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THE DEVELOPMENT OF A FULLY AUTOMATED IMMUNOASSAY FOR PHENYTOIN UTILISING FLUORESCENCE DETECTION.

By

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B.Sc. (Hons).

A Doctoral Thesis submitted in partial fulfilment of the requirements for the award of

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Of
Loughborough University

1996

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Finally I would like to thank Angela Moore for putting up with me during my Ph.D. and the help and support of my family, especially my mother, without who's constant encouragement this thesis and Ph.D. would not have been completed.
Phenytoin is used to prevent the seizures associated with epilepsy. The therapeutic range is generally between $9-21 \mu g/ml$, with toxic side effects common above $40 \mu g/ml$. Unfortunately phenytoin has non-linear pharmacokinetics in humans and hence the use of the drug must be monitored closely.

The aim of this research was to develop an automated assay for phenytoin in human plasma with fast sample throughput. The research was carried out in 3 phases:

a. The characterisation of the POROS IIa Protein A matrix.

b. The development of a manual assay for phenytoin utilising a pre-incubation and an on-line method.

c. The development of a fully automated immunoassay for phenytoin in human plasma.
ABSTRACT

For a number of years immunoassays have been a major tool in development and determination of drugs in biological fluids.

The immunoassay techniques commonly applied in industry at present are those based on micro titre plates consisting of 96 wells. These assays although more complicated, time consuming and labour intensive have for some time been available in an automated format. Immunoassays utilising flowing streams have proven to be quicker, less labour intensive and simpler to perform but until recently have not been available in automated formats. It has been possible with the development of a novel immunoaffinity matrix, POROS IIa, to improve the speed of immunoassays without loss of precision and to operate in an automated format. This format involved a AS3000 Hitachi auto sampler, a K1000 Hitachi automatic flow analyser operating a 16 way injection valve and a F4500 Hitachi fluorescence detector, all controlled using a 486 microprocessor. It was possible, using the system described above, to develop an automated immunoassay producing a sample through put of 40 samples per hour. This system combines the sensitivity and specificity of fluorescence immunoassay together with the automation of a simple flow injection system.
PUBLICATIONS AND PRESENTATIONS

Papers

Flow Injection Immunoassay for Serum Phenytoin using Perfusion Chromatography.

Rapid Fluorescence Immunoassay using a Novel Perfusion Chromatographic Matrix.

Thiophillic Gels: An Alternative to Protein A and G for use in Flow Injection Immunoassays.

Flow Injection Immunoassay for Albumin using Thiophillic Gels.

Presentations.

Pitcon 1993, Atlanta, US. Poster
Euroanalysis 1993, Edinburgh, UK. Poster
Flow Analysis 1994, Toledo, Spain. Poster
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CHAPTER 1

THERAPEUTIC DRUG MONITORING
1.1. Therapeutic Drug Monitoring.

Therapeutic drug monitoring (TDM) is a necessary part of patient study, to validate the benefits and risks in the use of therapeutic drugs. Drug determination in biological fluids can be undertaken for several reasons, for example, pharmacokinetic and bio availability studies, drug overdose (toxicological monitoring), screening for drugs of abuse, or compliance testing (whether or not the patient takes the prescribed medicine). The term therapeutic drug monitoring (TDM) is usually reserved for the routine measurement of therapeutic drugs in blood (plasma or serum) or other biological fluids as an aid to improving drug therapy (1-4).

By their very nature therapeutic drugs alter biological events, therefore any drug is potentially harmful. It is then obvious that the potential beneficial effects need to be separated from the possible harmful ones. This is possible by the development of new compounds which provide these beneficial effects at concentrations considerably lower than the levels at which toxic manifestations occur. For example, the relative success of benzodiazepines lies in the fact that they are relatively non-toxic, as compared to barbiturates used for the same illnesses (5).

Despite extensive research into the development of novel drugs it is still necessary to persist with those therapeutic drugs whose so-called therapeutic range is narrow and dosing must be carried out with extreme care (6). However, it is not the dose of the drug administered that is important but the concentration at its therapeutic site and this cannot always be predicted directly from the dose supplied (7). It is important to determine the plasma/serum concentration of therapeutic drugs, although it is not always true that the blood or plasma concentration of the drug is an indication of its activity, it is generally accepted so (8), if the side-effects are to be eliminated or at least reduced.
The main purpose of TDM is to monitor the plasma/serum levels with a view to altering the dose to provide the optimum effect with the minimum risk of toxicity (9).

The emergence of TDM as a specialist discipline owes much to recent advances in analytical techniques and instrumentation, which allow rapid determination of low concentrations of drugs in small samples of biofluids.

The principal techniques used in TDM are gas and high-performance liquid chromatography (GC and HPLC) (10-15) and immunoassays (16-20). This chapter explains some of the reasons for TDM and briefly outlines the techniques involved together with a comparison of the techniques available.

1.1.1. Therapeutic Range.

For a particular drug to produce the desired response or particular therapeutic effect in patients it must be administered in a recommended dosage regime. To establish this dosage regime requires lengthy trials first in test animals, then human volunteers and finally in patients. By monitoring a therapeutic response it is possible to discover not only the range at which the drug of interest works but also the nature of its action. There are a number of therapeutic responses possible. It may give a graded response whereby the effect of the drug gradually increases with concentration, producing a characteristic dose-response curve. Eventually a concentration will be reached where the drug has its maximum therapeutic effect.

In other cases the drug can have a response which is not continuous but occurs suddenly whether present or absent, i.e. an all or nothing response, a quantal response.
The easiest way to produce a therapeutic range is to monitor the plasma concentrations and then relate these to the administered dose, producing a series of plasma concentrations that provide the desired therapeutic effect with the reduction or elimination of side-effects.

Therefore in general terms the therapeutic range can be described as the upper and lower therapeutic dose (upper and lower plasma levels).

1.1.2. The Reasons for Therapeutic Drug Monitoring.

It is expensive and time consuming to measure drug levels in patients routinely, hence the reason for the study of a particular drug must be examined carefully before the task is undertaken.

In practice there is little reason to monitor the therapeutic level of drugs which can easily be monitored by simple observations or clinical measurements of the patient. For example, monitoring the blood pressure of a patient following anti-hypertensive treatment or the eradication of a fever following antibiotic treatment. There is also no need to routinely monitor the drug if it has a wide difference between therapeutic and toxic concentrations and has no serious side-effects (21).

However, in some cases the drugs used have narrow therapeutic indices (small differences between safe and toxic concentration range, i.e. therapeutic doses are close to toxic doses) and the interpretation of the measurable dose is not clear-cut, which makes dose adjustment much more difficult.

In such cases the measurement of the plasma concentration of the drug provides essential information on the likelihood of toxicity and adequacy of the dosage regimen, providing that
there is a correlation between the plasma concentration and therapeutic and/or toxic effect of the drug.

There are a number of criteria for undertaken drug level monitoring. Table 1.1. shows a list of these criteria.

1.1.3 Problems Associated with Therapeutic Drug Detection.

As the analysis of any therapeutic drug is normally performed on biological samples problems arise from the nature of the matrix and its contents, the most common biological fluids analysed being blood, plasma (or serum) and urine. Less common fluids analysed include bile, sweat, milk and saliva (22-25). Table 1.2. shows a number of body fluids which can be used in TDM listed in order of difficulty to obtain(26).

Blood, the most complex of the fluids mentioned in table 1.2. supplies the analyst with a number of specific problems. The major constituents are the erythrocytes (red blood cells) and these can be removed by simple centrifugation, leaving a buffered clear liquid which contains solubilised proteins, dissolved fats and salts (plasma) (27).

The major problem associated with plasma is the presence of large amounts of protein. Although the protein itself is physically and chemically different from the drug of interest there is a possibility of a strong affinity of such plasma proteins with drugs.
<table>
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<th>Criteria for Undertaking Drug Level Monitoring(26).</th>
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<tr>
<td>1.</td>
<td>A good correlation exists between the plasma drug concentration and therapeutic and/or toxic effect.</td>
</tr>
<tr>
<td>2.</td>
<td>Existence of an optimum therapeutic concentration range within which most patients experience some clinical benefit at minimum toxicity and minimal side-effects. Steady-state concentration usually falls within this range.</td>
</tr>
<tr>
<td>3.</td>
<td>Narrow therapeutic index with serious side-effects in the toxic range (digoxin, aminoglycosides).</td>
</tr>
<tr>
<td>4.</td>
<td>Considerable inter-subject variation in plasma levels from the same dose e.g. theophylline.</td>
</tr>
<tr>
<td>5.</td>
<td>Pharmacological and toxic effects not readily measurable (anticonvulsants, antiarrhythmics).</td>
</tr>
<tr>
<td>6.</td>
<td>Absence of an active metabolite (an active metabolite can complicate the concentration/side effect relationship).</td>
</tr>
<tr>
<td>7.</td>
<td>Special circumstances such as treatment of infants, presence of hepatic or renal disease, clinical trials.</td>
</tr>
<tr>
<td>8.</td>
<td>Availability of a valid assay method.</td>
</tr>
<tr>
<td>9.</td>
<td>Ability of clinician to understand results.</td>
</tr>
</tbody>
</table>
Table 1.2. A Number of Samples in Descending Order of Degrees of Difficulty to Obtain (26).

Urine
Bile
Plasma
Serum
Blood
Tissue
Sweat
Bone

Removing these proteins by methods such as ultrafiltration or dialysis could also lead to the removal of the drug (28). As a result of this additional sample manipulation, free drug assays are not performed routinely.

1.2. Analytical Methods Utilised in TDM.

An analytical method in TDM must be simple, fast and reliable, because generally results are required urgently. It must be specific and free from interferences from structurally related compounds.
There are a number of analytical techniques currently being used in TDM and these include HPLC, GC, TLC and immunoassays. A notable exception is that of lithium determination, which is carried out using atomic absorption spectroscopy (29).

There are almost as many diverse methods for the detection of therapeutic drugs as there are drugs themselves, each with their own advantages, but it is possible to place these methods into specific detection techniques. Each of the techniques described in the following section are diverse and require a more detailed description but in this case they will be briefly outlined together with a number of their limitations.

1.2.1. Chromatographic Techniques.

TDM utilising chromatographic techniques cover the whole range of chromatographic procedures available including TLC, GC and HPLC.

1.2.1.1. Thin-layer Chromatography (TLC).

Thin-layer chromatography is the least used chromatographic technique in TDM. Although widely used for the detection of substances of abuse (25), it is not well suited for TDM because for quantitative work it can be time consuming and labour intensive, together with its lower sensitivity and resolution as compared to HPLC and GC as well as also being difficult to automate.
1.2.1.2. Gas Chromatography (GC).

GC is a very sensitive technique, however, it can be difficult to perform on plasma/serum because the samples must be thermally stable (this problem is not limited to TDM) and GC is not always suitable for highly polar samples (the majority of drugs by their very nature are polar). It is possible to convert the samples to more thermally stable and less polar derivatives, but this has the obvious effect of increasing assay time and sample manipulation. Capillary columns made from fused silica have greater potential than conventional glass columns in TDM because they are more robust, offer higher resolution, sensitivity and faster separations (32).

1.2.1.3. High-performance Liquid Chromatography (HPLC).

Of the many versions of HPLC analysis (normal phase, reversed phase, ion exchange, size exclusion) (33), reversed phase liquid chromatography (RPLC) is the most widely used technique employed in TDM. A wide variety of drugs (polar, non-polar, ionic) can be separated by RPLC (34). In RPLC the stationary phase is non-polar (for example C₈ or C₁₈ hydrocarbon chains) and the mobile phase consists of polar solvents, such as water, methanol or acetonitrile. In addition, recently new stationary phases have been developed which allow the separation of enantiomers or direct injection of biological samples (35). HPLC offers greater flexibility than GC, it is quicker and has a wider range of applicability, for example, it can be used for polar, non-volatile and thermally labile compounds, which permits the determination of a wide variety of drugs, with a lower sensitivity compared to GC.
Together with the flexibility offered by HPLC the sample preparation is simpler to perform than GC, usually only requiring a single solvent extraction together with the possibility of using larger volumes than GC (up to 100μl as compared to 1μl for GC).

1.2.2. Immunoassays Techniques.

Immunoassays through their components (i.e. antibodies) can be performed on a large number of therapeutic drugs. Immunoassay methodology is described in more detail in chapter 3, but in the case of TDM can be divided into specific detection methods.

1.2.2.1. Radio-immunoassays (RIA)

Radio-labelled immunoassays use reagents incorporating radio-isotopes as tracers to monitor the distribution of free and bound antigen in radio-immunoassays (RIA) or free and bound antibody in immunoradiometric assays (IRMA). Radio-isotopes were introduced into immunoassays in 1960 (36) and have proved to be a major advance in detection limits. The majority of radio-labelled immunoassays for therapeutic drugs are heterogeneous as radio-labels do not lend themselves to non-separation protocols(37). Homogenous radio-immunoassays have been reported, for example, internal sample attenuator counting radioimmunoassays (ISAC) are homogenous immunoassays. In this assays, antibodies and adsorbed (attenuating) materials such as bismuth oxide are coupled or trapped to particles such as polyacrylamide materials and radioactivity is reduced when 1²⁵I labelled antigen binds
to the antibodies on the particles. Scintillation proximity radioimmunoassays (PROTEIN A) are also indirect homogenous immunoassays where particles are labelled with antibodies and fluorophores, which are excited when $^{125}\text{I}$ labelled antigens bind to the particles(38).

1.2.2.2. Spectroscopic Analysis.

It is possible to divide spectroscopic analysis into two areas, direct methods, where the sample is measured after dilution, separation i.e. UV/Vis analysis, and indirect methods, where the absorbance of a second substance, usually a chemically modified analyte is measured and related back to the analyte originally present, known as colorimetric analysis.

A great number of therapeutic drugs show natural UV/Vis absorption and a number of these are listed in table 1.3.

Unfortunately UV/Vis direct analysis shows important limitations in the detection of therapeutic drugs in that the majority of UV/Vis absorption bands are characterised by broad peaks with hills and valleys, therefore compounds with the same functional groups will show similar absorption spectra and interference is common. As a result UV/Vis direct methods of analysis are not suitable for unknown samples (30).

Colorimetric (indirect) analysis use the same principles of measurement as direct UV/Vis except as the name suggest, employs the use of colour. In the majority of cases a chromophore is introduced into or prepared from a substance of almost any chemical class using relatively simple chemical procedures (31).
Table 1.3. A Number of Therapeutic Drugs which show UV/Vis Absorbance.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Concentration giving absorption of 0.4 A.U. (mg/ml) (l=1cm)</th>
<th>Approximate Plasma Concentrations during Therapy (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbamazepine</td>
<td>15</td>
<td>10</td>
</tr>
<tr>
<td>Codeine</td>
<td>80</td>
<td>0.03</td>
</tr>
<tr>
<td>Diazepam</td>
<td>3</td>
<td>0.20</td>
</tr>
<tr>
<td>Digoxin</td>
<td>15</td>
<td>0.002</td>
</tr>
<tr>
<td>Haloperidol</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Imipramine</td>
<td>13</td>
<td>0.10</td>
</tr>
<tr>
<td>Lignocaine</td>
<td>300</td>
<td>5</td>
</tr>
<tr>
<td>Lorazepam</td>
<td>3</td>
<td>0.01</td>
</tr>
<tr>
<td>Methaqualone</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Neostigmine</td>
<td>200</td>
<td>0.10</td>
</tr>
<tr>
<td>Nitrazepam</td>
<td>3</td>
<td>0.03</td>
</tr>
<tr>
<td>Oxazepam</td>
<td>3</td>
<td>0.01</td>
</tr>
<tr>
<td>Phenacetin</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Phenytoin</td>
<td>130</td>
<td>20</td>
</tr>
<tr>
<td>Primidone</td>
<td>740</td>
<td>10</td>
</tr>
<tr>
<td>Procainamide</td>
<td>6</td>
<td>10</td>
</tr>
<tr>
<td>Quinidine</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Quinine</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>Sulphadiazine</td>
<td>5</td>
<td>30</td>
</tr>
<tr>
<td>Sulphapyridine</td>
<td>5</td>
<td>0.01</td>
</tr>
</tbody>
</table>
1.2.2.3. Fluorescence Analysis.

Fluorescence analysis is used by some workers because of its excellent sensitivity. It is possible to group fluorescence analysis with spectroscopic methods but because of the diversity of the technique it warrants specific mention. As in spectroscopic methods it is possible to divide fluorescence into direct (natural fluorescence by the drug of interest) and indirect methods (enhancing either chemically or by coupling with a fluorophore). In the case of direct methods there are a number of drugs which show natural fluorescence, for example, quinine (39), butaperazine (40), where measurements are possible down to 8 ng/ml. Unfortunately few drugs are so accommodating in their spectral characteristics but it is possible, however, to transform the drug of interest, either by direct chemical action (41), or by the formation of a fluorescent derivative with the appropriate coupling reagents either producing a shift in the fluorescent intensity to a more suitable wavelength or to increase the Stokes' shift (42). It is also possible to produce fluorescence by irradiation (43), but the procedure can be very time consuming. A final method to utilise fluorescence detection is to use a fluorescent probe i.e. chemically coupling a fluorescent compound to the drug of interest.

1.2.2.4. Enzymatic Analysis.

Enzyme immunoassays can be conveniently divided into heterogeneous and homogenous immunoassays. One of the first commercially successful homogenous immunoassay was based on an Enzyme multiplied immunoassay (EMIT) technique(44) and was developed for
morphine using lysozyme as the enzyme label. There have also been a number of successful substrate labelled fluorescent immunoassays (SLFIA) developed for therapeutic monitoring of anti-convulsant drugs in serum(45). Prosthetic group labelled immunoassays (PGLIA), also known as apoenzyme reactivation immunoassay systems (ARIS) have also been developed for therapeutic drugs(46). As well as the homogenous enzyme immunoassays mentioned there are many others which lend themselves well to therapeutic drug monitoring and these include cloned enzyme donor immunoassay (CEDIA)(47), liposome based enzyme immunoassays(48) and avidin biotin enzyme immunoassays(49) which are not as widely used and have only limited commercial value.

1.2.3. Comparison of Chromatographic and Immunoassay Techniques.

No single technique can be used reliably for all TDM but from a practical standpoint immunoassays have an advantage over chromatographic techniques in terms of speed and simplicity.

Table 1.4. shows a number of general comparisons between chromatographic and immunoassay techniques (2, 41-44). Immunoassays can be less sensitive and specific than chromatographic techniques, depending on the quality of the antibody. Antibodies may cross-react with metabolites structurally close to the parent drug, or endogenous substances present in the sample, giving high results, therefore the quality of the antibody determines the specificity of the immunoassay.

The major advantage of chromatographic techniques is their ability to measure a number of drugs simultaneously, which is advantageous when a number of drugs are prescribed together,
Table 1.4. General Comparison of Chromatographic and Immunoassay Techniques for TDM.

<table>
<thead>
<tr>
<th></th>
<th>GC/HPLC</th>
<th>Immunoassay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample size</td>
<td>0.1-1.0 µl</td>
<td>50-100 µl</td>
</tr>
<tr>
<td>Sample clean-up</td>
<td>Usually required</td>
<td>Not required</td>
</tr>
<tr>
<td>Derivatisation</td>
<td>Sometimes (HPLC)</td>
<td>Not required</td>
</tr>
<tr>
<td>Analysis time</td>
<td>Relatively slow</td>
<td>Fast*</td>
</tr>
<tr>
<td>Skill and experience required</td>
<td>Considerable</td>
<td>Minimal</td>
</tr>
<tr>
<td>Equipment cost</td>
<td>Moderate</td>
<td>Moderate</td>
</tr>
<tr>
<td>Reagent cost</td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>Method development cost</td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>Method development time</td>
<td>Short</td>
<td>Long</td>
</tr>
<tr>
<td>Running expenses</td>
<td>Low</td>
<td>Moderate</td>
</tr>
<tr>
<td>Ability to assay several compounds simultaneous</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Specificity</td>
<td>High</td>
<td>Moderate</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>High</td>
<td>Adequate</td>
</tr>
<tr>
<td>Accuracy</td>
<td>High</td>
<td>Moderate</td>
</tr>
<tr>
<td>Precision</td>
<td>High</td>
<td>Moderate</td>
</tr>
<tr>
<td>Automation</td>
<td>Possible</td>
<td>Possible</td>
</tr>
<tr>
<td>Sample through put (with automation)</td>
<td>Moderate</td>
<td>High</td>
</tr>
</tbody>
</table>

*Heterogeneous assays are relatively slow.
for example, antiepileptics, or when a drug and its active metabolite need to be measured (tricyclic antidepressants, benzodiazepines, procainamide or methotrexate) with selectivity, sensitivity, flexibility and overall versatility. In contrast to immunoassays, chromatographic methods for new compounds can be devised very quickly and often GC and HPLC are used as reference methods to validate new immunoassays. However, chromatographic techniques do show a number of disadvantages. Compared with immunoassays they are slow, labour intensive and require skilled operators and their throughput is usually lower, even with automation (although immunoassays themselves are not free from labour intensive steps). These factors somewhat limits their applications in TDM despite their significant advantages.

1.2.4. Mass Spectroscopic Methods of Therapeutic Drug Monitoring.

In recent years a great deal of effort has been put into the problem of monitoring therapeutic drugs using mass spectroscopy and a number of assays for commonly available barbiturates for example, amobarbital or barbital, have been reported(50). It is now possible to analyse biological samples utilising newly developed ionisation techniques which have been developed to give, as the only significant ion, a quasi-molecular ion which permits specific identification of many drugs(50). Utilising the high sensitivity of mass spectroscopy it is possible to monitor therapeutic drugs down to pg quantities, but unfortunately it can prove expensive to run and trained personnel are required which makes mass spectroscopic determination of therapeutic drugs a limited option.
1.3. Analytical Methods for the Determination of Phenytoin.

1.3.1. Introduction.

Phenytoin (5,5-diphenylhydantoin) is used for the prophylactic treatment of grand mal epilepsy, fig 1.1 shows the structure of phenytoin (45). It is an odourless white crystalline solid with a molecular weight of 252.26. It is partially soluble in water and is used in preference to phenylbarbitone (from which it was originally derived) as it does not cause drowsiness (46).

\[
\begin{align*}
\text{Fig 1.1. Structure of Phenytoin.} \\
C_6H_5 & \quad \text{NH} \\
\text{C}_6\text{H}_5 & \quad \text{O} \\
\text{NH} & \quad \text{Mwt 252.26}
\end{align*}
\]

1.3.2. Therapeutic level.

The therapeutic level of phenytoin in serum is between 9 and 21 mg/l (45) with concentrations in plasma of patients being treated varying between 2.5 and 30 mg/l (47), seizures being effectively controlled when concentrations are maintained between 10 and
20mg/\\. Most adult patients can tolerate doses of 300 to 400 mg daily without experiencing toxic side effects (47).

1.3.3. Mode of Action.

Phenytoin suppress epileptic discharges from the neurons in the brain by affecting the sodium gradients of the ion channels within the neurons themselves (48). This occurs by altering the voltage and frequency of the sodium channels by interacting preferentially with the inactivated state of the channel. It shows a selective action on the neurons undergoing epileptic discharges which increase the population of sodium channels in the inactivated state (48) (hyperpolarisation). Associated with the hyperpolarisation is a reduction of the intracellular calcium ions, this reduction causes the secretory cells to decrease their production of neurotransmitter hormones (an excess of neurotransmitter hormones causes epileptic fits), thereby reducing the possibility of a seizure (49).

1.3.4. Toxicity of Phenytoin.

As with a large majority of drugs phenytoin treatment must be monitored to ensure the correct dosage is prescribed. Unfortunately phenytoin has non-linear pharmacokinetics in humans (i.e. the time required for the drug to be removed from the body changes from patient to patient) therefore frequent monitoring is required. Each dose has to be manipulated to suit individual patients, otherwise a number of side effects can occur. The main short term side
Table 1.5. Plasma Half-life and Therapeutic Concentration Ranges for some Commonly Monitored Drugs.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Half life(average)/h</th>
<th>Therapeutic range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbamazepine</td>
<td>15</td>
<td>5-12 µg/ml</td>
</tr>
<tr>
<td>Digoxin</td>
<td>36</td>
<td>0.5-15 µg/ml</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>4</td>
<td>4-15 µg/ml</td>
</tr>
<tr>
<td>Lithium</td>
<td>22</td>
<td>0.1-1.0 µg/ml</td>
</tr>
<tr>
<td>Methotrexate</td>
<td>12</td>
<td>&lt;1 µg/ml</td>
</tr>
<tr>
<td>Phenobarbitone</td>
<td>96</td>
<td>20-45 µg/ml</td>
</tr>
<tr>
<td>Phenytoin</td>
<td>24</td>
<td>9-21 µg/ml</td>
</tr>
<tr>
<td>Procainamide</td>
<td>3</td>
<td>4-10 µg/ml</td>
</tr>
<tr>
<td>Sodium valproate</td>
<td>12</td>
<td>125-200 µg/ml</td>
</tr>
<tr>
<td>Theophylline</td>
<td>8</td>
<td>15-30 µg/ml</td>
</tr>
</tbody>
</table>

Effects of an excess of phenytoin include gingival overgrowth (a hyperplastic process induced by phenytoin's direct stimulatory effect on gingival fibroblasts, resulting in increased collagen synthesis, which is seen in 10 to 30% of patients treated), ataxia, dysarthria and nystagmus (involuntary jerking movement of the eyes). Long term side effects are also common and these include gum hypertrophy, acne, hirsutism and liver enzyme induction (48).

The obvious problem in under prescribing phenytoin is that epileptic discharges from the brain will not be suppressed resulting in epileptic fits.

Accurate and precise monitoring of phenytoin levels in serum is important to prevent the side effects outlined in section 1.3.4. Earliest methods for the determination of phenytoin plasma levels involved colorimetric (50) and chlorination (51) methods but these suffered from poor sensitivity (not enough sensitivity to monitor phenytoin blood plasma/serum levels therapeutically) and it was not until the development of more sensitive instrumentation in the 60's and 70's did the detection of blood plasma/serum levels of phenytoin in the therapeutic range become routinely possible. With this increase in sensitivity of analytical instrumentation the detection of phenytoin diversified into several areas and these will be discussed in the following sections.

1.4.1. Chromatographic Methods.

The majority of methods for the determination of anti-convulsant drugs in plasma/serum are, with the exception of immunoassay techniques, based on chromatographic techniques.

1.4.1.1. Thin Layer Chromatography.

Initial chromatographic methods for the detection of phenytoin in plasma/serum involved thin layer chromatography (52). High performance thin layer chromatography has also been
applied to the detection of phenytoin (53) in plasma/serum with detection limits of approximately 2 µg/ml.

1.4.1.2. Gas Chromatographic Methods.

Gas chromatography (GC) has been widely used in the detection of phenytoin in plasma/serum. A number of methods have been developed using gas chromatography and gas liquid chromatography (GLC) produced detection limits of 2 µg/ml (54-58). All the methods mentioned in the references show good reproducibility (S.D. = 3%), but prove to be time consuming, the quickest being 4-6 hours per 20 samples.

