Electrochemical Analysis of Some Drug Substances in Complex Matrices

by

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Declaration

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

Signed: Kamal Shafar Date: 25/5/93

Candidate

Dedicated to my parents, wife and son

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Abstract

This thesis describes the development of several analytical methods, based on electrochemical techniques, for the determination of various drug substances in formulations, biological fluids and environmental samples. The work included a study of the voltammetric behaviour of both sumatriptan succinate and salbutamol sulphate at a glassy carbon electrode. Based on these studies, simple, rapid and sensitive voltammetric methods were developed for the determination of these drugs in tablet dosage forms without any interferences from the excipients. Using differential pulse voltammetry, the dissolution profile of salbutamol tablets was also investigated. The principal criteria for designing electrochemical flow detectors, and the great advantages of carbon fibre electrodes, have been utilized in the development of a versatile electrochemical detector for HPLC that is based on a carbon fibre (14 μm diameter) placed in a polyethylene tube connected directly to the end of the chromatographic column. The analysis of salbutamol in human plasma was then developed based on extracting the drug from the plasma into an organic solvent and separation of the drug on a reversed-phase C_{18} column, followed by detection using the carbon fibre flow electrode. Several advantages of the carbon-fiber flow electrodes over conventional electrodes have also been demonstrated. The cell was then used for monitoring of terbutaline concentrations in human plasma in the range of 1-35 $ngml^{-1}$ using column-switching LC. The method involved direct injection of the plasma sample into a C_{18} extraction column for sample clean-up. This method was also applied for the simultaneous determination of both salbutamol and terbutaline at overdose levels in human plasma. A method was then developed to determine the suspected carcinogen gentian violet in human urine and in poultry feed. Liquid chromatography with electrochemical detection was also applied in environmental studies for the analysis of malachite green in drinking water and river water.

Chapter 1

Drug Development and Electrochemical Analysis

1. 1. INTRODUCTION

1. 1. 1. *Drug Development*

Analytical methods are required to assure the purity of starting materials and can be used to monitor a systematic process to determine if it has reached completion., and to, isolate and quantitate by-products of the reaction.

Analytical research on a drug substance starts after synthesis, with the characterisation of its physicochemical properties to ensure that all pharmacological testing is done with pure material and to establish specifications so that future production lots will yield a reproducible in vivo response. These specifications will include studies of the purity of the drug, identification and quantitation of other products of synthesis and degradation, the pK_a values of the drug , and physical characterisation such as thermal stability, optical activity, surface properties, particle size, and crystalline form. These studies will also include investigations of the stability and solubility of the drug in aqueous media as a function of pH. Most drugs are weak acids or bases and are present in solution as both the nonionized and ionized species. Dissolution constants of weak acids and bases are numerically small and a logarithmic notation is, therefore, convenient. The dissociation constant exponent, pK, is derived from the dissociation constant in a manner analogous to the derivation of pH from hydrogen ion concentration. Hence

$$pK_a = -\log_{10} K_a \qquad (1)$$

It follows from this expression that the higher the value of K_a the smaller the value of pK_a, so that the stronger the acid the smaller the pK_a value.

Upon completion of the analytical profile, the drug is released for preliminary pharmacological and toxicological testing in laboratory animals. At this stage of development, the drug is usually administered in a simple preliminary formulation by parentral and oral routes, to determine its pharmacological activity, its relative safety, and dose-response characteristics. During toxicological testing, blood, urine, and tissue samples are collected and analysed to correlate both pharmacological response and toxicity to the blood level. These data aid in the assessment of adequate absorption, dose-response behaviour, metabolic effects such as accumulation, enzyme induction or attainment of steady-state conditions, intra- and inter-species variations, and the relationship of drug levels to symptomatology.[1]

In addition to toxicological studies, various in vitro studies are then performed to determine if any physicochemical factors such as solubility, dissolution or release rate, permeability, gastrointestinal degradation, and rapid biotransformation will affect the drug's oral absorption characteristics after administration of a given dose in a pharmaceutical dosage form[2]. The absorption. distribution, biotransformation, and excretion of a drug all involve its passage across cell membranes. It is essential, therefore, to consider the mechanisms by which drugs cross membranes and the physicochemical properties of molecules and membranes that infleuence this transfer. Important characteristics of a drug are its molecular size and shape, solubility at the site of its absorption, degree of ionization, and relative lipid solubility of its ionized and nonionized forms. Since drugs generally are too large to pass through membrane channels, they must pass membrane barriers by diffusion through the lipid components of the membranes. The nonionized molecules are usually lipid soluble and can diffuse across the cell membrane. In contrast, the ionized fraction is usually unable to penetrate the lipid membrane because of its low lipid solubility.

When a drug permeates a cell, it must obviously traverse the cellular plasma membrane. Other barriers to drug movement may be a single layer of cells (intestinal epithelium) or several layers of cells (skin). Despite these structural differences, the diffusion and transport of drugs across these various bounderies have many common characteristics, since drugs in general pass through cells rather than between them. The plasma membrane thus represents the common barrier.

Assays must be devised to assess the solubility and dissolution rate of drugs as a function of pH within the physiological pH 1-8 at 37°C, and to evaluate the drug's permiability characteristics across the intestinal mucosa. The utility of these in vitro studies has been reported Kaplan[3]. Metabolic transformation is evaluated in vitro by incubating the drug in a liver microsomal enzyme fraction (900 x g supernatant) at 37°C, which was separated from the liver homogenates[4]. Analytical methodology is needed for these studies to quantitate the rate of biotransformation, and to isolate and quantitate the metabolites formed.

The design of a suitable dosage form at a clinically effective and safe dosage level is based on the data obtained from the pharmacological and toxicological studies, together with biopharmaceutical data gained from the in vitro studies. Such dosage forms are evaluated for bioavailability following oral adminstration to laboratory animals at a relatively high dose (typically body weight in the dog). The bioavailability, rate, and extent of absorption of this formulation are then assessed

by comparing its blood level, urine, and faecal elimination profile to that obtained from an intravenous and oral solution of the drug at the same dosing level[3]. The dosage form exhibiting optimal bioavailability is used for dosing animals in toxicity studies, in several animal species, in which blood, urine, and tissue levels of drug and/or metabolites are monitored. If the drug is shown to be safe, it is then ready for limited testing in humans. An Investigational New Drug application (IND) is filed with the Food and Drug Adminstration (FDA) which describes the drug formulation (list of components, quantitative composition), the analytical procedures employed to establish and maintain standards of identity, strength, and purity, and all the pre-clinical information pertaining to the administration of the drug to laboratory animals.

With approval from the FDA the drug proceeds into Phase 1 clinical testing, where it is adminstrated to humans to determine its safe dosage range, pharmacological activity, side effects, metabolism, and distribution, absorption, and elimination characteristics. From these studies, samples of blood, urine, and faeces are collected and analysed to assess the preliminary bioavailability and pharmacokinetic profile in humans. Pharmacokinetics is defined as the study of the kinetics of absorption, distribution, metabolism, and excretion of drugs and other endogenous substances. During Phase 2 and Phase 3 testing of a drug for the safety and effectiveness in the diagnosis, treatment, or prophylaxis of a given disease or condition, biological fluids are collected to obtain comprehensive pharmacokinetic information and to correlate such data with activity of the drug. At the completion of the clinical trials, all the data are accumulated about the drug from its initial synthesis to its clinical testing and submited to the FDA as a New Drug Application (NDA) for approval of the drug as a marketable product. Subsequent to marketing, constant quality control monitoring of the product from lot to lot must be maintained to provide the patient with a reproducible product. To guarantee this reproducibilty in the dosage form, it is necessary to develop assays that will analyse reagents, bulk chemicals, and intermediates which go into the dosage form, and also to obtain and analyse representative samples of the dosage form.

Analytical chemistry plays a critical role in the development of a compound from its synthesis to its marketing as part of a drug formulation. The instrumental methods for quantitation which are most commonly used in a pharmaceutical laboratory fall into four basic categories: chromatographic, spectrophotometric, electrochemical, and radiometric analysis.

The choice of the appropriate technique to solve an analytical pharmaceutical problem is often controlled by the sample matrix, and the amount of preparation that is required before the analytical measurement can be made. If the material is an organic bulk substance or intermediate, it is usually possible to dissolve the compound in a suitable organic solvent and analyse the sample by thin liquid chromatography (TLC), gas chromatography (GC) or high-performance liquid chromatography (HPLC). A dosage form may be dissolved in an aqueous medium, filtered, and analysed directly using an electrochemical technique or extracted into an organic solvent and analysed chromatographically or spectrophotometrically. The final selection for the determination step is dependent on the intrinsic physicochemical properties of the pure compound or its derivatives. Thus, compounds or their derivatives should be processed through an analytical screening program to examine their spectrophotometric, chromatographic, and electrochemical properties which might be amenable to chemical analysis. Based on the signal response per unit mass of a compound, a projected limit of detection (LOD) per milliliter or gram of sample can be calculated. Many analytical procedures can be eliminated at this stage of method development.

1. 1. 2. *Therapeutic drug monitoring*

Chemists and biochemists have been routinely analysing biological fluids since the early part of this century when they discovered that a relationship existed between the concentration of various endogenous substances, for example glucose, hormones, enzymes, etc., and the cause of a disease[5]. The analysis of drugs in biological media, however, has emerged relatively recently, and it is only in the last twenty years that it has recieved widespread attention[6]. With few exceptions, drugs are low molecular weight organic compounds of known structure and well defined physico-chemical properties, readily available in pure form[7]. The analysis of drugs in biological materials possess a serious challange to the analytical chemist due to difficulties imposed by the complexity of these media.

Growth in the field of biopharmaceutical analysis has paralleled the expansion in the number of new drug compounds introduced onto the market. Assays for proposed new drugs are essential with a view to establishing protocols for new drug registration. It is necessary for a drug company to develop methods capable of determining low concentrations of their products in blood, plasma, urine and other biological matrices in order to establish the pharmacokinetic profile of that product. Drug levels are also monitored in cases of drug abuse, overdose, and as a guide to

optimise individual drug-dose regimens, i.e. therapeutic drug level monitoring (TDM.).

TDM may be required in the following circumstances: (a) where there is a question of patient compliance, (b) where there is a lack of therapeutic effect, (c) where the drug has a narrow therapeutic range, (d) where there is danger of toxicity, and (e) where there is a need for medico-legal variation of treatment. In many cases quantitation of metabolites in addition to the parent drug will be required if these metabolites are pharmacologically active. TDM may involve quantitation in the microgram (ppm), nanogram (ppb) or picogram (ppt) concentration range.[8]

1. 2. DRUG ANALYSIS

Modern analytical chemistry must satisfy very diverse demands. Among the most difficult tasks are the continuous monitoring of substances and analyses of very complicated organic and inorganic systems, often at trace concentration levels (mainly in clinical analysis, pharmaceutical research, industry etc). Although the chemical structure of the drug will largely dictate the most suitable assay procedure for its determination[8], the magnitude and frequency of the doses administered and the pharmacokinetics of the compound, govern the ultimate sensitivity and selectivity required of the assay for its quantitation in biological fluids. The requirements of an assay are that it is selective, sensitive, reproducible, and applicable to a large range of compounds. It should also be relatively simple to develop and execute, and ideally should not involve lengthy and tedious sample preparation stages.

1. 2. 1. *Spectrophotometric Analysis*

Spectrophotometric absorption (UV-Vis) and emission (fluorescence and phosphorescence) methods have been the classical methods used extensively in drug analysis. They possess reasonable sensitivity, but lack high selectivity since spectral characteristics cannot usually differentiate between the parent drug from any metabolites present, unless used in conjunction with differential solvent extraction techniques using liquid-liquid or solid extraction separations. Most drugs exhibit moderate to strong absorption bands in the 215 - 300 nm region. While UV spectrophotometric methods are not as selective as chromatographic methods, they are simple and rapid, making them useful for screening purposes.

The most recent development in spectrophotometric detection has been the introduction of instruments based on a photodiode array detector[9]. By using

"reverse optics", all the light from a deuterium lamp is passed through the sample cell onto the monochromator, which spreads out the beam into a spectrum. This falls across an array of 230-350 photodiodes mounted on a silicon chip. These can readily be monitored virtually simultaneously by a microcomputer to provide the full absorption spectrum from 200 to 700 nm every 0.1 s. This detector can thus provide a continuous measurement of the full spectral absorbances of the eluent with time. Because they are very versatile and are supported by sophisticated computer software for the interpretation of the results, diode array detectors are relatively expensive (3-6 times more costly than a variable-wavelength detector).

1. 2. 2. *Immunoassay Techniques*

The discovery of competitive binding assays by Yalow and Berson[10] in 1959 has proved to be a significant development in the fields of both clinical and analytical chemistry. Drug immunoassays are simple to perform but complex to develop. In development of such an assay, the drug first has to be coupled as a hapten to a carrier protein, usually bovine serum albumin, in order to raise antibodies against them, since most drugs themselves are not immunogenic[11]. If the drug molecule does not have an appropriate group for direct conjugation reaction, a chemical derivative may have to be prepared prior to the conjugation step. After formation of the immunogenic species, an animal (usually a rabbit or a goat) is immunised and the appropriate antibodies isolated. The drug must then be labelled with the appropriate label. Typical labels include radiochemical, enzymatic, fluorescent and electrochemical labels. Finally when all the reagents are available, the assay procedure itself must be optimised in terms of variables such as antibody dilution, pH, temperature and time of incubation[12]. These assays are amongst the most specific methods of drug analysis, but complications can arise from factors such as cross-reactivity.

1. 2. 3. *Chromatographic Techniques*

The first person to use the term "chromatography" was the Russian botanist Tswett (1872-1919), who reported on the separation of coloured plant pigments into bands on a column of chalk[13,14]. Tswett (1906) stated that: " Chromatography is a method in which the components of a mixture are separated on an adsorbent column in a flowing system". Recently the International Union of Pure and Applied Chemistry has defined chromatography as:

"A method used primarily for the separation of the components of a sample, in which the components are distributed between two phases, one of which is

stationary while the other moves". The stationary phase may be a solid, or a liquid supported on a solid, or a gel. The stationary phase may be packed in a column, spread as a layer, or distributed as a film, etc.; in these definitions "chromatographic bed" is used as a general term to denote any of the different forms in which the stationary phase may be used. The mobile phase may be gaseous or liquid[15]. There are three principal elution procedures; isocratic(from the Greek, isochros, meaning equal strength); stepwise (or fractional); and gradient. The isocratic procedure is the operation of the chromatographic column by allowing a solvent mixture of unvarying composition to run through the column until separation is complete. If only one solvent is used ready elution of only some of the components of the original mixture from the column may result. To remove those which are more firmly held, a stronger eluting agent is required. Sometimes it may be necessary to use several different solvents of gradually increasing strength for the successive desorption of different components. This is known as stepwise elution. The technique of gradient elution analysis was first described in detail by Alm et al.[16]. It involves the use of a continuously changing eluting medium. The effect of this gradient is to elute successively the more strongly adsorbed substances and at the same time to reduce tailing.

It was not until the 1930s that chromatography in the form of thin-layer and ion-exchange chromatography became a regularly used technique. The 1940s saw the development of partition chromatography and paper chromatography with gas chromatography following in 1950. The 1960s saw a rapid rise in the routine use of chromatography as universal technique, particularly in chemistry, biology and medicine. Two nobel prizes have been awarded to chromatographers, Tiselius (Sweden) in 1948 for this research on "Electrophoresis and Adsorption Analysis", and Martin and Synge (UK) for the "Invention of Partition Chromatography".

Recent developments and technologies, such as microelectronics and microcomputers have enabled manufacturers to produce instruments that are reliable, with parameters that can be precisely set and measured, to give reproducible chromatograms and a vast expansion of our knowledge and understanding of this powerful analytical separation method. High-performance liquid chromatography (HPLC) and gas chromatography (GC) are the most widely used chromatographic techniques for quantitative analysis of therapeutic drugs in biological media.

1. 2. 3 1. *Gas Chromatography*

Gas chromatography coupled to a variety of detection modes, i.e. flame ionisation (FID), electron-capture (ECD) and nitrogen-phosphorous (NPD) detection, is a widely applied technique. Although analysis of an intact drug moiety (underivatised) is preferred to assure selectivity, derivatisation is often necessary. Simple derivatisation reactions such as alkylation or silylation can be used when needed to yield very sensitive and selective methods. When making a derivative, one must take account of the detection system to be used, so that during sample preparation contaminants are not introduced into the extract which could be deterimental to the determination. NPD is particularly susceptible to interferences by residues of silylating reagents and phosphate plasticizers contained in plastic syringes.

1. 2. 3. 2. *High-performance Liquid Chromatography (HPLC)*

In 1963, Giddings[17] showed that if the framework developed for gas chromatography applied equally to liquid chromatography, it would require high mobile phase inlet pressures. When such systems were demonstrated, high column efficiencies were obtained and high-performance liquid chromatography was described first by Kirkland[18], Huber[19] and Horvath et al.[20]. By operating at high pressure, these instruments overcame the effect of higher liquid viscosities relative to gas viscosities, and gave analysis times comparable with gas chromatography (GC). HPLC is an attractive approach in drug analysis for a number of reasons. Firstly, sample preparation is often minimal; a simple protein precipitation step followed by centrifugation and injection of the supernatant may be all that is required. Secondly, there is rarely a requirement for sample derivatisation prior to the chromatographic step, as there is in GC. Thirdly, it can be used for samples that are thermolabile and unsuitable for GC. Fourthly, most analyses can be performed successfully on reversed-phase systems. These systems are ideally suited to drug analysis. Most drugs possess sufficient hydrophobic structure to ensure retention by the stationary phase. However, many also contain hydrophobic groups which will limit this retention and allow the chromatographic process to occur in a reasonable time. Mobile phase "strength" can be readily controlled by adjusting the organic solvent to buffer ratio. Variations in pH, ionic strength and the use of ion-pairing agents provide additional versatility.

Detectors for drug analysis using HPLC include ultraviolet and visible absorbance, fluorescence and electrochemical detection, depending on the chemical properties

of the drug to be analysed. HPLC is of major importance to the analyst in the pharmaceutical, food and fine chemical industries and has also played a significant role in forensic, environmental, chemical and biochemical studies. In the latter field it has been used by molecular biologists to isolate gene probes, i. e. short lengths of DNA (ca 30 bases), from synthetic mixtures[21]. For the practising chromatographer, often a search of the relevant literature will identify a similar application which with some minor modification will prove suitable for the particular need.

1. 2. 4. *Guidelines for Bioanalysis using HPLC*

A bioanalytical method consists of several steps: sampling, sample treatment (clean-up and/or pre-concentration), derivatisation (if required), separation, (post-column) reaction/detection, quantitation, and data processing[22,23]. Sample pre-treatment and chromatographic separation are normally the most time-consuming steps. When developing a bioanalytical procedure, the problems and objectives should be clearly defined. This allows selection of the appropriate sample treatment step (STS), and chromatographic and reaction/detection techniques. In bioanalysis, the use of a guard column between the injection device and the analytical column is essential in order to avoid clogging of the latter by macromolecules and/or particulate matter. The internal volume of the guard column should be small enough (volume ratio less than 40% of the analytical column) to avoid additional band broadening. The following modes are frequently applied in modern HPLC.

(i) Normal-phase (NP) adsorption chromatography, using polar stationary phases (e.g. silica, alumina) in combination with non-polar solvents.

(ii) NP partition chromatography using chemically modified silicas. A wide variety of hydrophilic or polar modified silicas (e.g. cyanopropyl, diol, aminopropyl phases) are available.

(iii) Reversed-phase (RP) partition chromatography[24,25]. A wide selection of modified silicas (e.g. octadecyl, octayl, butyl, phenyl phases) is available for polar as well as non-polar compounds. It is now estimated that 60 - 80% of all HPLC separations are accomplished using RP packings[26,27].

(iv) Ion-pair (IP) chromatography is another technique for the separation of ionised compounds. This can be performed as a liquid-solid adsorption or a liquid-liquid partition technique, using either the RP or the NP mode.

(v) Chiral chromatography. It is now well understood that different enantiomers of compounds can have significant differences in their biological activity. Examples of this are commonplace, for instance, the (+) form of estrone is an estrogenic hormone whereas the (-) form is inactive. D-Penicillamine is an antiarthritic drug, but the L-form has been found to be extremely toxic. Sucrose can only be metabolised if it is in its naturally occuring D-form. The synthetic L-form tastes identical, but can not be metabolised and therefore has potential as a dietary sweetener. Since biological systems are generally stereospecific and stereoselective it is likely that the chemical or biological activity of a compound will depend on its stereochemistry. The aim of the chemical industry is to develop the most effective compounds for the market place that are both highly active and safe in the environment. Stereospecific compounds are therefore being developed with the aim that they will be more active than the racemates and safer in the environment because they will contain less inactive material.

The chirality of novel compounds is therefore examined at all stages in their development. During the early stages of the development of a chemical, different synthetic routes for stereospecific compounds need to be examined analytically. Individual enantiomers require biological and toxicological screening. During later stages of development and when the compound is in the market place, analytical methods are necessary for registration submissions, to provide enforcement methods for regulatory authorities and to monitor the quality of the product in manufacturing. Enantiomers have indistiguishable physical and chemical properties, except when they are in a chiral environment or in response to polarized light. They cannot therefore be resolved directly by conventional chromatography. Chiral high-performance liquid chromatography (HPLC) provides the chiral environment to enable enantiomer separation for analytical methods and, on a preparative scale, to isolate sufficient quantities for screening programmes. This can be achieved in essentially three ways, namely using chiral derivatization, chiral additives and chiral stationary phases.[28 - 31]

Chiral derivatizing reagents are treated with the chiral compound of interest before being separated chromatographically. The resulting derivatives are diastereoisomers and can therefore be separated using non-chiral chromatographic techniques. The derivatization reaction may be incomplete at the time of separation or the derivatization reagents or their impurities could interfere with the chromatography of the enantiomers of interest. An alternative is to use chiral additives that are dissolved in the mobile phase. The chiral complexes formed have different capacity factor so that the separation can again be achieved using non-chiral

chromatographic techniques. With chiral stationary phases, however, the enantiomers are separated by their interaction with a chiral entity that is bound to the stationary phase. This is proving to be a popular technique, since no pre-column derivatization is required and it is not necessary to optimize the concentration of any chiral additives during development.

Differences in stability between the diastereoisomers is reflected in differences in retention times, the enantiomer forming the less stable complex being eluted first. An interesting example of this stationary phase separation of the anti-inflammatory drug, ibuprofen after derivatisation to its amide is shown in Figure 1.1.

(vi). Size exclusion chromatography (SEC) is a widely used clean-up technique to remove high-molecular-mass components that could interfere with liquid or gas chromatographic determination of analytes, such as pesticides and other organic compounds, in environmental matrices[32 - 34]. SEC, though an established technique for the separation of macromolecules using open-column systems, met with limited success when applied to modern LC, as many of the commercially available packings did not meet the constraints and instrumental demands of HPLC. Recently (from 1982), a variety of packings have been developed which have led to a spectacular growth in the HPLC analysis of macromolecular samples, such as polydisperse polymer samples and biological materials, such as proteins and carbohydrates. SEC differs from other LC modes in that separation takes place exclusively because of differences in molecular size; consequently solvent selection is simpler, as the requirements of the solvent are simply that of sample solubility and packing compatibility.

(vii). Internal surface reversed-phase (ISRP). ISRP supports represent a relatively new concept in liquid chromatography[35], which combines the fundamental principles of size exclusion and bonded phase partitioning separations to produce a type of surface discriminating chromatography. The ISRP material has the ability to exclude large molecules, such as proteins, from the pores with negligible adsorption of these molecules on the external surface. Low-molecular-mass compounds, such as drugs, are separated with good capacity, selectivity and efficiency. Several methods have been described for direct injection of biological matrices, such as serum and plasma, for the determination of drugs[36 - 41] in which proteins did not interfere with the chromatographic analysis.

The physicochemical properties of the analyte primarily determine which chromatographic system is most appropriate, but the physicochemical properties of

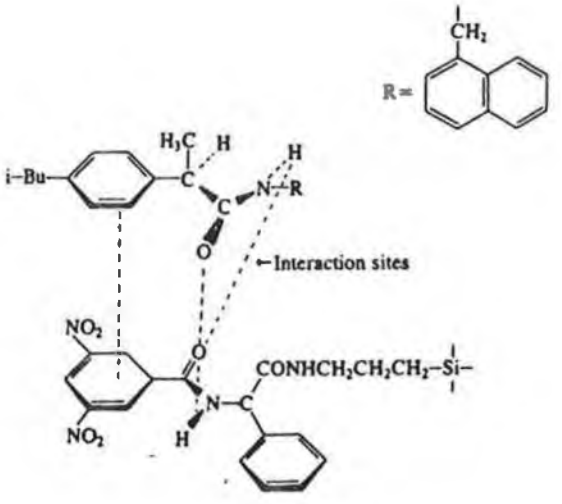

Figure 1. 1. Reaction between chiral stationary phase and amide derivative of (R) - ibuprofen. Reproduced from Braithwaite, A. and Smith, F. (Eds), Chromatographic Methods Chapman and Hall, New York, 1985, p. 267.

the biological matrix are also important. A major problem in developing bioanalytical procedures is the presence of proteins, which can clog the chromatographic system, especially when analyte-containing solutions are subjected to one or more freeze-thaw cycles.

1. 2. 4. 1. *Choice of Samples for Drug Analysis*

In drug analysis, the most commonly sampled body fluid is plasma or serum, because a good correlation between drug concentration and therapeutic effect is usually found.

Whole blood can be broken down into its general components as follows: plasma (which contains the serum and fibrinogen) and the cellular elements (which contain the erythrocytes, leucocytes and platelets). Plasma is the liquid portion of circulatory blood. The cells are separated from the blood by centrifugation of whole blood. If blood is allowed to clot, the fibrinogen is removed from plasma leaving serum. The majority of clinical analyses are performed on whole blood, plasma or serum. Urine is also often analysed. In urine, the analyte concentrations are sometimes higher than plasma or serum. However, many different types of solutes, including metabolites, are present in strongly varying concentrations. Urine analysis for drugs is most often used in connection with urinary excretion and bioavailability studies. Because of the constant ratio between the drug concentration in plasma and in saliva obtained for a number of non-protein bound analytes, saliva can be used for the analysis of samples from neonates. Drug concentrations in saliva are sometimes assumed to represent free plasma levels, but this is found to be true only for a few drugs (e. g. carbamezepine and phenytoin). For others the correlations are less satisfactory or apparently non existant[42 - 44]. Cerebrospinal fluid (CSF) is also analysed for drugs, but not frequently. It is not practical to analyse CSF samples routinely but occasionally CSF. levels may be required if damage to the blood brain barrier is suspected.

1. 2. 4. 2. *Collection and Storage of Samples*

Collection of samples at the correct time in relation to dose is important in drug analysis. If insufficient care is taken in the collection and handling of biological samples, the data generated will be invalidated. If plasma or serum is analysed, the expressions "blood samples" or "blood levels" should not be used in description of the analytical procedures unless whole blood samples are to be analysed for some reason (for example, for drugs with a high affinity for red cells such as chlorthalidone or cyclosporin). Sample collection tubes containing anti-coagulants

(for blood) or preservatives (for urine) are available. The choice of anticoagulant may affect assay results as may some plasticizers released from blood collection tubes. The selection of glass or plastic tubes for blood collection and subsequent separation of plasma or serum, and its effect on the drug in an in vivo sample with respect to adsorption losses, contamination by plasticizers, displacement of protein-bound drug by plasticizers and their effects on the plasma to haematocrit ratio, warrant investigation. Drug concentration data can be adversely biased by these phenomena, giving rise to poor pharmacokinetic data. For example, a significant decrease in plasma tricyclic antidepressant concentrations has been attributed to sample contact with the plasticizer tris-(2-butoxyethyl) phosphate (TBEP) which may be present in the rubber stopper of collection tubes. TBEP-free stoppers are now available[45].

On no account should a blood sample be frozen without pre-treatment, since this would result in haemolysis, preventing the subsequent separation of plasma or serum. After collection of blood (5 - 10 ml), a clot can be allowed to form and the supernatant liquid (serum) collected after centrifugation. Coagulation is complete in about 30 minutes at room temperature. Alternatively the blood can be collected in tubes containing an anticoagulant (e.g. heparin, EDTA) and the supernatant liquid (plasma) collected after centrifugation. Since the anticoagulant effect is temporary, collected specimens must be centrifuged quickly to prevent eventual clotting. Plasma is more frequently used than serum in drug analysis, since although similar results are obtained from both fluids, plasma is preferred since the anticoagulated blood can be centrifuged immediately, whereas serum cannot be sampled until coagulation is complete. Secondly it is relatively easy to centrifuge blood that has been treated with anticoagulant, since the plasma separates quickly and the maximal volume can be recovered if required. Drugs stored under conditions of heat, light, humidity and pH should be examined for possible decomposition. Fresh plasma or serum samples can usually kept for 6 hours at room temperature, 1-2 days in a refrigerator at 4^0C, or frozen at -20^0C for long term storage. The Council of the British Pharmaceutical Society has issued guidelines for a pharmacy-based pharmacokinetic service which includes instructions on sampling and storage of blood[46].

Urine drug analysis is carried out either on a single or a 24 h specimen. Both pH and volume are important factors in urine analysis. If urine is allowed to stand at room temperature, bacterial action causes the decomposition of urea into ammonium carbonate and then to ammonia, with a resulting change in pH. Urine may be preserved by freezing or by addition of preservative (toluene, boric acid or

concentrated HCl). Freezing should be done relatively fast to avoid losses during subsequent thawing: during the last stage of the freezing process an oversaturated solution will exist and a non-soluble structure of the analyte may then be formed. Frozen samples of plasma/serum or urine should be brought to room temperature and subjected to vortex mixing for 10 seconds to ensure homogeneity before analysis.

1. 2. 4. 3. *Direct Analysis*

In a few cases, direct injection of the biological fluid into a HPLC system is possible. For example in the case of theophylline, which is therapeutically present in relatively high concentration (mg/l) and possesses good UV absorption characteristics, a small volume of diluted serum can be injected directly onto the chromatograph, thus minimising the amount of non-eluting contaminants placed on the column with each injection. In this case the mobile phase and the column packing release the drug from protein binding sites as the sample enters the column. Direct injection has also been applied to analysis of salicylate and naproxin in serum[47].

Urine can also be directly injected onto the chromatograph following centrifugation to remove particulate matter, and suitable dilution, preferably with the mobile phase. For example, antibiotics[48] and other drugs[49] can be determined in urine by HPLC with on-column injection.

When using direct injection the analytical column should always be protected by a guard column to prevent irreversible adsorption and blockage. Biological samples contain materials such as lipids and proteins which deposit on the chromatographic column and lower its performance. Direct injection is limited in its application. Normally to improve both the sensitivity and selectivity of an assay, some form of sample clean-up prior to injection will be necessary.

1. 2. 4. 4. *Sample Pretreatment in Bioanalysis*

Sample pretreatment steps are one of the most important in any bioanalytical method. To ensure sensitivity and selectivity, interferences in the chromatographic separation should be eliminated. Moreover, the required reproducibility and repeatability can normally be achieved when relatively clean samples are analysed[50]. Since sample pretreatment is an integral part of the bioanalytical method, the most suitable pretreatment for an analyte can be totally different when

the matrix, the number of samples to be analysed, or the concentration, is changed. The objectives of a sample pretreatment step are:

(i) removal of macromolecules; (ii) stabilisation of the analyte(s); (iii) avoidance of irreversible adsorption; (iv) removal of interfering micromolecules, and (v) preconcentration (trace enrichment) of the sample.

However, these objectives can seldom be combined in a single step. Therefore, combining an initial or non-selective step, with a selective pretreatment step is a general strategy in the development of routine procedures.

A number of features of sample pretreatment which should be optimised are:

(i) stability of the analytes(s); (ii) interferences by impurities from vials, reagents, and/or solvents; (iii) compatibility of the solvent(s), used in the last steps, with the applied chromatographic mode; (iv) simplicity; (v) accuracy, reproducibility, repeatibility, and precision.

Non-selective pretreatment regimes, e.g. protein precipitation, dialysis, ultrafiltration, freezing, dilution, and microwave irradiation, can be used for sampling, stabilisation, storage, or pre-concentration of the sample. The selective pretreatment regimes e.g. liquid-liquid extraction (LLE) and solid-phase sorption (SPS) are used for isolation, trace enrichment, and/or cleanup of the samples[51]. In practice the distinction between selective and non-selective pretreatment regimes is not always obvious. For instance, LLE can be used to denaturate proteins and to pre-concentrate a sample as well as isolating the analyte from the matrix.

Before a method is developed, it should be considered whether the non-protein-bound (free) fraction, the protein-bound fraction or the total amount of the analyte has to be determined. Depending on the non-selective pretreatment regime chosen, analyte-protein bonds can be quantitatively, partially, or not broken at all. It should not be assumed that the applied non-selective pretreatment regime is able to destroy the analyte-protein bonds[52]. In addition the analyte stability (enzymatic, hydrolytic) should always be established, i. e. can the samples be stored without any precautions, or stabilisation steps (e.g. pH regulation, enzyme inhibition, addition of antioxidants), or should the sample be analysed within a certain period of time?

In many cases, it is advantageous to combine the pretreatment regime with the chromatographic separation. This can, for example, be acheived by using a

precolumn to retain the analytes, while the proteins are flushed through, and then switched on-line with a chromatographic column[53,54]. These techniques are especially attractive for relatively large numbers of samples.

1. 2. 4. 5. *Factors Affecting Column Performance*

1. 2. 4. 5. 1. Choice of Columns

For general clinical and biochemical HPLC analysis, and particularly for TDM, reversed-phase columns are the ones of choice[55]. Advantages of these columns are:(a) resistance to contamination and water-based mobile phases, (b) ease of equilibration, and (c) broad applicability. The most popular reversed- phase columns are C_{18} and C_8. The differences in chromatographic properties between both these columns are subtle, but at higher flow rates chromatographic efficiency decreases less with C_8 than with C_{18}. More polar bonded phases, such as cyano and phenyl, can also be used in the reverse- phase mode by incorporating adequate water in the mobile phase.

Other important factors influencing separation efficiency are column length and particle size of the packing. In order to obtain maximum efficiency, the column must be evenly packed, the column must be packed as uniformly as possible to minimize distortion of the chromatographic boundaries. If the particle size of the adsorbent is uniform, it is easier to get homogenous packing. On no account should any part of the column be allowed to run dry during packing or during separation. The most widely used columns are those 15 cm in length packed with particles 5 um in diameter. Gaining in popularity is "high speed liquid chromatography", which uses short columns 3 to 8 cm long of internal diameter 4.6 mm and packed with 3 um particles. These are operated at a high flow rate (2 to 5 ml/min), using a small volume flow cell (2.4 ml) and a detector system with a fast response time. Analysis time is significantly shortened and there is less solvent consumption, but since a higher than usual back-pressure is used, wear and tear on equipment can be greater.

Most analytical columns have an internal diameter ranging from 3 to 6 mm. These dimensions provide a good balance between (a) ease of packing, with minor contribution from wall effects[56,57] (b) compatibility of the sample and peak volumes on these columns with detectors in routine use, (c) limited elution volume of costly mobile phase, (d) limited amount of costly packing and (e) moderate pressure drops. As increased loading of the column (i.e. a higher amount of injected sample) results in a decrease in the column efficiency, a shift in the retention values and also, in some cases, in a change of the sample peak shape.

The stability of a column can be greatly improved by the use of a guard column before the analytical column providing protection from contamination and clogging. Modern columns can withstand more mechanical stress, but more work is required to investigate the possibility of regenerating columns contaminated by impure substances, such as the usefulness of a strong solvent injection between samples[58]. A major factor affecting the stability of columns is the pH of the mobile phase. The pH range within which bonded-phase silica-based packings can be used is typically 2 to 7. At lower pH values, the bonded groups are hydrolysed, and at higher pH, the silica matrix itself dissolves, leading to the release of bonded groups from the surface. The stability at higher pH may be extended by the use of a silica-packed pre-column between the pump and the sample valve, thus presaturating the mobile phase with silica before it enters the analytical column[59]. Mobile phases with a high pH can be used in normal phase HPLC with silica particles, because the high organic content of the mobile phase minimizes the solubility of the silica.

1. 2. 4. 5. 2. Column to Column Reproducibility

It is important that the same chromatographic conditions for a drug analysis give similar results with each new column. This is not often the case, and modification to certain parameters is often required in order to achieve separation on a new column, particularly for drugs that are susceptible to active sites on the column. It is important to develop mobile phase conditions not only to achieve separation but also to minimize column differences. Probably the most severe test of column to column reproducibility is the relative retention of acidic and basic substances analysed simultaneously. Although many drugs are neutral, avoiding major problems in column to column reproducibility when analysed by HPLC, some of the potential interferences may be acidic or basic, and so their retention may vary from column to column giving rise to interferences in the chromatogram.

1. 2. 4. 5. 3. Choice of Mobile Phase

A major advantage of HPLC over GC is the opportunity to influence the separation of drugs by changing the composition of the mobile phase, giving extra flexibility and control.

Reverse-phase chromatographic systems are the most popular for drug analysis. The most two most commonly used organic solvents in the reversed- phase mode are methanol and acetonitrile, acetonitrile being the less polar of the two. Another solvent which may be considered is tetrahydrofuran, because it offers further solubility properties (less polar, weaker dipole moment than acetonitrile, non

hydroxylic relative to methanol). The temperature of the mobile phase can also affect chromatographic behaviour. The primary effect of increasing the temperature is to increase the solubility of drugs in the mobile phase solvent, thus diminishing their chromatographic retention. A higher temperature also improves column efficiency, largely because the mobile phase is less viscous. A disadvantage of higher temperature is decreased column stability.

1. 3. ELECTROCHEMICAL TECHNIQUES IN DRUG ANALYSIS

In 1922, Heyrovsky[60] reported his results relating to electrochemical reactions at a dropping mercury electrode (DME). His discovery of the technique that he named "Polarography" was so notable that the discoverer was subsequently awarded a Nobel prize in Chemistry in 1959. Unfortunately, electrochemical techniques did not immediately become popular on a world-wide scale but, for many years, remained within the institution of the discoverer and those of nearby countries of Central Europe. Some years after World War II, there was a renewed interest in electrochemical techniques, with the concomitant appearance of more sophisticated commercial apparatus. The past twenty years have therefore seen a tremendous upsurge in the number and innovativeness of reports in all areas of polarographic and voltammetric procedures.

In 1983, the first International Symposium on Drug Analysis was held in Brussels. At that time, several reports were presented on the application of electrochemical techniques to the analysis of drugs and pharmaceuticals. Patriarche et al.[61] expounded on the use of some new, modified electrodes in the analysis of pharmacologically active substances. Bersier[62] dealt with the ways in which polarography and voltammetry could be used in industrial pharmaceutical laboratories. He pointed out that frequently these techniques were overlooked when, in actual fact, they can offer rapid and sensitive means for the measurement of drugs in pure solutions, dosage forms, bulk materials and biological fluids. At the same conference, based on some of his own work as well as the experience of others, Chatten[63] presented a report on applications of various electrochemical techniques to the analysis of pharmaceuticals.

1. 3. 1. *Voltammetry at Stationary Planar Electrodes*

Voltammetric techniques of analysis, i.e. those based on the measurement of current resulting from an oxidation or reduction at an electrode surface following the application of a potential difference to an electrochemical cell [64], have assumed an important place in the armoury of analytical techniques for the identification and

determination of trace concentrations (i.e. 10^2 to 10^{-4} ppm) of many organic, organometallic, and inorganic molecules of biological and environmental significance. Techniques such as differential pulse voltammetry (DPV), anodic and cathodic stripping voltammetry (ASV; CSV), adsorptive stripping voltammetry (AdSV) and electrochemical detection (ED) coupled with flow injection analysis (FIA-ED), ion chromatography (IC-ED) and high performance liquid chromatography (HPLC-ED) are particularly notable in this respect, and are used in the determination of compounds such as pollutants, drugs of abuse, food additives and contaminants, and agrochemicals.

Differential pulse voltammetry (DPV) at stationary electrodes has been increasingly used for electrochemical analysis of substances which are oxidised at such a positive potential that they can not be detected using a mercury electrode.

In 1962, starting from phenolic resins, Yamada and Sato [65] prepared a gas-impermeable carbon material which they called "glassy carbon". This glassy carbon has interesting physical properties in comparison with other carbon materials such as, for instance, impregnated carbon. An important feature of their method of formation was that artefacts of various shapes could be produced. Furthermore, glassy carbon exhibits a much lower oxidation rate at elevated temperatures, suggesting a greater inertness to chemical attack than other types of carbon materials, such as various graphites. This property, together with its very small pore-size, makes glassy carbon an attractive material for the preparation of inert electrodes. Glassy carbon electrodes were applied for the first time in electroanalytical chemistry by Zittel and Miller [66]. They showed that its usable potential range extends to more positive values compared to those obtained for platinum. The good resistance of glassy carbon against chemical attack makes it possible to apply the electrode in very corrosive media, such as concentrated hydrofluoric acid. The existence of reactive groups on its surface was confirmed by the work of Laser and Ariel [67], who studied the behaviour of glassy carbon following anodic polarisation and subsequent reduction in acidic media. From current-voltage and reflectance-voltage curves they concluded that the overall process of anodic polarisation was the result of three processes: formation of a redox couple caused by chemical adsorption of oxygen, irreversible redox reactions of existing surface groups, and, at sufficiently positive potentials, the evolution of oxygen.

Possible surface groups found on oxidation are, for instance, carbonyl groups, that subsequently can be reduced to hydroxyl groups at more negative electrode

potentials. Also, the possibility of the formation of a quinone/hydroquinone couple, as found on oxidized/reduced pyrolytic graphite [68], cannot be excluded. It is evident that glassy carbon electrodes need some kind of pretreatment to obtain reproducible results.

It is, however, virtually impossible to summarise all the pretreatment procedures described in the literature, because there seem to be many different procedures as there are investigators who have used glassy carbon electrodes (GCEs). However, some general aspects are considered later in the thesis (2. 1. 2).

1. 3. 2. *Cells, electrodes and potentiostats*

Modern voltammetric analyses are usually performed in a vessel containing a three-electrode system of the type shown in Figure 1. 2. This type of cell is commercially available in a range of sizes that can accomodate between 2-50 cm^3 of solution; however, smaller volumes can be investigated using specially constructed microcells[69]. The important considerations in the design are that the electrodes should be as close as possible to one another and there should be a simple means of deareation before analysis.

In polarography, the working electrode is the dropping mercury electrode, which consists of a glass capillary about 15 cm long and about 0.05 mm in internal diameter. The mercury electrode can also take the form of a hanging drop; this may be achieved by a micrometer arrangement where the mercury drop size is produced manually. However, most modern voltammetric instruments incorporate a special electrode assembly, which may be operated by gas pressure or by a solenoid; this allows the operation to select either the dropping mercury or stationary hanging drop mode. As the mercury (Hg) oxidises at +0.2 V, mercury electrode is not suitable for electrooxidises compounds. Oxygen (O_2) is easily reduced and must be completely removed and precautions should be made to prevent oxygen from reentering the system. Even at relatively low working electrode potentials, very large back-ground current are produced by the reduction of oxygen. Other electrode materials used to extend the anodic potential range include the noble metals (gold, silver and platinum), carbon paste, glassy (vitreous) carbon, and pyrolytic graphite.

The three-electrode configuration is standard in the modern potentiostats and galvanostats that are used for control of potentials and current, respectively. The electrodes consist of the (1) working electrode at which the redox process of interest occurs, (2) the auxiliary electrode, which is the second current carrying electrode in the cell, and (3) the reference electrode, which carries no cell current,

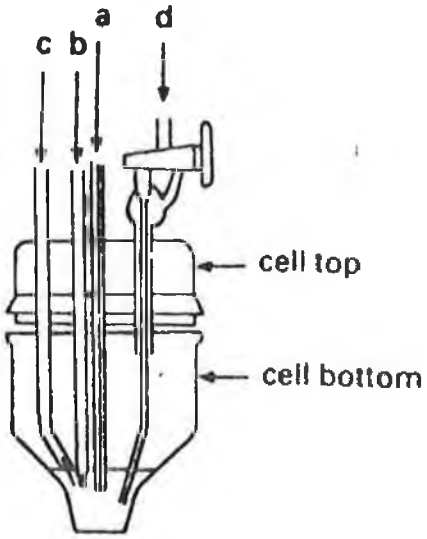

Figure 1. 2. Voltammetric cell containing: (a) working electrode; (b) reference electode; (c) auxiliary electrode; (d) a two way nitrogen line for deareation.Reproduced from Hart, J., Investigative Microtechniques in Medicine and Biology, Bitensky, I and Chayen, J. (Eds), Marcel Dekker Inc., 1981, p. 199.

to accurately monitor the potential applied at the working electrode so that, if necessary, adjustments may be made. The most commonly used reference electrodes are the saturated calomel electrode (SCE) and the silver/silver chloride (Ag/AgCl) electrode. The auxiliary electrode is normally made from platinum wire and serves as a means of applying the input potential to the working electrode.

The potentiostat is a device which can compensate for the voltage drop across a high resistance solution; if this was not done, the actual potential at the working electrode would be different to the applied potential. The potentiostat contains an operational amplifier which is connected to the reference and auxiliary electrodes, as shown in Figure 1. 3; the reference electrode has a constant potential and is placed as close as possible to the working electrode in the cell. The input potential waveform is applied through the auxiliary electrode to the working electrode; therefore, any voltage drop is experienced by both the reference and working electrode. In this case, the operational amplifier will apply sufficient compensating potential to the auxiliary electrode to ensure that the potential at the reference electrode tip (and therefore the working electrode) is the desired one[70].

1. 3. 3. *Solvents and Supporting Electrolytes*

All electrochemical phenomena occur in a medium which generally consists of a solvent containing a supporting electrolyte. There is no universal solvent. Generally, a solvent system is used whose merits outweigh its disadvantages for a particular application. A good solvent system for one type of experiment or compound may be totally unacceptable for other applications. In order to permit the widest latitude in application, the solvent system should not undergo any electrochemical reaction over a range of potentials from very positive (strongly oxidizing) to very negative (strongly reducing) ones.

The potential at which the electrochemical reaction relating to the solvent system commences is known variously as the background limit, decay of supporting electrolyte, or decomposition potential. In order to support passage of an electrical current, the solvent system should have low electrical resistance, and hence a moderately high dielectric constant (>10), since the prevalence of ion pairing, and even multiple ionic association in less polar solvents, can result in low ionic mobility and conductance in such solutions. An electrochemical solvent must be able to dissolve a wide range of substances at acceptable concentrations. This generally means that electrolytes must be soluble at least to the extent of 0.1 M, while the electroactive material must dissolve to form approximately millimolar

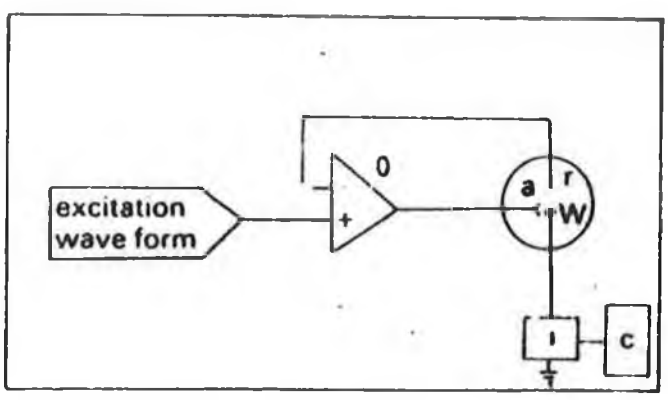

Figure 1. 3. Three electrode operation with potentiostatic control: (w) working electrode (r) reference electrode; (a) auxiliary electrode; (o) operational amplifier; (i) current-to-voltage converter; (c) chart recorder. Reproduced from Flato, J., Anal. Chem., **44**, 1972, 75A.

solutions (for application of the various electroanalytical techniques) or greater (for preparative electrolyses). The solvent should not react with the electroactive material, nor with intermediates or products of the electrode reaction under investigation. The solvent should be reasonably stable, so that purification, preparation, and storage of standard solutions will present no major problems.

1. 3. 4. ***Voltammetric Techniques in Pharmaceutical Analysis***

Over twenty years dedicated to the application and adaptation of voltammetric techniques to given problems, and to the development of quantitative voltammetric procedures for inorganic and organic compounds (as "main" and "secondary" methods), have shown that numerous realistic problems in pharmaceutical industry can be solved by these techniques. "Main" methods refer to procedures preferably used for routine work; "secondary" methods refer to procedures used as an alternative to an established one.

In pharmaceutical analysis, it is becoming increasingly desirable, or even advisable, to have two different methods of analysis for a particular compound. This procedure often represents the only way to ascertain the correctness of analytical data provided by a new analytical method. "Secondary" procedures are also used for checking established analytical procedures, such as chromatographic, spectrophotometric, or microbiological procedures in the field of organic analysis, and X-ray fluorescence and atomic absorption spectrometry in inorganic analysis. Another type of application of voltammetry is in the determination of physicochemical, thermodynamic, and kinetic parameters required for the elucidation of electrode reactions.

In terms of pharmaceutical analysis, voltammetry (i.e. direct current, alternating current, fast scan, and pulse polarography, along with voltammetry at solid electrodes), coulometry (constant current and potential), and potentiometry (including the use of ion selective electrodes), are the most commonly employed techniques. Voltammetric techniques are quite useful for the direct measurement of the stability of some pharmaceuticals in aqueous solution. Depending on the compounds involved, voltammetry can be used to either monitor the decomposition of a drug and/ or the formation of a decomposition product. This type of study is typically performed by applying a potential to the working electrode which is on the voltammetric plateau for the starting material or end product to be monitored. The resulting current vs. time data then provides the desired kinetic information. The

application of voltammetric techniques to pharmaceutical analysis has been reviewed by Bersier and Bersier[71].

1. 4. Development of Liquid Chromatography Electrochemical Detection (LC-ED)

Electrochemical detection in liquid chromatography is not new. In 1940, Troistskii[72] used a dielectric constant sensitive instrument to detect absorption boundaries in chromatography, and in 1952 Kemula et al. [73] attempted to use a DME for LC detection. In this case, the dead volumes were very large (many ml), and the polarograph was "low performance" by today's standards. As a result, only milligram quantities could be detected and chromatograms lasted for hours. Nothing of practical significance came of this early work, although it established the concept of using electrochemistry to follow chromatographic profiles. The development of electrochemical detectors only became really important with the wide application of HPLC techniques in the seventies. In 1974, several important clinical applications of LC-ED were reported[74]. Figure 1. 4 illustrates the components of a typical LC-ED system .

At present, some applications of HPLC can not be imagined without the use of amperometric or coulometric detection. Electrochemistry also offers other methods for detection of substances in flowing liquids, mainly, equilibrium potentiometry, conductometry and high-frequency impedance measurements, but these methods have much more limited application. In fact, most authors use the term "electrochemical" as a synonym for "amperometric" or "coulometric" measurements. Since amperometric detection was employed in this thesis, the following section deals with this mode of operation.

