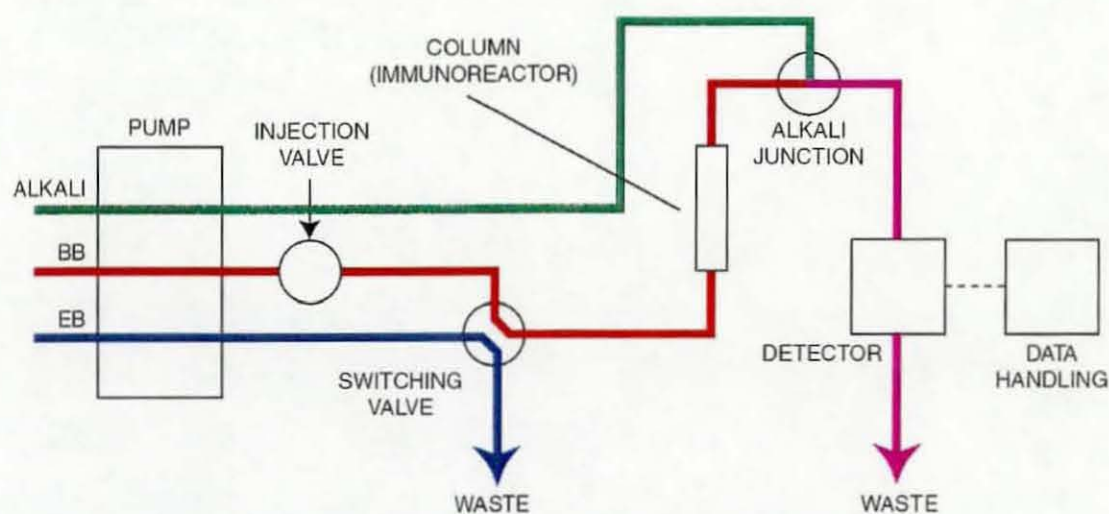
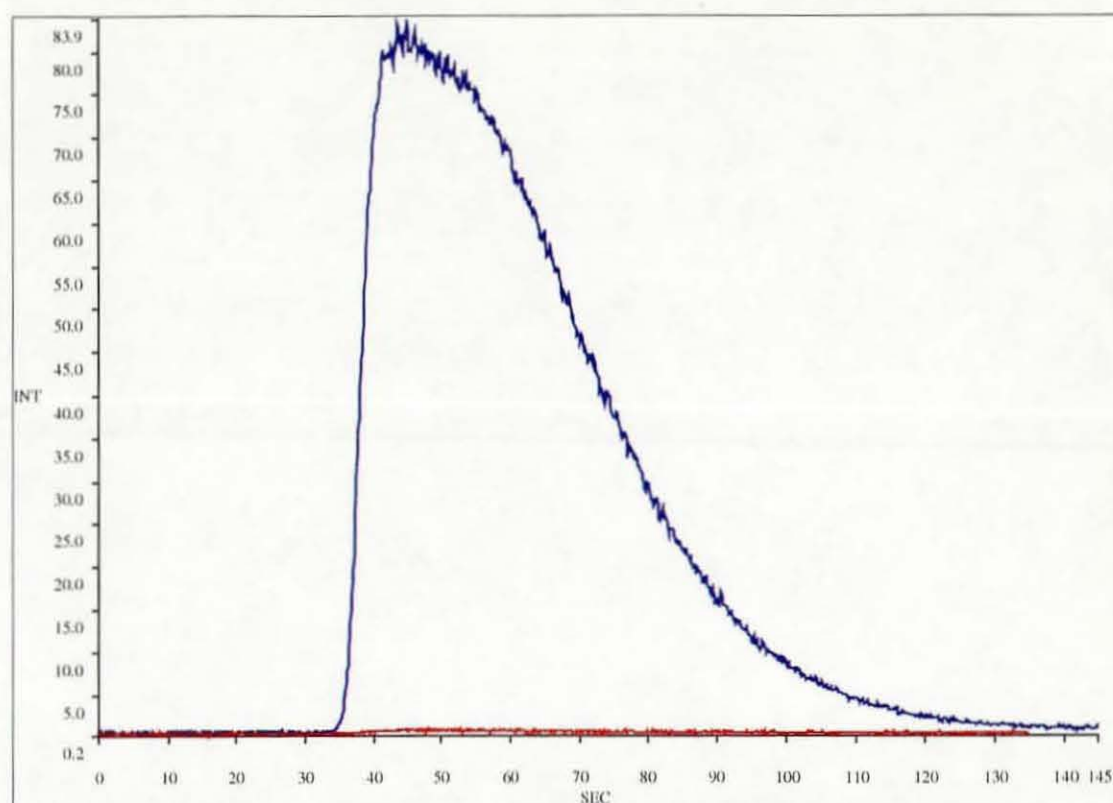


Figure 5.7 Diagram illustrating three stream flow-injection system facilitating introduction of alkali into the effluent stream.

Some simple experiments were conducted to verify the degree of quenching of fluorescein at low pH in a flowing stream. A 10 $\mu\text{g/ml}$ solution of fluorescein was injected into pH 10.2 TRIS buffer followed by injection into pH 2.5 citrate buffer (POROS elution buffer).



Key: Injection into pH 10.2 TRIS, Injection into pH 2.5 citrate.

Figure 5.8 Data showing quenching effect of pH on fluorescein fluorescence.

5.4.2 pH Change Experiments

A simple 'Y' shaped Omnifit junction, illustrated in Figure 5.9, was used to enable alkali to be bled into the effluent stream. The inlets and outlets of the junction were controlled by simple on or off valves. Note, it was not possible to precisely control the mixing of the alkali and effluent streams using this junction.

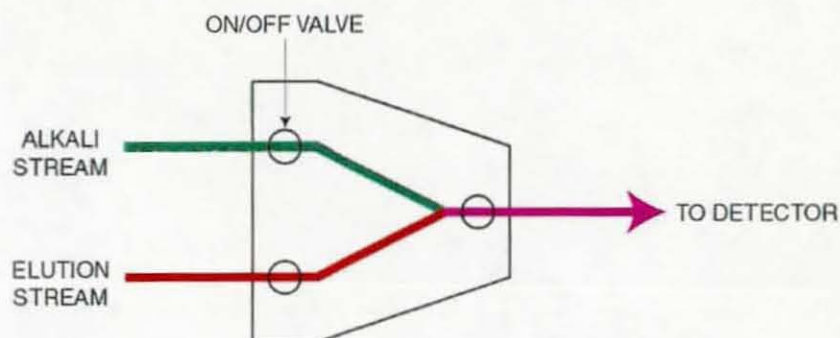


Figure 5.9 Simple 'Y' junction for introducing alkali into flow-injection system.

In order to recover the fluorescence of the quenched fluorescein it was necessary to devise a set of conditions which would raise the pH of the effluent stream from pH 2.5 to 7 or above. Two possible solutions were tested: 1) varying the composition and concentration of alkali, 2) varying the flow-rate of alkali relative to the effluent stream.

Solution two was quickly discounted as it proved impossible to get a significant difference in flow-rates between the two streams, despite varying the peristaltic pump tubing and tension settings. Note, that only one four stream peristaltic pump was available for this work. As mentioned previously, no fine-tuning of flow-rates was possible using the 'Y' junction. Varying the alkali composition and concentration proved more successful. The results are presented in the following section.

5.4.2.1 Alkali Optimisation

Various concentrations of sodium hydroxide were used to raise the pH of the effluent stream post-column. The data are shown in Table 5.3 and Figure 5.10.

Conc ⁿ of NaOH (M)	pH of effluent stream
0.031	3.3
0.063	3.6
0.13	4.7
0.18	5.5
0.21	6.4
0.25	12.4

Table 5.3 Effect of alkali concentration on post-column effluent pH.

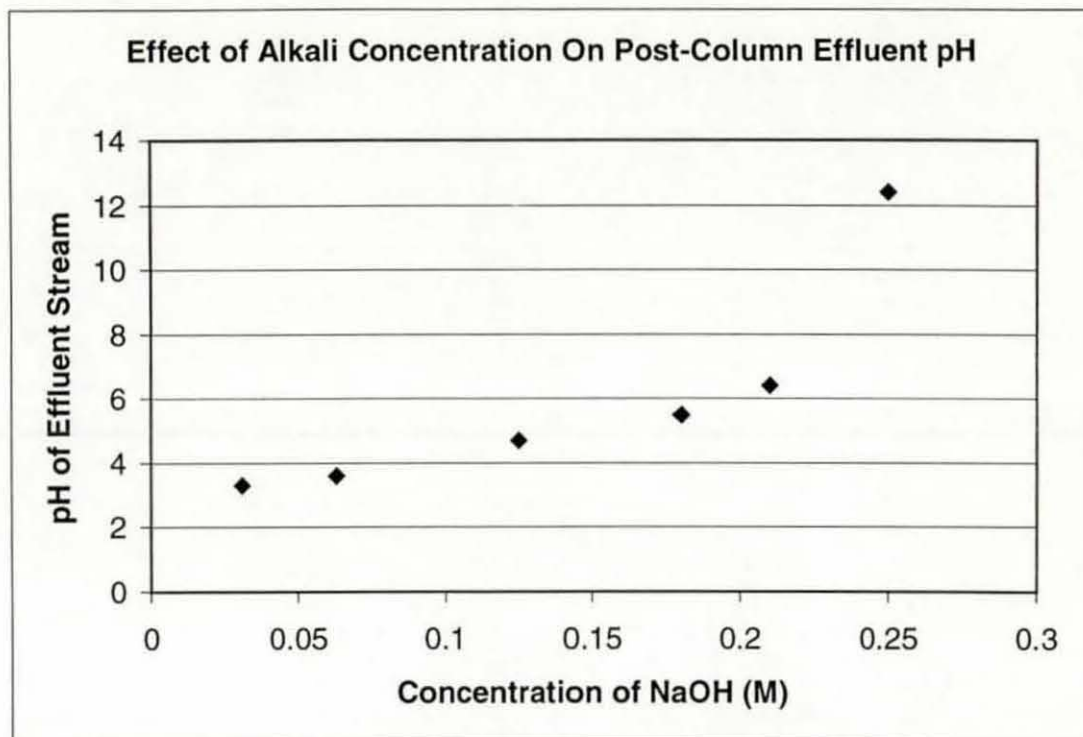


Figure 5.10 Effect of alkali concentration on post-column effluent pH.

After the alkali concentration had been optimised, some experiments were tried with native fluorescein, i.e. not the SMT-fluorescein conjugate. Unfortunately, the simple alkali junction caused too much sample dispersion resulting in broad signal peaks, such as the one in Figure 5.8. It was thought that the 'Y' junction used was too simplistic and more investigations were required to optimise the mixing of the alkali and effluent streams if the system were to be useful.

5.5 Pre-Incubation Assay For Sulfamethazine Using T-Gel

5.5.1 Flow-Rate

An experiment was conducted to determine the effect of flow-rate on antibody binding to t-gel as follows:

- 25 μ l of antisera (1:100) was injected into the FIA system, with a t-gel column in place, at various flow-rates. The column bed volume was approximately 3 mm x 6 mm ID.

- The areas of the unbound and bound peaks were recorded over the flow rate range 0.92 ml/min to 3.57 ml/min.

