SOME CHROMATOGRAPHIC AND ELECTROANALYTICAL STUDIES OF SPECIES OF BIOLOGICAL AND INDUSTRIAL IMPORTANCE

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.

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Dedicated to my parents, Patrick and Mary

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CONTENTS

ABSTRACT

Some Chromatographic and Electroanalytical Studies of Species of Biological and Industrial Importance

Following a short preview of electroanalytical techniques in drug analysis, a comparison was made between the use of mercury- and glassy carbon- based electrodes as electrochemical detection systems for the determination of the antineoplastic agent cisplatin and its major hydrolysis products following separation by ion-pair chromatography.

Differential pulse polarography was then applied to the determination of the antibiotic ciprofloxacin in formulated tablets. HMDEs and carbon paste electrodes (CPEs) were subsequently compared for the determination by adsorptive stripping voltammetry (AdSV) of ciprofloxacin in urine.

The electrochemical behaviour of four metabolites of ciprofloxacin at CPEs was then investigated, in addition to the development of a flow-injection analysis assay with glassy carbon-based electrochemical detection to determine the antibiotic compound in pharmaceutical preparations.

Following a brief introduction to the analysis of anaerobic sealants and adhesives, an investigation was made of the use of a cation exchange separation method, coupled to a detection system involving post-column derivatisation with 4-(2-pyridylazo)resorcinol (PAR), for the determination of transition metals in anaerobic sealants.

In order to elucidate further the role of selected transition metals in anaerobic cure mechanisms, a polarographic study of the reactions of elemental copper and iron in the presence of 1-acetyl-2-phenylhydrazine and 1-acetyl-2-phenyl-diazine based cure systems was carried out.

CHAPTER ONE

ELECTROANALYTICAL TECHNIQUES IN DRUG ANALYSIS

1.1. INTRODUCTION

The relentless pace of advances being made in such areas as medical and chemical research, and the ever increasing awareness of the deterimental effect of industrial progress on the environment, has increased the demand for highly sensitive and selective methods for the determination of analytes in a large variety of matrices.

Modern electroanalytical techniques, due to the possession of such attributes as being rapid, convenient and extremely sensitive, have made a significant contribution to meeting this demand for new analytical procedures in these areas. They have been found to be particularly useful in biomedical applications, where the determination of ultra trace levels of pharmaceutical compounds and their metabolites in biological tissues and fluids is often required. In particular, important information concerning the absorption, distribution and biotransformation of both established and new pharmacologically active compounds and their metabolites, has been obtained by using such sensitive methods.

The origins of electroanalysis dates back to the early 1920's when Heyrovsky discovered that the current flowing between the reference electrode and a dropping mercury electrode at a particular potential was related to the concentration of one of the species present in the solution through which a current was flowing [1]. Such experimental work was revolutionary in its time, as instrumental methods were then a rarity, and for the first time, a method other than time-consuming volumetric and gravimetric techniques was available for the determination of analytes at below millimolar concentration levels. The contribution of this technique to the advancement of analysis was considered sufficiently important to

warrant the awarding of the Nobel prize for chemistry to Heyrovsky in the late 1950's.

The original direct current (DC) polarographic technique developed by Heyrovsky suffered from a number of disadvantages including (1) experimental waveforms were difficult to interpret; (2) in very dilute solutions, the faradaic current produced by the reduction or oxidation of the species of interest was small in comparison to the charging current which charges the electrode to the respective potential; and (3) the early two electrode equipment could not cope with the higher resistance of non-aqueous solvent systems, so applications were limited to systems which were aqueous based.

Over the course of time DC polarography could not meet the increasing demands on sensitivity in trace analysis of heavy metals (mainly in environmental detection) or in trace analysis of organic compounds in pharmaceutical, clinical and biochemical applications. For this reason, the period covering the 1950s and 1960s was characterised by a sharp decline in the practical everyday use of the technique. Ironically, it was during this period that a variety of research groups made the greatest advancements in the technical and theoritical aspects of polarography.

The charging current was the crucial-limiting factor in polarographic and voltammetric trace analysis. In order to increase the sensitivity of polarography, better discrimination was required to differentiate between the charging current and the faradaic current. AC polarography based on phase-sensitive detection was developed as a unique way to solve this problem [2]. This technique comprised of polarising the sensor electrode by means of a ramp voltage with a superimposed AC voltage with an amplitude of several millivolts and frequency in the range 50-300 Hz. The capacitive current is suppressed by using a

phase-sensitive detector to measure only that portion of the alternating current which maintains a specific phase relationship with the applied potential. In such a manner the phase sensitive detector can descriminate between the capacitive current, which is 90^{o} out of phase with the applied voltage, and the faradaic current which differs from the applied potential by 45^{o}.