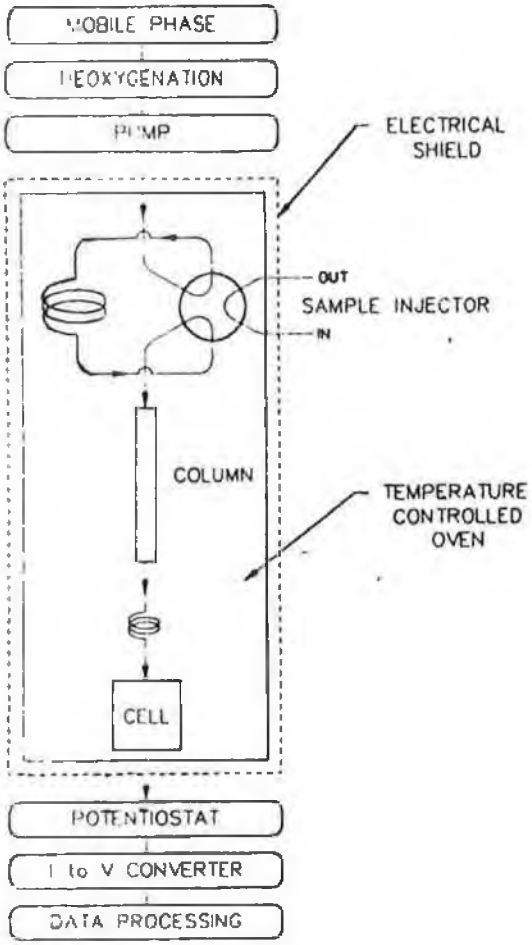

Figure 1. 4. Outline of a LC-ED system. Reproduced from ref. 64

1. 4. 1. *Controlled Potential Amperometry*

Unlike the commonly made potentiomteric pH measurement, electrochemical detection is usually based on amperometry at controlled potential. Electrochemical detection involves a chemical redox reaction, in contrast to ultraviolet or fluorescence detection where a passive, physical absorption of radiation occurs. The reaction occurs at an electrode surface, placed in or alongside the flow of eluent from the column. Either an oxidation or reduction may be forced to occur by judicious selection of a potential applied to the cell from the potentiostat. The potential is a source of electrochemical selectivity, in the same manner as the wavelength selected in spectrophotometric detection. In essence, the electrode acts as an oxidising or reducing agent of variable power. As with spectroscopic methods, molecular structure is the primary determinant for the electroactivity of an analyte. The accessability of various filled and unfilled molecular orbitals ultimately decides the thermodynamics and kinetics of the electrode process. As for spectroscopy, there is a great body of empirical information that can be drawn upon to predict the behaviour of individual compounds. In examining a candidate drug or metabolite for electrochemical analysis, several key questions must be answered. What functional groups are present? Does the parent structure permit delocalisation of the added positive or negative charge? Are there substituents present in the molecule that enhance or detract from electroactivity? Is the redox reaction pH-dependent? What is the solubility? All of these factors are important considerations in assessing electroactivity for LC-ED or any electrochemical technique. Electrochemical detection in liquid chromatography is in almost every case based on controlled potential amperometry. Coulometric detectors are defined as those where the conversion efficiency is 100%. For amperometric detectors, the efficiency is somewhat less (often 1-10%). Amperometric detectors presently have the advantage of giving better detection limits and being[75] less complex than coulometric detectors. But how can the limit of detection be better if less material is converted? This is often true in practice because of the geometry required to achieve complete conversion in a flowing stream. As more electrode surface area is added downstream to improve efficiency, each increment of surface area contributes proportionately less to the total amount of material converted but approximately equally to solvent breakdown.

Perhaps more important is the fact that the advantage of the precision coulometry in titrimetry is not often realized in practice for chromatography detection where

sample preparation and injection are normally the primary sources of error and relative measurements (internal or external standards) predominate.

The active region of most amperometric detectors consists either of a wall-jet electrode or a thin-layer cell. If one considers Faraday's law:

$$Q = n\,FN \qquad (2)$$

for each chromatographic zone the number of coulombs (Q) passed will related to the number of moles (N) converted to product in the cell and the number of electrons *(n)* involved in the reaction. A schematic diagram of a thin-layer electrochemical detector cell is shown in Figure 1. 5. For clarity, only the immediate vicinity around the working electrode is shown. Eluent from the column passes into the thin-layer zone as a parabolic profile (represented by the arrows). A potential difference is then applied to the cell between the working electrode and the reference electrode (which is not shown) and external to the thin layer. This potential difference is adjusted to provide a sufficient driving force for the redox reaction to occur. Suppose an oxidisable species R is eluting from the column into the detector and that the potential is sufficient to drive the conversion of R to its oxidation product O, yielding a release of *n* electrons to the electrode surface for every molecule converted, according to:

$$R \longrightarrow O + ne^- \qquad (3)$$

as the chromatographic band passes through the thin layer, those R molecules which are immediately adjacent to the electrode surface become oxidised due to the electric field imposed by the potential at the electrode solution interface Figure 1. 6. It is crucial to recognise that electrochemical detection is a surface technique; those molecules more distant to the surface may or may not be oxidised at all. At typical flow rates of 1-2 ml/min, the residence time of any molecule over the electrode is only a few tens of milliseconds, a much shorter time than necessary for most to diffuse laterally to the surface. In actual practice, therefore, only 3-5% conversion efficiency is achieved. Frequently the amount reacts is less than 5 **fEq**. Near the detection limit of a molecule with a molecular weight of 200 undergoing a two-electron transfer, about 0.5 pg of sample is converted into product.

The current measured at the electrode surface in DC hydrodynamic amperometry is the summation of several factors: (1) the background (faradaic) current, due to the redox reactions of impurities, solvents, etc. contained in the mobile phase; the background current is relatively small over a wide range of potentials (-0.8 to +1.1V

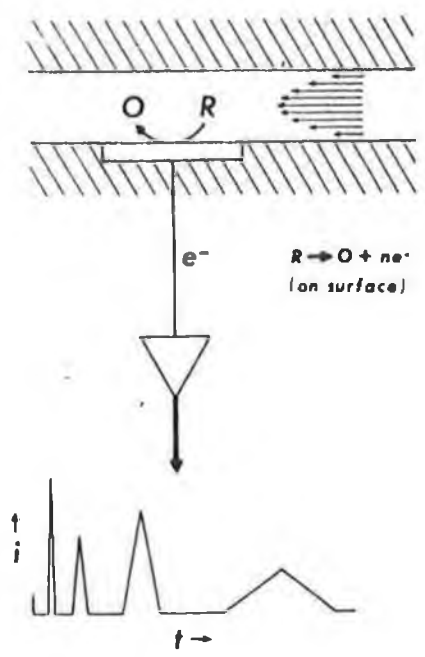

Figure 1. 5. Schematic diagram of thin-layer electrochemical transducer. Laminar flow developed in the channel (typically 50 um thick) passes over a planar electrode held at some dc potential with respect to a known reference electrode. Direct electrolysis current is measured. Reproduced from Shoup, R., Liquid Chromatography/ Electrochemistry in High Performance Liquid Chromatography, Horvath, C. (Ed), Academic Press, Inc., New York, 1986, p. 94.

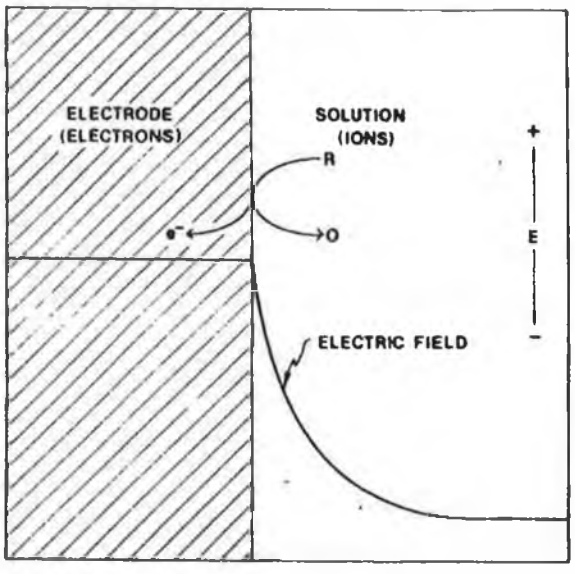

Figure 1. 6. Electric field imposed by the applied potential at the interface between the electrode surface and the bulk solution. The field is localized over the electrical double layer, which is only 10 - 20 A thick.Reproduced from Shoup, R., Liquid Chromatography/Electrochemistry in High-Performance Liquid Chromatography, Horvath, C. (Ed), Academic Press, Inc., New York, 1986, p. 95.

versus Ag/AgCl), before abruptly rising exponentially outside this "window"; (2) the signal (faradaic) current, arising from the oxidation of the analyte(s) of interest; and (3) the charging (capacitance) current, which occurs whenever the cell is turned on or the potential is changed. The applied potential is very dependent on the restrictions imparted by the LC working conditions. Since reversed-phase LC conditions (aqueous, pH 2 to 8) are most widely used, a typical example is an applied potential between + 1.2 and + 1.3 V (using glassy carbon electrode material vs a Ag/AgCl reference electrode). The applied potential, however, is highly dependent on the redox behaviour of the compound to be detected and varies due to experimental conditions. The oxidising strength (increasingly positive potential for stronger oxidising capability) or reducing strength of the electrode surface (increasingly negative potential for stronger reducing capability) is determined by the applied potential. As the potential of the working electrode (see Figure 1. 7) relative to the reference becomes more positive, the surface becomes a better oxidant (electron sink).

Likewise, as the working electrode becomes more negative, the surface becomes a better reductant. The potential is applied between the reference and working electrodes, and the current is passed between the auxiliary and working electrodes. Positioning the auxiliary electrode opposite to the working electrode (i.e. across the flow stream) reduces the uncompensated resistance to a negligible value even when low ionic strength (<10 mM) mobile phases are used. With a low uncompensated resistance, the interfacial electrode potential is not significantly influenced by sample concentration, and a wide linear dynamic range is obtained [76].

An LC-ED detector should have an active volume (that volume of the cell where the detection is taking place) which is small relative to the volume occupied by the concentration zone passing from the column. The detector should respond rapidly in order to accurately represent the shape of the concentration profile. LC bands with volume well below 100 nl are becoming quite common. Often the peak width is measured in seconds. Obviously, not all LC-ED detector need to be used with such highly efficient columns. Nevertheless, the future clearly points to the need for development of ED flow cells with very small dead volumes.

1. 4. 2. Hydrodynamic and Cyclic Voltammetry

The hydrodynamic voltammogram is used to help choose an optimum operating potential for detection. Hydrodynamic voltammograms (HDV) are generally developed by making repeated injections of standard solutions and increasing the

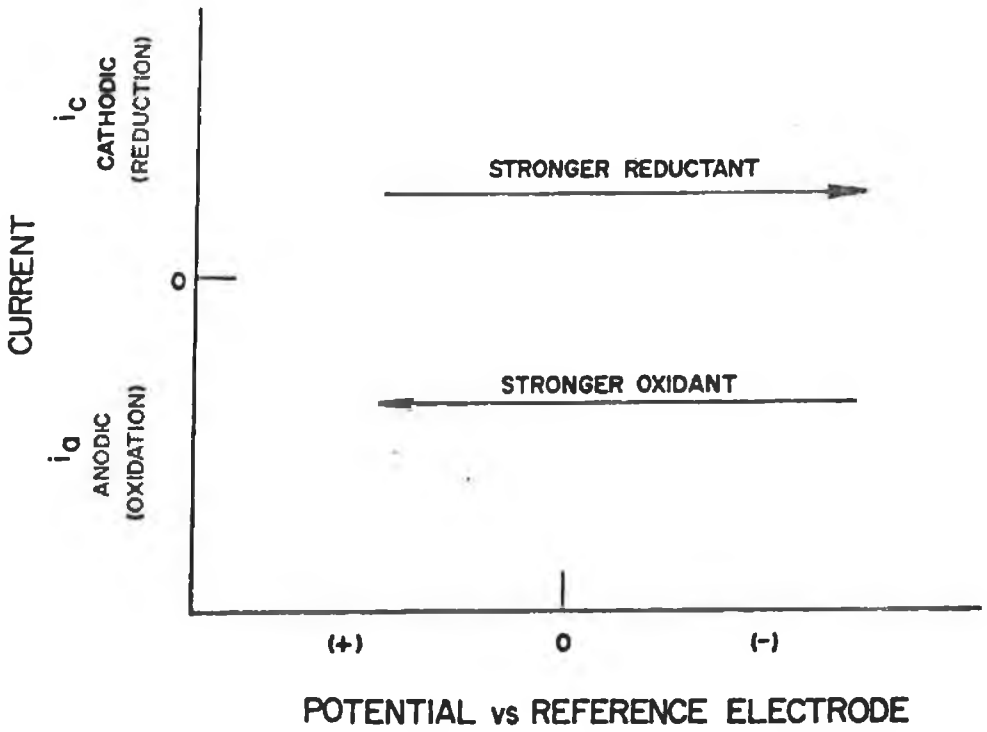

Figure 1. 7. Action of applied potential as the driving force in an electrochemical reaction. Reproduced from Radzik, D. and Lunte, S., Critical Reviews Anal. Chem., **20** (1989) 317.

potential of the detector between these injections. A typical HDV is shown in Figure 1. 8. Initially, at negative or very low positive potentials, the energy applied to the cell is insufficient to cause any reaction to occur. As the applied potential increases in the positive direction, the energy requirement for the reaction is now partially met, and a faradaic current arises. As the potential is further increased, the faradaic current rises until a potential is reached, past which no further improvement in the current response is noted. The curve now attains a plateau. All points in this zone yield essentially the same current response. The hydrodynamic voltammogram just constructed may be broken into these regions:

Region I: Zero current region. The potential is insufficient to drive the redox reaction to the right; no current results.

Region II: Potential-controlled region. For a reversible system, the response is dictated by the Nernst equation.

Region III: Diffusion-controlled region. Further increases in the potential are irrelevant, since the material is oxidised at the surface as fast as diffusion rates permit. The current is constant since the constant flow of mobile phase through the cell provides a steady-state flux of new electroactive material at the surface.

HDVs may be used in tandem with a stationary CV experiment to determine the optimum detector operating potential, in the same way as the UV absorbance spectrum is used to help choose an optimum wavelength for detection. In addition to retention times, these current-potential curves can be compared. In this way, identities of peaks can be confirmed based on the chromatographic and electrochemical properties of a compound. Figure 1. 9. illustrates the individual hydrodynamic voltammograms for several hypothetical molecules which can be oxidised (A, B, C) and one which can be reduced (E).

The selectivity of electrochemical detection is inversely related to the experimentally observed oxidation (or reduction) potentials. Measured half-wave potentials are often significantly greater than the corresponding thermodynamic E values (particularly for organic molecules). Although it is often desirable to operate an electrochemical detector on the limiting current plateau E_2 for compound A, in some cases it is advantageous to operate on the rising part of curve (E_1 for compound A).

The lower the potential chosen, the better the selectivity. If, for example, A and B are not well resolved chromatographically, it may be difficult to detect A at E_2 in

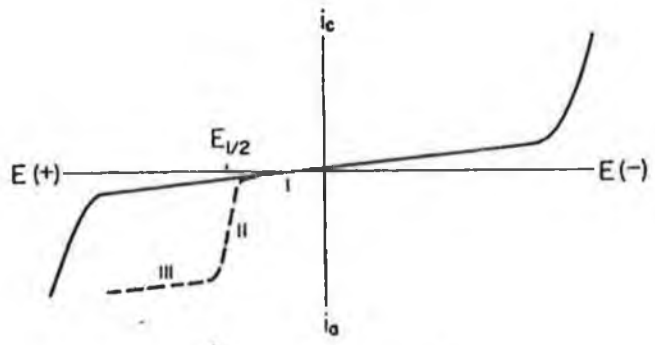

Figure 1. 8. Hydrodynamic voltammogram for background current (solid line) and injected solute (dashed line). Per electrochemical convention, positive potential, positive potential versus the reference electrode is to the left and negative potential to the right. Current response for reduction is in the $^{+}$y direction; current response for oxidation is in the $^{-}$y direction. Reproduced from Shoup, R., Liquid Chromatography/ Electrochemistry in High-Performance Liquid Chromatography, Horvath, C. (Ed), Academic Press, Inc., New York, p. 96.

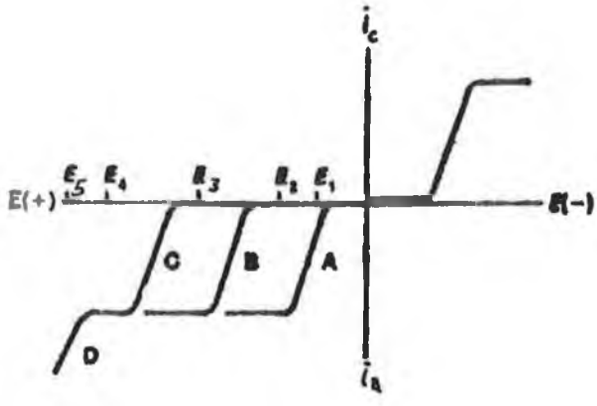

Figure 1. 9. E(+): positive potentials vs. reference electrode; E(-): negative potentials vs. reference electrode; i_c: cathodic (reduction) current; i_a: anodic (oxidation) current. Reproduced from Kissinger, P., Anal. Chem., **49** (1977) 445A.

the presence of a thousand-fold excess of B. Lowering the potential to E_1 will decrease the sensitivity to A severalfold, but sensitivity to B may decrease by many orders of magnitude. This principle has been used to detect[77] trace amounts of p-aminophenol in the presence of the analgesic drug N-acetyl-p-aminophenol (acetaminophen). At E_3, A and B will be detected with comparable sensitivity, while at E_4 it would also be possible to detect C. Clearly, the selectivity for C will not be good, and a greater reliance has to be placed on the chromatographic separation. The sensitivity for C can also be expected to be somewhat diminished when compared to more easily oxidised substances due to the greater background current (greater "baseline" in the chromatogram). At E_5 and beyond it is likely that the electrochemical detector will not be useful for trace analysis as the background oxidation (D) becomes very high. Hydrodynamic voltammograms can also be the result of bad flow-cell design, due to hydrodynamic or electrical factors[78]. Cyclic voltammetry (CV) rapidly provides useful preliminary information needed for the study of an electroactive compound[79]. This stationary solution experiment easily duplicates those conditions (electrode material, electrolyte, etc.) found in the LC-ED detector cell. CV may also used to evaluate the mechanism of redox reactions and subsequent chemical reactions[80]. Figure 1. 10 illustrates a cyclic voltammogram for todralazine, a benzodiazine drug of the phthalazine group frequently used in pharmaceutical preparations as an antihypertensive agent. The cyclic voltammograms show one oxidation peak (A) and one reduction peak (B). The cyclic voltammetric peaks which appear at distinctly separate potentials, Ep_a = 0.58 V and Ep_b = -0.17 V, are characteristics of an irreversible process[81].

1. 4. 3. *Current and Signal/Noise Ratio and Optimisation in LC-ED*

All determinations are accomplished with some degree of imprecision. The understanding of the causes of imprecision, or noise, is an important part of the optimisation of an analytical system. To be able to use any theoretical expression for signal intensity in the calculation of detection limits or optimum conditions, some knowledge of the noise in the system is needed. The noise is that part of the quantity measured that does not contain information. In an ideal detector, the quantity measured would be zero in the absence of the test substance; in a real detector, the quantity measured consists of the signal (i.e. the response to the test substance) and of several other components that make up the background (baseline). The background involves a constant component originating for example from reactions of the flowing liquid and the impurities in it, or from bias currents of the signal amplifiers, noise with a relatively high frequency (up to hundreds of Hz); which is mainly caused by the line frequency noise; low-frequency noise (with a

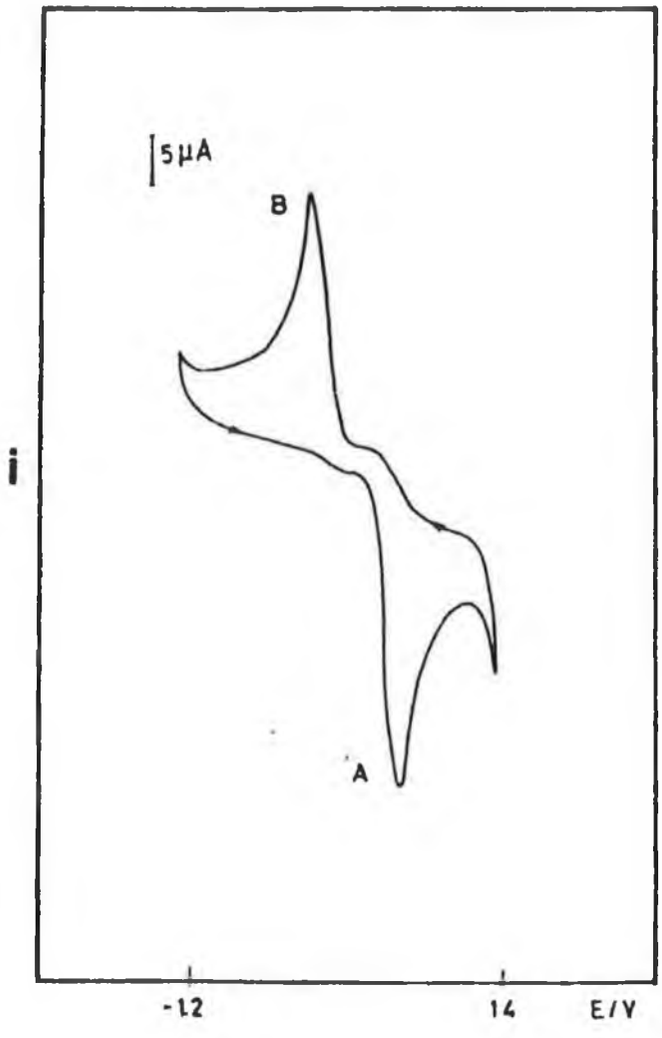

Figure 1. 10. Cyclic voltammogram of todralazine (5 x 10^{-4}M, pH 6.0, citric acid-phosphate buffer). The scan rate was 200 mvs^{-1}. Reproduced from Barrio, R., et al., Electroanalysis, **3** (1991) 429.

frequency similar to the variations in the signal, which is usually due to unsuitable design of the detector, and a systematic shift in one direction, called the drift. In additional, occasional spikes appear, which are rapid random pulses of the quantity measured, usually caused by fluctuations in the line voltage, bubbles in the liquid, and other disturbances. A drifting baseline does not affect the quality of the measurement much, but complicates the operation of measurement, as frequent zero adjustments must be made. The constant component can be compensated for and the high-frequency noise with spikes can be suppressed by suitable electronic filtering (RC filter). To calculate detection limits, quantitative information about the noise is necessary. To determine optimum conditions, qualitative information will suffice, i.e. knowledge of the major source (or sources) of noise. The electrode's size, shape, and material undoubtedly influence the signal and the noise obtained[82]. Noise in highly sensitive electroanalytical systems is not nearly as well characterized as noise in optical systems, so that some hypothesizing must be done. While it is relatively straightforward to model the faradaic current response as a function of cell parameters, the ultimate issue is the signal/noise (S/N) ratio, which determines detection limits. The relationship of cell design to these variables has not been defined rigorously; estimates have been made based on the assumption that noise is proportional to electrode area. Weber and Purdy[83] found that a properly optimised (i.e. maximum S/N ratio) cell would have the following characteristics: (1) the width of the electrode should equal the width of the flow channel; (2) the length of the electrode along the direction of flow should be as small as possible, and (3) the flow cell should be as thin as practible.

Initially, it would seem worthwhile to increase the electrode surface area and thereby increase the conversion efficiency of the detector. However, conversion efficiency per se is rather irrelevant[84], since S/N ratios determine detection limits, not conversion efficiencies. Figure 1. 11 graphically summarises this fact. As the electrode area is increased incrementally, more solute R will be oxidised and the overall conversion effeciency will increase. By the same rationale, the conversion efficiencies for mobile phase components which contribute to the detector's steady-state background current will also increase. Since the supply of mobile phase components is essentially unlimited, each corresponding area increment adds an equal contribution to the background current. In other words, this current is directly proportional to the electrode area. For the solute **R,** however, the supply of molecules to be reacted is not unlimited. Each area segment adds a smaller contribution to the peak current, since diffusion must supply new material to the surface from points more distant than before. Therefore, the net peak current does

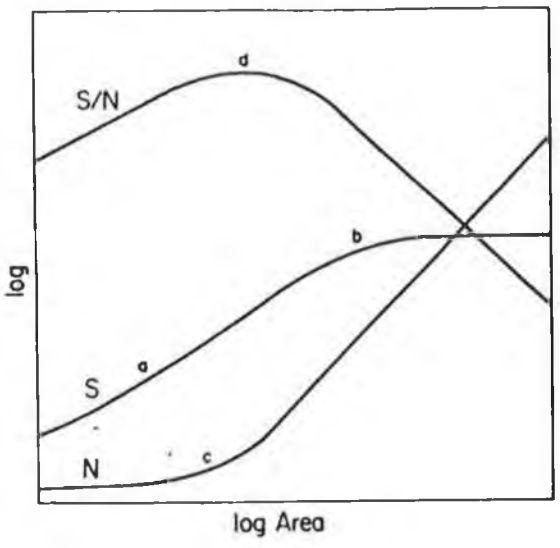

Figure 1. 11. Relationship of signal, noise, and signal/noise ratio versus electrode area.Signal (S) is dependent on two-thirds power from a to b and zero power thereafter, depending on conversion effeciency. Noise (N) is dependent of area below c due to electronic noise and increases with area thereafter. Maximum signal/noise ratio is somewhere in the thin-layer region (a to b) at point d.Reproduced from Morgan and Weber, Anal. Chem. **56** (1984) 2560.

not increase linearly with area (Figure 1. 11). Since noise is roughly proportional to the background current, the S/N ratio is actually maximal at an electrode of vanishingly small area. Of course, limitations outside the cell also play a part in these arguments. For example, secondary noise, such as electronic noise of the potentiostat/current amplifier, may become important with very small electrodes[85]. It is a natural tendency to think of residual current as the unwanted element in an analysis. In fact, if the residual current for a given system is large, but more reproducible and noise-free than another system with a low residual current, then the detection limit will necessarily be lower in the former case[86]. Other factors, however, play a decisive role in assessing conditions for maximum S/N ratios. Variations in temperature and flow rate may prove to be important[87]. As dual-electrode experiments have demonstrated, much of the long-term drift and short-term noise is not due to the vagaries of the working electrode *per se*, but rather to microscopic flow variations and irregular mobile-phase impurity levels[88]. Using appropriate pumps/pulse dampeners can avoid "flow noise". However, this may not be sufficient. For ordinary HPLC work the pump fluctuations can easily be filtered out electronically using a high-order filter[89].

1. 4. 4. *Detector Cell Design*

Over the last 5 years, increased activity has been directed to detector cell design. Well over 100 publications have illustrated changes to existing commercial transducers or developed entirely new concepts of design. Electrochemical detection is now sufficiently commonplace alongside ultraviolet absorption and fluorescence methods which have become accepted in LC. From a manufacturer or even inventor's point of view, transducers for sensitive LC-ED are not easily developed. In addition, the implementation of a working prototype into a reproducible, serviceable component is often fraught with difficulties. The design constraints may be summarized as follows:

(i). incoporation of a minimum of one working electrode, one reference electrode, and one auxiliary electrode within a cell volume sufficiently small to prevent chromatographic bandspreading;

(ii). the electrodes must be electrically isolated from each other and from earth ground;

(iii). the mobile phase must not contact external connections to the working electrode, else these in turn become part of the active working electrode;

(iv). expansion for multiple working electrodes measuring simultaneously, either in series or parallel orientations with respect to the flow;

(v). the active surface of the sensor must be sufficiently mechanically strong to withstand liquid flow;

(vi). interchangeability of electrode materials for specialised applications;

(vii). ultimate compatibility with microbore LC formats which are ideal for electrochemical methods.

These overlapping requirements can be troublesome to satisfy simultaneously. A majority of the developed prototypes satisfy requirements (i) to (iii) above, but major omissions of flexibility occur thereafter. The followings are the most common used cell designs:

1. 4. 4. 1. *Thin-layer Amperometric Flowcells*

The majority of applications have been worked out using this detector[90,91] and it was the first commercially available design. The cell design and operation have been discussed in page 28.

1. 4. 4. 2. *Wall-jet Transducers*

An alternative geometry to the "thin layer" design is to use a "wall jet," as developed by Fleet and Little which subsequently modified by other workers. A schematic representation is shown in Figure. 1. 12, which shows the direction of flow perpendicular to the working electrode surface. The mobile phase escapes radially, past the outside reference and working electrodes. Patthy et al.[93] compared thin-layer and wall-jet transducer designs under actual analytical conditions for the determination of rat brain catecholamines and concluded that the thin-layer design was more sensitive.

1. 4. 5. ***Scope and Limitation of Electrochemical Detection***

The suitability of electrochemical detection to a given problem ultimately depends on the voltammetric characteristics of the molecule(s) of interest in a suitable mobile phase and at a suitable electrode surface. All detectors limit mobile phase composition to some degree; however, in electrochemical detection one must be conscious of the fact that a complex surface reaction is involved which depends on the medium. Therefore, some effort is required to simultaneously optimize both the column and detector performance. Fortunately, it is possible to make a few

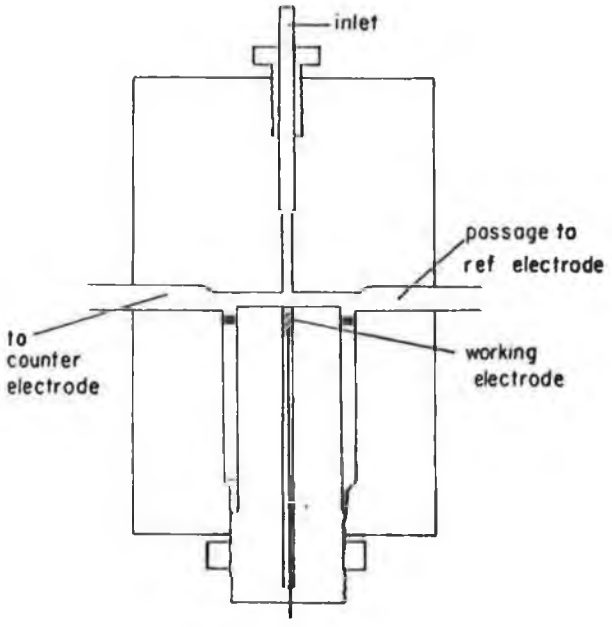

Figure 1. 12. Wall-jet design proposed by Fleet and Little (Ref. 92). Fluid impinges perpendicular to the working electrode surface.

generalisations. For all practical purposes, direct electrochemical detection is not likely to be useful in normal phase adsorption separations since non-polar organic solvents are not well suited to many electrochemical reactions. The HPLC stationary phases of choice clearly include all ion-exchange and reversed-phase materials since these are compatible with polar solvents containing some dissolved ions. The limitations of amperometric and coulometric detection can be overcome in some cases. Pre- and post-column derivatization are now being utilized to make some compounds better candidates for LC-ED. Chemical, enzymatic, and photolytic derivatizations in conjunction with both oxidative and reductive detection schemes are being used to determine species with normally unfavourable redox properties.

1. 4. 5. 1. *Mobile Phase Restrictions*

Electrochemical detection is different to ultraviolet absorption or, fluorescence measurements, in that it relies on a chemical reaction. As with any chemical reaction, conditions such as solvents, pH, and temperature play an essential role in successful LC-ED experiments. The requirements for the mobile are as follows: (1) it must be electrochemically inert (i.e. low background currents in the range of interest), and (2) it must be capable of supporting a dissociated electrolyte, usually 0.01 to 0.1 M in ionic strength, to minimize resistance and convey charge. These limitations are not generally restrictive, since most reversed-phase, ion-exchange, and some polar bonded-phase separations employ similar conditions. The most successful LC-ED determinations have typically utilised aqueous buffers (pH 2-7) with varying amounts of solvent (0-90% by volume). Ion-pairing agents have also been used very successfuly. When designing a mobile phase for LC-ED it is important to remember that the electrolyte must not only dissolve, but also dissociate, in order to guarantee conductivity. For a buffer salt such as KH_2PO_4, the essential processes are

$$KH_2PO_4 \longrightarrow [K^+ \, {}^-H_2PO_4^-]_S \longrightarrow K^+_{(S)} + H_2PO_4^-{}_{(S)} \qquad (4)$$

where (S) signifies the solvated species. The user should always ascertain the approximate ionic strength of the mobile phase using appropriate factors such as pK_a of the buffer salts, desired pH, buffer salt solubility products, etc. At least 5-10 mM ionic strength, preferably buffered, is desirable. For the above reasons, non-polar solvents such as hexane and chloroform are not compatible with LC-ED. Although a few papers have reported success with normal phase LC-ED[94 - 96] the detection limits do not approach those using reversed-phase separation, and in most

1. 4. 5. 2. *Matching Electrode Properties to the Analytical Problem*

The choice of electrode material is a powerful variable in optimising selectivity in LC-ED. The choice of electrode material is more critical in LC-ED than for the usual electroanalytical experiment, primarily due to the ruggedness and long-term stability required. Mechanically awkward devices such as the dropping mercury electrode will try the patience of the most stalwart chromatographer, although these have been used in several reports. Electrodes subject to complicated surface renewal problems (platinum, glassy carbon, and mercury films) may work well in some cases and are disastrous in others. In spite of these difficulties, it is possible to routinely detect picomole amounts (or less in some cases) of electroactive components separated by HPLC.

Electrochemical reactivity can be altered considerably by changing the electrode material. In many cases this can be highly advantageous. For example, although mercury has been the most popular electrode material in the past due to its applications in polarography, solid electrodes such as glassy carbon and platinum have largely replaced mercury as the electrode material of choice, since the most important applications of LC-ED have been developed in regions of potential were the residual currents on mercury would be prohibitive. However, the hydrogen overvoltage on Hg/Au electrodes is more negative, an advantage in properly deoxygenated systems. Besides a material's applicable potential range, the extent of surface conditioning necessary before stabilisation must be considered. The background current derives from three sources: mobile-phase impurities, electrode charging currents, and surface redox reactions.

1. 4. 5. 2. 1. Carbon Paste

Although the carbon paste electrode can be inherently sensitive, the main disadvantage of this material is that it is not very compatible with non-aqueous solvents. Solutions containing up to 25% (v/v) methanol or 5% (v/v) acetonitrile are tolerable, but these will decrease usable lifetimes. The semi-solid nature of carbon paste prevents its use in wall-jet flowcell configurations, a serious drawback to that design, since carbon paste has better S/N ratio than glassy carbon for several important applications.

1. 4. 5. 2. 2. Mercury drops, Pools, and Films

Mercury provides excellent hydrogen over-potential, to about - 1.1 V versus Ag/AgCl for trace analysis work. For this reason it has proved popular for moderate and easily reducible species such as azo, nitro, nitroso, organometallic, and imine functional groups. Several cell configurations have been reported for mercury

cases, the user would have been a better to employ simple ultraviolet detection. An electrolyte can be dissolved in the mobile phase normally without causing any deterious effects on the chromatographic separation. The use of non-aqueous reversed-phase chromatography[97] is of particular interest for the exploitation of electrochemical detection since the solvents used in such separations often allow greater potential to be employed, thereby extending the range of application of the technique. Table 1. 1 lists the accessible potentials for some of the solvents commonly used in reversed-phase chromatography. The ionic strength, pH, electrochemical reactivity of the solvent and electrolyte, and the presence of electroactive impurities (dissolved oxygen, halides, trace elements) are all important considerations. Using cyclic voltammetric data, existing HPLC information and the tables below, a chromatographic system can often be developed which meets the requirements of both separation and detection.

Table 1. 1. Accessible potential range for some solvents

Solvent	Cathodic limit V vs SCE	Anodic limit V vs SCE	Reference
Water	-2.7	1.5	98
Methanol	-2.2	1.8	99
Acetonitrile	-3.5	2.4	100
Dimethylformamide	-3.5	1.5	101
Tetrahydrofuran	-3.6	1.8	102
Methylene dichloride	-1.7	1.8	103
Acetic acid	-1.7	2.0	104

The above figures can only be taken as a guide since the actual potential limit is dependent on solvent purity, electrode material and electrolyte.

electrodes[105]. The early and some present designs incorporating the DME suffer from drop noise and baseline oscillation, high cell volumes, and cappilary clogging. Thin-film electrodes, such as mercury amalgamated onto gold or platinum, have been more extensively studied[106]. While usually much less noisy, these surfaces have limited lifetimes due to intermetallic formation at the electrode surface.

1. 4. 5. 2. 3. Platinum/Gold

Electrocatalytic groups on Pt and Au, and precise electrochemical recycling of those groups, from the basis for detection of sugars, amino acids, and alcohols otherwise transparent to amperometry. The advantages and disadvantages of the different kind of electrodes used for LC-ED are given in Table 1. 2.

1. 4. 6. *Selectivity in LC-ED*

Selectivity is one of the principal requirements placed on any analytical procedure. In order to obtain meaningful information from the measurement of a particular analytical property, the signal must be unambiguously attributable to the substance to be determined; that is, the analyte and other components of the sample must not influence it. Such a measurement can then be considered specific for the given substance. Of course, this is the ideal objective for real analytical procedures, for they are only selective to varying degrees for any particular analyte.In general, such interferences arise to at least some degree from other sample components interferents. There is no general way of quantitatively describing the degree of selectivity (that is, extent of interferences) for an analytical procedure, as the effects of the interferents are often interrelated and depend strongly on the nature of the analyte matrix and the experimental conditions. The simplest and most common method specifies the concentrations (activities, absolute amounts) of individual interferents that produce a preset change in the magnitude of the measured signal under particular set of experimental conditions and a given significance level. Like any other analytical procedure, a flow determination always involves a separation of the analyte(s) from other sample components followed by measurement of a suitable analytical property. The separation may be literal, e.g. in a column or reactor, or figurative as through the suppression of interferences (through masking of interferents, selective measurement at a particular wavelength or potential, etc.). Hence, any flow system can be considered as a kind of separation column followed by a more selective detector. The overall degree of selectivity attained depends on the relative strengths of physico-chemical interactions of the sample components ocurring in the 'column', on the number of elementary equilibria or steady states attained along the 'column', and on the selectivity of the column. The most common approaches for attaining the required selectivity can be summarised as follows:

Table 1. 2. Advantages and disadvantages of electrode materials used in LC-ED.

Electrode Material	Advantages	Disadvantages	Ref
Mercury electrode	used for electroreduction work	As the mercury (Hg) oxidises at + 0.2 V, it is not suitable for elect-reoxidisable compounds	107
		the current oscillating over the lifetime of the necessitates some form of damping	
		oxygen (O_2) is easily reduced and must be completely removed	
		the dropping process causes high noise level.	
Solid electrodes	Used for electrooxidzable and also for easily reduced compounds	Fouling of the electrode surface	108
Carbon paste	Low residual current and noise.	Different formulations of carbon paste exhibit variations in sensitivity	109
	Easy to prepare and replace	Solubility of the binder in organic solvents restrict usage to mobile phases that contain typically less than 20% of organic modifier	110

(i) masking of interferents, by employing either thermodynamic or kinetic principles of a suitable physico-chemical process (usually complex formation);
(ii) separation, either in a high performance system, or in a cell or reactor preceding the detection unit;
(iii) use of selective detection methods, including selective sensors, rapid-scan techniques and multiple sensors;
(iv) treatment of the detector signal, leading to deconvolution of overlapping signals and to differentiation of the signal from the background;
(v) combination of various detection methods

While the present-day LC column technology is at a when point one may quickly achieve excellent separation of closely related substances, this is often not sufficient. This is especially true when a determination involves a biological matrix, such as blood or urine. Often selectivity must be enhanced by a clean-up step prior to injecting material onto the column. This typically consists of one or more liquid-liquid extraction or liquid-solid extractions carried out in a batch mode.

The selectivity of amperometric detection has been shown to be useful in the determination of L-dopa in the presence of the decarboxylase inhibitor carbidopa. Levodopa and carbidodopa are used in combination to treat Parkinson's disease. Carbidopa is a dopa decarboxylase inhibitor which can significantly reduce the dosages of levodopa required for therapy. The selectivity of the column was also commonly augmented by the detector. This is a real strength of ED schemes. While a "universal detector" could be useful in solving some simple problems, a "selective detector" is much more applicable in difficult determinations. The ED detector is a tunable device that permits enhancement of selectitivity by changing the applied potential.

1. 4. 7. *Applications of LC-ED*

The types of compounds that can be determined by LC-ED are compounds that undergo electron transfer reactions. Characteristics of a small oxidation (or reduction) potential and fast kinetics are most desirable for sensitive and selective detection. The classes of organic compounds with these characteristics are phenols (catechols and catecholamines), thiols, nitro compounds, and quinones. Compounds such as aldehydes and ketones require too high a reduction potential, while alkylamines and carboxylic acid functionalities require too high an oxidation potential, for direct determination by LC-ED. There are other classes of compounds that are electroactive, as well as some clinically important compounds, such as ascorbic acid and uric acid, and organo-metallics (such as *cis*- platinum).

LC-ED is also uniquely applicable to some heterocyclic compounds of pharmacological interest (e.g. phenothiazines, imipramine). Many biochemicals, pharmaceuticals, food additives, pesticide residues, industrial antioxidants, plant phenolics, and so forth are ideal candidates for LC-ED method development. Simple aromatic amines and their derivatives (hydroxylamines, amides, quinoneimines) represent another class of important molecules of natural and commercial interest. Most of these are not well suited to GC and can often be handled with ease by LC-ED.

1. 4. 7. 1. *Applications in Bulk and Formulated Drug Analysis*

A great number of analytes of interest in all aspects of the pharmaceutical industry have been determined using LC-ED. The initial development of a pharmaceutical agent usually involves the development and characterisation of the bulk drug. Although this is not an area in which LC-ED is typically applied, recent applications have begun to demonstrate its utility, especially in the determination of the purity of reference standards. This issue is becoming more important as most current LC methods normally rely on single or perhaps dual UV wavelength detection to assign bulk drug purity. Auxiliary methods must be utilised to confirm the absence or the presence of non-UV-absorbing materials, which may elute during the normal course of a chromatographic method. LC followed by controlled potential coulometry has been utilized to determine the purity of electroactive drugs, such as acetaminophen[111]. The utility of the ED detector to screen for electroactive impurities has also been demonstrated[112]. When investigating the

purity of a bulk drug that is not electroactive, the presence of electroactive impurities can provide important information about the causes of contamination or the route of degradation. The combination of UV and ED detection is becoming routine in many cases like this. Often the UV detection is used to quantitate major components, while ED selectivity detects the low-level impurities.

An important new area of pharmaceutical research is the development of biotechnology products. The lack of previous background information or therapeutic applications have led to stringent restrictions on trace levels of impurities in these bulk drug substances. Prior to their use for therapeutic applications, recombinant DNA products must be evaluated for trace levels of nucleic acids derived from recombinant bacterial chromosomes and plasmids. Previous techniques for such low-level determinations have been time consuming, laborious, and prone to experimental error. Kafile et al. have devloped a highly sensitive and precise method for quantitation of DNA/RNA fragments[113,114]. The method is based on hydrolysis of nucleic acids followed by LC-ED.

Many smaller anionic species such as CN^-, HS^-, Br^-, I^-, $S_2O_3^{2-}$, and SCN^- may be determined in bulk drugs using LC-ED. Simultaneous determination of cyanide, sulfide, iodide and bromide by ion chromatography followed by electrochemical detection at a silver electrode has been demonstrated[115,116]. Such rapid and sensitive determinations are especially applicable to bulk drug and formulated drug preparations, where meta-bisulfite is sometimes added as an antioxidant, and the other species are considered contaminants. In an evaluation of UV absorption vs. amperometric detection for LC, Musch et al.[117,118] reported minimum detection concentrations (ED vs. absorbance detection) for 94 different drugs. Classes of pharmacological compounds included local anaesthetics, antipyretics, sulfonamides, sex hormones, beta-adrenoceptor blocking agents, phenothiazines, alkaloids, diuretics, and penicillins.

1. 4. 7. 2. *Applications in Biochemical Research*

LC-ED is well known as an extremely powerful analytical tool and for its direct application to biomedical analysis. Amperometric detection is a significant technique in biomedical research, largely because of aromatic hydroxylation in small molecule metabolism. Most thiols have to be derivatised to be detected by HPLC with UV absorption detection[119]. Unlike UV detection methods, LC-ED can be used to detect thiols in biological samples directly. The most popular electrochemical detector for thiols is one which was introduced by Rabenstein and

Saetre[120]. This detector has the advantage that mercury oxidizes at a lower potential in the presence of thiols as follows:

$$2RSH + Hg \longrightarrow Hg(SR)_2 + 2e^- + 2H^+ \qquad (5)$$

In the original papers, a mercury pool set at a potential of + 0.1 V was used to detect thiols as they eluted from the column. At this potential only thiols and other compounds that form complexes with mercury are detected. Saetre and Rabenstein used this detector for the determination of cysteine, homocysteine, glutathione, and penicillamine in a variety of biological samples, including serum, urine, and plasma[121 - 123]. Using the mercury pool electrode, less than 10 pmol of glutathione could be detected. This reaction can also take place at a gold electrode that has been amalgamated with mercury. Perret and Rudge reported the detection of D-penicillamine, thiomalate, and captopril using a gold electrode at + 0.8 V vs. Ag/AgCl[124]. Other endogenous analytes have been determined using LC-ED techniques, including prostaglandins[125]. The important enzyme cofactor, NADH, is readily oxidised at carbon electrodes[126] and provides a unique opportunity for enzyme immunoassays coupled to LC-ED. The pterins are of particular interest because of their possible role in several diseases[127]; abnormal levels of various pterins have been observed in several diseases such as phenylketonuria, rheumatoid arthritis, Parkinson's disease and kidney dysfunction. The electrochemical behaviour has been exploited for the sensitive measurement of a variety of pterins, and their different oxidation states, in biological samples. In one of the first reports by Kissinger's group[128] on LC-ED methods for pterin analysis, an amperometric detector equipped with a single glassy carbon electrode, operated in the reductive mode, was used. The high sensitivity of the method may be seen from the detection limits shown in Table 1. 3.

1. 4. 7. 3. *Therapeutic Drug Monitoring and Determination of Drugs in Biological Matrices*

The routine use of LC-ED for the selective detection of pharmacologically active substances in biological samples has been particullarly highlighted in its use for the determination of biogenic amines. This includes blood, plasma, urine, organic tissues, and subcellular fractions (e.g. microsomes). The advantages of sensitivity and specificity have led to further applications, such as the study of metabolism of biologically active compounds. Lavrich and Kissinger[129] have reviewed LC-ED as it relates specifically to therapeutic drug monitoring. In most cases, if a compound

is electroactive, it will be detected selectively and generally with better detection limits than those found for UV spectrophotometric detection.

Table 1. 3. Detection limits for selected pterins.

Pterin	LOD/pmol*
Biopterin	0.19
6-Hydroxymethylpterin	0.45
erythro -Neopterin	0.15
theo - Neopterin	0.14
Pterin	0.37
Pterin-6-aldehyde	0.20
Pterin-6-carboxylic acid	0.33
Xanthopterin	0.28
Tetrahydrobiopterin	0.75

*Limit of detection at a signal-to-noise ratio of 3.

Homogentisic acid (H6A; 2,5-dihydroxyphenylacetic acid) is an important compound in the primary catabolic pathway for tyrosine in man. In the LC-ED determination of HGA in serum it is clearly advantageous to operate the detector at a low potential where many possible interferences will not react (Figure 1. 13). This degree of selectivity is rarely possible with a UV spectrophotometric detector since the electronic spectra of aromatic compounds are broad and usually less sensitive to variation in substituents than oxidation potential. Since, in essence, the same detection principle has been applied for most pharmacologically active substances (buffered mobile phase followed by detection at a working electrode), the following are some examples of applications for some specific classes of compounds:

1. 4. 7. 3. 1. Analgesics

As a group, analgesics have been the drugs most widely studied by LC-ED techniques. The most widely studied of this group have been acetaminophen[130], salicylates[131], codeine[132], and morphine[133]. LC-ED has been investigated in

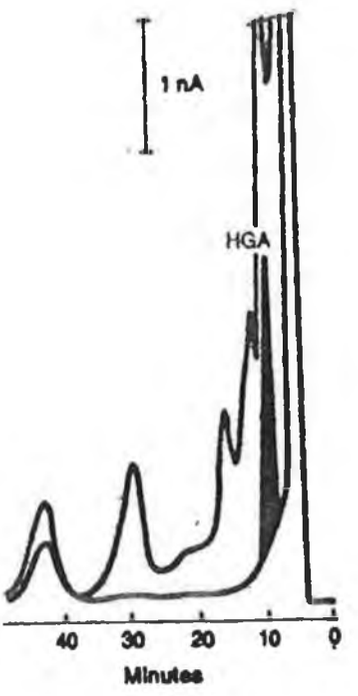

Figure 1. 13. Assay of homgentisic acid in serum at 100 $ngml^{-1}$. Two samples of extract (7ng HGA) were injected using different electrode potentials (upper trace: E= +0.75 V, lower trace: E= +0.45 V). Mobile phase: 0.5 M pH 4 acetate buffer at 0.2 ml min^{-1}.Reproduced from Kissinger, P., Anal. Chem. **49** (1977) 445A.

more detail recently as a method for opiate alkaloids of abuse, including heroin, cocaine, and related compounds[134 - 136]. Detection limits were reported to be in the nanogram range for morphine, heroin and cocaine. Ciramadol and dezocine are synthetic opiod analgesics of the agonist-antagonist type and have a phenolic group that allows selective detection in plasma at the 10 μg/ml level[137]. In a simillar manner, the narcotic analgesic ketobemidine (1-[4-(3-hyroxyphenyl)-1-methyl-4-piperidyl]-1-propane) is another new narcotic analgesic and phenolic that is amenable to EC detection at the 1 ng/ml level in plasma[138].

1. 4. 7. 3. 2. Antibiotics

While many antibiotics are not electrochemically active, several elegant applications have been developed, including the derivatization of β-lactams[139]. Chloramphenicol is an antibiotic that can be assayed conveniently by LC-ED at negative potentials at mercury film electrodes, like other compounds containing an aromatic nitro group. Since there are very few reducible compounds found naturally in blood, this determination is highly selective[140]. Abou-Khalil et al.[141] reported the determination of chloramphenicol and four other analgesics using both oxidation and reduction modes at a glassy carbon electrode, as well as by UV detection (Figure 1. 14). Both trimethoprim and sulfonamides can be detected utilizing LC-ED methodology at a greater sensitivity than is possible with UV detection, although the applied potential is high, generally about + 1.1 to + 1.2 V. Tetracyclines have several moieties that make them amenable to electrochemical detection (typically at about + 0.60 V)[142]. Other antibiotics determined have included erythromycin and related macrolide antibiotics[143,144], enviroxime[145] and amoxicillin.