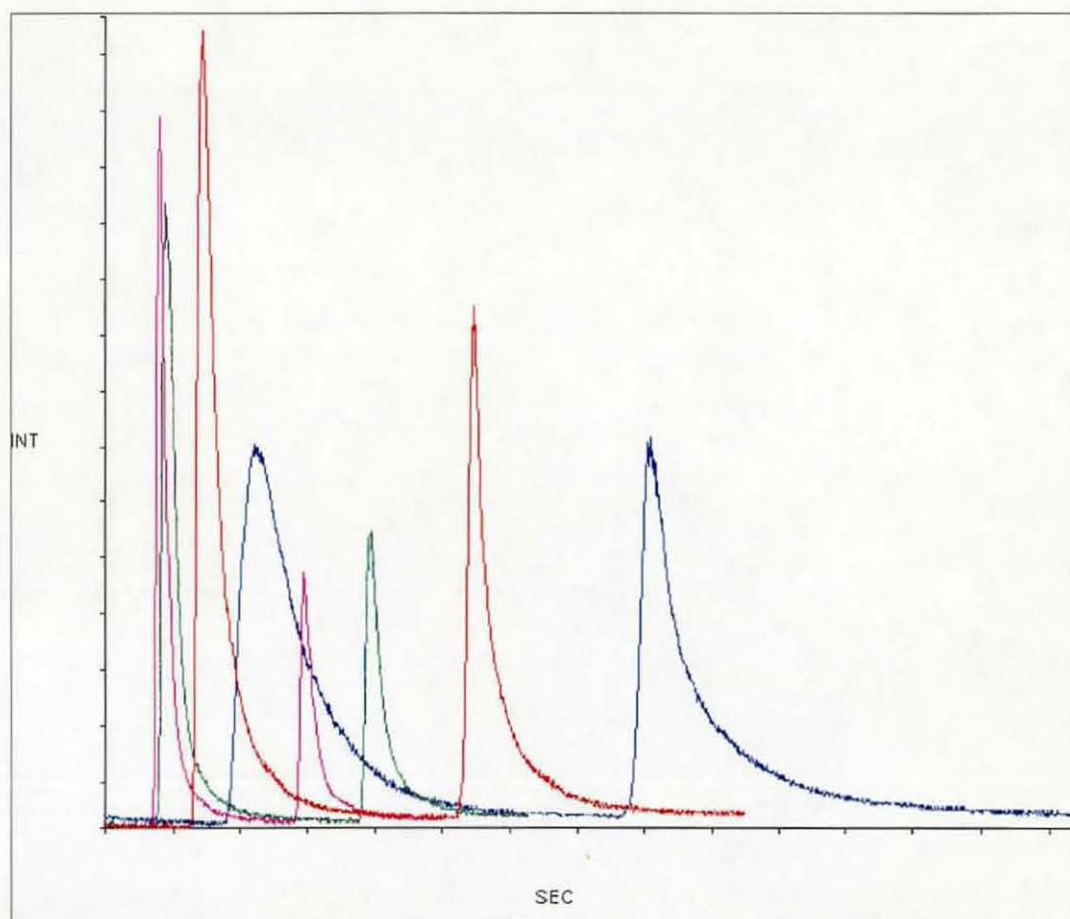
Figure 5.11 shows the effect of flow-rate on antibody binding and peak shape. The data are summarised in Table 5.4.

Flow (pump units)	Flow-rate (ml/min)	Average area [†]		% of Maximum binding [‡]	
		Unbound	Bound	Unbound	Bound
5.0	0.92	7117	6128	100	100
10.0	1.82	4320	2848	61	46
15.0	2.65	3273	1639	46	26
20.0	3.57	2617	1107	37	18

[†] Average of two injections.

[‡] Assuming maximum binding occurs at lowest flow rate.

Table 5.4 Effect of flow-rate on antibody binding.



Key: 0.92 ml/min, 1.82 ml/min, 2.65 ml/min, 3.57 ml/min.

Figure 5.11 Effect of flow rate on antibody binding.

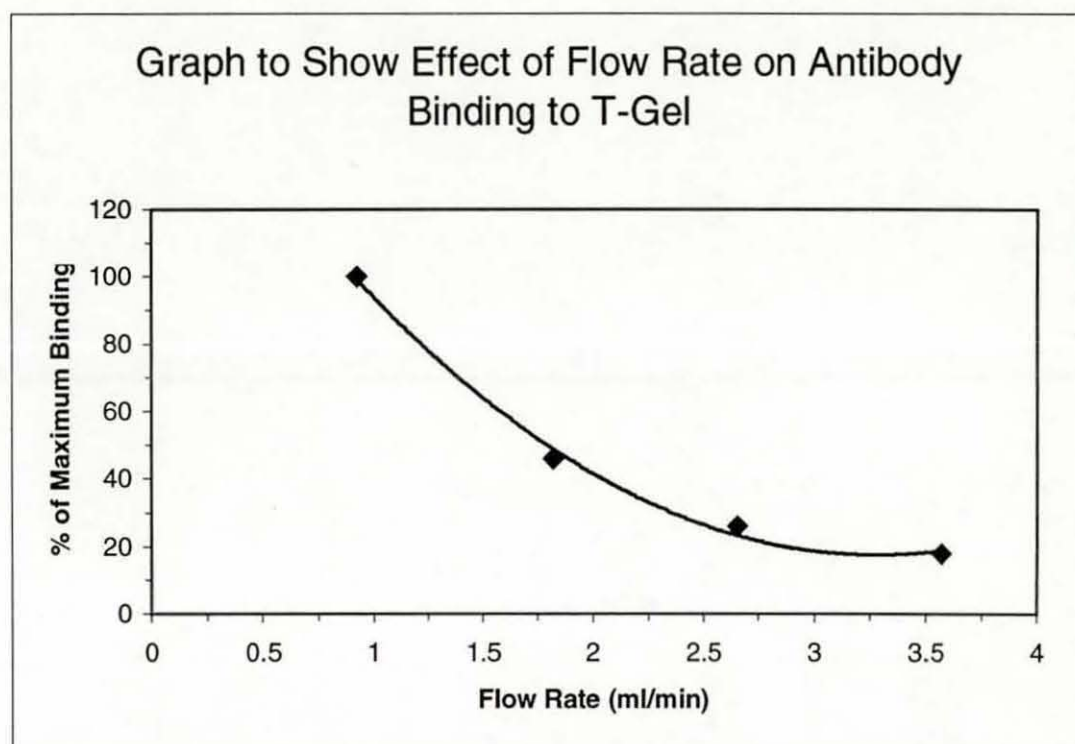


Figure 5.12 Graph to show effect of flow-rate on antibody binding to t-gel.

5.5.2 Incubation Time

An experiment was conducted to test the effect of antibody-antigen incubation time on the analytical signal. The aim of the experiment was to determine if the incubation time had to be carefully controlled in order to get good signal reproducibility. An incubation mixture was set up as follows:

50 μ l 1:100 conjugate

50 μ l t-gel binding buffer (BB)

50 μ l 1:10 Ab

- The conjugate mixture was left to stand in the dark at room temperature for the allotted time period.
- After the allotted time period, each sample was injected in duplicate and the size of the bound peak was recorded.

- The following incubation times were investigated: 0, 5, 30, 60 minutes and 24 hrs.

Incubation time (mins)	Bound peak area			
	Injection 1	Injection 2	Average	% CV
0	199	209	204.0	3.5
5	199	230	214.5	10.2
30	214	232	223.0	5.7
60	208	212	210.0	1.3
Overnight	206	232	219.0	8.4

Table 5.5 Effect of incubation time on signal reproducibility.

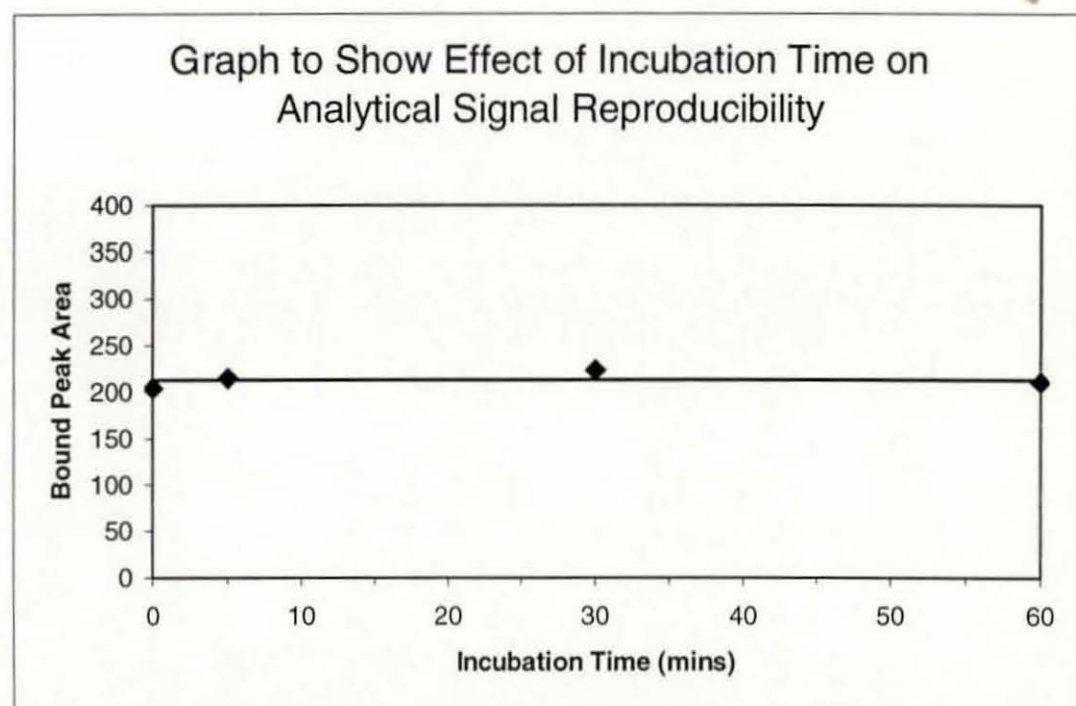


Figure 5.13 Graph showing effect of incubation time on signal reproducibility (data for time = 24 hrs omitted for clarity).

5.5.3 Antibody Concentration

An experiment was conducted to gauge the effect of antibody concentration on signal size.

An incubation mixture was set up as follows:

50 μ l 1:100 conjugate

50 μ l t-gel binding buffer (BB)

50 μ l Ab

The antibody concentration was varied from 1:10 to 1:40 and each sample was injected in duplicate.

Table 5.6 and Figure 5.14 show the data in tabular and graphical form respectively.

Antibody concentration	Bound peak area			
	Injection 1	Injection 2	Average	% CV
1	164	192	178.0	11.1
1:10	219	226	222.5	2.2
1:20	169	174	171.5	2.1
1:40	89	93	91.0	3.1

Table 5.6 Variation of bound peak area with antibody concentration.

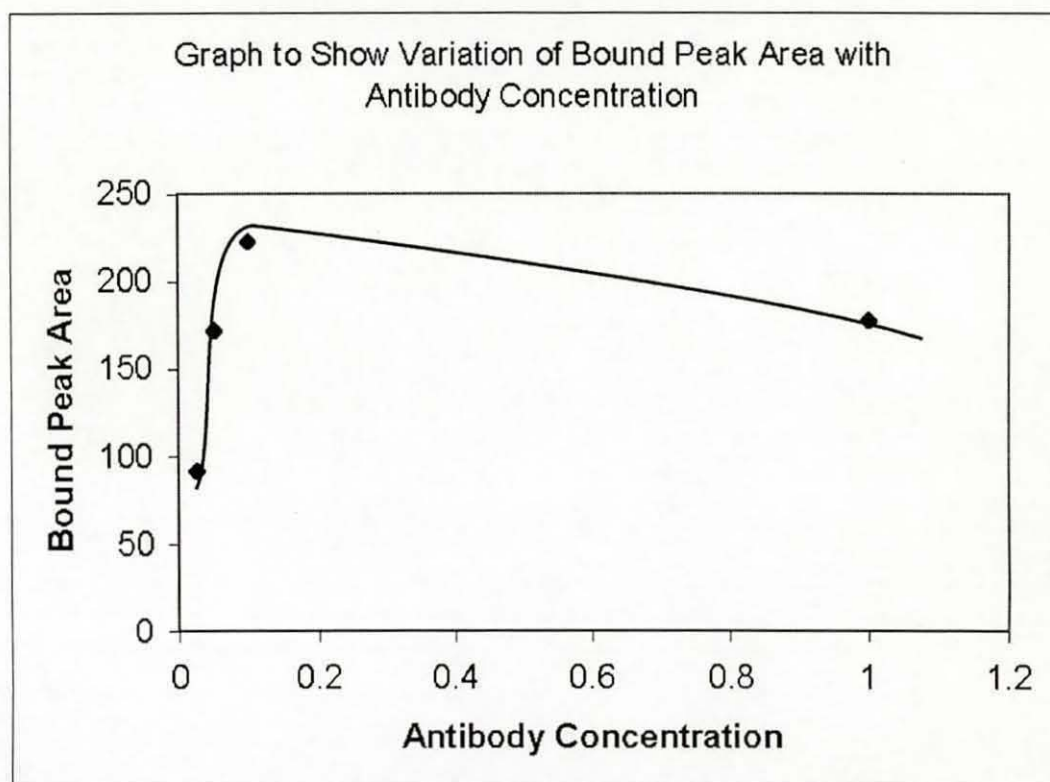


Figure 5.14 Graph to show variation of bound peak area with antibody concentration.