1.4.1.3. High Pressure Liquid Chromatographic Methods.

High pressure liquid chromatography (HPLC) has a particular advantage in the analysis of phenytoin over other methods in that it is possible to analysis the sample without initial derivatisation. As with GC it is possible to analyse phenytoin in plasma/serum, using in the majority of cases a reverse phased HPLC system, with the exception of Curry et al. who describe a method for the detection of phenytoin using a normal phase partition (59). The reversed phase provides greater analytical flexibility than the adsorption column, giving shorter analysis time and permitting the use of largely aqueous mobile phase, consisting of aqueous acetonitrile (85ml acetonitrile in 415ml deionised water) (60). It is also possible to use various preparative techniques such as solvent extraction, protein precipitation, direct
sample injection, filtration, centrifugation and chromatographic pre-column separation before injecting into the HPLC system (61). Utilising HPLC methods it is possible to obtain a working range for phenytoin between 1-100μg/ml with intra assay CV’s of 4.8%, inter assay CV’s of 7.3% (61) with analysis time of approximately 15 mins per sample.

There are a number of shortcomings associated with the HPLC determination of phenytoin in plasma/serum. The columns used are rapidly deteriorated by the mobile phase (which has a pH of 8), multi step extraction gives poor recovery together with being time consuming. Also the HPLC determination of phenytoin suffers from a number of interferences which are explained in more detail in section 1.4.3. (62).

1.4.2. Immunoassay Methods.

As mentioned in section 1.2.2. immunoassay methods can be used in the determination of therapeautic drugs and have been applied to the detection of phenytoin in plasma/serum. As with all immunoassay methodology it is possible to divide the determination of phenytoin into a number of specific areas, depending on the immunoassay technique employed. All the techniques mentioned in the following sections will be discussed in more detail in chapter 3.

1.4.2.1. Radioimmunoassay Determination of Phenytoin.

Radioimmunoassays (RIA) have been developed for the detection of phenytoin in plasma/serum with detection limits as low as 300 pg/ml in plasma (63), with the radio-label
most commonly used for the detection of phenytoin being tritium (\(^3\)H). The only disadvantage with using tritium is that it is a \(\beta\)-emitter and liquid scintillation techniques are required but there are a number of commercial radioimmunoassay kits available for the detection of phenytoin (64). The RIA of phenytoin does have a number of disadvantages over other methods, which include, sample throughput can be slow (times for 20 samples can be as long as 8 hours), together with the obvious problems associated with radio-labels. Interferences from 5-(4-hydroxyphenyl)-5-phenylhydantoin (see section 1.4.3.) are common and the cost of the RIA of phenytoin is 6 times that of the equivalent GC method (64).

1.4.2.2. Enzyme Immunoassay Determination of Phenytoin.

Since the mid 70's both EMIT and ELISA assays have gained acceptance in the clinical laboratory as a reliable tool for the detection of phenytoin in plasma/serum and a commercially available kit developed by SYVAET (EMIT assay) has been available since 1975 (65). EMIT determination of phenytoin has been developed, by several workers, into an automated instrument and reagent system designed specifically for the primary screening of drugs(66). The EMIT assay for phenytoin is based on the competition between the drug in the sample and the drug labelled with the enzyme glucose-6-phosphate dehydrogenase (G6PDH), for the antibody binding sites. G6PDH converts nicotinamide adenine dinucleotide (NAD) to NADH, with the resulting absorbance change being measured spectroscopically. Other enzymes may also be used, for example, in the apoenzyme reactive immunoassay (ARIS) the enzyme is adenine glucose oxidase, which converts flavin dinucleotide (FAD) to FADH, \(^2\) (67). These assays rely on the change in absorbance caused by the decrease in the amount of substrate, but it is also
possible to use fluorimetric detection for enzyme immunoassays, i.e. it is possible to detect fluorometrically the enzyme activity which changes on addition of the drug (68). There are also substrate labelled fluorescent immunoassays available (SLFIA) (69). This technique involves the coupling of phenytoin to a substrate which is non-fluorescent, but becomes fluorescent when the enzyme is present, but when the drug-substrate is bound to the antibody the enzyme is unable to hydrolysis the substrate and produce the fluorescence, for example, galactosyl-umbelliferone covalently linked to phenytoin becomes fluorescent when catalysed by the enzyme B-galactosidase (70).

1.4.2.3. Fluorescence Methods.

There are a number of fluorescence immunoassays which compare favourably with HPLC and enzyme immunoassay methods (71), for example, Kamel et. al. developed a separation fluoroimmunoassay for phenytoin based on the separation of the free and bound antibody fractions, in a direct analogue to the radioimmunoassays, using a salt precipitin procedure (72) providing a limit of detection of serum phenytoin of 1μg/ml. Other fluorescence immunoassays described by the same workers utilises a magnetic separation technique which involves the use of fluorescein labelled phenytoin as the tracer and coupling the phenytoin antibodies to magnetisable cellulose/iron azide particles (73), providing a limit of detection of 1μg/ml.

A double antibody fluoroimmunoassay has been developed by Kurtz et. al. (74) for a number of therapeutic drugs, including phenytoin, which follows the classical double antibody
technique (section 2.1.2.) except that a fluorescently labelled analyte (fluorescein isothiocyanate) is used as the label.

A number of fluorescent polarisation immunoassays (FPIA) have also been developed for the detection of phenytoin (75) using sulphonamide derivatives and a succinimide ester of carboxyfluorescein, providing a limit of detection of 0.25µg/ml (76).

Flow injection fluorescent immunoassays for phenytoin have also been developed by Evans et al. with a limit of detection of 100pg/ml (77).

1.4.3. Interferences Associated with Phenytoin Detection.

As mentioned in section 1.1.3. detection of compounds in biological fluids provide the analyst with a number of problems, usually as a result of the matrix being analysed i.e. plasma, blood urine etc. But in some classes of compounds interferences can be caused by metabolites and other drugs. The following sections describe the interferences associated with phenytoin.

1.4.3.1. Interferences with Other Drugs.

Concurrent administration of phenytoin with some drugs can show an increased plasma/serum concentration. This is thought to be due to the inhibition of its deactivation (the inability of the body to breakdown the drug) and this process is known to occur with chloramphenicol, dicumarol, disulphiram, isoniazid, cimetidine and certain sulphonamides(78). Also some drugs, which compete for sites on the plasma proteins, can enhance the concentration of free
phenytoin in the blood, these include sulphisoxazole, phenylbutazone, salicylates and valproate. Phenytoin can interact with phenobarbital which may increase the biotransformation of phenytoin by induction of the hepatic microsomal enzyme system, but may also decrease its deactivation, apparently by competitive inhibition (79).

1.4.3.2. Interferences Caused by Phenytoin Metabolites.

The major metabolic pathway of phenytoin in humans is oxidation to 5-(p-hydroxyphenyl)-5-phenylhydantoin (HPPH), then conjugation to HPPH-glucuronide (80), which in patients with renal disease can attain concentrations of 10-100μg/ml in plasma/serum. Concentrations of HPPH in normal patients is <1μg/ml (81) which HPPH causes the recorded level of phenytoin in blood to be higher than the correct level (depending on the assay), the higher the HPPH level the greater the interference (81). HPPH-glucuronide accumulates in the plasma/serum (82) and 8μg/ml of HPPH-glucuronide corresponds to an apparent increase in phenytoin concentration of up to 1 μg/ml, using an EMIT assay system (82). The increase in apparent phenytoin concentration caused by the HPPH-glucuronide depends not only on the HPPH-glucuronide concentration but also the phenytoin concentration, for example, at lower phenytoin concentrations the interference of HPPH-glucuronide is greater than at high phenytoin concentrations (83). The reason for the interference of HPPH-glucuronide of the EMIT assay is because it reacts with the phenytoin antisera (84) and therefore is not a problem in the other phenytoin determination formats, for example, HPLC, GC, fluorescent polarisation, etc. (85).
1.4.3.3. Interferences Caused by Phenytoin Binding to Serum/Plasma.

Phenytoin binds strongly to approximately 90% of plasma proteins and on average 10% of the phenytoin administered binds to these plasma proteins, therefore any condition or drug which affects the percentage of bound protein will affect the expected results of free phenytoin. Valproic acid co-treatment is an example of a compound which affects the plasma protein binding of phenytoin, by displacing the phenytoin from the protein increasing the amount of free phenytoin. This causes the administered phenytoin to have a greater therapeutic effect because only free phenytoin in plasma/serum can cross the blood-brain cell wall barrier. In some cases the free phenytoin in the presence of valproic acid can be up to 50% higher (86).
References.


CHAPTER 2

IMMUNOASSAYS
2.1. History of Immunoassays.

Since the beginning of the twentieth century antibodies have been purified and used as analytical reagents(1), and as far back as 1930 Reigen suggested the use of labelled antibodies for qualitative measurements of an immunoreaction. By 1932 Heidleberg et. al. were measuring polysaccharides of pneumococcus by antibody-augmented precipitation reactions, and by 1941 fluorescent labelled antibodies were being used by Coons et. al. for the detection of bacterial strains. In 1953 the first homogenous, quantitative immunoassay was performed by Stavitsky and Arquilla (based on the heamaggulation reaction(2)). Therefore the components of modern immunoassay techniques were in existence(3) before the renowned publication by Berson and Yalow in 1959 but not until the early sixties did immunoassay begin to gain wide acceptance and the reason for its rapid increase in popularity during this period was the development of radio-labels, which now made it possible to produce highly sensitive immunoassays. During the next two decades radio-immunoassays (RIA) held the dominant position in the field of immunoassays, therefore it is possible to suggest that the great revolution of immunoassays which took place during the 60's and 70's occurred via RIA. It was not until the late seventies that new, non-isotopic labels were used which have since developed new perspectives in technological research.
2.1.1. Principles of Immunoassays.

The principles of immunoassays are quite simple and are based largely on the reversible non-covalent interaction between an antigen with its specific binding partner, for example, antibodies.

\[ \text{Ag} + \text{Ab} \leftrightarrow \text{Ag-Ab} \]

\[ \text{equation 1.} \]

\( \text{Ag} = \text{antigen}. \)

\( \text{Ab} = \text{antibody}. \)

\( \text{Ag-Ab} = \text{antibody-antigen complex} \)

Immunoassays derive their unique and specific characteristics from two important properties of antibodies (1), firstly an unparalleled specificity for the substance to which they bind and secondly the strength of the bond once formed. Examples of substances with specific binding properties (i.e. molecular recognition) include enzymes, antibodies, lectin, transport proteins and cell surface receptors (2-4). The most commonly used binding proteins presently used in immunoassays are antibodies, both monoclonal and polyclonal (section 2.2.2.) which bind specifically to a target molecule, known as an antigen.

Antigens are substances which are foreign to the animal to which they been introduced, once introduced they will cause the animal to produce an immune response resulting in the production of antibodies, termed an antigenic response.

Small molecules like peptides and drugs do not in themselves produce this antigenic response but can be made to do so by coupling them to macromolecule carriers (i.e. proteins or...
synthetic peptides) before injecting them into the animal so that antibodies will also be raised for these smaller molecules attached to the larger carrier molecule, such small molecules are known as haptens. As a result immunoassays can be developed for large molecular weight molecules, for example, hormones, adrenocorticotrophin (5), together with other proteins of interest, for example, thyroglobulin (6), as well as small molecular weight drugs, for example, phenytoin (7).

Unfortunately it is not possible to detect the reaction products in equation 1 sensitively enough to produce a working immunoassay, therefore a system is required to distinguish between the unbound antigen and the antigen bound to the antibody, as well as the antibody itself. This problem can be overcome if a label is attached to one of the constituents of the assay to distinguish it from the other components, thereby making it possible to measure its relative concentration.

An example of this, shown in equation 2, is the labelling of the antigen involved in the reaction. Labelled antigen, Ag*, is added to the reaction mixture, the labelling enabling its concentration to be monitored.

Note: it is important that the label used does not affect the antibody-antigen binding site.

\[
\text{Ag}^* + \text{Ag} + \text{Ab} \leftrightarrow \text{Ag}^* - \text{Ab} + \text{Ag-Ab} + \text{Ag} + \text{Ag}^* \quad \text{Equation 2.}
\]

\(\text{Ag}^* = \text{labelled antigen}\)

In the situation illustrated in equation 2 there is a mixture of unlabelled and labelled antigen competing for the binding sites on the antibody, known as a competitive binding assay, as the
labelled and unlabelled antigen are competing for a limited amount of antibody binding sites. The first immunoassay developed by Berson and Yalow (8) used an antigen labelled with an isotope in a competitive assay format, as was Ekins's assays for serum T4 and vitamin B12 which followed in 1960 (9-10). By utilising the information provided by the labelled antigen it is possible for the analyst to make accurate determinations of the concentration of the unlabelled antigen (this is discussed in more detail, with examples in chapter 5.) Conversely, it is also possible to label the antibody rather than the antigen (11-12). Equation 3 shows the basic principle:

\[ \text{Ag + Ab}^* \leftrightarrow \text{Ag-Ab}^* + \text{Ab}^* \]  \hspace{1cm} \text{equation 3} 

\( \text{Ab}^* = \text{labelled antibody} \)

It is then possible, after this initial reaction, to remove the labelled antibody from the solution which has not bound the antigen, for example, by addition of a large excess of solid phase bound antigen, such a system is shown in equation 4:

\[ \text{Ab}^* + \text{Ag-Ab}^* + \text{Ag-}] \leftrightarrow \text{Ag-Ab}^* + \text{Ab}^*-\text{Ag-}] \]  \hspace{1cm} \text{equation 4} 

\( \text{Ag-}] = \text{solid phase bound antigen} \)

Note: this format is also possible when using labelled antigen and binding the antibody to a solid phase (discussed in chapter 3).
There are two possible formats for this labelled antibody immunoassay design, although they in fact represent two extremes of the same thing. The first variant is analogous to the antigen labelled competitive assay in that the antibody concentrations are limited (12), for example, immunoradiometric analysis of insulin (13) and human growth hormone (14) (see fig 2.1). The second technique which employs labelled antibodies is known as a reagent excess immunometric assay, i.e. the antibody is in excess and due to the large excess of antibody the reaction between the antibody and the antigen is driven much more to completion than it is possible for the competitive assay (equation 1 is driven to the right) which in theory offers greater sensitivity over assays with competitive designs (fig 2.2).

In practice one-site immunometric assays using large concentrations of labelled antibody seldom offer any advantage over competitive assays and as a result have never gained widespread popularity (15). From this a two site immunometric assay was described by Wide et al. in 1967 (16) with working assays developed by Addision and Hales (15) in 1970 (fig 2.3). Such immunoassays have the obvious advantage of increased specificity and a number have been produced for larger molecules, for example, glycoprotein hormone thyrotropin (17), human growth hormone (18) and follicle stimulating hormone (19). Two site immunometric assays are often called sandwich assays. Immunometric assays which have their antibody or antigen coated onto a solid phase and use an enzyme as the label are also known as enzyme linked immunosorbent assays (ELISA).

As mentioned previously the major advantage of all types of immunoassays are their specificity, versatility and sensitivity. Typical radio-immunoassays have limits of detection in the pmol\(^{-1}\)(10\(^{-9}\)) range (20), while detection limits in the attomolar (10\(^{-18}\)) range have been reported using enzyme immunoassays (21-22).
Fig 2.1. Antibody Limited Immunometric Assay.
Fig 2.2. Antibody Excess Immunometric Assay.
Fig 2.3. Two Site Immunometric Assay (Reagent Assay).
2.1.2. Immunoassay Nomenclature.

Immunoassays can be divided into two groups, separation (heterogeneous) and non-separation (homogeneous) assays.

2.1.2.1. Homogeneous Immunoassays.

Homogeneous immunoassays do not require a separation step of the free and antibody bound fraction of the analyte. Separation of the components of the immunoassay is not required because the detection systems employed can distinguish between the bound and unbound analyte. They generally have shorter incubation times, are simpler to perform and as a direct consequence of this are easier to automate than heterogeneous assays. A number of homogeneous assay detection systems have been developed and these include agglutination (23), turbidimetry (24), nephelometry (25), enzyme multiplied techniques (26), fluorescent polarisation (27), scintillation proximity assays (28) and cloned enzyme donor (29) immunoassays (CEDIA).

2.1.2.2. Heterogeneous Immunoassays.

Heterogeneous assays require a separation step to remove the unbound tracer (and analyte) so that the antibody bound fraction or the free fraction can be isolated and detected. There are a variety of separation techniques available and some of the more common ones include:-
a) Column Separation: examples include gel filtration, ion exchange (30), immunoaffinity (7).

b) Adsorption: powdered charcoal has the ability to absorb molecules of free tracer into small crevices on its surface, for example, free insulin (31).

c) Electrophoresis: paper electrophoresis has been developed as a separation system for a number of immunoassays, for example, thyroglobulin (32), however electrophoretic methods are now mainly obsolete as a separation step in immunoassays.

d) Precipitation by Salts, Organic solvents and Polyethylene Glycol:

   Immunoglobulins are not particularly soluble proteins at neutral pH, it is therefore not difficult to precipitate antibodies by increasing the salt concentration, the most commonly used salt is ammonium sulphate (33).

e) Double (second) Antibody Precipitation:

   This technique uses the specificity of an antibody for an immunoglobulin of the species in which the first antibody was raised. For example if an assay uses goat antiserum, then the appropriate second antibody would be a rabbit or sheep anti-goat immunoglobulin. This technique was first described by Utiger et. al. in 1962 (34).
f) Surface Coated Solid Phases:

It is possible to bind antibodies to glass and plastic particles, for example, Sephacryl (35), to tubes and micro titre plates (36), magnetisable particles (37) and membranes, for example, nylon (38).

Whatever method is used all heterogeneous immunoassays require some type of separation step which can make them labour intensive and slower than their corresponding homogenous immunoassay.

2.1.3. Comparison of Heterogeneous and Homogeneous Immunoassays.

Each assay procedure whether it be hetero/homogeneous has certain characteristics which lend themselves to a particular assay method. The separation step in heterogeneous immunoassays results in additional manipulations and longer assay times but they do tend to be more specific than corresponding homogeneous assays and are easier to automate (39). Table 2.1. shows some comparisons between homogeneous and heterogeneous immunoassays.

2.2. Antibodies.

Antibodies are formed in response to the introduction of a foreign substance, an immunogen, into the body of most mammalian species and all antibodies belong to a group of serum
proteins known as immunoglobulins (40). The availability of antibodies with the desired affinities and specificity is the most important factor governing immunoassay performance.

2.2.1. Structure of Antibodies.

Antibodies are primarily synthesised by specialised plasma cell lymphocytes, the final product of B-cell differentiation. Production of the immune response can occur in two ways, the first involves phagocytosis of antigen by a number of antigen-presenting cells (these include macrophages, Langerhans cells in the skin, dendrite cells in the spleen, and lymph nodes and circulating monocytes) and the second, antigen specific response, is by direct internalisation of the antigen by the B-cells displaying specific antibodies on their cell surface. Both processes act synergistically in the immune response.

There are five main types of immunoglobulins:

IgG, IgM, IgE, IgA and IgD.

The most common antibodies and the one which is the major component of molecular immunity is IgG. As well as being the most common, IgG antibodies are the most important and widely used class of antibodies used in immunological techniques.
Table 2.1. Comparison Between Homogeneous and Heterogeneous Immunoassays.

<table>
<thead>
<tr>
<th>Homogeneous</th>
<th>Heterogeneous</th>
</tr>
</thead>
<tbody>
<tr>
<td>Easy to perform.</td>
<td>Mechanisation difficult.</td>
</tr>
<tr>
<td>Robust procedure.</td>
<td>Performance may contain critical steps.</td>
</tr>
<tr>
<td>Limited sensitivity.</td>
<td>High sensitivities</td>
</tr>
<tr>
<td>Limited dynamic range.</td>
<td>Wide dynamic range.</td>
</tr>
<tr>
<td>Limited Menu(small Ag).</td>
<td>General applicability.</td>
</tr>
<tr>
<td>Sensitive to interference derived from sample.</td>
<td>Less prone to matrix differences.</td>
</tr>
</tbody>
</table>
2.2.1.1. Immunoglobulin G (IgG).

The structure of IgG can be divided into three regions, two identical regions which are the immunoreactive, antigenic regions (Mw 25,000 each) (Fab) and one constant fragment (total Mwt 100,00) (Fc). The two Fab sites consist of regions which have the ability to vary the primary structure and can be generated for a number of antigens. These variable regions are linked to the constant regions by disulphide bonds forming a bifunctional molecule. The Fab region forms the basis for specific antigen interaction and the Fc portion for linking the primary immunologic binding to secondary reactions, including complementary binding. There are also a number of IgG subclasses, which are distinguished by the position and number of disulphide bonds present, as a result they can be expressed for different types of antigen, for example, IgG1 and IgG3 are mainly expressed against protein antigens and IgG2 is mainly produced in the T-cells against carbohydrates (41).

A schematic diagram of the structure of IgG is shown in fig 2.4. and shows the structure is a basic Y shape with the two Fab variable regions situated on the same end of the molecule. IgG is the major immunoglobulin consisting of 70 - 75% of the total immunoglobulin pool.

2.2.1.2. Other Immunoglobulins.

The other classes of immunoglobulins are not commonly used as immunological reagents and as a result will only be discussed briefly here. Of the other four only IgM is used with any frequency as an immunological reagent (consists of 10% of the immunoglobulin pool).
and is produced in the body as the first immunorespnses to an antigen, being more prevalent in neonates.

It is a multivalent pentameric molecule with a molecular weight of 950,000, has high avidity and is an effective activator for antibody mediated response. Of the other three classes of antibodies IgA (15-20% of immunoglobulin pool) is found in secretions, IgE is concerned with allergic reactions and the function of IgD has not been fully elucidated. Antibodies used as immunological reagents can be either polyclonal or monoclonal (section 2.2.2.).
2.2.2. Polyclonal and Monoclonal Antibodies.

As the two names suggest monoclonal antibodies are uniform homogenous proteins produced by a single cell line (B lymphocyte) clone, where as polyclonal antibodies are produced from a number of cell lines.

Each type of antibodies have specific advantages but monoclonal antibodies are beginning to replace polyclonal antibodies in immunological research because they do show some advantages compared to that of polyclonal antibodies. The most important and major advantage is the unlimited supply of well defined homogenous antibodies, which makes them perfect as analytical reagents. Their low affinities (which initially limited their use) can be overcome by careful selection of high affinity antibody producers and currently many monoclonal antibodies have affinities in the range of $10^{-10}$ to $10^{-12}$ l/mol. Table 2.2. describes some of the advantages of monoclonal antibodies.

2.2.3. Antibody Purification.

For a wide number of applications antibodies can be used following a straightforward dilution without the need for purification, but in other cases, for example, where the antibody is to be labelled with a detectable reagent, purification is required. There are four basic methods of purification and these include:-

1) Salting out (usually with ammonium or sodium sulphate) followed by dialysis.
2) Gradient ion-exchange techniques.
<table>
<thead>
<tr>
<th><strong>Advantages</strong></th>
<th><strong>Disadvantages</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Constant well defined characteristics.</td>
<td>Often low affinities.</td>
</tr>
<tr>
<td>Can be produced against small amounts of antigen.</td>
<td>Do not form precipitates.</td>
</tr>
<tr>
<td>Specificity can be unwelcome selected.</td>
<td>Can express individual properties</td>
</tr>
<tr>
<td>Indefinite supply.</td>
<td>May be too specific:- polymorphism of antigen.</td>
</tr>
<tr>
<td>Ease of purification.</td>
<td>Special techniques required for production.</td>
</tr>
<tr>
<td>Low non-specific binding in immunoassays.</td>
<td></td>
</tr>
</tbody>
</table>
3) By specific and reversible binding to lectins, Protein A and G.

4) Immunoaffinity purification.

Immunoaffinity purification isolates specifically the IgG antibodies reactive to the antigen of interest (the other three techniques isolate all IgG).

2.2.4. Fluorescence of Antibodies.

Antibodies being proteins show marked fluorescence especially at lower wavelengths with maximum excitation at 280nm and maximum emission at 335nm (but they can exhibit fluorescence up to 600nm). This fluorescence is caused by specific aromatic amino acids present in the antibodies and these include tryptophan, tyrosine and phenylalanine.

2.3. Antibody-Antigen Interaction.

The forces which hold the antibody and the antigen together are non-covalent and a number of excellent reviews on the nature and strength of the bonds have been published (40-45), but in this case it is sufficient to say that there are four general types present, hydrogen and hydrophobic bonds, ionic and van der Waals forces.
2.4. Detection in Immunoassays.

As will be explained in chapter 5 it is possible to derive the bound/free (B/F) ratio of labelled antigen for a given concentration of unlabelled antigen. Therefore by the production of a calibration curve it is possible to measure the concentration of the antigen in unknown samples. This principle formed the basis for the first immunoassay described by Berson and Yalow in 1959 (8). In the work of Berson and Yalow the two fractions were determined by a radioactive label, $^{125}\text{I}$-Insulin, but since the publication of this paper a number of labels have become available. The following section describes a number of the most commonly used labels together with their advantages and shortcomings.

2.4.1. Properties of Immunoassay Labels.

All compounds used as labels in immunoassays have a number of properties which enable them to be used and these include:-

Detection Limit - all labels should show good limits of detection (from $10^{-9}$ to $10^{-15}$ M) and be detectable by simple, rapid systems with high selectivity.

Coupling Properties - the coupling of the label to the compound of interest should be simple and not effect the immunological properties of the compound of interest.

Stability - the label should provide a stable constant signal for the period of time required to perform the detection of the compound, as well as remain stable for extended periods of time.
in storage. Labels should also be inert against the effects of the sample, for example, quenching.

Availability - to be widely used in the clinical and research environment the label should be easily accessible and require little modification, providing a constant signal for detection.

Safety - with the general trend in the clinical environment towards personal safety all labels should be relatively safe to handle using normal laboratory safety procedures.

As stated in section 2.1.1. the majority of immunoassays require the use of a specific label for signal generation, with a wide variety of labels presently being used which can be conveniently divided into two specific groups, depending on whether or not a radio isotope is used as the signal generator, isotopic or non-isotopic immunoassays.

2.4.2. Isotopic Immunoassays.

The development of radioisotopes as labels in immunoassays is one of the most important events in analytical biochemistry. From their development in 1959 they have become a major force in most of if not all clinical sciences. Radio-immunoassays utilise radio-isotopes as the label of choice and have a number of distinct advantages over other labels which has led to radio-immunoassays becoming the most extensively used form of immunoassays (46).

These advantages include:-
a) small size - being small they cause minimal disruption to the biological properties of the molecule that they are attached too.

b) high sensitivity and selectivity - in the range of $10^{-12}$ to $10^{-17}$ mol, for example, $^3$H sensitivity is approximately $10^{-16}$ mol (47), $^{125}$I is approximately $10^{-17}$ mol (48).

c) wide applicability - radio-immunoassays have been developed for a wide range of compounds, for example, peptides (49), steroid hormones (50), drugs (therapeutic and drugs of abuse) (51), together with various macromolecules of clinical interest, for example, alpha-fetoprotein and thyroglobulin (52).