1. 4. 7. 3. 3. Chemotherapeutic Agents

Many of the diverse chemical agents used to treat various cancers have favourable redox properties for very selective LC-ED methods. Cis-platinum complexes have been widely studied by LC-ED with various degrees of success. These species have been detected following LC at dropping-mercury and hanging-mercury drop electrodes (using differential pulse amperometry), thin-layer Au/Hg electrodes, glassy carbon, and by a halide-catalyzed oxidation platinum electrode[146, 147]. Although detection limits tend to be very dependent upon the legends associated with the Pt (thus altering oxidation states and molcular geometry) and are also higher (typically in the micromolar range) than those usually expected with EC detection, most of the species lack significant UV activity, and LC-ED provides an

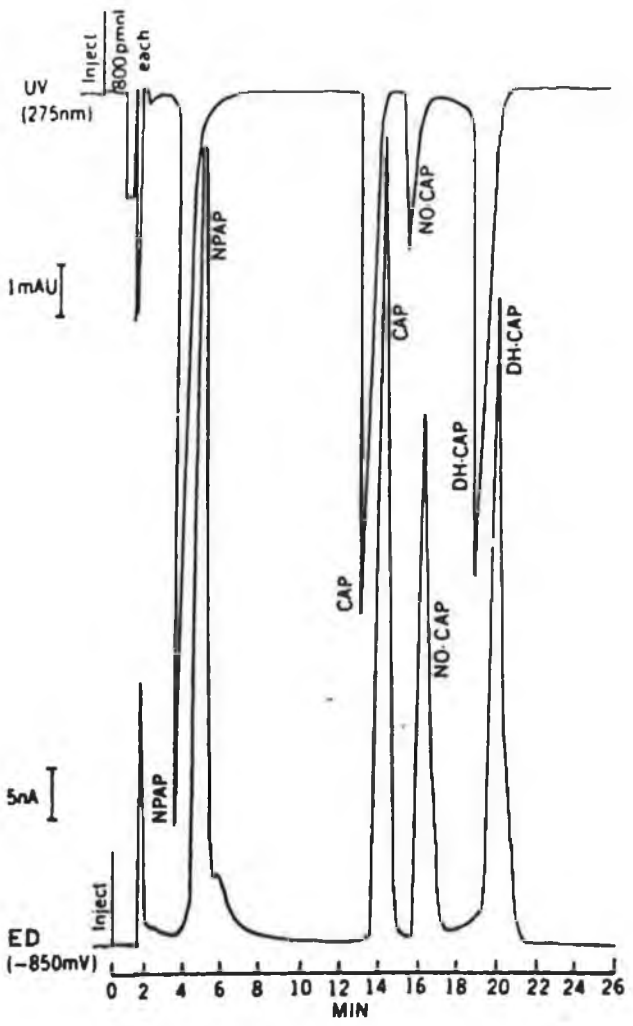

Figure 1. 14.HPLC separation and dual detection of a saturated mixture of (NPAP) Nitrophenylaminopropanedione: (CAP) Chloramphenicol; (NO-CAP) Nitrosochloramphenicol; (DH-CAP) Dehydrochloramphenicol. Reproduced from Abou-Khalil, S., et al. J. Chromatogr., **417** (1987) 11.

excellent method for their determination. Many anti-cancer drugs are based on a quinone or hydroquinone structure. This makes them very amenable to electrochemical detection. Table 1. 4 gives some of the quinone anticancer drugs and their metabolites that have been detected using LC-ED.

The anthracycline antibiotics, adriamycin and daunomycin, can be detected either by oxidative or reductive LC-ED due to the presence of both a quinone and hydroquinone moiety. Akpofure et al.[148] have detected daunomycin and adriamycin and their metabolites in serum and plasma using oxidative LC-ED at a potential of + 0.65 V. The detection limits using electrochemical detection were comparable with those of fluorescence detection (2 ng on-column). Riley et al.[149] compared the use of coulometric and amperometric detection for doxorubicin. They found that amperometric detection was more selective than coulometric detection.

1. 4. 7. 3 . 4. Vitamins

There is a growing need for sensitive, selective and reliable methods for the analysis of vitamins in areas as nutrition and pharmaceutical chemistry, as well as in clinical chemistry and research[156]. However, these determinations may present difficult analytical problems for a variety of reasons; for example , in blood, interferences between structurally similar vitamers can occur; also, interference from the presence of other naturally ocurring compounds may exist. In addition, the low endogenous levels present put considerable constraints on both the sensitivity and selectivity of potential analytical procedures[157].

The electroreduction of vitamin A has been exploited for the determination of the vitamin in an oil-based pharmaceutical product containing a ten-fold excess of vitamin D_2[158]. In a recent study, Hart and Jordan[159] investigated the possibility of using amperometric detection to monitor retinol in pharmaceutical compounds. Vitamin D has been found to undergo oxidation reactions at carbon electrodes. Atuma[160] utilised this reaction and employed a glassy carbon electrode to monitor the vitamin D present in admixture with vitamin A. A number of vitamins have been determined using LC-ED. Vitamin E has been determined in plasma using LC-ED, the detection limit for being approximately 0.6 ng at a S/N of 10 at an applied potential of + 0.70 V.

Table 1. 4. Electrochemical Characteristics of Anticancer Drugs

Compound	Electrode	Potential	Ref.
Doxorubicin (adriamycin)	GC	+0.80	154
Daunorubicin (daunomycin)	GC	+0.80	150
Teniposide (VM26)	GC	+0.75	151
Etoposide (VP16)	GC	+0.75	151
4'-Epidoxorubicin	GC	+0.80	152
Mitomycin C	Hg	-0.60	153
Daunorubinol	GC	+0.65	148
1-Napthol (napthoquinone)	GC	-0.40	154
Mitoxantrone (novantrone)	GC	+0.75	155

1. 4. 7. 3. 5. Tricyclic Antidepressants

The tricyclic antidepressants used in the treatment of endogenous depression have been widely prescribed in recent years. Since there is a strong correlation between the plasma levels of these compounds and their therapeutic efficacy, there is a great demand for routine laboratory procedures to assay plasma samples for these drugs and their metabolites. Laboratories performing therapeutic drug monitoring require techniques which are sensitive, selective, and simple. Present methodologies include GC-MS, LC-UV, and LC-ED. With GC-MS, assays are often long, requiring the synthesis of derivatives. LC-UV methodogies lack sensitivity; other methodologies cannot achieve the required detection limits and also have a problem with interfering endogenous compounds. LC-ED (Figure 1. 15) offers the advantage over GC procedures of measuring the compounds directly. Both the parent drug and the metabolites can be measured in a single chromatogram with LC-ED (Figure 1. 15). This is a critical because some of these drugs produce active metabolites which should be monitored. The standard protocol for assay of these compounds utilises reversed-phase liquid chromatography with electrochemical detection at an applied potential of + 1.05 V.

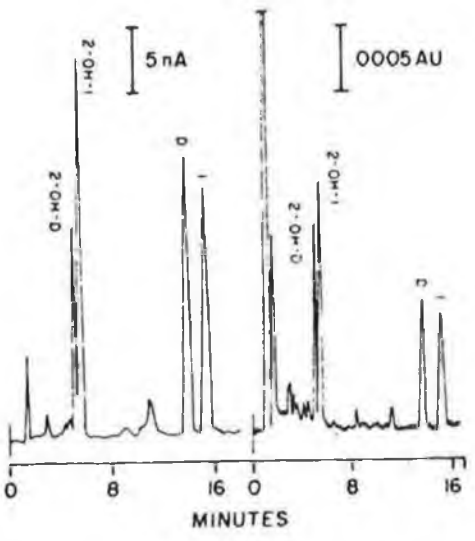

Figure 1. 15. Tricyclic antidepressants in plasma. Comparison of spiked chromatograms obtained simultaneously by EC and UV detection. Legend:2-OH-D = 2-hydroxydesipramine, 32 ng injected; 2-OH-1 = 2-hydroxyimipramine, 38 ng injected. As can be seen, EC detection has less baseline noise and a better signal response than UV detection. The potential is =0.05 V. Reproduced from ref. 133.

1. 4. 7. 3. 6. Biogenic Amines

The influence of a drug on a disease state or the metabolic functions of an organism is an important aspect in the determination of drug efficacy. Currently, developments in chromatographic technology have enhanced the manner in which biogenic amines are determined using EC detection. Lin et al.[161] reported the separation of a series of 18 biogenic amines in 4 to 7 min following extraction from brain tissue. Detection limits for some biogenic amines have been reported[162] in the range of 1 to 15 fmol. Future use of LC-ED in the neurochemistry area will be linked to development of improved sampling technology. By uniting microdialysis with LC-ED, the number of analytes that may be determined far exceeds that attainable using other in vivo techniques such as biosensors. Microdialysis is a new methodology that allows for the monitoring of chemical events in the extracellular space where chemical transmission takes place[163].

1. 4. 7. 4. ***Methods Involving Chemically Modified Electrodes and Mediated Reactions in Pharmaceutical Analysis***

Analytical, biological, and clinical chemists have for decades concerned themselves with the development of instruments and techniques capable of determining the identity and concentration of chemical substances that affect living things. Biosensors can be used to detect biologically important substances in vivo or in vitro. Tailoring electrode surfaces to exact the desired electroanalytical response is now being intensely investigated in electrochemical circles. All the important analytical properties of electrodes; namely sensitivity, selectivity, reproducibility and even applicability, have been shown to be capable of enhancement by the judicious use of chemical modification. Selective electrochemical sensors can be classified into:

(i) ISEs and related potentiometric sensors;
(ii) voltammetric electrodes whose selectivity can be regulated within certain limits by adjustment of the experimental conditions;
(iii) physically or chemically modified electrodes; and
(iv) sensors whose selectivity depends on biochemical processes, such as enzyme catalysis or immunological interactions.

For LC-ED, modified electrodes must respond rapidly to the analyte of interest on the time scale of the experiment and remain stable over a period of weeks for reproducibility's sake. Physical adsorption of the mediator to the surface provides

only limited stability, but the system is easily renewed; covalent attachment has not fared significantly better. Over the past few years there has been interest in the application of amperometric sensors for the determination of purine derivatives, which act as drug substances; such methods have generally been developed to enhance selectivity while maintaining simple experimental set-ups and procedures.

1. 4. 7. 5. ***Scope in Environmental Analysis and Monitoring***

The biosphere contains a myriad of substances which can influence, inhibit, aggravate or simulate various aspects of the health and behaviour of whole living organisms or systems isolated from them. The endeavours of technologically advanced societies are increasingly reliant on the estimation, monitoring and control of chemical species. This requirement for analytical information applies to all sectors of activity, including health care and veterinary medicine, the food, pharmaceutical, bioprocessing and petrochemical industries, environmental monitoring and control, defence and agriculture.

Environmental pollution is an age-old trademark of man, and in recent years as technology has progressed, populations have increased and standards of living have improved, so the demands on the environment have increased, with all the attendant problems for the earth's ecosystems. Combustion of fossil fuels, disposal of waste materials and products, treatment of crops with pesticides and herbicides, have all contributed to the problem. Technological developments have enabled man to study these problems and realise that even trace quantities of polluants can have detrimental effects on health and on the stability of the environment. There is a vast amount of literature on the use of ED for studying a wide variety of these problems[164 - 166].

Chlorinated phenols and naphthals, alkyl phenols used as antioxidants, and hydroxy derivatives of biphenyls are present in the wastes from the manufacture of formaldehyde resins, lacquers and binders, pharmaceuticals and pesticides etc., and constitute a major part of environmental pollutants; they can be found in waste waters from cocking plants and brown coal distillation plants, in industrial effluents, and in the soil as degradation products of various pesticides.

Aromatic amines enter the environment either directly, as industerial wastes, or indirectly as degradation products of pesticides and through bacterial conversion of azo compounds into their precursors, amines. Many of these substances are highly toxic and/or mutagenic and occur in the atmosphere, in biological materials and in wastes. Because of the polar character of amines, their determination by gas

chromatography requires derivative formation to be performed in order to prevent irreversible sorption of the analytes on the column packing and consequent peak tailing. A number of workers[167 - 169] have demonstrated good results which can be obtained by HPLC with amperometric detection.

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Chapter 2

Differential Pulse Analysis of Sumatriptan Succinate (1:1) and Salbutamol in Tablet Dosage Forms

2. 1. INTRODUCTION

Whereas potentiometric and coulometric titration methods are fairly universal and can be applied to a wide variety of problems, voltammetric methods are distinctly limited to easily reducible or oxidisable compounds. On the other hand, voltammetric methods are clearly the most useful for low-level quantitation and have a wide linear dynamic range. Solid electrode voltammetry, which is typically employed for anodic (oxidation) processes, can be carried outusing a rotating platinum electrode, a wax-impregnated graphite electrode, a carbon paste electrode, or a glassy carbon electrode.

Electroanalytical methods of measurement have a property that is usually considered as a serious drawback; namely, the sensor is in direct contact with the test medium and the measured signal is produced by a heterogeneous redox reaction occuring at the sensor surface. Consequently, it is very difficult to maintain the sensor surface in a reproducible, electroactive state because of contineuous interactions between the sensor and the test medium, for example, through adsorption, ion exchange, or redox processes. However, these interactions may also be very useful, as demonstrated by the broad field of chemically modified electrodes or techniques such as electrochemical stripping analysis.

2. 1. 1. *Solid Electrode Surfaces*

In most voltammetric measurements it is desirable that the electrode surface be as smooth as possible, for two reasons: the faradaic currents are proportional to the geometric surface area, whereas the background current, consisting mainly of the charging current, is proportional to the microscopic surface area; hence the signal-to-noise ratio deteriorates with increasing roughness of the electrode. Furthermore, the hydrodynamic pattern becomes poorly identified at rough surfaces and local turbulance often develops; the behaviour of the electrode then deviates from theoretical predictions and the precision becomes worse. Well-polished solid electrodes exhibit roughness factor values of a few units, while coarse surfaces, may attain values close to 1000.

Glassy carbon has a very low porosity but, depending on the quality of the glassy carbon, microscopic pitting can be observed at the polished surface. Presumably, these cavities are due to excessively fast heating during the carbonisation process so that the gases formed has too little time to diffuse to the surface. The more careful the heating procedure, the fewer the cavities and the better the electrode surface

will function.

An understanding of the deactivating processes at solid electrodes requires an insight into the surface reactions involved in the electrochemical conversion of the analyte as well as into other processes and reactions occuring at the electrode surface. However, little is known about general surface chemistry, and the field of electrochemistry at solid electrodes is no exception.

2. 1. 2. *Pretreatment of Solid Electrodes*

In physicochemical measurements, the test systems are made as simple as possible. Unfortunately, analytical systems are inevitably widely varied and often very complex and poorly defined. Therefore, the electrode pretreatment and surface modification procedures are empirical to a considerable extent, and specific conditions must usually be found for particular analytical tasks.

Techniques whose main purpose is to bring the electrode surface to a defined, active state are usually called the "electrode pretreatment." However, even these procedures are often carried out not only to attain a sufficiently sensitive and reproducible voltammetric signal, but also to improve the measuring selectivity through a suitable modification of the electrode surface. Many species dissolved in solution exhibit a tendency to adsorb on the electrode surface, a phenomenon that can markedly affect the results of electrochemical experiments. Adsorption is responsible for much "unusual" electrochemical behaviour and is frequently blamed for unexplained results. The efficiency of electrochemical methods for electrode activation depends mainly on; (i) electrode material, (ii) solution composition (pH, complexing agent), (iii) potential of the cleaning pulse, (iv) convection of the solution in the close vicinity of the electrode.

A wide variety of electrochemical pretreatment procedures have been recommended in the literature. Most of them have been developed through trial and error. This is understandable, since it has been shown that the optimum pretreatment is dependent on the electroactive species and on the type of supporting electrolyte used[1]. Besides, the reason for the increased activity of the electrodes after pretreatment is still not understood very well. The effects of these pretreatment procedures are not all beneficial. The background current becomes larger, as does the noise level. So far, application in trace analysis the pretreatment procedure must be chosen carefully. This has prompted us to investigate several electrochemical pretreatment procedures. Among the applicable methods of surface activation for laboratory measurement are mechanical and electrochemical methods.

2. 1. 2. 1. *Mechanical Grinding and Polishing*

The first step in pretreatment of a solid electrode is mostly mechanical polishing to a mirrorlike finish. Certain solid electrode types—for example, wires, fibres, or classical carbon pastes—are difficult or impossible to polish, leading often to poorly defined geometry and uncertain roughness. Disk, ring and ring-disk electrodes are undoubtedly the most suitable geometric systems from this point of view[2]. Common metallographic procedures are employed in electrode polishing: emery wheels, papers, clothes, and polishing suspensions of alumina and diamond are used, with gradually decreasing grain size, down to values of around 50 nm.

Mechanical cleaning by polishing with abrasive material ranks among the oldest methods and have been used by many authors up to the present. It can be easily done manually, while an automatic mechanical cleaning is quite a complicated procedure. The reason is that the electrode and the cleaning tool must not be in constant contact but move independently against each other to prevent the deposition of the abraded electrode material on the tool. The accumulated abraded material adhering to the tool is in electrical contact with the electrode and increases its irreproducibility. The electrode is then degreased by washing with a suitable organic solvent and thoroughly washed with water. During use the electrodes must be repolished at certain intervals, depending on the electrode material, test solution composition, and measuring technique used; brief repolishing with a damp synthetic fabric is often sufficient. The efficiency and success of mechanical polishing with abrading paste or very fine emery paper depends on the skill of the operator: abrading must not either change the microstructure of the electrode surface nor its wetting angle by, for example, touch of fingers. The controversial results in the literature support the fact that commercially available electrodes are sometimes damaged after a certain time of use.

The results of mechanical polishing can usually results in: an even, smooth surface, which, however, contains a certain amount of loose microscopic particles of the electrode material and of the abrasive[3 - 10]. This is, of course, more pronounced with softer carbon materials. A thin layer of carbon macroparticles produced by polishing causes large variations in the double-layer capacity[11]. The loose particles may act as adsorption and catalytic sites. In fact, a catalytically active electrode has been prepared by deliberate mechanical pressing of α-alumina particles into the polished surface of a glassy carbon electrode[12]. Most contaminants are mechanically removed from the surface during polishing, but others may be introduced. Furthermore, polishing uncovers a fresh, highly reactive surface of the

electrode material which interacts with the ambient atmosphere; this interaction is affected by the heat of friction. It has been shown that polishing of glassy carbon in air causes an increase in the amount of chemisorbed oxygen[12], whereas polishing in an inert atmosphere decreases this amount[13,14]. Thus it can be seen that even the simplest procedure of mechanical polishing leads not only to mechanical changes, but also to chemical modification of the electrode surface. However, electrode polishing gives a surface that is in an unstable condition. This is manifested by often ill-defined and non-reproducible voltammograms obtained at freshly polished electrodes. Besides, electron-transfer rates are, in that case, relatively small[15] and thus a further activation step is usually employed.

2. 1. 2. 2. *Chemical and Electrochemical Pretreatment*

Chemical, and especially electrochemical procedures are used by far most often to clean solid electrodes and bring them to an active state. In fact, virtually any electrochemical measurement with a solid electrode is preceded by polarization of the electrode under suitable conditions in a suitable solution. This approach is attractive because it is very simple, can be carried out without additional instrumentation and without dismantling the measuring cell, or often directly in the test solution and the pretreatment procedure can readily be incorporated in the measurement program, either at the beginning of the experiment, or periodically during the measurement. It is hoped that two principal goals are achieved during a chemical and/or electrochemical pretreatment of the electrode: The electrode surface is freed of passivating layers and brought to a state of the highest possible sensitivity (and often also an improved selectivity) for particular analyte(s). The electrode surface can attain three possible states: the reduced state in which it may be covered by adsorbed hydrogen; the oxidized state, chararacterised by the presence of various oxygen-containing species; and in between these two states, when the electrode is supposed to be "clean."

Repeated oxidation and reduction of the electrode surface in a suitable solution removes many passivating layers; however, some strongly adsorbed or chemically bound layers can only be removed by repolishing of the electrode or by a suitable heat (irradiation) treatment. Chemical pretreatment of solid electrodes strongly depends on the experimental conditions and sometimes it is useful to combine chemical and electrochemical pretreatment.

2. 1. 3. ***Assay of dosage forms***

Since voltammetric techniques demonstrate a large linear dynamic range (10^{-3} - 10^{-}

^{8} M), any one method can readily be applied to the analysis of pharmaceuticals in bulk and in dosage forms. The appeal of the voltammetric methods of analysis for organic compounds is attributable to their simplicity and rapidity. The functional groups which show excellent voltammetric properties include the nitro, nitroso, quinone, azo, azoxy, activated double bonds[16]. For the most part, the voltammetric activity of a compound can be deduced from an examination of its functional groups. However, to ensure that electrochemical activity of a compound is not missed, the analytical screen should include voltammetric scans of the compound in aqueous supporting electrolytes (it is convenient to use 0.1 M HCl, 0.1 M NaOH, and buffers with pH values of 3, 5, 7, 9, and 11) using either the DME or glassy carbon electrode.

If the compound is insoluble in aqueous media, then organic co-solvents such as alcohols, dioxane, acetonitrile, dimethyl sulfoxide, and dimethyl formamide must be employed. Occasionally, pure non-aqueous solvents of high dielectric constant, such as dimethyl formamide, acetonitrile , dimethyl sulfoxide, pyridine, or glacial acetic acid, containing tetraalkyl salts may be required to attain highly negative potentials (greater than -1.8 V vs. SCE) without supporting electrolyte decomposition. The voltammetric analysis of drugs in pharmaceutical products is by far the most common use of electrochemistry for analytical pharmaceutical problems. A better understanding of the electrode processes of compounds of pharmaceutical interest (active agents, excipients, additives, antioxidants, etc.); and application of oxidation waves are required. The USP (volume XX) quotes 16 drugs and formulations assayed by electrochemical techniques.

As a rule, many of the active constituents of formulations in contrast to excipients can be readily oxidised or reduced[17, 18]. Thus sample preparation usually consists of dissolving out the active ingredient from the particular formulation with a suitable solvent (non-aqeous, if necessary) and performing direct analysis on an aliquot of this solution without prior separation of the active substance from the formulation matrix[19]. The selectivity of the method is usually excellent because the compound can be identified by its voltammetric half-wave potential. Examples of voltammetric determination of organic compounds in pharmaceutical preparations include many classes of drugs: tranqulizers, sedatives, hypnotics, antibiotics, steroids, antihistamines, diuretics, muscle relaxants, anticoagulants, and others. For instance, disulfiram [bis(diethylthiocarbomyl) disulfide] a drug used in the treatment of alcoholism, has been assayed directly by differential pulse polarography in an aliquot of a solution of a ground-tablet dissolved in ethanol-acetate buffer (pH4.5)[20]. A mechanism for the electrode process was proposed

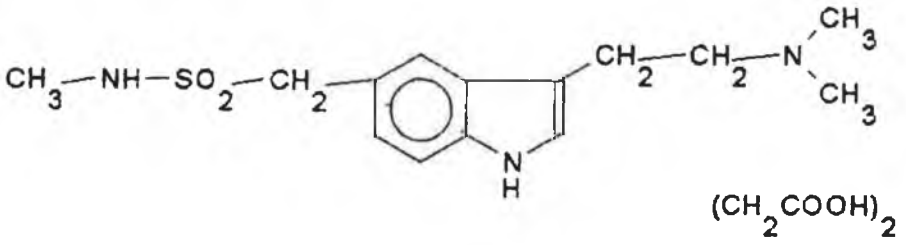
involving the reaction of the disulfiram with the mercury drop to form an insoluble mercuric salt, which then underwent reduction at the electrode surface.

Carbon, in its particular pyrolytic and glassy carbon forms, has found common usage as a working electrode for determination of oxidisable drug substances [21,22]. Its popularity stems from the fact that it has compartively good electrolytic properties, and is, moreover, less suscepetible to surface poisoning (for example, as a result of surface oxidation) than other materials such as platinum and gold. Schwartz and Benjamin[23] carried out differential pulse voltammetric (DPV) determinations of morphine in poppy straw concentrates at a staionary glassy carbon electrode and obtained results similar to those obtained by HPLC[24] and gas chromatography[25].

2. 2. Differential pulse voltammetric determination of Sumatriptan Succinate (1:1) in a tablet dosage form

2. 2. 1. *Introduction*

Sumatriptan succinate(1:1) [I] is a 5-hydroxytryptamine agonist under development for use in human medicine for the treatment of acute vascular headaches[26,27].



I. Structural formula of Sumatriptan succinate (1:1)

Oral administration of the drug is an effective and well tolerated treatment for acute migraine, with a dose of 100 mg appearing optimal in terms of efficacy: side effect ratio[28]. A review of the animal pharmacology, bioavailability, metabolisim and initial clinical studies using intravenous, sub-cutaneous and oral administration of the drug has been made by Oxford and Lant[29]. Existing liquid chromatographic and spectrophotometric procedures for this compound possess one or more disadvantages. These include a time consuming sample preparation, and long chromatographic elution times. Owing to the presence of several potentially oxidisable groups in its molecular structure, it was decided to investigate the voltammetric behaviour of this compound at a glassy carbon electrode, with a view to developing a simple and precise method for its determination in a tablet dosage form.

2. 2. 2. ***Experimental***

2. 2. 2. 1. *Reagents*

Sumatriptan succinate (1:1) tablets (containing 100 mg of active ingredient) were obtained from Glaxo Group Research Limited. A $1x10^{-3}$M solution of sumatriptan succinate (1:1) was prepared in isotonic saline and stored in the dark under refrigeration. The graphite used in the construction of the carbon paste electrode was obtained from Aldrich Chemical Company. Deionised water was used to prepare all solutions. This water was obtained by passing distilled water through a Milli-Q water purification system. All other reagents were of analytical grade. A stock Britton-Robinson(BR) buffer solution was prepared which was 0.04M in each of glacial acetic acid, orthophosphoric acid and boric acid. Buffer solutions of varying pH were then prepared by the addition of 0.2M sodium hydroxide.

2. 2. 2. 2. *Apparatus*

For voltammetric studies, a three-electrode cell was used in which a calomel electrode served as the reference electrode, a platinum wire served as the auxillary electrode, and a glassy carbon electrode was used as the working electrode. For coulometric studies, a carbon paste electrode was used as the working electrode. The potential of the working electrode was controlled using an EG&G Princeton Applied Research (PAR) Model 264A potentiostat connected to an Omnigraph 2000 X-Y recorder . Rotating disc electrode experiments were carried out using a glassy carbon electrode connected to an analytical rotator and a PINE model ASRE 416 speed controller. The pH values of the solution was measured using a Corning Model 240 digital pH meter. The pH electrode was standardised with the use of two buffers (phosphate buffer, pH 7.05 and phthalate buffer, pH 2.15). The accuracy of pH readings was equal to 0.02 unit.

2. 2. 2. 3. *Methods*

2. 2. 2. 3. 1. *Electrode Activation Procedures*

The glassy carbon electrode was first hand polished on silicon carbide paper (No. 240), followed by polishing using an alumina slurry. Residual polishing material was removed from the surface by sonication of the electrode in a methanol bath for 15 minutes, during which the methanol was changed every 5 minutes. As reported previously[30], activation of glassy carbon using a high anodic potential is highly effective. Activation of the glassy working electrode used in this study was achieved by holding the electrode at +1.5 V under stirring for 90 sec before

recording each voltammogram. The appropriate initial potential was then selected and the electrode allowed to stabilise for 30 seconds; a scan was then initiated from +0→+1.1 V using a scan rate of 10 $mVs.^{-1}$

2. 2. 2. 3. 2. *Assay of Sumatriptan in Tablet Dosage Form*

A single tablet was dissolved in 900 ml of isotonic saline solution. A series of serial dilutions was then performed to give a nominal final concentration of $6x10^{-4}M$. An aliquot of this solution was then transferred to the voltammetric cell containing 20 ml of BR buffer, pH 5.0, to yield a final concentration of $2x10^{-6}M$ sumatriptan. The voltammogram was subsequently recorded by employing a scan rate of 10 mVs^{-1}. To quantify the unknown amount of sumatriptan in solution, a multiple standard addition procedure was employed.

2. 2. 2. 3. 3. *Preparation of carbon paste electrode*

The carbon paste electrode used for potentiostatic coulometry was made by pressing the active paste into the well of the body of plastic syringe (20 mm in diameter, 5 mm deep). The paste surface was manually smoothed by polishing it on clean paper and a new surface was prepared for each experiment by removing the paste from the electrode body, which was washed with absolute methanol and water, then dried with tissue paper, and subsequently packed with a fresh portion of the required carbon paste. To simplify this procedure, the syringe plunger provided a screw system to slide the brass disk in and out which thus pushed the paste out of the electrode body for surface renewal.

Unmodified carbon paste was prepared by careful hand-mixing of 5 mg of UCP carbon graphite powder (Spectropure grade, Fluka Chemical Co.) and 3 mg of Nujol oil (McCarthy Scientific Co., Fullerton, CA) using a glass mortar and pestle, and blending the mixture into a paste. In its design the paste was in direct contact with a disk made of brass which was attached to a copper wire of about 3 mm o.d. providing electrical contact to the measuring circuit. The carbon paste electrode was stored in 0.05 M sodium dihydrogen phosphate, pH 5.0, at room temperature and at open circuit.

2. 2. 3. ***Results and Discussion***

2. 2. 3. 1. ***Activation Procedure***

The surface of the glassy carbon working electrode used in this investigation was fouled due to adsorbed products of the oxidation process. Different electrochemical

pretreatment procedures were examined, and it was found that good reproducibility could only be achieved by holding the potential of the working electrode at a potential more positive than the oxidation potential of the drug. The optimum conditions were found to be at +1.5 V for 90 sec under stirring. The precision (expressed as the relative standard deviation) of the signal obtained for a $8x10^{-6}M$ solution of sumatriptan in Britton-Robinson buffer (pH5) under these conditions was 1.1% (n=10).

2. 2. 3. 2. ***Effect of pH***

Sumatriptan gave rise to a single oxidation process at the glassy carbon electrode in BR buffers of pH 2-11 using DPV. The effect of pH on the the peak potential (Ep) and peak current (ip) of the oxidation peak of sumatriptan is shown in Figures 2. 1 and 2. 2 respectively. The graph of Ep versus pH clearly indicates that the peak shifts to more negative potentials with increasing pH. Between pH 2 and 5 the slope of the graph was 95 mV pH^{-1}. A break in the graph then occurred at pH 5.0, and the slope of the graph subsequently became 50 mV pH^{-1} between pH 5-11. The position of this break is close to the pK_{a1} and pK_{a2} values of succininc acid at pH 4.16 and 5.61 [Glaxo Group Research, personal communication]. Sumatriptan succinate(1:1) has pKa values (determined by UV spectroscopy) associated with the succinic acid ion pair at 4.21 and 5.67, with the tertiary amino group at 9.63 and the sulphonamide group at pH>12. The effect of pH on i_p shows a maximum peak current at pH 5.0, which coincides exactly with the polarographic pK_a value obtained from the plot of E_p vs pH. Below pH 4.0, the drug exists mainly in the monoprotonated form since the succinic acid will be mostly in the deprotonated state and the drug will be protonated at the tertiary amino group. On approaching the pK_{a1} value of succinic acid, the acid begins to dissociate into the hemisuccinate form which forms a neutral ion pair with the monoprotonated form of the drug. As the pH is increased beyond 5.0, and nears the pK_{a2} value of succininc acid, the ion pair becomes negatively charged as the concentration of succinate increases in solution. Only one polarographic pK_a value was seen because of the relative closeness of the pK_{a1} and pK_{a2} values of succinic acid. The highest current value was obtained for the neutral species over either the positively or negatively charged moieties.

2. 2. 3. 3. *Optimisation of Operating Parameters*

2. 2. 3. 3. 1. *Variation of Peak Current with Pulse Amplitude*

The application of the differential pulse waveform (pulse amplitude = 50 mV)

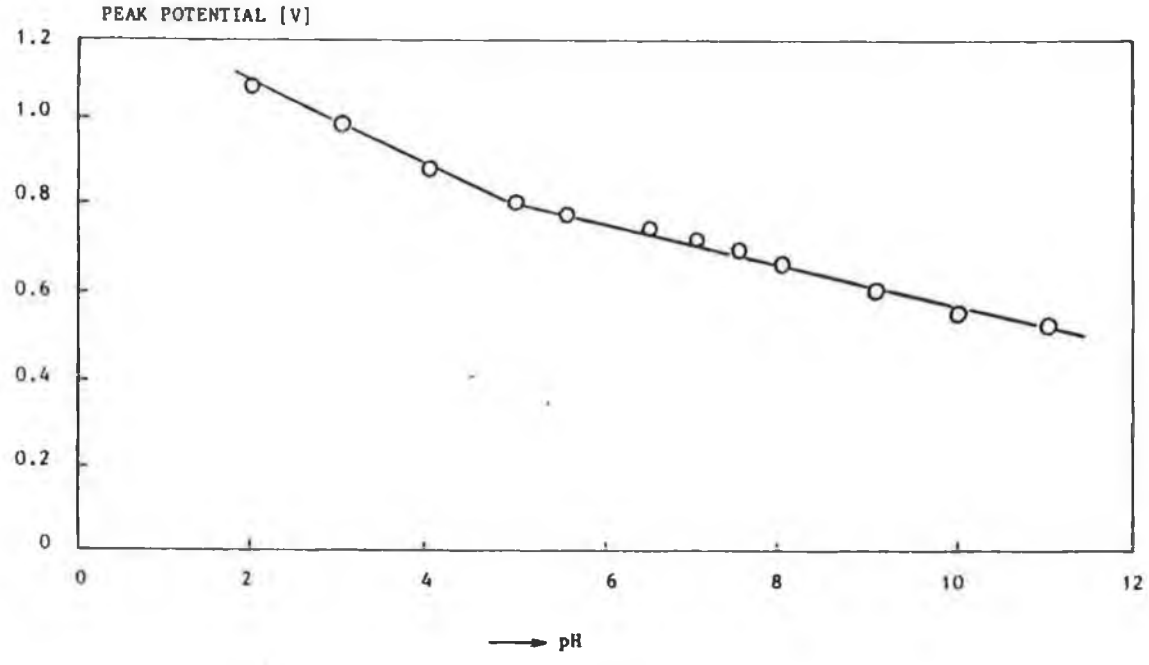


Figure 2. 1. Influence of pH on peak potential of sumatriptan succinate (1:1) ($1x10^{-3}$ M). Scan rate = 20 mVs^{-1}.

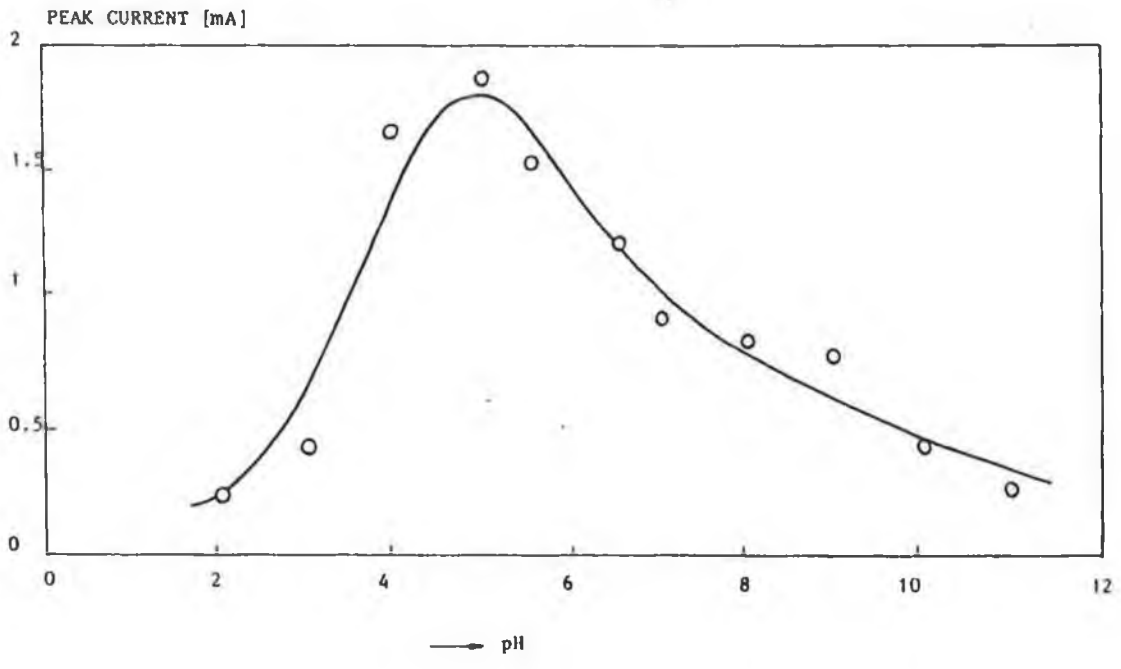


Figure 2. 2. Influence of pH on peak current of sumatriptan succinate (1:1) ($1x10^{-3}$ M). Scan rate = 20 mVs^{-1}.

yielded voltammograms in which the peak currents were on average twice as sensitive as those obtained by linear sweep voltammetry(LSV). The peak current intensity increased as the pulse amplitude was increased and a pulse amplitude of 100 mV provided the most sensitive signal. However, the use of this pulse amplitude resulted in an increased capacitive current and the analytical process merged to a certain extent with the electrolyte discharge. A value of 50 mV was therefore used for further studies.

2. 2. 3. 3. 2. *Variation of Peak Current with Pulse Interval*

Differential-pulse voltamograms recorded at various "pulse intervals" showed that the peak current increased as the pulse interval was increased. A pulse interval of 0.25 sec gave rise to the most sharp and symmetrical peak shape.

2. 2. 3. 3. 3. *Variation of Peak Current with Scan Rate*

The oxidation process was studied at a different scan rates, 2 mVs^{-1} → 100 mVs^{-1}). The optimum scan rate was found to be 20 mVs^{-1}. Higher scan rates gave rise to broader peaks and resulted in the oxidation process merging with the electrolyte discharge. The following operational conditions therefore gave rise to the best sensitivity and selectivity for the determination of sumatriptan : pulse height 50 mV, scan rate 20 mVs^{-1} and pulse interval 0.25 sec.

2. 2. 3. 4. *Mechanism of Oxidation Process*

The cyclic voltammetric behaviour of sumatriptan succinate at a glassy carbon electrode in Britton-Robinson buffer, pH 5 is shown in Figure 2. 3, indicating that the drug is irreversibly oxidised at the glassy carbon electrode. Studies were then undertaken to investigate the rate-controlling step of this process. Linear sweep voltammograms obtained for increasing values of the scan rate showed the existence of a linear dependence of the peak intensity upon the unit power of the scan rate between 5 and 100 mVs^{-1}.The characteristics of this graph were slope 4.00 $\mu AmVs^{-1}$, current intercept 0.25 μA, and correlation coefficient r= 0.9992. When this data was plotted vs the 1/2 power of the scan rate, a non-linear fit was obtained. These considerations pointed to an adsorption-controlled process, rather than a diffusion-controlled one. In order to further support this finding, hydrodynamic voltammograms were obtained for concentrations ranging from as low as $5x10^{-7}$ up to $1.5x10^{-6}$ M sumatriptan at rotation speeds between 200 to 1000 r.p.m. The resulting voltammograms failed to show the typical plateaus expected. Instead peaks were obtained throughout, whose magnitude did not depend markedly on the rotation speed applied to the electrode. This evidence constituted

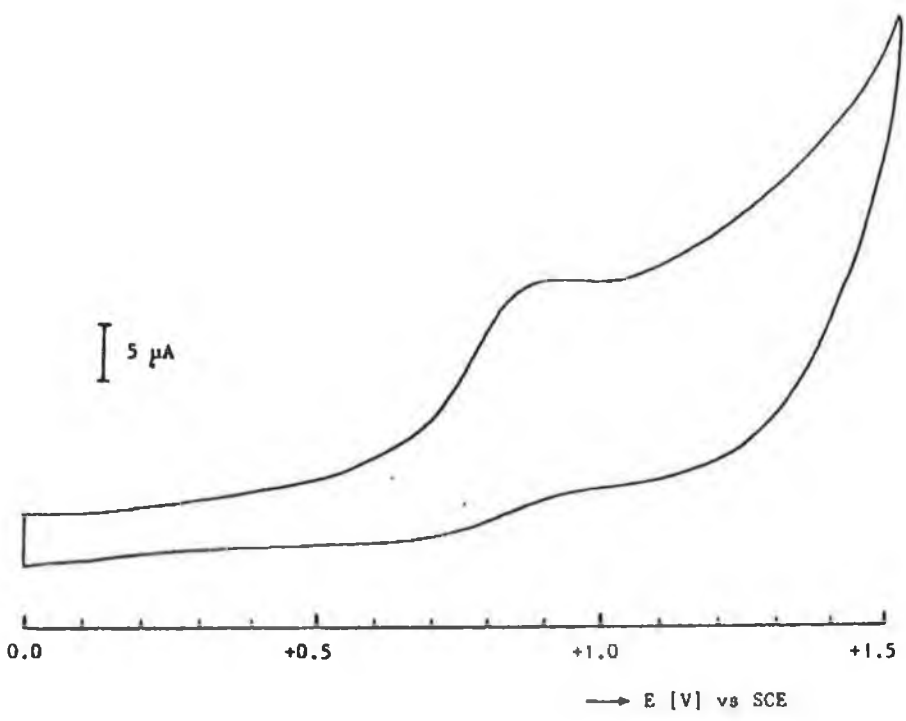


Figure 2. 3. Cyclic voltammogram of sumatriptan succinate (1:1) ($1x10^{-3}$ M), at glassy carbon electrode in Britton-Robinson buffer, pH 5. Scan rate = 200 mVs^{-1}.

further proof of a non diffusion-controlled oxidation process. Potentiostatic coulometry experiments were then carried out for a $1x10^{-3}M$ solution of sumatriptan succinate in Britton-Robinson buffer, pH 5.0, using a carbon paste macroelectrode (diameter 2 cm) at a fixed potential of +0.90 V. The number of electrons involved in the oxidation process was found to be 0.80, indicating that one electron was transferred in the oxidation process. A comparison with the oxidation wave obtained for indole-3-acetic acid, which occured at a similar potential to that of sumatriptan indicated that the oxidation occurred at the N atom in the indole ring of the molecule.

2. 2. 3. 5. *Assay of Sumatriptan Succinate (1:1) in a Tablet Dosage Form*

2. 2. 3. 5. 1. *Effect of Excipients*

The effect of excipients on the DPV behaviour of sumatriptan succinate (1:1) was investigated by adding in the relative concentration of each excipient into a pure solution of the drug in BR buffer, pH 5.0. The magnitude of the peak current for sumatriptan succinate increased after the addition of each the additives. Interference was found to be caused by cellulose, lactose and magnesium stearate. A cumulative effect for these compounds was also noticed when the analysis was carried out using placebo tablets (Figure 2. 4). This effect could be nullified using the activation procedure described in the Experimental section.

2. 2. 3. 5. 2. *Effect of Concentration*

Using the optimum conditions described, a linear calibration curve was obtained for sumatriptan in the range $1\text{-}8x10^{-6}M$. The characteristic of this graph were slope 1.75 μA $mol^{-1}dm^3$, current intercept 0.23 μA and correlation coefficient r=0.999. The limit of detection of the procedure was found to be $5x10^{-7}$ M. Nine samples from different dissolved tablets were analysed using the proposed voltammetric method. Appropriate dilution to produce solutions within the linear range was used in the analysis. The values found ranged from 96 to 99 mg per tablet. The relative standard deviation for the analysis of nine tablets was 1.86%.

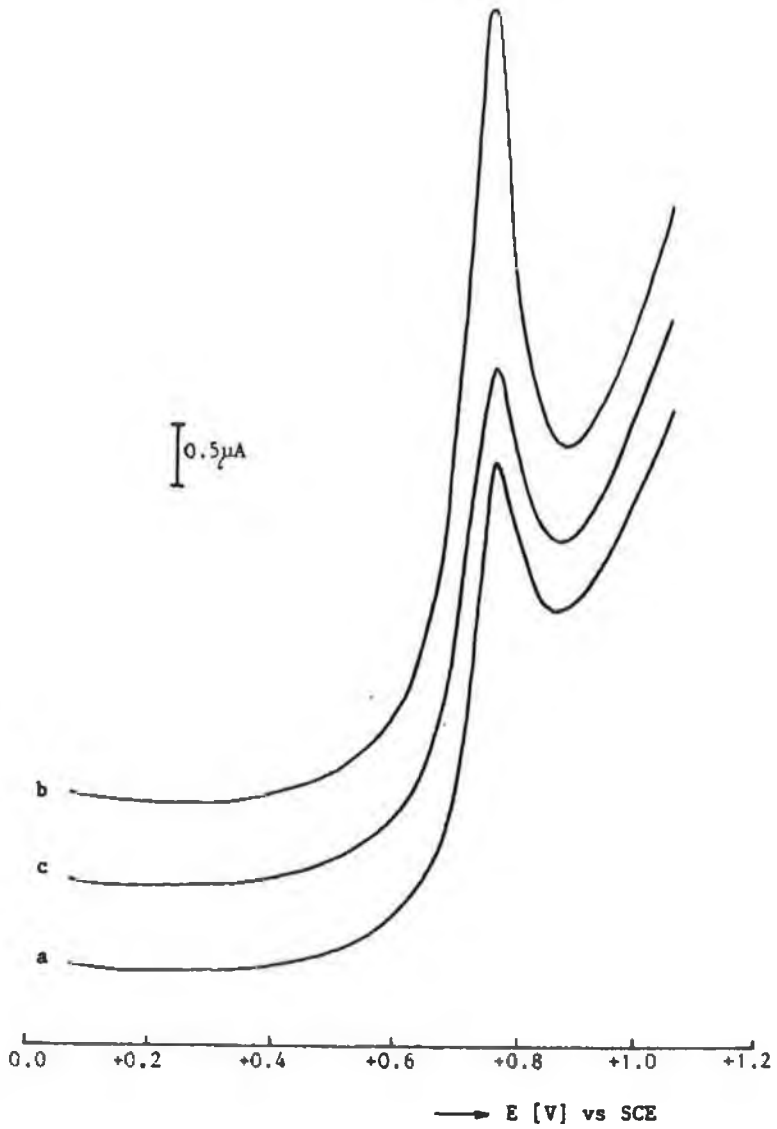
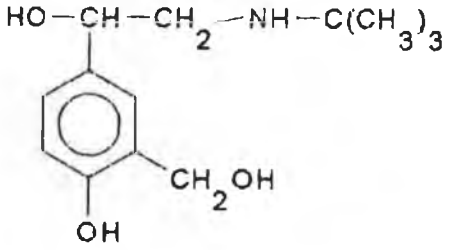


Figure 2. 4. Effect of the excipients on the DPV determination of sumatriptan succinate (1:1) ($1.5x10^{-6}$ M); (a). $1.5x10^{-6}$ M sumatriptan succinate,(b). in presence of dissolved placebo tablet with no pretreatment, c. in presence of dissolved placebo tablet following electrode pretreatment.

2. 3. VOLTAMMETRIC STUDY OF THE ELECTROCHEMICAL BEHAVIOUR OF SALBUTAMOL AND ITS DETERMINATION IN A TABLET DOSAGE FORM AND DISSOLUTION PROFILES FOR THE DOSAGE FORM.

2. 3. 1. *Introduction*

Salbutamol, 1-(4-hydroxy-3-hydroxymethylphenyl)-2-tertbutylamino-ethanol, also known as albuterol [II], is a sympathomimetic amine (or adrenergic drug) which affects those cell chemicals that mediate sympathetic nerve transmissions[31]. It has clinical application primarily as a bronchodilator and it is widely used in the prophylaxis of bronchospasms[32], where it has more prolonged actions than other bronchodilators and has been reported to have a more selective action[33]. The drug is also used in obstetrics for the prevention of premature labour[34] and as a nasal decongestant.



II. Structural formula of Salbutamol

Methods for the assay of salbutamol in tablet dosage forms are usually based on spectrophotometric[35] or colorimetric determinations[36]. Liquid chromatographic methods have also been developed for determination of this sympathomimetic drug in pharmaceutical preparations[37]. For such applications, however, HPLC, while having the advantage of requiring minimal sample preparation, is relatively slow and expensive, requiring filtration, degassing and expensive grades of reagents, eluents and equipment. The objective of the work described in this section was to investigate the electrochemical behaviour of salbutamol and devise a suitable differential pulse voltammetric method for the analysis of salbutamol in a tablet dosage form without employing a preliminary separation step, and to study the dissolution profile for the drug.

Dissolution rate testing is an important aspect of the development of a drug. It can be serve as a guide to formulation development and may be correlated to the absorbability potential of some poorly soluble drugs. Voltammetric methods are ideal for these studies since they are selective and the electrode used to monitor the rate of dissolution may be placed directly in the dissolution apparatus obviating the

need for respective sampling. The silicone-rubber-based graphite electrode has been employed as an appropriate voltammetric sensor to measure the rates of dissolution of several oxidisable pharmaceutical compounds[38,39]. The recirculating dissolution measuring arrangement with this sensing electrode and an Ag/AgCl reference are shown in Figure 2. 5. A peristaltic pump continuously samples the system in the thermostatically controlled dissolution vessel. A suitable constant potential is applied between the two electrodes and the current vs. time curve is recorded. An example of this system was demonstrated for the dissolution of aminopyrine tablets[40]. A similar design experimental set-up using a dropping mercury electrode in a flow cell had been applied to the determination of drug dissolution of chlordiazepoxide, trimethoprim, ornidazole, and isoniazide using polarography[41]. The dissolution rate of L-dopa in 0.1M HCl has been determined employing a tubular carbon electrode (TCE)[42]. A liter sample was pumped through a filter unit to the TCE and returned to the beaker. After setting the electrode potential at +0.9V vs. SCE and establishing a baseline current, a tablet was added and the limiting current was recorded as a function of time.

2. 3. 2. ***Experimental***

2. 3. 2. 1. *Reagents and Solutions*

Salbutamol and the different excipients used in the interference studies were kindly supplied by Glaxo Group Research Limited (Park Road, Ware, Hertfordshire, UK). Analytical grade sodium dihydrogen phosphate was obtained from BDH Chemicals Ltd (Poole, England). The compound and the supporting electrolyte were used without further purification. Stock solutions (1.0×10^{-3} M) of salbutamol were prepared by dissolving the compound in deionised water obtained by passing distilled water through a Milli-Q water purification system (Millipore). The solutions were stored in the dark under refrigeration to minimise decomposition. More dilute solutions were prepared daily from the stock solution. The supporting electrolyte used for all the voltammetric studies was prepared from a 0.1 M stock solution of sodium dihydrogen orthophosphate. This was subsequently diluted with deionised water to give the required concentration and adjusted to the required pH with 0.2 M sodium hydroxide.