5.5.4 Conjugate Concentration

To optimise the conjugate concentration an incubation mixture was set up as follows:

50 μ l Conjugate

50 μ l Buffer

50 μ l 1:10 Ab

- A visibly high conjugate concentration was chosen for the first injection. The incubation mixture was then injected into the flow system and the size of the bound signal recorded.
- The conjugate was then diluted and the injection repeated. The size of the bound signal was again recorded.
- This process was repeated until a noticeable drop in the size of the bound peak occurred. This was the point at which the antibody in the incubation mixture system was no longer saturated with conjugate.

5.5.5 Amount Of Buffer In Incubation Mixture

An experiment was performed to assess the effect of buffer volume on analytical signal size.

An incubation mixture was set up as follows:

50 μ l 1:100 conjugate

50 μ l t-gel binding buffer (BB)

50 μ l 1:10 Ab

- An incubation mixture containing 50 μ l of buffer was left to stand for 5 minutes in the dark at room temperature.

- The incubation mixture was then injected into the flow-system with the t-gel column in place.
- After elution of the unbound conjugate, the flow was switched to elution buffer in order to elute the antibody bound conjugate fraction.
- The area of the bound peak was then recorded.
- The experiment was repeated with incubation mixtures containing 100, 150 and 300 μl of binding buffer. The volumes and concentrations of both conjugate and antibody were kept constant during the experiment.

Table 5.7 and Figure 5.15 show the data in tabular and graphical form respectively.

Buffer volume (μl)	Bound peak area			
	Injection 1	Injection 2	Average	% CV
50	136	143	139.5	3.5
100	109	114	111.5	3.2
150	87	84	85.5	2.5
200	72	70	71.0	2.0

Table 5.7 Effect of amount of buffer in incubation mixture on analytical signal size.

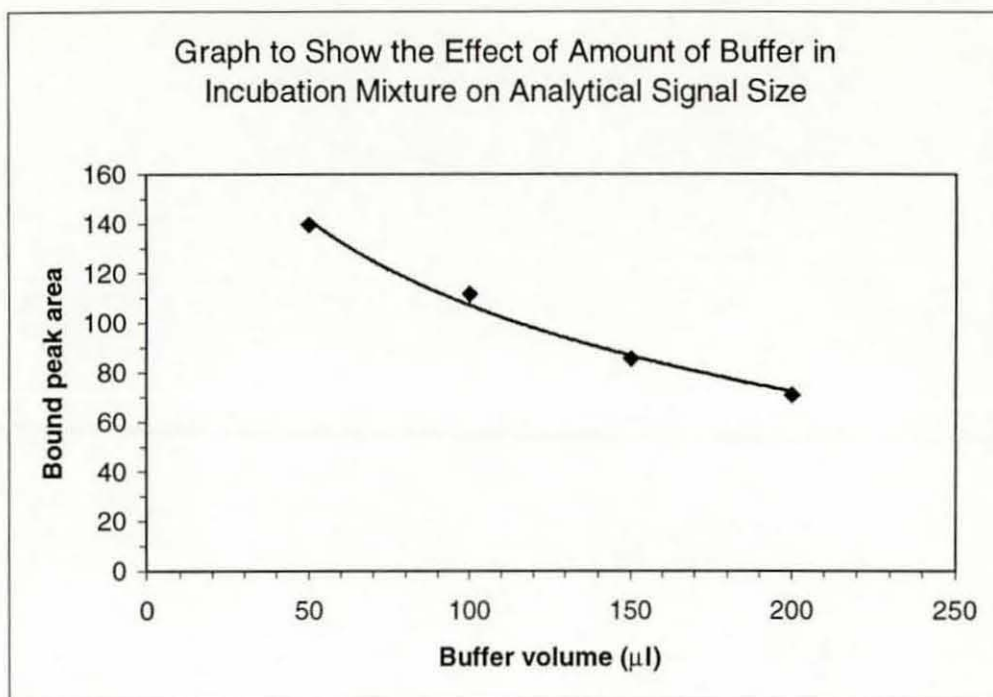


Figure 5.15 Graph to show the effect of amount of buffer in incubation mixture on analytical signal size.

5.5.6 Reproducibility Of Antibody Binding And Elution

An experiment was conducted to investigate the binding and elution efficiency of the sheep anti-SMT antibody on t-gel. The aim of the experiment was to establish if the antibody was being fully eluted, when the buffer was switched to elution buffer, leaving the column binding sites free for the next injection.

- 25 μl of 1:10 Ab was injected onto the t-gel column.
- After the unbound antibody had passed through the detector, the flow through the column was switched to elution buffer and the bound antibody eluted.
- The area of the unbound and bound peaks were recorded.
- Once it appeared the bound antibody had been fully eluted (i.e. the signal had returned to the baseline), the flow was then switched back to binding buffer prior to the next injection of antibody.

A solution of 1:10 Ab was used throughout the experiment and the flow rate was kept constant. LS 50 B fluorimeter was used with the following settings: $\lambda_{\text{Ex}} = 280 \text{ nm}$, $\lambda_{\text{Em}} = 335 \text{ nm}$. The excitation and emission slits were both set at 10 nm.

The data are presented in Table 5.8 and Figure 5.16. Figure 5.17 shows the sample traces for injections 1 and 25.

Injection number	Peak area	
	Unbound	Bound
1	4693	2681
2	4712	2671
3	4542	2595
4	4540	2484
5	4568	2606
6	4618	2624
7	4562	2509
8	4442	2593
9	4616	2473
10	4616	2420
11	4614	2435
12	4558	2427
13	†	†
14	4458	2340
15	4480	2293
16	4561	2226
17	†	2214
18	4347	2039
19	4289	2177
20	4487	2212
21	4458	2159
22	4303	2106
23	4475	2109
24	4584	2016
25	4481	2137

† Accurate integration of these peaks was not possible due to the presence of air bubbles.

Table 5.8 Unbound and bound peak areas for repeat injections of antibody on t-gel.

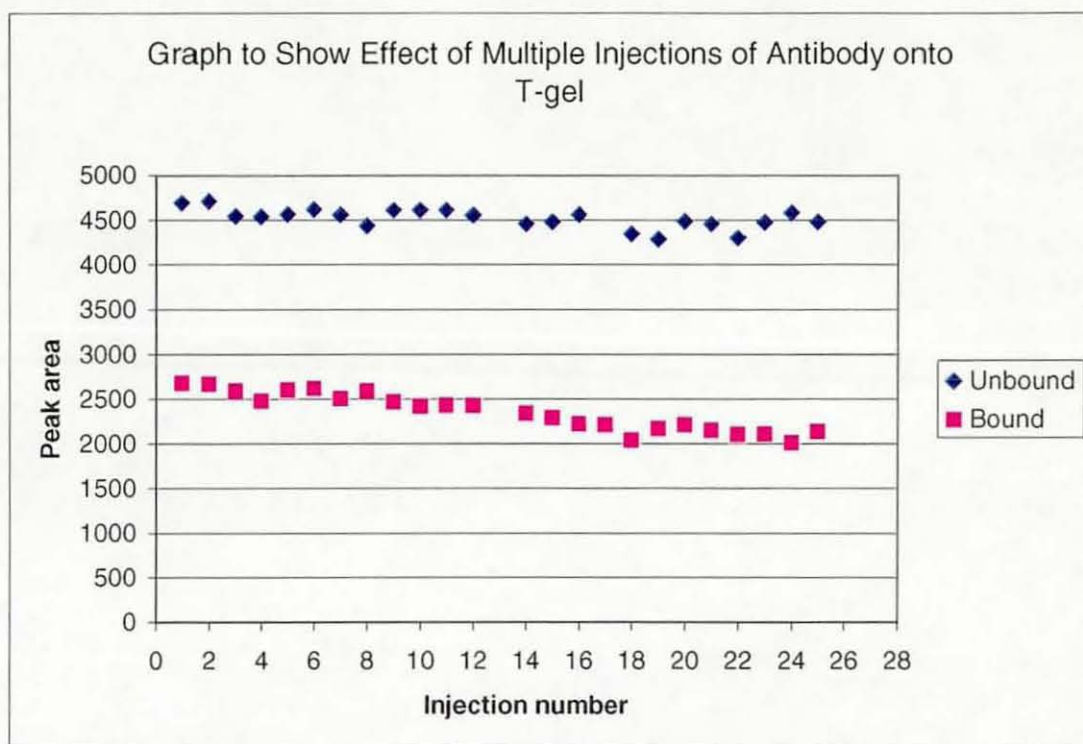
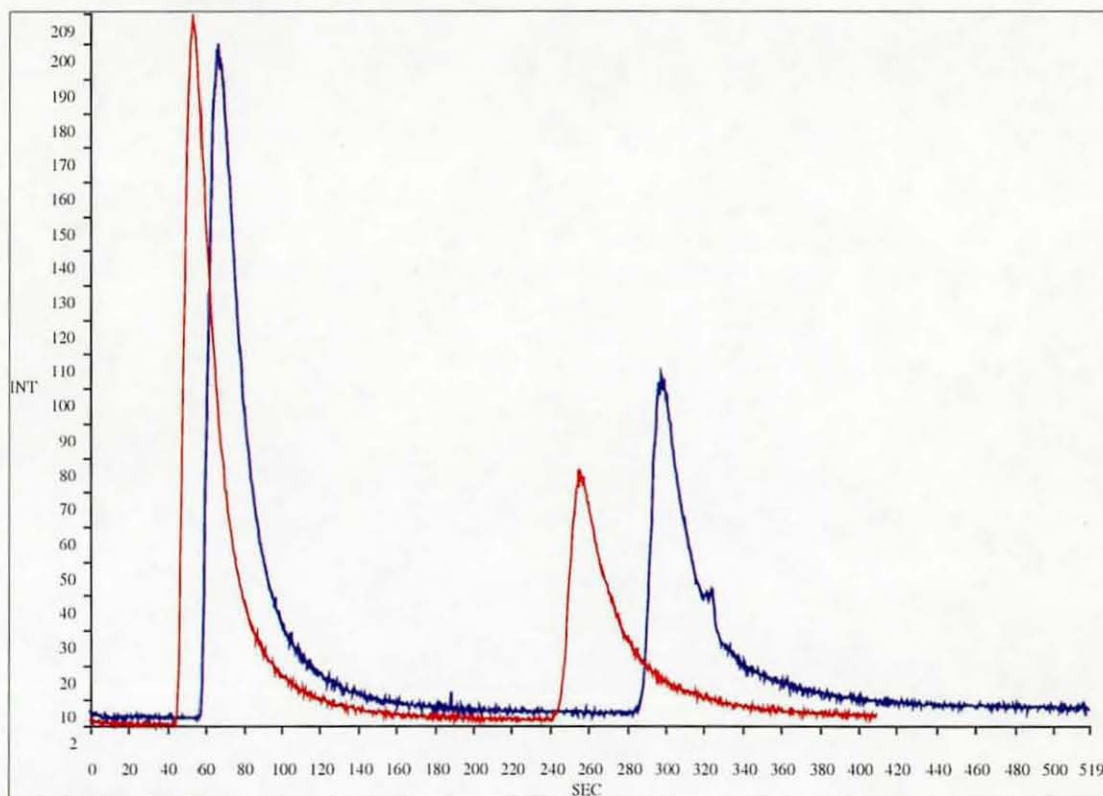


Figure 5.16 Graph to show effect of multiple injections of antibody onto t-gel.



Key: Injection 1, Injection 25.

Figure 5.17 Example detector traces showing the effect of multiple injections of antibody onto t-gel.

5.5.6.1 The Effect Of Instrumental Drift On Antibody Binding And Elution Reproducibility

An experiment was conducted to assess the effect of instrumental drift, of the LS 50 B fluorimeter, on signal reproducibility as follows:

- The same method was used as in section 5.5.6, but the fluorescence spectrum of a fluorophore immobilised in a perspex calibration block was recorded after every three injections of antibody, to check the instrument for drift.
- The calibration block used was block 6, set 1. The instrumental settings for measurement of the immobilised fluorophore were $\lambda_{Ex} = 560 \text{ nm}$; $\lambda_{Em} 560 - 590 \text{ nm}$; excitation and emission slits both set at 4 nm. Five emission spectra were recorded and averaged.

The results are shown in Table 5.9 and Figure 5.18. Figure 5.19 shows how the instrument drifts over the same time period.

The same calibration control block was used to test a sister LS 50 B instrument in an adjacent lab. The results are shown in Table 5.10.