Some of the more common labels used in radio-immunoassays are listed in table 2.3. Despite their widespread use radio-immunoassays have a number of disadvantages which limits their use and has provided a platform for the development of non-isotopic labels. These disadvantages are well documented but briefly they include:-

a) Considerable concern is often expressed about the radiation hazards associated with radio-immunoassays, for example, isotopes can be absorbed through the skin or inhaled (53), regulations are therefore enforced during production, handling, transport and disposal of radioactive materials.

b) short shelf lives of some isotopes limit their storage (as compared to other non-isotopic labels, for example, enzymes (section 2.4.3.1.). This is due to the short half
lives of some isotopes ($^{125}$I has a half life of approximately 60 days) and the rapid
degradation of some of the labelled antigens.

c) the specialised equipment required, i.e. gamma counters for gamma emitting isotopes
and liquid scintillation counters which can be used with either gamma or beta emitting
isotopes (54) are not available in many laboratories.

d) a separation step is normally required in radio-immunoassays (with the exception of
scintillation proximity assays) because the properties of the radio-label are the same
whether the labelled molecule is bound to the antibody or not. These separation
methods include adsorption onto charcoal or dextran coated charcoal (55), use of
solid phase antibodies adsorbed onto polystyrene tubes etc. (56) and the use of
second, precipitating antibodies forming insoluble complexes with the anti-antigen
antibodies (57) and Protein A (see chapter 3).

e) the radiation damage to the immunoreactants (58).

However, sensitive radio-immunoassays popularity is waning, mainly due to the increasing
pressure to abandon methods which use radioactivity. As a result there is an ever increasing
number of alternatives to radio-isotopes, which will be discussed in section 2.4.3.
<table>
<thead>
<tr>
<th>Isotope</th>
<th>Half Life(^a)</th>
<th>Specific activity(^b) (Ci/atom)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(^3)H</td>
<td>12.6y</td>
<td>29</td>
</tr>
<tr>
<td>(^{14})C</td>
<td>5760y</td>
<td>0.062</td>
</tr>
<tr>
<td>(^{32})P</td>
<td>14.3d</td>
<td>-</td>
</tr>
<tr>
<td>(^{60})Co</td>
<td>5.3y</td>
<td>1,488</td>
</tr>
<tr>
<td>(^{75})Se</td>
<td>120.4d</td>
<td>-</td>
</tr>
<tr>
<td>(^{125})I</td>
<td>59.7d</td>
<td>1,080</td>
</tr>
<tr>
<td>(^{131})I</td>
<td>8.1d</td>
<td>2,220</td>
</tr>
<tr>
<td>(^{35})P</td>
<td>87d</td>
<td>16,100</td>
</tr>
</tbody>
</table>

\(^a\)y = years, d = days.
\(^b\)Theoretical at 100% abundance.

Table 2.3. Common Isotopic Labels Used in Immunoassays.

2.4.3. Non-Isotopic Immunoassays.

Due to the inherent problems expressed in section 2.4.2. immunoassays have been developed which do not require the use of radio-labelled materials. Current methodology has divided non-isotopic immunoassays into those which use labelled material for detection and those which do not.

Several immunoassays have been developed which do not require a label for detection (58), rather they can depend on a number of physical factors, an example of such a techniques is
nephelometry, which measures the light scattering of particles present in solution. When large analytes, such as proteins, are incubated with antibodies, the presence of an insoluble antigen-antibody complex greatly increases the level of light scattering and there are a number of assay kits available which employ nephelometry (59). Turbidimetric assays are similar to those of nephelometry assays, except they effectively measure the cloudiness of the solution rather than the light scattering changes (59). Nephelometry is more sensitive than turbidimetry, with laser nephelometry providing the best results. Further increases in sensitivity are possible by using a system available from TECHNICON which was developed by Cambiaso et. al. (60) in 1977, a particle counting immunoassay (PACIA). In this case the antibodies are coated onto fine latex particles (size range 0.3-0.9mm) by simple physical adsorption (61) or chemical methods (62), which agglutinate in the presence of the antigen, thus the number of particles passing through the flow cell is inversely proportional to the antigen concentration. The full potential of this approach has been limited by non-specific agglutination caused by other factors.

Unfortunately non-isotopic immunoassays which do not require labels suffer from poor sensitivity and as a result are rarely used.

Immunoassays incorporating non-isotopic labels have become more widespread in recent years. There are now many possible alternatives to radio-isotopes, for example, enzymes, metals, fluorescent or luminescent compounds, viruses, free radicals etc. each satisfying the criteria required to be useful labels in non-isotopic immunoassays, these include:-

a) Do not effect the Ab-Ag reaction.

b) Freedom from hazards.

c) Ease of introduction into sample molecules.
d) Free from interference from biological samples.

e) Cost effective and available instrumentation.

f) Suitable for homogeneous assays and automation.

f) Suitable for homogeneous assays and automation.

g) Possibility of combination with another label to develop multicomponent analysis.

There are a number of compounds which satisfy this criteria and these will be discussed in greater detail in the following sections.

2.4.3.1. Enzymes as Labels in Immunoassays.

Enzymes can function as labels in immunoassays because their catalytic activity allows the detection and quantification of extremely small amounts of labelled immune reagents. Enzyme immunoassays were first developed independently by Engvall et al. (63) and Van Weeman et al. in 1971 (64). A large number of enzymes are used in immunoassays and table 2.4 shows a list of the most common. Enzymes have a number of advantages over other types of labels and these include:-

a) They are relatively cheap, are readily available in purified form with a long shelf life.

b) The enzyme labelled antigen and antibody are stable compounds.
c) A range of assays are available for the measurement of enzyme activity and these can be used on readily available equipment.

d) Detection limits for enzymes are very low (65) due to the ability of enzymes to catalyse reactions.

e) Enzyme activity can be modulated, thus non-separation enzyme immunoassays are possible (66-68).

Enzymes have also a number of inherent disadvantages:

a) The activity of the enzyme may be reduced by exposure to elevated temperatures or may be inhibited by substances present in the sample.

b) Enzymes are susceptible to interferences by changes in assay conditions during the signal generation stage.

c) There are two incubation stages in enzyme immunoassays (EIA), antigen-antibody binding and signal generation, each incubation must be carefully controlled to prevent imprecision.

d) Some reagents used during EIA are hazardous, for example, some peroxide chromogens are carcinogenic and mutagenic (69).
Table 2.4. Some Common Enzymes used in Immunoassays.

<table>
<thead>
<tr>
<th>Heterogeneous EIA</th>
<th>Homogeneous EIA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Horse-radish peroxidase.</td>
<td>Glucose-6-phosphate dehydrogenase.</td>
</tr>
<tr>
<td>Alkaline phosphatase.</td>
<td>Lysozyme.</td>
</tr>
<tr>
<td>β-D-Galactosidase.</td>
<td>β-D-Galactosidase.</td>
</tr>
<tr>
<td>Glucoamylase.</td>
<td>Malate dehydrogenase.</td>
</tr>
<tr>
<td>Carbonic anhydrase.</td>
<td>Lactate dehydrogenase.</td>
</tr>
<tr>
<td>Catalase.</td>
<td>Lipoamide dehydrogenase.</td>
</tr>
<tr>
<td>Acetylcholinesterase.</td>
<td>Hexokinase.</td>
</tr>
<tr>
<td>Penicillinase.</td>
<td>Glucose Oxidase.</td>
</tr>
</tbody>
</table>

Despite their disadvantages enzyme labelled non-isotopic immunoassays are used for a large number of compounds (64-69).
2.4.3.2. Luminescent Labels.

Luminescence is a well established analytical technique and is a generic term that covers a range of processes which produce light (70), these include fluorescence, phosphorescence, chemiluminescence and bioluminescence. All these techniques have been applied to immunoassays but by far the most common are fluorescent labels (phosphorescence, chemiluminescence and bioluminescence are discussed in an excellent review by Kricka and Thorpe (71)).

The development of modern, precise optical instrumentation in recent years has been the catalyst for an increased interest in fluorescent labels for immunoassays.

As well as the properties required by labels for immunoassays (section 2.4.2.) fluorescent labels have a number of additional requirements(71):

\( a \) High relative fluorescence.

\( b \) Large Stokes' shift and/or long lifetime.

\( c \) Good solubility with low affinities to serum proteins or solid-phase surfaces.

\( d \) Low non-specific binding to surfaces.

\( e \) No steric hindrance.

In recent years fluorescent labels have been divided into two distinct groups (72), those conventional labels which fluoresce in the wavelength region 280-600nm (visible), and those which fluoresce at wavelengths above 600nm, the near infrared (NIR) or long wavelength fluorophores (73).
Fluorescent labels utilise the energy emitted when a substance returns from an excited state, or higher energy state, to a ground state. In the case of fluorescence and phosphorescence the substance is raised to a higher energy state, usually by the absorption of radiant energy, for example, ultraviolet rays. During this high energy state the excited molecules either remain in the singlet state or are transferred by intersystem crossing to the triplet state (this triplet state requires a spin inversion with the loss of some energy). It is this transfer, which takes approximately $10^{-12}$-$10^{-14}$ secs, which delays the emission of the excited light and separates fluorescence from phosphorescence. If the emission of energy ceases after approximately $10^{-8}$ sec it is referred to as fluorescence, but if it persists for a longer period of time it is referred to as phosphorescence.

This absorption of energy occurs in discrete units or quanta equivalent to the energy difference between the ground state and an upper electronic excited state. This absorption of energy and the path which the excited molecules follow is best described using an energy diagram, shown in fig 2.5.

In most cases the absorption of energy will result in the first singlet state, $S_1$, if the highly excited states of $S_2$, $S_3$ etc. are reached these rapidly lose their energy ($10^{-12}$ secs) by passing vibrational energy onto other molecules in a process known as internal conversion. Once the molecules drops down from the excited $S_1$ state to the ground state much of its energy is emitted as fluorescence at a longer wavelength (and hence less energy) than the absorbed energy. This shift in wavelength is known as Stokes' shift. The molecule can also lose small amounts of energy in other ways, for example, non-radiant transition and intersystem crossing (previously mentioned).

Fluorescent labels are essentially fluorescent dyes which have been utilised in protein chemistry for a number of years, for example, fluorescent dyes have been used as labels for
Schematic representation of the energy transitions possible when a molecule capable of fluorescence absorbs energy. $S_0$ = electronic ground state; $S_1$ = first excited singlet state; $S_2$ = second excited singlet state; $T_1$ = first excited triplet state; $T_2$ = second excited triplet state

Fig 2.5. A schematic Diagram of Energy Transfer
proteins as far back as 1940, benzanthryl isothiocyanate was first used as a label for proteins by Creech et al. in 1940 (74), antibody labelling with fluorescent dyes by Coons et al. in 1941 (75).

The most popular conventional fluorescent label (250-600nm) historically has been fluorescein but because of the small Stokes' shift (i.e. 20nm) and the difficulty of obtaining the dye in pure form (commercial fluorescein is normally a mixture of the two isomers), alternative fluorescent dyes have been developed. These dyes include 4,5-dimethoxy-6-carboxyfluorescein (76), tetramethyl- and tetraethyl rhodamine isothiocyanate, rhodamine -B isothiocyanate, lissamine-rhodamine B sulphonyl chloride (77-79), dansyl chloride (80), fluoram (81), methoxy-2,4-diphenyl-3(2H) furanone, umbelliferones and lucifer yellow (82).

Fluorimetry in the near infrared or long wavelength region (600nm -1000nm) is beginning to gain support with a number of workers (83-84). The reason for NIR’s appeal is due to the advantages these labels show over conventional labels, such as less background noise, lower interference, less scattering, less photo-decomposition and the development of cheap portable solid state detectors and light sources. The advantages described are due to a number of factors, for example, less interference present when using NIR labels is because only a few families of compounds are able to absorb and fluoresce at such long wavelengths (84), the development of smaller, cheaper light sources such as semi conductive lasers (85) have made it possible to produce cheap instrumentation to detect femto gram levels of fluorescent labels (86). At longer wavelengths which are used for these labels less photo-decomposition takes place because there is less energy required to make the compound fluoresce together with the less energy available at longer wavelengths (i.e. the longer the wavelength the less the energy).

A final advantage of working in the NIR region is the reduction of interference from plasma proteins, i.e. proteins fluoresce from 250 to 600nm causing interference when using
conventional fluorescent labels, this interferences is considerably reduced at longer wavelengths. Many of the Polymethine cyanine dyes absorb and fluoresce in the NIR region (87), for example, Indocyanine green (IGG), a symmetrical carbocyanine dye with a sulphonylic acid group at the end of the butyl aliphatic chains attached to the indole rings (excitation wavelength 750 nm, emission wavelength 770 nm), cyanine dyes (88) and phthalocyanines (83). There is a great deal of work at present being done on the development of longer wavelength labels with the majority of workers moving away from the more conventional labels.

As well as the labels previously mentioned there are a variety of other compounds which are used to provide a signal in immunoassays, not as popular as enzyme or fluorescence, they still provide the analyst with a viable alternative to radio-isotopes. These labels are numerous, but some of the more common are included in table 2.5.

2.4.3.3. Miscellaneous Non-Isotopic Labels.

As well as the labels mentioned in table 2.5, other more obscure labels have also been used, for example, apoenzymes (89), coenzymes (90), liposomes (91). Whatever system is finally chosen for the specific analyte the fundamental immunoassay procedure remains the same. Some labels have specific advantages over others but the label finally chosen for a particular assay normally comes down to personal choice or available instrumentation. With the development of non-isotopic labels and the volume of publications accompanying them it seems the domination of radio-labels in immunoassays is at an end. Although the use of radio-labels will reduce slowly over the next few years it is doubtful that radio-immunoassay work
will cease, in fact it will probably continue long after new techniques have been developed, this in itself shows the importance of the discovery and subsequent development of radio-immunoassays by Berson and Yalow in 1959.
<table>
<thead>
<tr>
<th>Type of Immunoassay</th>
<th>Label</th>
<th>Detection Methods</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metallo-immunoassay</td>
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CHAPTER 3

AFFINITY CHROMATOGRAPHY
3.1. Introduction.

Of all the separation mechanisms currently used in bio-separation sciences, affinity chromatography (or more exactly, bioaffinity or biospecific affinity) is considered to be the most specific, since it is based on the unique specificity inherent in a ligand bio-molecular interaction. Thus affinity chromatographic techniques exploit the property of ligands to bind specifically and reversibly to other substances. These ligands are generally called affinants or affinity ligands (1). If an insoluble ligand is prepared, usually by covalently coupling to a solid support and a solution containing biologically active products to be isolated is passed through a column of this solid support matrix, then all the components which, under given experimental conditions, have no affinity for the ligand will pass through the column unbound. The products which do show affinity will be bound or retarded, to be released later, either by changing the pH, ionic strength, temperature, or by using dissociation agents (2).

The principle of affinity chromatography has been known since the early 1950's (3) but the method was not used extensively until the development of solid supports which could not only bind the ligand of interest but also eliminate or at least reduce the non-specific adsorption of the biomolecule of interest.

Such supports were developed by Porath et. al. (4) in 1967 and Cuatrecasas et. al. (5) in 1968. Both workers showed that it was possible to attach a ligand binder to agarose, which showed all the characteristics of the ideal support matrix, from these initial experiments it was possible to develop a variety of matrices, each with their own unique characteristics.
3.2. Affinity Chromatographic Matrices.

A successful affinity chromatographic matrix should mimic the interaction that would occur if the components were in free solution (6) and there are increasing numbers of solid supports becoming commercially available as the level of interest in affinity chromatography increases. There are a number of considerations when choosing the solid support and these include price, area of research, compound required to isolate etc. Ideal matrices should adhere to the following guidelines:-

a) Insolubility.

Complete insolubility is essential not only for the prevention of losses of affinity matrix but also to ensure that there is no contamination of the product isolated.

b) Loose porous network.

Required for unimpaired and uniform entry and exit from the matrix.

c) Particles should be spherical and rigid.

Solid, rigid particles show good flow characteristics which provide an even controllable flow rate which is important in affinity chromatography.

d) Sufficiently permeable with a large surface area.

A large surface area enables a large area of interaction between the solid surface and the ligand.
e) Little or no adsorption capacity.

To ensure that no non-specific binding occurs, i.e. binding of the ligand with an area of the matrix other than the specific ligand binder.

g) Stable ligand binding.

The capacity of the solid support depends on the number of ligand binding groups present. These groups should remain mechanically and chemically stable under the conditions required for the attachment of the ligand.

f) Ease of activation and modification.

It should be possible to attach and remove specific groups for specific ligands (under controlled conditions), for example, ligand binders.

h) Chemically and biological inert.

The support should not be susceptible to attack from micro-organisms, enzymes or chemicals used in the attachment of the ligand.

I) Hydrophilic.

Due to the nature of most affinity chromatographic procedures the support should be hydrophilic (most biological compounds are denatured by organic solvents).

There are a number of supports available, produced from natural and synthetic materials. Historically natural materials have been the most popular but as synthetic engineering has
improved so has the quality of the synthetic materials available. In sections 3.2.1 and 3.2.2, a number of these matrices will be discussed.

3.2.1. Natural Solid Supports.

3.2.1.1. Carbohydrate Polymers

Agarose is the most popular solid support used at present for column chromatography and is derived from a repeated agarobiase unit consisting of alternative 1,3-linked β-D-galactopyranose and 2,4 linked 3,6-anhydro-α-L-galacto-pyranose residues (fig 3.1) (7). The beaded derivative Sepharose has many of the properties of the ideal matrix and is available from several commercial sources, normally in a 1 to 12% gel, although custom preparations are available (8). It is possible to alter the chemical and physical stability of the gel by cross linking the polymer, for example, with 1,4-butanediol diglycidyl ether, to increase both the thermal and chemical stability but this may decrease the capacity (9). Increasing the rigidity of the gel enables it to be used in HPLC systems (10), whilst ultra rigid gels are available from PHARMACIA, in the form of Superose and Sepharose fast flow gels (11).

Cellulose, another carbohydrate used as a solid support has only historical significance in modern affinity chromatography and was initially used by Campbell et al. in 1951 to purify mushroom tyrosinase using a p-phenylazophenyl coupled to a p-aminobenzylcellulose (12). Cellulose has been particularly effective in the field of nucleotide chemistry, for example, phospho and DNA cellulose (13-17). However, the usefulness of cellulose has been limited
by its fibrous and non-uniform character, which impedes proper penetration of the macromolecules, although novel cellulose supports have been developed by Dean et. al. which are both porous and spherical (18).

![Fig 3.1. Structure of Agarose](image)

Dextran, an alpha-1,6-linked glucose polymer is also available as a solid support matrix. Two main types of dextran gels are available, Sephadex (cross linked by glyceryl bridges) and Sephacryl (allydextran cross linked by N,N-methylenebisacrylamide). Both are more commonly used in the chromatography of biomolecules, Sephacryl being used mainly for the affinity chromatography of very large molecules, for example, Sephacryl S-1000 used for separation of factor VIII (19), at low pH Sephacryl S-200 adsorbs proteins (20). Sephadex is available in a number of porosity sizes from the largest G200 to the smallest G10 and is usually used in affinity chromatography as a purification step, for example, purification of
glucoamylase using G200. Other applications for dextrins in affinity chromatography (22-23) are possible but normally they are mechanically too weak (G75-G200) or insufficiently porous (G10-G75) to provide an ideal matrix.

3.2.1.2. Glass and its Derivatives.

Glass has been the most important inorganic support matrix used in affinity chromatography in recent years (24) and is available commercially as porous granules of high silica glass permeated by interconnecting pores of uniform and precisely controlled size i.e. controlled pore glass (CPG). Glass is mechanically quite, thermally stable and is chemically compatible with organic solvents. Columns packed with CPG are ridged, insoluble (when derivatised) and are unaffected by changes in the eluent or solvent system, pressure, flow rate, pH or ionic strength. Also glass has the added advantage of being inorganic and therefore not susceptible to microbial attack, as a result each column can be used many times. CPG is the most commonly employed glass matrix for the immobilisation of biological products and consists of approximately 4% borate plus traces of other inorganic oxides (18). The pores, produced by acid treatment, are uniform and can be between 25-75 microns, providing uniform porosity. Underivatised glass is known to absorb enzymes non-specifically and therefore all glass used in this way requires derivatisation (24). It is also possible to coat glass with an inert polymer (25) to prevent non-specific adsorption caused by the hydroxyl groups on the surface of the glass exhibiting a slight negative charge in aqueous solution.
3.2.2. Synthetic Solid Supports.

As well as natural polymers there are a number of synthetic polymers used in affinity chromatography. They have a distinct advantage over natural supports in that they can be engineered to produce the desired solid support, without the weakness associated with natural polymers. New synthetic polymers for affinity chromatography are produced at regular intervals and any study could not hope to list them all, as a result the following section deals only with the more widely used synthetic solid supports.

3.2.2.1. Polyacrylamide (PAA).

Polyacrylamide is the most widely used of all the synthetic polymer solid supports and is available under a number of trade names, but the main suppliers are Bio-Rad Laboratories under the trade name of Bio Gel-P (26). They are composed of a hydrocarbon skeleton onto which carboxyamide groups are bound, i.e. a copolymerisation of acrylamide and a cross linking reagent, usually N,N'-methylenebisacrylamide (fig 3.2.) (27). Polyacrylamide (PAA) was first used in protein chemistry in 1962 by Hjerten et. al. (28) and can be used with most of the common elution agents, is stable over a wide pH range (pH1-11) and does not contain any charged groups (exchange interactions with chromatographic substances are minimised) (29). On reaction with a number of suitable compounds PAA gels can be converted into solid carriers suitable for binding a series of ligands (30) and is supplied in a number of pore sizes, 50-100, 100-200, 200-400 and 400 mesh (31).
The main disadvantage of PAA is, that like Sephadex, the gels are either too soft or the pores are too small (32).

3.2.2.2. Trisacryl.

The trisacryls (fig 3.3.) are a range of synthetic gels which can serve as chromatographic polymer supports which are very hydrophilic and suitable for the separation of biological macromolecules, especially proteins and cells. They provide a distinct advantage over PAA.
gels which are very hydrophobic. They are resistant to moderate pressure (up to 3 bar) which makes them ideal for use in high performance systems. There are a number of trisacryl gels available, depending on the functional group:-

\[ R = \text{COOH}, \quad \text{CM-Trisacryl.} \]

\[ R = \text{NH} (\text{CH}_2) _2 \text{NEt}_2, \quad \text{DEAE-Trisacryl.} \]

\[ R = \text{NH-CMe}_2 \text{-CH}_2 \text{-SO}_3 \text{H, SP-Trisacryl.} \]

\[ R = \text{NH}_2, \quad \text{unsubstituted Trisacryl.} \]

All exhibit similar properties, hydrophilic, non-biodegradable, stable at low (-20°C) and high (121°C) temperatures, unaffected by denaturing agents, stable at acidic and alkaline pH (pH 1-13) and all types are available with bead sizes 40-80μm.

3.2.2.3. Hydroxyalkyl Methacrylated Gels.

Hydroxyalkyl methacrylated gels were introduced in 1960 (33) and are synthesised by the copolymerisation of 2-hydroxyethyl methacrylate and ethylene glycol dimethacrylate (fig 3.4.) (34) which can change its porosity and specific surface area by altering the number of reactive
groups present. The gel forms regular beads with excellent chemical and physical properties (as compared to PAA) (34) and withstands pressure well (which is unusual for a gel), the structure is unaffected by heating (150°C for 8 hours in 0.1M sodium glycolate solution (34)), biologically inert, not attacked by micro-organisms and can be employed in organic solvents. It can be hydrophobic (like PAA) and as a result makes them unsuitable for many applications, but they can be used for the separation of chymotrypsin and proteolytic inhibitors (35).
3.2.2.4. Thiophilic Gels (T-Gel).

Thiophilic adsorption is based on the selective affinity of proteins, especially immunoglobulins, towards the β-mercaptoethanol derivative of divinylsulphone activated agarose (fig 3.5.) which binds proteins at high concentrations of lyotropic salt or sodium chloride (37). The operational principle behind thiophilic adsorption chromatography resembles that of hydrophobic interaction chromatography, although thiophilic gel leads to higher yield and sharper separations than the traditionally used hydrophobic matrices (for example, phenyl-Sepharose and octyl-Sepharose) (38).

\[ \text{O-CH}_2 \text{-CH}_2 \text{-SO-CH}_2 \text{-CH}_2 \text{-S-CH}_2 \text{-CH}_2 \text{-OH} \]

Fig 3.5. Structure of T-Gel

Thiophilic gels and thiophilic interactions are a viable alternative for the purification of immunoglobulins under conditions, which preserve biological activity, they offer selectivity, good protein recovery, preservation of antibody function, gentle elution conditions and high purity (39). Unfortunately it is only available in a gel format and as a result suffers the same problems associated with other gel solid phases, for example, attrition, unable to withstand high flow rate etc (40).
3.2.3. POROS

Since the development of the science of separation of biological macromolecules by liquid chromatography began in the late 1950's, with the introduction of polysaccharide based supports (41), a variety of matrices have dominated for extended periods of time. For the first twenty years, after the initial experiments with polysaccharide supports, they dominated the chromatography of proteins. This domination was ended in 1976 with the development of HPLC supports based on silica (42). Steady progress was made over the next two decades or so but separation scientists were finding that to increase the amount of ligands bound to the matrix the particle size had to be reduced, so as to increase the surface area. Unfortunately as particle size is reduced, problems mount exponentially, for example, as the particles are smaller greater pressure is required through the columns.

It has been possible to reduce the particle diameter down to approximately 2μm before these problems outweighed the advantages (43,44). As a result the era of decreasing particle size has probably come to an end (45). It is currently accepted that the transport efficiency between the stationary phase and the mobile phase is the most dominant variable controlling the resolution in liquid chromatography (46). Resolution is the subject which has preoccupied separation scientists for more than four decades (the reduction of resistance to mass transport, which increases resolution, through the use of small particles was the main reason for the success of HPLC), this variable transport efficiency, or intraparticle solute transport is controlled by a number of factors because solute transport through a chromatographic column occurs predominately by two mechanisms:-

(i) convective transport
As the solutes flow between the individual particles of the column.

(ii) diffuse transport

As the solutes flow through the stagnant pools of liquid which have formed around and in the column packing.

Obviously diffusion occurs within the flowing mobile phase but this is deemed insignificant.

As a result of these two mechanisms dominating solute transport through a chromatographic column, it is obvious solute transport to the surface of the matrix is an important prerequisite in surface mediated separations (46). Simply, the solutes must first diffuse across an immobilised film surrounding the surface of the matrix and then through the stagnant pools into the matrix pore system, i.e. the matrix beads become coated with the transport buffer covering them in a film which makes it difficult for the ligands in solution to transfer from the transport buffer to the ligand binders situated on the matrix. This process is known as stagnant mobile phase mass transport and it is this which has been identified as one of the major contributors to peak dispersion and poor resolution in liquid chromatography (47). There have been a variety of techniques and matrices which have been experimented with to reduce the problems associated with stagnant mobile phase mass transport. Some of these approaches have had limited success, for example, analytical separations have been achieved in less than 60 secs with 2-3 \( \mu m \) non-porous particles at mobile phase velocities of 3-5mm/s (48-49). Unfortunately removing the pores is not a realistic option as this significantly reduces the surface area. A more viable and alternative approach to the problem of stagnant mobile phase mass transport, caused by intraparticle diffusion is by altering not only the porosity of the particle but also the permeability of the packing material in order to allow controlled liquid
flow through as well as around the particle (50), such a method has been developed and is known as perfusion chromatography (section 3.2.3.1.) and an example of such a perfusion chromatographic matrix is POROS.