The standard polishing kit and the glassy carbon electrode were supplied by EG&G Princeton Applied Research (PAR). The saturated calomel electrode was obtained from Metrohm (Herisau, Switzerland). For chromatographic experiments, the mobile phase used was 0.067 M sodium phosphate, pH 5.0; methanol; 40 g/L

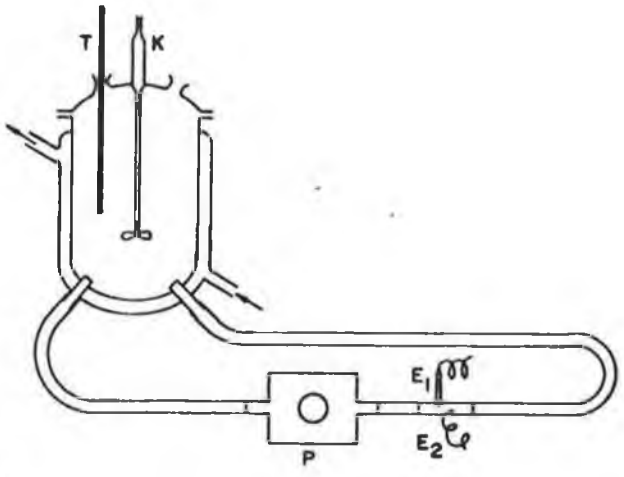


Figure 2. 5. Measuring setup for investigation of the dissolution of drugs from pharmaceutical preparations. T, thermometer; K, stirrer; P, peristaltic pump; E_1, silicone-rubber-based electrode; E_2, silver-silver chloride reference electrode. Reproduced from ref. 39.

sodium dodecyl sulphate (SDS) and diethylamine (DEA) in a ratio of 45: 55: 0.5: 0.02 (v/v). The drug was separated on a LC column (2.5 cmx4.6 mm i.d.), packed with reversed-phase octadecylsilane, (10 μm spherical particles) obtained from HPLC Technology (Macclesfield, Cheshire, UK).

2. 3. 2. 2. *Apparatus*

For voltammetric studies, the experiments were carried out in an all-glass cell designed for a three-electrode potentiostatic circuit. A platinum wire and a saturated calomel electrode were used as auxilliary and reference electrode, respectively. The potential of the working electrode was controlled using an EG&G Princeton Applied Research (PAR) Model 264A potentiostat connected to a JJ Instruments Model PL4 X-Y recorder. Rotating disc electrode experiments were carried out using a glassy carbon electrode connected to an analytical rotator and a PINE Model ASRE 416 speed controller. For coulometric studies, a carbon paste electrode was used as the working electrode. Chromatographic experiments were carried out using a Waters Model 501 liquid chromatograph. The dissolution test was performed in a basket-stirrer recommended by the United States Pharmacopeia (USP)[43] (Figure 2. 6).

2. 3. 2. 3. *Procedures*

2. 3. 2. 3. 1. *Activation of Glassy Carbon Electrode*

The electrode surface was first polished successively with small-particle-size silicon carbide paper containing a light covering of distilled water to lubricate the glassy carbon-paper interface, wet and dry emery paper (1200 grade) and finally with slurries prepared from 3, 1 and 0.03-μm aluminium oxide on a felt polishing mat. Residual polishing material was removed from the surface after each polishing step by sonication of the electrode in a water bath for 15 min, during which the deionised water was changed every 5 min. Prior to use, the electrode surface was washed with a jet of deionised water and dried with tissue paper. This polishing procedure was followed by electrochemical pretreatment involving cyclic voltammetry where the electrode was cycled between -1 V and 0.6 V for 5 min at a scan rate of 100 mVs^{-1}.

2. 3. 2. 3. 2. *Differential pulse and cyclic voltammetry*

The voltammetric conditions for a typical anodic differential-pulse scan were as follows: initial potential, 0.0 V; scan rate, 10 mVs^{-1}, and final potential, 1.0 V.

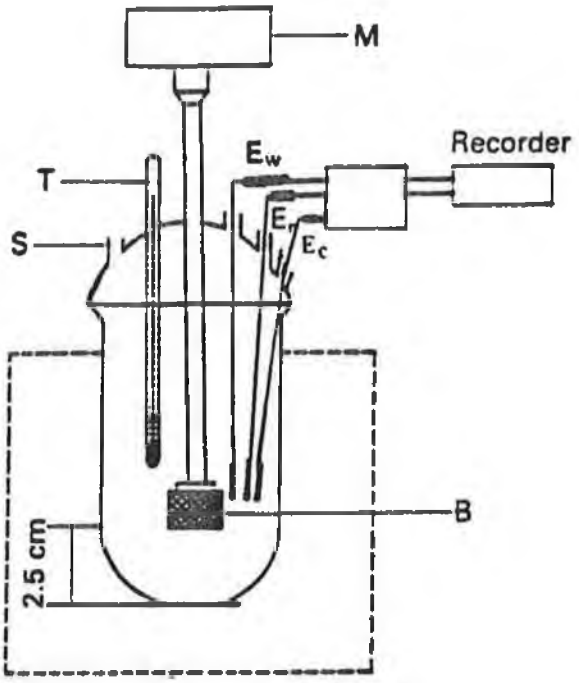


Figure 2. 6. Adapted USP basket stirrer apparatus for determination of dissolution profile of salbutamol tablet, E_W = working electrode (glassy carbon); E_T = reference electrode (SCE); E_C = counter electrode (platinum wire); M = stirring motor; B = basket stirrer; T = thermometer; S = ampling orifice.

From the voltammetric data, the optimum supporting electrolyte was found to consist of 0.05 M sodium dihydrogen phosphate. The following operational conditions gave rise to the best sensitivity and selectivity for the determination of salbutamol: pulse amplitude 25 mV, scan rate 10 mVs^{-1} and pulse interval 0.25 s. A blank solution without addition of salbutamol was used to obtain the base current. Standard cyclic voltammetry experiments were performed on plain 0.05 M sodium dihydrogen phosphate pH 5.0 solutions and those containing $4x10^{-5}$ M salbutamol using a glassy carbon electrode and the instruments described above. The voltammetric conditions were as follows: initial potential 0 mV; final potential 1.0 V, and sweep rate $20mVs^{-1}$.

2. 3. 2. 3. 3. *Determination of Salbutamol in Commercial Preparations*

For bulk drug determination, 4 and 8 mg portions of drug were accurately weighed and transferred to two separate 100 ml volumetric flasks. The drug was dissolved and diluted to volume in deionised water. The prepared solutions were used within two days.

For tablets ten 8 mg tablets were selected at random, weighed, and the average value was determined. The tablets were then ground into a fine powder, an accurately weighed quantity of powder equivalent to the average mass was dissolved in deionised water, and transferred to a 100 ml volumetric flask. The resulting mixture was shaken for 15 minutes to ensure that salbutamol was completely dissolved. A 200 µl aliquot of the dissolved sample was placed in an electrolytic cell containing 25 ml of 0.05 M sodium dihydrogen phosphate, pH 5.0 and the voltammogram was recorded by employing a scan rate of 10 mVs^{-1}. To quantify the unknown amount of salbutamol, successive 50 µl aliquots of a standard $1x10^{-3}$ M salbutamol solution were added to the cell and the voltammograms were recorded.

2. 3. 2. 3. 4. *Dissolution Profile Studies using Glassy Carbon Electrode*

The dissolution test was carried out according to the USP XXII method[43] with use of a USP basket stirrer type of apparatus Figure 2. 6 in 900ml of 0.05M sodium dihydrogen phosphate, pH 5.0 at a stirring rate of 50 rpm. The temperature of the cell was controlled at 37 $\pm$0.05°C by use of a thermostatic bath (AGB Ltd, Ireland). The tablet was placed in the basket which was rotated at 50 rev min^{-1}. Using the voltammetric technique, the current values were recorded at an appropriate time interval and the amount of salbutamol released was determined from a calibration graph.

2. 3. 3. *Results and Discussion*

2. 3. 3. 1. *Study of the Differential Pulse Behaviour of Salbutamol*

The discharge current of the background electrolyte in the potential region close to salbutamol oxidation gave rise to inaccurate measurements of the currents using linear sweep voltammetry (LSV). DPV signals were preferred in order to determine salbutamol in real dosage forms. Of the four electrolytes investigated, i. e. 0.05 M boric acid, 0.9% sodium chloride, 0.05M sulphuric acid and 0.05M sodium dihydrogen phosphate, the last gave rise to the best response with regard to oxidation peak current sensitivity, morphology, etc. Thus for further investigations, a solution of sodium dihydrogen phosphate at constant ionic strength (Ц= 0.05M) was used.

2. 3. 3. 2. *Effect of pH*

The DPV behaviour of the drug were investigated at various pH values. The glassy carbon working electrode was rinsed with deionised water prior to each analysis. The results obtained indicated that the peak potential (Ep) is strongly influenced by the pH. Figure 2.7 shows the dependence of Ep on pH. The peak shifts towards negative potentials with an increase in pH, in such a way that two straight lines with different slopes can be observed. The two lines intersect at pH 9.0 , which coincides with the pK_a value of salbutamol (Glaxo Group Research Data). The slopes above or below this pH were 0.004 and 0.002 V pH^{-1}, respectively. The peak obtained at pH 5.0 was found to be symmetrical in shape and easily measurable, and could be used to determine low concentrations of the drug. Hence, a pH of 5.0 was chosen for further study.

2. 3. 3. 3. *Variation of Peak Current with Pulse Amplitude and Interval*

The peak obtained for salbutamol at pH 5.0 was found to vary with pulse amplitude and interval. Differential pulse voltammograms recorded at various pulse intervals and with various pulse amplitudes showed that the peak current increased as the pulse interval and pulse amplitude increased. The application of the differential pulse wave form (pulse amplitude = 25mV) yielded voltammograms in which the peak currents were on average twice as sensitive as those obtained by LSV. A pulse amplitude of 100 mV provided the most sensitive signal. However, the use of this pulse amplitude resulted in an increased capacitive current and the analytical signal merged to a certain extent with the electrolyte discharge current. Optimum

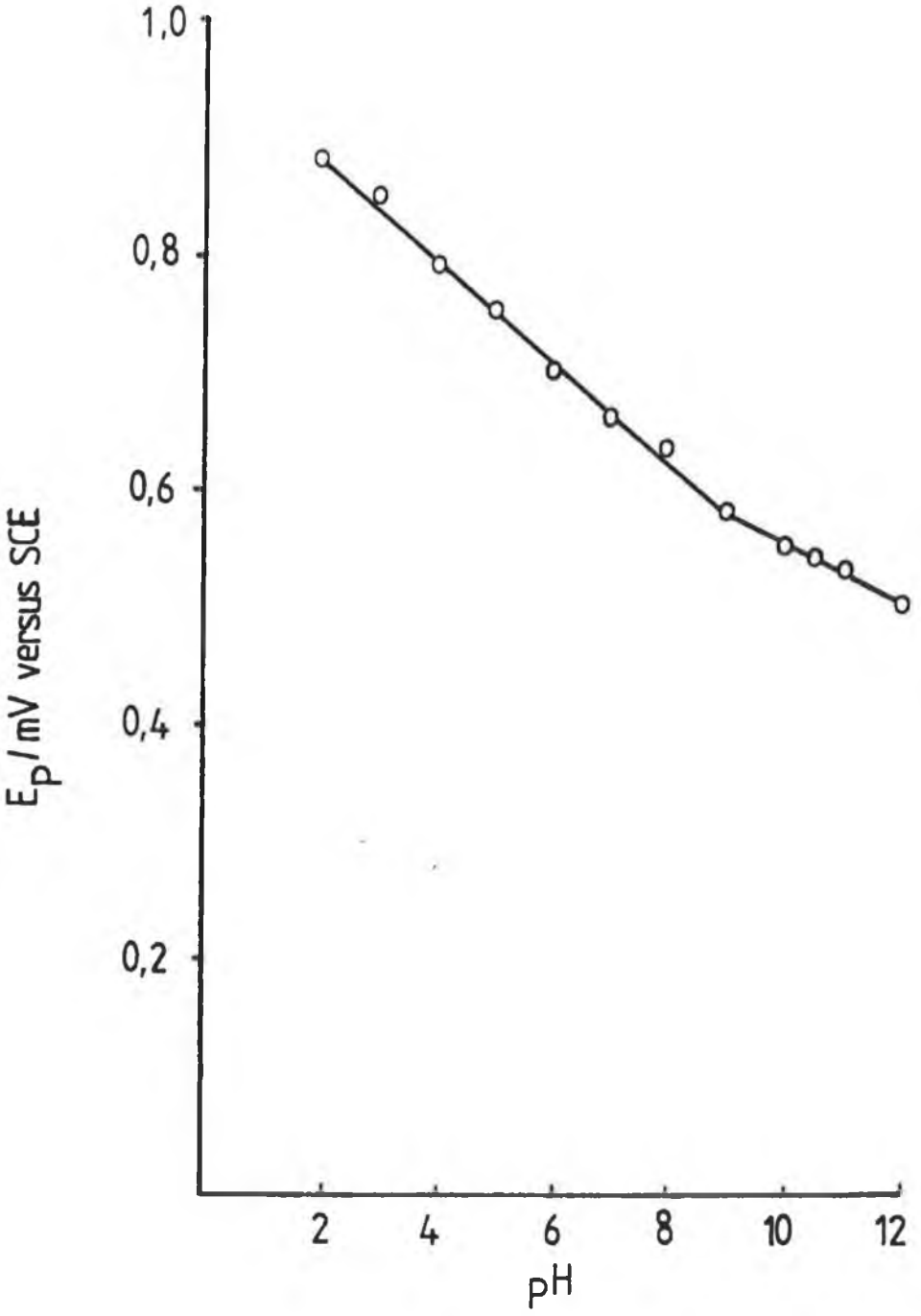


Figure 2. 7. Dependence of peak potential on pH.

conditions of pulse amplitude and pulse interval were found to be 25 mV and 0.2 s, respectively.

2. 3. 3. 4. *Variation of Peak Current with Scan Rate*

Figure 2. 8 examines the effect of the potential scan rate on the DPV heights of salbutamol at the glassy carbon electrode at different scan rates from 2 mVs^{-1} to 100 mVs^{-1}. The response was increased markedly upon increasing the scan rate until it started to level off at 50 mVs^{-1}. The increase in peak height was accompanied with broadening and distortion of the response (see inset). As a result, the optimum scan rate was found to be 10 mVs^{-1}, and this was used subsequently throughout the study.

2. 3. 3. 5. *Mechanism of Oxidation Process*

The cyclic voltammetric behaviour of salbutamol at an activated glassy carbon electrode in 0.05 M sodium dihydrogen phosphate, pH 5.0, is shown in Figure 2. 9, illustrating a single voltammetric oxidation peak. On the reverse sweep, no distinct reduction wave was observed, indicating that the drug is irreversibly oxidised at the glassy carbon electrode. Studies were then undertaken to investigate the rate-controlling step of the process, in order to determine whether the oxidation process was adsorption or diffusion controlled. Linear sweep voltammograms were obtained at different scan rates, and plots of i_p versus $v^{1/2}$, where v is the scan rate (mVs^{-1}), were constructed. The dependence of the peak current (i_p) on the square root of the scan rate ($v^{1/2}$) was found to be non-linear in the range 1 to 50 mVs^{-1}. When the peak current was plotted versus the unit power of the scan rate (v) it was found to be linear in the range 1 to 50 mVs^{-1}, according to the equation

$$ip\ (\mu A) = 0.004\ \mu A\ (mVs^{-1}) + 0.046\ (r= 0.9996\ ,\ n= 6) \qquad (6)$$

These considerations pointed to an adsorption-controlled process rather than a diffusion-controlled one. In order to further investigate this, studies were carried out under hydrodynamic conditions and hydrodynamic voltammograms were obtained for concentrations ranging from as low as 9×10^{-7} M up to 1×10^{-5} M at different rotation speeds between 200 to 1000 rpm. The resulting voltammograms failed to show the typical plateaus expected for diffusion controlled process and to increase linearly with the 1/2 power of the rotation speed ($w^{1/2}$). Instead, peaks were obtained throughout whose magnitude did not depend markedly on the rotation speed applied to the electrode. This evidence constituted a further proof of a non-diffusion-controlled oxidation process. The effect of accumulation time is shown in

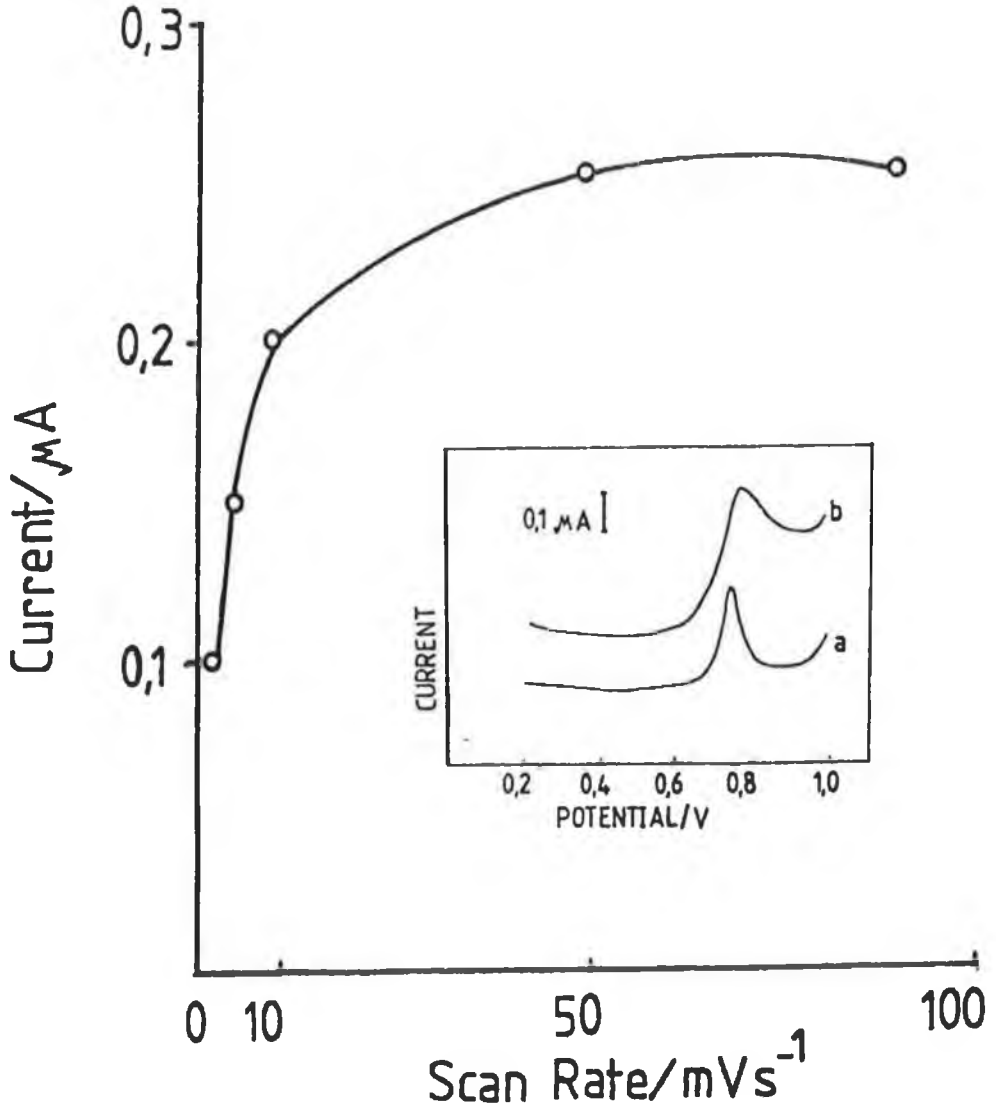


Figure 2. 8. Dependence of peak current on the scan rate of the differential pulse wave form. Also shown (inset) typical voltammograms of salbutamol at 10 (a) and 100 mV s^{-1} (b).

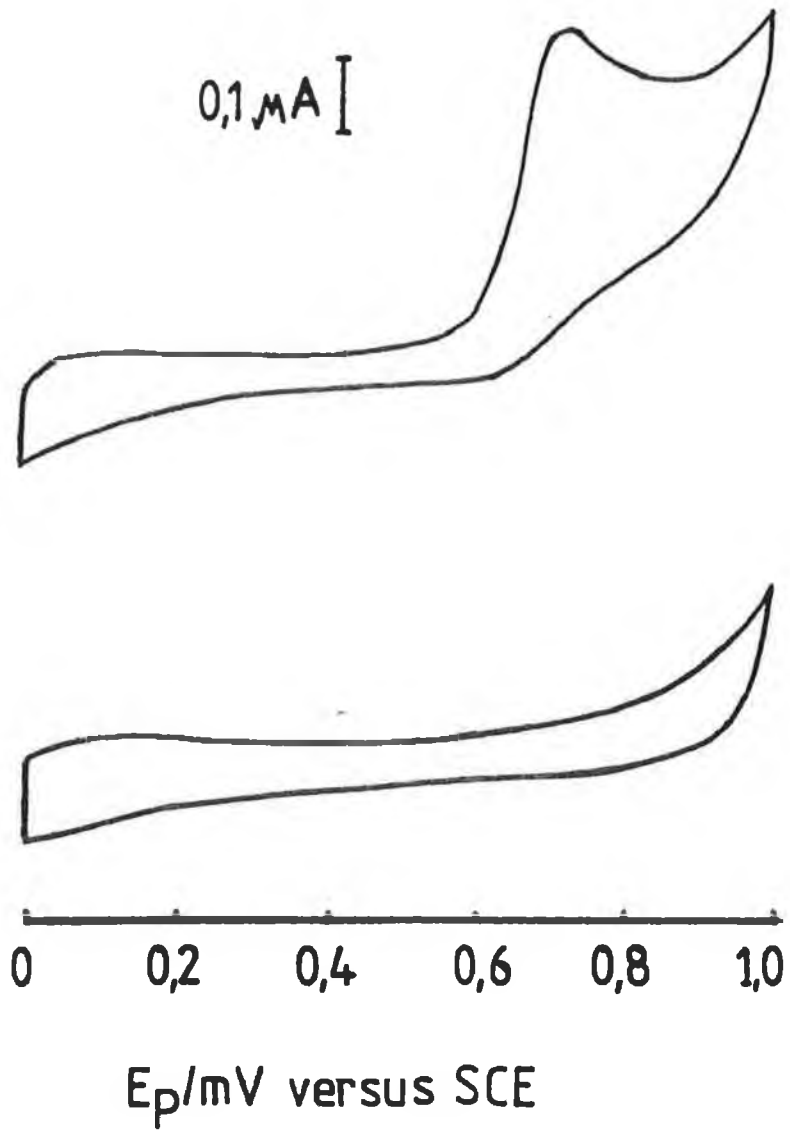


Figure 2. 9. Cyclic voltammogram of a 4 x 10^{-5} M solution of salbutamol in 0.05 M sodium dihydrogen phosphate, pH 5.0, obtained at a glassy carbon electrode and blank scan; initial potential, 0.0 V; final potential, + 1.0 V; reference

Figure 2. 10. Greater sensitivity was achieved by using a 30 s accumulation time. This points to adsorption of the drug compound on the surface of the electrode. Longer times of accumulation time gave rise to the same response. A potentiostatic coulometry experiment[44] was then carried out for a $1x10^{-3}$ M solution of salbutamol in 0.05 M sodium dihydrogen phosphate, pH 5.0, using a carbon paste macroelectrode (diameter 2 cm) at a fixed potential of +0.85 V. The coulometric analysis of the drug showed that two electrons were involved in the oxidation process.

2. 3. 3. 6. *Electrode Activation*

Glassy carbon electrodes used in electrochemical analysis are often pretreated in some way before measurements are performed. The voltammograms shown in Figure 2. 11 (b, c) and (d) represent the formation of an adsorbed film by the reaction product. Accordingly , a gradual loss in the electrode activity is observed on successive use. The reason for the low activity may result from the lack of functional groups on the surface or formation of passivating film[45] , gradually blocking the access of analyte to the surface and hindering the oxidation reaction. To provide a reproducibly active surface, and to improve the sensitivity and resolution, the glassy carbon electrode was polished to a mirror finish with a 1 μm diamond paste, carbide paper, and alumina and then rinsed with ethanol and deionised water. The electrode was then cleaned by ultrasonication.

The effect of electrochemical pretreatment was systematically evaluated with respect to the scan range, scan rate, and the duration of pretreatment. The pretreatment procedure found to give rise to the optimum response for salbutamol was cyclic voltammetry scanning between -1.0 to +0.6 V for 5 min at a scan rate of 100 mVs^{-1}. During this time, the electrode was kept in a quiescent solution. The current was then switched off, and the solution then stirred for 30 s. Before potential scanning the current was stabilised for 30 s at the required initial potential. Under this electrochemical pretreatment, no loss of electrode activity was observed (Figure 2. 11 "e") for similar analyses. Clearly, the "poisoning" effect appears to have been eliminated and the peak current was restored to its original height. (Figure 2. 11 "a").

The electrode pretreated in this way also exhibited a dramatic reduction in the background charging current. Some possible reasons for enhancement of the heterogenous electron transfer rates and the increase of the apparent rate of the electrode process are: (i) surface cleanliness or lack of impurities to adsorb on the

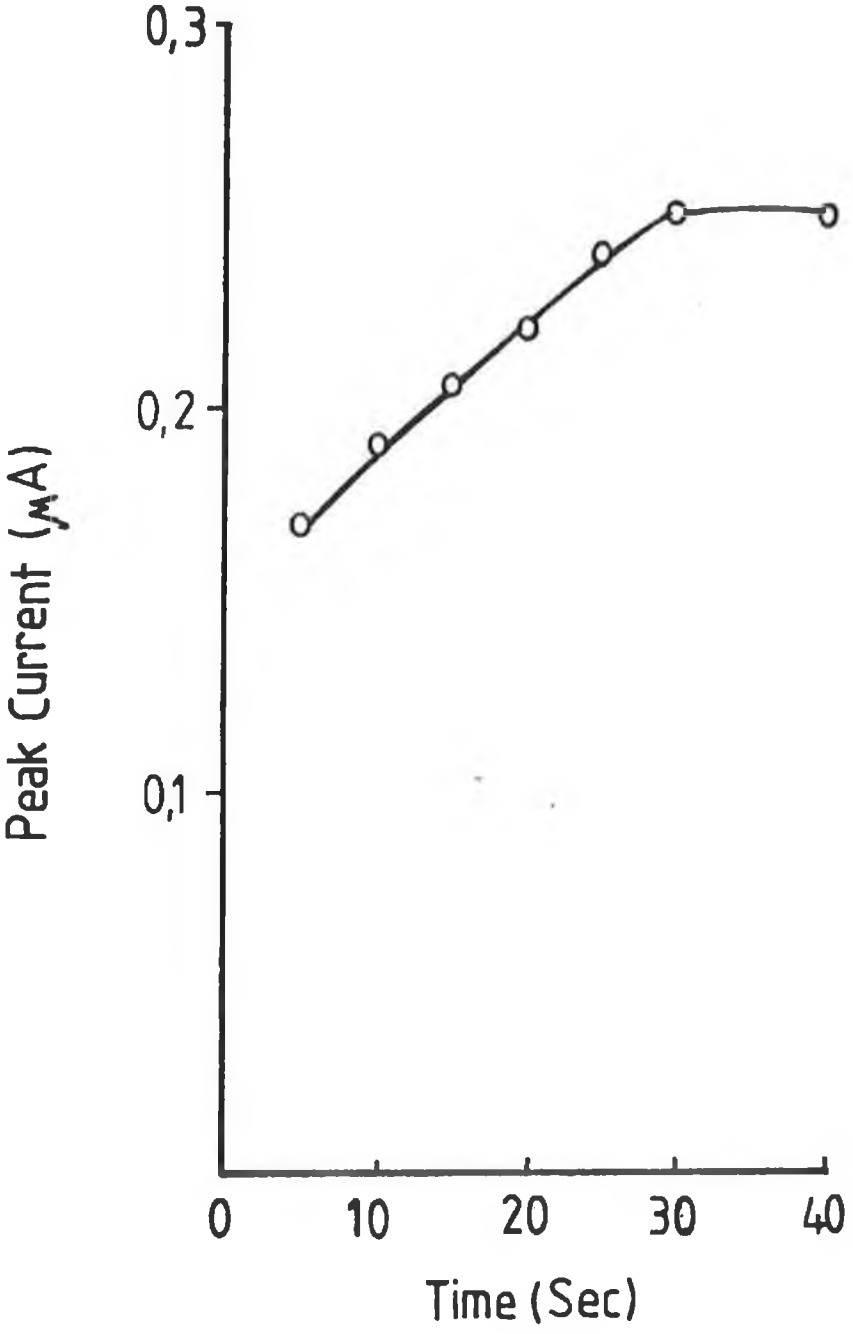


Figure 2. 10. Plot of peak current (μA) versus accumulation time (s).

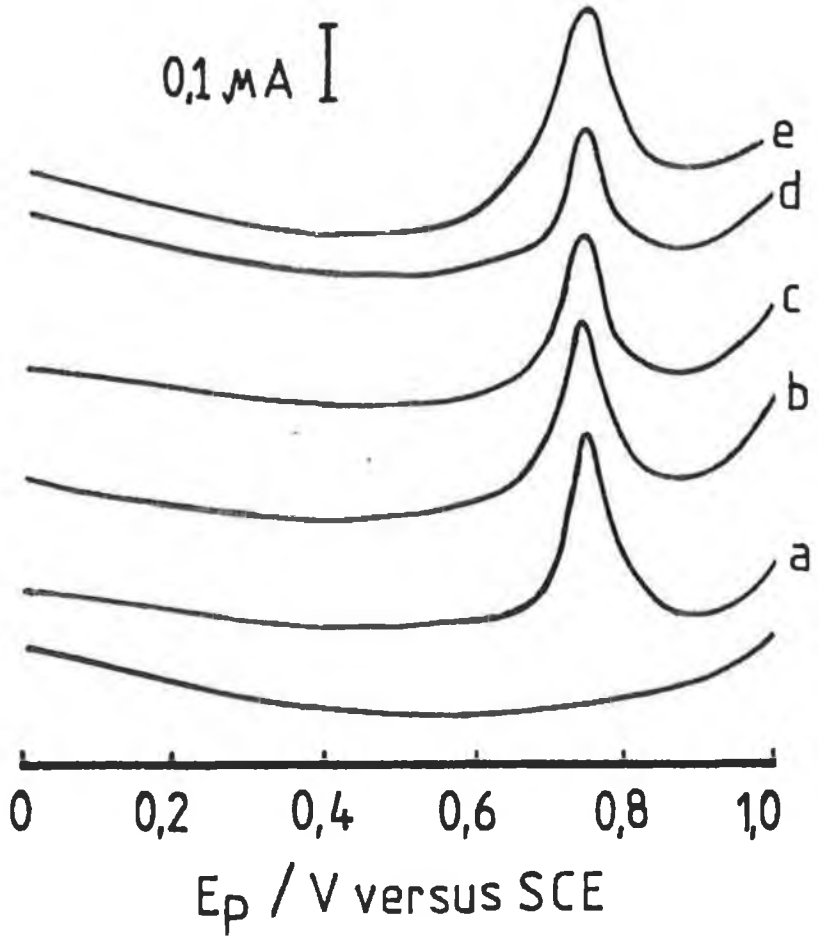


Figure 2. 11. The effect of the pretreatment of glassy carbon electrode on the DPV curves. (a) original response, (b) response after three scans (c) response after five scans, (d) response after six scans, (e) response after pretreatment.

surface blocking active sites; (ii) surface roughness causing the effective area for electron transfer to be greater than the geometric area; (iii) formation of "oxide" layers on the surface. The voltammetric experiments, described below, were performed with no further polishing to avoid the likelihood of drastically changing the physicochemical charateristics of the carbon surface, and the electrochemical treatment was performed daily prior to use of the electrode, whereas the polishing procedure was performed weekly. Precautions were taken to keep potential limits at which the electrode was operating, to within the range -1.0 to +1.0 V versus SCE, which ensured that significant alteration of the carbon surface did not take place.

Background cyclic voltammograms were routinely run between experiments to check the state of the surface. From these voltammograms it appeared that the glassy carbon surface remained reasonably constant throughout the entire course of the experiments. The electrode was soaked overnight in distilled, deionised water. When the above-mentioned pretreatment procedures were followed, reproducible voltammetric signals were obtained. Figure 2. 12 demonstrates the reproducibility of seven succesive voltammograms of 8×10^{-5} M salbutamol in NaH_2PO_4, pH 5.0, where a standard deviation of 2.4% was obtained. In view of the simplicity and speed of the pretreatment procedure step, it is recommended to carry out the pretreatment at the beginning of each analysis.

2. 3. 3. 7. *Effect of Concentration*

The peak current at +0.75 V was found to increase linearly over two orders of magnitude from 8×10^{-7} M to 8×10^{-5} M. The characteristics of the calibration graph of peak current versus concentration obtained by linear regression were: slope, 0.027 μA μM^{-1}; current intercept, 0.002 μA; and correlation coefficient, 0.9998. The minimum detectable concentration for salbutamol at the pretreated glassy carbon electrode was found to be 2×10^{-7} M.

2. 3. 3. 8. *Interference Studies*

In order to investigate the analytical application of this method, the effect of the excipients present in the dosage form was examined by carrying out the determination of 3×10^{-5} M salbutamol in the presence of each of the different excipients at conentrations that can be found in the tablet dosage form. Analysis of salbutamol was also carried out in presence of a placebo tablet. A deviation of more than 2% from the peak current of the solution containing no interfering additives was taken as a sign of interference. These studies showed that none of the

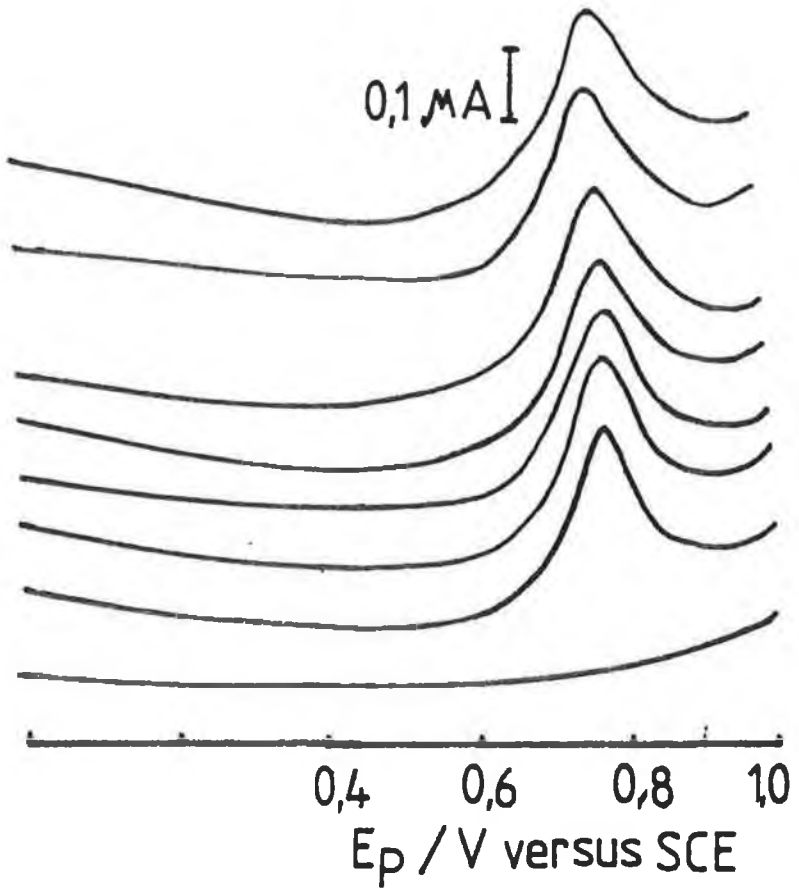


Figure 2. 12. Reproducibility of the DPV signals. Seven successive voltammograms were recorded following electorde pretreatment. [Salbutamol] = 8 x 10^{-5} M; scan rate 10mVs^{-1}.

excipients at the concentration level existing in the dosage form caused a positive or a negative error indicating that there were no serious interferences to the method.

2. 3. 3. 9. *Assay of Salbutamol in Bulk Drug and Tablet Dosage Form*

Differential pulse voltammetry for the determination of pharmaceutical products is by far the most common electroanalytical technique employed in pharmaceutical analysis. The proposed procedure described under Experimental was used for salbutamol determination in 4 and 8 mg tablets and for the bulk drug. Such determinations yielded mean values of 3.87 and 7.80 mg of salbutamol for 4 and 8mg tablets respectively. The accuracy of the assay is illustrated in Table 2. 1; Mean relative errors lower than 4 and 3%, and relative standard deviations lower than 2 and 3% (n= 7) were observed, respectively. The results for the bulk drug samples showed mean values of 3.91 and 7.85 mg of salbutamol; the relative mean error was lower than 4%, and the relative standard deviation was lower than 3% (n = 7), respectively. The recovery was in excess of 95% at the concentration levels studied. This is in a good agreement with official methods using high-performance liquid chromatography with UV detection[46,47].

Table 2. 1. Evaluation of Accuracy of the assay

Theoretical Concentration (mg)	Mean absolute error	Mean relative error (%)	RSD (%)
4	0.13	3.3	1.4
8	0.20	2.7	2.5

2. 3. 3. 10. *Comparison Studies*

Three different samples were analysed using a standard addition (Figure 2. 13) procedure and the proposed voltammetric method was checked by HPLC with carbon fibre electrochemical flow cell detection. This detection method is described in greater detail in chapter 3. The results obtained by both methods are summarised in Table 2. 2. As can be seen, the results obtained with the voltammetric method are in good agreement with those given by LC-EC. However, the voltammetric method

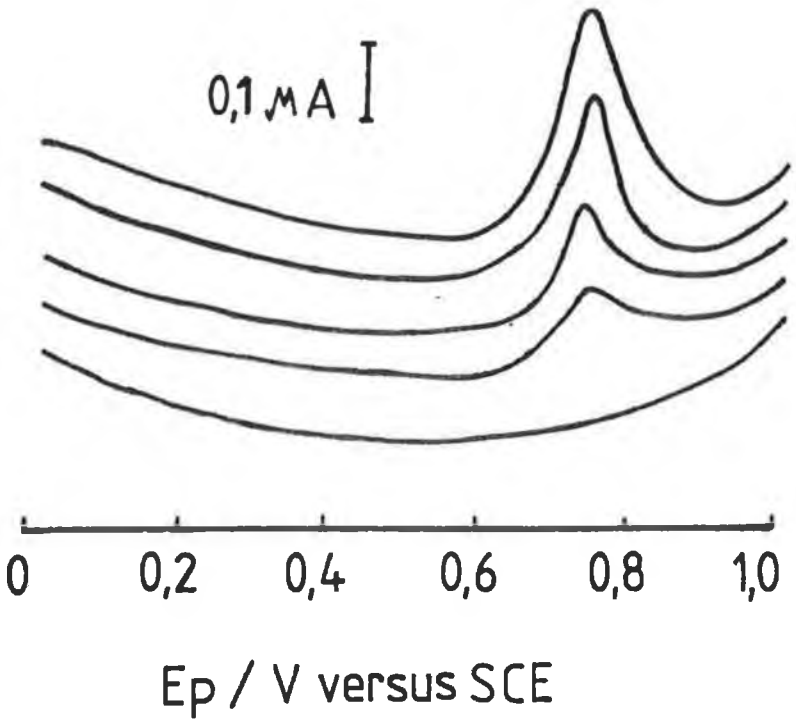


Figure 2. 13.Application of the standard additions method to a tablet dosage form: background current; sample and three additions of 50 µl of a 1 x 10^{-3} M standard solution of salbutamol.

is simpler, faster and requires less expensive equipment than the chromatographic method.

Table. 2. 2. *Comparative results for the determination of salbutamol in tablet and bulk preparations with voltammetric assay and HPLC.*

Sample	Voltammetric assay*		HPLC	
	Tablet, % found	Bulck, %found	Tablet, %found	Bulck, %found
1	96	97	98	97
2	97	96	97	96
3	95	95	96	98

* Results are the means of three replicate determinations.

2. 3. 3. 11. *Dissolution Studies Using Glassy Carbon Electrode*

Figure 2. 14 shows the dissolution profiles of salbutamol tablets obtained using the differential pulse voltammetric method. The glassy carbon electrode used in the monitoring of the drug from the tablet dosage form was pretreated between analyses while it was immersed in the dissolution medium, this procedure showed the advantage of continuously monitoring the concentration of the active ingredient in the standard dissolution cell without the need of withdrawing aliquots for analysis purposes, as it is the case in UV detection.

2. 4. ***Conclusions***

Using differential pulse voltammetry, methods for determination of sumatriptan succinate(1:1) and salbutamol in a tablet dosage forms and the dissolution rate of salbutamol tablets have been described. The activation procedures used in these studies permitted determination of these drugs without any interferences from the additives present in the dosage forms. The proposed methods are rapid, involves no sample preparation other than dissolving and transferring an aliquot to the supporting electrolyte, and do not require filtration, degassing and expensive grades of solutions that are needed for HPLC procedures. The methods also has the

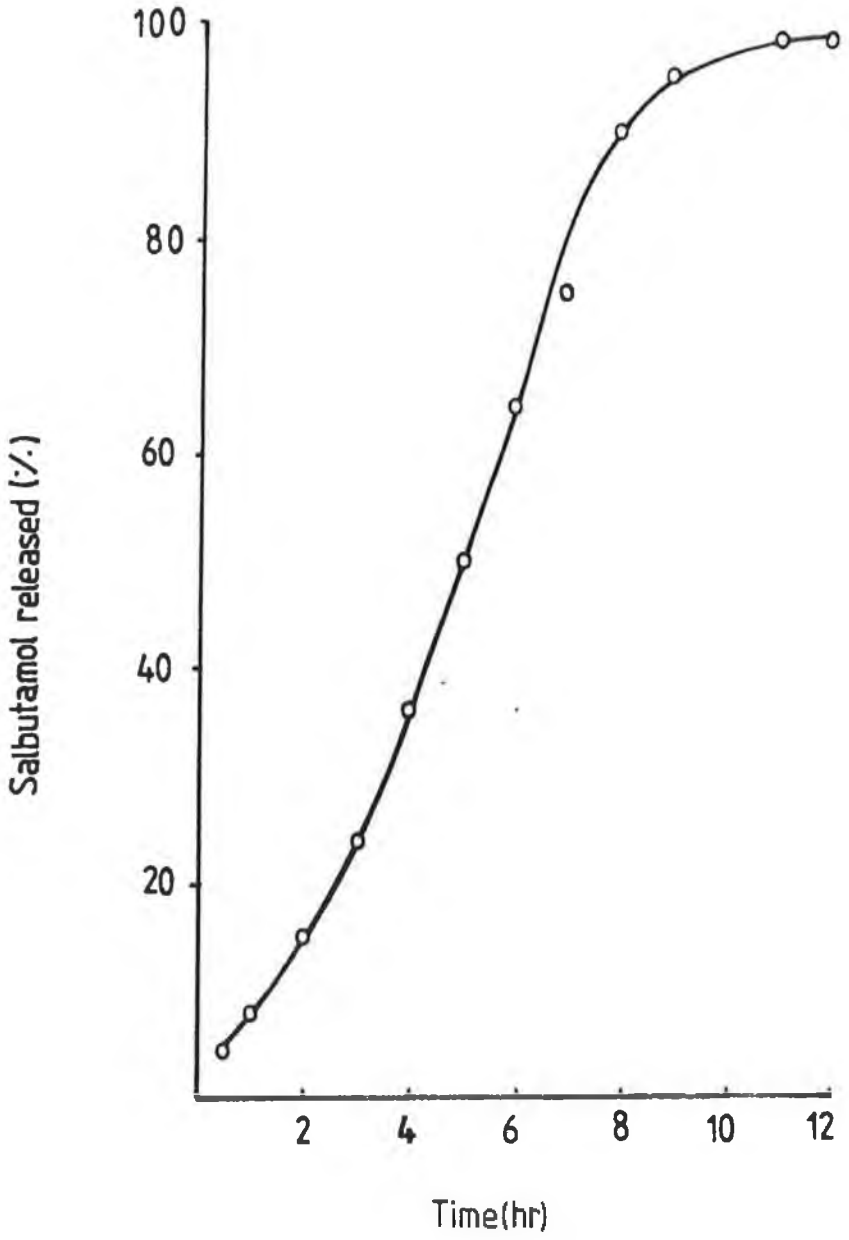


Figure 2. 14. Dissolution profile obtained with glassy carbon electrode for salbutamol tablets.

advantage of good recovery and is able to continuously monitor the concentration of the active ingredient in the standard dissolution cell without the need of withdrawing aliquots for analysis purposes.

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Chapter 3

Analysis of Salbutamol in Human Plasma by High Performance Liquid Chromatography with Electrochemical Detection using a Micro-electrochemical Flow Cell

3. 1. INTRODUCTION

The analysis of salbutamol in plasma is important in pharmaceutical research and clinical chemistry, and because the drug is widely prescribed, it can sometimes be taken in over-dose. Until recently, the only methods sufficiently sensitive for the measurement of this sympathomimetic drug in plasma were based on gas chromatography-mass spectrometry[1 - 3] and were beyond the scope of the instrumentation available in most hospital laboratories. However, the development of detectors with improved selectivity and sensitivity has facilitated the measurement of salbutamol by HPLC using more conventional detection schemes[4 - 6]. Because of the difficulty of extracting this drug free from endogenous interfering compounds, coupled to the fact that it occurs in low concentrations in plasma, means that its determination usually requires a selective extraction scheme and sensitive detection using spectrofluorimetric[5] or electrochemical methods[7].

3. 1. 1. *Solvent Extraction*

The analytical chemist has a difficult task arising from increasing requirements for versatility and accuracy of analysis. Bioanalysis is normally performed on blood serum or plasma, urine. The matrix used depends on the analytical objectives, the type of testing (invasive or non-invasive) and the information required. For extraction of collected samples, a three-step procedure is usually employed, but as little as one step may be sufficient for some applications. Liquid-liquid extraction is by far the most popular method used for the clean up of biological fluids, mainly due to its versatility[8 - 11]. It provides chromatographically clean extracts and allows for concentration of the sample by evaporation, thus enhancing sensitivity. It should be noted that a drug peak may often be accompanied by other peaks, because the likelihood of interfering peaks is probably about the same as with direct injection, because most if not all of the drugs and analogous physiological solutes will be included in the supernatant of these samples. Moreover, late eluting peaks from previous samples may co-elute with the drug peak, thereby distorting peak measurements and diversely affecting precision. The potential for late eluting peaks to degrade assay precision in therapeutic drug monitoring has been pointed out[12].

The size of the sample chosen will depend on the sensitivity required of the assay. Generally, however, 1 ml of plasma or serum is used and extracted into one 5 ml or more of organic solvent by vortex mixing followed by centrifugation. The addition of a large amount of extractant minimizes emulsion formation. Glassware

used in extractions must be scrupulously clean and free from any residual detergents. Polarity is usually the most important factor in the choice of extracting solvent and generally as this increases the range of components extracted also increases. The solvent should be selected with minimum polarity consistant with high recovery of the drug. Drugs of high polarity are difficult to extract and require strongly polar and hence non-selective solvents. Polar solvents that are miscible with water (e. g. n-propanol) can be forced to form a separate layer by saturating the aqueous phase with an inorganic salt, a technique useful for very polar drugs. Salts can be important in other ways, as they help to form better phase bundaries (i. e. avoid emulsion) and can also reduce the water content of the organic phase. Furthermore, the addition of salts can be used to increase the selectivity of an extraction by aiding the transfer of a drug from the aqueous to the organic phase. A solvent used for extraction should be of the highest purity, non-toxic, not highly flammable and have a suitable volatility. Modification of the aqueous phase is also most important in the development of an extraction scheme. Various solvents have been applied in this respect namely, diethyl ether, chloroform, n-hexane, n-heptane and ethyl acetate.

3. 1. 2. *Electrochemical Detection using Microelectrophotometric*

In the analysis of biological samples using HPLC, selective detection can be very welcome, especially when the analytical signal is to be differentiated from the sample matrix[13,14]. In electrochemical detection micro-or ultra-microelectrodes[15 - 18] can be used, either as single electroodes or as electrode arrays to improve selectivity. These electrodes have various advantages over conventional macroelectrodes[19,20]: (i) their small dimensions render them especially suitable for detection in micropacked and capillary column sparations, (ii) spherical or cylindrical symmetry of mass transport and electric field lead to fast establishement of steady state and extremely rapid electrode charging; consequently rapid scan techniques can readily be used, (iii) very low ohmic drop values permit measurements in poorly conductive media, often in the absence of base electrolyte[21,22]; the accessible potential range and selection of solvents (e.g. in acetonitrile) are thus widened, (iv) the signal -to-noise ratio is improved .

All these properties have obvious effects on the measuring sensitivity and selectivity. The extremely low signals produced by microelectrodes place great demands on the measuring apparatus and its shielding against external noise. The properties of ultramicroelectrode detectors have been examined[23] and special attention paid to the signal to noise ratio with microelectrode detection[24].

Selectivity is achieved by the choice of appropriate operating potentials which allow the detection of compounds of interest whilst other electroactive species requiring a higher potential are not "seen". Even at the upper limit of the available potential range, the electroactive compounds in biological material are far less in number than those which absorb UV light. This means that samples such as blood, serum or urine require less pretreatment before injection into the chromatographic system. Although modern analytical instrumentation is a powerful tool for bioanalytical determination, the selective detection and/or separation of the target analytes from the host, inert and possibly interfering species, are important in order to avoid errors due to matrix effects.

In many laboratories, EDs are not used routinely. One reason for this is that, although several instruments are now commercially available, relatively little has been published on their application. The design, fabrication and application of novel ED cells[25 - 33] have been well characterised experimentally and throughly reviewed and discussed. Such devices would complement (and sometimes replace) the use of costly machines. In addition, they offer the potential for continuous on-line analysis, for example, in critical care units and in non-medical applications such as industrial process monitoring and hydroponics.

Figure 3. 1 shows an electrochemical cell developed by Matysik et, al. for voltammetric measurements in flowing solutions. The cell body (d) consists of a glass tube (inner diameter 10 mm), which is closed by two PTFE plugs (c). They are pressed to the planar ends of the glass tube by PVC tightening devices (i). The inlet tube (a) with an inner diameter of 0.71 mm and the working electrode assembly (j) are fixed with the help of pierced brass screws (b). The home-made microelectrodes were produced using fine platinum with disk diameters between 0.7µm and 100 µm was directly sealed into glass. A silver-coated platinum coil (g) serves as a pseudo reference electrode and is contacted by a thicker platinum wire (h) melted into the glass body. The cell was used in a vertical position, that is, the solution level was fixed by the position of the outlet (e). The platinum disk microelectrode (f) is located a few millimeters within the inlet tube.