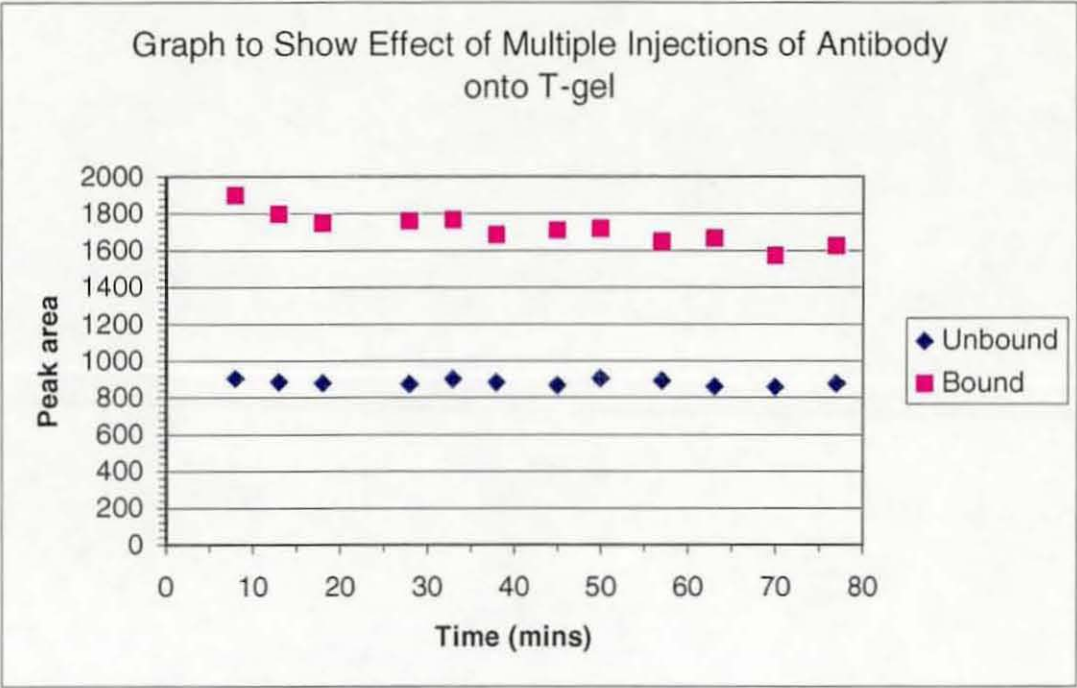


Figure 5.18 Graph to show effect of multiple injections of antibody onto t-gel.

Injection number	Time (mins)	Peak area		I_{\max} control
		Unbound	Bound	
	0			859
1	8	906	1900	
2	13	889	1798	
3	18	881	1749	
	23			842
4	28	875	1761	
5	33	903	1766	
6	38	886	1686	
	43			823
7	45	871	1710	
8	50	907	1720	
9	57	895	1648	
	62			817
10	63	863	1668	
11	70	861	1573	
12	77	878	1628	
	86			814

Table 5.9 Unbound and bound peak areas for repeat injections of antibody on t-gel showing how instrument drifts with time.

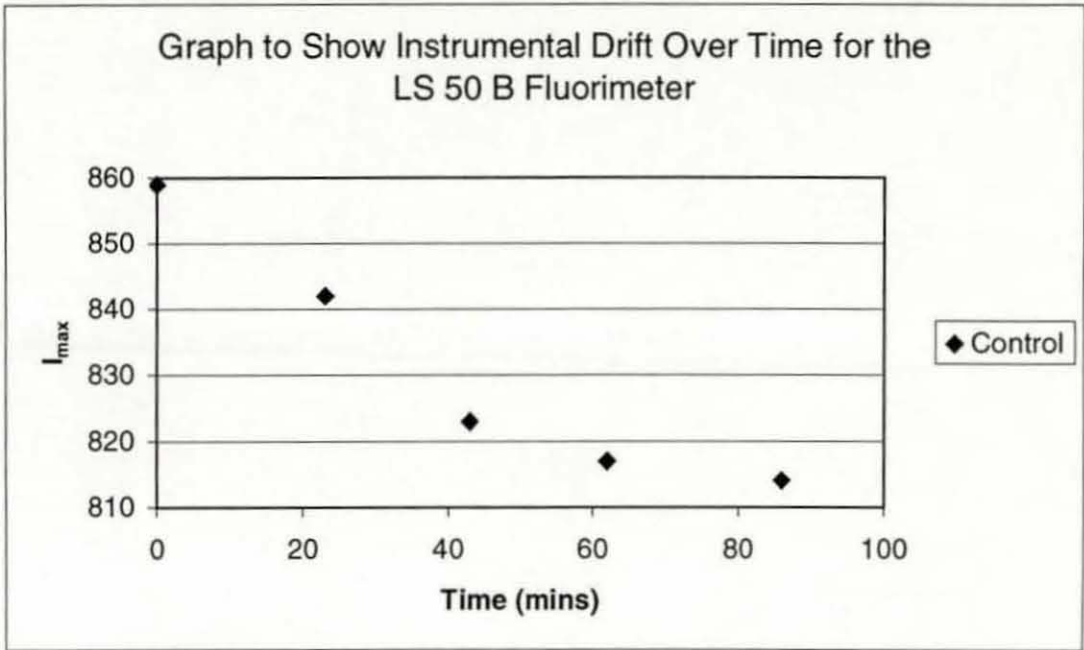


Figure 5.19 Graph to show instrumental drift over time for the LS 50 B fluorimeter.

Time (mins)	I_{max}
0	511
55	508
95	515

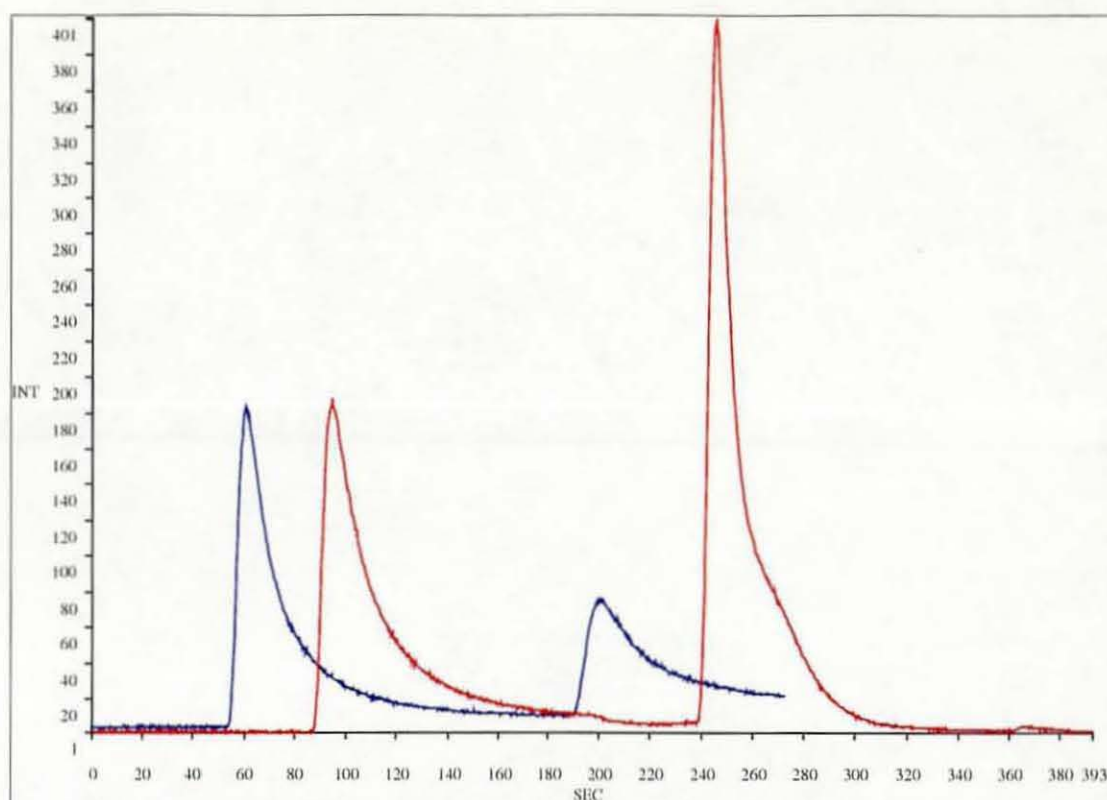
Table 5.10 Instrument drift for sister LS 50 B fluorimeter.

5.5.6.2 Optimisation Of Elution Buffer To Improve Antibody Binding And Elution Reproducibility

Two alternative buffer systems were tested to determine if the binding and elution profile could be improved.

MOPS acetate buffers were tried but showed no advantage over the standard t-gel buffers used previously.

6M urea, initially intended for use as a column wash solution, was used in place of the standard elution buffer. Figure 5.20 Shows the sample incubation mixture eluted using standard t-gel EB and urea.



Key: standard t-gel EB, urea used as EB.

Figure 5.20 Sample traces showing effect of using urea in place of standard elution buffer.

5.6 The Development Of A Calibration Curve For Sulfamethazine

Using the methodology described above, it was possible to produce a calibration curve for sulfamethazine. Curves were produced using the Perkin-Elmer LS 50 B and the more modern and sensitive Hitachi F4500 fluorimeter.

5.6.1 Perkin-Elmer LS 50 B Fluorimeter

A rough calibration curve for the LS 50 B fluorimeter was produced as follows:

- 0.1, 1.0, 10.0 and 100.0 $\mu\text{g/ml}$ sulfamethazine standards were produced by serial dilution.

An incubation mixture was set up as follows:

75 μl 1:100 conjugate

75 μl BB or unlabelled SMT

75 μ l 1:10 Ab

- The incubation mixture with no unlabelled SMT, called B_0 , was injected in triplicate. The area of the bound peaks were recorded.
- The standards were then each injected in triplicate and the area of the bound peaks were recorded.

Table 5.11 shows the data and the calibration curve is shown in Figure 5.21.

The experiment was repeated using a wider range of SMT standards. The data are shown in Table 5.12 and Figure 5.22.

SMT Conc ⁿ (μ g/ml)	Bound peak area			Average	% CV	B/ B_0 %
	Injection 1	Injection 2	Injection 3			
B_0	298	298	311	302.3	2.5	100.0
0.1	315	286	306	302.3	5.0	100.0
1.0	144	160	173	159.0	9.1	52.6
10.0	36	39	39	38.0	4.6	12.6
100.0	12	11	12	11.7	5.0	3.9

Table 5.11 Rough calibration data for SMT using LS 50 B fluorimeter.

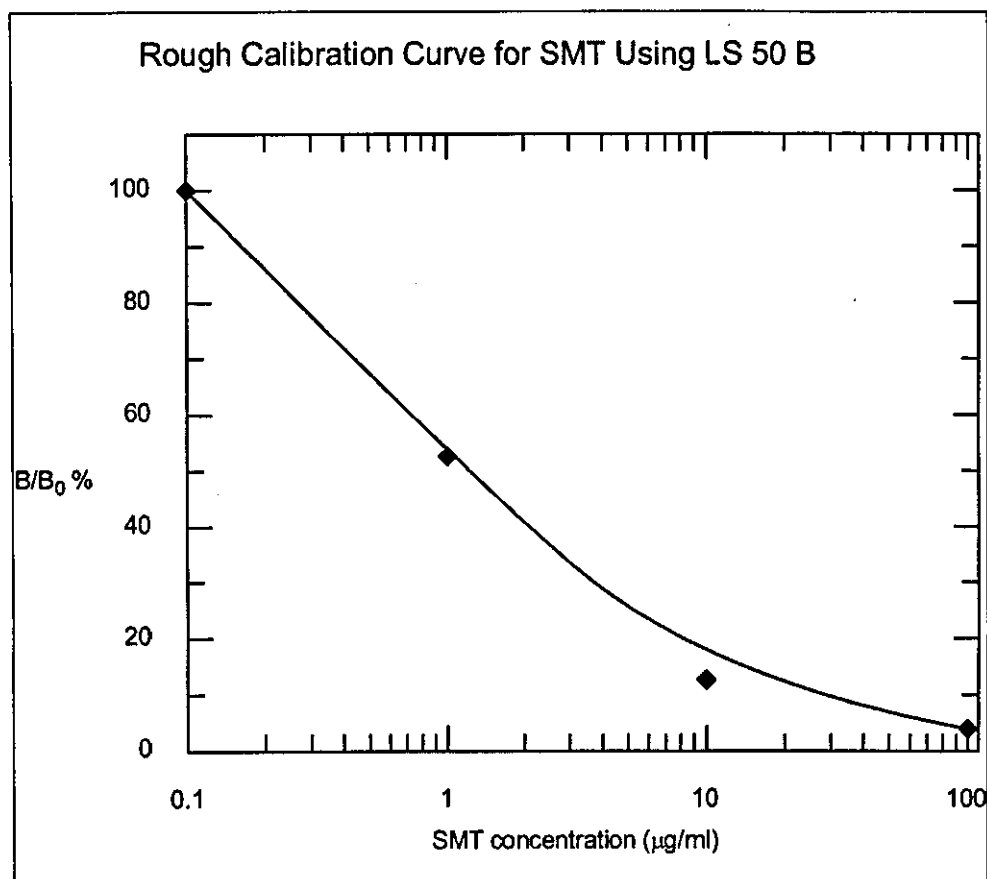


Figure 5.21 Rough calibration curve for SMT using LS 50 B.