3.2.3.1. Structure of POROS.

The structure of POROS can be described as an agglomeration of microspheres which are fused together into a continuous structure (43) to create the pore matrix. The matrix itself consists of polystyrene divinyl benzene, developed in 1960 (51), containing two types of pores, the largest of these being approximately 600 -800 nm in diameter with the second more abundant set of pores 80 -150 nm in diameter and approximately 1 µm deep (fig 3.6.). The larger through-pores transect the particles, allowing the mobile phase to flow directly through the particles, hence the name, the smaller diffuse pores intersect the larger through-pores. This set up allows liquid flow through the particles, via the larger pores, into the smaller pores, allowing fast solute diffusion from the mobile phase to the ligand binder. As a result the speed of ligand attachment to the solid phase is increased because the distance the solutes have to diffuse is greatly reduced by the combination of pore sizes and the elimination of mobile phase mass transport.
Fig 3.6. The Structure of the POROS Beads.

This type of system is referred to as a perfusion system (this is because the system of transport is similar to that which occurs in the kidneys).

3.3. Flow Injection Analysis.

In a chemical laboratory any liquid materials which are to be measured analytically undergo a number of procedures which are performed by the analyst. These include, solution handling,
sample detection, data collection and storage of results. With the ever increasing use of computers the handling of samples manually has become outdated. However automated the analysis the precision, speed and accuracy still depends largely on the skill of the analyst and therefore can vary markedly from lab to lab and from analyst to analyst. Therefore there has been an increased interest in the automation of analytical procedures involving liquids, particularly over the last 30 years.

The development and automation of such procedures has been reviewed in-depth by several workers (52,53) and therefore shall be discussed briefly here.

It is possible to automate sample handling procedures with the use of robots although these are only justified if large numbers of repetitive operations are to be handled over prolonged periods, for example, ZYMARK robots for the colorimetric determination of formaldehyde in water (53). Such robots can prove expensive, therefore a cheaper method of automation is required for routine analysis. A major breakthrough in the automation of analytical procedures was made by Skeggs in 1957 (54) with the development and use of segmented streams in segmented continuous flow analysis (SFA). By 1975 the inherent problems associated with SFA prompted two groups of workers, Ruzicka and Hansen in Europe and Stewart et al in America to develop flow injection analysis (FIA). There are a number of excellent papers describing the development, theory and applications of FIA (55) and several hundred papers have been published since the introduction of this versatile technique. From its introduction in 1975, FIA very quickly gained wide acceptance as an analytical tool with a wide range of workers which is illustrated by two reviews by Ruzicka and Hansen, the first in 1980 (56), lists nearly 80 papers covering several applications, the second, in 1986 (57), contains over 800 references covering 8 major application areas, measuring over 200 analytes and using 50
different analytical techniques. More recent reviews about the development of FIA are also available (58-60).

Flow injection analysis (FIA) is simply a technique which enables a liquid sample to be injected into a continuous non-segmented flowing steam. Its success and wide use can be attributed to the reproducibility of the operations involved in the system, such as injection, pumping and timing. This leads to the possibility of carrying out quantitative analysis without the necessity to complete the chemical reaction, for example, in techniques such as titrimetry quantitative analysis is possible on strong oxidising and reducing agents where reactions do not have to reach equilibrium using FIA, which under normal conditions has proven impractical. A basic scheme of a FIA system is shown in Fig 3.7.

FIA was initially developed from segmented flow analysis and the two systems do show some similarities which include hardware such as pumps, tubing, detectors, reaction coils etc. and both systems can incorporate additional separation techniques, for example, dialysis, solvent extraction. But the fundamental difference between FIA and SFA is the absence of air bubbles in FIA. Aspiration systems are used in SFA (introduction of bubbles of air) to ensure separation of the samples and to facilitate mixing of sample and reagent, which occurs between two plugs of air. This system therefore produces a steady state signal.
In the case of FIA, where no aspiration takes place, the signal is more transient due to dispersion of the sample with the carrier stream (fig 3.7.). As a FIA system does not require an aspiration system the manifold used is somewhat less complex and therefore produces more reproducible results on the premise that simpler systems have less variables to affect results. FIA has been transformed in the last decade from a relatively new concept of rapidly conducting serial assays to constituting a generalised approach of solution handling in the chemical laboratory.
As a result, has moved on from not only being a tool for fast serial analysis but has also diversified to become:

a) an impulse response technique where the sample is a species situated in the carrier stream and bombarded by zones of reagent solutions, for example, simultaneous determination of molybdenum (VI) and tungsten (VI) (61).

b) a tool for continuous monitoring where the sample source is the monitored process, for example, determination of soil nutrient status (62).

c) a vehicle for conducting physicochemical measurements, where the species under study replaces what originally was viewed as the sample, for example, fluorescent labelled immunoassays (63).
FIA is based on the information gathered from a concentration gradient formed while a well-defined zone of fluid disperses into a continuous non-segmented stream of carrier (57).

This definition, by Ruzicka and Hansen, though not complete, describes the unique features of FIA, i.e. the exploitation of a matrix of concentrations against time, shown schematically in fig 3.9.

The essential features of FIA can be summarised as follows:-

(a) The flow is not segmented by air bubbles, which is the fundamental difference from SFA.

(b) The sample is being injected or inserted directly into the flowing stream instead of being aspirated into it.

(c) The injection plug is transported within the system. A physicochemical process (chemical reaction, dialysis, liquid liquid extraction, etc.), may occur in addition to transport.

(d) The partial dilution or dispersion of the analyte during transport can be manipulated by controlling the geometric and hydrodynamic characteristics of the system.

(e) A continuous sensing system yields a transient signal which is suitably recorded.

(f) Chemical equilibrium need not have been attained when the signal is detected.
Fig 3.9. A Schematic Diagram of Concentration against Time for a FIA System.

(g) The operational timing must be highly reproducible because the measurements are made under non-steady state conditions.

The concentration gradient produced (obtained through exactly reproducible timing) is initiated by injection, then formed by controlled dispersion of the originally homogeneous zone into the carrier.
3.3.1. Sample Dispersion.

Sample dispersion has historically been ignored in most FIA reviews but is a key issue in the understanding and applications of FIA. The nature and magnitude of the dispersion depends on physical flow parameters, mechanical manifold parameters, the geometry of the reactor and chemical factors, therefore by controlling these factors the dispersion can be controlled. The purpose of dispersion control is to optimise the chemical reaction which takes place. Over a number of years refinements to the basic FIA system have proceeded gradually, especially as knowledge and studies of dispersion in narrow tubes have increased. Hardware refinements have resulted in a variety of sample injection systems which have often been claimed to surpass each other with respect to performance, reliability, simplicity and price. Exploration of reproducible timing in FIA has led to gradient techniques such as stopped flow (64) and titration techniques (65). However, all the FIA techniques, especially those mentioned, rely on the fact that the dispersion process can be controlled so that the conditions of the chemical reaction can be optimised.

When a sample is injected into a flowing stream it forms a sample zone (bolus) which is transported to the detector, which continuously monitors the absorbance, luminescence, electrode potential or other physical parameter of the flowing stream, by means of a flow cell, positioned in the detector.

There are two main mechanisms occurring:

(a) Convective Transport.

This occurs under laminar flow conditions that are present in FIA. The convective transport mechanism produces a parabolic velocity profile, in which the sample
molecules exhibit a distribution in linear velocity. This is close to zero at the walls of the tube and twice the average velocity at the tube centre.

(b) Diffusional Transport.

This occurs from the concentration gradients that are initiated by the convective transport processes. Radial diffusion is caused by concentration gradients that are perpendicular to the direction of the flow; it has a significant effect on the overall dispersion. Axial diffusion which has a negligible effect on the overall dispersion, occurs at the leading and trailing edges of the sample bolus and is caused by the presence of concentration gradients that are horizontal to the direction of the flow.

Fig 3.10 shows a schematic diagram of the two types of transport along a tube in FIA.

As the quality of readout in FIA determines the usefulness of the technique and the quality of readout depends on the "form" of the travelling sample zone, control of the dispersion of the sample becomes one of the most important tasks.

Dispersion is a result of diffusion and convection in axial and radial directions and can be divided into three main ranges, limited, medium and large dispersion, each corresponding to a value range of the practical dispersion parameter:-
Response

\[ h_{\text{max}} \]

\[ \text{i.p.} \]

\[ t_p \]

\[ t_w \]

\[ t_a \]

\[ \Delta t \]

Flow

Response

(a) (b) (c) (d)

Time

i.p. = Injection point.
h_{\text{max}} = Peak height at maximum.
t_a = Peak appearance time.
t_p = Residence time.
t_w = Return time.

Fig 3.10. A Schematic Diagram of the Transport of Samples along a FIA System.
(\(D = \frac{C_0}{C_{\text{max}}}\))

where:

\[ D \] = Dispersion
\[ C_0 \] = initial sample concentration.
\[ C_{\text{max}} \] = peak height concentration at the detector.

Limited dispersion occurs in narrow tubes where the dispersion of the sample zone will be limited (for example, \(D=2\)) and will result in the sample reaching its maximum value in the detector within seconds of injection. Therefore as this implies this type of limited dispersion system is unsuitable for assays based on chemical reactions but is ideal for transporting a sample to the detector with controlled dispersion and exact timing. Examples of detectors used in limited dispersion are ion-selective electrodes and atomic absorption spectrophotometer which use FIA techniques to produce higher sample rates and better reproducibility than conventional manual batch methods.

Medium dispersion (\(D=2-10\)) are the most interesting from an analytical view point because they can be applied to a large number of analytical procedures, for example, fluorescence, electro-activity. In this type of determination mixing aids are employed to produce the required dispersion level, for example, mixing coils.

Large dispersion (\(D=>10\)) systems are used to produce a concentration gradient with the help of FIA producing a substantial degree of mixing between the sample and the flowing stream,
examples of such systems are continuous flow titrations or sample/stream interface experiments.

In normal experimental conditions dispersion levels are not as clear cut as those stated but there is normally a compromise depending on degree of mixing required, the sample interval accepted, the degree of completeness that the reaction is required to reach and most critically the type of analytical readout, for example, peak height or area. Whatever compromise is reached, any degree of dispersion will have an adverse effect on sample frequency and sensitivity.

There are a number of variable components in FIA which affect dispersion which have been studied extensively by several workers, but briefly they are:-

(a) Flow rate (66,67).
(b) Tube length (66-68).
(c) Sample volume (66,67,69).
(d) Reactors (70-72).
(e) Temperature (73-78)
(f) Multiline system (79-83).

As previously mentioned FIA has diversified into nearly all fields of analytical chemistry incorporating a variety of detection systems. In this case we are concerned with the development of flow injection utilising immunoassays as the analytical technique, which is discussed in greater detail in section 3.3.2.
3.3.2. Flow Injection Immunoassays (FIA).

With the ability of FIA to provide an easily automated, precise control of reagent addition, reaction times and reliability, coupling the technique with immunochemical methods provides the analyst with an extremely powerful tool. As suggested in section 3.2 typical solid phase matrices utilised in immunoassays are far from ideal and therefore the vast majority of immunoassays are performed using microtitre plates although, in the last 15 years, an increase in the development of flow injection immunoassays utilising small immunoaffinity columns has occurred (84-86).

These immunoreactors can contain a number of rigid supports to covalently couple the antibody or ligand binder and there are a number of solid supports available, sepharose (87), non porous silica (86,87), trisacryl (88,89), divinylbenzene (90), polymeric beads (91), activated nylon membranes (92), controlled pore glass (93), etc, (these supports are discussed in more detail in section 3.2.).

A number of solid phase immunoassays have been developed employing a range of binders (87, 93-94). Antibodies are the most common binders employed (i.e. a molecule that exhibits molecular recognition for another molecule, a ligand) in ligand binding assays but there are others available, these include, protein A (95), protein G (94), thyroxine binding globulin (96), riboflavin binding protein (97), avidin (98), DNA (99), concanavalin A (100) and cell surface receptors (100).

The coupling of antibodies directly to the solid phase in flow injection immunoassays occurs in a random fashion, i.e. not all the antibodies are attached directly or in the right orientation
for ligand binding, therefore reducing the antigenic binding capacity. This problem can be largely removed by using Protein A coated matrices (section 3.4.) because it binds the Fc region of the antibodies and orientates them for maximum ligand binding. Also, as table 3.1. shows it can bind a whole range of antibodies (101) which ensures not only high affinity but also extremely flexibility in their use.

These FIA based immunoassays generally provide greater speed and precision as compared to the more conventional assays, for example, ELISA assays based on microtitre plates or plastic tubes, because they utilise the reproducibility associated with flow and a wide range of flow injection immunoassays have been published using enzymes (102), immunoprecipitation (103), liposomes (104), and fluorescence (105).

The use of flow injection immunoassays is not as popular as such a flexible and simple technique would suggest (61), but the advent of automation and the development of novel matrices with increased sample throughput should generate increased interest in this versatile system.

3.4. Protein A (SpA).

Staphylococcal protein A (Protein A), a protein component found in nearly all strains of *staphylococcus aureus* (106) and was first described by Verwey in 1940 (107) as a protein antigen present in type A Staphylococci (coagulase-positive, α-toxin-positive, mannitol fermenting and pathogenic) but not in type B staphylococci (those lacking these characterises). The study and characterisation of Protein A did not begin in earnest until nearly 20 years later in the 1960's when together with the proposal of the designation of protein A
to avoid confusion with the antigenic polysaccharide A, it was found that Protein A had the ability to bind non-specifically with the majority of mammalian immunoglobulins (108).

3.4.1. Biochemical Structure of Protein A.

Protein A is a 42,000 molecular weight protein, highly stable and retains its activity when exposed to 4M urea, 4M thiocyanate, 6M guanidine hydrochloride, together with pH and temperature extremes (109).

It has a markedly extended, rather elongated shape (this can be determined by its frictional ratio of 2.1-2.2 and its intrinsic viscosity of 29 m/g (110)) with very few if any sugar residues. The secondary structure, as estimated by Lindmark et. al., consists of 31% α-helix (which is present as two helices containing 10 of the 11 residues known to make close contact with the Fc region of immunoglobulins), 13% β-structure and 51% random coil (110).

3.4.2. Reactivity of Protein A with Immunoglobulins.

Protein A binds to serum immunoglobulins of almost all mammalian species (111) with the affinity markedly different depending on the species and class of immunoglobulin (112), for example, Protein A binds IgG from human, pig and rabbit with high affinity, horses and cows IgG with lower affinity and binds rat IgG only very weakly (Table 3.1). The different classes and sub-classes of human IgG also show varying binding affinities. IgG1, IgG2, IgG4 bind strongly, IgG3 does
Table 3.1. The Affinities of Some of the common IgG Species to Protein A.

<table>
<thead>
<tr>
<th>Species</th>
<th>IgG-Protein A Binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>S</td>
</tr>
<tr>
<td>Mouse</td>
<td>S</td>
</tr>
<tr>
<td>Rabbit</td>
<td>S</td>
</tr>
<tr>
<td>Goat</td>
<td>W</td>
</tr>
<tr>
<td>Rat</td>
<td>W</td>
</tr>
<tr>
<td>Sheep</td>
<td>W</td>
</tr>
<tr>
<td>Cow</td>
<td>W</td>
</tr>
<tr>
<td>Guinea Pig</td>
<td>S</td>
</tr>
<tr>
<td>Pig</td>
<td>S</td>
</tr>
<tr>
<td>Horse</td>
<td>W</td>
</tr>
<tr>
<td>Dog</td>
<td>S</td>
</tr>
<tr>
<td>Chicken</td>
<td>nb</td>
</tr>
</tbody>
</table>

W = Weak Binding.
S = Strong Binding.

not bind (comprises only 1-3% of human immunoglobulins), IgA₂ and IgM also bind well.
An important characteristic of the Protein A-IgG interaction at the Fe region of the immunoglobulin (116) is that the antigenic activity of the IgG is not affected (117), conversely antigen binding to the Fab portion of the IgG probably does not cause conformational changes in the Fc region because Protein A binding does not seem to be modified when the antigen is present with the IgG (118), however enhanced interactions between antigen-antibody and Protein A has been reported if the complexes are generated by polyvalent antigens (119). It has also been reported that the antigen-antibody reaction has been enhanced when the IgG is bound to solid phase Protein A as compared to a non-specifically bound antibody free in solution (120).

3.4.3. Use of Protein A.

The use of Protein A in immunoassays can be considered to fall into three major areas defined by the way in which it is used. Protein A can be used as an immunoadsorbent either in its purified form or bound to a carrier such as Sepharose or as intact staphylococcal cell which is then used in solution which then requires centrifugation to remove it from the incubation mixture. It can also be used either by having the Protein A-carrier complex packed into a column or immobilising Protein A onto a solid surface. Finally the most common use of Protein A in immunoassays is as a labelled binder for IgG, acting as a pseudo labelled anti-immunoglobulin utilising a whole range of labels isotopes, enzymes, metals and proteins. It is their applications in immunoassays which is of main interest here.
3.4.3.1. Protein A as an Immunoadsorbent in Solution.

Protein A is commercially available from a number of sources either in its pure form, as a recombinant product, as killed whole cells or covalently linked to a variety of carrier molecules. Methods for preparing whole killed, fixed *S. aureus* cells (121-122) for isolating and purifying Protein A (123-124) and for conjugating to Sepharose (125) and controlled pore glass beads (126) have been reported. Protein A bound to carrier molecules has been used as an immunoadsorbent in a whole range of assays that fall into two main types, in the first antibody (Ab) is bound by the Protein A after incubation with the antigen (Ag), (the antigen must be large enough to have more than one recognition epitope), a second labelled antibody binds to the antigen, separated and the bound labelled antibody monitored. This method has been used to measure human serum albumin (127) and Pituitary growth hormone (PGH) synthase (128). Secondly, it is possible to attach a label to the antigen which is then used in a competitive assay (with the antibody and unlabelled antigen) using the Protein A-binder to precipitate out the bound antigen. This method has been used to develop assays for antibodies produced in response to grass allergen components (129), staphylococcal enterotoxins in foods (130) and the products of cell lysates (131). In addition Sepharose-Protein A has been used to remove endogenous anti-insulin antibody from the plasma of patients with insulin dependent diabetes prior to the determination of free insulin (132). Protein A can also be used as an immunoadsorbent in its pure form, acting as a bridge between specific antibody to cell surface antigen and labelled antibody, binding both by their Fc regions (133).
3.4.3.2. Protein A as an Immobilised Immunoadsorbent.

The use of Protein A as an immobilised immunoadsorbent falls into two main groups, the first and most common involves packing a column with a Protein A-carrier matrix and using this in a flowing system, for example, Protein A-Sepharose. This approach has been used to determine the levels of serum antibodies for a number of analytes including insulin and human thyroglobulin (125). In this assay, serum is incubated with enzyme labelled antigen and then applied to the Protein A column. The retained antibody complex then eluted off by passing phosphate buffered saline (PBS) containing 25mM dithiothreitol through the column which splits disulphide bonds between Protein A and the Sepharose carrier matrix. A drawback of this method is that Protein A is removed from the matrix and so the column is not re-usable. Assays with re-usable columns are obviously more attractive and this approach has been used to isolate and purify membrane components from cell lysates by preparing a number of columns with different monoclonal antibodies covalently bound to Sepharose (134). Protein A coated glass beads have also been used in a similar way (126) with rabbit IgG covalently linked to the Protein A beads which are then packed into a column and used in a HPLC systems for the analysis of human serum albumin and human IgG. At Loughborough University competitive flow injection immunoassays have been developed for the immunosuppressive drug cyclosporin A and the anti asthmatic agent theophylline (135) using a controlled pore glass Protein A immunoreactor. In the former case monoclonal mouse IgG2a antibodies were used with rhodamine labelled cyclosporin to produce a fluorescence based assay with a working range of 10-1500 ng/ml and for the theophylline method sheep antisera was used in conjunction with alkaline phosphatase labelled theophylline with electrochemical detection as the end point to producing an assay with a working range
between 2.5 and 40 μg/ml. In both of these assays the antibodies were incubated with either buffer/standard solution of the analyte and labelled analyte for a fixed period of time (from 2 to 20 min) before injecting onto the controlled pore glass Protein A column using either PBS pH 7.4 or Tris buffer pH 8.8. The antibody labelled analyte complex and antibody unlabelled analyte complex were then eluted off with citrate buffer pH 2.5 to generate the analytical signal. This signal was then used in the construction of the standard curve.

The second method for using immobilised Protein A as an immunoadsorbent involves solid surfaces rather than beads as the carrier. This method allows for the development of biosensors as demonstrated by the use of a piezoelectric quartz crystal (136) which resonates at a specific frequency, dependent upon the mass bound to its surface. In this assay Protein A is deposited onto one of a pair of gold electrodes attached to either side of the crystal which forms the base of a well in a Plexiglass block. When IgG is bound by the Protein A the resonance frequency decreases by a quantity related to the mass of the IgG bound, i.e. the addition of anti-IgG causes an additional decrease in resonance frequency. Changing the buffer to 0.1M sodium acetate pH 3.0 displaces the antibody from the Protein A allowing the sensor to be re-used. A biosensor based on a semiconductor and Protein A has also been developed (137), this sensor has an immunochemical membrane of IgG or Protein A bound to a support biopolymer matrix covalently linked to the surface of a semi-conductor device. In this case the membrane antigen specific antibody is incubated with the device and the antigen is determined by labelling.
3.4.3.3. Protein A as a Labelled Binder.

By far the most common use to date of Protein A in immunoassays is as a labelled binder of IgG, acting in a manner analogous to the traditional labelled second antibody or immunoradiometric methods. A range of labels have been used with a bias towards traditional isotopes and enzymes, however recently developed labels have enabled the development of new and sometimes simpler Protein A methodologies.

3.4.4. Advantages of Protein A over Anti-Immunoglobulins.

Regardless of the assay format Protein A offers a number of distinct advantages over anti-immunoglobulins, the most important being its ease and flexibility of use. Protein A is a well defined molecule readily available in pure form, stable and can be conjugated to a number of labels without loss of activity (138), for example, erythrocytes, $^{125}$I, $^3$H, fluorescein, gold, glucose oxidase, biotin.

Protein A's ability to bind many of the mammalian sera enables the development of indirect immunoassays with immunoglobulins in which anti-immunoglobulins are unavailable. This property also permits the detection of multiple antigens in indirect immunoassays utilising antisera from several different species (139). The reported problems of anti-immunoglobulins non-specifically binding to glass, plastics and uncomplexed antibodies appears to be lower with Protein A (139).

Finally anti-immunoglobulins do posses some advantages over Protein A, for example, anti-immunoglobulins can be raised for those sub-classes of IgG and IgM which do not bind to
Table 3.2. A Number of Commons Labels Used with Protein A.

Label Used with Protein A.

- Erythrocytes.
- \( ^{125}\text{I} \).
- \( ^{3}\text{H} \).
- Fluorescein.
- Ferritin.
- Gold.
- Invertase.
- Glucose Oxidase.
- Peroxide.
- Alkaline Phosphatase.
- Biotin.
Protein A, sensitivities of some assays using Protein A are not as great as those obtained using anti-immunoglobulins (140).

Despite these disadvantages antibody capture by Protein A seems to be the method of choice by the majority of workers.
References.


26. Bio-Rad laboratories, Richmond, California, USA.


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4.1. Introduction

The next three chapters describe the development of an immunoassay for phenytoin utilising a novel affinity chromatographic matrix, POROS, to perform the assay. This matrix, initially designed for protein separation has not been used in immunoassays until the development of the assay for phenytoin. All the work described in the following sections is therefore novel and as a result a number of new techniques have been used which until the development of POROS could not be utilised. Therefore each section contains not only novel work concerning immunoassays, but also new affinity chromatographic procedures, which as a result produce very few references and work to complete the results with. Therefore a detailed description of each individual instrument and process is required.

4.1. Instrumentation and Apparatus.

4.1.1. Flow Injection Equipment.

A schematic diagram of the single and dual stream flow injection systems incorporated in the manual assay are shown in fig 4.1. and 4.2. respectively.
4.1.2. Peristaltic Pump.

A Minipuls 3 peristaltic pump from Gilson (Villiers, France) was employed to propel the binding and elution buffer through the flow injection system. The pump head consists of ten rollers and was capable of propelling two channels of buffer via the peristaltic pump tubing, which was made of PVC (Anachem Ltd, Luton, UK.). Different flow rates could be achieved by either altering the rpm of the pump head or altering the diameter of the PVC pump tubing.

4.1.3. Flow Rate Calculations.

Each channel of the peristaltic pump was switched, in turn, to pump buffer through the column and flow cell. This flow was then collected for one minute and the weight of the buffer measured. From this it was possible to determine the flow rate of the pump in millilitres per minute (ml/min).

![Fig 4.1. Schematic Diagram of a Single Stream FIA System.](image)
4.1.4. Transmission Tubing.

The manual assay set-up consisted of 0.8mm ID Teflon transmission tubing (Anachem, Luton, UK.).


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, Cambridge, UK.).

The manual injection valve used for both the one stream and two stream manifolds 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 polypropylene. 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 the inlet and outlet of the eluent towards the detector.

4.1.7. Flow Cell.

The flow cell used in the F4500 spectrophotometer was supplied by Hellma (Essex, UK) and has a volume of 100μl. A schematic diagram of the cell used is shown in fig 4.3. The flow cell was housed in the sample compartment of the detector (see section 4.3.1.).

4.1.8. Column Equipment.

The borosilicate columns used for packing the CPG matrix were supplied by Omnifit with the dimensions 50mm length, 3mm bore. A number of borosilicate columns were used for the POROS IIa 50 and 20 microns and these are:-

- 50mm length, 3mm bore
- 20mm length, 3mm bore
Fig 4.3. Diagram of the Flow Cell used in all the Immunoassays.

100mm length, 6.5mm bore.

Each column is sealed with replacement end pieces containing porous Teflon frits (pore sizes 10, 20, 25, 40, 70 and 100 µm), porous polyethylene frit (pore size 25 µm) or porous stainless steel frits (pore size 0.5 µm).

Note: The varying sizes of Teflon and polyethylene frits were used for all the columns, the stainless steel frits were used only with the 50mm and 20mm columns.

Adjustable end pieces were used for the 100mm length 6.5mm bore column (variable from 6mm to 60mm, i.e. by screwing the adjustable end pieces closer or further apart the volume can be altered) resulting in an adjustable bed volume.
4.1.9. Fluorescence Detector.

The detector used for all the experiments was the Hitachi F4500 Fluorescence Detector. This is discussed in more detail in section 4.3.1.

4.2. Reagents

4.2.1. Buffer Solutions.

Phosphate Buffer (0.1M, pH 7.4).

The binding buffer was produced by dissolving the following analytical grade reagents (Fisons, Loughborough, UK.) in distilled water (supplied by a Maxima Ultra pure water system by Elga Maxima, Prima Systems, UK.).

8.0g Sodium Chloride (130mM).
0.2g Potassium Dihydrogen Orthophosphate (1.4mM).
2.9g Disodium Hydrogen Orthophosphate (8mM).
0.2g Potassium Chloride (2.6mM).

The pH was adjusted to 7.4 by either 0.5M hydrochloric acid or 0.5M sodium hydroxide. After the buffer was adjusted it was then filtered, using a vacuum flask, though 0.45 micron filters (Millipore, Bedford, MA. USA.).
Citrate Acid Buffer (pH 2.5).