The construction of an electrochemical detector requires a solution of three main problems: (i) the working electrode must be chosen and the working electrode constructed, so that the accessible potential range suffices for the given purpose, and the residual signal and noise are sufficiently low and reproducible for as long a time as possible, to achieve a high sensitivity and reproducibility of measurements; (ii) the active surface of the working electrode must be sufficiently

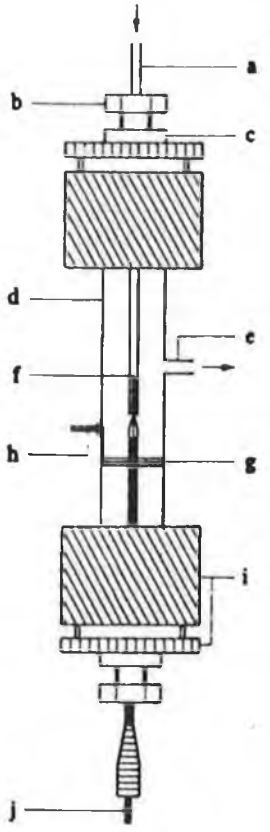

Figure. 3. 1. Flow cell construction: a) inlet tube, b) brass screw, c) PTFE plug, d) cell body, e) outlet, f) microdisk electrode, g) silver coated platinum coil, h) external contact oh the pseudo reference electrode, i) PVC tightening devices, j) external microelectrode contact. Reproduced from Matysik, F., et al., Electroanalysis, **4** (1992) 501.

mechanically strong to withstand liquid flow and compatible with a wide range of aqueous and organic matrices; (iii) the measuring cell must have a very small working volume and favourable hydrodynamics to avoid post-column band broadening and distortion. Thus the tubes must be short, narrow, must not hold up the eluate, and the detector must have the smallest possible dead volume[34]. Common industrial analysers often have effective volumes of the order of 100ul, or even less. High-performance macrocolumns require effective cell volumes not exceeding a few ul, microbore packed columns a few hundred µl and open tubular capillary columns about 10 nl or even less; (iv) a suitable measuring technique must be selected, from the point of view of measuring sensitivity, reproducibility, selectivity and ease of signal handling; (v) a wide dynamic range (ideally linear) of response and a long term stability of the electrode's activity towards the analyte; (vi) simple and reliable fabrication that results in consistency of response from one electrode to another; (vii) fast response of the sensor, to attain a low time constant for the detector. This time is defined as the time necessary for the signal to reach 63.2% of the maximum value[35]. In addition to these criteria other factors can affect the detector applicability and performance. For example, it is advantageous if the detector exhibits the same sensitivity for all the solutes detected and if its signal depends as little as possible on the experimental conditions, mainly on the temperature and the mobile-phase flow rate and composition.

From the electrochemical point of view, an optimum arrangement of the electrodes should minimize the voltage drop between the working and reference electrodes by placing them as close as possible and enable homogeneous charge distribution on the working electrode (uniform polarisation), which is best achieved by placing the working electrode and counter electrodes against one another in the channel through which the mobile phase flows. However, it is simultaneously required that the geometric volume of the detector cell should as small as possible, i.e. the channel thickness must be very small (0.02 - 0.1 mm). With such a small distance between the surfaces of the working and counter electrode, the products of the electrochemical reaction on the counter electrode might interfere in the reaction on the working electrode.

3. 1. 3. *Electrochemistry of Carbon Fibres*

Carbon fibres are miniaturised chemical sensors, smaller in diameter than a human hair, and because of their very small size they exhibit very different voltammetric properties than do electrodes of conventional size. Carbon fibres are

formed from the pyrolysis of pitch or polyacrylonitrile. The detailed picture of the chemical groups on the carbon fibre surface is confused, although some major points seem fairly clear[36]. The surface of carbon fibers is populated with oxide functions, typically carbonyl, carboxyl, hydroxyl and ester groups predominate[37].

Anodic oxidation increases the surface density of all these groups, but the degree and method of oxidation alter their relative proportions. Anodic oxidation in solution favours the formation of carbonyl and hydroxyl groups. Evidence from other carbon surfaces suggests that these functional groups may be assigned to aromatic structures such as quinone, hydroxyquinone and phenols. Aromatic and aliphatic carboxylates are also present. These functional groups may be formed in the bulk of the fibre as well as just on the surface; i.e. prolonged oxidation will increase not only the surface density of functional groups, but also the surface thickness.

3. 1. 4. *Fabrication of Single Fibre Electrodes*

Carbon fibers of high modulus have been produced by the pyrolysis of polymer textiles since 1964[38,39]. Carbon fibres are manufactured from a range of precursors, but three predominate: polyacrylonitrile (PAN), cellulose (Rayon) and PVC pitch. Most fibers from the UK are made of PAN, whereas those from the USA are made from rayon. The PNA fibres, produced by wet spinning, are stretched and heated to 200^0C. A ladder polymer is formed of linked hydrogenated naphthylpyridine rings. This process increases the thermal stability of the polymer by preventing easy formation of small volatile fragments, and by inhibiting melting. The fibres are heated to 220^0C, when some of the CH_2 groups are oxidised to ketones. After this final oxidation, an inert atmosphere is introduced into the heating chamber and the temperature is taken to 300 - 400^0C. Further cross-linking takes place between adjacent polymer chains, as water, hydrogen cyanide and nitrogen are eliminated. At this point the fibre consists of ribbons of largely carbon atoms, arranged in aromatic ring structures. These ribbons begin to resemble the basal planes of true graphitic structures although the distance between them is much greater than in graphite, and the atoms in a plane are not ordered with respect to those in neighbouring planes. Once the evolution of gases has decreased, the fibres are heated to above 400^0C. Further evolution takes place of inter-ladder hydrogen, hydrogen cyanide and nitrogen, until by 1000^0C the fibre has lost about 50% of its weight. This process, known as carbonisation, yields fibres that have a non-graphitic carbon structure, are of low modulus and high strength. Heating at 2000^0C causes the degree of perfection to

increase so that the fibre begins to look more like graphite. These fibres are most frequently encountered in structural materials and in analytical applications.

Clearly, for a material as fine and fragile as carbon fibre, a method of mounting the fibre must be used to produce reasonably rugged electrodes. There are several problems in handling carbon fibres, not the least being the safety aspect. The fibres are easily inhaled, and care should be taken to prevent this happening. The fibers also acquire a static charge, which can make placing or positioning them in capillaries particularly difficult. The fibers may well be coated in a sizing material that should be removed by washing in acetone. Finally, the fibres are very small and the use of a microscope and high-quality forceps for manipulations is recommended.

3. 1. 5. *Practical Applications of Carbon Fibre Electrodes*

During the past few years, significant progress in voltammetry has been achieved with the application of micro or ultra microelectrodes[40,41]. Due to the unique features of such working electrodes with at least one dimension in the micrometer or submicrometer range, new possibilities were opened in the fields of electrochemical kinetics, voltammetry in highly resistive media or in vivo electrochemistry. Recently, promising applications of microelectrodes in the field of analytical chemistry, particularly for HPLC or flow injection were reported[42 - 45]. Carbon electrodes are particularly useful for studying anodic processes in cases where the use of platinum or gold electrode is complicated by the adsorption of organic materials[46].

Neurotransmitters have a very important role in the brain since they are the key link in communication between neurons. A major challenge of the neuropharmacologist is to relate physiolgical functions to the numerous ligand binding sites identified in brain tissue. There are, for example, at least five postulated 5-hydroxytryptamine (5-HT) receptors and associated sub-types. However, carbon fiber electrodes figure 3. 2 have the most suitable charecteristics for neuropharmacological work. In particular, their tip small size (8 - 20 μm o.d.) make them ideal for use in small brain nuclei e. g. in the suprachiasmatic nucleus (SCN) and chemical and electrical pretreatment of these electrodes enables them to be used for measurement of amine neurotransmitter metabolites and , more recently, the neurotransmitter itself in the case of 5-HT[47].

A part from the oxidation of neurotransmitters and related species, various other voltammetric determinations have been reported. Miniature, amperometric

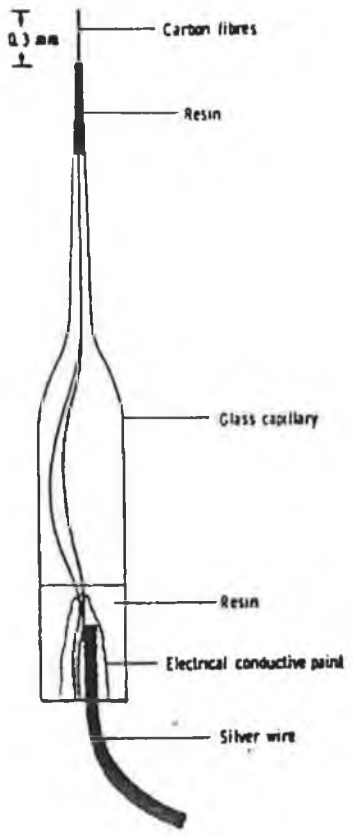

Figure 3. 2. Diagram of a completed carbon fiber electrode in which electrically conductive paitn has been used to form electrical contact between fibers and .silver wire. Reproduced from Martin, K., et al., Trends in Anal. Chem., 7. (1988) 334.

glucose sensors were constructed for implantation tissue of normal and insulin-dependent diabetic subjects[48]. Implantable biosensors are being developed for use in clinical medicine because they allow continuous measurement of rapidly varying analytes, without the need for added reagents or withdrawal of body fluids[49].

3. 1. 6. *Development and Validation of a Chromatographic Assay*

The most logical approach in the development stage of an assay involves opposite chronology to the final protocol. Although isolation precedes measurement, it can be more convenient to develop the analytical technique first. This is followed by selection of an internal standard and devlopment of the isolation procedure[50]. Samples of drug-free material (serum, urine) spiked with known drug concentrations should always be employed for calibration. Alternative "external" approaches on assaying or even just injecting (in the case of chromatography) pure standard solutions are unacceptable because they ignore the specific peculiarities of the biological matrix.

Most regulatory agencies, like the Food and Drug Adminstration, have mandated Good Laboratory Practice (GLP) regulations for clinical and non-clinical laboratory studies[51,52]. Based on these regulations, the fundamental criteria required for validation of chromatographic methods to check their reliability and overall performance are the evaluation of drug stability, specificity, limit of detection, accuracy, precision, linearity and recovery. Each of these individual criteria will now be discussed.

3. 1. 6. 1. *Drug Stability*

Drugs stored under different conditions of heat, light, humidity and pH (stock solutions and spiked biological samples) should be examined for possible decomposition. The stability under prolonged storage conditions for possible adsorption onto glass or other materials, such as plastics, must be determined. In certain instances, plasticisers from plastic syringes and containers and certain polymers from vacutainer stoppers have been known to interfere with drug measurement. The stability of the drug must also be checked under the conditions of analysis, since any losses or decomposition due to experimental manipulations can significantly affect the overall recovery, reproducibilty and precision of the assay. The method chosen should be sufficiently sensitive to detect low levels of decomposition products and sufficiently precise to observe small changes in the assay of the drug.

3. 1. 6. 2. *Selectivity*

The selectivity of a measurement is a rather vaguely defined criterion, expressing the ability of the sensor to respond to a particular substance without interference from other substances present in the test system. A sensor is said to be specific if no substance other than the particular analyte produces a response; this is an unattainable ideal, approached more or less closely by some sensors, such as the glass pH electrode. The selectivity requirements vary according to the purpose of the measurement. In industrial and environmental continuous monitors and in FIA instruments, the selectivity for a given analyte should be as high as possible, to decrease the number of operations that have to be performed on the sample prior to the detection; however; in some simple monitors (e.g. for the total content of salts in a solution, the overall acidity of a solution, etc.) the selectivity requirements are not very high. Chromatographic detection requires both selective and non-selective detectors. When a mixture of many substances is completely separated in the column, then a universal, non-selective detector suffices to detect all the components; on the other hand, when a complex mixture cannot be completely separated, a selective detector is advantageous as it provides a further means of differentiating and identifying the poorly resolved components, especially in trace analyses, when small amounts of the test substances must be determined in the presence of a great excess of other compounds.

The biological sample should be examined for the presence of endogenous components and/or other drugs which might interfere with the drug or its metabolites in the assay system. Should any peak interfere with the assay, a systematic search should be undertaken to find the origin of the interference. For example, an interfering peak eluting at the same retention time as phentermine was found during its GC analysis in plasma[53]. This was subsequently found to be due to the use of plastic pipette tips. However, selective extraction procedures, use of specific detectors or change in chromatographic parameters should normally eliminated interferences of this kind.

3. 1. 6. 3. ***Limit of detection***

The limit of detection (LOD) is defined as the lowest concentration of an analyte that the analytical process can reliably detect. In mathematical terms, the most popular quantitative approach seems to be that which defines LOD as 3s (s = standard deviation of the peak to peak noise). The detection limit must be statistically defined, below which no data should be reported.

The limit of detection, given in terms of the concentration or amount of the analyte, is directly related to the sensitivity and the noise. In the batch analysis, the concepts of the limit of detection and the limit of determination (limit of quantification, LOQ) are usually different, the former denoting the concentration (amount) of the test substance that can be qualitatively detected, whereas the latter is the lowest concentration (amount) that can be determined. However, in flow measurements the term limit of detection is usually used for quantitative analysis and will be employed in that sense in this study.

3. 1. 6. 4. *Accuracy and Precision*

The reliability of the measurement is expressed in the same way as for any other measurement, i.e. in terms of the precision and accuracy, obtained by well-known statistical methods. In measurements in flowing liquids, long-term signal stability and reproducibility are especially important for obtaining reliable results. The term accuracy denotes the nearness of a measurement to its accepted value[54] and is expressed in terms of error. The absolute error is the difference between the observed and the accepted value. The relative error is expressed as a percentage of the accepted value and is often used to express the accuracy of a chromatographic assay. In real terms, accuracy should be tested by comparison of results to another reliable method. Correlation of the two methods against each other by regression analysis ($r > 0.98$) is the most acceptable form of validation.

The term precision is used to describe the reproducibility of the method[54]. The precision of a measurement is given by the percentage coefficient of variation of replicate experiments performed under identical conditions.

In chromatographic methods of drug analysis, the precision and reproducibility of the method usually described both in term of intra-assay (within-day) and inter-assay (between-day) results. In performing intra- and inter-assays, at least four replicate analyses over the entire concentration range of calibration should be performed. For intra-assay, the mean values of the peak height or peak area should be obtained and a linear regression analysis performed by plotting the mean values versus the accepted value (the accepted value is based on the amount of test drug to the drug-free biological fluid). Then the concentration based on each individual value, expressed either as a peak height or peak area is calculated from the generated regression curve and precision defined based on these results. For inter-assay, a linear regression analysis is performed for each day of calibration, and then three or more of these calibration curves are used for

statistical calculations and for expressing reproducibility. Long-term signal stability and reproducibility are especially important for obtaining reliable results. Many of these calculations will be shown in following chapters.

3. 1. 6. 5. *Linearity*

The linearity of the assay should be defined by the linear regression analysis of replicates of spiked biological standards in the expected concentration range of the "unknown". In practice the linear regression curve obtained in intra-assay may be used to demonstrate the measure of linearity of the curve.

3. 1. 6. 6. *Recovery*

The overall recovery of a given drug from spiked biological standards is calculated from the limit of detection to the upper end of the linear calibration curve by two different methods[55]. The first method compares the peak heights/area of a series of spiked biological samples which have taken through the entire analytical procedure with the peak heights/area of a series of authentic standards. The second method compares the slope (determined by linear regression analysis) of a processed standard curve to that of the reference standards.

3. 1. 6. 7. *Quality Control*

The performance and reliability of routine drug assays should be checked with each batch of samples. Quality control standards are prepared by accurately spiking drug-free biological fluids with known amounts of the test drug by a person other than the analyst. At least 10% of these standards should be subjected to quality control and the results of these should be within - 10% of their accepted value, or the batch repeated[52].

If a method is modified it should be statistically revalidated even if one assumes that it had been correctly validated in the first place. A method might have to be modified in the following cases.

If the initial method had been developed on a used column and then is repeated on a "new" column, the two columns may behave differently for any of the following reasons:

(i) The lack of readily available identical column, e. g. not all C_{18} columns behave simillarly.

(ii) If the chromatographer decides to use a column which may perform differently, he or she may be forced to change other chromatographic conditions.

(iii) The chromatographer may have different requirements from the method in terms of sensitivity, and may be forced, for example, to change the extraction technique.

All the above points necessitate revalidation.

The above strategy for the development and validation of chromatographic methods of analysis of drugs in biological fluids forms the foundation for the analytical work in the following sections. In this chapter, an LC-ED method analytical method using a microelectrochemical flow cell based on carbon fibre for the determination of salbutamol in human plasma is described, and validated. This approach permits the determination of salbutamol at levels down to 1 ng/ml in this biological fluid. A comparison was also made with a conventional macro-glassy carbon based flow cell for this particular application.

3. 2. EXPERIMENTAL

3. 2. 1. *Materials*

Salbutamol sulphate was kindly supplied by Dr. J. Bloomfield (Trinity College Dublin). Plasma obtained from Beaumont Hospital (Dublin,Ireland) was frozen until required, and thawed at room temperature. Analar grade sodium dodecyl sulphate (SDS), sodium dihydrogen phosphate, and reagent grade diethylamine were obtained from BDH (BDH Ltd., Poole, Dorset,UK). HPLC grade methanol and ethyl acetate were supplied by Labscan Analytical Sciences, Dublin, Ireland. Water was distilled and then further purified using a Milli-Q water purification system (Milford, MA, U.S.A.). Carbon fibres, 13 μm in diameter, were obtained from Kureha Chemical Company, Tokyo, Japan. Graphite powder was obtained from Aldrich Chemical Co.Ltd, (Gillingham-Dorset, England). Silver epoxy was purchased from RS Components, Corby , Northants, U.K. All glassware was cleaned in chromic acid and then thoroughly rinsed with triply distilled water. The glassware was stored in a drying oven at 150°C.

3. 2. 2. *Construction of Carbon fibre flow cell*

The carbon fibre working electrode was inserted through the centre of a 25-mm-length polyethylene tubing (2 mm x 0.5 mm I.D.) as demonstrated in figure 3. 3 (a). The silver phosphate reference electrode was prepared by connecting a silver

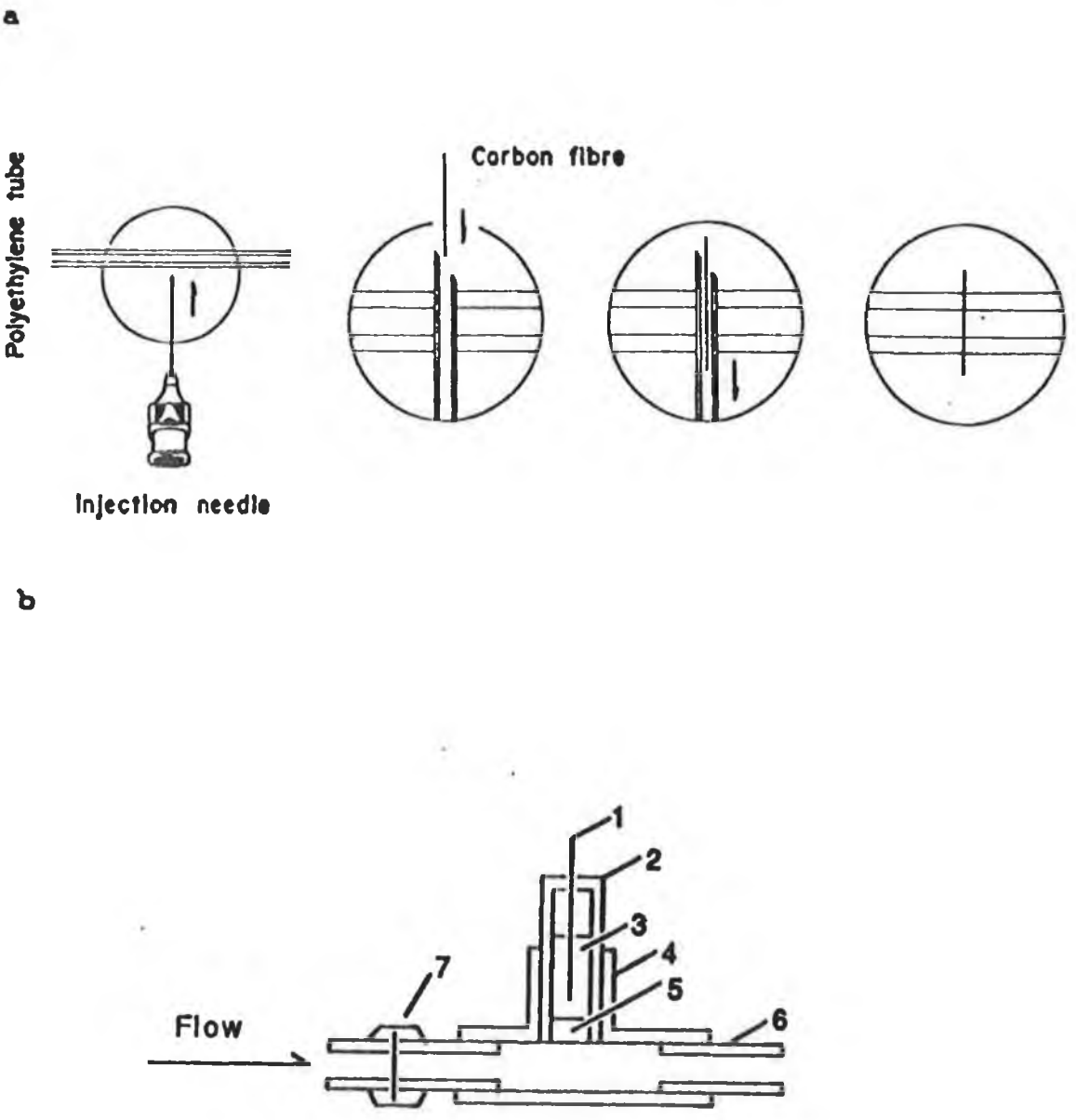

Figure 3. 3. (a) Method of fixing a carbon fiber into polyethylene tube. (b) The structure of the carbon fibre micro-flow cell consisting of: 1, silver wire coated with .Ag_3PO_4 ; 2, reference electrode body; 3, internal reference solution; 4, T .tube; 5, ceramic rod; 6, stainless steel counter electrode; 7, fibre flow electrode.

wire (0.1 mm in diameter) to the anode and a platinum electrode to the cathode of a 1.5 V battery while immersing the assembly for 2 minutes in a solution of 1 M phosphoric acid. The wire was then inserted into a polyethylene tube, which was 15 mm long, 1 mm in diameter and a porous rod 2 mm long, 1mm in diameter was fitted to one end of the polyethylene tube. The reference electrode tube was then filled with an internal reference solution containing 1 M phosphoric acid, and this end was closed by heating. A length (2 cm) of stainless steel tubing (1 mm x 0.2 mm i.d.), served as the counter electrode. The working, reference and counter electrodes were mounted in a T-tube arrangement and the working electrode was connected to the outlet of the HPLC column (downstream of the working electrode). Hence the mobile phase eluent passed first through the working electrode and then via the counter electrode to waste. A diagram of the flow cell is shown in figure 3. 3 (b).

3. 2. 3. *High-performance Liquid Chromatography*

The separation reported in this section was based on the work of Jarvie al.[7] following a slight modification in the methanol:water ratio to obtain a suitable retention time for salbutamol. The drug and plasma extracts were separated on a Spherisorb ODS (10 μm) column (25 cm x 4.6 mm i.d.) and were introduced onto the column via a Rheodyne Model 7125 (Cotati, CA, U.S.A.) 6-port injection valve fitted with a 20 μl loop. The mobile phase consisted of 0.067 M phosphate buffer, pH6: methanol: sodium dodecyl sulphate (40g/l): diethylamine (250:250:2.5:0.1), and was delivered by a Waters Model 501 HPLC pump at a flow rate of 0.9 ml/min. The eluted compounds were detected using the carbon fibre electrode connected to an EG&G Princeton Applied Research (PAR) Model 400 EC detector and recorded using a Philips Model PM8261 X-T recorder. Before use, the column was conditioned by passing 100 ml mobile phase adjusted to contain 10 times the standard concentration of sodium dodecyl sulphate and 2.5 times the standard concentration of diethylamine. Prior to the addition of SDS and diethylamine, the mobile phase was filtered through a 0.45 μm membrane and degassed by sonication. A conventional PAR Model 400 glassy carbon detector was used for comparison studies.

3. 2. 4. *Sample Preparation*

2 ml aliquots of plasma standards in a stoppered 10 ml glass tube were spiked with 2 $\mu g ml^{-1}$ salbutamol. 0.1 ml of 0.1 M sodium dodecyl sulphate was then added to each sample and the pH adjusted to 2.0. The samples were then extracted

with 4 ml 0.1 M ethyl acetate by vortex mixing for 2 min. The phases were separated by centrifugation (1000 g for 10 min) and the ethyl acetate phase was transferred to another tube, dryed under a stream of nitrogen and reconstituted in 40 µl mobile phase. A 20 µl aliquot was injected into the chromatograph. For calibration, drug-free plasma aliquots were spiked to give a series of salbutamol standards in the concentration range 0-55 ng drug per ml plasma.

3. 3. RESULTS AND DISCUSSION

3. 3. 1. Conostruction of Microflow Cell

The major drawback with microelectrodes is that fabrication difficulties increase as one decrease the size of the microelectrode[22] and so far they have relatively limited commercial availability. Hence, most workers normally prepare their own microelectrodes by sealing thin wires of fibres in glass capillaries either by melting the glass or by using an epoxy resin[55 - 59]. Sealing wires or fibres into glass is more technically demanding, and there is always a risk that the relatively high temperatures needed to melt the glass might change the characteristics of the electrode material. It has been reported that carbon fibres which had been in contact with a flame were more fragile and needed longer electrochemical conditioning[60]. The carbon fiber electrodes used in potentiometric stripping analysis (PSA)[57 - 59] is constructed by sealing a carbon fiber in a poly(vinyl chloride) tube. However, this kind of electrode is not suitable for LC because the epoxy glue used for sealing the needle holes on the electrode tube is not stable in organic solvents. The heat sealing of the needle holes on the microelectrode used in this work circumvented the use of epoxy glue, which is liable to attack by organic solvents. In addition, the method was easy and rapid to perform and did not change the characteristics of the electrode material.

3. 3. 2. *Hydrodynamic Studies*

The hydrodynamic voltammogram for salbutamol was obtained by injecting 16 ng of the drug into the chromatograph with the detector set at different working electrode potentials vs. Ag/Ag_3PO_4. The hydrodynamic voltammograms shown in Figure.3. 4 indicates that a working potential of +1.45 V was required for maximum response. When the potential was greater than +1.3 V, however both background current and the noise level increased rapidly. Hence a potential of +1.3 V was choosen for detection purposes.

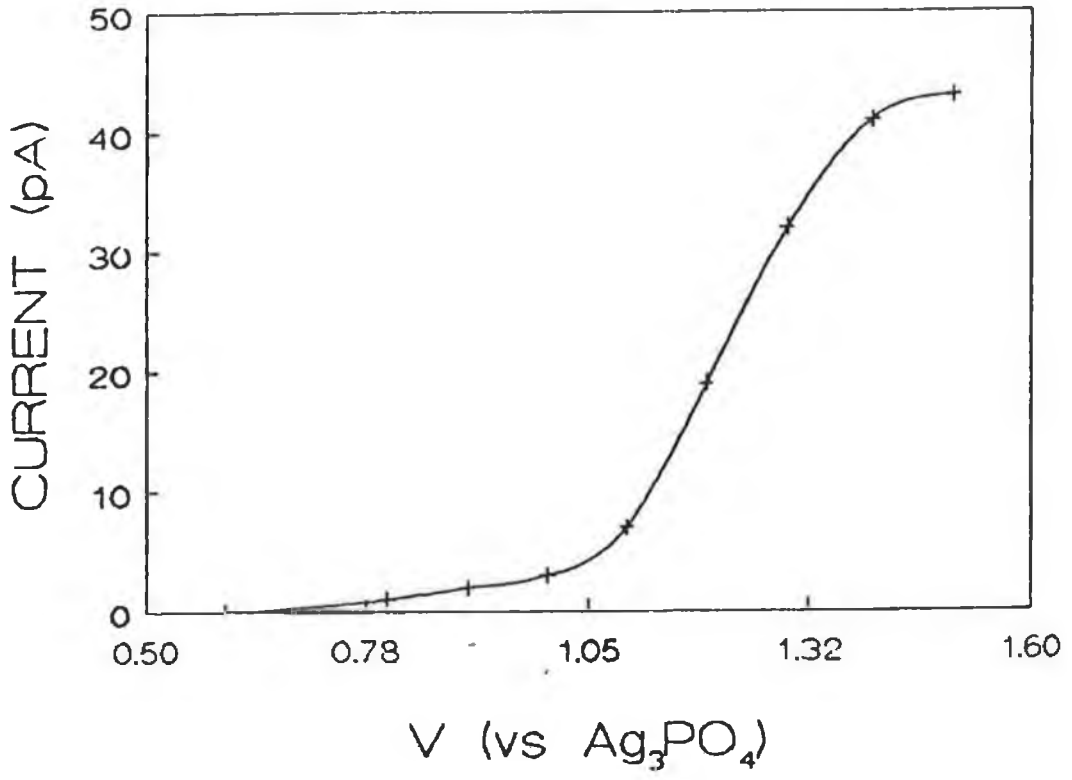

Figure 3. 4. Hydrodynamic voltammogram of salbutamol obtained by injections of 16ng into the chromatograh with detection at carbon fibre working electrode at differentpotentials (vs.Ag/Ag_3PO_4).

3. 3. 3. *Sample Preparation*

Several organic solvents, such as chloroform, dichloromethane, and ethylacetate, could be used for the extraction of salbutamol from plasma samples. Ethyl acetate was tried first because of its lower toxicity, and the extraction recovery was found to be more than 90%. Other solvents were therefore not investigated. The extraction of salbutamol was studied at different pH values; the optimum pH was found to be 2.0. A typical chromatogram of a plasma extract spiked with salbutamol at the 20 ng ml^{-1} level is shown in Figure 3. 5. This figure shows that peaks due to endogenous plasma components did not interfere with the salbutamol peak.

3. 3. 4. *Assay Performance*

The linearity of the method was determined by constructing a calibration curve in the concentration range 0-55 $ngml^{-1}$ salbutamol in plasma . As shown from the data in Table 3. 1, the method is linear over this range with correlation coefficients of greater than 0.999 over three days during which this experiment was carried out.

Table 3. 1. Calibration Plots Data For Salbutamol

	Equation of the regression line	correlation coefficient,r
Day 1	y= 0.0020 + 0.00028x	0.9998
Day 2	y= 0.0019 + 0.00013x	0.9997
Day 3	y= 0.0019 + 0.00021x	0.9994

Analysis with good reproducibilities are often possible, without the use of internal standards[61]. The reproduciblity of the overall method was determined by extracting and injecting quadruplicate plasma standards at each of six concentrations, i.e. 1, 5, 10, 20, 40 and 50 $ngml^{-1}$, and by calculating the amount of "drug found" by interpolation of the y-values (peak current) on the individual regression lines. The values of "amount found" were then used in the calculation of the mean, standard deviation SD and coefficient of variation CV, and as shown by the results in Table 3. 2, the method had an overall mean coefficient of variation of 5.40%. The recovery of the extraction method was then estimated by

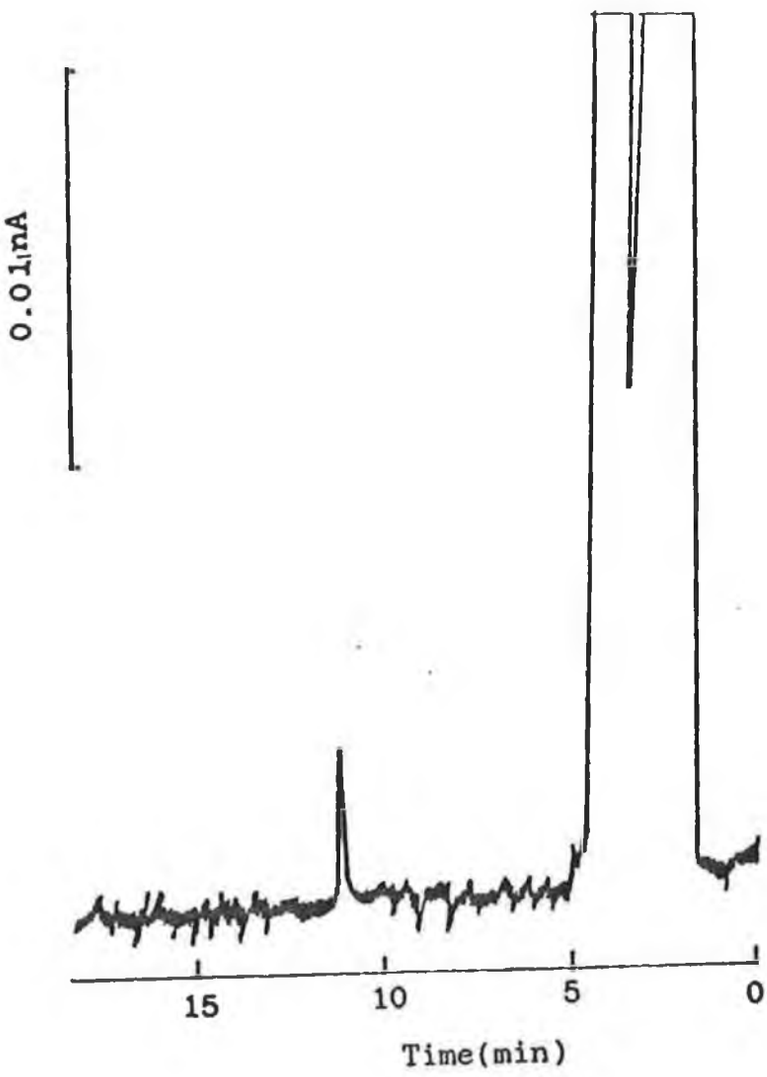

Figure 3. 5. HPLC chromatogram obtained by analysis of a plasma extract spiked with 20 ng ml-1 of salbutamol.

comparing the peak current of extracted standards at the 3, 5, 10, 20, 40 and 50 $ngml^{-1}$ levels with the peak current of authentic standards, which were injected at the same concentration levels in reconstituted residues, assuming 100% recovery.

Table 3. 2. Reproducibility Studies

Day I

Amount added (ng)	Mean amount found (ng)	Coefficient of variation(%)
1	0.85	12.00
5	4.40	8.20
10	9.00	8.00
20	18.50	5.84
25	23.87	3.58
40	38.88	2.20
50	48.75	2.14

Day II

Amount added (ng)	Mean amount found (ng)	Coefficient of variation(%)
1	0.75	11.25
5	4.00	7.20
10	9.30	6.08
20	19.00	4.55
25	24.00	3.90
40	38.58	1.43
50	48.50	0.98

Day III

Amount added (ng)	Mean amount found (ng)	Coefficient of variation%
1	0.8	9.3
5	4.5	7.0
10	9.2	7.4
20	18.9	4.8
25	24.2	4.4
40	38.9	2.1
50	49.2	1.1

The results in Table 3. 3 show that, at these concentration levels, drug recovery was always greater than 85%, and usually greater than 90%. The limit of detection was found to be 1 ng of drug per ml of plasma at which the signal-to-noise ratio was greater than 3. The limit of quantitation was approximately 3 ng ml^{-1}, at which the signal-to-noise ratio was greater than 10. This limit of detection compares favourably with previous methods for the determination of salbutamol in biological fliuds and the method is capaple of monitoring the therapeutic range of the drug[62].

Table 3. 3. Recovery Studies

Concentration(ng)	Peak current (nA)		Recovery(%)
	Extract(E)	Authentic(A)	
3	0.005	0.007	85
5	0.0087	0.01	87
10	0.018	0.02	90
20	0.036	0.039	92
40	0.077	0.08	97
50	0.0975	0.10	98

3. 3. 5. *Electrode pretreatment*

A variety of methods have been devised for pretreating carbon fibre electrodes, including laser and electrochemical [63 - 67] and the methods need to be evaluated individually for the problem at hand. Electrochemically treated electrodes have been found advantageous, e.g. in flow detection of hydrazines[68], ascorbic acid[69] cathecholamines[70] or tetracycline antibiotics[71]. The requirements for pretreatment is that the surface of the electrode changes with time due either to the adsorption of species from solution or chemical changes on the electrode surface itself. These changes often result in variations in sensitivity , reproducibility and selectivity.

The amperometric oxidation of salbutamol is particularly troubled by poisoning of the electrode surface, owing to a build-up of a polymeric film, which is an accumulation of electrode reaction products. Various attempts at overcoming the electrode poisoning associated with the detection of salbutamol and their suitability for continuous monitoring were investigated. One protocol which would improve the peak current and reduce the noise to its original level involved the anodisation of the microelectrode at +1.4 V for 5 sec, then cathodisation at -1.4 V for 5 sec vs. Ag/Ag_3PO_4 followed by equilibration for 5 min. It has been proposed[72] that the improved performance is as a result of the removal of surface contaminants or inhibitory layers which hinder electron transport. The electrochemically modified carbon fibre electrode was less susceptible to poisoning than a non modified one and prolonged lifetime of the electrode was obtained after electrochemical pretreatment.

The effect of electrochemical pretreatment is shown in Figure 3. 6. The current response of the electrode was found to increase after the pretreatment regime. This is probably due to activation of the surface produced quinoidal functionalities[73]. It also seemed to stabilise the contribution due to the charging current. One of the sources of noise in the system might be the potentiostat, which was designed for a conventional glassy carbon working electrode, and tailored to analyses at higher currents. A lower noise level would be expected with a potentiostat more suitable for the lower current encountered with carbon fibre electrodes.

3. 3. 6. *Comparison with Conventional Glassy Carbon Based Detector*

The performance of the microelectrochemical flow cell was compared to a conventional macro-glassy carbon electrode flow cell, the electrode of which was polished successively with small particle size silicon carbide, alumina, diamond paste and then subjected to ultrasonic cleaning[74]. As shown in Figure.3. 7, the current scale for the fibre electrode is 200 times smaller than that for the glassy carbon electrode. This is consistent with the surface area ratio between them. The surface area of the glassy carbon electrode is about 170 times greater than that of the carbon fibre electrode.

The detection limit of the carbon fibre electrode was, however, shown to be about three times better than that obtained using glassy carbon electrode. Similar findings have been reported by Luscombe et al.[75] for the determination of copper in urine using platinum disc microelectrodes instead of a conventional carbon disc

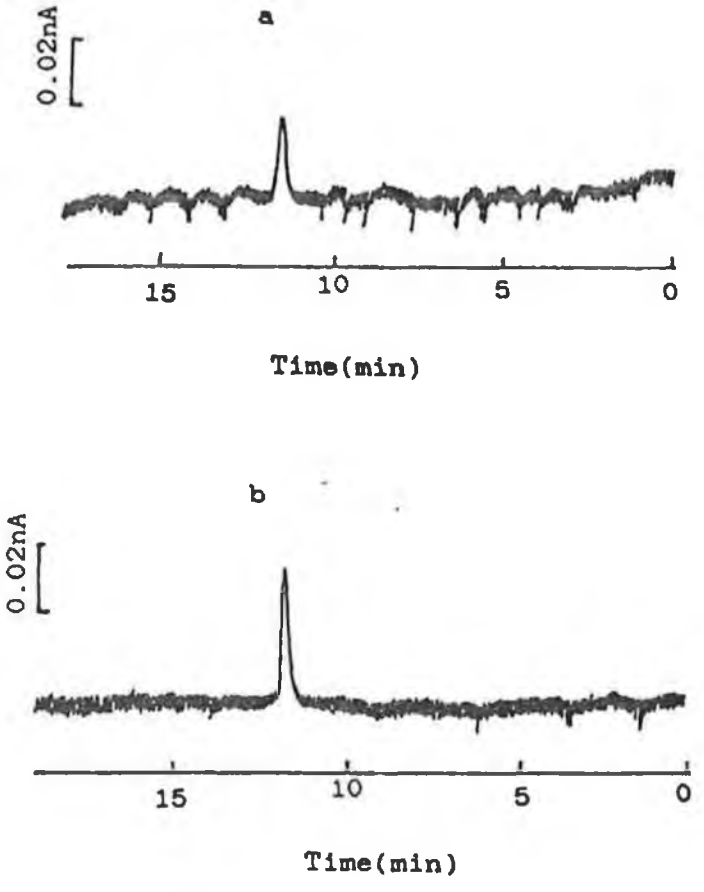

Figure 3. 6. HPLC chromatograms of 20 ng salbutamol obtained by analysing the drug before (a) and after (b) electrochemical pretreatment of the working electrode .surface.

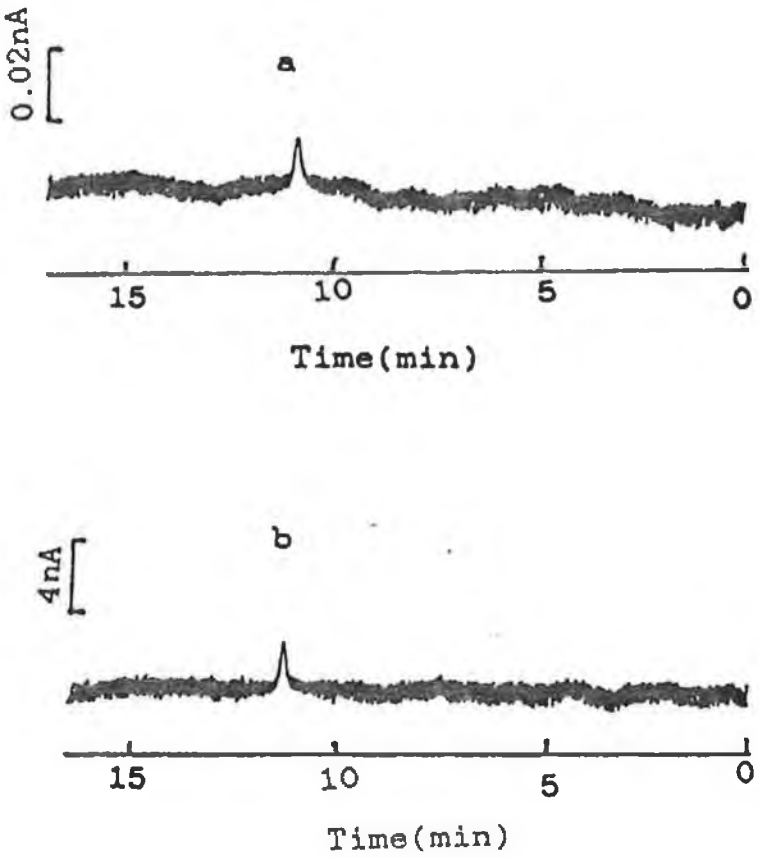

Figure 3. 7. Chromatograms obtained by analysis of (a) 1 ng salbutamol using the carbon fibre microelectrode at +1.3V vs Ag/Ag_3PO_4; and (b) 3ng salbutamol using a .macro-glassy carbon electrode at +1.3V vs Ag/Ag_3PO_4.

macroelectrode. In addition, carbon fibre electrodes can overcome many problems associated with a conventional glassy carbon based detection system. For example, the glassy carbon electrode requires frequent polishing, which can be quite tedious; fluid can leak from the cell, air bubbles can form in the cell, and a high electrolyte concentration is needed in order to reduce the iR drop in the cell caused by the large surface area. A lower limit of detection could be obtained using a potentistat tailored for measurements using microelectrodes because the noise levels are undesirably high when small currents are meaured under the potentiostatic control provided by commercial instruments[76].

3. 4. CONCLUSION

A reversed-phase HPLC method has been developed for the assay of salbutamol in human plasma, based on liquid-liquid extraction and detection using a carbon fibre flow cell. The cell is simple to prepare and easy to manage. Carbon fibre electrodes have the advantages that the rate of mass transport to and from the electrode increases as the electrode size decreases, iR drops are very small and the capacitive charging currents are reduced to insignificant proportions. The limit of detection of the method was found to be 1 $ngml^{-1}$, the mean overall coefficient of variation was 5.4% even without using an internal standard, and drug recovery was in excess of 85% at all concentration levels studied. The limit of detection reported in this paper is approximately 2-3 times lower than that reported for ferrocene by Bixler and Bond[77] using a microdisk electrochemical wall-jet flow cell, and could be further improved by lowering the electronic component of the noise.

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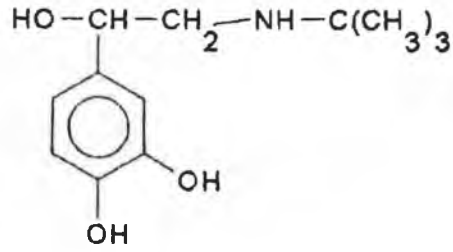
Chapter 4

Analysis of Terbutaline in Human Plasma by on-line Solid-Phase Extraction with Amperometric Detection

4. 1. INTRODUCTION

Terbutaline (1-(3,5-dihydroxyphenyl)-2-(t-butylamino) ethanol) is a sympathomimetic agent affecting those neurotransmitters which mediate sympathetic nerve transmissions[1]. Its clinical application is primarily that of a bronchodilator, and it is widely used in the prophyllaxis of bronchospasms. Plasma concentrations of unchanged drug associated with effective therapy[2] are in the range 10-30 pmol/ml. The analysis of terbutaline [III] is important in pharmacuetical research and clinical chemistry, and because the drug is widely prescribed, it can sometimes be taken in over-dose. Because of the low concentrations encountered in biological fluids, the complexity of the sample matrix and the reactivity of the drug to trace impurities, sensitive and selective detection is necessary for its determination in plasma.



III. Structural formula of Terbutaline

As has been mentioned in the previous chapter, HPLC has become a widely applied analytical technique, particularly in the determination of pharmaceutically active compounds. Coupled with this growth there has been a rapid development in the quality of chromatographic supports and equipment available[3]. However, particularly in the case of biological samples, extensive sample clean-up is often required prior to analysis and this has been a limiting factor in the past.

The column switching technique[4,5] offers the analyst a powerful technique allowing him to combine the separative powers of HPLC with simple sample clean-up procedures[6 - 8]. The term "column switching" is used in modern liquid chromatography for different operating modes without a strictly defined sense. Synonymous expressions such as "multiple column chromatography", isomodal, bi- or heteromodal chromatography", etc., are used for column switching, but with different objectives for chromatographic separations. The term "column switching"[9] includes in the widest sense all techniques by which the direction of the mobile phase is changed by valves, so the effluent from a primary column is passed to a secondary column for a defined period of time. The use of valves means that the chromatographic system involves not one, but a number of columns forming a

network. Switching within this network may be effected manually or by automated controllers.

In the following section, valve switching, and in particular column-switching, will be presented as on-line method for sample preparation which allows the analyte to attain the goals set out above. The following section includes a through review of important research in this area to date and highlights the versatility, and efficiency possibility of employing this technique in particular for sample clean-up and trace analysis in biological fluids.

4. 1. 1. *Column Switching*

A generalised set-up of multidimensional HPLC is shown in Figure 4. 1. Mobile phase compatibility of different modes of columns, and the additinal extracolumn effects introduced by the switching valves, tubings, size of transfer volumes, etc., will determine the optimum configuration for a particular application. In most cases, a much simpler setup than in Figure 4. 1 will be adequate to do the job. The set-up in Figure 4. 1 basically reveals two important modes of operation commonly used in column switching automated sample preparation and enhancement of system resolution. The small columns, L1, L2, etc., located in the switching valve loop are commonly used for automated sample preparation such as trace enrichment, peak compression, sample cleanup, etc.; since they are located on the valve loop, they are sometimes refer to as loop columns. Pre-concentration is usually carried out on relatively short (2-30 mm long) precolumns, with similar diameter to the analytical column, packed with a sorbent employed in HPLC, such as chemically bonded silicas[10]. Depending on the applications, packings of these columns can be different from the analytical column. The analytical columns, A1, A2, etc., however, are connected in series to provide multidimentional separation.

4. 1. 2. *Valve Switching*

In general, valve switching is a method of rerouting chromatographic eluents by means of valves. When combined with the automatic switching of pumps, samplers, detectors and other apparatus, valve switching allows automation of those areas of chromatography that can be time consuming and require large numbers of man hours[11].

The type of valve commonly used in valve switching is the Rheodyne 6-port, 2 way valve (Model 7000) which operates at high pressure (figure 4. 2). This valve has two internal pathways connecting ports 1 and 2, 3 and 4 and 5 and 6 in the nonswitched

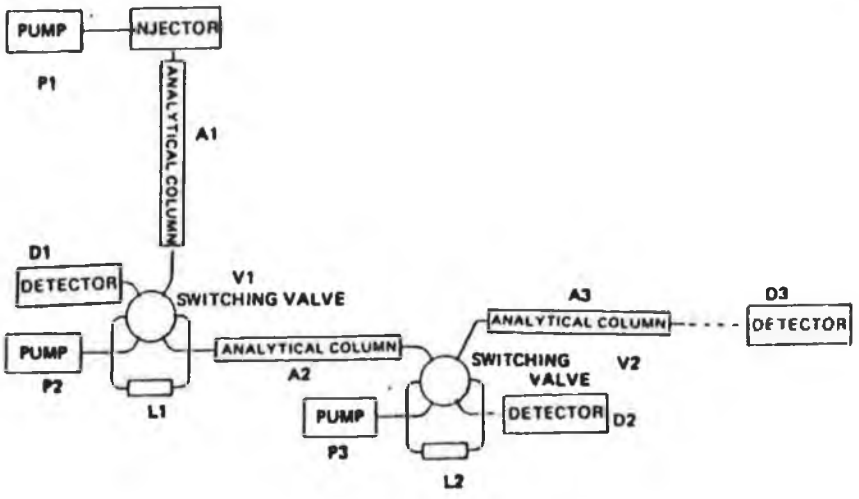


Figure 4. 1. Generalized setup of column switching. Reproduced from Chow, F., in HPLC in the Pharmaceutical Industry, Fong, G. and Lam, S. (Eds.), Marcel Dekker, Inc., New York, p. 47.

Figure 4. 2. Rheodyne 6-port switching valve.

or RESET position and in the SWITCHED position parts 2 and 3, 4 and 5 and 6 and 1 are connected. Depending on the valve position, different external connections to the valve are selected. Valve switching finds application in areas such as sample clean-up, trace enrichment, method development, sample identification, boxcar chromatography, multi-column chromatography, and incremental gradient elution[12]. Of these, sample clean-up and trace enrichment are major areas of interest.

Valve switching may be used to carry out column switching, column selection, solvent switching, solvent selection, zone cutting, auxillary pump on/off, trap solute in detector, recycle, fraction collection, sample injection, and detector selection

4. 1. 3. ***Applications of Column Switching***

Analytical samples are often so complex that one or several of the target component must be determined within a matrix of a very large number of other components that are present at higher or lower concentrations. Multi-setup methods are necessary with several purification steps before the chromatographic determination. Column switching systems should permit the multi-step to be transformed into single-step procedures by on-line purification. Since off-line techniques require further handling of the sample between the trace enrichement and the separation step, they are not highly suitable for fully automated techniques. In addition, they suffer from the disadvantages of possible sample loss and/or decomposition and are often complex and time- consuming.