SMT Conc ⁿ (µg/ml)	Bound peak area			Average	% CV	B/B ₀ %
	Injection 1	Injection 2	Injection 3			
B ₀	354	349	348	350.3	0.9	100.0
0.0001	358	350	361	356.3	1.6	101.7
0.0005	331	320	344	331.7	3.6	94.7
0.001	329	354	348	343.7	3.8	98.1
0.005	321	314	338	324.3	3.8	92.6
0.01	347	323	340	336.7	3.7	96.1
0.05	308	334	326	322.7	4.1	92.1
0.10	274	286	309	289.7	6.1	82.7
0.50	206	198	208	204.0	2.6	58.2
1.00	141	139	161	147.0	8.3	42.0
5.00	70	80	80	76.7	7.5	21.9
10.00	57	64	56	59.0	7.4	16.8
50.00	38	43	44	41.7	7.7	11.9
100.00	38	33	40	37.0	9.7	10.6

Table 5.12 Full calibration data for SMT using LS 50 B.

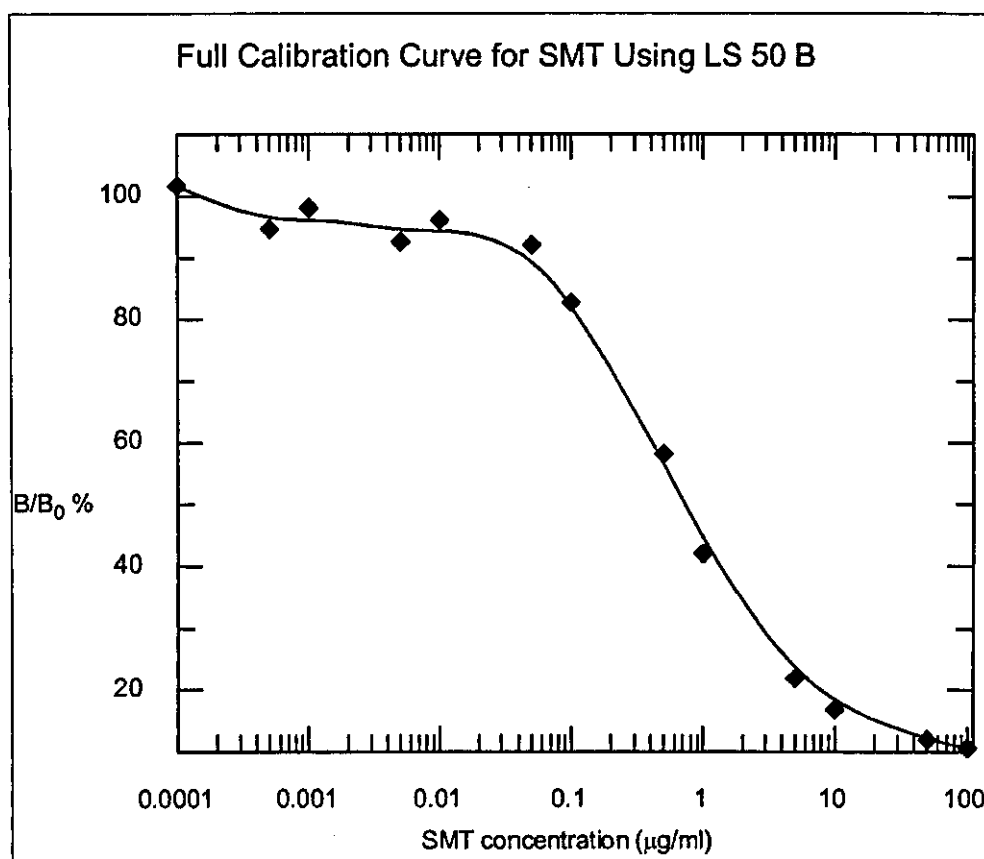


Figure 5.22 Full calibration curve for SMT using LS 50 B.

5.6.2 Hitachi F4500 Fluorimeter

A rough calibration curve for the F4500 fluorimeter was produced as follows:

- 0.0001, 0.001, 0.01, 0.1, 1.0, and 10.0 $\mu\text{g/ml}$ sulfamethazine standards were produced by serial dilution.

An incubation mixture was set up as follows:

75 μl 0.35 $\mu\text{g/ml}$ conjugate[†]

75 μl BB or unlabelled SMT

75 μl 1:100 Ab

[†] Absolute concentration of conjugate was measured for this experiment.

- The incubation mixture with no unlabelled SMT, called B_0 , was injected in triplicate. The area of the bound peaks were recorded.
- The standards were then each injected in triplicate and the area of the bound peaks were recorded.

The data are shown in Table 5.13 and Figure 5.23.

SMT Conc ⁿ ($\mu\text{g/ml}$)	Bound peak area			Average	% CV	B/ B_0 %
	Injection 1	Injection 2	Injection 3			
B_0	1450	1458	1452	1453.3	0.3	100.0
0.0001	1492	1509	1555	1518.7	2.1	104.5
0.001	1461	1396	1410	1422.3	2.4	97.9
0.01	1390	1257	1339	1328.7	5.1	91.4
0.1	771	790	767	776.0	1.6	53.4
1.0	389	405	398	397.3	2.0	27.3
10.0	256	300	267	274.3	8.3	18.9
100.0	187	200	225	204.0	9.5	14.0

Table 5.13 Rough calibration data for SMT using F4500 fluorimeter.

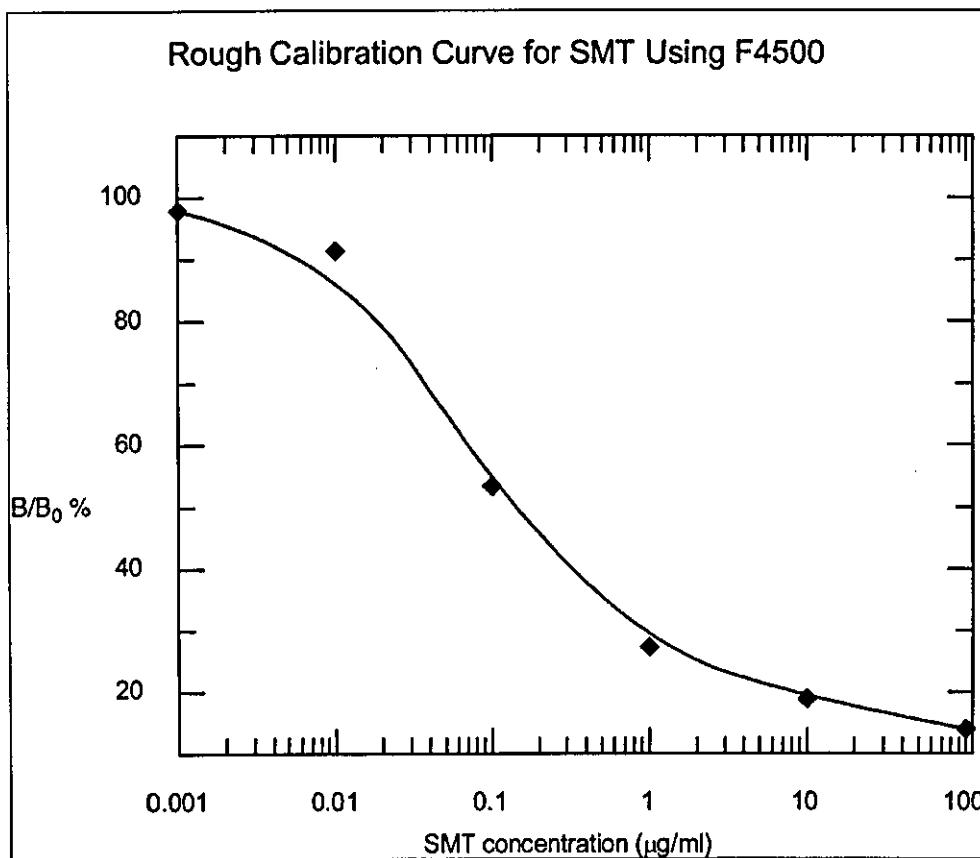


Figure 5.23 Rough calibration curve for SMT using F4500 fluorimeter.

5.6.2.1 Assay Controls

A calibration curve was obtained using the same method as described in section 5.6.2. The curve was tested with SMT samples made up separately from the calibration standards.

Table 5.14 shows the calibration data obtained for the SMT standards and Table 5.15 shows the data obtained for the control samples. Figure 5.24 shows the final calibration curve for SMT using the Hitachi F4500 fluorimeter. The controls are overlaid.

SMT Conc ⁿ (µg/ml)	Bound peak area		Average	% CV	B/B ₀ %
	Injection 1	Injection 2			
B ₀	1036	941	988.5	6.8	100.0
0.001	919	946	932.5	2.0	94.3
0.01	882	893	887.5	0.9	89.8
0.05	756	761	758.5	0.5	76.7
0.5	543	530	536.5	1.7	54.3
1.0	441	427	434.0	2.3	43.9
10.0	135	157	146.0	10.7	14.8

Table 5.14 Calibration data for SMT using F4500 fluorimeter.

SMT control Conc ⁿ (µg/ml)	Bound peak area		Average	% CV	B/B ₀ %
	Injection 1	Injection 2			
0.025	795	795	795.0	0.0	80.4
0.1	658	667	662.5	1.0	67.0
2.5	260	271	265.5	3.0	26.9

Table 5.15 Control data for calibration curve produced using F4500 fluorimeter.

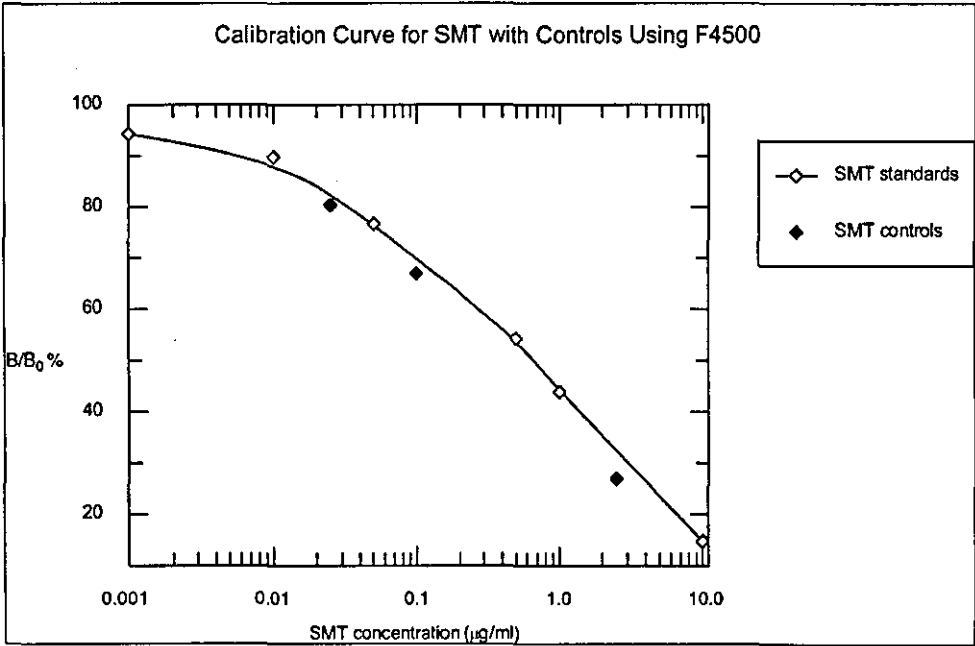


Figure 5.24 Calibration curve for SMT produced using the F4500 fluorimeter showing controls.

5.7 Life-Time Of The T-Gel Column

No long term stability trials were conducted, however it was clear from the results of the assay optimisation experiments that the t-gel was highly robust if cleaned and stored effectively. In excess of 250 injections of antibody could be executed before renewal of the t-gel was necessary.