The elution buffer was produced by dissolving the following analytical grade reagents (Fisons, Loughborough, UK.) in distilled water (supplied by a Maxima Ultra pure water system by Elga Maxima, Prima Systems, UK.).

21g Citric Acid (0.1M).

29.26g Sodium Chloride (0.5M).

The pH was adjusted using disodium hydrogen orthophosphate (5.7g in 20ml, approximately 50ml required). After the buffer was adjusted it was then filtered, using a vacuum flask, though 0.45 micron filters (Millipore, Bedford, MA. USA.).

4.2.2. Antisera.

Phenytoin (Dilantin) antisera (sheep) (conc 25.8 mg/ml) was supplied by IGi Ltd. Gateshead, UK.

Phenytoin (Dilantin) antisera (sheep) affinity purified (conc 25.8 mg/ml.) supplied by IGi Ltd. Gateshead. Theophylline antisera (sheep) was supplied by IGi Ltd. Gateshead, UK.
4.2.5. Labelled and Unlabelled Phenytoin.

The Rhodamine Phenytoin conjugate (Diphenylhydantooin HFK, DPH (oe)-Rhod (sr-101 plp) (C_{53}H_{52}N_{6}O_{5}S_{2}), (Mwt. 981.7), was supplied by Boeringer, Mainheim, Germany. The label was Sulfurhodamine 101 linked to the Phenytoin by a propionic acid piperazide linkage at the 3N coupling position (fig 4.4.). The free phenytoin (5,5-diphenyl-2,4-imidazolidine-dione) was supplied by Sigma Chemical Company Ltd. (Poole, Dorset, UK) (fig 4.5.).
Fig 4.5. Structure of Phenytoin.

4.2.6. Cyclodextrins.

The cyclodextrins used were \( \alpha \) cyclodextrin (Mwt. 972.8), \( \beta \) cyclodextrin (Mwt. 1135.0), \( \gamma \) Cyclodextrin (Mwt. 1297.1). The cyclodextrins (analytical grade) were supplied by Sigma Chemical Company Ltd (Poole, Dorset). Controlled Glass protein A (CPG) was supplied by Waters Associates, Inc, UK. The POROS IIa (20 and 50 micron particle size) was supplied by Perceptive Biosystems (Cambridge, MA, USA).

4.2.7. Column Matrices.

Control pore glass protein A (CPG) was supplied by Bioprocessing Ltd. (Co. Durham, UK) and POROS IIA from Perceptive Biosystems, (Cambridge, MA, USA).
4.3. Instrumentation.

The instrumentation systems described in the development of the immunoassay require detailed descriptions because the mode of interfacing the separate pieces of equipment is crucial to the assays performance. Each instrument used during the project will be described schematically with their function in the final complete assay outlined in more detail. The three separate instruments described are the AS3000 auto sampler, the K1000 auto analyser and the F4500 fluorescence spectrophotometer (all supplied by Hitachi, Wokingham, UK.). They are

Fig 4.6. A Schematic Diagram of the Automated System.
connected in series with the order being AS3000-K1000-F4500. Fig 4.6 shows a schematic diagram of the system.

4.3.1. F4500 Fluorescence Spectrophotometer.

The general layout of the spectrophotometer is shown in fig 4.7. The F4500 is composed of the main unit and a data processing unit, i.e. a personal computer. A schematic diagram of the processing system and the control system of the F4500 is shown in Fig 4.8.
4.3.1.1. Spectrophotometer Mainframe.

Fig 4.8. shows a schematic diagram of the spectrophotometer mainframe and is explained in detail in the following paragraph. The beam from the Xenon lamp is incident on the excitation monochromator and after wavelength selection is directed to the sample. Part of this beam is filtered to the monitor detector by means of a beam splitter. The fluorescence emitted from a sample is incident on the emission monochromator and after wavelength selection, the intensity of the fluorescence is measured via the photomultiplier. In the fluorescence mode, the output of the photomultiplier is divided by the output of the monitor detector to obtain data for the purpose of light quantity compensation of the light source. Between the beam splitter and the sample cell is a mechanism (shutter or chopper) which shuts off or chops the excitation beam to prevent excess light and scattering effects causing interference. The sample compartment, in this case, contains a 100μl flow cell (see section 4.1.7.). The personal computer incorporates a 3.5 inch floppy disc driver, a single 20M bytes hard disc drive, and a memory of 1M bytes, together with a colour monitor, a keyboard and mouse. Parameters for the measurement with the spectrophotometer and for recording with the plotter are controlled by either the mouse or keyboard. The measurement parameters are set into the mainframe via communication. The received measurement data can be displayed and recorded on the monitor in a multiple of colours. Data can be smoothed, differentiated, expanded, contracted and saved onto hard or floppy disc. The program operations are run through the MS-Windows and MS-Dos.
4.3.2. AS3000 Intelligent Auto Sampler.

The AS3000 autosampler was essentially developed to be combined with a spectrophotometer to automate analysis. Its ability to be programmed to perform specific functions enables a variety of methods to be performed and stored within the auto sampler. Fig 4.9. shows the configuration of the AS3000 intelligent auto sampler with the rack configuration being shown in fig 4.10 The needle which is used for injecting the samples can
be moved in 3 directions, X, Y and Z. A diluter is connected to the needle for use in aspirating and dispersion of the samples. The aspiration and dispersion of the samples is controlled by a 2.5 ml volume syringe. Fig 4.11. shows the flow path of the auto sampler syringe, from the sample vials to the injection port. The auto sampler can be programmed to perform two modes, each with various functions. The first is a sequential mode where the samples placed on the rack are successively injected, and the second is a programming mode where the samples can be pretreated. In this case the later mode is used. In the programmable mode the program is created by a number of commands and these commands are listed in table 4.1.

The specifications of the AS3000 are as follows:-

<table>
<thead>
<tr>
<th>Specification</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of standard samples</td>
<td>150.</td>
</tr>
<tr>
<td>Syringe capacity</td>
<td>2.5 ml.</td>
</tr>
<tr>
<td>Needle moving range</td>
<td>244mm in X direction,</td>
</tr>
<tr>
<td></td>
<td>163mm in Y direction,</td>
</tr>
<tr>
<td></td>
<td>108mm in Z direction.</td>
</tr>
<tr>
<td>Sample injection volume</td>
<td>0-2500μl.</td>
</tr>
<tr>
<td>Injection rate</td>
<td>set at 187.5 μl/s.</td>
</tr>
<tr>
<td>Data storage</td>
<td>battery backed-up memory.</td>
</tr>
<tr>
<td>Communicating function</td>
<td>RS-232C.</td>
</tr>
<tr>
<td>Input terminal</td>
<td>8 bits (photo coupler input: 3 bits, TTL level input: 5 bits)</td>
</tr>
</tbody>
</table>
Fig 4.9. The AS3000 Autosampler.

Fig 4.10. Rack Configuration of the AS3000.
Table 4.1. Commands Used for the Programming of the AS3000.

<table>
<thead>
<tr>
<th>Keys for Program Creation</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>TUBE</td>
<td>Moves the needle to a specific test tube.</td>
</tr>
<tr>
<td>ASP</td>
<td>Aspirates via needle a specified volume of liquid.</td>
</tr>
<tr>
<td>DISP</td>
<td>Dispenses a specified volume of liquid.</td>
</tr>
<tr>
<td>HOME</td>
<td>Returns needle, valve and entire mechanism to its original starting point.</td>
</tr>
<tr>
<td>WASH</td>
<td>Washes needle, syringe and injection port.</td>
</tr>
<tr>
<td>HEIGHT</td>
<td>Moves needle to specified height.</td>
</tr>
<tr>
<td>VALVE</td>
<td>Switches syringe valve.</td>
</tr>
<tr>
<td>TIMER</td>
<td>Set the auto sampler to perform a function for a specified time period.</td>
</tr>
<tr>
<td>WAIT</td>
<td>Pauses the program of the auto sampler for a specified period of time.</td>
</tr>
<tr>
<td>OUT</td>
<td>Sends a signal through a specified output port at a specified level.</td>
</tr>
<tr>
<td>DO</td>
<td>If the program requires repeating it is possible by using the DO command to specify the number of times the program needs to be repeated.</td>
</tr>
<tr>
<td>LOOP</td>
<td>This command is placed at the end of a series of commands and is set to return the auto sampler to the DO command to repeat the series of commands required.</td>
</tr>
</tbody>
</table>
Fig 4.11. The Flow Path of the AS3000 Autosampler.

Output terminal: 8 bits (relay contact
output: 3 bits, TTL level
output: 5 bits)

Ambient temperature: 15-35°C.

Ambient humidity: 45-85%.

Power consumption: 150 VA.
The programming and sequence of sample addition is explained in more detail in section 4.5.

4.3.3. K1000 Flow Injection Analyser

The K1000 is a continuous flow analyser based on the concept of flow injection analysis (FIA) (see chapter 2).

The flow path of the K1000 is shown in fig 4.12. and the measurements are made by connecting the outlet (shown in fig 4.12.) to the F4500 spectrophotometer.

The K1000 consists of a number of different parts and these include.

4.3.3.1. Main Pump.

A 2-channel piston pump is used for sending and controlling the flow of the equilibrium buffer. The flow rate is controllable for each channel independently.
4.3.3.2. 16-way Injection Valve.

The injection valve incorporated in the K1000 works as a manual injection valve would, i.e. the valve alternates from a loading position (charge status), where it is able to load the sample into the sample loops, to the inject position, where it injects the sample into the flowing stream by bringing the loops in line with the flowing stream. At charge status, sample and
elution buffer can be introduced into their respective loops. At injection status the sample and elution buffer are introduced into the flowing equilibrium stream. Fig 4.13. illustrates the initial set-up of the 16-way valve. The valve is mounted on the front panel of the K1000 and its drive section is located in the K1000. The operating control of the valve also appears on the panel. The stainless steel sheath on the moving section of the valve is fixed on to the drive shaft and is turned forward or reverse at 45° by the drive section motor depending on whether the valve is in the charge position or the inject position. Fig 4.14. shows the internal structure of the injection valve. The set-up of the injection valve during the immunoassay varied greatly from the original set-up. The set-up and a discussion of the alterations are shown and explained in section 4.8.

4.3.3.3. Peristaltic Pump.

The peristaltic pump supplies the sample and reagents to the injection loops, i.e. controls the filling of the sample and elution buffer loops by pumping the elution buffer from the reservoir, and the sample from the auto sampler injection port. It consists of two channels with the liquid flow being regulated by controlling the pump operation timer, which is situated on the front panel.

4.3.3.4. Oven.

This is not used in this particular instance but can heat the flowing stream up to 150°C.
Fig 4.13. The Initial Set-up of the K1000 Injection Valve.
4.3.3.5. Interval Timer.

The interval timer controls the interval between individual charging of the sample and elution buffer loops.
4.3.6. Charge Timer.

The charge timer controls the length of time the peristaltic pump charges the injection valve. This timer is situated on the front of the K1000 above the injection timer.

4.3.7. Injection Timer.

The injection timer controls the length of time the injection valve remains in the injection position before return back to the charge position.

4.4. The Combination of the Instrumentation for the Automated Immunoassay.

One of the most important tasks of the project was to construct the instrumentation to work together as one instrument. This development will be discussed in the following sections. All the instrumentation used in the set-up of the automated immunoassay can be used independently to perform specific tasks, but in this case are linked together, both chemically (i.e. the immunoassays wet reagents are passed from instrument to instrument) and also electrically (i.e. all the instruments can be controlled by signals generated through their specific computer outlets).

Therefore for ease of explanation the two specific areas (chemical and electrical) will be discussed separately, although they are in fact two parts of the same thing.
4.4.1. Flow Path of the Reagents in the Immunoassay through the Instrumentation.

Fig 4.6. shows a schematic diagram of the instrumentation in the order in which they are set-up. As there are three instruments involved there will be two areas of communication:-

a) the connection between the AS3000 auto sampler and the K1000 auto analyser.

b) the connection between the K1000 and the F4500 spectrophotometer.

4.4.1.1. The Connection between the AS3000 Auto Sampler and the K1000 Auto Analyser.

The AS3000 auto sampler has the capability to prepare samples ready for injection onto the POROS column and the K1000 auto analyser has the capability to inject a number of samples into a flowing stream, therefore by linking the two instruments together it was possible to have an instrument which could prepare samples and inject them onto the POROS column. This involved the connection between the auto sampler injection port (fig 4.11) and the number 1 port of the injection valve on the auto analyser (fig 4.13). This connection is done by using 300mm of Teflon transmission tubing (I.D. 0.8mm) which enables the samples to pass from the sample vials to the injection valve, ready for injection into the flowing stream.

4.4.1.2. The Connection between the K1000 and the F4500 Spectrophotometer.

As explained in section 4.3.3. the main functions of the K1000 is to provide a constant flow of the equilibrium buffer and to inject the sample into the flowing stream. Therefore the flowing
stream supplied by the K1000 must be connected to the F4500, or more specifically the flow cell housed within it. This is achieved by connecting the outlet of the K1000's flowing stream to the inlet of the flow cell using 200mm of Teflon transmission tubing (0.8mm I.D.) (fig 4.6).

4.4.2. Computer Communications.

As with the sample flow the computer connections of the instruments can be divided into two areas:-

a) the connection between the AS3000 auto sampler and the K1000 auto analyser.

b) the connection between the K1000 and the F4500 spectrophotometer.

4.4.2.1. The Computer Connection between the AS3000 Auto Sampler and the K1000 Auto Analyser.

In this instance the auto sampler is required to initiate the injection sequence of the auto analyser by a specific signal programmed into the auto sampler's sequence. This signal, as explained in more detail in section 6.2.2., is sent just before the needle injects the sample into the injection port. The connection is made by using an interface cord (P/N 080-2096) connected between the communication mode switch setting on the AS3000 and the accessory
RS-232 terminal on the K1000. This allows signals to be sent from the auto sampler to the auto analyser. The signal generation is programmed into the method of the AS3000 so that the K1000 receives the initiation signal at specific times during the assay, i.e. when the sample has been prepared and is ready for injection.

4.4.2.2. The Computer connection between the K1000 and the F4500.

The K1000 auto analyser provides the signal for the F4500 to initiate signal recording. This signal is sent via a 1 volt communication cord (P/N 155-1159) which is connected to a contact signal unit accessory (a relay box, P/N 155-1160) situated on the K1000 which generates a signal when the injection timer starts (section 4.3.3.7.) which initiates signal recording on the F4500 (fig 4.15). This relay box was prepared in Japan by Hitachi.

Fig 4.15. The Computer Connection between the K1000 and the F4500.
4.5. Automation of the Immunoassay.

The automation of the phenytoin immunoassay involved modification and programming of the instrumentation mentioned in the previous section. The specific reactions, incubation periods, methods used and concentrations used will be discussed in more detail in chapter 6., but at present we are concerned with the instrumentation set-up and modifications, which will be explained in more detail in the following sections. The automated immunoassay can be divided into a series of events, each controlled by one or more of the instruments. This series of events, in chronological order are:-

a) Preparation of the immunoassay mixture.
b) Transport of the reagents to the flowing stream.
c) Charging of the sample and CHAPS loops with the reagents.
d) Charging of the elution loop with the elution buffer.
e) Injection of the sample and elution buffer into the flowing stream.
f) Measurement of the signal.
g) Repeating the process a specified number of times.

This sequence of events are described in a series of steps but it must be emphasised that they are in fact all parts of one flowing movement, with some steps occurring at the same time. As a result the assay should not be looked upon as a series of separate events but as one continuous assay. These steps are described in more detail in the following sections.
4.5.1. Preparation of the Immunoassay mixture.

This involves the mixing of the conjugate, standards and antisera. This function is performed manually in both the manual and semi-automated immunoassay and by the auto sampler in the fully automated immunoassay. The exact volumes and procedure are explained in more detail in section 6.2.

Once the samples have been prepared they must then be transported to the POROS column, via the flowing stream.

4.5.2. Transport of the Reagents to the Flowing Stream.

Once the samples have been prepared and incubated they must then be injected, together with the CHAPS, into the flowing stream. This is performed by both the AS3000 and the K1000. The AS3000 is programmed to pick up 300μl of the sample mixture, 300μl of surfactant and inject these into the injection port of the auto sampler. The auto sampler then sends an electronic signal (section 4.4.2.1.) to the K1000 to initiate the loop charging procedure. This charge procedure fills both the sample and CHAPS loops with the sample and the CHAPS solutions respectively.

The filling of sample and CHAPS loops is performed by the peristaltic pump (section 4.3.3.3.) which pulls the sample and CHAPS from the auto sampler injection port to the injection valve.
4.5.3. The Charging of the Elution Loop with Elution Buffer.

The modifications of the injection valve (section 4.8.) produced an injection valve consisting of four loops, the sample loop, the surfactant loop, a timing loop and the elution loop. The elution loop is 1000µl and is situated in such a way that the elution buffer flows onto the column after the sample but before the CHAPS. The charging of the elution loop (fig 4.16.) is initiated by a signal generated from the auto sampler and is performed by the K1000. The filling of elution loop is performed by the peristaltic pump (as explained in section 4.3.3.3.) which pulls the elution buffer from the elution buffer reservoir (fig 4.12.) to the injection valve. The charge time of the peristaltic pump is controlled by the charge timer (section 4.3.3.6.).

4.5.4. Injection of the Sample, CHAPS and Elution Buffer into the Flowing Stream.

Injection of the sample, CHAPS and elution buffer into the flowing stream follows after the charge timer and peristaltic pump has completed the charging of the injection valve loops. The modifications to the injection valve (section 4.8.) ensures that the sample, elution buffer and CHAPS are injected into the flowing stream in the correct order, i.e. the sample followed by the elution buffer and finally the CHAPS. As described in section 4.4.1.2. the injection valve of the K1000 is connected, via the POROS, to the F4500 so that when injection of the samples takes place they will pass, via the column, to the F4500, generating a signal. When the samples are injected into the flowing stream the K1000 generates a signal (section 4.4.2.2.) which initiates data collection (peak area calculation is discussed in section 7.4.).

The signal measurement is performed by the F4500 spectrophotometer. As explained in section 4.4.5. once the injection valve, on the K1000, has been charged, it then injects the sample, elution buffer and CHAPS into the flowing stream. Upon injection the K1000 sends an electronic signal (via the communication cord described in section 4.4.2.2.) to the F4500 to initiate signal measurement. The signal is then recorded by the F4500. Therefore the signal measurement is initiated by the K1000 and performed by the F4500. The specifications for the F4500 during the immunoassay for phenytoin are described in chapter 5, with the time drive being set at 3600 secs. The peak areas can be calculated using the software provided by the F4500 (section 7.4.).

4.7. Repeating the Procedure.

Fig 4.17 shows a block diagram of the events during the automated immunoassay for phenytoin. It is possible, as fig 4.17 shows, to repeat the procedure a number of times, depending on the number of samples and this is possible because once the auto sampler has injected the sample into the injection port it is then programmed to wait for 70 seconds to allow the process of injection and signal generation to be performed before the next sample is injected. The auto sampler then initiates a repeat of the series of events described in fig 4.17. The pathway of the original injection valve did not provide the correct injection sequence required to perform the immunoassay, therefore a number of modifications were performed (fig 4.16). These modifications include:-

165
1. LOAD

0.1M PBS
Timing loop = 3 ml

0.1M
Elution loop = 1 ml

from autosampler
to column

from pump
from autosampler
to column

Column environment

2 INJECT

Timing loop

Elution loop

Sample loop

Column environment

Fig 4.16. The Modified Injection Valve.

166
4.8. A Block Diagram of the Events in the Automated Immunoassay.
a) A sample loop, 30μl in volume, inserted to receive the sample from the auto sampler first.

b) This sample loop was connected by 100μl of transmission tubing (0.8mm ID) to the CHAPS sample loop.

c) The CHAPS sample loop, 300μl in volume, was situated at such a point in the injection valve that it would flow onto the POROS column last (fig 4.16).

The filling of the sample and CHAPS loops, which are connected, is performed by the peristaltic pump described in section 4.3.3.3. and controlled by the charge timer described in section 4.3.3.6. with the volumes of being controlled by the autosampler.

d) The injection valve also contains an elution buffer loop, 1000μl in volume, which is filled from the elution reservoir situated on the K1000. This loop is also filled by the peristaltic pump and controlled by the charge timer. This loop is situated just before the CHAPS loop.

e) The injection valve also contains a timing loop, 3000μl in volume, which is situated between the sample loop and the elution buffer loop.

The function of this loop is to allow sufficient time for the sample to pass through the POROS column before the elution buffer enters, allowing the signal generated by the unbound peak of the sample to return to background levels before the elution peak begins. During loading there is no liquid movement in this timing loop.
Fig 4.16. shows the position of the individual loops in the injection valve during loading and injection, together with a key which describes the environment of each individual loop during the loading and injection of the injection valve.

By using the modified injection valve it is possible to flow the sample, CHAPS and elution buffer onto the column in the correct order required for the immunoassay. As a result it is the injection loops and the reproducibility of the charge process which controls the success and precision of the immunoassay (this is discussed in more detail in chapter 6).
CHAPTER 5

PRELIMINARY INVESTIGATIONS AND CHARACTERISATION OF THE POROS IIa PROTEIN A SOLID PHASE MATRIX
5.1. Binding of Antibodies onto Protein A Matrices.

Two matrices were investigated for use as solid phases in a flow injection immunoassay system illustrated in section 4.1.1, in order to examine and compare their antibody binding properties under the same conditions. These matrices were Controlled Pore Glass Protein A (CPG) (section 3.2.1.2.) and POROS IIa Protein A (section 3.2.3.).

5.1.1. Binding onto and Elution of Antibody using Controlled Pore Glass (CPG).

The CPG matrix consists of staphylococcal Protein A (section 3.4) immobilised onto CPG which has been developed to maximise IgG binding, particular murine IgG subclasses that do not bind to traditional affinity chromatographic matrices.

Matrix Characteristics:

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maximum Binding Capacity</td>
<td>Approximately 40-45mg mouse IgG, per ml of settled gel.</td>
</tr>
<tr>
<td>Particle Size</td>
<td>75-125 micron.</td>
</tr>
<tr>
<td>pH range</td>
<td>1.5-9.0.</td>
</tr>
<tr>
<td>Temperature Stability</td>
<td>4°C-37°C.</td>
</tr>
</tbody>
</table>
The CPG is supplied packed in 0.05M acetate buffer, pH 5.0 containing 0.5M NaCl and 0.01% thimerosal. After packing it is recommenced that the matrix be washed with at least 5 column volumes of equilibrium buffer, to remove the preservative.

Column Size.
There are a variety of column sizes available but in this series of experiments a 3mm internal diameter by 50mm length column was used.

Column Preparation.
The columns are prepared by filling with the matrix and pumping equilibrium buffer (with a peristaltic pump) through the column until the matrix has settled. This procedure was then repeated until the column is filled with the matrix.

Method of Immobilisation of the Antisera.
1. Equilibrate the column with the equilibration buffer.

2. Load the antibody, via the injection valve, onto the column. The column flow direction should be upwards to prevent possible cracking of the column.

3. Continue to flow the equilibrium buffer through the column for approximately 10 column volumes.
4. The antibody can then be eluted from the column by running elution buffer, by turning the switching valve, until the elution peak has returned to background.

5. Re-equilibrate the column with at least 5 column volumes.

The buffer solutions used were:

- Equilibrium Buffer: Phosphate Buffer Saline (pH 7.4).
- Elution Buffer: Citrate Buffer (pH 2.5).

Fig 5.1 shows the binding and elution profile of a 1:10 dilution of Testosterone antisera with the detection parameters of the F4500 set at:

- Excitation Monochromator: 280nm.
- Emission Monochromator: 335nm.
- PMT: 750V
- Slit Widths (EX/EM): 10/10

And the conditions of the flow injection system were:

- Transmission tube diameter: 0.8mm.
- Injection loop volume: 25μl.
- Flow rate: 0.5 ml/min.
- Flow cell volume: 100μl.
Fig 5.1. Binding and Elution of Testosterone Antisera onto CPG Protein A

Flow rate = 0.5 ml/min
Testosterone Antisera = 1:10
Ex (WL) = 280 nm
Ex (WL) = 335 nm
5.1.2. Binding and Elution of Antibody onto POROS Protein A matrix.

The POROS matrix consists of staphylococcal Protein A immobilised onto polystyrene divinyl benzene beads. This matrix has been developed to incorporate the binding ability of the Protein A with the enhanced binding supplied by a perfusion chromatographic matrix (section 3.2.3).

Matrix Characteristics:

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maximum Binding Capacity</td>
<td>Approximately 28-32mg mouse IgG, per ml of settled gel.</td>
</tr>
<tr>
<td>Particle Size</td>
<td>25 micron.</td>
</tr>
<tr>
<td>pH range</td>
<td>1.5-9.0.</td>
</tr>
<tr>
<td>Temperature Stability</td>
<td>4°C-37°C.</td>
</tr>
</tbody>
</table>

Column Preparation.

The columns were prepared using the method described in section 5.1.1.

Note: the preparation of the POROS columns required only 10% of the time that the CPG column required, due to the finer nature of the POROS particles which made them settle quicker once the equilibrium buffer was flowed through the column.

Method of Immobilisation the Antisera.

The antisera was immobilised onto the column by the method described in section 5.1.1.
Fig 5.2. shows a binding and elution profile of a 1:10 Testosterone antisera onto the POROS column.

Flow rate = 0.5 ml/min
Testosterone Antisera = 1:10
Ex (WL) = 280 nm
Ex (WL) = 335 nm

Fig 5.2. Binding and Elution of Testosterone Antisera onto POROS Protein A.
5.1.3. Comparison of the Binding of Testosterone Antisera for the CPG and POROS Columns.

Fig 5.3 shows that the testosterone antisera appears to have greater affinity for the POROS as compared to the CPG for the same flow rates and instrument parameters. Each column was injected 10 times with 1:10 of testosterone antisera at a flow rate of 0.5ml/min and their elution peaks recorded. The CV's of these peak areas were then calculated and found to be 2.9% for the CPG and 1.9% for the POROS.

5.1.4. Comparison of Increasing Flow Rate on the CPG and POROS Columns.

The flow rate of the system was increased in a number of steps from 0.5 to 2 ml/min and at each individual flow rate a 1:10 concentration of testosterone antisera was injected, in replicates of 3, into the system and eluted off, the peak areas recorded and compared for both the CPG and the POROS matrices (fig 5.4.). As the flow rate is increased the elution peak area decreases for both matrices, suggesting that as the flow rate increases the binding of the antisera decreases, but fig 5.4. also shows that the decrease in peak area is more pronounced in the CPG matrix than the POROS matrix. Therefore by utilising the faster flow rate it will be possible to increase the sample throughput.
Fig 5.3. A Comparison of the Binding of Testosterone Antisera to CPG and POROS Protein A.
Fig 5.4. The Effect of Increasing the Flow Rate of the FI System on the Binding of Testosterone Antisera onto CPG and POROS Protein.

5.1.5. Effect of Antibody Binding with Flow Rates Above 2ml/min.

At 2ml/min the binding of the antisera to the CPG became unmeasurable, therefore to experiment with CPG at flow rates above 2ml/min would prove unfruitful, but it is still possible using the POROS matrix. Therefore the flow rate was increased in steps up to 8ml/min for the POROS column and the effect on binding observed (fig 5.5.). The
testosterone antisera 1:80 was injected onto the column, in replicates of ten, at each individual flow rate under the parameters and conditions outlined in section 5.1.1. (a dilution of 1:80 was used in this case because it was found that due to the improved antibody capturing abilities of the POROS column less antibody was required to produce a large elution peak area).