The goal of all sample preparation system therefore must be to cope with these problems. The following points have to be considered if the method is to be used for routine determinations.

(i) The total analysis time should be short.
(ii) To facilitate the operation, the sample preparation should on-line to the HPLC separation system.
(iii) The sample preparation should be suitable for automation.
(iv) The sample preparation should be mild so that sensitive compounds can be handled.
(v) To minimize the problem of interferences, no loss of the separation power of the chromatographic systems is acceptable; on the contrary, an improvement in selectivity is desirable.
(vi) To reduce the time for development and optimisation of a given analytical problem the sample preparation should be generally applied in such a way that the experimental parameters can be changed on a rational basis with high flexibility.

4. 1. 3. 1. *Trace Enrichment*

Trace enrichment or preconcentration by on-line chromatographic techniques are based on the fact that the components will be retained in a narrow zone on the top of the column when a large volume of sample is pumped through the column[13,14]. Overloading can be overcome by diluting the sample before injection. If trace enrichment has to be effected for less strongly adsorbed components, the sample volumes must be smaller or the column volume must be increased to prevent "breakthrough".

Trace enrichment allows on-line concentration of samples and can be of particular use when dealing with samples from enviromental media such as sea water or mineral water[15]. A large volume of the sample is passed through a concentration column (10-100 ml) under chromatographic conditions where the sample does not elute and is adsorbed onto the column. A reversed-phase column and water are typically used. After concentration, the precolumn is switched in line with a different solvent, so that it is rapidly eluted onto another column where separation can take place. Unfortunatly trace enrichment also concentrates other materials in the sample and this may cause interference and lack of sensitivity. One way to overcome this problem is to combine trace enrichment with automatic sample clean-up.

4. 1. 3. 2. *Pharmaceutical Formulations*

Many pharmaceutical formulations such as creams, ointments, injectables, syrups, suppositories, and transdermal patches contain complex matrices. Analysis of the active components in these formulations, in many cases, requires substantial sample preparation such as liquid-liquid partition, dissolution, filtration, open column chromatography, minicolumn and cartidges, evaporation and reconstitution of sample residues[16]. These manual sample preparations are time-consuming and limit sample throughput. Obviously, it is highly desirable to automate the sample preparation. Column switching offers a great opportunity to automate these manual procedures. Kenely et al. reported on the use of a microprocessor controlled, tandem reversed-phase HPLC method for analysing both cream and ointment formulations[17 - 19].

4. 1 3. 3. *Sample Clean-up in Biological Fluids*

The use of column switching has been applied to the determination of drugs in biological matrices with much success. A survey of literature showed that more than

80% of column switching were in the area of analysing drugs in biological fluids. In many cases the biological fluid is directly injected onto the precolumn with no sample preparation whatsoever[20 - 34]. If this is the case care should be taken that there are no particles present which might clog up the column. This offers obvious advantages in terms of speed of analysis and sample throughput. Some workers favour centrifugation of plasma prior to injection[35 - 38], followed by injection of the supernatant onto the precolumn.

Sample clean-up of biological samples is critical for the following reasons[39]:

(i) complicated sample clean-up is time consuming and expensive;

(ii) there is a risk of loss of interesting compounds;

(iii) sensitive compounds may decompose during the sample treatment.

In order to prevent clogging of the precolumn and reduce the viscosity of samples, plasma may be diluted with water [40] or aqueous buffer[41] prior to injection. This procedure will increase the limit of detection and may not be possible in the case of drugs which are present in the very low nanogram range. Plasma may be deproteinized prior to injection[42 - 44] with an organic solvent such as acetonitrile which causes precipitation of plasma proteins which are then separated by centrifugation and the supernatant injected. This procedure usually results in low recoveries for the drugs since acetonitrile acts as a strong eluent and the drugs do not become trapped on the precolumn.

Of growing importance therefore in clinical applications is the principle of adsorption of the aqueous plasma matrix on a special precolumn followed by:

(i). water wash to remove proteins;
(ii). Solvent change and backflush of drugs onto the analytical column;
(iii). Isolation of the precolumn for clean-up and re-equilibration. The need here is to remove strongly adsorbed species such as lipids and fats[45].

4. 1. 4. *Considerations in Setting up a Column Switching System for Biopharmaceutical analysis*

In order to optimise the recovery and selectivity of an assay major consideration must be given to (i) size and design of the precolumn; (ii) precolumn packing material; (iii) eluent for the wash phase; (iv) frits and tubing; (v) backflush vs forward flush mode (vi). sample pretreatment; (vii) lifetime of the precolumn; (viii)

protein binding, and (viii) memory effects.

4. 1. 4. 1. *Size and Design of the Precolumn*

Of major importance is the construction and geometry of the precolumn into which the adsorbent will be loaded. The design of the precolumn can be quite diverse, ranging in length from 2 mm to 3 cm and an inner diameter anywhere from 1 mm to 4.6 mm. It is best to use as short a precolumn as possible to save on expensive adsorbent, enable easy and manual packing and to reduce backpressure[46].

In a study by Werkhoven-Goewie et al[47], different size precolumns were examined for their effects on band broadening ranging from (1.45 mm x 4.6 mm) to (41 mm x 1.1 mm) to (30 mm x 4.6 mm). Under the conditions of the study the on-line coupling of the precolumn to the analytical column has a negligible effect on band broadening and the system performance was barely dependent on the precolumn design used. For more polar compounds the design of the precolumn may be more critical, with shorter columns being preferred. Other workers[20] when comparing a precolumn of dimensions 40 x 4.6 mm with cartidges 5, 10 and 20 mm long obtained full recovery of the analyte with all four types but found the number and amount of interfering peaks was reduced considerably with shortercolumns, the 5 mm cartidge giving the best result.

For determination of clobazam and its active metabolite desmethyl clobazam, a micro precolumn was designed[48]. A hole 4.5 x 1 mm i.d. is drilled within the axis of a valve and filled with suitable packing material. This, when used in conjunction with a narrow bore column, allowed a limit of detection of 2.5 $ngml^{-1}$ for each drug in plasma after direct injection of the biological fluid, with little contribution to band broadening.

So in general best results are obtained with a small precolumn leading to fewer contribution to band broadening.

4. 1. 4. 2. *Precolumn Packing Material*

The precolumn packing material chosen should display a high affinity for the analyte during the sampling step, a low affinity during the desorption stage and have high loadability. The most widely used packings are commercially available alkyl modified silica (C_{18}, C_8 and C_2), ion-exchange resins.

Particle size exerts a profound effect on precolumn performance. Although it is known that larger size particles cause greater band dispersion,[49] most workers

favour particles with size 25-50 µm as precolumn packing in order to prevent clogging of the precolumn with particles from plasma. Employing a direct injection of plasma using particles in the range 25-50 µm with Corasil C_{18} has been chosen by a number of workers[25,50 - 54] and equivalent results obtained using these and three step method have been demonstrated[55]. In studies where a smaller particle size packing is used in the precolumn some form of sample pretreatment of plasma prior to injection was generally required.

4. 1. 4. 3. *Choice of Eluents*

The eluent must be chosen in such a way that the drug and its metabolites are retained while the bulk of unwanted constituents from plasma are flushed through. The mobile phase for flushing must be pure otherwise impurities will be concentrated and later eluted onto the analytical column where they will cause interferences. Care must also be taken that breakthrough does not occur. This is a function of the sample capacity of the concentration column which depends on the composition of the sample and the eluting strength of the solvent.

4. 1. 4. 4. *Frits and Tubing*

It is recommended[25] that the steel capillaries from the injector to the precolumn should be of wider bore than those from the precolumn to the analytical column. This is especially important for viscous plasma samples. Zech and Huber has discussed to the importance of using sieves (although with an adequate filter function) instead of frits in order to avoid column blocking[36]. Sieves of the precolumn should not be too fine, sieves of at least 18 um pore size are recommended[25]. Also to guarantee a homogenous distribution of the adminstered plasma to the top of the guard column, crossed grooves should be fitted onto the surface of the end fitting which crosses the top of the precolumn[25]. Other workers have also found screens to be better than frits[56]. We found in our laboratory that if frits are used they need to be changed frequently.

4. 1. 4. 5. *Back flush/Forward flush*

Backflush mode for the desorbtion of the drugs from the precolumn to the analytical column is by far the favoured mode[20,35,44,45]. Backflushing prevents contamination of the precolumn and minimizes band broadening. It can be shown by the use of a strong fluorescent substance that the analytes are concentrated in a small region at the top of the precolumn so that their removal by backflushing minimizes their dispersion[57]. In the "elute through" mode these drugs initially in a narrow band

at the top of the precolumn must pass through say the full 2 cm of packing, experiencing all the band dispersion effects of the column[23]. In the case of the few studies[26,36,42,22] where forward flushing has been employed, it was advocated noted that the precolumns should be very small, less than 1 cm in length, so that the overall efficiency does not deteriorate as one might expect in the straight flush mode. Forward flushing does prevent the build up of contaminants at the head of the analytical column. However, in general, backflushing is to be favoured.

4. 1. 4. 6. *Precolumn Lifetime*

Although the lifetime of the precolumn will depend on a number of factors such as the particle size and type of the packing, the porosity of the frits, sample pretreatment and the plasma in general, each precolumn will be able to handle between 10-20 ml of plasma with the average being 15ml. The lifetime of the column depends on the total amount of plasma injected rather than the size of each injection. A precolumn therefore that can handle 400 x 50 µl injections will only handle 80 x 250 µl injections. With larger injection volumes, the reconditioning of the precolumn between injections is particularly important. Eventually the surface of the adsorbent in the precolumn will become modified with matrix elements from plasma resulting in a loss of effeciency and a decrease in recovery.

4. 1. 1 4. 7. *Memory Effects*

Memory effects from the precolumn ("substance bleeding") may be checked by injecting blank solutions between approximately every 10 injections. No memory effects should be observed. The switching procedures for clean-up of biological fluids in our laboratory will be described in Section 4. 2. 2. 2.

In this chapter, an analytical method for the determination of terbutaline in plasma is described and, validated. The method allows direct injection of plasma using a column switching technique. The method is based on the enrichment of the drug on a reversed-phase concentration column packed with Corasil RP C_{18}. The enriched drug was then separated, using the backflush mode on a Spherisorb ODS column using an isocratic phosphate buffer-methanol-sodium dodecyl sulphate-diethylamine in a ratio of 45:55:0.5:0.02 (v/v). Hitherto, only methods based on gas chromatography- mass spectrometry (GC-MS)[58 - 60] and electrochemical detection[2] using a thin layer amperometric detection employing a macro glassy carbon electrode, have proved suitable for the determination of low levels of terbutaline in human plasma. The method was found to compare favourably with a glassy carbon electrode when applied to the same conditions of analysis and

separation and providing a limit of detection of at least 0.8 $ngml^{-1}$. In addition, the on-line method of sample preparation is less laborious and time consuming than methods employing liquid-liquid extraction, thus providing an assay which is sensitive, cost-effective and expedient in its execution.

4. 2. EXPERIMENTAL

4. 2. 1. *Materials and Reagents*

Terbutaline was obtained from Sigma (Poole, Dorset, England), and analytical grade sodium dihydrogen phosphate from Merck (Darmstatd, Germany). Orthophosphoric acid and diethylamine (Analar grade) were supplied by BDH (BDH Ltd., Poole, Dorset, UK) and HPLC-grade methanol was supplied by Labscan Analytical Sciences, Dublin, Ireland. Human plasma, obtained from Beaumont Hospital (Dublin, Ireland) was frozen until required, and thawed at room temperature. Water was distilled and then further purified using a Milli-Q water purification system (Millipore, Milford, MA, USA). The preconcentration column was packed with Corasil (Waters, Milford, MA, USA) C_{18} material (37 µm).

Carbon fibres, 14 µm in diameter, were obtained from Avco, Lowell, MA, USA. The surface of these fibres has no external coating. Silver epoxy was purchased from RS components, Corby, Northants, UK, and the glassy carbon used in the comparison studies was obtained from Princeton Applied Research (Princeton, NJ, USA).

4. 2. 2. *Instrumentation and Operating Conditons*

4. 2. 2. 1. *Chromatography*

Chromatographic separation was based on the work of Jarvie et al.[61] following a slight modification to the ratio of methanol to aqueous component in order to obtain a sutitable retention time for terbutaline. The drug was separated on a Spherisorb ODS (10 µm) column (25 cmx4.6 mm i.d.) which was protected by a guard column packed with C_{18} packing material. The mobile phase consisted of a mixture of 0.067 M phosphate buffer, pH5, methanol, 40 g/l sodium dodecyl sulphate (SDS) and diethylamine (DEA) in a ratio of [45:55:0.5:0.02]. Prior to the addition of SDS and DEA the mobile phase was filtered and degassed by sonication. The prepared eluent was delivered by a Waters (Milford,MA,USA) Model 501 HPLC pump at a flow rate of 1 ml/min. Before use, the column was conditioned by passing 100 ml mobile phase adjusted to contain 10 times the standard eluent concentration of SDS and 2.5 times the standard eluent concentration of DEA. Sample introduction was via a Rheodyne (Cotati, CA, USA) Model 7010 injection valve, fitted with a 20 ul loop

for direct injection.

4. 2. 2. 2. *Column Switching Extraction Procedure*

For the purposes of extraction by column switching, the injector was fitted with a 1 ml loop and a second pump (pump A) and the concentration column were connected to the analytical assembly via a Rheodyne Model 7000 six-port switching valve. The loading/washing eluent delivered by pump A was filtered degassed deionised water. The spiked plasma sample was injected through the injector port and washed by the water in pump A onto the concentration column (position 1, Figure. 4. 3). The drug was held on the concentration column while the other components in plasma are eluted to the drain. Meanwhile the eluent from pump B is passing through the analytical column and out to waste. On switching the valve after a pre-determined wash time (position 2, Figure. 4. 3), the eluent from pump B elutes the drug in backflush mode, which have been held on the concentration column, onto the analytical column where it was separated. In using the column switching valve the following variations in sample handling were studied: (i) sample pretreatment, (ii) concentration column packing, (iii) wash time and flow rate and (iv) analytical column.

4. 2. 2. 3. ***Amperometric Measurements***

Oxidative amperometric measurements were performed using an EG&G Princeton Applied Research (Princeton, NJ, USA) Model 400 EC potentiostat connected to the flow cell described in Section 3. 2. 2 by crocodile pins. The drug was detected amperometrically by employing potential of +1.3 V at the working electrode. The resultant signals were recorded on a Philips Model PM8261 x-T recorder (Eindhoven, the Netherlands) at a chart speed of 300 $mmhr^{-1}$. The peak currents as a function of concentration were then measured for quantitative analysis.

4. 2. 2. 4. ***Standard Solutions and Calibration Curves***

Stock solutions equivalent to 0.2 mg/ml of the drug in water were freshly prepared. These were diluted and added to drug-free plasma aliquots to generate spiked plasma standards in the concentration range 1-35 $ngml^{-1}$. Each calibration point was run in triplicate over three consecutive days.

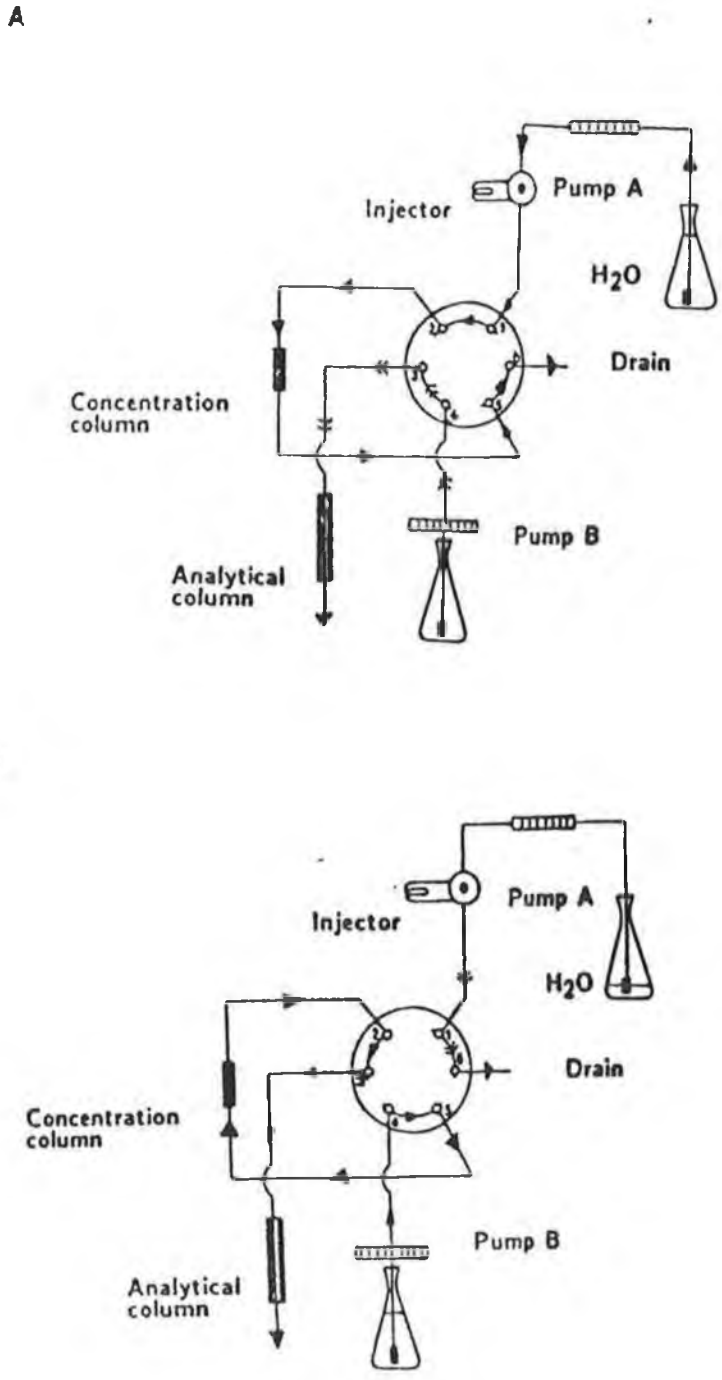


Figure 4. 3. Column switching assembly, used for retaining terbutaline in the pre-concentration column (a) and eluting the drug from the pre-concentration column (b). Reproduced from Kelly, M. and Smyth, M., J. Pharm. Biomed. Anal., 7 (1989) 1757.

4. 3. RESULTS AND DISCUSSION

4. 3. 1. *Hydrodynamic Voltammetry*

The hydrodynamic voltammogram for terbutaline was obtained by injecting 35 $ngml^{-1}$ of the drug into the chromatograph with the detector set at different working electrode potentials vs. Ag/Ag_3PO_4. The hydrodynamic voltammogram shown in Figure 4. 4 indicates that a working potential of +1.4 V was required for maximum response. When the potential was greater than +1.3 V, both background current and the noise level increased rapidly. Accordingly, a potential of +1.3 V was used in subsequent experiments.

4. 3. 2. *Optimisation of Extraction Procedure*

4. 3. 2. 1. *Pre-column Selection*

Terbutaline can be extracted into organic solvents, either as a zwitterion[59], or as an ion-pair[62,63]. Such off-line methods are time consuming, and may cause loss of the analyte through the formation of emulsions or artefacts, adsorption onto glassware or volatilisation at the evaporation stage. Solid-phase extraction was chosen to circumvent some of these problems and the column switching technique described in this chapter provided the requisite extraction selectivity in a reasonable time frame.

The first step in the set-up of the switching system involved selection of a suitable pre- (or concentration) column which would retain terbutaline completely, and which would allow the interfering substances in plasma to be completely removed during the wash step. The diameter of the precolumn should be similar to or smaller than that of the analytical column, and the precolumn should be as short as possible. The retention of terbutaline on the precolumn should ideally be similar to, or lower than, that on the analytical column in order to prevent band-broadening problems during the desorption and actual separation step. As regards the small size of the precolumn, this aspect is of importance because of supression of band broadening due to the precolumn, a low pressure drop and ,thus, a high flow rate during the separation step, ease of packing and replacement, and material saving. A short stainless-steel pre-column (10 mm x 1.5 mm i.d.) was chosen for this purpose. The precolumn served as a preconcentration and clean-up column and as a guard column to prevent contamination of the analytical column. The use of a precolumn on which the sample compound is preconcentrated before elution onto the analytical column provides a precise means of sample introduction and leads to high precision in quantitative determinations[23,64]. It is essential that terbutaline has a greater affinity

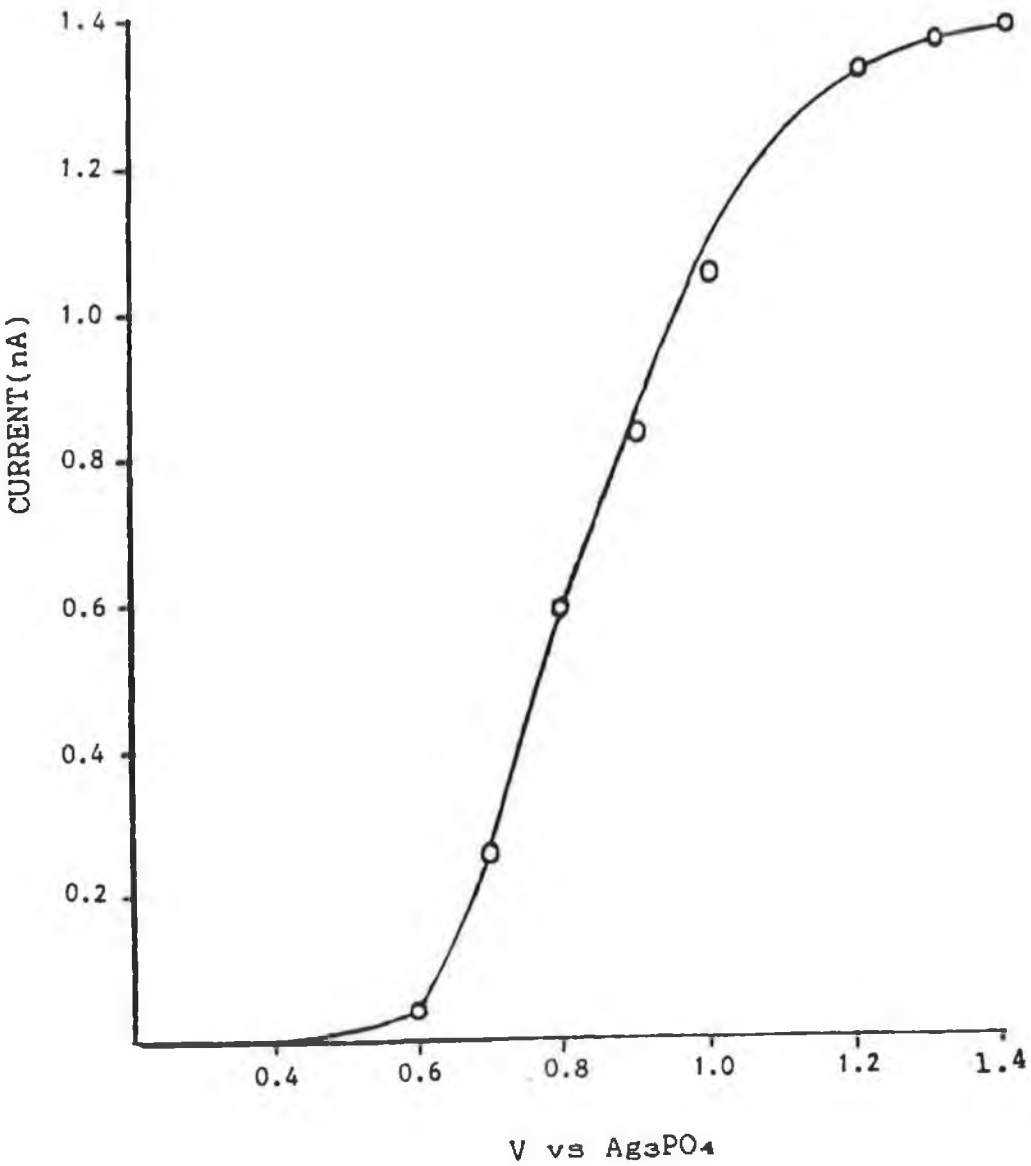


Figure 4. 4. Hydrodynamic voltammogram of terbutaline obtained by injections of 35ng into the chromatograph with detection at carbon fibre electrode at different potentials (vs.Ag/Ag_3PO_4).

for the sites on the surface of the packing than for protein in plasma. Otherwise recovery of the drug from the precolumn will be low due to plasma protein binding. In order to asses the extent of binding, the peak current obtained for a series of recovered authentic standards were compared with the peak currents obtained for a series of recovered plasma standards from the precolumn. Pellicular C_{18} material showed the most favourable retention characteristics for terbutaline when compared to C_8 or cyano packing materials and proved to be the best among the packings tried in terms of recovery and cleanliness of chromatogram, and was therefore chosen as the precolumn packing.

The next stage was to find two compatible eluents of different elutropic strengths[65]; one weakly eluting solvent to concentrate terbutaline on the pre-column and a second strongly eluting solvent to elute terbutaline off the pre-column and onto the analytical column. The choice of solvents is also important in terms of their mutual miscibility, as even slight incompatibility could result in a slug of solvent travelling down the analytical column partially carrying sample components which may cause band spreading. The washing/loading eluent (designated mobile phase A) ideally would have poor elution capability on the pre-column in order to ensure maximum concentration of the sample with minimum band broadening. Degassed deionised water was found to provide adequate concentration and was compatible with the aqueous based analytical mobile phase (mobile phase B) which effected full elution of the drug from the concentration column, and any possible band broadening was minimised by eluting in a backflush direction. Some workers may add a small amount of some organic modifier e. g. 4% acetonitrile to the wash phase but this may result in lower recovery for a drug[66].

Wash times were varied from 1 minute up to 10 minutes, and flow rates from 0.5 to 1.5 $mlmin^{-1}$. Variation in these factors did not affect the chromatograms obtained to a large extent, but the sharpest peaks and cleanest chromatograms were obtained for a wash time 90 seconds and a flow rate of 1 $mlmin^{-1}$. Shorter wash times result in very large plasma peaks while longer wash times gave rise to band broadening. Typical chromatograms of drug-free plasma and a plasma extract spiked with terbutaline at the 30 $ngml^{-1}$ level are shown in Figure 4. 5a and 4. 5b respectively.

4. 3. 2. 2. *Boundary conditions*

Consequently, after preconcentration of the samples, a wash step is absolutely necessary to remove a major part of interesting compounds. To determine the optimum wash time for the samples on the concentration column, plasma aliquots containing 10 $ngml^{-1}$ of the drug were injected onto the concentration column. The

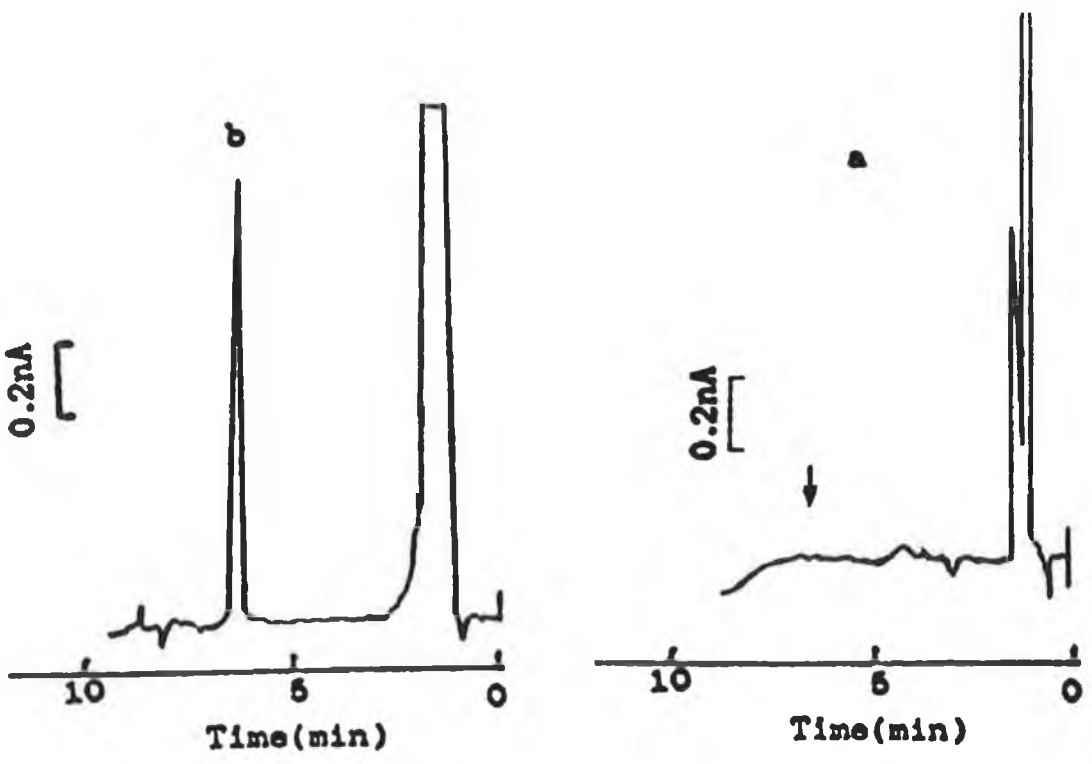


Figure 4. 5. HPLC chromatogram obtained by analysis of drug-free plasma extract (a), and an extract of plasma spiked with 30ngml^{-1} of terbutaline (b).

wash time (defined as the length of time between injection and switching of the valve) was varied between 1 minute and 3 minutes, and it was found that a maximum response was obtained at 1.7 minutes, in that it provided good clean up of the plasma components without causing the drug to elute. Seven replicate injections of plasma samples containing 10 $ngml^{-1}$ terbutaline yielded a RSD of 5%.

4. 3. 3. ***Electrode pretreatment***

The reason electrode pretreatment is required is that the surface of the changes with time due either to the adsorption of species from solution or chemical changes on the electrode surface itself. These changes often result in variations in sensitivity, reproducibility and selectivity.

The electrochemical pretreatment regeim which improved the peak current and reduced the noise to its orginal level, involved the anodisation of the microelectrode at +1.4V for 10 sec, then cathodisation at -1.4V for 10 sec vs. Ag/Ag_3PO_4 followed by equilibration for 5 minutes and the effect of the procedure described above is shown in Figure 4. 6b. The effect of pre-treating the electrode is to increase its current response, which is probably due to activation of the surface leading to the production of quinoidal functionalities. It also seemed to stabilise the noise contribution due to the charging current.

4. 3. 4. ***Assay Performance***

The linearity of the method was determined by constructing a calibration curve in the concentration range 0-35 $ngml^{-1}$ terbutaline in plasma, and as shown from the data in Table 4. 1, the method is linear over this range with correlation coefficients of greater than 0.999 on each of three successive days.

Table 4. 1. Calibration Data For Terbutaline

Day	Equation of the regression line	Correlation coefficient,r
1	y= 0.0390 + 0.0067x	0.9997
2	y= 0.0384 + 0.0041x	0.9998
3	y= 0.0370 + 0.0027x	0.9999

The precision of the assay was assessed by carrying out seven replicate runs over the

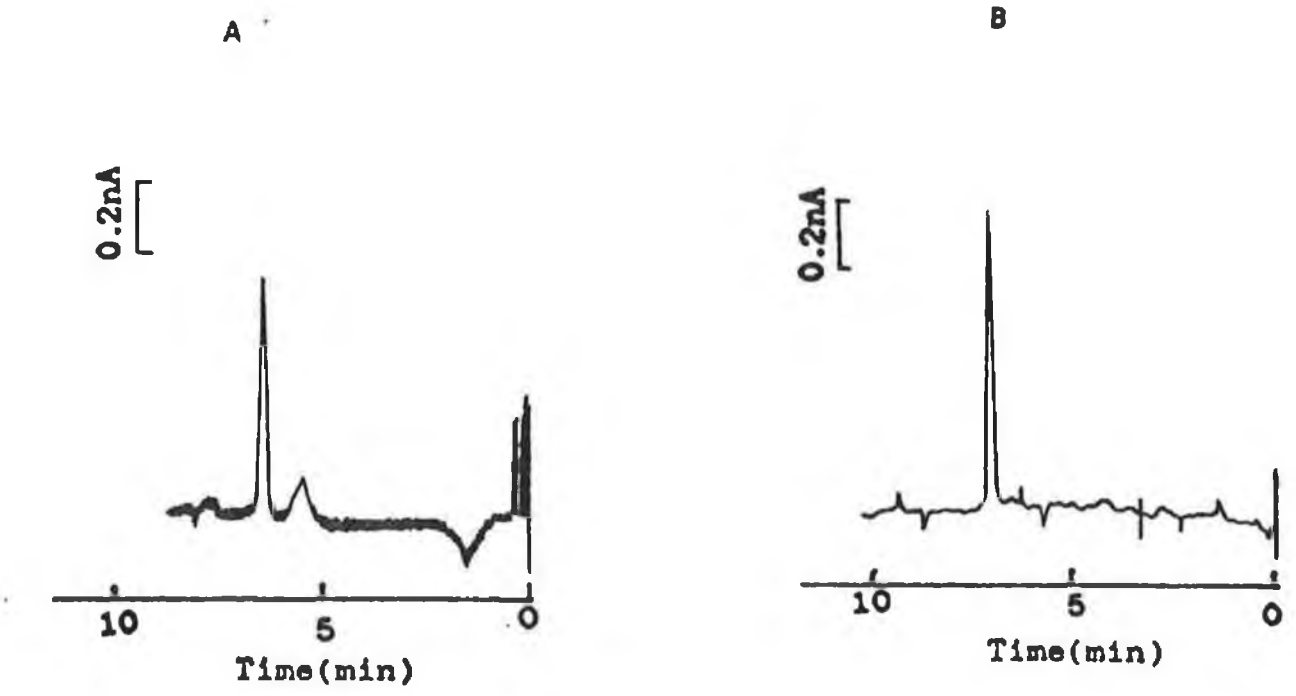


Figure 4. 6. HPLC chromatograms of 20ng terbutaline obtained by the analysing the drug before (a) and after (b) electrochemical pretreatment of the working electrode surface.

concentration range 3, 5, 10, 20 $ngml^{-1}$ on each of three consecutive days. Between batch or intra-assay variability was assessed by constructing a calibration curve based on the mean (of seven) detector response values at each concentration level in the range 3, 5, 10 and 20 $ngml^{-1}$. Individual peak current values then interpolated on the regression line to yield 7 new values of x (i.e. "amount found") at each concentration. The mean, SD and CV for each amount found were then calculated with the intra-assay being expressed as the mean CV over the entire concentration range. Between-batch variability was calculated by constructing a calibration curve on each of 3 consecutive days in the same concentration range as above. Peak current values were interpolated on the individual regression lines to yield three new values of amount found at each concentration level. Between-batch variability was then calculated by obtaining the mean CV over the calibration range. Results from this experiment are presented in Tables 4. 2(a) and (b) and they show that the within-day CVs were 7.2, 5.3, 3.0 and 2.25% for the 3 ,5 , 10 and 20 $ngml^{-1}$ cocnetrations, respectively with a mean coefficient of variation of 4.4%. The corresponding CV values for intra- (between-day) assays were 8.1, 7.0, 4.3 and 3.0% respectively with a mean coefficient of variation of 5.6%. Recovery of terbutaline from plasma was assesed by comparing the peak current of extracted plasma samples containing 3, 8, 15, 30 $ngml^{-1}$ terbutaline with the peak current of authentic (unextracted) standards, which were directly injected (i.e. without column-switching) onto the analytical column and at the concentration levels. The results in Table 4. 3 show that drug recovery was greater than 86%, and usually greater than 91%. The minimum detectable concentration was found to be 0.8 ng of terbutaline per 1 ml of plasma, at which the signal to noise ratio was 3:1. This limit of detection compares favourably with previous methods for determination of terbutaline in biological fluids and the method is capable of monitoring the therapeutic range of the drug.

TABLE 4. 2(a). Intra-Assay (Within-batch variation)

Amount added ($ngml^{-1}$)	Mean amount found(ng)±SD n=7, $ngml^{-1}$	Coefficient of variation(CV),%
3	2.9±0.20	7.2
5	4.7±0.24	5.3
10	9.6±0.32	3.0
20	19.9±0.40	2.3
Mean		4.4

Table 4. 2(b). (Between-batch Variation)

Amount added ($ngml^{-1}$)	Mean amount found(ng)±SD n=3, $ngml^{-1}$	Coefficient of variation(C.V), %
3	2.96±0.21	8.1
5	5.20±0.33	7.0
10	10.10±0.42	4.3
20	20.20±0.50	3.0
Mean		5.6

Table 4. 3. Recovery Studies

Terbutaline concentration ($ngml^{-1}$)	Detector response(nA) Extract	Authentic	Recovery (%)	CV (%)
3	0.12	0.14	86	9.2
5	0.59	0.63	93	7.4
8	0.31	0.34	91	6.0
30	1.21	1.26	96	3.5

4. 3. 5. ***Comparison with Conventional Glassy Carbon Based Detector***

The performance of the microelectrochemical flow cell was compared to a conventional macro-glassy carbon electrode flow cell, the electrode of which was polished successively with small particle size silicon carbide, alumina, diamond paste and then subjected to ultrasonic cleaning[67].

As shown in Figure 4. 7, the detection limit of the carbon fibre electrode was shown to be approximately half that obtained using the macro glassy carbon electrode. Similar findings have been reported by Luscombe et al. in the determination of copper in urine using platinum disc microelectrodes instead of a conventional carbon disc macroelectrode[68].

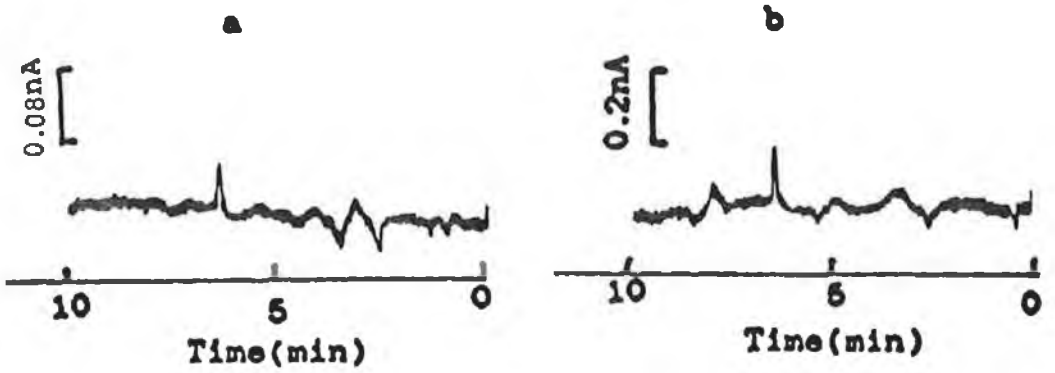


Figure 4. 7. Chromatograms obtained by analysis of 1ngml^{-1} terbutaline using (a) the carbon fibre microelectroe at +1.3V vs Ag/Ag_3PO_4; and (b) 2ng terbutaline using a macro-glassy carbonelectrode at +1.3V vs Ag/Ag_3PO_4.

4. 4. CONCLUSIONS

An HPLC method for the determination of terbutaline in human plasma based on pre-column switching and electrochemical detection using a carbon fibre flow cell has been described. The column switching technique permitted selective retention of terbutaline on the pre-column and provided satisfactory clean-up to avoid any interferences due to endogenous plasma components. The limit of detection of the method was found to be 0.8 $ngml^{-1}$, the mean overall coefficient of variation was 5.60%, and drug recovery was in excess of 86% over the concentration levels investigated.

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Chapter 5

Simultaneous Determination of Salbutamol and Terbutaline at Overdose Levels in Human Plasma by HPLC with Electrochemical Detection

5. 1. INTRODUCTION

The monitoring of drugs in cases of overdose is one of the oldest areas of drug measurement in clinical medicine. In cases of overdosage with drugs the prime reason for an analysis is to establish which drug has been overdosed in order to apply the appropriate remedial action. The need will arise if the patient is comatose and cannot tell the physician himself and there is no evidence (medicine bottles, known history) to suggest the immediate cause.

Salbutamol and terbutaline are directly acting sympathomimetic amines, their main action being on the adrenergic receptors in the bronchi and respiratory tract. Both drugs induce bronchodilation and inhibit bronchospasm in doses which do not produce marked cardiac acceleration. They are extensively used in the treatment of asthma, chronic bronchitis, emphysema, and other bronchopulmonary disorders involving bronchospasm[1]. Overdoses of these drugs can cause palpitations and tachycardia, and excessive use of sprays containing these drugs can be so severe, that a relationship between intensity of action and drug dose is usually evident. That is as the dose is increased, the intensity of the undesirable side-effects also increases, sometimes resulting in fatal consequences[2]. It was suggested that parenteral β-adrenergic simulants, including salbutamol, should be used with caution in severe bronchial asthma since high plasma-concentration of free fatty acids had been associated with the development of ventricular arrythmias and other cardiotoxic effects[3].

In clinical toxicology, the first goal of the analyst must be to identify the drug or toxic agent. In clinical situations, when rapid and accurate analyses are available, they have proven very helpful to the healthcare team. These results have helped guide therapy for acutely poisoned patients, have facilitated adjustments in drug therapy, and have provided reliable evidential information for rehabilitation, probation, and medicolegal programmes[4]. In most cases, analysis of plasma concentrations is usually taken since this can normally be related to the concentration of the drug at the site of action[5]. LC-ED has the ability to measure several compounds simultaneously[6] and the potential recovery of 90% of the analytes.

For salbutamol and terbutaline, simultaneous methods based on gas chromatography-mass spectrometry[7,8] and HPLC with fluorescence detection[9] have been described in the literature, but the instrumentation is complex and sample preparation can be lengthy. A previous method based on high-performance liquid

chromatography with electrochemical detection (LC-ED) using a glassy carbon electrode following a solvent extraction procedures has been described in the literature[10]. Simultaneous isolation, separation and determination of salbutamol and terbutaline in human plasma at overdose levels is described in this chapter.

The method described below circumvents the "classical" sample purification approach involving extraction, by direct purification and enrichment of the sample on the pre-column. The good recovery, low cost, speed and simplicity of distinguishing the two drugs based on differing retention times, without changing the chromatographic conditions between samples, is particularly important for a proper study of the features of poisoning in relation to plasma concentrations of poisoned patients and the choice of the appropriate levels of antidote necessary for treatment of such cases.

5. 2. EXPERIMENTAL

5 2. 1. *Materials and Reagents*

Epinepherine, diazepam, methyl dopa, nor-epinepherine, paracetamol, phenylepherine and terbutaline were obtained from Sigma (Poole, Dorset, England). Salbutamol was kindly supplied by Dr. J. Bloomfield (Trinity College Dublin). Human plasma, obtained from Beaumont Hospital (Dublin, Ireland) was frozen until required, and thawed at room temperature. Water was distilled and then further purified by passing through a Milli-Q water purification system (Millipore, Milford, MA, USA).

5. 2. 2. *Instrumentation and Operating Conditions*

5. 2. 2. 1. *Construction of Carbon Fibre Flow Cell*

The preparation of the carbon fibre microelectrode flow cell was carried out using a procedure described previously in Section 3. 2. 2. Oxidative amperometric measurements were performed using an EG&G Princeton Applied Research (Princeton, NJ, USA) Model 400 EC potentiostat connected to the flow cell by crocodile clips. The resultant signals were recorded on a Philips Model PM8261 X-t recorder (Eindhoven, The Netherlands) at a chart speed of 300 mm/hr. The experimental techniques employed in flow measurements are discussed in section 4. 2. 2. 2 and specialized data on the design and operation of the detector are also given in chapter 3.

5. 2. 2. 2. *Standard solutions and calibration curves*

Stock solutions equivalent to 0.2 mg/ml of these drugs were freshly prepared in deionised water. These were diluted and added to drug-free plasma aliquots to generate spiked plasma standards in the concentration range 20-100 ng/ml. Each calibration point was run in triplicate over three consecutive days.

5. 2. 2. 3. *Optimisation of Detection Potential*

In order to determine the optimum applied potentials for salbutamol and terbutaline, hydrodynamic voltammograms were obtained by repeated injections of salbutamol and terbutaline standard solution containing 60 ng/ml of each drug into the chromatography system and recording the peak current, at fixed applied potentials.

5. 2. 2. 4. *Examination of Interferences*

The effect of interference of caffeine, diazepam, epinepherine, isoprenaline, metadrenaline, methyldopa, nor-epinepherine, nor-metadrenaline, orciprenaline, paracetamol, phenobarbiton, phenytoin phenylepherine, propranolol, salicylate and theophylline in the proposed method was studied by the addition of such drugs to the plasma samples already spiked with salbutamol and terbutaline at a concentration levels similar to those of both drugs (70 ng/ml plasma).

5. 3. RESULTS AND DISCUSSION

5. 3. 1. Optimisation of Chromatography/Detection Conditions

Figure 5. 1 shows the hydrodynamic voltammograms of salbutamol and terbutaline in the chromatographic system with 0.067 M phosphate buffer, pH5, methanol, SDS and DEA 45:55:0.5:0.02 (v/v) as mobile phase, The plots show a distinct sigmoidal dependence upon applied potential. A working potential of +1.30 V was thus chosen for the quantitation of both drugs. The required sensitivity, selectivity and reproducibility for this analysis were achieved by pre-anodisation of the microelectrode at +1.40 V for 10 s, then cathodisation at -1.40 V for 10 s vs. Ag/Ag_3PO_4 followed by equilibration for 5 min. Figure 5. 2 shows the effect of this electrochemical pretreatment.

Pretreatment of plasma was not required before injection into the chromatographic system, due to the column switching operation (Figure 4. 3). It was necessary that the mobile phase and the precolumn packing be chosen in such a way that the drugs were completely retained on the precolumn while the interfering components of

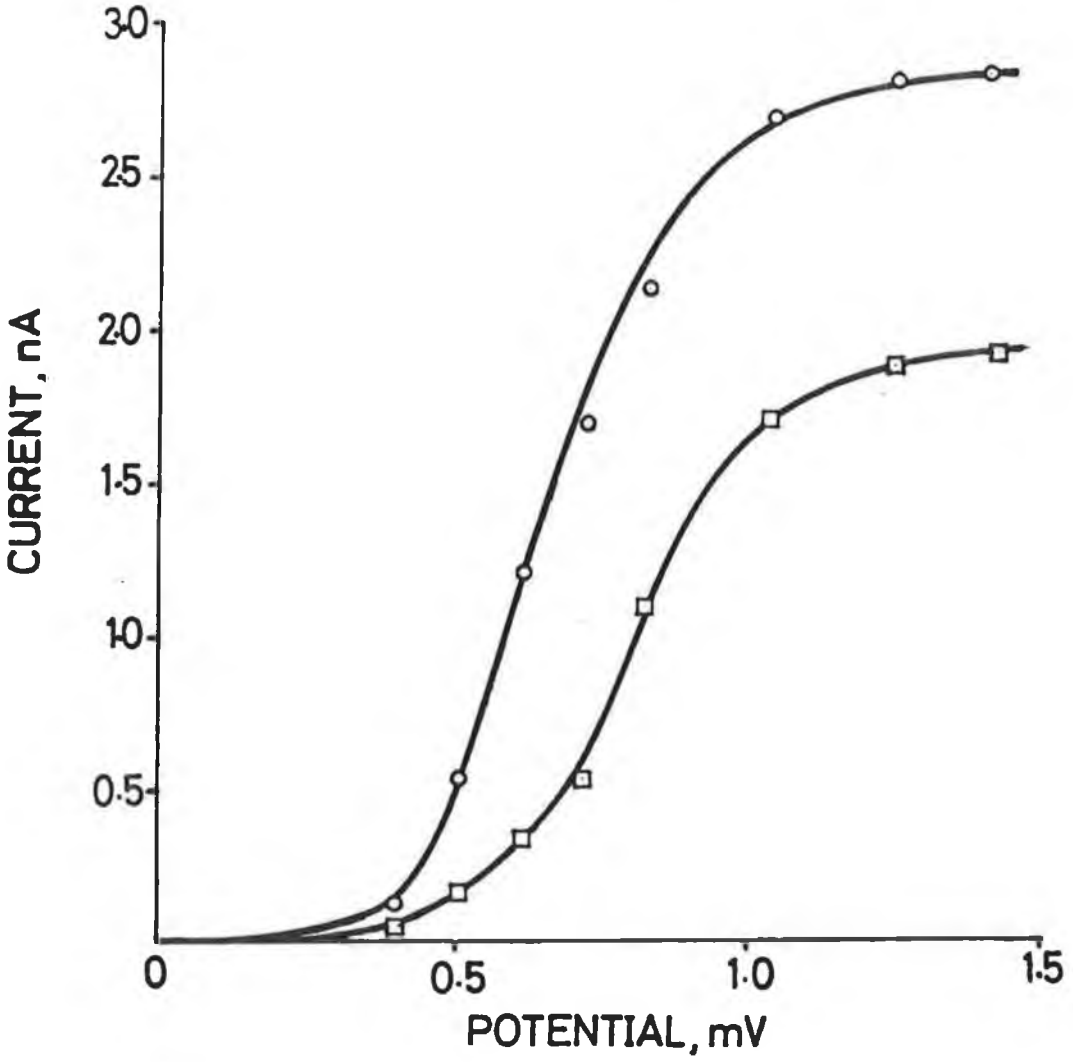

Figure 5. 1. Hydrodynamic voltammograms of salbutamol (o) and terbutaline (□) obtained by injections of 60ng drug substance into the chromatograph with detection at carbon fibre working electrode at different potentials (vs. Ag/Ag_3PO_4).