5.8 Assay Performance

It is worth, at this stage, putting the assay performance in context with the figures set out in the initial aims of the work. The aim was to develop a screening assay capable of detecting SMT at its MRL of 100 mg/kg in kidney tissue, which translates to 0.1 µg/ml if extracted directly in an equivalent volume. The assay was optimised to detect 0.1 µg/ml SMT at the mid range of the calibration curve without any pre-concentration steps, but it should be noted that the limit of detection was over one order of magnitude lower than this. The shape of the calibration curve at this point is designed to reduce the chance of any false negative results as these would pass through the regulatory process unconfirmed. Any false positive samples would be detected at the confirmation stage.

Chapter 6

Discussion And Conclusions Of The Immunoassay For Sulfamethazine

6. Introduction

The discussion of the experimental work described in chapter five is presented, along with conclusions and suggestions for further work.

6.1 Comparison Of Protein A And T-Gel

Work by Evans¹⁵³ had shown that POROS protein A was an excellent candidate for use as a solid phase in FIIA, having excellent mechanical rigidity, coupled with a bimodal pore system, which allowed high flow rates (typically above 5 ml/min). Also, POROS protein A binds IgG with a greater specificity than t-gel. However, immunoglobulins from different animal species bind to protein A to different extents, ranging from no binding for IgG raised in chickens to strong binding for IgG raised in rabbits (Table 2.10). IgG raised in sheep was known to bind only weakly to protein A. It was envisaged that these binding phenomena would be reflected in the results of the simple antibody-solid phase binding experiments.

This was indeed the case. For POROS protein A, the bound antibody fraction only represented $\approx 5\%$ of the total peak area. In the case of t-gel, the results were much more encouraging as the bound peak area represented almost 50 % of the total peak area.

Although Evans¹⁵³ has successfully developed a FIIA for phenytoin using POROS protein A, the low level of binding of the Enviromed, sheep-raised anti-SMT to POROS coupled with the fact that a low pH is required to elute antibodies from POROS (fluorescein is quenched at low pH) initially made this matrix unattractive for use in an assay for SMT using this particular antibody. However, one cannot deny the advantages of using POROS, as described above, and before this matrix was shelved in favour of t-gel, several experiments were carried out to investigate whether it was feasible to use POROS for further assay development. These investigations are described in the following section.

6.1.1 POROS Protein A Feasibility Experiments

It is well known that fluorescein is completely, but reversibly, quenched at low pH and if POROS were to be used then this would be the first problem to tackle, as the buffer used to elute antibodies from POROS is pH 2.5 citrate. The degree of quenching of fluorescein, at low pH is illustrated in Figure 5.8. In order to recover the fluorescence of the quenched fluorescein it would be necessary to raise the pH of the eluent stream from pH 2.5 to 7 or above.

A third reagent stream was added to the simple flow-injection system illustrated in Figure 2.9. The aim of this stream was to introduce alkali to the system, post-column in order to raise the pH of the effluent stream before detection. The resulting system is shown schematically in Figure 5.7. A simple 'Y' shaped Omnifit junction was used, the inlets and outlets to which, were controlled by simple on or off valves. It was not possible to precisely control the mixing of the alkali and elution streams using this junction.

The pH change experiments (section 5.4.2) clearly showed that it was possible to raise the pH of the citrate stream from 2.5 to 7 or above, using this simple system. A sodium hydroxide concentration of 0.25 M raised the pH from 2.5 to 12.4. However, when similar experiments were tried using native fluorescein it was found that the fluorescence was indeed recovered, but the alkali junction caused a high degree of dispersion. If the system were to be used in a real assay, it was clear that the alkali junction would need further development to minimise the sample dispersion caused by the introduction of the alkali.

The advantage of using POROS protein A over t-gel would be that the POROS matrix is rigid, facilitating high flow-rates, coupled with a higher specificity for IgG than t-gel. However, it was thought that the advantages gained by utilising the superior binding capabilities of the t-gel, coupled with the fact that the pH remained constant throughout, would far out-weigh those gained by using POROS protein A. The primary aim of the development stage is to produce an assay capable of detecting SMT at its MRL. Increasing the speed of the assay is an important, but secondary consideration.

It was noted that with further development, it would be feasible to use POROS protein A with the fluorescein conjugate. However, it was thought that thiophilic gel would provide the quickest and most effective route to a successful assay and it was therefore selected for use in all further method development.

6.2 Choice Of Assay Mode – Pre-Incubation Vs. On-line Incubation

In flow-injection immunoassay, there are two principle assay modes. The first involves incubation of the antibody with the analyte of interest prior to injection onto the immunoreactor, hence the name pre-incubation. In this way, it is possible to perform the reaction in a small sample vial and use the t-gel column to separate the reacted and unreacted material. This method is illustrated in Figure 6.1.

The second method involves the injection of the antibody onto the column followed by the analyte of interest. The antibody would be first bound to the t-gel and then the analyte binds to the antibody. The reaction, or incubation is occurring on the column itself, and this approach is therefore referred to as on-line incubation and is illustrated in Figure 6.2.

Once again, the choice of approach involves a trade off between speed and sensitivity. The pre-incubation method is inherently more sensitive because antibody and analyte are allowed to reach equilibrium prior to injection into the flow-injection system. However, in the case of on-line incubation, the antibody, analyte and conjugate are in contact for only a very short time. This is determined principally by the flow-rate, but column bed volume is also important.

Evans¹⁵³ investigated both assay modes using a fully automatic sample handling system. However, in the absence, at this stage, of an automatic sample handling system, it was felt that the pre-incubation method would provide the most effective route to a sensitive assay. The pre-incubation method was therefore selected as the mode of choice.

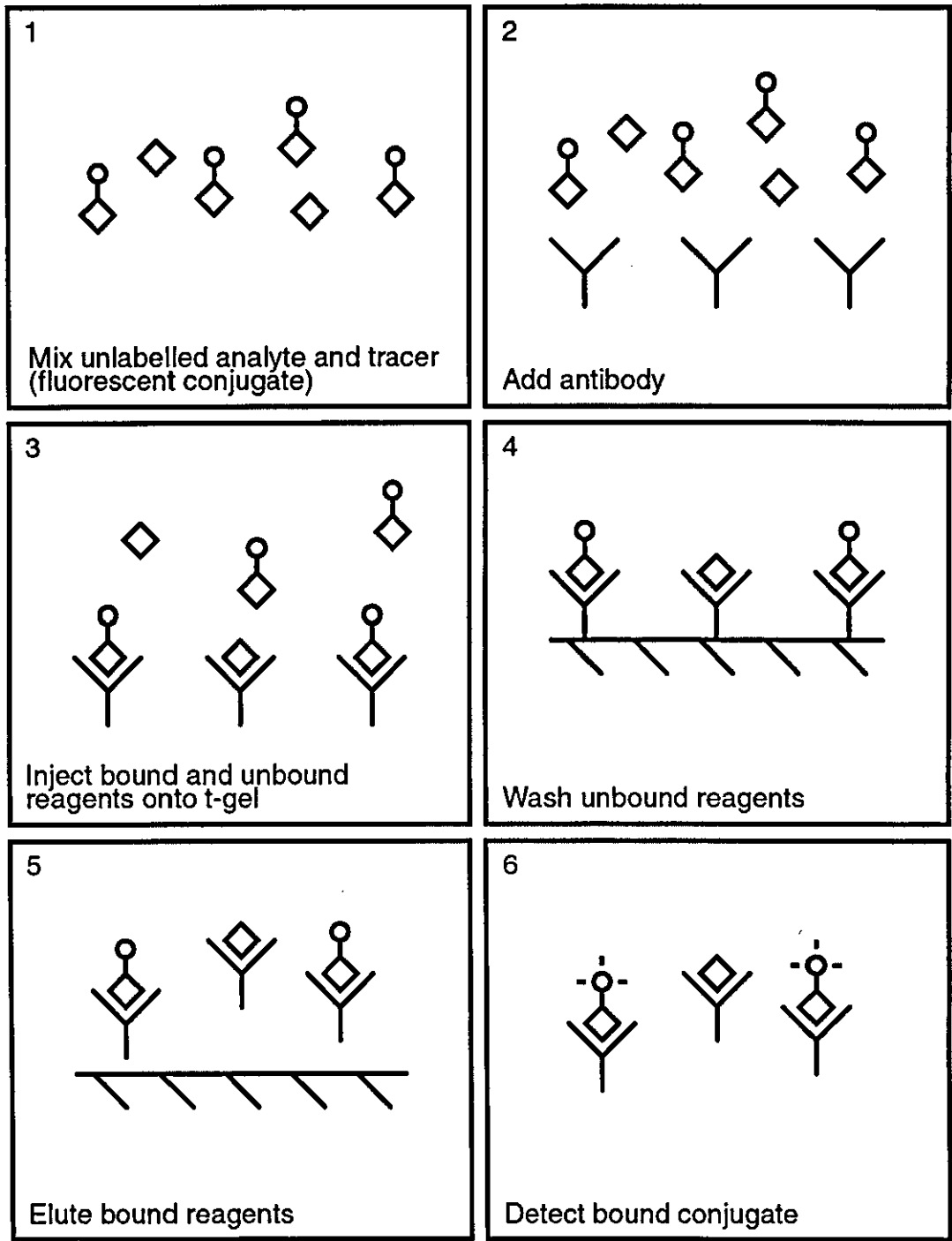


Figure 6.1 Schematic diagram illustrating pre-incubation assay mode.

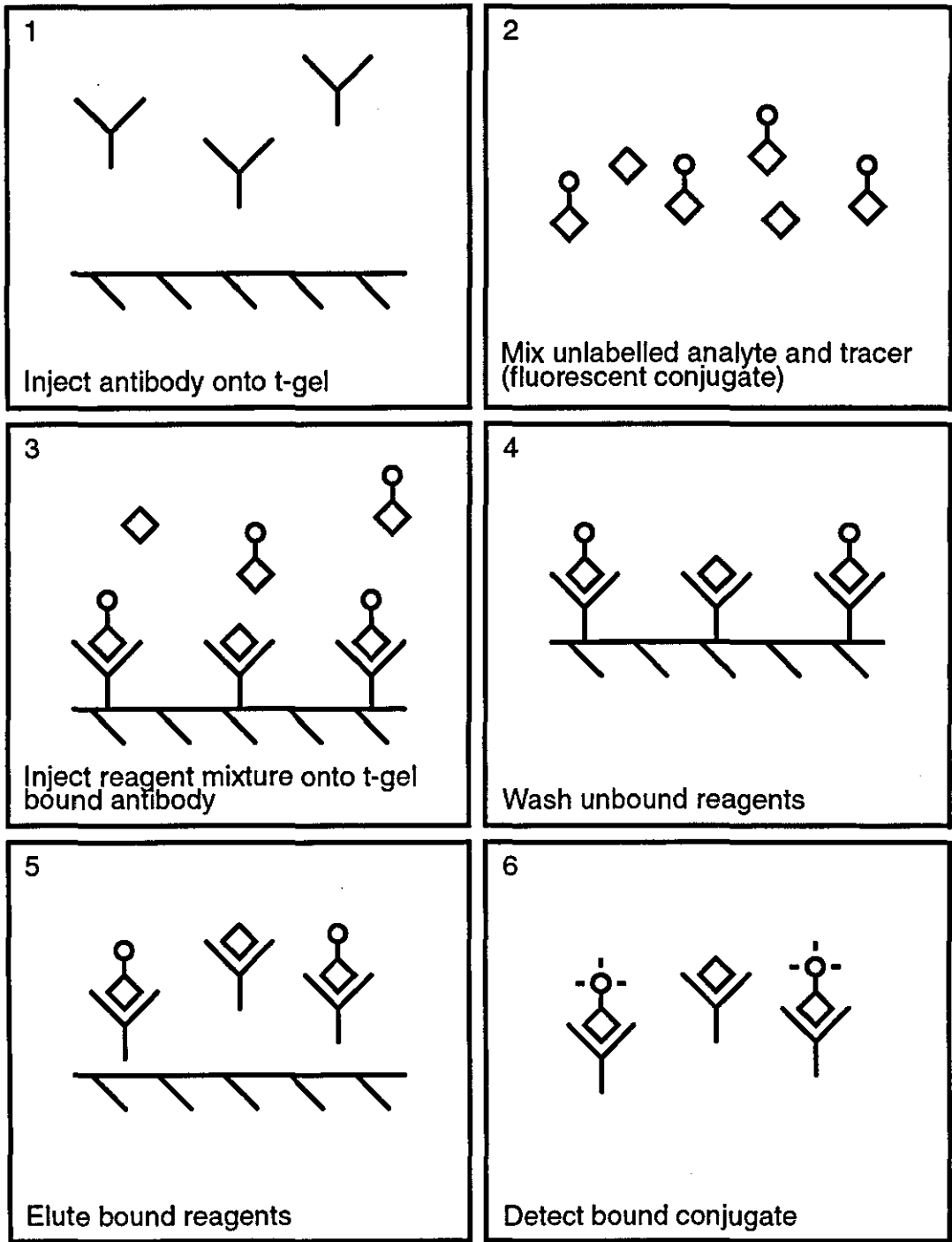


Figure 6.2 Schematic diagram illustrating on-line incubation assay mode.