Fig 5.5. The Effect on Antisera Binding onto the POROS Column of Increasing the Flow Rate up to 8ml/min.
Fig 5.5. shows that as the flow rate increases the antibody elution peak area decreases, for example, at 8ml/min the elution peak area of the testosterone antibody is 11% of that of the elution peak area at 0.5ml/min.

5.1.6. The Effect of Antisera Concentration on the Elution Peak Areas using the POROS Matrix.

A number of different antibody concentrations were injected (using the 25µl injection loop) onto the POROS column using the procedure described in section 5.1.1. Fig 5.6. shows the effect of decreasing the antibody concentration from 1:40 to 1:400 of phenytoin antisera and shows that as the concentration decreases the relative elution peak area also decreases. In this series of experiments the antisera used was phenytoin as it will be the antisera used in the production of the assay. The results of these experiments are discussed in section 5.6.

5.1.7. Pre-Incubation and On-line Immunoassays.

It is possible to develop immunoassays for flow injection systems using two methods, the first involves incubation of the antibody with the analyte of interest before injecting onto the Protein A matrix, hence the name pre-incubation. In this way it is possible to perform the reaction in a test tube and use the protein A column to separate the reacted and unreacted material. The second method involves the injection of the antibody onto the column followed by the analyte of interest. In this case the antibody is first bound to the Protein A and then the
analyte binds to the antibody, before being eluted off. Therefore the reaction, or incubation is occurring on the column itself, rather than in a test tube before hand and this approach is referred to as an on-line incubation. Obviously the antibody-antigen reaction has little time to occur as they flow past one another which results in lower sensitivities associated with the on-line assays, but the major advantage of such a method is the speed and the ease of automation this method provides.

Fig 5.7. shows a schematic diagram of both the pre-incubation and the on-line methods.
1. PRE-INCUBATION

Sample in binding buffer → Binding buffer → Elution buffer → Binding buffer

Buffer flowing through the column

2. ON-LINE INCUBATION

Sample in binding buffer → Binding buffer → Elution buffer → Binding buffer

Buffer flowing through the column

Fig 5.7. A Schematic Diagram of Pre-incubation and On-line Assay Methods.
5.2. The Development of a Pre-incubation Immunoassay for Phenytoin using the POROS Matrix.

5.2.1. The Binding of the Phenytoin Rhodamine Conjugation.

50µl of Phenytoin antisera (1:80) was incubated with 50µl of the phenytoin rhodamine conjugate (0.025mg/ml) and 100µl of PBS for 2 minutes in a test tube. 25µl of this was then injected into the flow injection system shown in section 4.1.1 (fig 4.2.) with the instrument set up:-

- **Excitation Monochromators**: 579nm.
- **Emission Monochromators**: 595nm.
- **Slit widths (EX/EM)**: 10/10nm.
- **PMT**: 950V
- **Response**: 0.04s
- **Flow rate**: 2ml/min.

Fig 5.8. show a binding and elution profile for the pre-incubation of the labelled phenytoin with the phenytoin antisera. This procedure was then replicated 10 times providing data on the precision of individual injections, i.e. a CV of 2.4%.
Fig 5.8. Binding and Elution Profile of the Phenytoin rhodamine Conjugate Utilising the Pre- incubation Method.

5.2.2. The Effect of the Flow Rate on the Pre- incubation Immunoassay for Phenytoin using the POROS Matrix.

The flow rate was increased in steps to 8ml/min and at each individual flow rate 10 injections of the pre- incubation mixture described in section 5.2.1. were performed and the elution peaks recorded. Fig 5.9. shows the effect of increased flow rate on the binding of a pre- incubation mixture on the POROS column from 0.5 to 8ml/min and shows that as the flow rate is increased the binding of the pre- incubation assay mixture is decreased until at a flow rate of
Fig 5.9. The effect of Increasing the Flow Rate on the Binding of the Phenytoin Conjugate Pre-incubation Mixture.

8ml/min the relative peak area of the incubation mixture is only 15% of the relative peak area of that of the incubation mixture loaded at flow rate of 0.5ml/min.

5.2.3. The Development of a Calibration Curve for Phenytoin in Buffer Using a Pre-incubation Method.

By varying the concentration of unlabelled phenytoin it is possible to construct a calibration curve of peak area against concentration. The peak area calculations were recorded as:-

\[(B/Bo)\%\]
where $B =$ peak area of known unlabelled concentration.

$B_0 =$ peak area when there is no unlabelled drug present.

The following stock concentrations of free phenytoin in methanol were made up:

$0.5, 2.5, 5, 10, 20, 30, 50 \mu g/ml.$

together with the stock control concentrations:

$2, 4, 12.5, 25 \mu g/ml.$

Note: the controls and standards were made up from separate stock solutions. These standards and controls were then diluted 50 fold in PBS to produced the following working standard and control solutions:

| standards:      | 0.01, 0.05, 0.1, 0.2, 0.4, 0.6, 1 \mu g/ml |
| controls:       | 0.04, 0.08, 0.2, 0.5 \mu g/ml.              |

Note: as described in section 1.3. the therapeutic range of phenytoin is 9-21 \mu g/ml, therefore the standards should cover this range, also the samples are further diluted 50 fold to reduce the matrix effects caused by the plasma (although not using plasma samples in this case the assay must work within the range where monitoring of plasma samples is possible).

$200 \mu l$ of each standard and control was then added to $50 \mu l$ of phenytoin rhodamine conjugate ($0.025 \text{mg/ml}$), then $50 \mu l$ of phenytoin antisera (1:80) added and incubated for 2 minutes.
Each sample is then, in turn, injected into the system with the process being repeated two times for each sample and four times for each control. After each sample has been eluted off the column a solution of 10% CHAPS is injected into the system, via the injection valve, to remove any non specific binding of the rhodamine and the phenytoin to the POROS matrix, tubing and flow cell. From the results obtained from each sample, control and blank (which substituted the unlabelled free phenytoin with an equal amount of PBS buffer) a calibration curve is produced (fig 5.10) which shows the characteristic sigmoidal calibration curve expected, over several orders of magnitude.

5.2.4. Intra Assay CV's for the Pre-incubation Immunoassay for Phenytoin.

The CV's for the respective concentrations, controls and blank are shown in table 5.1, together with the overall CV for the assays providing information on the precision for each sample in the assay.

5.2.5. Inter Assay CV's for the Pre-incubation Immunoassay for Phenytoin.

Four calibration curves were produced on different days for phenytoin using the method described in section 5.2.3. (using replicates of 2 for each standard) and are shown in fig 5.11, with the inter assays CV's shown in table 5.2. The inter assay CV's provide information on the precision of the assay and will be discussed in more detail in section 5.6.
Fig 5.10. Calibration Curve for Phenytoin utilising the Pre-incubation Method and the POROS Protein A Matrix.
<table>
<thead>
<tr>
<th>Conc(µg/ml)</th>
<th>Peak area</th>
<th>Average</th>
<th>Intra Assay%CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standards</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bo</td>
<td>97</td>
<td>101</td>
<td>99</td>
</tr>
<tr>
<td>0.01</td>
<td>74</td>
<td>77</td>
<td>75.5</td>
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<td>63</td>
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<td>64</td>
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<tr>
<td>0.1</td>
<td>52</td>
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<td>51.5</td>
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<tr>
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<td>37</td>
<td>35</td>
<td>36</td>
</tr>
<tr>
<td>0.4</td>
<td>28</td>
<td>30</td>
<td>29</td>
</tr>
<tr>
<td>0.6</td>
<td>28</td>
<td>26</td>
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<td>19</td>
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<table>
<thead>
<tr>
<th>Controls(µg/ml)</th>
<th>Measured Conc (µg/ml)</th>
</tr>
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<tbody>
<tr>
<td>0.04</td>
<td>64  68  71  74  69.25</td>
</tr>
<tr>
<td>0.08</td>
<td>53  53  54  56  54</td>
</tr>
<tr>
<td>0.25</td>
<td>36  33  32  33  33.5</td>
</tr>
<tr>
<td>0.5</td>
<td>27  26  28  27  27</td>
</tr>
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</table>

Table 5.1. The Standards and Controls for the Production of a Calibration Curve for Phenytoin Utilising the Pre-incubation Method.
<table>
<thead>
<tr>
<th>Conc(µg/ml)</th>
<th>INTER ASSAY %CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standards</td>
<td></td>
</tr>
<tr>
<td>Bo</td>
<td></td>
</tr>
<tr>
<td>0.01</td>
<td>3.1</td>
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<td>0.05</td>
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</tr>
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<table>
<thead>
<tr>
<th>Controls(µg/ml)</th>
<th>Measured Conc(µg/ml)</th>
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<tbody>
<tr>
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<td>0.25</td>
<td>0.26</td>
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<td>0.5</td>
<td>0.61</td>
</tr>
</tbody>
</table>

Table 5.2. The Inter Assay Coefficients of Variation from the Production of Four Calibration Curves for Phenytoin Utilising the Pre-incubation Method.
Fig 5.11. Four Calibration Curves for Phenytoin Produced by the Pre-incubation Method.
5.3. The Development of an On-line Immunoassay for Phenytoin using the POROS Matrix.

As outlined in section 5.1.7, a second method for the production of an immunoassay is possible, an on-line immunoassay. This was performed for phenytoin, using the POROS column and following the method described below:

1. Equilibrate the column with the equilibrium buffer at a constant flow rate of 0.5 ml/min.

2. 25μl of phenytoin antisera(1:80) was injected onto the POROS column, via the injection valve.

3. 25μl of a mixture of 25μl phenytoin conjugate (0.025mg/ml) and 200μl buffer/standard was injected onto the column.

4. Run the equilibrium buffer through the column until the unbound signal has returned to background.

5. Run elution buffer through the column until the elution peak returns to background.

6. Re-equilibrate the column with equilibrium buffer.
The flow rate, instrument set up and conditions were the same as for the pre-incubation assay described in section 5.2.1.

Fig 5.12. shows a binding and elution profile of an on-line phenytoin assay using a POROS matrix. This process was repeated 10 times producing a CV of 3.7% for the elution peak area which, suggests that the on-line assay can produce reproducible results.

5.3.1. The Effect on Flow Rate on the On-line Immunoassay for Phenytoin using the POROS Matrix.

The antibody loading flow rate was kept constant with the loading flow rate of the conjugate mixture being increased in steps up to 8ml/min and the elution peaks recorded. Fig 5.13. shows the effect of increasing the flow rate on the binding of an on-line assay using the POROS column, from 0.5 to 8ml/min which shows that as the flow rate increases the binding
Binding and Elution Profile of the Phenytoin Rhodamine Conjugate Utilising the On-line Incubation method.

of the on-line assay mixture decreases until at a flow rate of 8ml/min the relative peak area of the labelled phenytoin is only 16% of that loaded at flow rate of 0.5ml/min.
Fig 5.13. The Effect of Increasing the Flow Rate on the Binding of the On-line Phenytoin assay keeping the Antibody Concentration and loading Flow Rate Constant at 2.54 ml/min.

5.3.2. The Development of a Calibration Curve for Phenytoin in Plasma Using the On-line Method.

As with the pre-incubation immunoassay (5.2.3.) a number of standards and controls were produced, in this case in human plasma, over the range:

0.5, 2.5, 5, 10, 20, 30, 50 µg/ml.

with the following stock free phenytoin in plasma controls:
2, 4, 12.5, 25 μg/ml.

Note: as with the pre-incubation assay the controls and standards were made up from separate stock solutions but in this case both were made up in human plasma.

These plasma standards and controls were then diluted 50 times in phosphate saline buffer to produced the following working standard and control solutions:

standards: 0.01, 0.05, 0.1, 0.2, 0.4, 0.6, 1 μg/ml
controls: 0.04, 0.08, 0.2, 0.5 μg/ml.

Note: the dilutions of the samples in this case is more important than those performed in the pre-incubation assay because this time plasma samples are used and the matrix effects and interferences caused by the plasma must be reduced before injection.

200μl of each plasma standard and control was then added to 50μl of phenytoin rhodamine conjugate (0.025mg/ml).

Then the samples were injected onto the column, after the injection of a 1:80 concentration (25μl) onto the column, using the method described in section 5.3.2. After each sample has been bound then eluted off the column a solution of 10% w/v CHAPS was injected into the system, via the injection valve, to remove any non specific binding of the rhodamine and the phenytoin to the POROS matrix, tubing and flow cell. From the results obtained from each sample, control and blank (which substituted the unlabelled free phenytoin with an equal amount of PBS buffer) a calibration curve was produced and this is shown in fig 5.14.
5.3.3. Intra Assay CV's for the On-line Immunoassay for Phenytoin.

Each sample and control was repeated 3 times to provide individual CV's of each standard providing information on the precision of the individual samples and controls. These CV's are shown in table 5.3.

5.3.4. Inter Assay CV's for the On-line Immunoassay for Phenytoin.

4 calibration curves were produced for phenytoin using the method described in section 5.3.2. and are shown in fig 5.15. with the inter assay CV's being shown in table 5.4. The precision, accuracy and comparison of the two assays will be described in more detail in section 5.6.
Fig 5.14. Calibration curve for Phenytoin Utilising the On-line Incubation method.
### Table 5.3. The Standards and Controls for the Production of a Calibration Curve for Phenytoin Utilising the On-line Method.

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<thead>
<tr>
<th>Conc (µg/ml)</th>
<th>INTRA ASSAY %CV</th>
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<tbody>
<tr>
<td>Standards (n=2)</td>
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<tr>
<td>Conc (μg/ml)</td>
<td>INTER ASSAY %CV</td>
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<tr>
<td>-------------</td>
<td>-----------------</td>
</tr>
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<th>Controls (μg/ml)</th>
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</table>

Table 5.4. The Inter Assay Coefficients of Variation from the Production of Four Calibration Curves for Phenytoin Utilising the On-line Method.
5.4. Life Time of the POROS Column.

Previous work performed on CPG estimated that only 70 (1) or so runs could be performed on the matrix before the column required changing, therefore an experiment was set up to investigate repeated injections on the POROS matrix. 25μl of phenytoin antisera (1:80) was injected onto the same POROS column for a period of 4 hours. Conditions and instrument set up was the same as those described in section 5.1.1. with a flow rate of 4 ml/min.

Fig 5.16. shows a number of the repeated injections of the phenytoin antisera which also shows the fluctuations in peak height over the four hours providing a CV of 4.8%. By monitoring the injection of antisera onto the POROS column over several days it was noted that the matrix can maintain a constant elution peak area in excess of 500 runs, even when the column was regularly disconnected from the system and stored at 4°C overnight.

5.4.2. Recovery Time Required Between Each Injection for the POROS Column.

Each matrix requires a specific amount of equilibrium buffer before binding is returned to pre-ligand binding level. 25μl of phenytoin antisera (1:80)) was loaded onto the POROS column and then eluted off. The time between each loading of antisera onto the column was kept constant, as was the amount of elution buffer used, 500μl. This was replicated 4 times and the elution peak areas recorded. The time intervals between repeated injections were set at 120, 60, 50, 40, 30, 20 secs, at a flow rate of 4.95ml/min, for example in the case of 120 secs the equilibrium buffer was allowed to flow through the column for 120 secs before a
5.16. Repeated Injection of Phenyltoin Antisera.

Fig 5.17. Comparison of the Effect of Repeated Injections of Antisera onto the POROS Column at Different Recovery Time Intervals.
repeated injection of antisera was performed. The experiment was repeated using several
different flow rates and injection times. Fig 5.17 shows a comparison of the effect of repeated
injections at the time intervals mentioned and shows that if the column is not flushed through
with sufficient equilibrium buffer the binding of the antisera decreases temporarily, to return to
expected levels after a larger volume of equilibrium buffer. From these experiments it is
possible to suggest that at a flow rate of 5 ml/min it was possible to inject antibody after 30
seconds without loss of peak area suggesting that a volume of 2.5-3ml of buffer is required to
return the POROS matrix's binding back to pre-elution levels (this is approximately 10 column
volumes. Obviously at slower flow rates more time would be required for 3ml of elution
buffer to pass through the column.

5.5. Single Stream Flow Injection Immunoassays.

Section 4.1.1. shows a system containing a two stream system incorporating an equilibrium
buffer stream and a elution buffer stream controlled by a switching valve. With the speed and
recovery time available from the POROS matrix it is possible to simplify this system and
utilise one stream by using an additional elution injection loop rather than the second elution
stream.
5.5.1. The Development of a Single Stream Flow Injection Immunoassay.

25µl antisera (1:80) was loaded onto the POROS column as described in section 5.1.1. The antisera was then eluted off using the elution buffer controlled manually by the switching valve for a specific period of time before the column was returned to the equilibrium buffer and re-equilibrated for 120 secs, with a constant flow rate of 4.95ml/min. The switching time i.e. the period of time in which the elution buffer was flowing through the column was reduced in steps until no elution peak is observed. From these experiments it is possible to calculate the amount of elution buffer required to remove the antisera from the column. In the case of a 1:40 dilution of phenytoin antibody, 400µl of elution buffer was required to remove the antisera as a result it is possible to introduce an elution loop into the system by the introduction of a second injection valve, with a loop size of 400µl. Fig 5.18 shows two binding and elution peaks, the first pair produced by a system incorporating an elution loop, the second pair using the conventional two stream system. This showed that the elution loop produces the same result as the two stream system.

5.6. Discussion.

CPG and POROS were essentially developed to perform the same function, i.e. as solid phase matrices coated with protein A, to capture antibodies in flowing stream systems, i.e. the matrices are used to provide a solid phase to aid the protein A to capture the antibodies more efficiently.
Fig 5.18. Two Binding and Elution Peaks produced from the Single Stream and Double Stream FI Format.

Sections 5.1.1. and 5.1.2. illustrate that both the matrices perform this function well, producing solid phase immunoreactive columns which can be utilised to capture and release antibodies repeatedly. Fig 5.1. shows that the CPG matrix performs well at low flow rates (i.e. below 2 ml/min) but suffers from poor binding at higher flow rates (fig. 5.4.) and as a result sample throughput can be slow, approximately 1 sample every 10-15 minutes (1). At
corresponding flow rates the POROS matrix binds more of the antibody than the CPG and this is illustrated in the peak area differences observed in fig 5.3. POROS also shows relatively high binding at higher flow rates (fig 5.4.), thereby enabling the possibility of increasing the sample throughput above that achievable by the CPG matrix and making it possible to investigate the binding of antibodies at flow rates up to 8 ml/min. Fig 5.5. illustrates that as the flow rate is increased the binding of the antibody decreases, which is to be expected because the increased flow rate would decrease the time the antibodies come into contact with the matrix, this decrease in binding is such that at 8 ml/min the peak area is 11% of the peak area obtained at 0.5 ml/min. Although the peak area has decreased markedly at higher flow rate the POROS matrix still provides enough binding at higher flow rates to perform the assay. Also, as shown in fig 5.19. the coefficients of variation (CV) at each flow rate do not show any trend, i.e. as the flow rate is increased the CV's do not increase. This increased binding at corresponding flow rates (over CPG) and the ability to work at higher flow rates is due to the perfusion system employed in the capture of the antibodies in the POROS matrix. This reduces stagnant mobile phase mass transport and also provides a much greater surface area for antibody capture, which not only increases the antibody binding but also improves the CV's for the POROS. For example, the CV for 10 repeated injections of antisera is 1.9% as compared to the CPG CV's of 2.9% at the same flow rates. There are a number of possible explanations for this improved precision, firstly the POROS matrix capture more antibody at corresponding flow rates than the CPG, i.e. the peak areas are much bigger, providing a greater margin of error thereby reducing the CV's (section 7.3.), and secondly the perfusion system utilised by the POROS matrix is more efficient in controlling the attachment of the antibodies to the protein A, i.e. the design of the matrix brings the antibodies into closer contact with the Protein A. Fig 5.6. illustrates that the binding of the antisera at different
concentrations follows the same trend in peak area decrease with increased flow rate which allows the analyst a choice of antibody concentration, allowing the possibility to increase sensitivity or signal, depending on the assay required.

Fig 5.8. shows that it is possible to pre-incubate the antibody with the phenytoin rhodamine and bind the antibody to the POROS detecting at the rhodamine wavelengths, i.e. at an excitation wavelength of 579 nm and emission wavelength of 595 nm, therefore it is possible to develop an immunoassay utilising the labelled phenytoin, the phenytoin antibody and the POROS matrix. The pre-incubation method was employed to perform an immunoassay using the buffer standards with the controls set at low, middle and high concentrations on the calibration curve. Fig 5.10. and 5.11. show the calibration curves utilising the pre-incubation method. Tables 5.1. and 5.2. show the intra and inter assay CV's of the curves and show that method provides good precision at each individual (intra CV's) concentration as well as for the good precision between assays (inter CV's) and the controls show that the assay has good
accuracy. It was also possible to inject a number of spiked samples (i.e. samples made up in plasma and then diluted 50 fold and injected into the system) which also showed good recovery allowing the possibility of performing a full immunoassay in plasma.

The pre-incubation procedure was the initial procedure investigated for a number of reasons, firstly the pre-incubation of the antibody with the rhodamine phenytoin conjugate for two minutes ensured that adequate binding of the antibody with the antigen had occurred and secondly the pre-incubation procedure is one of the simplest immunoassay techniques available which allowed the initial production of a working immunoassay to provided information on how the POROS is likely to perform under more severe conditions and increased speeds. The pre-incubation immunoassay using the POROS matrix performed well and produced excellent intra and inter assay CV's (table 5.1. and 5.2.), together with the ability to be perform in human plasma. The speed of the assay was also improved by using the POROS matrix but the two minute incubation period was obviously a limiting factor in the overall speed. Therefore to remove this two minute incubation an on-line incubation method was investigated.

The on-line immunoassay method as the name suggests performs the incubation of the antibody with the rhodamine-phenytoin conjugate on the column itself. The principles of the immunoassay remain the same, except for the incubation period, and in theory should produce similar results. Fig. 5.13. shows it is possible to perform the on-line method using the POROS matrix and as Fig. 5.14. shows it is possible to perform this at increased flow rates without a significant loss of peak area. The decrease in peak area of the on-line method follows the same trend as that of antisera, which is to be expected, as the antibody is required to bind to the
matrix before the phenytoin rhodamine conjugate is injected, causing the peak area decrease observed.

From the results produced in fig 5.12 it was possible to develop an on-line assay utilising plasma samples, prepared by the method described in section 5.3.2. These samples were injected into the system, after an injection of antibody and a calibration curve produced (fig 5.14.). Table 5.3. lists the intra assay CV's for the plasma samples and controls which shows that the majority of the CV's are under 5%, but are noticeably higher than those produced by the pre-incubation assay. A loss of precision is expected with the on-line assay for two main reasons, firstly the samples do not undergo a pre-incubation thereby reducing the amount of phenytoin rhodamine/free phenytoin bound to the antibody, and secondly the samples have to flow past the antibodies introducing another variable in the assay, resulting in the expected precision decrease. Fig 5.15. shows four calibration curves produced using the on-line method and table 5.4. lists the inter assay CV's for the plasma standards and controls. Table 5.4. shows that in the majority of cases the assay shows good accuracy, but these are again not as good as those produced by the pre-incubation method. This can be explained in the introduction of the samples/controls after the initial injection of the antibody and the absence of the incubation period. But it must be stressed that the results shown in figs 5.14. and 5.15., tables 5.3. and 5.4. correspond well to a number of plate assays available producing presentable and acceptable CV's for an assay for phenytoin.

The advantage of using the on-line method as compared to the pre-incubation method is one of speed, obviously the incubation period is removed and therefore the assay can be performed with a higher sample throughput, which in this case was one sample per minute, 60
samples per hour, producing the similar precision and accuracy results shown by the pre-incubation method.

What the two methods do show is that it is possible to produce two immunoassays, using the two methods, providing results showing good accuracy and precision. The on-line assay was by far the quicker (1 min as compared to 3 min) but the accuracy and precision suffered as a result. What the analyst must decided is whether the speed generated by the on-line assay is required or the greater accuracy of the pre-incubation method.

All the assays performed to date used a simple two stream system incorporating an injection and switching valve, a peristaltic pump and a flow cell. The next series of experiments was to investigate the possibility of a one stream system by removing one of the flowing streams altogether.

Section 5.5. describes a series of experiments to investigate the amount of elution buffer required to remove a specific concentration of antibody from the column. This type of system is possible using the POROS matrix because the speed of flow rate and the perfusion system within the POROS allowed easy access of the elution buffer to the protein A-antibody bonds, as a result using the two stream system it was found that the switching valve had to be used for only brief periods of time during a singular run, i.e. almost immediately switched from binding buffer to elution buffer and back again. Therefore a system was set up which removed the two stream system and incorporated an extra injection valve with a loop size estimated from the experiments performed with the switching valve. This system is illustrated in fig 4.1. and shows the injection valve now performs the function of the second flowing stream, the
elution stream. This enable a one-stream system to operate, performing the elution of the antibody from the column by operating the injection valve.

Fig 5.18. shows two elution peaks, one operated in the conventional way, via a two stream system and a switching valve, the second by the one stream system and injection valve and shows that the injection valve introduction of the elution buffer has the same effect as the two stream system. There are a number of reasons for introducing this system into the assay, initially, as Fig 5.17. shows, protein A requires a period of time to recover before the next sample is injected and the more elution buffer passing through the matrix the longer the recovery time between each injection, therefore by reducing the elution buffer requirement to a minimum it was possible to decrease this recovery time. Thus the one stream system was developed for two main reasons, firstly a system with only one stream and two injection valves would be easier to automated with the instrumentation available and secondly, the reduction in the amount of elution buffer passing through the column can only increase the life time of the column.

As stated previously (1) a major draw back of using the CPG is the fact that it can only perform approximately 70 runs before the binding deteriorates, as a result assays requiring large numbers of samples and controls are not possible. The life time of the POROS columns were investigated in section 5.4. and were found to be in excess of 500 runs, using plasma and buffer samples, providing more than sufficient lifetimes to perform large numbers of samples without the column deteriorating during the analysis (fig 5.16.), providing constant binding for over four hours. The lifetime of the POROS cannot be over emphasized as if further investigation into automated systems is to be performed the lifetime of the columns produced must be well in excess of those provided by CPG.
This section describes the development of a number of assays for phenytoin which are far superior to those developed using CPG in both speed and precision. The assay developed were over a number of orders of magnitude, well within the therapeutic range required and were performed in human plasma. The controls used were set according to the samples run.

The one stream system developed could now be used in the development of an automated immunoassay system for phenytoin, which is described in chapter 6.
References.

CHAPTER 6

THE DEVELOPMENT OF A FULLY AUTOMATED IMMUNOASSAY FOR

PHENYTOIN IN HUMAN PLASMA
6.1. Introduction.

The validation experiments performed on the POROS matrix were impressive compared to those already achieved by CPG matrices but probably the most important feature of this work was the development of an automated assay which required very little operator input, together with the ability to provide rapid and reliable results from serum/plasma samples.

The developments outlined in chapter 5, together with the increased speed of the assay (as compared to those of CPG) were important factors in suggesting that automation of an immunoassay is possible. This next section describes the development, the directions taken and the results provided by an automated immunoassay for phenytoin, which describes the individual instrumentation, modifications and programming carried out to provide the completed assay.