Barker [3,4] has been credited with making great strides in the design of modern polarographic instrumentation. His initial techniques of square wave and pulse polarography were based upon the fact that after applying a voltage pulse to the electrode, the charging current decays with time more rapidly than does the faradaic current. A current measurement is therefore taken at the end of the duration of the voltage pulse, as only the faradaic component of the signal is recorded in that period.

1.2. DIFFERENTIAL PULSE POLAROGRAPHY (DPP)

Differential pulse polarography was developed as an offshoot of square wave polarography [5]. This technique differs from normal pulse polarography in that the current is sampled prior to the application of the voltage pulse, as well as at the end of the pulse. The difference between the two sampled currents is recorded in the form of a peak, rather than the usual polarographic wave. By virtue of this pulsing and sampling process, which minimises the influence of the capacitative current, DPP is an extremely sensitive technique and can detect analyte concentrations down to 10^{-8} M. Other features that are associated with DPP include its applicabilty over a wide concentration range, the ability to differentiate between different valencies of the same element, and the determination

of several elements or species in a single run.

1.2.1. Vitamins

Most of the organic substances determined by DPP have been of pharmaceutical importance. According to Linquist and Farroha [6], one of the first class of pharmaceutical compounds to be studied by DPP was the vitamins. Riboflavin can be determined in phosphate buffer (pH 7.2), with a linear concentration range of 0.1 to 1.0 ug ml^{-1} and a detection limit of 0.1 ug ml^{-1}. Lithium hydroxide was the supporting electrolyte of choice in the analysis of nicotinamide, with a linear concentration range similar to that of riboflavin and a detection limit of 0.01 ug ml^{-1}. The vitamins K_1 and K_3 were determined in an electrolyte containing a mixture of sodium acetate buffered to pH 5 and methanol. Detection limits of approximately 0.1 and 0.02 ug ml^{-1} were reported for vitamins K_1 and K_2 respectively.

1.2.2. Antibiotics

DPP has been successfully applied to the analysis of samples containing antibiotics such as chloramphenicol, tetracycline, rifamycin, streptomycin and adriamycin [7]. Patriarche et al. have reviewed the electrochemical behaviour of B-lactam antibiotics such as penicillins, cephalosporins, penems and carbapenems, which are one of the most frequently prescribed class of antibiotics in medical practice today [8]. Penicillins demonstrate no inherent polarographic activity and as such must be converted to electroreducible derivatives before analysis [9-11]. Penicillins can be assayed polarographically as their

penicilloic acid derivatives following basic or enzymatic hydrolysis [12], as 2-hydroxy-3-phenyl-6-methylpyrazine following acid hydrolysis in the presence of 0.1% formaldehyde [10], or as nitroso derivatives [12]. Unlike the penicillins, the cephalosporins are electroactive at the dropping mercury electrode (DME). Ogorevc et al. [13] reviewed and subsequently divided the literature dealing with their electroactivity and resulting analytical applications into two parts: (1) those papers concerning direct polarographic activity of cephalosporins which contain one or two electroreducible groups and (2) those papers dealing with polarography of their degradation products, after acidic, neutral or alkaline hydrolysis. It is thought that the polarographic activity of the cephalosporins may be due to the reduction of the double bond at position 3 in the cephem nucleus. However, this activity is dependent on the presence and nature of the substituent at position 3.

1.2.3. Tranquillisers

The popularity of determining the pyschopharmaceutical compounds containing 1,4-benzodiazepine by DPP is based on the ease of reduction of the azomethine group (C=N-) contained in their molecular structure [14]. Diazepam, bromazapam, flurazepam and other compounds containing this functional group have been successfully determined by DPP, although reduction is complicated when other electroactive groups are present, such as the aromatic nitro group in nitrazepam and the hydroxyl group in the 3-position in oxazepam. Although these compounds and their decomposition products formed during storage have been primarily determined by DPP in the pharmaceutically formulated state, metabolites of these substances have been polarographically determined in biological fluids [15].

1.2.4. Other drugs

Brooks et al. [16] have reported the determination of the antibacterial agent trimethoprim alone or in combination with sulphamethoxazole in biological fluids. The agents are initially extracted from blood or urine with chloroform after pH adjustment to 11.5. In the case of blood analysis, the compounds are back-extracted into 0.5 M H_2SO_4 and polarographed directly. For the determination of these substances in urine, trimethoprim is separated from N-oxides and hydroxylated metabolites by thin-layer chromatography with ethanol as the mobile phase and determined by DPP in 0.005 M H_2SO_4. Concentrations of about 1-3 ug ml^{-1} and 0.5 ug ml^{-1} have been determined in blood and urine respectively.