6.3 Pre-Incubation Assay For Sulfamethazine Using T-Gel

6.3.1 Flow-Rate

As discussed briefly above, flow-rate has a large effect on the amount of antibody binding to the solid phase. This is not surprising when one considers that the faster the flow-rate, the less time the antibody and solid-phase (t-gel) are in contact, therefore, there is less overall binding than at a slower flow-rate.

The experiment to determine the effect of flow-rate on antibody binding was carried out with a column bed volume of 3 mm x 6 mm ID. This bed volume was chosen to allow a range of flow-rates to be tested without the back pressure of the column becoming such that it impeded the flow. The data presented in Table 5.4 and Figure 5.11 clearly show two important phenomena. Firstly, as the flow rate is increased, the total area of the bound (and unbound) peak decreases. A doubling of the flow-rate from 0.92 ml/min to 1.82 ml/min results in a 50 % decrease in the area of the bound peak. This is because of the shorter residence time of the peak in the flow-cell.

Secondly, as the flow-rate increases the total elution time of the unbound and bound peak decreases. This effect is accompanied by a marked sharpening of the peak shape. A sharp peak shape is desirable in chromatography because the assay time is shortened and the integration of the peak is more accurate and reproducible.

Once again, there is a trade off between assay sensitivity and speed. As in the previous discussion (section 6.1) it was decided, at this stage, to err on the side of sensitivity rather than speed. Therefore, a flow-rate of 1.82 ml/min (10.0 pump units) was chosen for further work.

6.3.2 Incubation Time

When using the pre-incubation format, it is important that one carefully controls the length of the antibody-antigen incubation time, particularly if it is known that the antibody-antigen equilibrium time is long. The aim of the

incubation time experiments was to determine if this was the case with the Enviromed anti-SMT and the synthesised conjugate.

The results of the experiment (Table 5.5 and Figure 5.13) clearly show that equilibrium between antibody and conjugate is reached very quickly, in fact virtually straight after mixing. The result for bound peak area with no incubation time (i.e. simply mix antibody, conjugate and inject) is the same as when the incubation mixture is left for 5, 30, 60 minutes or 24 hours.

The results mean that any assay error from the mixing step will probably be small. An incubation time of 5 minutes was chosen for all further experiments.

6.3.3 Antibody Concentration

An obviously important consideration in the development of an immunoassay is the most efficient use of the antibody. This is principally because antibodies are extremely expensive to purchase or raise. The effective use of antibody is particularly important when one is developing a FIIA method because much larger quantities of antibody are used than in for example a micro-titre plate type assay.

Reducing the amount of antibody in the incubation mixture, while keeping everything else constant, will have two principle effects. The first of which is that the size of the analytical signal will be reduced. The second and equally important effect is that the assay sensitivity will increase. This is because there will be greater competition between labelled analyte (conjugate) and unlabelled analyte for the limited amount of antibody present (section 2.4.1).

The antibody concentration experiments were conducted to gauge the effect of antibody binding on analytical signal size and not to chose the final concentration of antibody for the assay, as it was noted that this concentration may need to be further optimised to achieve the desired assay sensitivity.

The results showed that a 1:10 dilution of the original stock solution of antibody gave, at this stage, the best signal size. There was no advantage in

using a higher concentration of antibody as this led to a decrease in signal size. Using a lower concentration of antibody, predictably also resulted in a decrease in signal size.

Therefore a 1:10 dilution of the original antibody stock solution was preliminarily chosen for use in further assay development.

6.3.4 Conjugate Concentration

The conjugate concentration was optimised as described in section 5.5.4. The concentration of conjugate was chosen such that the finite amount of antibody in the incubation mixture was just saturated with conjugate. This meant that even a small amount of unlabelled antigen would displace conjugate from the antibody and give a notable decrease in the size of the bound peak.

6.3.5 Amount Of Buffer In Incubation Mixture

Some simple experiments were carried out to determine the effect of buffer volume on the size of the bound peak. Initially, a volume of 50 μl for each of the conjugate, buffer (which would ultimately contain the desired concentration of unlabelled analyte), and antibody were chosen. This allowed two injections of each sample to be made easily.

The results in Table 5.7 and Figure 5.15 show that increasing the amount of buffer in the incubation mixture, caused a decrease in the signal size. This is principally due to dilution effects.

It was therefore decided to keep the volumes of conjugate, buffer and antibody constant at 50 μl for further assay development as this minimised the amount of reagents used and provided a volume which could be pipetted easily and accurately. To comfortably get 3 injections, 75 μl of each constituent of the incubation mixture was required.

6.3.6 Reproducibility Of Antibody Binding And Elution

The use of a solid phase such as thiophilic gel enables antibodies to be captured and released under mild conditions. However, if the assay is to be

reproducible, the binding and elution of antibodies to and from the solid phase also needs to be highly reproducible. Having consistent binding and elution will improve the accuracy of the assay and prolong the life of the column. It is desirable that the column cleaning and storage procedure be as simple as possible.

The experiments in this section are designed to investigate and optimise the antibody binding and elution the t-gel column. Initially, the standard t-gel binding and elution buffers were used (see section 3.3.4) and the column cleaning step consisted of flushing the column for 15 minutes with elution buffer prior to storage in the fridge at 4 °C.

Using this buffer system, it was observed (Figure 5.16) that the size of the bound peak decreased quite markedly over the period of 25 injections. This presented a serious problem because in a real assay, there would need to be a minimum of 18 standard injections plus 9 control injections, plus samples.

It was suspected that 2 factors were responsible for the decrease in the size of the bound peak over time. The first possible reason was that the LS 50 B fluorimeter was drifting (i.e. losing sensitivity) over the course of the experiment. The second reason is that the elution step is not effectively removing all of the bound antibody from the column. Therefore the binding capacity of the column is being reduced over time.

The following sections outline the investigation and solution of these problems.

6.3.6.1 The Effect Of Instrumental Drift On Antibody Binding And Elution Reproducibility

To check the instrumental drift, a fluorescence calibration standard was used as described in section 5.5.6.1. The results show that over a period of 12 injections (approximately 80 minutes) the instrument sensitivity does drift significantly downwards from 859 fluorescence intensity units to 814. This would, of course, be more over a larger number of injections.

A similar experiment was conducted on a sister LS 50 B being used in an adjacent laboratory. This instrument exhibited no drift over a comparable time period.

It is therefore safe to conclude that instrumental drift is partly, if not wholly, responsible for the decrease in the size of the bound peak over time. This presented a problem, as it was not considered viable to call an engineer to remedy the fault due to the age of the instrument and the potential cost involved.

The solution would simply be to use an internal standard and adjust the results accordingly or more simply, use a different instrument. It was decided, at this stage, to continue the method development on the LS 50 B without use of an internal standard as it was known that a more modern and sensitive fluorimeter (Hitachi F4500) would soon become available.

6.3.6.2 Optimisation Of Elution Buffer To Improve Antibody Binding And Elution Reproducibility

It was suspected that the decrease in the size of the bound antibody peak, over time, was not simply due to instrumental drift alone and an attempt was made to improve the elution conditions to ensure all of the antibody was removed from the t-gel matrix prior to the next injection. However, if an aggressive cleaning solution were to be used, it is important that it has little or no detrimental effect on the chemical stability of the column.

Work done in 1998 by Berna *et al.*¹⁹⁸ had indicated that a solution containing a high concentration of urea was an effective cleaning agent for thiophilic-gel. When the t-gel column was cleaned at the end of a days work with a 6 M urea solution, a large protein peak was observed. It was immediately clear that the hypothesis was indeed true, not all of the antibody had been effectively removed from the column between each injection. However, simply using the urea prior to column storage would not solve the in-day variation observed.

It was therefore decided to use the 6 M urea wash solution in place of the traditional t-gel elution buffer. Along with improving the reproducibility of the antibody elution, another unexpected effect was observed. The elution peak was sharper and taller, making quantitation far more accurate. This also meant that the total time per assay was also shortened.

6.4 Assay Development Summary

Action	Effect	Overall effect on assay
↑ flow-rate.	↓ amount of Ab binding, but sharpens peak shape.	↓ sensitivity, but ↑ assay speed.
↓ flow-rate.	Maximised Ab binding, but broadens peak shape thus lengthening assay time.	↑ sensitivity, but ↓ assay speed.
↑ or ↓ incubation time.	No measurable effect.	No effect on sensitivity
↑ Ab concentration in incubation mixture.	↑ signal size (up to a point) but ↓ competition between conjugate and unlabelled analyte.	↓ sensitivity and ↑ overall assay cost.
↓ Ab concentration in incubation mixture.	↓ signal size, but ↑ competition between conjugate and unlabelled analyte.	↑ sensitivity and ↓ overall assay cost.
↑ conjugate concentration in incubation mixture.	↑ signal size, but needs to be carefully optimised to ensure good competition between conjugate and unlabelled analyte.	Can ↑ or ↓ sensitivity.
↑ buffer volume in incubation mixture.	↓ signal size, but ↑ competition between conjugate and unlabelled analyte. Needs to be carefully optimised to allow 3 sample injections without significantly affecting sensitivity.	Can ↑ or ↓ sensitivity.
↓ or eliminating instrumental drift.	Signal size is maintained and reproducibility ↑.	Ultimately ↑ sensitivity and accuracy.
Ensuring complete elution of Ab between sample injections.	Maximum t-gel is available for Ab binding each column cycle.	Ultimately ↑ sensitivity and accuracy.

Table 6.1 Assay development summary.

It is clear from Table 6.1, and the preceding sections, that there are numerous and conflicting experimental factors to be optimised if a flow-injection fluoroimmunoassay is to function effectively at the desired sensitivity level. It is the application of these rules, sometimes simultaneously, that leads the analyst to the best possible set of experimental conditions for each assay.

Indeed it is not possible to produce a bullet point recipe to arrive at the best overall method, but it is the application of a series of trade-offs which will lead the analyst to the best solution.

The following sections describe the application of these rules to produce working calibration curves for sulfamethazine on two instruments of different sensitivity, the Perkin-Elmer LS 50 B and the Hitachi F5400.

6.5 The Development Of A Calibration Curve For Sulfamethazine

6.5.1 Perkin-Elmer LS 50 B Fluorimeter

The development of the calibration curves took place in two phases. Firstly, a calibration curve was produced with roughly optimised experimental factors. This rough calibration curve gave an indication of the assay sensitivity and working range.

The results of the rough calibration curve (Table 5.11 and Figure 5.21) were better than expected for a first attempt. The % CV's were typically 5 % or below and the assay was reasonably sensitive; the 1.0 $\mu\text{g/ml}$ calibrator giving a 40 % drop in B/B_0 %.

The calibration curve was then repeated with a wider range of SMT standards and the concentration of conjugate was optimised to give the best signal response. The results show (Table 5.12 and Figure 5.21) that the SMT standard of 0.10 $\mu\text{g/ml}$ did cause a significant drop in the B/B_0 % value, from 100.0 % to 82.7 %.

Several attempts were made to improve the assay sensitivity, i.e. optimising instrumental parameters, reducing the amount of Ab to improve competition between conjugate and unlabelled SMT and optimising the flow-rate, but it proved impossible to increase the sensitivity further.

The principle reasons for the lack of assay sensitivity with the LS 50 B are: 1) the instrument is simply outdated and less sensitive than modern fluorimeters and 2) the flow-cell arrangement in the fluorimeter was not ideal. It was not possible to adjust the position of the flow-cell in the fluorimeter and only one flow cell was available, itself not optimised for use in this instrument.

6.5.2 Hitachi F4500 Fluorimeter

The results obtained using the Perkin-Elmer LS 50 B fluorimeter were encouraging, but it was clear that the sensitivity of the assay would have to be increased by at least one order of magnitude of the assay were to be useful as the MRL for SMT is 0.1 µg/ml.