6.1.1. Instrumentation.

The instrumentation used for the automation of the immunoassay comprised of:-

a) AS3000 Intelligent Auto sampler.
b) K1000 Auto Analyser.
c) F4500 Fluorescent Spectrophotometer.

Section 4.5. describes the linking and specifications of the separate pieces of instrumentation.
Initial experiments, outlined in section 6.2., describe in detail, a semi-automated immunoassay system incorporating manual sample handling. This system incorporated all the features of the manual assay combined with a degree of automation.

From this system, and the promising results it provided led to the development of a fully automated immunoassay.


Initial investigations of the system involved the development, programming and validation of a semi-automated immunoassay. In this system the immunoassay was only partially automated with some sample manipulation still being required. The samples and controls were made up manually (as described in section 6.2.1.) using blank human plasma (provided by and screened by GlaxoWellcome, Beckenham, Kent. UK.).

The concentrations of the stock phenytoin plasma solutions used were:

\[ 0.5, 2.5, 5, 10, 20, 30, 50 \mu g/ml. \]

which were then diluted 50 fold to produce working standards of:

\[ 0.01, 0.05, 0.1, 0.2, 0.4, 0.6, 1.0 \mu g/ml. \]
As in the manual assay internal controls were used to monitor the performance and validate the immunoassay and these were set at low, middle low, middle high and high, with concentrations of:-

2, 4, 12.5, and 25 μg/ml respectively.

which were then diluted 50 fold to produce working standards of:-

0.04, 0.08, 0.25 and 0.5 μg/ml.

The controls were set at the concentrations stated to ensure that the assay was working correctly over the entire range of concentrations. Replication analysis of each control concentration was used to determine values for its respective precision and accuracy levels, which were required to be within specified limits for the assay to pass (section 6.5.)

6.2.1. The Manual Production of Samples and Controls.

The technique employed in the production of samples and controls was the one advised by GlaxoWelcome and were produced using the following procedure:-

1ml of the 1:50 diluted sample/buffer/standard was mixed manually with 40μl (0.0125mg/ml) of phenytoin rhodamine conjugate. This mixture was then added to 500μl (1:80) of phenytoin antibody, then incubated at room temperature for two minutes.
All the mixing of the solutions was performed by hand in the vials provided for use in the autosampler.

After manual manipulation of the samples the assay was then performed automatically, using the program described in the section 6.2.2.

6.2.2. AS3000 Programming for the Semi-automated Immunoassay for Phenytoin.

The AS3000 auto sampler, which is described in more detail in chapter 4, provided the assay with a simple programmable loading device which can produce accurate and reproducible sample loading. It is possible to program the auto sampler to perform a number of functions and reproduce these actions for a specified number of times, which is ideal for immunoassays, where repeated mixing, incubations and injections are required.

The auto sampler was required to perform a number of functions which included the injection of the surfactant into the system followed by the injection of the sample into the system (the elution of the bound peak was performed by the K1000 auto analyser and will be discussed in section 6.2.3).

Therefore the AS3000 auto sampler was programmed to perform the following functions in the order specified:-

a) Locate the 10%(w/v) CHAPS solution vial in the sample tray of the autosampler. The sample tray layout is shown in Fig. 4.10.

b) Aspirate with 50μl of air.
Air is introduced into the sample needle.

c) Aspirate with 300μl of CHAPS.

The air is required to prevent mixing of the CHAPS with the carrier buffer in the auto sampler needle.

d) Locate the sample in the auto sampler sample tray.

e) Aspirate the auto sampler needle with 50μl of air.

The air is required to prevent mixing of the sample of interest with the CHAPS solution in the auto sampler needle.

f) Place the auto sampler needle into the sample required.

g) Aspirate the auto sampler needle with 300μl of sample.

h) Remove the autosampler needle from the vial

i) Send a signal to the K1000 to initiate the loading procedure.

j) Wait 20 seconds.

k) Move the autosampler needle to the injection port, which is connected to the K1000's injection valve (fig 4.14).

l) Inject 700μl (300μl of sample, 300μl of CHAPS and 100μl of air) into the injection port at a speed of 187.5 μl/sec. 300μl of sample is required to ensure that the 30μl loop was filled.

m) Return the needle to the reservoir.

n) Wash the needle with 1ml of equilibrium buffer.

o) Wait 70 seconds.

p) Repeat the process, in duplicate for the samples, in quadruplicate for the controls.
6.2.3. Functions of the K1000 Flow Injection Analyser in the Semi-automated Immunoassay.

The K1000 flow analyser provides the system with a controllable, reproducible flow of equilibrium buffer together with an automatic injection valve for repeated injections into the flowing stream and as a result performs several functions in the process of the semi-automated immunoassay. The K1000 is initiated by a simple voltage signal generated by the AS3000 and transferred to K1000 by the signal cord connecting the two instruments (fig 4.6.). On receiving the signal from the auto sampler the K1000 then initiates a pre-set loading procedure. As described in section 4.3.3.3. the peristaltic pump is situated on the front panel of the K1000 and it is this pump which fills the sample/CHAPS/citrate buffer loops. The set-up, loop sizes and loading procedures are described in detail in section 4.5.

As fig 4.16. shows the sample loop is considerably smaller than the elution buffer loop (30μl as compared to 1000μl) and as a result does not require as much time for loading. It is for this reason that the AS3000 auto sampler is instructed to wait 20 sec after the peristaltic pump begins before injecting the sample, so as to allow the larger citrate buffer loop to be filled before loading the sample/CHAPS into the system.

The time of the loading procedure is set at 60 seconds, i.e. the charge timer is set at 60 secs (section 4.3.3.6). Once the loading procedure is complete the injection procedure is then initiated.

This involves the injection valve switching from the loading position to the inject position to allow the sample and CHAPS to enter the flowing stream and onto the POROS. Fig 4.13.
shows the injection valve in the loading position and injection position respectively. This injection procedure is set at 42 seconds by the injection valve timer (section 4.3.3.7.).

Fig 6.2. A Schematic Diagram of the Order of Injection of the Reagents/Sample and CHAPS onto the Column.

At the same time the injection valve is switched from the load position to the inject position the K1000 initiates the recording procedure of the F4500 (section 4.4.1.2.).
After the injection period of 42 secs has elapsed the injection valve returns to the charge position ready to receive the next sample. A schematic diagram of the order of CHAPS/sample/citrate buffer flowing onto the column is shown in fig 6.2.

6.2.4. Function of the F4500 in the Semi-automated Immunoassay System.

On receiving the initiation signal from the K1000, the F4500 automatically monitors the fluorescence of the flowing stream, via the flow cell (section 4.1.7.). The conditions and, instrument set-up are the same as those described for the manual assay (section 5.1.1.) The time drive of the F4500 is set at 3600 secs.

6.2.5. Binding And Elution Peak Profile Produced by the Semi-automated Immunoassay.

Fig 6.3. shows a binding and elution profile produced by the semi-automated immunoassay system and it can be seen that the binding and elution peaks produced correspond to those produced by the manual method (fig 5.12.).
Fig 6.3. Binding and Elution Profile Produced by the Semi-automated Immunoassay for Phenytoin.
6.2.6. Calibration Curves Produced by the Semi-automated Immunoassay.

The next step was to produce a calibration curve for phenytoin using the same samples and experimental conditions used for the manual assay. The samples and controls were prepared using the same method as described in section 6.2.1. Fig 6.4. shows a calibration curve for phenytoin using a semi-automated fluorescence flow injection immunoassay, in human plasma and fig 6.5. shows four calibration curves run on separate days. As with the manual assay each standard was repeated in duplicate and each control was repeated in quadruplicate.

Table 6.1. shows the range of CV's produced from the individual samples and controls, together with the overall CV's for the assay.

6.3. Interpretation of the Data Produced by the Semi-automated Immunoassay for Phenytoin.

6.3.1. Intra Assay CV's.

The intra assay CV's are concerned with data produced from the same calibration curve and provide useful information on how reproducible the standards and controls are within the same assay. The semi-automated immunoassay was repeated on a daily basis for four days. Table 6.1. shows intra assay %CV's for both standards and controls for four separate calibration curves and shows all the CV's produced are under 10% (i.e. the coefficient of variation expressed as a percentage).
Fig 6.4. Calibration Curve produced by the Semi-automated Immunoassay
Fig 6.5. Four Calibration Curves produced by the Semi-automated Assay
<table>
<thead>
<tr>
<th>Conc (µg/ml)</th>
<th>INTRA ASSAY %CV (n=2)</th>
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<tr>
<td>0.1</td>
<td>2.35</td>
</tr>
<tr>
<td>0.2</td>
<td>3.14</td>
</tr>
<tr>
<td>0.4</td>
<td>1.70</td>
</tr>
<tr>
<td>0.6</td>
<td>7.61</td>
</tr>
<tr>
<td>1</td>
<td>4.81</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Controls (µg/ml)</th>
<th>Measured Conc (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.04</td>
<td>0.039</td>
</tr>
<tr>
<td>0.08</td>
<td>0.089</td>
</tr>
<tr>
<td>0.25</td>
<td>0.26</td>
</tr>
<tr>
<td>0.5</td>
<td>0.44</td>
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</tbody>
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Table 6.1. The Standards and Controls for the Calibration Curve produced by the Semi-Automated for Phenytoin.
<table>
<thead>
<tr>
<th>Conc(µg/ml)</th>
<th>INTER ASSAY %CV (n=2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standards</td>
<td></td>
</tr>
<tr>
<td>Bo</td>
<td>11.5</td>
</tr>
<tr>
<td>0.01</td>
<td>2.20</td>
</tr>
<tr>
<td>0.05</td>
<td>4.59</td>
</tr>
<tr>
<td>0.1</td>
<td>9.44</td>
</tr>
<tr>
<td>0.2</td>
<td>8.47</td>
</tr>
<tr>
<td>0.4</td>
<td>9.26</td>
</tr>
<tr>
<td>0.6</td>
<td>6.74</td>
</tr>
<tr>
<td>1</td>
<td>10.3</td>
</tr>
<tr>
<td>Controls(µg/ml)</td>
<td>Measured Conc (µg/ml)</td>
</tr>
<tr>
<td>0.04</td>
<td>0.35</td>
</tr>
<tr>
<td>0.08</td>
<td>0.07</td>
</tr>
<tr>
<td>0.25</td>
<td>0.29</td>
</tr>
<tr>
<td>0.5</td>
<td>0.51</td>
</tr>
</tbody>
</table>

Table 6.2. The Inter Assay Coefficients of Variation from the Production of Four Calibration Curves for the Semi-Automated Method.
Note: %CV's are statistical calculations based on, in this case, individual peak areas. The formula used for the calculation of the individual %CV's is shown in fig 6.5. %CV's provide the analyst with information concerning the reproducibility of the assay, i.e. whether the result produced by the assay method is precise enough to have confidence in it.

Table 6.1. shows good correlation between the CV's of individual samples in the same run showing that the assay method and automation were reproducible during the same assay.

6.3.2. Inter Assay CV's.

Table 6.2. shows the inter assay CV's for the semi-automated immunoassay. Inter assay CV's are a comparison of data between separate assays, to investigate the assays performance. The inter assay CV's do not have to be produced on separate days, but in this case are, but the solutions have to be different. As table 6.2. shows the inter assay CV's are higher than those obtained from the intra assay CV's. This is to be expected because of a number of factors explained in section 6.3.3.

6.3.3. Discussion of the Data Produced by the Semi-automated Immunoassay.

Tables 6.1. and 6.2., figs 6.3. and 6.4. show the intra and inter day variation for the semi-automated immunoassay.

Table 6.3. shows a comparison between a semi-automated immunoassay calibration curve and one produced manually. The system employing the semi-automated procedure would be
### MANUAL ASSAY

<table>
<thead>
<tr>
<th>Standards (μg/ml)</th>
<th>(n=2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bo</td>
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<tr>
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<td>2.80</td>
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<td>2.20</td>
</tr>
<tr>
<td>0.1</td>
<td>1.37</td>
</tr>
<tr>
<td>0.2</td>
<td>3.92</td>
</tr>
<tr>
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<td>4.87</td>
</tr>
<tr>
<td>0.6</td>
<td>5.23</td>
</tr>
<tr>
<td>1</td>
<td>0.00</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Controls (μg/ml)</th>
<th>Relative conc (n=4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.04</td>
<td>0.05</td>
</tr>
<tr>
<td>0.08</td>
<td>0.07</td>
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<tr>
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<td>0.24</td>
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<td>0.5</td>
<td>0.60</td>
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</tbody>
</table>

### SEMI-AUTOMATED ASSAY

<table>
<thead>
<tr>
<th>Standards (μg/ml)</th>
<th>%C.V. (n=2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bo</td>
<td>3.74</td>
</tr>
<tr>
<td>0.01</td>
<td>0.91</td>
</tr>
<tr>
<td>0.05</td>
<td>6.38</td>
</tr>
<tr>
<td>0.1</td>
<td>2.35</td>
</tr>
<tr>
<td>0.2</td>
<td>3.14</td>
</tr>
<tr>
<td>0.4</td>
<td>1.70</td>
</tr>
<tr>
<td>0.6</td>
<td>7.61</td>
</tr>
<tr>
<td>1</td>
<td>4.81</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Controls</th>
<th>Relative conc (n=4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.04</td>
<td>0.039</td>
</tr>
<tr>
<td>0.08</td>
<td>0.089</td>
</tr>
<tr>
<td>0.25</td>
<td>0.26</td>
</tr>
<tr>
<td>0.5</td>
<td>0.44</td>
</tr>
</tbody>
</table>

Table 6.3. A Comparison of Calibration Curves produced Manually and Semi Automatically Utilising the Pre-incubation Method.
expected to produce improved CV's as compared to those of the manual method because the instrumentation would be expected to remove the experimental error which occurs when performing the assay manually. Also the injection timings, elution timing and equilibration timing would remain constant for each injection. This would have the effect of ensuring the POROS column is in the same condition to receive each sample, therefore be expected to provide the same results for the same samples.

But as table 6.3. shows the CV's are not improved greatly from assay to assay as would be expected. This is because the K1000 is running at the limits its capability and as a result did not provide CV's in the an automated system would be expected to produce, i.e. it was not originally designed to inject samples below 500µl. The redesign of the injection valve required accurate loading to be performed by the peristaltic pump with small volumes of sample and surfactant. Unfortunately the peristaltic pump mechanism which controls the loading of the samples mentioned was unreliable, i.e. on occasions, for no obvious reason, the pump would not provide enough pressure to pull the liquids through the transmission tubing, which resulted in the samples not always being in the desired place, therefore, as shown in fig 6.3. and 6.4., adversely affecting the CV's.

But with the limitations associated with the K1000 the assay intra and inter CV's provided good results to suggest the assay was working correctly. Also analysis of the controls used in the assay did not suggest the samples followed any specific trend, for example, concentration, in sample injection. All the controls remained within the specifications required providing concentrations close to those expected (tables 6.1. to 6.3.).
6.4. The Development of a Fully Automated Immunoassay.

The next logical step of the assay was to produce a system which was fully automated, i.e. a further reduction in manual sample handling. The system remained essentially the same except the samples did not require any pre-treatment, reducing the operator input to a minimum. The standards, controls and concentrations of the conjugate and antibody remain the same and are prepared using the same method as described in section 6.2.

6.4.1. AS3000 Programming for the Fully Automated Immunoassay for Phenytoin.

The AS3000 autosampler was programmed to perform specific tasks during the immunoassay which were not present during the semi-automated assay. These functions included:

a) Locate the vial in the auto sampler that contains the phenytoin antisera (1:80). This vial is always located in position 1,1 (see fig 4.7).

b) Aspirate 50\,\mu l of air.

c) Aspirate with 500\,\mu l of phenytoin antisera.

d) Move auto sampler needle to the first mixing vial.
Note: the location of the mixing vials depends on the number of samples due to the fact that the first mixing vial is situated directly after the last sample vial, for example, if seven samples are to be analysed the configuration of the rack would consist of:

<table>
<thead>
<tr>
<th>Position</th>
<th>Contents</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,1</td>
<td>phenytoin antisera.</td>
</tr>
<tr>
<td>1,2</td>
<td>phenytoin conjugate.</td>
</tr>
<tr>
<td>1,3</td>
<td>CHAPS solution.</td>
</tr>
<tr>
<td>1,4-1,10</td>
<td>plasma samples.</td>
</tr>
<tr>
<td>1,11-1,17</td>
<td>mixing vials.</td>
</tr>
</tbody>
</table>

e) Disperse the phenytoin antisera into the first mixing vial, i.e. in the example stated in section d the first mixing vial is situated at position 1,11.

f) Return the needle to the wash position (see fig 4.11.)

g) Wash the needle with 1ml of equilibrium buffer from the autosampler reservoir, to prevent cross contamination.

h) Aspirate 50μl of air.

i) Move the needle to the vial containing the conjugate. This vial is always located in position 1,2 (see fig 4.10.).

j) Aspirate with 40μl of conjugate.
k) Aspirate with 50μl of air.

l) Move the needle to the first sample.

The first sample is always located in position 1,4.

m) Aspirate with 1000μl of sample.

n) Move the needle to the first mixing vial i.e. the vial which contains 500μl of the phenytoin antisera (1:80).

o) Inject both the conjugate and the sample into the mixing vial.

p) Return the needle to the wash position.

q) Wash the sample needle with 1ml of equilibrium buffer.

r) Repeat the process for each sample and control.

This process described above removes the need for the analyst to mix the samples manually thereby removing possible errors when sample handling. After each sample has been mixed the auto sampler is then programmed to inject each of the samples in turn into the K1000.

This process of sample injection, signal transfer and K1000 initiation is as described in section 6.2.
6.4.2. Binding and Elution Profile Produced by the Fully Automated Immunoassay for Phenytoin.

Fig 6.6. shows the binding and elution profile produced by the fully automated system and can be seen that the binding and elution peaks correspond to those produced by the manual method (fig 5.12.).

6.4.3. Calibration Curves for Phenytoin produced by the Fully Automated Immunoassay.

By utilising the system described in section 6.4.1. it was possible to produce a number of calibration curves for phenytoin using the same samples and controls which were used for the production of the semi-automated immunoassay. Fig 6.7. shows four calibration curves produced by the fully automated system and table 6.4. shows the intra and inter assay CV's for both the controls and the samples, produced by the calibration curve.

6.4.4. Interpretation of the Data Produced from the Fully Automated Immunoassay.

6.4.4.1. Intra Assay CV's.

As explained in section 6.3.1. the intra assay CV's provide information on how reproducible the samples are within the same assay. As with the semi-automated immunoassay the calibration curve was repeated a number of times to produce intra assay CV's at all standard
Fig 6.6. Binding and Elution Profile Produced by the Fully Automated Immunoassay for Phenytoin.
Fig 6.7. Four Calibration Curves Produced from the Fully Automated Immunoassay for Phenytoin.
concentrations. Table 6.4. shows the intra assay CV's for four calibration curves and shows that all of the CV's for the samples were under 8%. The intra assay data suggests, as it did for the manual and semi-automated immunoassays, that the assay methodology and the system itself is stable and produces reproducible results during the production of a calibration curve and controls in plasma.

6.4.4.2. Inter Assay CV's

As explained in section 6.3.2. inter assay CV's are read from separate calibration curves and provide information on possible trends and continuous errors of the assay. They also provide the analyst with useful information on the day to day variation of both the method and the instrumentation utilised. As seen in the manual and semi-automated immunoassay the inter assay CV's are higher than the intra assay CV's which is once again to be expected because it is very difficult to ensure the same assay conditions over extended periods of time, i.e. the instrumentation should provide the same functions in each separate assay (except for the small degradation of the xenon lamp as it gets older) but the POROS protein A column will not remain exactly the same over extended periods of time due to general wear and tear of the beads and the protein A. As stated in the initial investigation of the media in chapter 5 the column is very reliable over extended periods of time but small changes are likely to occur as the column is disconnected, stored away etc., providing small changes on a daily basis, although if used continuously then this problem would not be a factor. Other more important factors also affect the inter (and intra) assay CV's and these will be discussed in section 6.6.
### Table 6.4

The Inter and Intra assay Coefficients of Variation for the Fully Automated Immunoassay.

<table>
<thead>
<tr>
<th>Conc (μg/ml)</th>
<th>INTRA ASSAY %CV (n=2)</th>
<th>INTER ASSAY %CV (n=2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standards</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bo</td>
<td>1.63</td>
<td>11.5</td>
</tr>
<tr>
<td>0.1</td>
<td>7.31</td>
<td>2.20</td>
</tr>
<tr>
<td>0.2</td>
<td>7.93</td>
<td>4.59</td>
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<td>0.4</td>
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<td>0.6</td>
<td>3.84</td>
<td>8.47</td>
</tr>
<tr>
<td>1</td>
<td>5.03</td>
<td>9.26</td>
</tr>
<tr>
<td>Controls (μg/ml)</td>
<td>3.14</td>
<td>6.74</td>
</tr>
<tr>
<td>0.04</td>
<td>6.70</td>
<td>10.3</td>
</tr>
<tr>
<td>0.08</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Relative conc (n=4):

<table>
<thead>
<tr>
<th></th>
<th>0.037</th>
<th>0.90</th>
<th>0.23</th>
<th>0.49</th>
</tr>
</thead>
</table>

Leave space before brackets.
6.5. Analysis of the Variation of the Results obtained from the Fully Automated Immunoassay (ANOVA).

Analysis of variance (ANOVA) is an extremely powerful statistical technique which can be used to separate and estimate the different causes of variation. In this case it is used to determine whether the variations between the individual samples is due to random error, or due to other factors, for example, sample degradation, equipment loss of performance, assay technique breakdown etc., and to examine the samples and controls to see if there are any trends in the variance of the samples, or are the variations due to experimental/random error. In this case the ANOVA plots are used to see if the calibration controls are within the confidence limits of the calibration curves i.e. it is not possible to predict with 100% probability that the control will produce exactly the result expected because there is always some fraction of risk (a) or percentage probability (100-100a) involved in such a prediction. Therefore to formulate the confidence limits, the standard deviation $s$ of the sample is calculated, and a value of $t$ is found by consulting table A (fig 6.8.) The constant $t$ depends on the fraction of risk $a$ and on $n$, the degrees of freedom. From this it is possible to calculate the confidence limits using the following equation:

$$H = \pm ts / \sqrt{n}$$

where:

$H$ = confidence limits.

t = is found by consulting table A in the appendix.

$n$ = degrees of freedom.

$s$ = standard deviation.
<table>
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<tr>
<th>Number of Measurements</th>
<th>Degrees of Freedom</th>
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<th>0.01</th>
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<td>$n - 1$</td>
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<td>95%</td>
<td>99%</td>
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<td>63.657</td>
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<td>2.920</td>
<td>4.303</td>
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<td>3.182</td>
<td>5.841</td>
</tr>
<tr>
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<td>4</td>
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<td>4.604</td>
</tr>
<tr>
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<td>5</td>
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<td>2.571</td>
<td>4.032</td>
</tr>
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<td>6</td>
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<td>11</td>
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<td>15</td>
<td>1.753</td>
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<td>31</td>
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<td>2.750</td>
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<tr>
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<td>2.660</td>
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<td>$\infty + 1$</td>
<td>$\infty$</td>
<td>$\infty$</td>
<td>1.645</td>
<td>1.960</td>
</tr>
</tbody>
</table>

Fig 6.8. Degree of Risk Calculation Table(2).
Fig 6.9. ANOVA Plot of Automated Immunoassay.
Therefore it is possible to say that the fraction of risk \( z \) lies outside these confidence limits of \( a \). Obviously, \( a/2 \) is the fraction of risk that \( z \) is either larger or smaller than the confidence limits. It may also be said that the probability that \( z \) lies inside these limits is \( 100-100a \). The type of analysis described above, i.e. where there is one factor, either controlled or random, in addition to the random error, is known as one-way ANOVA. From the ANOVA plot shown in fig 6.9, it is possible to calculate the percentage bias, the upper and lower confidence limits the degrees of freedom and the CV's of the controls, which are all shown in tables 6.5.-6.7. The ANOVA plot results confirm that the assay controls are within the limits of confidence set by the equation providing evidence to suggest that the immunoassay is producing the expected results and the errors, in the CV's and in the controls are caused by random error rather than the method or the instrumentation.

The individual CV's of the controls (QC's) are not as good as expected and are higher than they should be for this type of assay and the manual assay suggests These high CV's are caused by a number of factors which will be discussed in more detail in section 6.6.

6.6. Discussion of the Data Produced by the Fully Automated Immunoassay.

The fully automated fluorescence immunoassay for phenytoin in human plasma produced results which correlate well with the manual and the semi-automated immunoassays. What is probably the most disappointing factor is the CV's produced for the assay. By removing a great deal of the operator input the CV's should exceed the manual and semi-automated assay CV's by some margin, but unfortunately as the results show (tables 6.1.-6.5.) there is no significant difference in the CV's of the fully automated immunoassay as compared to the
### Table 6.5. Non-transformed Data for the Phenytoin Assay

<table>
<thead>
<tr>
<th>Theoretical &amp; Actual QC Values</th>
<th>QC (2)</th>
<th>QC (4)</th>
<th>QC (12.5)</th>
<th>QC (25)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Target QC</td>
<td>2</td>
<td>4</td>
<td>12.5</td>
<td>25</td>
</tr>
<tr>
<td>Overall mean</td>
<td>1.69</td>
<td>3.73</td>
<td>12.23</td>
<td>24.31</td>
</tr>
<tr>
<td>% Bias</td>
<td>-15.50%</td>
<td>-6.80%</td>
<td>-2.20%</td>
<td>-2.80%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Confidence Limits of QC Means at 95% level</th>
<th>QC (2)</th>
<th>QC (4)</th>
<th>QC (12.5)</th>
<th>QC (25)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lower limit</td>
<td>1.52</td>
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<td>11.44</td>
<td>21.56</td>
</tr>
<tr>
<td>Upper limit</td>
<td>1.86</td>
<td>3.99</td>
<td>13.01</td>
<td>27.07</td>
</tr>
</tbody>
</table>

### Table 6.6. Results obtained from the 1-Way ANOVA

All concentrations are shown in units of µM.

<table>
<thead>
<tr>
<th>Assay:</th>
<th>QC (2)</th>
<th>QC (4)</th>
<th>QC (12.5)</th>
<th>QC (25)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Replicate 1</td>
<td>4.90</td>
<td>10.72</td>
<td>24.95</td>
<td>36.67</td>
</tr>
<tr>
<td>Replicate 2</td>
<td>4.44</td>
<td>18.16</td>
<td>20.32</td>
<td>24.31</td>
</tr>
<tr>
<td>Replicate 3</td>
<td>4.57</td>
<td>14.63</td>
<td>18.19</td>
<td>21.56</td>
</tr>
<tr>
<td>Replicate 4</td>
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<td>12.35</td>
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### Table 6.7. Validation Analysis
manual assay method. This is due to a number of factors. It is true that the automation of the system does reduce the human error associated with the assay but the instrumentation itself, can in some cases, cause errors in the assay. As explained in section 6.3.3. the K1000 was not reliable enough to produce the reproducible results expected from the instrumentation and as a result increased the CV's to levels not expected by an automated system. The AS3000 proved to be an excellent sample preparation instrument but did not provide enough power in its simple syringe pump (section 4.3.2.) to propel the sample (once prepared) to the K1000 resulting in some errors occurring during sample transport between the two instruments. These two problems mentioned would, of course, also effect the semi-automated immunoassay system and the results produced by both the systems seem to confirm this. It is also possible that although operator input is always a problem in manual systems the repetitive nature of the analysis outlined in chapter 5. allowed the operator to gain a high degree of efficiency when handling the samples and controlling the manual systems, possible giving lower CV's than would be expected.