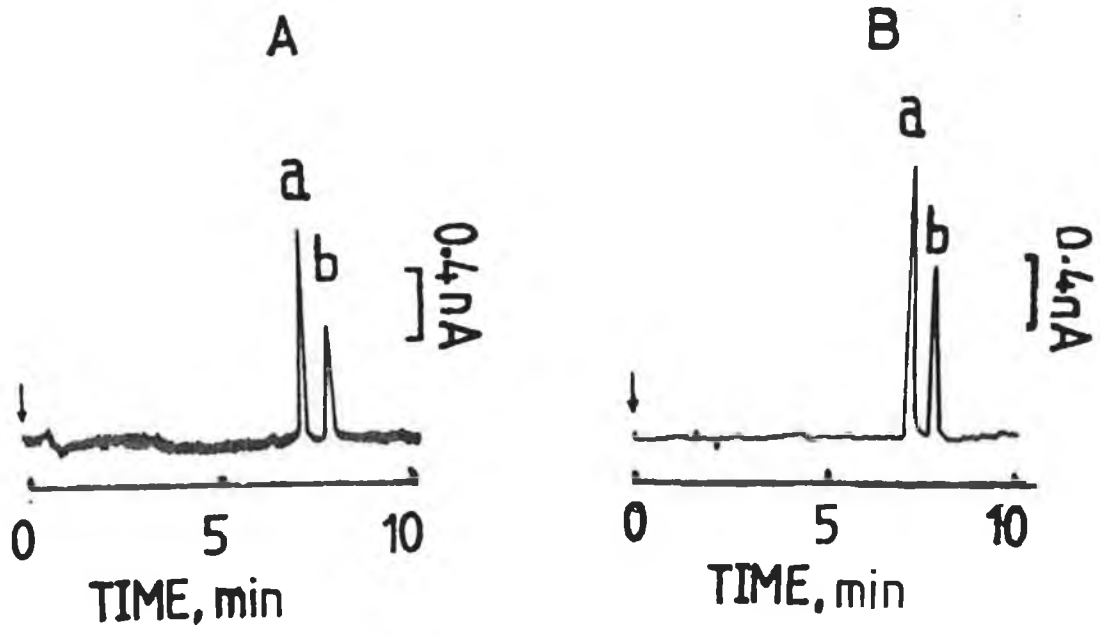

Figure 5. 2. HPLC chromatograms of 40ng/ml terbutaline (a) salbutamol (b) obtained by analysis before (A) and after (B) electrochemical pre-treatment of the working electrode surface.

blood plasma eluted to waste. Pellicular C_{18} material exhibited the most favourable retention characteristics for both drugs when compared to C_8 or cyano packing materials and was therefore chosen as the pre-column packing. The loading/washing eluent delivered by pump A was filtered degassed deionised water. Degassed deionised water showed good compatibility with the aqueous-based analytical mobile phase. It also provided adequate washing in 1.7 min of gross plasma interferents. Seven replicate injections of plasma samples containing 30 ng/ml plasma salbutamol and terbutaline yielded a RSD within 5% for both drugs.

5 3. 2. ***Study of Analytical parameters***

The drugs were detected amperometrically by employing a potential of +1.3 V at the working electrode. The peak currents (measured as peak heights on the recorder) as a function of concentration were then measured for quantitative analysis. The peak current of both drugs were plotted separately versus concentrations in the range 20-100 ng/ml plasma, Regression analysis of the data was used to establish the calibration curve. As shown from the data in Table 5. 1, the method is linear over this range with correlation coefficients of greater than 0.992 over three days during which this experiment was carried out. The intra-assay precision (Table 5. 2) was within 7.8% for salbutamol and 5.6% for terbutaline, and the inter-assay precision (Table 5. 3) was within 6.7% for salbutamol and 5.5% for terbutaline. The accuracy, which was defined as the percentage difference between the mean concentration, was 6.8 and 5.5% or better for salbutamol and terbutaline respectively.

Table 5. 1. Linear regreession data (y= mx + b) and correlation coefficients (r) for plots of salbutamol and terbutaline.

(a) Salbutamol

	Equation of the regression line	correlation coefficient, r
Day 1	y= 0.027 + 0.022x	0.9998
Day 2	y= 0.016 + 0.024x	0.9980
Day 3	y= 0.040 + 0.021x	0.9990

(b) Terbutaline

	Equation of the regression line	Correlation coefficient,r
Day 1	y= 0.033 + 0.034x	0.9990
Day 2	y= 0.032 + 0.036x	0.9998
Day 3	y= 0.084 + 0.032x	0.9920

Table 5. 2. Intra-assay precision

(a) Intra-assay precision for salbutamol

Amount added $ngml^{-1}$	Mean amount found ±SD n=6, $ngml^{-1}$	Coefficient of variation(CV),%	Accuracy %
20	18.4±0.7	4.3	7.8
50	47.5±1.7	3.9	5.0
70	66.4±3.0	5.1	5.1
80	75.8±2.0	3.1	5.2
100	97.2±4.5	5.1	2.8

(b) Intra-assay precision for terbutaline

Amount added $ngml^{-1}$	Mean amount found±SD n=6, $ngml^{-1}$	Coefficient of Variation(CV)%	Accuracy %
20	19.2±0.7	3.8	3.9
50	47.5±1.1	2.6	5.0
70	67.4±2.5	4.0	4.0
80	76.2±2.6	3.8	4.7
100	94.4±2.8	3.2	5.6

Table 5. 3. Inter-assay precision

(a) Inter-assay precision for salbutamol

Amount added ($ngml^{-1}$)	Mean amount found±SD n=3, $ngml^{-1}$	Coefficient of Variation(CV),%	Accuracy %
20	18.4±0.8	5.2	6.8
50	47.7±1.5	3.9	4.7
70	67.0±4.3	6.0	4.3
80	75.4±4.0	7.0	5.8
100	95.7±3.4	4.4	4.3

(b) Inter-assay precision for terbutaline

Amount added ($ngml^{-1}$)	Mean amount found±SD n=3, $ngml^{-1}$	Coefficient of Variation(CV), %	Accuracy %
20	19.0±0.7	4.5	4.8
50	47.6±1.2	3.4	4.9
70	66.9±2.7	5.0	4.4
80	76.0±2.5	4.0	5.3
100	94.5±3.0	3.7	5.5

Interference studies on plasma samples co-spiked with caffeine, diazepam, paracetamol, phenobarbiton, phenytoin, propranolol, salicylate and theophylline gave chromatograms free from any interference. Epinepherine, isoprenaline, metadrenaline, methyldopa, norepinephrine, nor-metadrenaline, orciprenaline and phenylepherine were detected with retention times different from those of salbutamol and terbutaline. A typical chromatogram for a mixture of norepinephrine, salbutamol and terbutaline in plasma at the 80$ngml^{-1}$ level is shown in Figure 5. 3. The retention times of epinephrine, methyldopa and phenylephrine were 5.0, 1.0 and 6.5min respectively.

The total absolute recovery of the method was tested on spiked plasma samples, by comparison with directly injected aqueous samples, The recovery of both drugs from human plasma at 30, 60, and 90 $ngml^{-1}$was always more than 94%. The limit of detection for the assay was set at 1ng and 0.8 ng/ml plasma for salbutamol and terbutaline respectively (signal-to-noise ratio of 3:1)

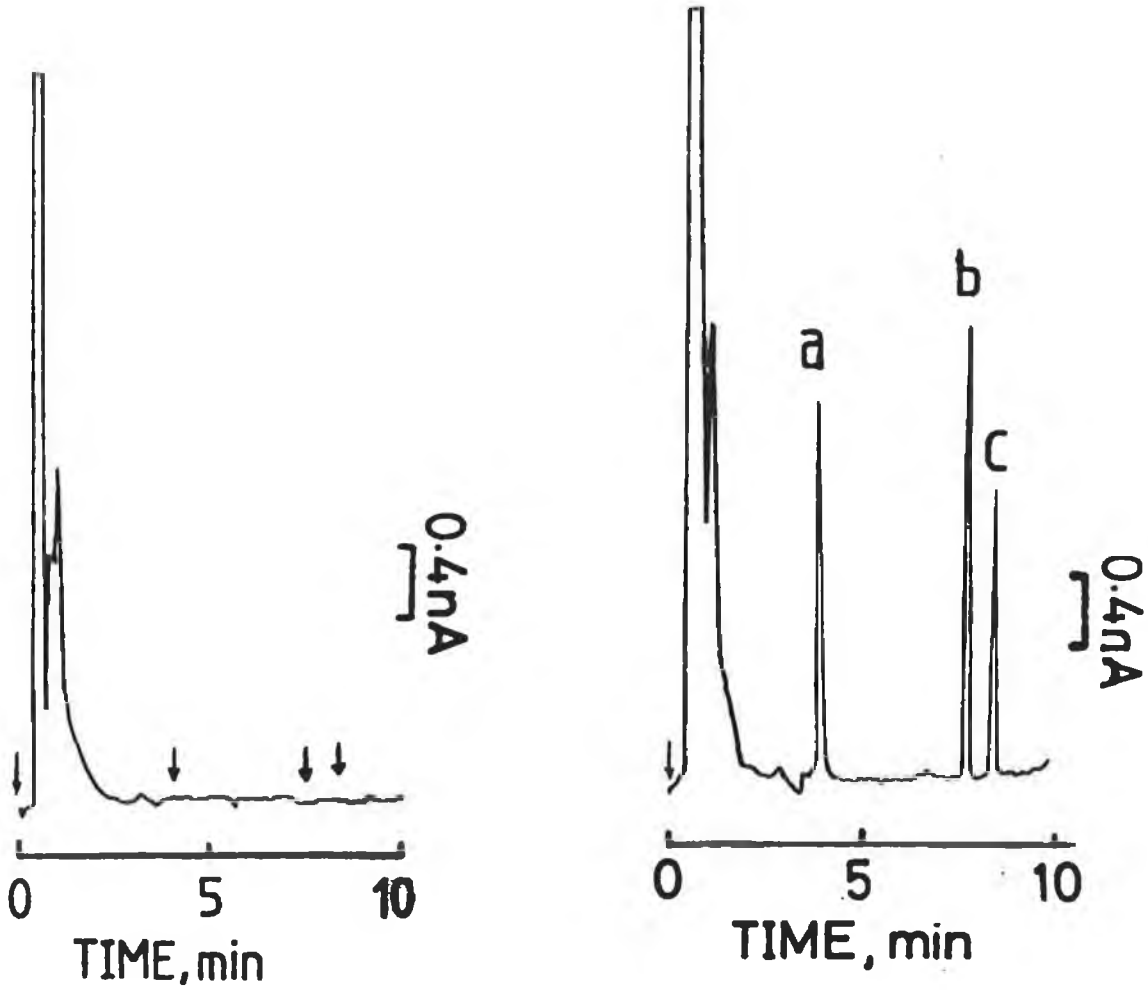

Figure 5. 3. Chromatograms obtained by analysis of plasma containing 80ng/ml of norepinephrine (a), terbutaline (b) and salbutamol (c).

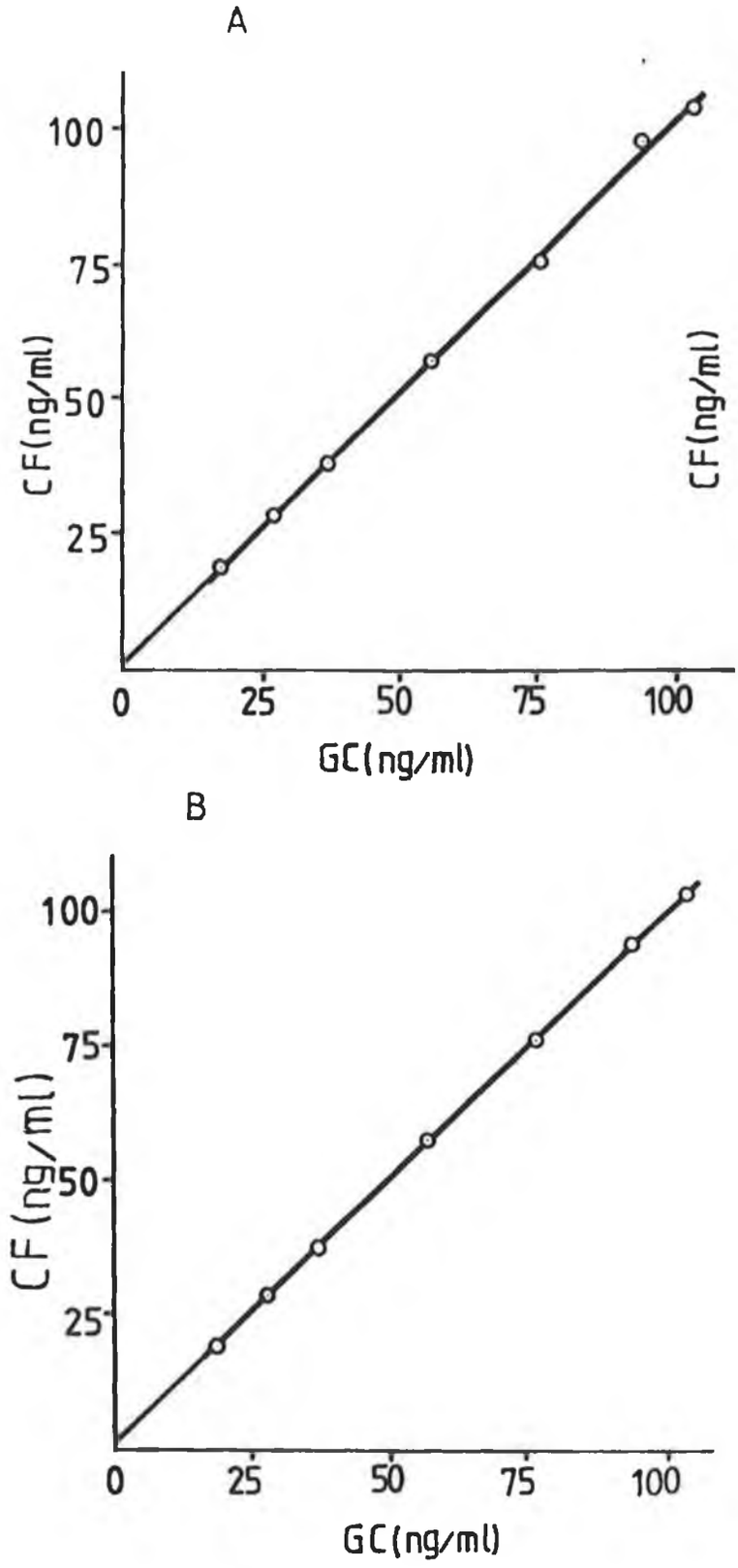

Figure 5. 4. Plots of the mean values found with carbon fibre microelectrode flow cell (FC) against the mean values found using glassy carbon macroelectrode flow cell (GC) for salbutamol (A) and terbutaline (B).

The proposed method was compared with detection involving the use of macro-glassy carbon electrode. A convenient way to compare the two methods is to plot the mean values found using the carbon fibre flow cell against the mean values obtained using the conventional glassy carbon electrode. Plots for both salbutamol and terbutaline are shown in Figure 5. 4, and the data are presented in Table 5. 4. Linear regression gave the following equations; $y = 0.013 + 1\ x$, $r = 0.9996$ for salbutamol and, $y = 0.051 + 1\ x$, $r = 0.9999$ for terbutaline. The results clearly show that although slightly higher values are obtained with the microelectrode flow cell at low concentrations, a good agreement between the methods was obtained. When the results were compared by calculating the respective limits of detection, the use of the microelectrode flow cell allowed salbutamol and terbutaline to be detected at lower levels than with the conventional flow cell.

Considering the laborious preparation time required for polishing the glassy carbon electrode, the detection with the microelectrode flow cell is labour saving and less time consuming. These parameters are highly considered in hospital routine work for studying of the features of poisoning in relation to plasma concentrations of poisoned patients. In conclusion, the necessary assay sensitivity and selectivity were achieved with amperometric detection using a microelectrode flow cell. Optimisation of the extraction procedure using the column-switching technique maximised the recovery and resolution of the drugs, and provided a chromatogram free from interference by endogenous substances and other commonly ingested drugs. The resulting method proved simple without using extensive sample purification, and was fast and reliable in the measurement of the drugs in plasma at overdose levels.

Table 5. 4. Comparison of the carbon fibre microelectrode flow cell (CF) with conventional glassy carbon detector (GC)

(a) Salbutamol

Amount added ($ngml^{-1}$)	Mean amount found ($ngml^{-1}$)	
	CF	GC
20	18.4	17.9
30	28.2	27.0
40	37.7	37.2
60	56.8	56.0
80	75.8	75.6
100	94.4	94.0
110	103.6	103.1

(b) Terbutaline

Amount added ($ngml^{-1}$)	Mean amount found ($ngml^{-1}$)	
	CF	GC
20	19.2	18.8
30	28.5	27.9
40	37.3	37.0
60	57.7	56.9
80	76.2	76.5
100	94.4	93.9
110	103.4	103.0

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Chapter 6

Determination of Gentian Violet in Human Urine and Chicken Feed

6. 1. INTRODUCTION

Gentian violet (GV; hexamethyl pararosaniline), also known as crystal violet, is classified as a triphenyl methane dye, and has been marketed since 1951 for a variety of uses. Crystal violet has anthelmintic properties and has been used in the treatment of strongyloid infestation [1]. The compound has also been used in humans, as an antimicrobial agent in the treatment of burn victims, to treat umbilical cords of infants, as a vaginal cream, for various purposes in veterinary medicine, and as a pH indicator substance. At present, the U.S. Food and Drug Administration (FDA) permits the use of GV (8 ppm) in poultry feeds as a mould inhibitor. Crystal violet was determined to be mutagenic to Bacillus Subtilis, Escherichia Coli, and Salmonella Typhimurium [2], and cytotoxic to mammalian cells [3]. Au et al. [4,5] demonstrated its genetic cytotoxicity to chinese hamsterovary (CHO) cells. Concern about the health risks and carcinogenicity associated with the use of GV requires that a routine laboratory method be developed to monitor the drug and its metabolite.

Typical approaches for determining GV have involved the use of potentiometry [6], spectrophotometry [7] and mass spectrometry[8], and lately by HPLC with amperometric detection using a glassy carbon electrode [9]. Microelectrodes, however, have a lower dependency on flow rate and thus lower noise associated with flow rate fluctuations[10 - 13].

The method described in this chapter was used to determine GV in human urine and chicken feed. The method could also determine GV in the presence of the metabolite leuco gentian violet.

6. 2. EXPERIMENTAL

6. 2. 1. *Reagents and Materials*

Carbon fibres, 14 μm in diameter, were obtained from Avco, Lowell, MA, USA.. Chemicals were used without further purification. All chemicals were of analytical-reagent grade, unless otherwise indicated. Gentian violet (USP[14], crystal violet 96 %) was obtained from Sigma and was used to prepare reference standards.

Chicken basal diet containing GV at 0 ppm and fortified feed containing GV at 2.5, 5.0, and 10.0 ppm were kindly supplied by the National Food Centre (Dublin, Ireland). Human urine for control and recovery analyses were provided by healthy volunteers. The urine specimens were collected in polycarbonate bottles and were

stored at - 4°C until analysis. Stock standard solutions equivalent to 0.20 mg ml^{-1} GV were freshly prepared in LC grade methanol, mixed and then stored in a cool place (ca 4°C) and protected from light. These were diluted and added to GV-free urine aliquots to generate spiked urine standards in the concentration range 1 - 30 ppb. The "spiked" samples were left for at least 1 hr before they were analysed to permit any possible interactions between the drug and urine constituents.

6. 2. 2. *Cyclic Voltammetry*

Cyclic voltammetric experiments were initially recorded on blank solutions of 0.05 M acetate buffer, followed by solutions containing 1 x 10^{-3} M GV dissolved in 0.05 acetate buffer solution (pH 5.5) at a glassy carbon electrode. These experiments were performed with a three electrode system consisting of a glassy carbon electrode (EG&G Princeton Applied Research) as working electrode, a saturated calomel electrode (SCE) and a platinum wire counter electrode connected to an EG&G Princeton Applied Research (PAR) Model 264A potentiostat, and an Omnigraph 2000 X-Y recorder. Before any electrochemical measurement, the glass cell and the counter electrode were soaked in HNO_3 solution, and then rinsed throughly with triplydistilled water. Voltammetric conditions were as follows: initial potential 0 V; final potential 1.6 V and scan rate 50 mVs^{-1}. Prior to scanning, the electrode was cleaned by washing with distilled water, polishing the surface with aluminium oxide powder (0.3 μm), rinsing again with distilled water followed by acetone and finally drying with a tissue-paper. The presence of any adsorption process was investigated by multiple CV scanning without pretreatment of the glassy carbon electrode.

6. 2. 3. *Extraction and Clean-up Procedures*

6. 2. 3. 1. *Poultry Feed*

The sample preparation and clean-up procedures were reported previously by Roybal et al [9]. Blank feed and sample feed were ground to pass through a 1 mm sieve (Pascal Eng. Co. Ltd., Crawley, U.K). Ground chicken feed was mixed, stored in a quart glass jar, and kept in a cool, dry place. 20 g ground feed was then accurately weighed into a 500 ml centrifuge bottle. 200 ml methanol/1 M HCl (99+1) was added and the mixture was shaken vigorously for 1 h on a mechanical shaker, then left to stand overnight. An appropriate aliquot of the supernatant was then pipetted into a 15 ml centrifuge tube according to the following schedule: GV (ppm), aliquot (ml)): 10 ppm, 1 ml; 5 ppm, 2 ml; and 2.5 ppm, 4 ml. The aliquot was then diluted to 10 ml with mobile phase and mixed. The solution was then

filtered through a disposable, 5 um polytetrafluoroethylene (PTFE) membrane (Millipore Corp., Bedford, MA) into a 25 ml Erlenmeyer flask. 20 µl of filtered solution was then injected into the liquid chromatograph. The concentration of GV (ppm) in feed was calculated as follows:

$$ppm = (P/S) \times (C/W) \times (D) \qquad (7)$$

where P = peak height (mm) obtained from injection of filtered solution, S = average peak height (mm) obtained from injection of standard, C = concentration (ppm) of injected standard, D = final volume (ml) of filtered solution, and W = initial weight of feed sample taken for analysis.

6. 2. 3. 2. ***Human Urine***

The procedures for extracting GV from urine speciemens were based on the work of Rushing and Bowman [15]. A 100 ml portion of spiked human urine in a 165 ml culture tube was adjusted to pH 7.0 with 6 M NaOH. After the addition of 50 ml of dichloromethane the tube was gently shaken by hand for 3 min with care taken to prevent emulsification of the contents. The tube was then centrifuged for 5 min at 500 rpm and the dichloromethane layer removed by syringe, percolated through 20 g of sodium sulfate in a glass funnel (3.0 cm diameter), and collected in a 250 ml round-bottom flask. The extraction process was repeated with two additional 20 ml portions of dichloromethane, the sodium sulfate washed with 5 ml of dichloromethane, and the combined extracts evaporated to dryness on a 30 ^{o}C water bath using water pump vacuum. The dry residue was transferred to a 50 ml round-bottom flask by using 4 successive 1 ml rinses of the dichloromethane which were then evaporated to dryness as described. The residue was finally dissolved in 1.0 ml of methanol and 100 µl (10g equivalents of urine) were injected for analysis.

6. 2. 4. ***HPLC***

The HPLC separation used in this study for the chromatographic separation of the triphenylmethane dye is similar to those reported by Roybal et al [9]. The system consisted of a stainless steel column (250 x 4.6 mm i.d.) packed with cyano packing material (particle size 5 µm) (Hichrom Ltd, Reading, Berkshire, RG7 4PE, U.K.). The mobile phase was acetonitrile-acetate buffer (60 + 40); the acetate buffer was prepared by adjusting 0.1 M sodium acetate solution (8.2 g sodium acetate/1000 ml deionised water) to pH 4.5 with glacial acetic acid (ca 8 ml). The resulting solution had a pH of 6.0. Sample injections to the column were effected via a syringe-loading Rheodyne valve (Cotai, CA, U. S. A.). The analytical column was protected

by a 20 mm direct-connect guard column, (LC-18-DB, Supelco, Inc., Bellefonte, PA 16823) packed with CN packing material (Lichrosorb 10 CN, HPLC Technology, Macclesfield, Cheshire, U.K.). All solvents used in the HPLC system were filtered through a disposable 0.5 µm fluoropore filter (No. FHLP 04700, Millipore, Bedford, MA, U. S. A.). Oxidative amperometric measurements were performed using an EG&G Princeton Applied Research Model 400 EC potentiostat connected to the flow cell by crocodile pins. GV was detected amperometrically by employing a potential of + 1.3 V at the working electrode. The resultant signals were recorded on a Philips Model PM8261 X-t recorder (Eindhoven, The Netherlands) at a chart speed of 300 mm hr^{-1}.

The peak currents as a function of concentration were then measured for quantitaive analysis. Operating conditions : LC mobile phase at room temperature; the mobile phase flow rate was maintained at 1.0 ml min^{-1} through the analytical column; column temperature, ambient; column pressure, ca. 2500 psi; the normal operating current range was set at 20 nA and 2 nA for full-scale deflection for the analysis of GV in human urine and polutry feed respectively.

6. 3. RESULTS AND DISCUSSION

6.3. 1. *Cyclic Voltammetric Behaviour*

A typical cyclic voltammogram for a 1 x 10^{-3} M solution of GV obtained using a glassy carbon working electrode in 0.05 M acetate buffer (pH 5.5) supporting electrolyte is shown in Figure 6. 1. The voltammogram showed one peak on anodic scanning between 0 to +1.4 V, with no peak on the reverse scan. The appearance of a single anodic peak in the first cycle only, with no cathodic peak on the reverse scan, is indicative of the irreversible nature of the oxidation process. Further cycling without reactivation of the electrode (scan B) showed complete suppression of the anodic peak indicating that the reaction product is strongly adsorbed on the electrode surface.

6. 3. 2. *Optimisation of HPLC-ED Conditions*

In order to determine the optimum applied potential for amperometric detection following liquid chromatography, a hydrodynamic voltammogram was constructed for GV (Figure. 6. 2) by injecting 100 µl of 30 ppb GV standard solutions of the compound and recording the detector response at intervals of 0.10 V over a range of + 0.40 V to 1.40 V. The voltammogram verifies that GV undergoes a single oxidation within this potential range. The maximum currents were achieved at a

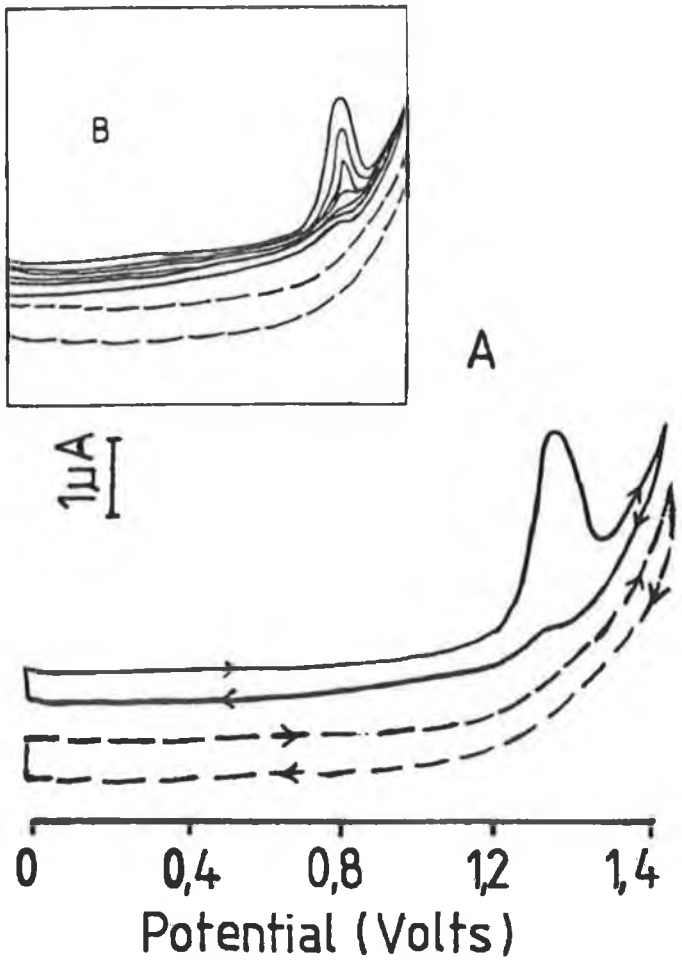

Figure 6. 1. Cyclic voltammogram of 1 x 10^{-3} M GV in o.o5 M acetate buffer (pH6) at a stationary glassy carbon electrode (A). The inset in (B) shows the cyclic voltammograms obtained after recycling without intervening activation of the electrode.

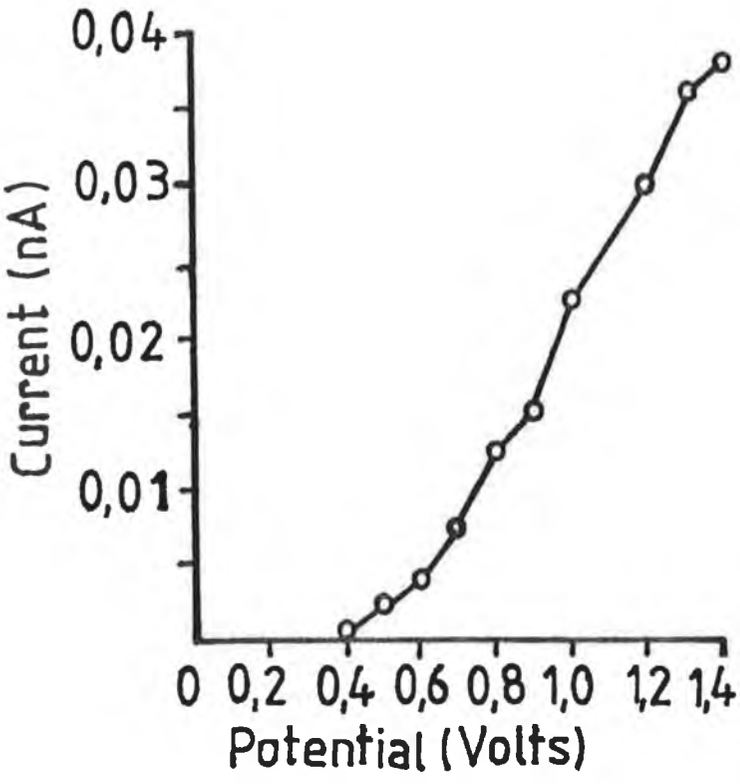

Figure 6. 2. Hydrodynamic voltammogram obtained for 30 $ngml^{-1}$ GV standard solution. Mobile phase: acetonitrile-acetate buffer (60 + 40), pH 5.5; working electrode: 14 μm carbon fibre.

potential of + 1.40 V; however, the magnitude of the background current was about 0.004 nA at this applied voltage. It was found that reducing the potential to + 1.30 V afford a significant reduction in background current, which resulted in better signal-to-noise ratios; therefore, an applied potential of + 1.30 V was selected for the remainder of the LC-ED studies. This was in accord with the observations performed with cyclic voltammetry. Using cyclic voltammetric data, existing HPLC information and the hydrodynamic voltammogram, the chromatographic system was developed to meet the requirement of both separation and detection of GV in poultry feed and in human urine.

6. 3. 3. *Pretreatment of the Carbon Fibre Working Electrode*

The electrode response is usually affected by the features of the sample matrix. Urine is a complex medium that contains many complex biological materials that can interfere with the performance of the analysis. In the proposed procedure, the occurrence of matrix effects (defined as the relative sensitivity in the presence of a matrix to that in the absence of of a matrix) during urine analysis was significant. It was necessary to counteract the matrix effect when the carbon-fibre electrode surface became contaminated. Electrochemical pretreatment was used to solve this problem. The working electrode was treated by applying - 1.40 V for 40 s and then + 1.40 V for 40 s (vs. Ag/Ag_3PO_4) followed by equilibration for 3 min. When the upper potential limit was extended to + 1.5 V, many fibres were either broken or provided noisy and erratic oxidation peaks. When this pretreatment procedure was applied in the analysis of the urine specimens obtained from healthy volunteers, the interferences from other urinary constituents were effectively eliminated (Figure 6. 3).

Chemical pretreatment of the carbon fiber electrode were also tried. The working electrode was immersed for 20 min in HNO_3, H_2SO_4, H_2O_2 and concentrated acid dichromate solution. In all these cases the resulting test voltammograms showed poor defined peak shapes. The flow system used provides short contact between the sample and the active surface allows minimal contamination of the electrode surface.

Table 6. 1"a" shows a comparison of precision determined with and without using the pretreatment procedure. From this it can be seen that, the absolute and the relative standard deviation were significantly lower using the pretreatment procedure. The pretreatment regime also improved the correlation coefficient for the calibration graph and the sensitivity of the method. Table 6.1"b" shows that the

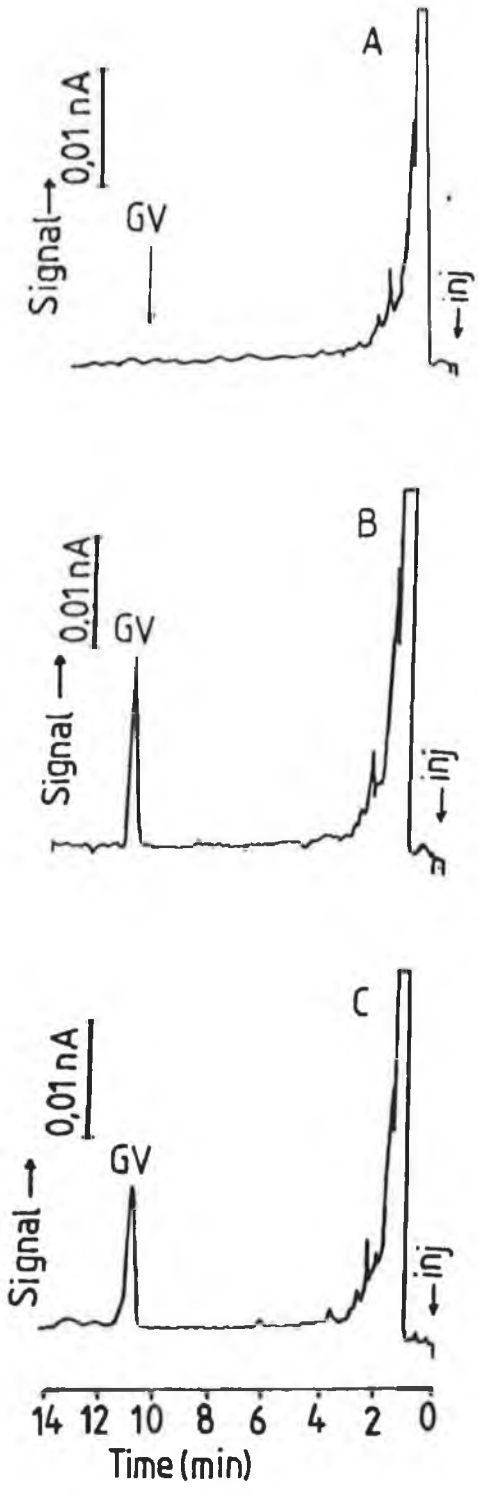

Figure 6. 3. Influence of electrochemical pretreatment on the current response of the electrode: (a) Blank urine extract; (b) current response for 5 $ngml^{-1}$ spiked urine speciemen after electrochemical pretreatment and (c) current response before electrochemical pretreatment. See experimental for additional details.

Table 6. 1 (a). Comparison of performance using the electrochemically modified and unmodified electrode for determination of 4 ppb GV in human urine.

	GV concentration/ppb	
	With pretreatment	Without pretreatreatment
Absolute error*/ppb	0.35	1.30
R. S. D.(%)	3.49	9.82

* The absolute error is defined as the difference between the measured and the certified value of GV.

Table 6. 1 (b). Urine concentrations of GV (ppb) before and after pretreatment procedures from urine samples

	Concentration of GV found / ppb	
Urine sample concentration	No pretreatment	After pretreatment
4.0 ppb	2.81	3.65
7.5 ppb	5.06	7.08
12.5 ppb	8.91	11.88
25.0 ppb	16.74	23.25

measured concentration of GV in urine is reduced significantly before using the pretreatment procedures, making the pretreatment of the electrode an essential step for the determination of GV using the microelectrode flow cell. A similar improvement was observed when the pretreatment procedure was applied during the analysis of the animal feed. One explanation of this behaviour is the increase of the effective rates of electron transfer by generating electrocatalytic functional groups at the surface of the electrode due to the pretreatment.

6. 3. 4. ***Determination of Gentian Violet in Human Urine and Poultry Feed.***

The analytical procedure was then applied to control human urine and poultry feed samples to establish the limit of detection and recovery of the method.

6. 3. 4. 1. *Linear Range*

A series of external GV standards were prepared in a mixture consisted of methanol: water (95:5), and a calibration graph constructed by injecting between 1 and 30 ppb of GV for standard solutions for urine samples analysis and between 1 and 20 ppm for polutry feed analysis. Typical chromatograms of the blank and of a 1 ppm solution of GV are shown in Figure 6. 4. Peak currents were measured and graphs of peak current versus amount injected were constructed. The linear calibration curves indicated that the oxidation of GV at the carbon fibre working electrode is reproducible over these ranges using the microelectrode flow cell and the chromatographic conditions, with correlation coefficients of greater than 0.996 for both studies. The characteristics of these curves were slope: 1.2×10^{-3} nA/ppb, and 9.1×10^{-2} nA/ppm and intercept 1×10^{-3} nA, 2×10^{-3} nA for human urine and poultry feed analysis respectively. These calibration ranges were considered suitable for the applications being undertaken in this study.

6. 3. 4. 2. *Precision*

The method described has been applied to a number of samples of human urine and poultry feed. The LC-ED chromatograms obtained for GV extracted from human urine and chicken feed as described earlier, showed a well defined peak for GV with a retention time of 11 min (Figure 6. 5 and 6. 6). The precision was measured as the relative standard deviation of a repeated injection of the same quantity of a sample at 4, 7.5, and 10 ppb urine samples and at 2.5, 5, and 10 ppm feed samples. The results summarized in Table 6. 2"a" and "b" display an acceptable level of accuracy and precision, with RSD ranging between 2.33 to 5.07 %. Using a second detector (glassy carbon detector), whose precision was more accurately known, in

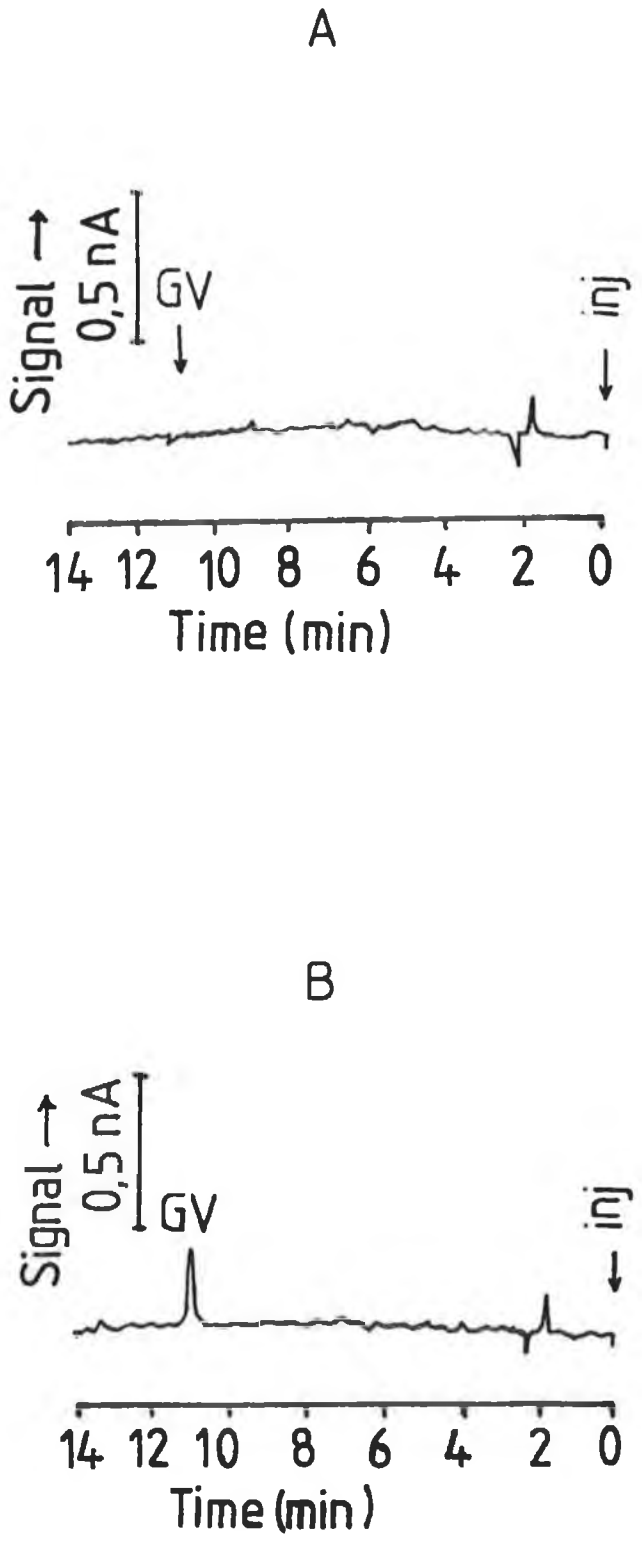

Figure 6. 4. Typical chromatogram of peak separation of a calibration standard containing GV 1 $\mu g ml^{-1}$ (b) and chromatogram for balnk standard solution.

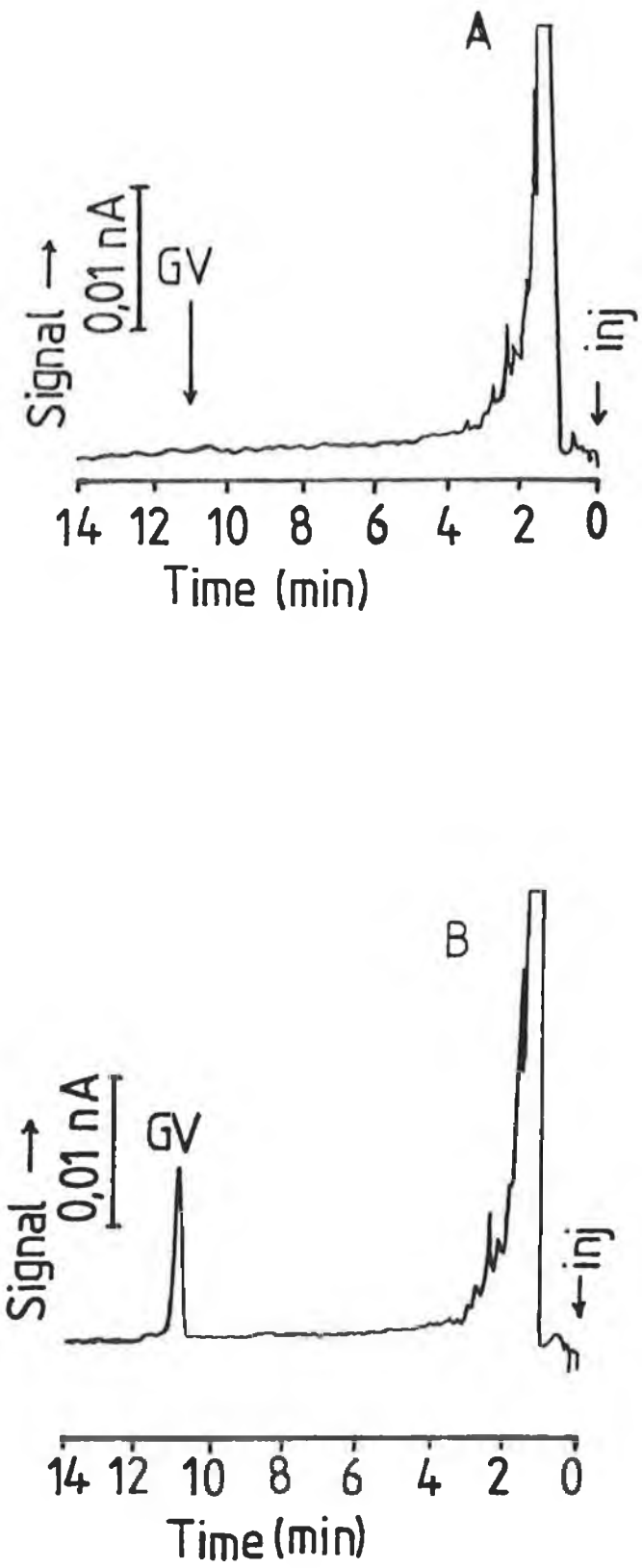

Figure 6. 5. HPLC of GV-free human urine (a) and (b) the same urine spiked with 5 $ngml^{-1}$. Chromatographic conditions (see text).

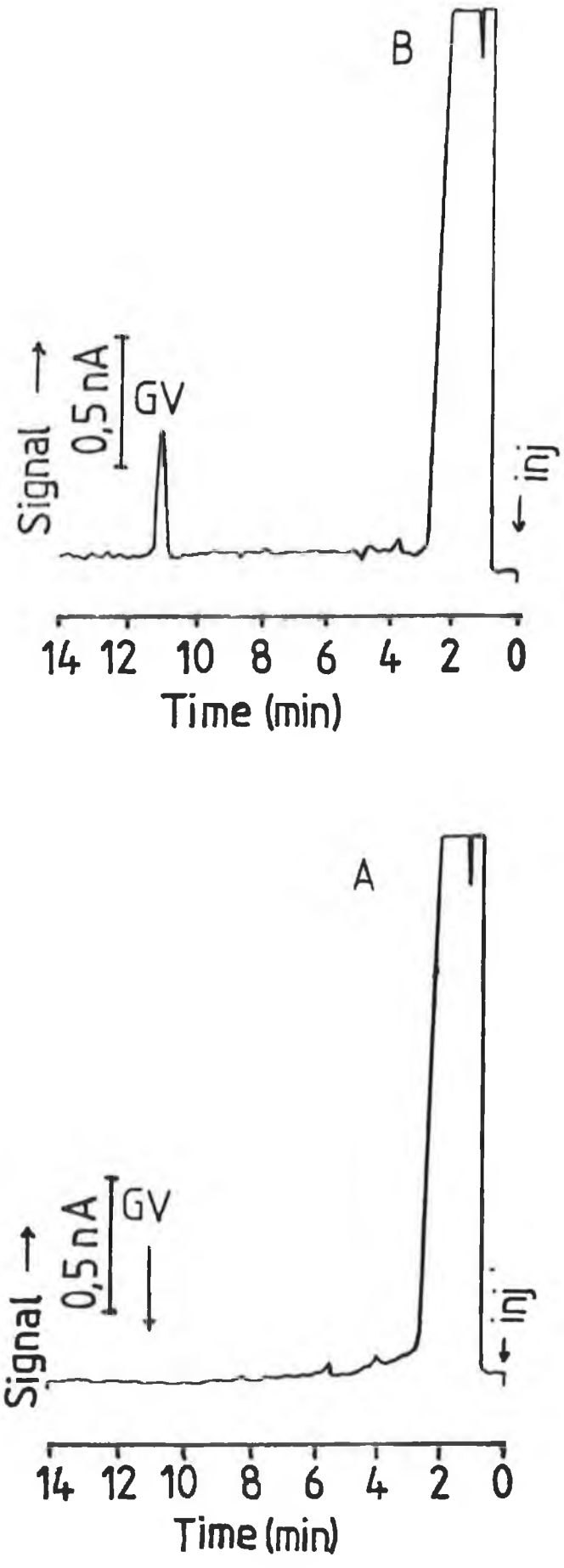

Figure 6. 6. Sample chromatograms of (a) GV-free chiken feed, (b) chicken feed containing 2 $\mu g ml^{-1}$ GV See text for conditions.

Table 6. 2 (a). Precision of the assay in urine

Concentration spiked/ppb	Mean concentration found*/ppb	SD	RSD(%)
4.0	3.65	0.11	3.49
7.5	7.08	0.14	2.33
10.0	9.45	0.30	5.07

Table 6. 2 (b). Precision of the assay in chicken feed

Concentration spiked/ppb	Mean concentration found*/ppb	SD	RSD(%)
2.5	2.38	0.10	5.27
5.0	4.73	0.12	3.22
10.0	9.60	0.16	2.63

*n = 5

series with the carbon fibre detector gave 2.0 % RSD for 5 ppb urine injections of GV compared with 2.7 % RSD for the microflow cell. Although the figure for the microflow cell is slightly worse than for the conventional glassy carbon electrochemical flow detector for the majority of trace level determinations this precision will be perfectly adequate. Using an automated sample injector it should be possible to improve the precision figures for both detection systems. These results are in good agreement with those published with other authors.

6. 3. 4. 3. *Recovery Studies*

The extraction procedures produced a "clean" extract and consequently good recoveries of GV. Table 6. 3 shows the overall recovery of GV, which was assessed by comparing the representative peak currents for spiked urine specimens and poultry feed sample taken through the study with the peak current for corresponding standards. The recoveries of GV from spiked urine blanks and from chiken feed ranged from 91.69 to 94.09 % at ppb levels and from 94.85 to 95.70 % at ppm levels and averaged 94.06 %. These recovery levels are satisfactory, and the concentration of the "spiked samples" did not appear to influence the recovery of GV residues in urine specimens. Data represented in Table 6. 3 indicates that the developed method has good precision.

6. 3. 4. 4. *Interference Study*

In many cases quantitation of metabolites in addition to the parent drug will be required if these metabolites are pharmacologically active. Intestinal microflora, under anaerobic conditions, are capable of metabolizing GV by reduction to its leuco (colorless) form[16]. According to the USA National Center for Toxicological Research [17], this leuco derivative (LGV) is then "structurally similar to the classical aromatic carcinogens". The possible potential interference of the leuco derivative in the detemination of GV was tested using the proposed method.

Figure 6. 7 shows the chromatogram observed for simultaneous determination of GV and LGV. It can be seen from this figure that both forms are well resolved and there were no significant effects due to the added interferent. This metabolic product would not be expected to interfere in real samples as the level of spiking was considerably higher than that normally found in human urine.

Table 6. 3 (a). Recovery of spiked GV from urine specimens.

	GV concentration / ppb		
Sample	Present	Mean amount found*	Mean recovery (%)
A	2.0	1.83	91.69
B	5.0	4.63	92.66
C	15.0	14.11	94.09

*n = 5.

Table 6. 3 (b). Recovery of GV from chicken feed

	GV concentration / ppb		
Sample	Present	Mean amount found*	Mean recovery (%)
A	2.5	2.39	95.7
B	5.0	4.74	94.85
C	10.0	9.63	95.38

*n = 5.