Two closely related compounds used in gynaecological practice, 1-(2-nitro-1-imidazol)-3-methoxy-2-propanol and chloromethyl-2-methyl-5-nitro-1-imidazolethanol have been simultaneously differentiated and determined by DPP [16]. After deproteinisation of the blood filtrate, the pH is adjusted to 7, which is followed by extraction with ethyl acetate and the subsequent evaporation of the organic phase. The residue is dissolved in 0.1 M NaOH and polarographed. The detection limit for the procedure is about 0.2-0.3 ug ml^{-1} and the procedure is more sensitive than spectrophotometric determination (1-2 ug ml^{-1}) but less sensitive than gas chromatography with electron capture detection (1-2 ng ml^{-1}).

The polarographic reduction of the cardiovascular drugs digoxin and digitoxin can occur at the C=C bond in their five membered ring structure that also contains a conjugated C=O group. A detection limit of 2.5×10^{-6} M was reported for both drugs in the DPP assay using propan-2-ol/0.1 M tetrabutyl-ammonium bromide as electrolyte [17].

1.3. VOLTAMMETRY AT CARBON-BASED ELECTRODES

When electrodes other than the standard dropping mercury electrode are employed, the technique is then referred to as voltammetry. The use of solid electrodes in voltammetry is necessitated by the need to enlarge the scope of detectable compounds by expanding the attainable anodic potential range. Although the most common electrode materials are carbon based, gold and platinum have also been used as working electrodes.

Since their introduction more than thirty years ago, electrodes based on carbon paste [18] and glassy carbon [19] have received considerable attention by electroanalytical chemists. In recent years interest has focused on the enhancing the sensitivity and selectivity of these electrodes by modifying their surface with either a suitable chemical or biological species. The modified electrodes are then normally employed as selective electrochemical detectors for both flow systems and liquid chromatography [20]. Numerous publications have also in the last ten years reported in particular on the extraction [21] and adsorption capabilities [22] of carbon paste electrodes.

Carbon paste electrodes are noted for their unique properties, such as low background currents, favourable anodic potential range, extraction and adsorption capabilities and easily renewable electrode surfaces. Among the more important features of glassy carbon is its greater inertness to chemical attack than other types of carbons such as graphite paste electrodes, its very small pore size (1×10^{-2} mm), and in comparison to other carbonaceous electrodes, it has the largest available potential span.

1.3.1. Linear Sweep Voltammetry

Linear sweep voltammetry, which involves the application of a rapid linear potential sweep in the range of 10-1000 mVs^{-1}, is a widely applied technique to solid electrodes because rapid analysis times can be achieved [23]. Although the fast sweep technique provides a marked increase in sensitivity over DC polarography, it is not as sensitive as DPP.

1.3.1.1. Analgesics

Phenolic analgesics have been determined by linear sweep voltammetry at glassy carbon electrodes by Chan and Fogg [24]. In aqueous media, no cathodic and anodic waves were observed but in ethanol a suitable anodic wave appeared. Linear calibration curves were found down to a concentration level of 10 ug ml^{-1}. Barreira Rodriguez et al. [25] reported the determination of methadone, a narcotic analgesic, in urine using differential pulse voltammetry at carbon paste electrodes. After the extraction of the methadone from the biological matrix using cyclohexane, the voltammetric signal was recorded on the extract dissolved in Britton-Robinson buffer (pH 7.0). Samples containing methadone in the concentration range of 10^{-4} to 10^{-6}M could be determined by linear sweep voltammetry or differential pulse voltammetry.

1.3.1.2. Antimicrobial agents

The voltammetric behaviour of sulphanillic acid and eight sulphonamides at the glassy carbon electrode was studied

by Momberg et al. [26]. These compounds, used in the treatment of bacterial infections of the gastrointestinal tract, were found to undergo a $2e^-$ process which appeared to involve the formation of iminobenzoquinone as the final product. Differential pulse voltammetry was then applied to the determination of a typical sulphonamide, i.e. sulphaguanidine, in a pharmaceutical formulation. Calibration graphs obtained in Britton-Robinson buffer, pH 7.0, were linear within the concentration range 1 to 8 X 10^{-5} M.

1.3.2. Adsorptive Stripping Voltammetry

In order to determine trace concentrations of compounds of biological importance, stripping voltammetry is increasingly being applied to carbon based electrodes. This technique involves two separate stages; the first involves a preconcentration step to accumulate the analyte onto the working electrode, which is then, in the second step, electrochemically stripped back into solution and the measured current related to the analyte concentration present in the electrolyte. The preconcentration or accumulation step can occur with or without electrolysis depending on the analyte to be determined.

Organic compounds, which possess surface-active properties, can adsorb onto the surface of the working electrode, without electrolysis, and thus permit their measurement at subnanomolar concentration levels. This technique involving this form of accumulation at the working electrode, followed by voltammetric measurement of the surface species, is known as adsorptive stripping voltammetry (AdSV) [27].