6.7. Comparison of the Fully Automated Immunoassay for Phenytoin in Human Plasma with the Semi-automated Method.

The two methods (described in section 6.2. and 6.4.) use the same basic method except for the increased sample manipulation of the fully automated assay. The advantage of the fully automated immunoassay is its ability to pretreat the samples, making them ready for injection onto the column. Probably the most important advantage of using the auto sampler to perform the mixing of the analytes is that the two minute incubation period is not wasted time,
i.e. the samples are mixed in turn, with each mixing taking 40 secs, therefore if a number of samples are to be analysed then the time required to mix all the samples is significantly longer than two mins. By the time all the samples have been prepared, using the method described in section 6.4.1., the samples have had more than enough time to incubate, i.e. more than two minutes (Note: the assay performance is not influenced by incubations above 2 mins) and can be injected straight onto the column once premixing has been completed by the AS3000.

Also, once the last sample has been prepared the samples in turn, starting from the first sample are injected in sequence onto the column and eluted off. This procedure of injection and elution takes over two minutes, therefore the final samples will also have more than enough time for incubation.

Tables 6.1.-6.3. and tables 6.4.-6.7. show the CV's for the semi-automated and the fully automated assays respectively and as expected show no striking difference between the two.

The analyst might expect a slight improvement in the fully automated procedure as the samples are mixed by the auto sampler, but in fact the sample preparation does not normally contribute much to the overall variation of the CV's, i.e. other factors, such as the injection valve and determination of the area (chapter 7.) effect the CV's to a greater extent.


The initial aim of this project was to provide a fully automated immunoassay for drugs of pharmaceutical interest with precision and levels of confidence equal to or in excess of those possible using a manual method. It would be expected that as the automation of any system is
increased the precision of the system would also increase but in this case that has not found to be true. The figures and tables show that there is good correlation between the individual methods, with the ANOVA plots showing the percentage bias (i.e. the difference between the measured value and the true value) is not constant but is not dramatically different for each control.

Each system, manual and automated, provided their own distinct advantages, which will be summarised below.

The manual method provided quick, reliable results, with good accuracy and precision. The system is uncomplicated, easy to use and although manual is not physically demanding, i.e. the system does not require repeated injection quickly after each other, but the system does require constant operator input. The manual system is quicker than either of the automated systems due to the fact it uses an on-line method for the production of the results, providing results at approximately one sample per minute. The automated methods used the pre-incubation technique (the on-line technique was not used because the instrumentation was incapable of injecting different samples in series, i.e. the antibody, followed by the sample, followed by the citrate and finally CHAPS) which slowed down the sample throughput to approximately one sample every two minutes. Although the fully automated assay is slower than the manual it does have a number of distinct advantages, mainly associated with its automation. Being an automated system the immunoassay requires little operator input, allowing the analyst the freedom to perform other tasks. In this case the fully automated immunoassay is far from ideal but as the results show it does produce results which are reproducible enough to be considered a working assay. As stated a number of times in the preceding chapter the majority of the errors are caused by the K1000, which is not as reproducible as the assay would require to produce better precision and accuracy. There are
also other factors which cause the automation of the system to produce poorer results than
would be expected from initial investigations of the POROS matrix and these will also be
discussed in chapter 7., together with the conclusions and a discussion on the relative poor
precision of the assay.
References.


CHAPTER 7

DISCUSSION AND CONCLUSIONS OF THE IMMUNOASSAY FOR

PHENYTOIN
7.1. Introduction.

The POROS matrix, described in section 3.2.3., does provide the analyst with an affinity chromatographic matrix which produces quicker, more precise immunoassays than the conventional matrices available. The assay for phenytoin outlines a number of advantages of the POROS affinity matrix but also provides a number of problems which although not directly the fault of the matrix occurs as a direct result of higher flow rate, sample throughput and the label utilised. An important point to emphasise before describing a number of the problems encountered is that the majority of the problems associated with the immunoassay would occur with any matrix employed and are a result of the conditions imposed on the assay and the reagents used rather than limitations of the POROS matrix.

7.2. Flow Rate Deviations.

Flow rate (section 5.1.4. and 5.1.5.) is an important factor in determining the size of the signal produced by the immunoreactive column, i.e. as the flow rate decreases the size of the signal increases (fig 5.1.4.). Previous matrices and columns have used flow rates of less than 0.5ml/min and therefore flow rate deviations have been minimal and have gone undetected. But during the production of high flow rate immunoassays incorporating the POROS matrix flow rate deviations became a major problem. These flow rate deviations took the form of a decrease in flow rate over time (fig 7.1.) i.e. the flow rate of the system decreases rapidly to approximately 50% of its original speed after 60 mins.
Fig 7.1. Decrease in Flow Rate through the Column over Time.

Fig 7.2. shows the effect this flow rate decrease has on the elution peak area which illustrates that as the flow rate decreases the peak area increases (section 5.1.4.). As a direct result of these flow rate deviations it is not possible to run the assay with any degree of confidence because each sample would be introduced to the column under different conditions, therefore prevention of the flow rate decrease is essential. As the flow rate deviations were first observed when the flow rate of the assay was increased it was possible to suggest that the decrease in flow rate was caused by this increase in flow rate, therefore a number of experimental conditions were examined including changing the pump tubing, changing the end pieces and frits etc. (section 4.1.8.). Initially the deviations were thought to be caused by the
stretching with time of the PVC transmission tubing which propels the buffer, via the peristaltic pump, through the system. This proved not to be the case because when equilibrium buffer was pumped through the system at 9 ml/min, which did not contain the column, no flow rate deviation was observed (fig 7.3. i.e. the was no loss of flow rate observed), which meant that the flow rate deviations were caused by a physical problem of buffer flow through the column. It was noted that if the frits (section 4.1.8.) contained within
Fig 7.3. Flow Rate Deviations through the System without the Column.

the end pieces of the column were changed at regular intervals the flow rate and therefore the elution peak areas remained constant, therefore the flow rate deviations were due to the frits contained within the end pieces of the column. The solution was simpler than expected and was found in the amount of buffer flowing through the column rather than mechanical effects of the flow rate on the system or the matrix. As mentioned previously, until the experiments with the POROS matrix the flow rates used in flow injection immunoassays were always below 2 ml/min, as a result only small volumes of buffer pass through the column and no decrease in flow rate was observed, but at higher flow rates large volumes of buffer pass through the column, simply, this increased volume increases the speed of the frit blocking of the end pieces which results in the decreased flow rate. This frit blocking occurs when minute particles of debris, in the buffer, for example, dust, block the frits, reducing the access of the buffer and as a consequence reducing the flow rate. So the simple solution of preventing the
decrease in flow rate is to filter the buffer solution (both the binding and elution buffer) using a Gilson vacuum filter pump system containing a 0.45 micron filter, before flowing through the column. Fig 7.4. shows the effect of filtering the buffer before flowing through the column on the flow rate for a period of 4 hours and shows there is no deviation in flow rate.

Note: All systems which employ high flow rates such as those mentioned will require some form of filtration method to prevent the flow rate deviations.

Fig 7.4. Flow Rate Deviations of Filtered Buffer through the System.
7.3. Non-Specific Binding (NSB).

NSB is the binding of analytes and reagents to tubes, matrices, flow cells, frits etc. non-specifically and can be a major problem with a variety of analytes and labels. In this case the NSB was caused by the phenytoin, both the unlabelled and the labelled.

Phenytoin (section 1.3.1.) is only sparingly soluble in water and therefore can precipitate out of solution easily, this causes the phenytoin to non-specifically bind to the flow injection system matrix, being more noticeable on the matrix due to the large surface area compared to the tubing but probably occurs in the same percentage on the other materials of the flow injection system.

This NSB became more noticeable when the on-line method is used and the reasons for this are explained below.

Fig 7.5 shows the affect of NSB on the elution peak area of labelled phenytoin using the on-line method for a number of injections of an identical sample and shows the peak area increase over a number of injections until it reaches a maximum after approximately 3 injections. The reason for this is because when the first injection of antibody passes through the column it travels to the Protein A attached on the POROS and becomes bound to it. The labelled phenytoin is then injected, the vast majority passes through the column, either becoming bound to the phenytoin antisera, or passing through to the detector, producing the unbound signal, however some of the labelled phenytoin becomes non-specifically bound to the matrix, frits and column etc.

The elution buffer is then passed through the system, eluting off the
Fig 7.5. The Effect of NSB on Elution Peak Area showing the Increase in Peak Area after Repeated Injections.

antisera and bound labelled phenytoin from the Protein A and producing the elution peak. Once this signal has been generated the process is repeated, by again injecting the phenytoin antisera into the system, this time the antisera binds to some of the non-specifically bound labelled phenytoin before itself becoming bound to the Protein A. This process occurs because the affinity of the labelled phenytoin for the phenytoin antisera is greater than the labelled phenytoin's affinity for the walls of the tubes, matrix etc. As a result some of the phenytoin antisera already contains labelled phenytoin before the next injection of
labelled phenytoin is performed. Therefore when the labelled phenytoin is injected it will once again bind to the antisera. The percentage of newly injected labelled phenytoin binding to the phenytoin antisera will remain relatively constant for each injection and will not be affected by the loss of some of the antisera binding sites being already occupied by the once non-specifically bound labelled phenytoin because the antibody is in excess and the perfusion system allows the maximum amount of binding possible between the labelled phenytoin and the antisera. Therefore as fig 7.5. shows the peak areas will increase until the antisera picks up as much non-specifically bound labelled phenytoin as the flow rate allows. This was more noticeable, as stated previously, using the on-line method because during the pre-incubation method all the antibody has bound to the labelled phenytoin before it is injected into the system, therefore the antisera will not bind to the non-specifically bound phenytoin in the system, unless the antisera is in a large excess.

The unlabelled phenytoin also suffered from the same non-specific binding problems but are not as noticeable as the labelled phenytoin because it is only the label which provides the signal, showing the increase in peak area after repeated injections. This theory is further strengthened by the increasing size of the antisera elution peak at rhodamine wavelengths, i.e. when antisera is injected onto the column and eluted off before the addition of any unlabelled or labelled phenytoin then the interference peak produced is small (as compared to the label signal) and can be calculated for in the final calibration curve, but upon repeated injection of the labelled phenytoin this antisera interference peak increases in size which can cause increasing interference to the label signal. This increase in antisera peak can be attributed to the labelled phenytoin non-specifically bound to the POROS so that when the antisera removes some of the non-specifically bound phenytoin it results in a larger antisera peak on
elution. This theory can also explain the increase in signal over repeated injection, which is in fact the antisera peak increasing rather than the analytical signal increasing (as well as the ratio of the label signal to the antisera peak decreasing).

There are a number of possible solutions to the problem of non-specifically bound phenytoin including the use of a solvent, cyclodextrins or surfactants.

7.3.1. The Use of Solvents to Prevent NSB.

It is possible to use small percentages of a number of solvents to assist the solubility of the phenytoin. These solvents can be introduced into the system in a number of ways, firstly small percentages of the solvents can be added to the buffer streams allowing a continuous amount of solvent present throughout the assay or, secondly introducing the solvent by means of an injection loop to flush the system. A number of solvents were used in various concentrations.

7.3.1.1. Methanol.

Used in concentrations between 0.1 to 10% v/v in both the binding and elution buffer. Fig 7.6. shows that methanol in a 10% v/v concentration in the binding and elution buffer did prevent some of the NSB, but did not completely remove it resulting in erratic peak areas.

Injecting the undiluted methanol into the stream by means of a 300μl injection valve also reduced the NSB but once again did not provide a constant solution to the problem and
Fig 7.6. The Effect a 10%v/v concentration of methanol has on NSB by initially preventing NSB in the first 3 injections but failing to continue to prevent the elution peak area from increasing.

repeated injections of neat methanol would possibly have an adverse effect on the lifetime of the matrix.
7.3.1.2. Acetonitrile.

Used in concentrations between 0.1 and 10% v/v in both the binding and elution buffer. Unfortunately even small percentages of acetonitrile gradually affects the binding between protein A and the antibody, resulting in a decreased elution peak area (fig 7.7.). Therefore it is not possible to use acetonitrile to remove the NSB.

Note: The binding of the antibody to the protein A returned to normal levels after the solvent was removed from the binding buffer suggesting that the acetonitrile unfolds the tertiary structure of the protein A preventing binding of the antibody.

7.3.1.3. Other Solvents.

Other solvents were also tried and these included ethanol and propanol, both in the buffer and in the injection valve format.

Both solvents provided some reduction of the NSB with the order of reduction of NSB for the solvents used being:

methanol > ethanol > propanol > acetonitrile.

Unfortunately none of the solvents mentioned removed the NSB completely and as a result other methods were investigated.
Fig 7.7. The Effect of Acetonitrile on Elution Peak Area Showing that as the Column is Exposed to more Acetonitrile the Binding Decreases at a Flow Rate of 6 ml/min.

7.3.2. The Use of Cyclodextrins to prevent NSB.

It is possible by the use of cyclodextrins to enhance the solubility of a number of compounds by encapsulating them inside the ring formed by the cyclodextrin and so prevent NSB. There are a number of cyclodextrins available and these include:

- α- Cyclodextrins (C_{36}H_{60}O_{30})
- β- Cyclodextrins (C_{42}H_{70}O_{35})
- γ- Cyclodextrins (C_{48}H_{80}O_{40})
All three cyclodextrins were examined and were found to produce a small decrease in NSB, but not enough to provide a working assay.

Note: There are a number of substituted cyclodextrins available which have been reported to increase the solubility but these were not tried.

7.3.3. The Use of Surfactant to Prevent NSB.

Surfactants have been used successfully in a number of plate immunoassays to prevent non-specific interaction between a variety of compounds and there are a number available, both charged and neutral. The surfactants investigated in this case were:

Triton X-100 (Octylphenoxypolyethoxyethanol).
Tween 80 (polyoxyethylene(20) sorbitan mono-oleate).
CHAPS ((3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate).

Both the Triton X-100 and the Tween 80 produced too much background fluorescence when added to the buffer stream in concentrations greater than 0.1% w/v and therefore could not be used to prevent the NSB. CHAPS did not produce an appreciable increase in the background fluorescence therefore it is possible to use high concentrations in the immunoassay system. CHAPS is a non-denaturing zwitterionic detergent, usually used in membrane biochemistry (1) which does not effect the binding of the antibody, both to the matrix and to the molecule of interest.
7.3.3.1. The Use of CHAPS to Prevent NSB.

Experiments involving CHAPS in the buffer solution were unsuccessful, in that the NSB was not reduced significantly in concentrations up to 1% w/v of CHAPS. To increase the concentration to higher levels would make the immunoassay financially unworkable, therefore investigations into the injection of higher concentrations of CHAPS were performed. These experiments were concerned with the concentration of CHAPS required to remove the NSB of the labelled and unlabelled phenytoin from the POROS Protein A matrix. Initial experiments using a variety of solvents injected into the system used a number of injection loop sizes, the most successful being the 300μl loop, therefore it was decided to inject the surfactant into the system also using a 300μl injection loop. Experiments which involved the injection of CHAPS into the stream ranged from a 1% w/v to a 10% w/v solution.

1% Solution - did not seem to effect the NSB.

2% Solution - this solution did show some reduction in NSB but repeated injection of labelled phenytoin followed by injections of 2% solution of chaps produced an increase in peak area of the labelled peak of 25% over 4 injections.

5% Solution - there is again an increase in the Bo peak over a period of 4 injections.

7.5% solution - there is an increase in signal as repeated injections using a 7.5% solution in a 300μl loop but in this case the increase is not as marked and does actually fluctuate through the injections.
Therefore this would suggest that some of the NSB is being removed but not all. An interesting point to make is that with repeated injections of labelled phenytoin onto the column the size of the CHAPS injection peak is increasing (fig 7.8.) which would again suggest that some of the NSB is being prevented but not all.

Fig 7.8. The Increase in CHAPS Peak Area over Repeated Injections.
10% solution Fig 7.9. shows the effect of using a 10% solution of the chaps in the 300μl injection loop. This solution seems to remove the majority of the NSB over a period of 10 repeated injections. This result would suggest that a 10% solution of chaps injected into the system removes the problem of NSB because:

a) The antisera elution peak does not increase in size over repeated injections.

b) The CHAPS injection peaks remain constant after each injection which suggest that the majority if not all the non-specifically bound phenytoin has been removed.

c) The label signal remains constant after repeated injections.

d) On addition of alternate injections of solutions of unlabelled and labelled phenytoin the label signal peak does not fluctuate (fig 7.10.).

In conclusion the CHAPS at a 10% w/v solution injected, via the 300μl injection valve, seems to prevent the NSB of the phenytoin to the POROS.

7.4. Peak Area Data Collection.

Data collection in any analytical system must be precise and uniform, i.e. each time the data must be collected under the same conditions and timings. Unfortunately the system of peak
Fig 7.9. Repeated Injection Of Phenytoin Rhodamine Conjugate between Injections of a 10%w/v Solution of Chaps.
Fig 7.10. Alternating Injections of Bo and 2μg/ml of Unlabelled Phenytoin.

area collection employed by the F4500 software does not allow the analyst to collect the data reproducibly, i.e. it is not possible to collect the data from the same points on each peak because the end points are chosen by the analyst and can be subject to error (fig 7.11. shows a schematic diagram of the process involved in data collection). This error in data collection can
be illustrated when examining the data collected from the peak areas of repeated injections of antibodies (both automated and manual) and repeated injections of the labelled samples. As stated in chapter 5 it is possible to produce %CV's of under 2% for a series of ten injections of antibody with this figure increasing to 3% when the automated system is used, this slight increase in %CV is explained in detail in section 7.5. When the labelled samples are repeatedly injected and recorded 10 times the %CV's increase up to 5 and 6% respectively. This obvious increase in %CV has occurred because the peak area calculation for the antibody injections are large, for example in their thousands, but the peak area calculations for the labelled samples are very much smaller, being less than 100 in most cases, so that if there is some deviation in the collection of the antibody peak areas it would go undetected because of the large figures involved but becomes very important when the small sample areas are calculated,
resulting in the increased %CV's observed, in both systems. This data collection, together with the problems outlined in section 7.5, are the reasons why the results obtained initially for the antibody peak areas could not be achieved by the labelled samples.


Chapter 6 describes the conversion of a manual immunoassay to an automated system. By utilising the instrumentation described in chapter 4 it was possible to provide an assay which provided comparable results to those achieved by the manual assay. The conversion from a manual to a fully automated system would be expected to produce improved precision and accuracy data but, as illustrated in chapter 6, is not the case in this assay. The reasons for this are an accumulation of a number of factors which are explained below.

7.5.1. Sample Transfer from the AS3000 to the K1000.

As with all clinical analysis sample handling and preparation are important if reliable results are to be obtained, therefore any area which alters the condition and introduction of the sample to the system warrants specific mention. Although the AS3000 auto sampler is simple to program, provides reproducible volumes and adequate control of the solution mixing procedures during sample preparation, it did provide a number of problems in the injecting of the sample into the injection port and onto the K1000. Two problems arose in this area, firstly when the injection needle entered the injection port (fig 4.11.) it did not provide an air tight
seal, therefore the sampler could not be injected very quickly into the injection port and even at slow injection rates often would fill the injection port before the liquid was carried off to the K1000, providing an area for both contamination and air introduction. The second problem associated with the AS3000 was the inability of the pump to propel the sample to the K1000, i.e. the syringe pump which was provided for the aspiration and dispersion of samples did not provide enough pressure to push the sample along the connecting transmission tubing to the injection loop contained within the K1000. It was possible to overcoming by precise timing of the K1000's peristaltic pump, which then pulled the sample the required distance to the injection loop but the AS3000 auto sampler did not propel the sample reproducible distances along the transmission tube causing different areas of the sample to be injected each time (although the sample should in fact be the same throughout the 300μl it is unlikely to be so because the sample will disperse along the transmission tubing).

The K1000 also provide the assay with one or two problems which were mainly concerned with the reproducibility of the system rather than the method employed or the set up of the instrument. The timing of the peristaltic pump is controlled by a number of timing devices situated on the front (section 4.3.3.) of the K1000, unfortunately these timers are not accurate and are difficult to precisely time, i.e. the timer does provide reproducible timing but the setting of the exact time is difficult. This difficulty of exact timer setting on the K1000 allied with the poor reproducibility of the AS3000 syringe pump did cause deviation in the assay affecting the precision and accuracy.

The result of this was data comparable to that achieved by the manual assay when in theory they should have been slightly better.
7.5.2. Sample Throughput.

The development of the automated assay provided a system which had a sample throughput of 1 sample every 3 minutes, i.e. an increase of two minutes from that obtained by the manual assay. This decrease in sample throughput was because the auto sampler could not inject the samples as quickly as it was possible manually, therefore increasing the time for each sample. This slight decrease in sample throughput was not a problem because the system could be left to perform the assay and controls unattended, enabling the operator to perform other tasks.

Another factor in increasing the time required to process the samples was the method employed, a pre-incubation immunoassay. Obviously this method requires a two minute incubation period before injection which will slow the assay down but, in the case of the automated assay this did not provide a problem because it was possible to programming the auto sampler to mix all the reagents (antibodies, unlabelled and labelled phenytoin) in series taking 25 secs for each sample, therefore it is possible to incubate the first sample while the others are being prepared. When all the samples have been prepared the first is then injected in the system, which has had more than enough time to incubate. As each sample takes longer than two minutes the other samples will also have adequate time to incubate. An important point to make at this stage is that incubation periods of longer than two minutes do not affect the result of the sample analysis. Therefore in the case of the automated system the incubation period is not wasted time as the auto sampler does performing other functions while the sample is being incubated.
As stated previously the pre-incubation method is used because of the instrumentation available but from the manual information available it would be possible to perform an on-line assay providing even quicker results than those mentioned in chapter 6.


As mentioned in chapter 1 there are a variety of techniques used in the determination of phenytoin, each providing a specific level of detection and analysis time.

As the therapeutic range of phenytoin in serum is between 9 and 21 µg/ml(2) each method of analysis is required to cover this range. As suggested in section 1.4.1. the majority of methods used for the determination of phenytoin are based on chromatographic techniques. These techniques include TLC, GC and HPLC methods, with detection limits of 2µg/ml for GC and TLC and as low as 1µg/ml for HPLC methods(3-13). In the case of the automated format described in chapter 6 a level of detection of 0.01 µg/ml is possible. This is possible because the technique uses the sensitivity associated with both immunoassays and fluorescence detection. By the use of immunoassay methods alone it is possible with the aid of radio-labels to achieve detection limits of 300pg/ml and by using enzymatic detection 0.01µg/ml(14-21). A number of workers have combined the techniques of immunoassays and fluorescence and have produced a number of microtitre plate based assays(22-27) which provide detection limits ranging from 0.25-1µg/ml. Therefore it is possible to say that the detection limit of...
0.01μg/ml obtained using the automated flow injection fluorescence immunoassay compares favourably with the techniques quoted.

Of equal importance to the analyst is the sample throughput. Workers (3-5) have suggested that chromatographic methods can be slow with the quickest being 15 minutes per sample for some HPLC methods (6-7). Microtitre plate immunoassays have always suffered from slow sample throughput and as a result are time consuming. The flow injection system incorporated in the assay described allows quick analysis time with an approximate sample throughput of 40 samples per hour.

Many of the techniques quoted suffer from interferences, whether from phenytoin metabolites (28-33), other drugs (34) or the phenytoin binding to plasma (35). During the development of the automated a number of serum samples were used but interferences and the effect on the sensitivity was not investigated fully which will be discussed in further work. It is probable that this technique will suffer from the same interferences that affect micro titre plate assays, for example, phenytoin serum/plasma binding (35).
7.7. Conclusions of the Study.

The initial aim of the project was to develop a rapid and automated assay for a therapeutic drug. Initial work by French and Palmer (36-37) had laid down the foundations of the technique, i.e. assays for drugs using flow injection immunoassays. They encountered a number of problems including poor sample throughput, labour intensive methods and poor reproducibility. These problems had to be overcome if the technique was to become a viable alternative to the existing drug analysis methods. It was possible, by the use of the POROS material, which until the early stages of the project had never been used as a matrix in flow injection immunoassays, to develop a fast reproducible method. This was achieved by increasing the flow rate through the system and in addition enabling the technique to become quicker and more robust, by providing a chromatographic media which could perform many hundreds of analysis without loss of performance. Once this problem of robustness was overcome it was then possible to develop an automated system which could utilise the increased lifetime of the assay. The automation of the system itself took a number of stages which are outlined in chapters 5 and 6, and it is important to note that the assay produced compares favourably with other techniques even though the conditions were not ideal. The problems encountered with the assay, outlined in the previous sections, were overcome by a number of changes in the conditions which enabling the assay to perform routinely for a number of hours. The sample throughput provided by the assay is probably the most important aspect of the development work, i.e. the initial aim of the work was to develop rapid immunoassays with high throughput, but it is somewhat disappointing that the initial sample throughput achieved by the manual assay could not be matched by the automated system, although the fact that the automated assay can be performed unsupervised
counteracts the decrease in speed. Although, as described in chapter 5 and 6, the assay run at flow rates of 6 ml/min with fast sample throughput, it should be possible to increase the speed of the assay further, up to and beyond 8 ml/min. The speed, in this case, was limited by instrumentation speed and not the limitations of the POROS matrix. Further development work could yield quicker assays than those mentioned here. An important point to emphasise is that the speed of the assay should not be the only concern of assay development because increased speed is likely to affect both the precision and the accuracy. The somewhat disappointing results provided by the automated assay were caused, as explained in section 7.5., by the instrumentation not being as reproducible as suggested in the manuals provided and resulted in the assay not performing as well as it would be expected to. From the results obtained by the manual assays, although this disappointment must be put into context. A fully automated assay was developed for phenytoin, requiring little operator input providing standards and controls well within confidence limits in both buffer and human plasma. This development itself was the initial aim of the work and can be said to have succeeded in producing a working assay. The final point to make about the assay is that the problems associated with the assay are a result of the instrumentation and the reagents used and further development with improved instrumentation and reagents will undoubtedly yield improved results.
There are a number of areas in which this work could be fully developed. The POROS itself as explained in chapter 5 can be run at increasing flow rates which in turn would make the automated assay quicker, even speed of up to 10 ml/min could be possible.

Also if quicker and more reliable instrumentation could be utilised it would then also be possible to not only increase the sample throughput but also improve the CV's of the system. Real plasma samples also need to be investigated to ensure that interference associated with other forms of phenytoin detection does not become a problem.

Probably the one feature which would improve the assay the most would be the introduction of a new labelling system. As stated in chapter 4 the label used in this case was rhodamine which does have a tendency to cause non-specific binding and effect the results obtained from the assay. Therefore a label which would not cause non-specific binding would be an advantage. There is an increasing demand for these types of fluorescence labels and I expect fluorescence assays of the future to utilise them.
References.