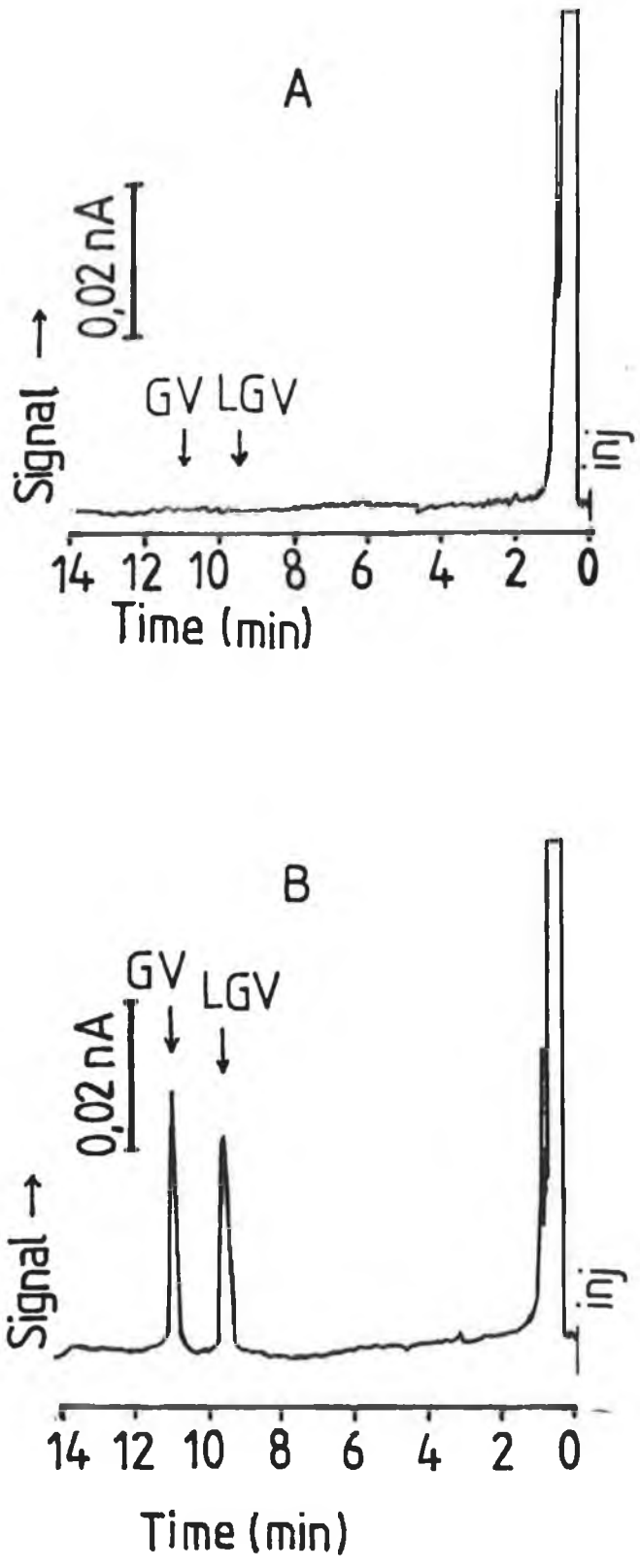

Figure 6. 7. Typical chromatogram of the separation of the leucogentian violet from gentian violet at + 1.3 V. A: blank urine extract; b: 30ngml^{-1} spiked human sample.

6. 3. 4. 5. *Sensitivity Comparison*

The sensitivity of the proposed method was compared with detection involving the use of macro-glassy carbon electrode. In principle, the smaller the working electrode the better, because smaller electrodes reach steady states more quickly [18]. The limit of detection was taken to be the concentration at which the signal was three times greater than the noise of the base line. A comparison of the limits of detection obtained using the microflow cell for determination of GV in human urine with that obtained with the conventional cell is illustrated in Table 6. 4. It was anticipated that decreasing the size of the electrode would lead to lower limits of detection (more favorable signal-to-noise "S/N" ratios) using the microflow cell, compared to the conventional flow cell. The minimum detection limit for the method was estimated to be 0.5 ppb; this limit of detection compares favourably with previous methods. A secondary source of noise ,other than the interferents found in the matrix could be the electronic noise of the potentiostat/current amplifier [19] and a lower noise level would be expected with a potentiostat more suitable for the lower currents encountered with carbon fibre electrodes. The carbon-fiber working electrode has a much smaller surface area, so that the currents flow through it is much smaller than that through the glassy carbon electrode.

6. 3. 4. 6. *Flow-rate Dependencies*

From the analytical point of view, flow rate independent amperometric detection may help to reduce the background noise level, particularly in the case of FI and HPLC detection where electroactive impurities lead to baseline fluctuation depending on flow rate pulsations. Practically, most HPLC pumps cause unavoidable pulsations of the mobile phase[20,21].

The flow-rate dependency of the carbon fiber microelectrode and 50 um carbon disk electrode has been examined by injecting 5 ppb GV in methanol through the flow cell. Data illustrating the dependency of peak currents upon the flow rate of the running solvent for the electrodes are presented in Figure 6. 8 for oxidation of GV in mobile phase. Each plotted data point is the average of five or more replications. From this data the 14 μm carbon fiber electrode is clearly superior to the 50 um glassy carbon electrode, which has a larger flow rate dependency of peak current. Previous studies demonstrated that an improved signal-to-noise ratio was obtained using carbon fiber microelectrodes (compared a conventional glassy carbon electrode) at positive potentials[22]. At these potentials, the oxidation of buffer impurities gave rise to noisy baselines at glassy carbon electrode because of

Table 6. 4. Detection limits[a] for the determination of GV using the microelectrode and macroelectrode methods coupled with HPLC analysis[b].

	Limit of detection / ppb
Conventional flow cell	0.80 ppb
Carbon fiber flow cell	0.50 ppb

a Detection limits (S/N ratio of 3:1) calculated in units of concentration of GV added to human urine specimens

b Conditions: mobile phase, acetonitrile-acetate buffer pH 6; (60+40); applied potential, + 1.3 V vs Ag/Ag_3PO_4 reference electrode; flow rate, 1.0 ml min^{-1}.

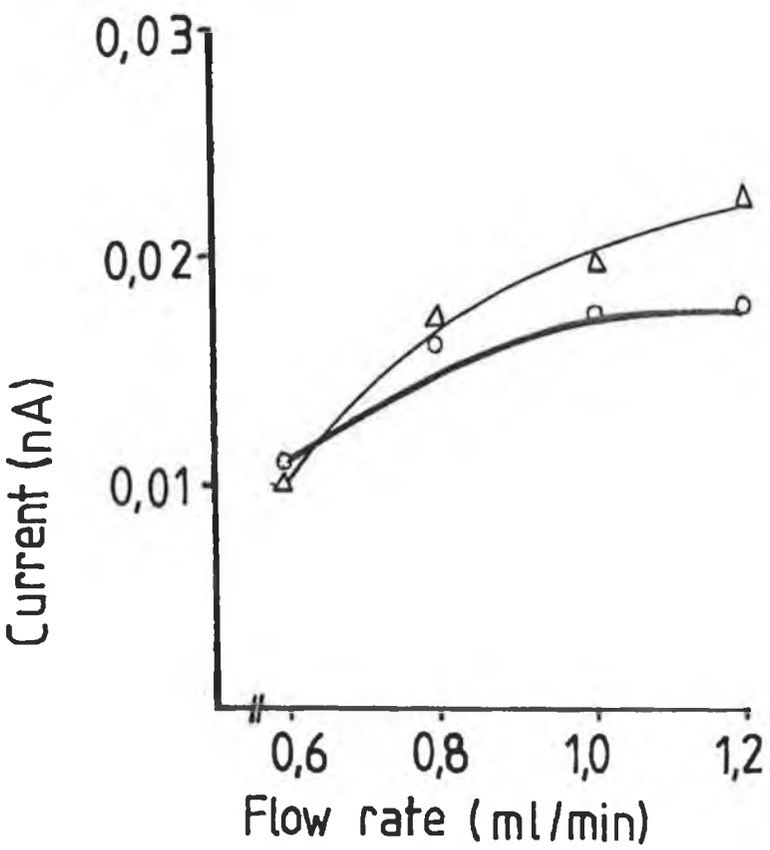

Figure 6. 8. Flow rate dependency of peak current for oxidation of GV in mobile phase: E = +1.3 V vs. Ag_3PO_4; (o) carbon fiber microelectrode; (▲) conventional glassy carbon macroelectrode.

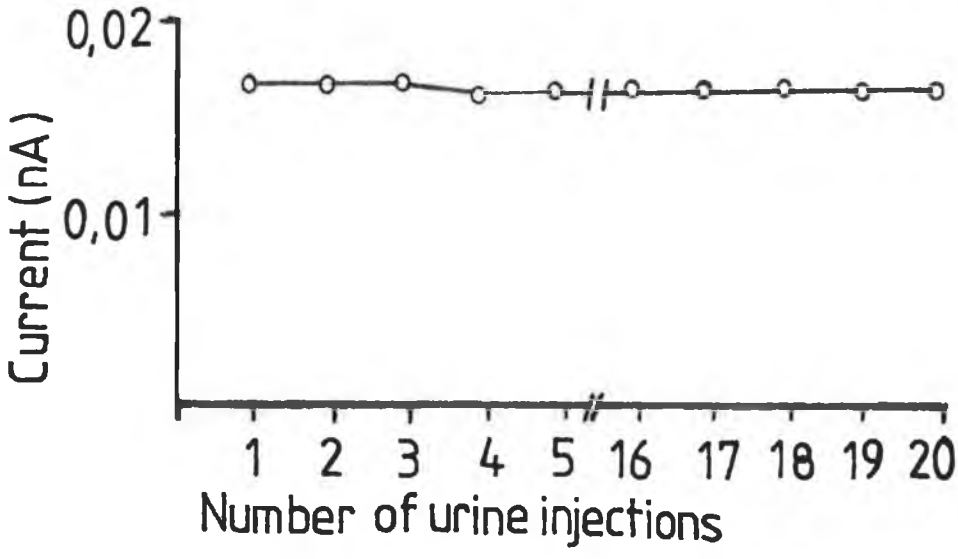

Figure 6. 9. Peak currents obtained from the first five and the last five urine samples of 20 consecutive injections over a period of 10h. GV was spiked into human urine at 5 $ngml^{-1}$.

the strokes of the liquid chromatography pump; the microelectrode flow cell was much less susceptible to this noise.

6. 3. 4. 7. *Stability Studies*

The stability of the urine samples and the carbon fibre electrode was examined. The stability of the samples at room temperature was tested by making 20 consecutive injections of the same urine over a period of approximately 10 h. As demonstrated in Figure. 6. 9 , there were no significant changes in the peak current between the first five and the last five. In fact, there was no significant changes in the chromatograms after the consecutive injections. This indicated that the urine samples containing the drug were stable at room temperature during the test period. In addition, urine samples spiked with the test compound were stable for more than 20 days at -4^0C.

The stability study results also indicated the carbon fiber was performing extremely well even after a large number of injections. In fact, the same carbon fiber had been used for 10 different urine samples before the first sample of the stability study was injected. These results suggest that the carbon fiber electrode could endure more than 30 injections of urine samples before replacement was needed.

6. 4. CONCLUSION

This research describes a sensitive LC-ED method for quantitative analysis of GV in human urine and chicken feed using an electrochemical microelectrode flow cell. The detector has been found to exhibit a considerable improvement in the limit of detection and in some instances decreased dependence on flow rate over that found with the conventional method under these experimental conditions. It is considered that the method will be useful for both clinical and pharmacokinetic studies.

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Chapter 7

Determination of Malachite Green in Environmental Samples

7. 1. INTRODUCTION

Malachite green, like most dyes, is known by a number of synonyms; most of these are trade names and the dye may be sold under synonyms such as Victoria Green B or WB, New Victoria Green Extra O 1 or II, Diamond Green B, BX or P extra, Solid green O, Light Green N and Astramalachite Green. Despite the active use of malachite green in fisheries practice for 50 years, the history of its use has been punctured by continuing series of misunderstandings and confusion. A detailed history of the development and manufacture of malachite green was described by Karr[1]. There is considerable scope for further research, particularly into areas such as possible tetragenic effects, effect on host and pathogen, and environmental persistence. The use of malachite green will undoubtedly continue[2], since no alternative effective compound has so far been discovered. Provided that due care is excercised in its use, no significant problems should be anticipated. Treatment side effects were first reported by Mazuranich and Nielson[3], who noticed that the incidence of "white spot" disease in silver salmon increased when eggs were treated with 5 mg/L (1:200,000) malachite green for one hour every other day. Malachite green reacts with oxygen in the water, thereby lowering dissolved oxygen. High initial oxygen levels will help prevent anoxia during treatment[4]. The bacteriostatic action of malachite green is due to its three six-membered (phenyl) rings and the two substituted amino groups, each with two ethyl radicals[5]. The triaminotriphenyl methane dyes (crystal violet, parafuschin and new magenta) have increased activity because of the alkylated amino groups on the three phenyl rings, allowing resonance among all three rings instead of only two[6]. Enzyme studies indicated that the dye causes irreversible inhibition of thiol enzymes, and irreversible inhibition of non-thiol enzymes[7]. In the case of trypsin, malachite green combines mainly with the imidazole groups of histidine residues.

The leuco (colourless) form of malachite green is non-toxic , possibly because of its non-polar structure[8]. Leucomalachite green tends to reduce the toxic effects of the latter. Evidently, MG is detoxified by reduction to LMG[8]. Malachite green injected intravenously into male rats (3.0 to 3.5 mg/100 gm body weight) was acutely toxic, producing clinical signs of asphixiation[9]. Histological examination revealed excess fluid in the lungs and electrocardiograms indicated a slower heart rate. Cytochrome C injections (2mg per mg malachite green) abolish intoxication symptoms. In vitro, malachite green did not affect oxygen saturation of haemoglobin or intact human erythrocytes. There was no marked reduction in blood oxygen in samples taken

after poisoning, indicating that the hypoxic effect of the dye is probably of endogenous origin.

The lethal oral dose of malachite green for rabbits is 75 $mgkg^{-1}$ daily; death occurs within six to ten days[10]. Ingestion causes diarrhea and abdominal pain. Intraperitoneal injections of 0.1875 mg malachite green in physiological saline are lethal to mice; similar injections of 1.5 mg malachite green are lethal to rats.

Because of the staining properties of MG, careful handling is necessary; preparation of stock solution helps to minimize handling difficulties. Users should wear protective clothing and avoid contact with the chemical since it is carcinogenic[11].

Today's environmental sample may be either aqueous, organic liquid, organic or organic solids, sludge or slurry, or a combination thereof. The one thing they have in common is that each sample represents a small part of the whole and may vary from a one-second grab to a several-day composite sample. The laboratory should be familiar with the sample type, source, and sampling procedure as well as with any safety information required for handling. This knowledge will prevent the analysis of a sample of questionable validity and assist the laboratory in taking a subsample for analysis. The laboratory should have written procedures for proper praparation of equipment and containers for sampling, and description of sampling procedures for use by field sampling personnel.

Besides the original acquisition of the sample, the taking of the subsample for use in the laboratory is most critical and often overlooked or treated lightly, e.g. shaking a sludge sample for 2 min and pouring or pipeting off 25 ml will not yield a representative sample. A representative sample normally requires the use of a high speed blender from which the sample is withdrawn at different liquid levels while being blended. Therefore, procedures to assure that possible errors in sampling and subsampling should be addressed and minimized.

The Environmental Protection Agency (EPA) in the USA have produced a manual[12] of standard methods for analysis of polluants in waters. These include methods for multiclass analysis, for example, analysis of about 40 chlorinated, 40 organophosphorous and seven carbamate polluant species. A problem in the analysis of residues in water is whether the residue is actually in the water or in suspended particles of silt, soil, plant material, or even aquatic animal life. Water samples may vary from the crystal-clear water of a mountain stream to the deep green water of a stagnant lake or the brown of flood water saturated with suspended solids. If only the residue in the water is of interest, other material must be

removed. Filtration is adequate for most water samples, but where there is a suspension of colloidal material, the addition of a small amount of a divalent salt such as calcium chloride to flocculate the clays is recommended. In some cases centrifugation will also be necessary. Residues in water are usually very low. This means that a considerable concentration is required before the residue can be detected by HPLC. This may take the form of a partition into a water-immiscible solvent or removal of the residue on to a column either by adsorption or ion-exchange and subsequent elution in a smaller volume. Problems may arise with each of these systems. If a solvent that is lighter than water is used, then the emulsion can sometimes be dispersed by adding sodium chloride, which increases the difference in density and also affects the surface tension of the water. It is usually necessary to partition more than once for complete recovery of the residue. The use of a solvent that is heavier than water makes the process easier since it can be run out of the bottom of the separating funnel.

Slow flow rates are the main problem associated with traditional adsorption columns although a pump can be used. Waters Sep-Pak columns are reproducible, disposable, and easy to use.

Enviromental analysis for trace phenols are among the most important applications of HPLC with electrochemical detection, especially of waters, as the samples can be injected into aqueous mobile phases without any pretreatment, provided that the analyte concentration is not less than ca. 20 $ngml^{-1}$. At lower concentrations, off-line or on-line enrichement is necessary, e.g. by adsorption on silica gel, liquid-liquid extraction, ion-exchange or trapping in cartidges containing a reversed phase packing. For example, traces of phenols (0.1-10 $ngml^{-1}$) have been determined in potable water[13,14]. Microarray working electrode[15] was used for determination of carbamate pesticides (e.g. amino carb, carbendazine, chlorporpham, desmediphan) in river water, these compounds can be oxidised at about +1.1 V[16]. The detection limits varied from 40 to 150 pg (2-7 $ngml^{-1}$) and the response was linear over three concentration decades[17]. The sample pretreatment was minimal and the detection limit was lower by a factor of 60 than that with glassy carbon electrode. Most phenols are electroactive and either oxidised at solid electrodes within the potential range from + 0.7 to +1.1 V (Figure 7. 1), or reduced at mercury electrodes (e.g. nitrophenols).

In Europe, malachite green is regularly used for the control of Saprolegnia parasitica and also in the treatment of proliferative kidney disease (PKD). Its use is controlled within the EEC by guidelines laid down in each of the individual

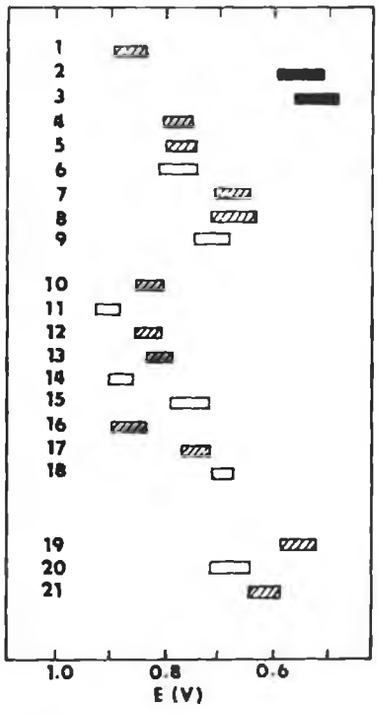

Fig. 7. 1. Potential regions of the oxidation of some phenols of environmental interest. Empty boxes — irreversible oxidation; shaded boxes — a reducible product is formed, which is not the principal oxidation product; solid boxes — reversible oxidation. Carbon paste electrode, medium of 90% v/v 0.1*M* citrate, pH 4.0, and 10% ethanol. Potential scan rate, 200 mV/sec. 1 — Phenol; 2 — catechol; 3 — hydroquinone; 4 — 2-methylphenol; 5 — 4-methylphenol; 6 — 3,5-dimethylphenol; 7 — 2,4-dimethylphenol; 8 — 2,3,6-trimethylphenol; 9 — 2,3,5-trimethylphenol; 10 — 2-chlorophenol; 11 — 3-chlorophenol; 12 — 4-chlorophenol; 13 — 2,4-dichlorophenol; 14 — 2,5-dichlorophenol; 15 — 2,4,6-trichlorophenol; 16 — 2,4,5-trichlorophenol; 17 — 2,3,4,6-tetrachlorophenol; 18 — Pentachlorophenol; 19 — 1-naphthol; 20 — carbofuran phenol; 21 — 4-methylthiophenol.

Reproduced from Stulik, K. and Pacakova, V., Electroanalytical Measurements in Flowing Liquids, Wiley, New York, 1987, p. 239.

member states. In Ireland, these state that the concentrations of malachite green in fish farm water effluent and water extracted for drinking water purposes should not exceed 100 µg/l and 1.0 µg/l respectively Under the fisheries and agriculture research programme (FAR), an EEC project into the the environmental fate of malachite green is presently being carried out in Trinity College Dublin.

In response to concerns regarding the health risks associated with the use of malachite green, an increasing number of methods have been developed in the past decade for its determination in environmental samples and food fish tissue.

A thin-layer chromatographic (TLC) method was first used for the analysis of malachite green residues in edible fish[18]. A methanolic solution of material extracted from fish was analysed by TLC on silica gel followed by densitometric detection and quantitation at 610 nm. An extension of this work[19] made use of a high performance liquid chromatographic (HPLC) separation on a LiChrosorb RP-8 or Spherisorb Octyl column with subsequent spectrophotometric detection and quantitation at 600 nm.

The simultaneous determination of malachite green and methylene blue in rainbow trout and amago muscle using high performance liquid chromatography with subsequent spectrophotometric detection[20] has recently been reported. Residues of malachite green were determined, using a colorimetric method, in muscle, eggs, and fry of Atlantic salmon (Salmo salar) and chinook salmon (Oncorhynchus tshawytscha) which had been treated with the chemical at fish hatcheries[21]. Allen and Meinertz[22] reported on a post-column reaction for the simultaneous analysis of chromatic and leuco forms of malachite green and crystal violet by high-performance liquid chromatography with spectrophotometric detection at 588 nm. This system made use of a post-column reaction chamber containing PbO_2 situated between the HPLC column and the spectrophotometric detector. Separation of the leuco and chromatic forms of the dye was effected on the HPLC column, after which they passed through the post-column reaction chamber and subsequently into the spectrophotometric detector. This method has recently been applied to the analysis of the chromatic and leuco forms of malachite green in water[23]. The method proved successful for the determination of the chromatic form of malachite green in river and drinking water samples. In addition, it was also possible to carry out the simultaneous analysis of the chromatic and leuco forms without the need for post-column modification.

7. 2. EXPERIMENTAL

7. 2. 1. *Chemicals and Reagents*

Malachite green oxalate and leuco malachite green were obtained from Aldrich (Gillingham, Dorset, UK) and from BDH (Poole, UK) respectively. Acetic acid (Analar grade) was supplied by BDH and HPLC grade methanol was supplied by Labscan Analytical Sciences (Dublin, Ireland). Analytical grade sodium dihydrogen orthophosphate was obtained from BDH. This compound and the supporting electrolyte were used without purification.

Deionised water was used throughout this work and was obtained by passing distilled water through a Milli-Q water purification system (Millipore, Milford, MA, USA).

7. 2. 2. *Apparatus*

Voltammetric studies were performed using an EDT potentiostat (EDT Research, London, UK) in conjunction with a JJ X-Y Model PL4 recorder (JJ Lloyd Instruments Ltd, Southampton, UK). A three electrode cell system was employed, incorporating a 0.28 cm^2 area glassy carbon electrode (EG&G Princeton Applied Research), a saturated calomel reference electrode and a platinum counter electrode.

Amperometric measurements were performed using an EG & G Model 400 EC potentiostat (EG&G Princeton Applied Research) connected to the flow cell by crocodile pins.

Malachite green was detected amperometrically by employing a potential of +1.2 V at the working electrode. The resultant signals were recorded on a WPA Model CQ 95 X-t recorder (WPA, Linton, Cambridge, UK). The peak currents (measured as peak heights on the recorder) as a function of concentration were then measured for quantitative analysis.

Malachite green was separated on a 250 x 4.6 mm i.d. cyano (CN) stainless steel column, particle size 5 µm, (Hichrom Ltd, Reading, Berkshire, UK) using a 70/30 methanol/0.1 M sodium acetate, pH 4.5. A Waters Model 501 HPLC pump was used for mobile phase delivery and sample introduction was via a Rheodyne (Cotati, CA, USA) Model 7010 injection valve, fitted with a 20 µl loop for direct injection. For extraction purposes, Techelut 100 mg/1.0 ml cyano solid phase extraction cartridges (HPLC Technology, Cheshire, UK) were used in conjunction with a Techelut 12 position manifold.

7. 2. 3. *Voltammetric Procedures*

The supporting electrolytes used for pH and cyclic voltammetric studies were prepared from a 0.1 M stock solution of sodium dihydrogen orthophosphate. All solutions were prepared in deionised water and were adjusted to the required pH with 0.2 M orthophosphoric acid or sodium hydroxide.

Cyclic voltammetry was performed on solutions containing $1x10^{-3}$ M malachite green dissolved in the supporting electrolyte. Voltammetric conditions were as follows: initial potential 0.0V; final potential +1.3 V; scan rate 50 mV/s; current range 20 µA.

Hydrodynamic voltammetric studies were carried out by injecting 5 mg/l of malachite green, dissolved in 95 % methanol containing 0.05 M sodium acetate, pH 4.5, into the chromatograph with the detector set at different working potentials *vs* Ag/Ag_3PO_4.

Cleaning potentials of +1.3 V and -1.3 V were applied for 60 seconds respectively between each injection. Hydrodynamic voltammograms were constructed by plotting the peak current against the applied potential. The optimum potential for malachite green determination was found from the position of the plateau on the hydrodynamic wave.

7. 2. 4. *Extraction Procedure*

Drinking water samples were collected from the Dublin city water supply and river water samples were collected from Arklow, Co. Wicklow. The samples were stored in plastic containers at 4°C on return to the laboratory prior to analysis on the same day.

Drinking water and river water samples were adjusted to pH 5.0 with acetic acid and spiked with 1 ug/l malachite green prior to extraction and preconcentration on the solid phase extraction cartridges. These cartridges were preconditioned with 2 ml of methanol followed by 5 ml of deionised water. 250 mls of the spiked water sample was eluted through the column at a flow rate of 7 ml/min after which the retained malachite green was eluted with two 0.5 ml volumes of mobile phase. These 0.5 ml aliquots were combined and used for chromatographic analysis.

7. 2. 5. *Chromatographic Separation of Chromatic Malachite Green*

The liquid chromatographic analysis of the chromatic form of malachite green was based upon the method of Roybal et al.[24]. The chromatography of malachite green was investigated by varying the methanol content and the pH of the mobile phase.

The chromatographic separation of the chromatic and leuco forms of malachite green was carried out on a cyano column using 70/30 methanol/0.1 M sodium acetate, pH 4.5 with amperometric detection at +1.2 V vs Ag/Ag_3PO_4.

7. 2. 6. *Calibration, Recovery and Reproducibility*

Calibration graphs were constructed by plotting peak current against concentration for a series of chromatic malachite green standards, prepared in mobile phase, at a potential of +1.2 V *vs* Ag/Ag_3PO_4 and a mobile phase flow rate of 0.9 ml/min.

Aliquots of malachite green standards, made up in 0.1 M sodium acetate, pH 4.5, were added to drinking water and river water samples to give final concentrations of 1.0 µg/L. Replicate spiked samples and the corresponding blanks were extracted according to the extraction procedure previously described. Recoveries of chromatic malachite green from drinking water and river water samples were assessed by comparing the peak currents obtained for the extracts with those of standard solutions.

The reproducibility of the method was ascertained by determining the relative standard deviation of five successive measurements of extracts of water samples.

7. 3. RESULTS AND DISCUSSION

7. 3. 1. *Voltammetric Behaviour of Malachite Green*

The voltammetric behaviour of the chromatic form of malachite green was studied over the pH range 4 - 12 in 0.05 M phosphate buffer using differential pulse voltammetry (DPV) at a glassy carbon electrode. The single anodic peak obtained in the range studied was found to be pH dependent.

The equation relating peak potential to pH for those pH values up to 6.5 was calculated to be:

$$Ep = 1.03 - 4.36 \times 10^{-2}\ pH\ (V) \qquad (8)$$

The equation describing the relationship between peak potential and pH for those pH values over 6.5 was found to be;

$$Ep = 0.87 - 1.81 \times 10^{-2} \text{ pH (V)} \qquad (9)$$

A plot of pH against peak current indicated a pKa value of 6.5. This value is in close agreement with the value of 6.9 previously reported[25].

In the cyclic voltammogram of malachite green, no cathodic peaks were present in the reverse scan, thus indicating that the oxidation process was irreversible in nature.

7. 3. 2. *Chromatographic Analysis of Chromatic Malachite Green*

In the initial chromatographic studies, variations were made in the mobile phase composition to optimise the analysis time and peak width. A mobile phase composed of 70/30 methanol/sodium acetate buffer, pH 4.5 yielded the optimum results.

In order to determine the working potential used for the amperometric detection of malachite green, hydrodynamic voltammograms were constructed.

The hydrodynamic voltammogram shown in Figure 7. 2 exhibited one wave, a finding which was in accordance with the results obtained from the pH and cyclic voltammetric studies. The hydrodynamic voltammogram indicates that a working potential of +1.3 V was required for maximum response. However, at potentials greater than +1.2 V, both the background current and noise levels increased rapidly. Consequently, a potential of +1.2 V was used throughout the study, as this gave the optimum signal to noise ratio.

A pretreatment protocol which improved the peak current involved the pre-anodisation of the microelectrode at +1.3 V for 60 seconds followed by cathodisation at -1.3 V for 60 seconds *vs* Ag/Ag3PO4, followed by equilibration at +1.2 V for 3 min.

7. 3. 3. *Precision of Method*

Calibration curves prepared on each of three successive days were linear over the range 0-15 mg/l with correlation coefficients of greater than 0.999 as shown in Table 7. 1.

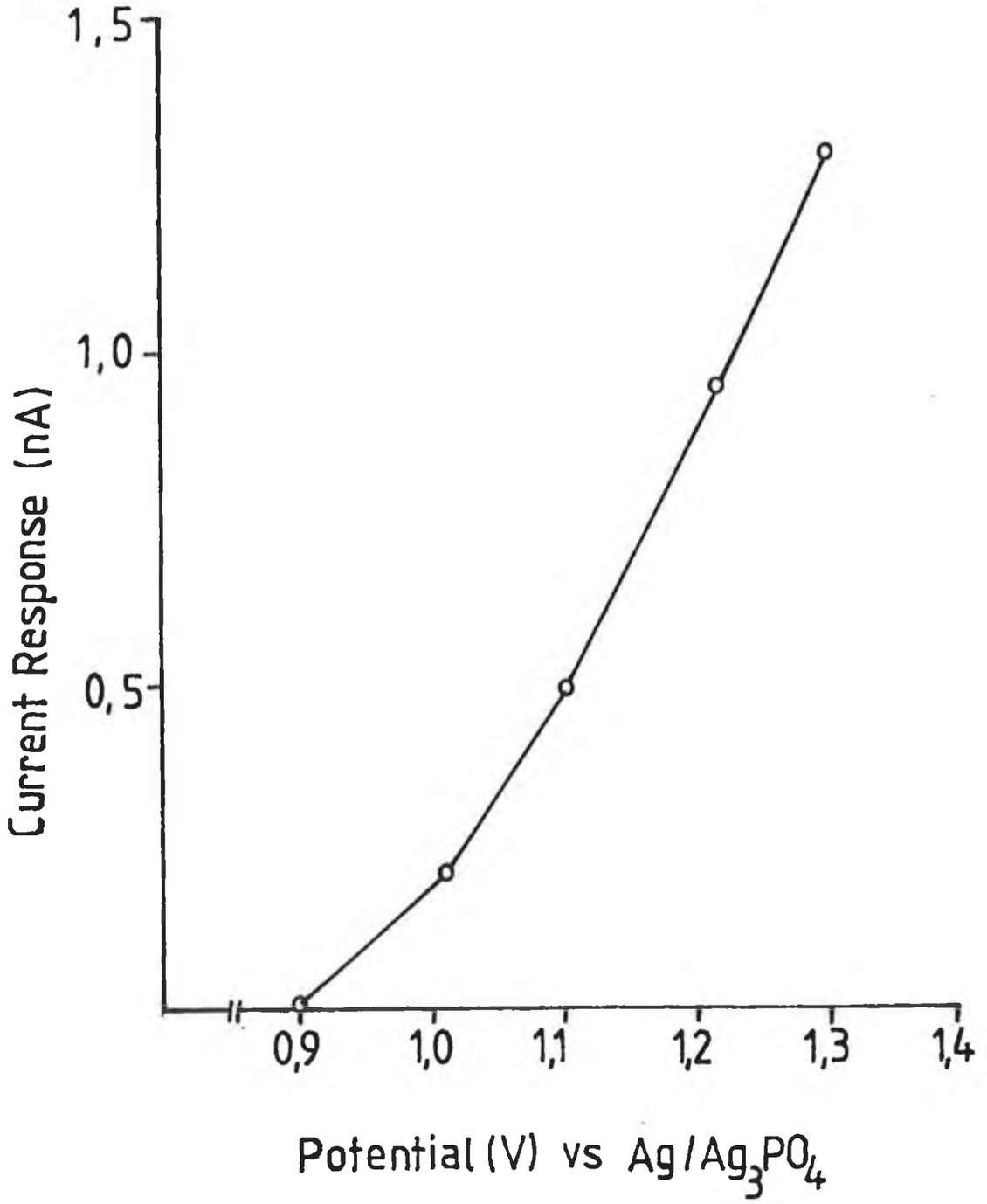

Figure 7. 2. Hydrodynamic voltammogram of malachite green obtained by injecting 5mg/l malachite green into the chromatogram with subsequent detection at the carbon. fibre working electrode at different potentials (vs Ag/Ag_3PO_4).

The reproducibility of the method was determined from five replicate injections of 5 mg/l and 0.25 mg/l standard solutions. Typical reproducibilities, determined from the coefficient of variation of the peak currents, of 3.6% and 5.4% were achieved at the 5 mgL^{-1} and 0.25 mgl^{-1} levels respectively.

The detection limit, defined as three times the signal to noise ratio, was 0.1 mgl^{-1}. However, as previously mentioned, a lower detection limit should be possible with the use of a potentiostat designed to specifically measure the low currents at carbon fibre microelectrodes.

Table 20. Regression equations and correlation coefficients for calibration curves in the range 0 - 15 mg/L prepared on each of three successive days

Day	Regression equation	Correlation Coefficient
1	y= 0.01068 + 0.20155x	0.9997
2	y= 0.02328 + 0.21557x	0.9998
3	y= 0.01821 + 0.21392x	0.9998

7. 3. 4. ***Analytical Application***

The chromatographic method was applied to the analysis of drinking water and river water samples spiked with 1.0 µg/L chromatic malachite green. In Figure 7. 3, chromatograms are shown for a blank drinking water extract and a spiked drinking water extract.

The chromatograms show well defined peaks for the chromatic form of malachite green with a retention time of 5.9 minutes. There were no late eluting peaks in the analysis, thus enabling successive injections to be made without the need for a wash period.

The mean recoveries for malachite green were found to be 82% and 41% in drinking water and river water samples respectively. The reproducibility of the determination was measured by carrying out successive injections for drinking

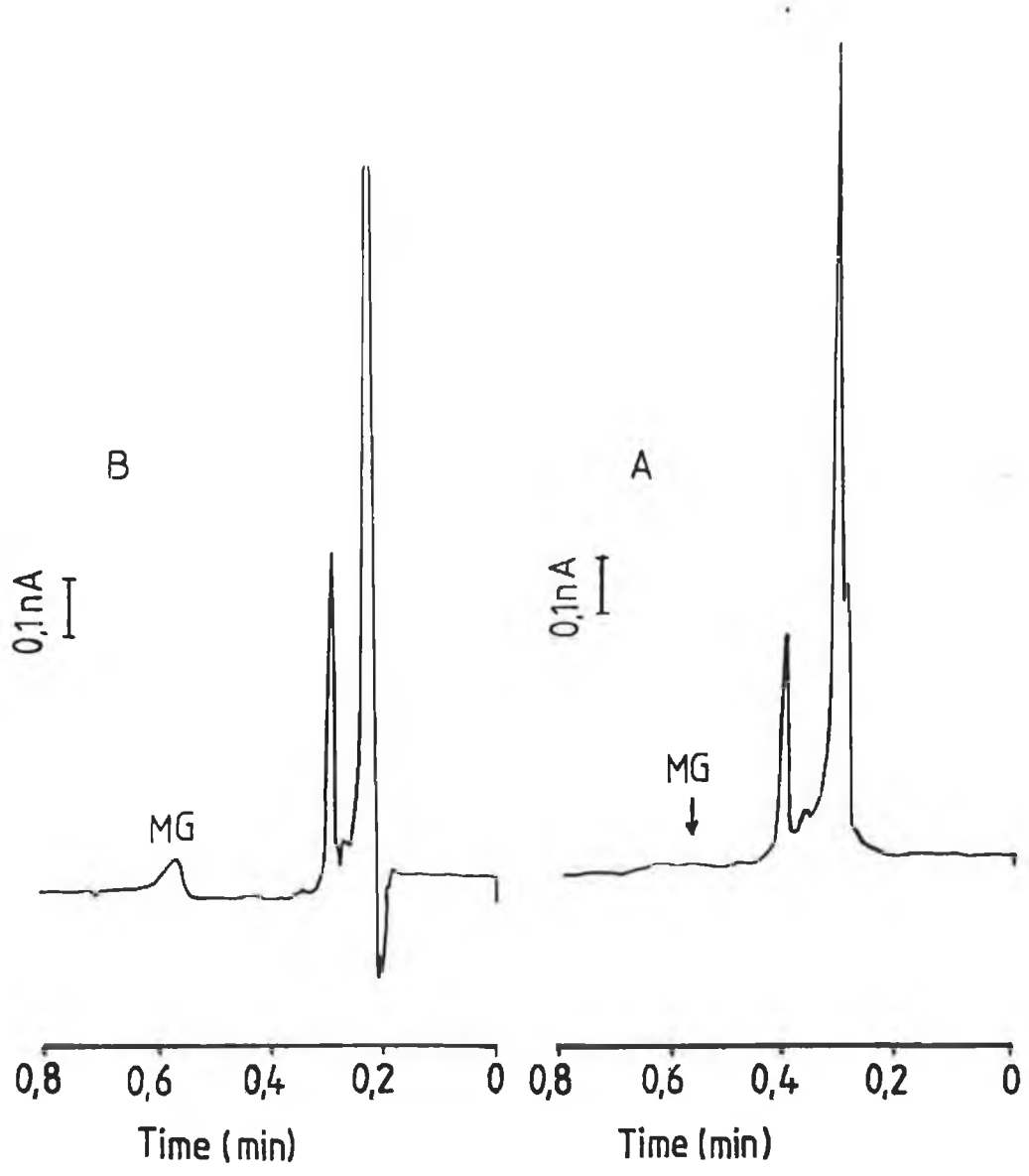

Figure 7. 3. HPLC chromatograms of drinking water extracts,(A) blank drinking water sample (B) drinking water sample spiked with 1 µg/l malachite green.

water extracts. The reproducibility, as determined by the relative standard deviation, was 6.5%.

In Ireland, the current directives on malachite green in water state that the maximum permissible levels in fish farm effluent water and water extracted for drinking purposes should not exceed 100 μgl^{-1} and 1.0 μgl^{-1} respectively. The chromatographic method described here is capable of determining malachite green at the 1.0 μgl^{-1} level.

The sample analyses were carried out in the nanoampere (nA) current range. The use of lower current ranges was prohibited by the presence of early eluting electroactive species which in the low current ranges produced large chromatographic peaks that obscured the analyte peak of interest. As part of continuing research in Trinity College Dublin, the incorporation of an extraction procedure into the analytical methodology is being investigated. It is hoped that this will eliminate these electroactive species thus allowing sample analyses to be carried out in the lower current range, and as a consequence improve the detection limits.

The low recoveries of malachite green from river water samples was thought to be due to its sorption on organic material in the water column. The fraction of dissolved or colloidally dispersed organic macromolecules, such as humic substances or non-filterable particles in natural waters, have been shown to exert significant sorptive properties towards organic compounds. Harrison et al. indicated that organic compounds of low solubility exist in the water column primarily sorbed on suspended solids[26]. The accumulation of such organic compounds in sediments has been attributed to their sorption on suspended solids and subsequent settling[27]. Similar findings were reported by Rushing and Bowman[28] for the analysis of gentian violet in wastewater. The low recoveries encountered during this analysis were attributed to the adsorption of gentian violet onto particulate matter present in the wastewater.

Further chromatographic work is in progress to determine the extent to which malachite green is sorbed onto organic matter. This speciation work is of considerable importance in elucidating the distribution of malachite green in a water system.

7. 3. 5. *Electrode Stability*

The stability of the electrode is of importance in order to define the period of time over which the electrode can be used without a significant decrease in the sensitivity. The response of the electrode to a 5 mg/L malachite green standard solution was measured over a nine day period. As shown in Figure 7. 4, a significant decrease in the response was observed after the fourth day.

The carbon fibre flow detector yielded peaks whose currents had relative standard deviations of 5.4 %, 4.8 %, 6.1 % and 5.6 % on each of the first four days respectively. After 6 and 9 days of continuous use, the electrode response was 61% and 38% respectively of its initial value, with relative standard deviations of 9% and 11%. There was no leakage from the electrode during its operating lifetime and the nature of its construction made it very stable in the mobile phase.

As the carbon fibre working electrode can be easily and cost effectively replaced, this decrease in sensitivity is not of major consequence. The use of a glassy carbon macroelectrode would otherwise require extensive physical cleaning and polishing steps to renew the electrode surface and consequently regain a sensitive response.

7. 3. 6. *Analysis of Chromatic and Leuco Malachite Green*

The chromatographic separation and detection of the chromatic and leuco forms of malachite green, shown in Figure 7. 5, was achieved on a cyano column using a mobile phase composed of 70/30 methanol/0.1 M sodium acetate, pH 4.5 and amperometric detection at +1.2 V. Under these conditions the leuco and chromatic forms of malachite green separated into two well resolved peaks with retention times of 5.0 and 5.9 minutes for the leuco and chromatic forms respectively.

This ability to simultaneously analyse the chromatic and leuco forms is of great importance in studying the biotransformation of malachite green into its leuco form. Werth et al.[29], have shown that the organs of rats, injected with malachite green, contain a considerable proportion of leuco malachite green two hours after injection. Although never tested, the leuco form of malachite green is structurally similar to classical aromatic amine carcinogens. In their study on the detoxication of malachite green in rats by the formation of the leuco form of malachite green, the authors determined the distribution of leuco malachite green in tissues by carrying out the photometric titration of leucomalachite green with potassium permanganate in a weak acid solution. However, extreme care had to be exercised in order to ensure that there was quantitative oxidation whilst at the same time avoiding excess

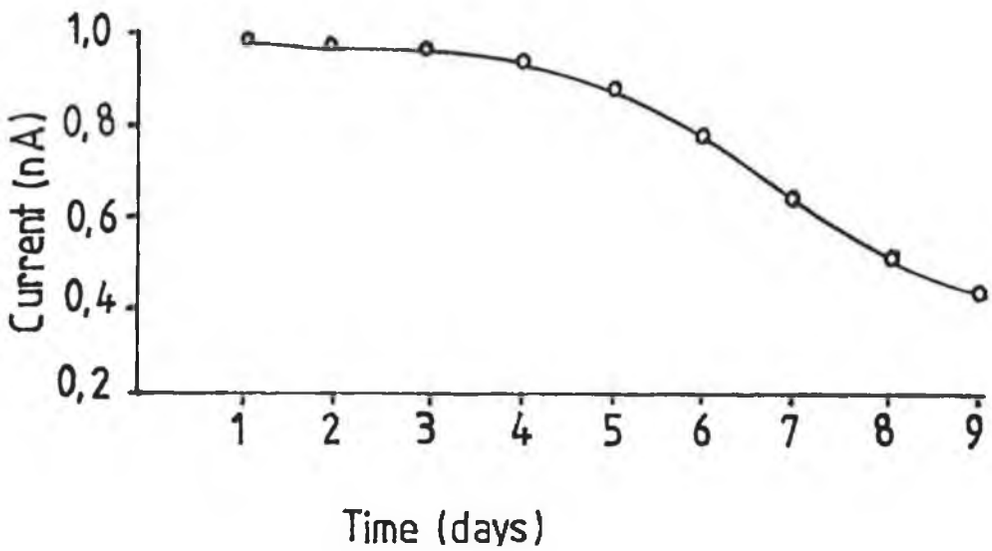

Figure 7. 4. Current response of carbon fibre working electrode to a 5 mg/l malachite green .solution over a nine day period.

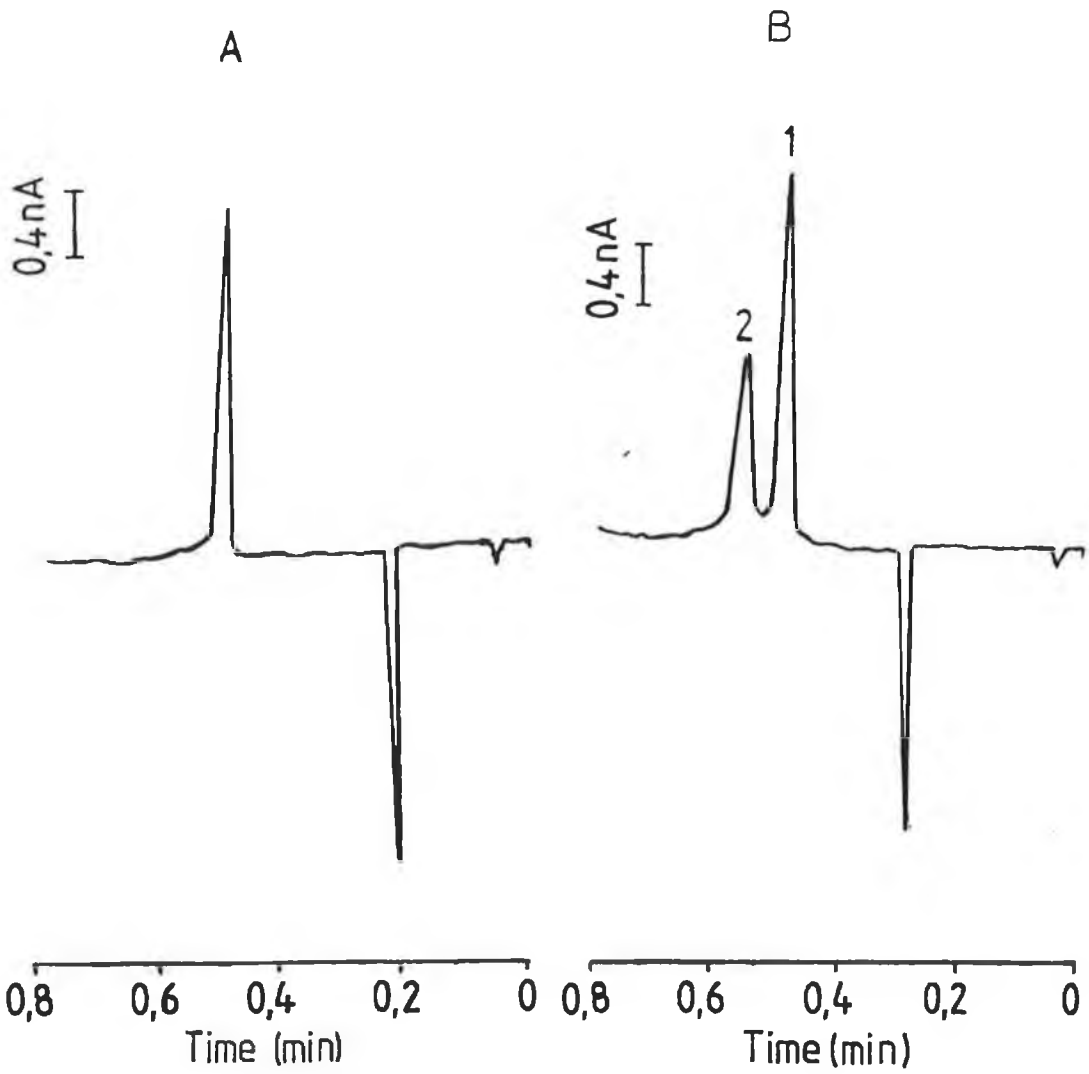

Figure 7. 5. Typical chromatograms for (A) 5 mg/l leuco malachite green and (B) a mixture .containing 5 mg/l of chromatic and leuco forms of malachite green.

titration of potassium permanganate which would otherwise cause oxidative destruction of the molecule. In addition, the background presence of the chromatic form of malachite green in the sample extract meant that its concentration had to be determined prior to the titration.

Allen et al.[22,23] have previously reported on the use of a post column oxidation step to allow the determination of the chromatic and leuco forms by spectrophotometric detection. This involved the packing and incorporation of a lead dioxide column into the chromatographic apparatus.

The use of the chromatographic method described here should allow direct analysis of a sample extract for chromatic and leuco forms of malachite green without the need for any post column modification procedure, as the use of the carbon fibre flow cell represents a small volume, easily incorporated system which can be simply maintained and replaced when the sensitivity of the response decreases.

At the present time, work is being carried out as part of ongoing research in Trinity College Dublin to adapt this methodology to the simultaneous analysis of the chromatic and leuco forms of malachite green in tissue samples.

As the leuco form is one of the final intermediates in the production of chromatic malachite green, it is therefore a potential contaminant in commercial supplies. The use of the HPLC method described is ideally suited for rapid quality control of the final product to determine possible contamination from leuco malachite green.

7. 4. CONCLUSIONS

A high performance liquid chromatographic method has been developed for the analysis of chromatic malachite green in drinking water and river water, based on solid phase extraction, reversed phase chromatographic separation on a cyano column and amperometric detection using a carbon fibre flow cell. The method compares favourably with that reported by Allen et al.[23]. The use of electrochemical detection in conjunction with high performance liquid chromatography permits the sensitive and reproducible determination of the chromatic form of malachite green in drinking water samples at those levels required under current legislation. In terms of detection limit, a lower value should be obtainable with the use of a potentiostat specifically designed for the measurement of the small currents associated with microelectrodes.

The use of electrochemical detection for the analysis of malachite green shows potential for the simultaneous determination of its chromatic and leuco forms. Both the parent drug and the metabolite can be measured in a single chromatogram using LC-ED.

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Appendix A: Publications

1. "Differential pulse voltammetric determination of sumatriptan succinate (1:1) in a tablet dosage form", Sagar, K., Fernandez Alvarez, J., Hua, C, Smyth, M. R. and Munden, R., J. Pharmaceutical and Biomedical Analysis, **10** (1992) 17.

2. "Development of a microelectrochemical flow cell using carbon or gold fibres for voltammetric and amperometric analysis", Hua, C., Sagar, K., McLaughlin, K., Manuel, J., Meaney, M. and Smyth, M. R., Analyst, **119** (1991) 1117.

3. "Analysis of salbutamol in human plasma by HPLC with electrochemical detection using a microelectrochemical flow cell", Sagar, K., Hua, C., Kelly, M. and Smyth, M. R., Electroanalysis, **4** (1992) 481.

4. "Analysis of terbutaline in human plasma by on-line solid phase extraction with electrochemical detection using a microelectrochemical flow cell", Sagar, K., Kelly, M. and Smyth, M. R., J. Chromatography, **577** (1992) 109.

5. "Simultaneous determination of salbutamol and terbutaline at overdose levels in human plasma by high performance liquid chromatography with electrochemical detection", Sagar, K., Kelly, M. and Smyth, M. R., Biomedical Chromatography, **7** (1993) 29.

6. "Voltammetric study of electrochemical behaviour of salbutamol in determination of tablet dosage form and dissolution profiles for the dosage form", Sagar, K., Smyth, M. R. and Munden, R., J. Pharmaceutical and Biomedical Analysis, in press.

7. "Determination of gentian violet in human urine and animal feed by high performance liquid chromatography with electrochemical detection", Sagar, K., Smyth, M. R., McLaughlin, K. and Wilson, J., submitted to Analyst.

8. "High performance liquid chromatographic determination of malachite green using amperometric detection at a carbon fibre microelectrode", Sagar, K., Rodriguez, M., Smyth, M. R., McLaughlin, K. and Wilson, J., submitted to J. Chromatography.