The assay was roughly optimised for use on the F4500 instrument and similar experiments were carried out as with the LS 50 B. The fact that the F4500 instrument was more sensitive than the LS 50 B allowed lower concentrations of Ab and conjugate to be used.

Firstly, a rough calibration curve was constructed (Table 5.13 and Figure 5.23) and results showed that the assay sensitivity had increased by one order of magnitude with no significant loss of assay precision. The 0.10 µg/ml SMT standard now caused a drop of 45 % in the B/B_0 value where previously, a 1.0 µg/ml SMT standard was required to cause this response.

The second stage was to produce a full calibration curve and test it with independently made up controls. The data for the final calibration curve are shown in Table 5.14, Table 5.15 and Figure 5.24. The controls show good agreement with the calibration curve.

The extra sensitivity gained by using the F4500 instrument was due to: 1) the F4500 being a more modern and sensitive instrument (complete with much improved peak quantitation software). This meant that much lower concentrations of antibody and conjugate could be used without loss of signal intensity or assay precision and 2) the flow cell was optimised for this particular instrument.

6.6 Assay Development Conclusions

The assay was developed using a Perkin-Elmer LS 50 B fluorimeter but despite extensive assay optimisation it was not possible to reach the desired sensitivity of 0.10 $\mu\text{g/ml}$ of sulfamethazine. However the assay developed did have respectable % CV's for a manual injection manifold, typically around 5 %. These would doubtless be improved by use of an automatic sample handling system.

It was possible, however, to reach the desired assay sensitivity using the more sensitive Hitachi F4500 fluorimeter. This was made possible because of the inherent greater sensitivity of the F4500 over the older LS 50 B, coupled with the use of a flow-cell, which was optimised for that particular instrument. This allowed lower concentrations of antibody and conjugate to be used, thus providing a route to the increased sensitivity required. A significant benefit of using the F4500 is that the quantity of antibody used is reduced 10 fold, therefore the price per assay is also reduced.

The assay was tested using independently made up controls, but due to time constraints, a full assay validation was not possible.

6.6.1 Sample Throughput

Typical sample throughput was in the order of 5 minutes per sample. This included adequate time for the column to re-equilibrate prior to the next injection. Use of an integrated autosampler and injection manifold would doubtless reduce this time.

6.6.2 Repeatability

Assay reproducibility was good considering a fairly crude manual injection manifold was used throughout this work. % CV's of 5 % were typical and in some cases, even lower.

When the % CV's worsen or the binding capacity of the column is visibly reduced, a renewal of the t-gel is usually required. The t-gel itself is extremely robust and the column can be cycled for at least 250 injections before matrix

renewal is necessary. This is not a prohibitively small number of injections as the t-gel itself is very cheap.

6.6.3 Field Use Of The Assay

The data presented prove the assay works and is capable of detecting sulfamethazine at and below its maximum residual limit, using a modern bench top fluorimeter. However, a full validation is required before the assay can be used industrially. This would include testing the assay on real sulfamethazine samples extracted from meat. Work by Patel *et al.*²²⁰ showed that sulfamethazine can be easily and effectively extracted from meat samples using a simple process involving homogenisation and sonication.

At the time of writing, my colleague Jill Hancox, had successfully completed the development of the test-strip assay and submitted the work to Loughborough University in a thesis entitled 'Sulfamethazine In Food: A New Approach To Screening'.

6.7 Further Work

The flow-injection fluoroimmunoassay developed was capable of detecting SMT below its maximum residual limit. However there is potential for the assay to be made many times more sensitive thorough the use near-infrared (NIR) labelling and diode laser excitation. The increase in sensitivity using this type of system could be in excess of three orders of magnitude.

Much work has already been done within this project on the development of Cy5 and Cy7 derived SMT conjugates but, unfortunately, these showed no activity in the flow-injection immunoassay. However, Hancox²²¹ has shown a Cy5 conjugate to work in a solid-phase test-strip assay.

It is clear that there is enormous potential in the use of NIR fluorescence for use in environmental monitoring applications such as FIIA and this merits further investigation. The present difficulty in the use of NIR fluorophores is that they are extremely expensive to purchase and difficult to synthesise and purify. One could also run into considerable patent difficulties if using a

commercially available or 'in house' synthesised version of a commercially available dye.

The Cy7 derived labels, in particular, are very interesting because they can be excited by cheap and compact, long wavelength (>750 nm) laser diodes. This gives great scope to reduce the size of the instrumentation such that even a flow-injection system would become portable. Indeed, a commercial venture, spawned from work carried out in our research group, is already underway.

6.8 Final Conclusions

The immunoassay developed in chapters four and five utilises the cheap and robust antibody purification matrix thiophilic gel, coupled with a visibly labelled fluorescent sulfamethazine analogue to perform rapid assays with good precision and accuracy. This accuracy and precision would doubtless be improved with the use of automatic sample handling equipment.

The principle problem encountered with the assay, was the development of the analyte-fluorophore conjugate itself. In previous work in this field, suitable model analytes, usually easy to derivatise (or often bought in ready labelled), have been chosen to illustrate the potential of the method. In this case, commercial pressures indicated that sulfamethazine was the target analyte of choice.

It took a disproportionately large amount of time to produce a suitable fluorescently labelled version of sulfamethazine as several labelling strategies were investigated before the FITC-SMT conjugate was successfully synthesised. A lot of time was also spent in an attempt to synthesise a novel five carbon chain cyanine dye based on the work done by Mank *et al.*^{207,208}. The synthetic route consisted of seven steps, the latter two of which, furnished highly unstable oils. The instability of these intermediates was so acute that formation of the final active dye (Cy5.4a.1A) proved impossible. The work is not reported in this thesis.

Once this first phase of work was completed, the optimisation of the assay itself was relatively straightforward, not withstanding the problems with antibody binding and elution reproducibility. For the effective second phase of the work, much credit must be given to the work done by Evans, who pioneered this area of work in our laboratory. His work is presented in an excellent thesis entitled 'The Development Of A Fully Automated Immunoassay For Phenytoin Utilising Fluorescence Detection'¹⁵³.

The net result of the project is that as a collaboration, we have achieved the aims which were set out at the start of this thesis:

- 1) To produce a field portable assay capable of screening for sulfamethazine 'on farm', using 'test-strip' type technology and
- 2) To produce a flow-injection fluoroimmunoassay capable of detecting sulfamethazine at and below its maximum residual limit.

Neither of these aims could have been achieved with either of the parties working in isolation. It was the successful technology and information crossover from academia to industry and visa-versa that has facilitated the successful conclusion of this project.

Appendices

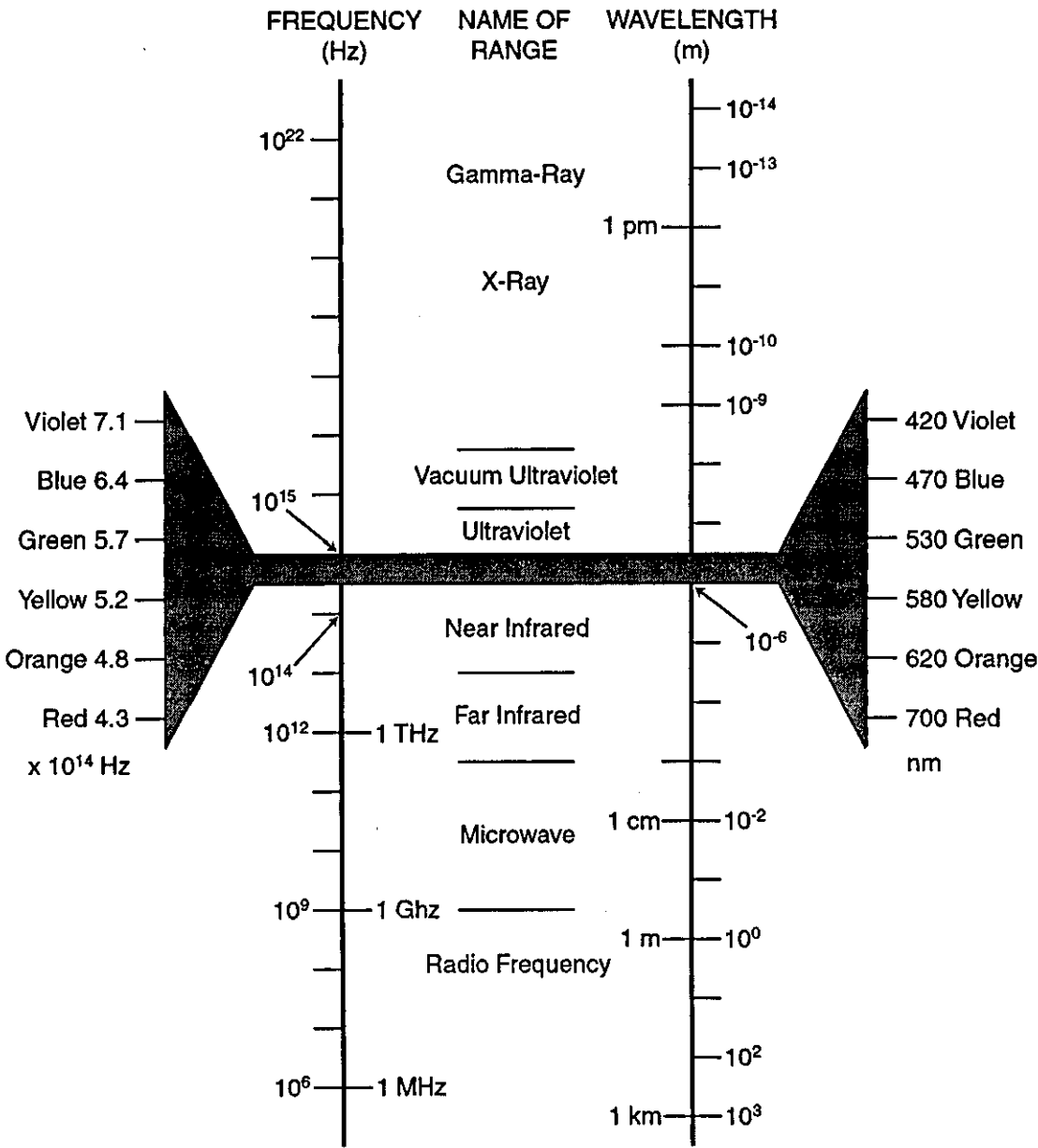
Appendix 1 – Reagents

Reagent	Supplier
Acetic Acid, 99.8%	Aldrich
Acetic anhydride	Aldrich
Albumin, fraction V	Sigma
Biotin-LC-hydrazide	Pierce Warriner
Bodipy FL labelled avidin	Molecular Probes
CHAPS (zwitterionic detergent)	Sigma
Citric acid, AnalR	Fisher
Disodium hydrogen phosphate, AnalR	Fisher
Disodium hydrogen phosphate, AnalR	Fisher
EDC	Sigma
FITC isomer I	Sigma
HRP	Biozyme
Lysine	Sigma
MES buffer	Sigma
Ovalbumin	Sigma
p-bezoquinone	Sigma
Potassium dihydrogen phosphate, AnalR	Fisher
Potassium sulphate, AnalR	Fisher
Sephadex G-10	Pharmacia
Sodium chloride, AnalR	Fisher
Sodium dihydrogen phosphate, AnalR	Fisher
Succinic anhydride, AnalR	Fisher
Sulfamethazine, 99%	Sigma
Triethylamine hydrochloride	Sigma

Appendix 2 – Supplier Addresses

Supplier	Address
Aldrich Chemical Company	Poole, Dorset, UK.
Altec	Alton, Hampshire, UK.
Amersham Life Science	Arlington Heights, USA.
Fisher Chemicals	Loughborough, Leicestershire, UK.
Fluka Chemicals	Gillingham, Dorset, UK.
Gilson Medical Electronics Ltd	Villiers, France.
Hellma	Essex, UK.
Hitachi Instrument Division Ltd	Tokyo, Japan.
Molecular Probes	Cambridge, UK.
Omnifit	Cambridge, UK.
Perceptive Biosystems Ltd	Cambridge, MA, USA.
Perkin-Elmer	Beaconsfield, Buckinghamshire, UK.
Pharmacia	Milton Keynes, UK.
Pierce and Warriner Ltd	Chester, UK.
Pye-Unicam	Cambridge, UK.
Sigma Chemical Company	Poole, Dorset, UK.

Appendix 3 – Spectrum Of Electromagnetic Radiation



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