1.3.2.1. Vitamins

Hart et al. [28] studied the accumulation behaviour of vitamin K_1 at carbon paste electrodes. The adsorption of the vitamin on the working electrode, without electrolysis, depended on the the type of graphite and the pasting agent used. In order to determine the concentration levels of the vitamin K_1 in plasma, a solvent extraction procedure using hexane and methanol was employed prior to adsorptive stripping analysis. After a preconcentration step of 15 minutes, calibration graphs were found to be linear in the concentration range of 300 to 2000 ng ml^{-1} in the supporting electrolyte of ethanolic acetate buffer, pH 5.0. The detection limit was about 180 ng ml^{-1} in the supporting electrolyte (450 ng ml^{-1} in plasma).

1.3.2.2. Tranquillisers

The adsorptive behaviour of benzodiazepines has been investigated in depth, since this group of pharmaceutical agents exhibits good adsorption properties. Although, normally determined on mercury electrodes because of the easily reduced azomethine bond within their molecular structure, there have been some reports in the literature of the determination of these compounds with carbon-based electrodes. The dibenzodiazepine clozapine has been oxidised after its accumulation, without electrolysis, at a carbon electrode [29]. Although a detection limit of 7.1 ng ml^{-1} using a glassy carbon electrode has been reported with this procedure, the latter electrode was not able to accumulate selectively the dibenzodiazepine from human serum samples.

Jarbawi et al. [30] determined the phenothiazine

derivatives, promethazine, diethazine, trifluoperazine and fluphenazine in urine and plasma using a wax-impregnated graphite electrode. By covering the electrode with a Spectropor membrane to prevent fouling and protein adsorption, the aforementioned tranquillisers could be directly determined without preliminary separation from the biological matrix. Sensitivity was enhanced by the use of this membrane from 1 X 10^{-5} to 5 X 10^{-8} M using a 15 min accumulation time.

1.3.2.3. Antidepressants

Through a process of medium exchange, whereby the preconcentration step is carried out in the sample solution and then the electrode removed to a blank supporting electrolyte for the voltammetric step, the tricyclic antidepressants, imipramine, trimipramine and desipramine were determined in urine [31]. After 4 min preconcentration, detection limits of 1.4 X 10^{-8} M trimipramine, 1.5 X 10^{-8} M imipramine and 1.7 X 10^{-8} M desipramine were obtained.

1.3.2.4. Chemotherapeutic agents

A rapid and simple AdSV procedure for the determination of the antitumour agent adriamycin in urine has been developed by Chaney et al. [32]. The carbon paste electrode was immersed in the sample containing adriamycin for a 3 min period, rinsed and then the differential pulse voltammetric measurement made in a blank supporting electrolyte, pH 4.5. Limits of detection of the order of 10^{-8} were achieved with linear calibration in the range 10^{-5} to 10^{-7} M and a relative standard deviation of approximately 10%. This analysis

procedure, which takes less than 10 min, permitted the determination of the drug in a urine sample from a cancer patient 2 h following intravenous administration. Although the principal metabolite of the drug, adriamycinaglycone has strong adsorptive properties, other chemotherapeutic agents, including cisplatin, mitoxanthrone, mitomycin C and vincristine do not adsorb strongly.

1.3.2.5. Other drugs

The spontaneous adsorption of 9,10-phenanthrequinone (9,10-PAQ) and other structurally similar organic compounds on the carbon paste electrode was utilised by Cheng et al. [33] to develop a selective preconcentration step for the trace analysis of these compounds. Relatively linear calibration graphs were obtained with 9,10-PAQ, over the concentration range of 10^{-6} to 10^{-8} M, using perchloric acid as the electrolyte and an accumulation time of 15 minutes. A detection limit of 1×10^{-9} M was reported for this neuroactive compound, when differential pulse voltammetry was used after the preconcentration step.

1.4. LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

The rapid progress of high performance liquid chromatography (HPLC) in the last twenty years has created greater demands for the development of more sensitive and selective detectors. Electrochemical detection, following separation by HPLC, has been shown to possess great potential for the determination of trace quantities of electroactive species of biomedical and biological interest in complex matrices [34]. Although, it is not as widely used as ultra

violet spectrophotometric detection or fluorimetric detectors, it has become unrivalled in certain areas of biomedical research in terms of sensitivity and linear dynamic range.

The initial reports of using the electrochemical or electrical properties of chromatographic eleuents as the basis of detection date long before HPLC. In 1940, Troitski [32] detected the adsorption boundaries in liquid chromatography by measuring the dielectric constants, while the first applications of the combined use of polarographic detectors and liquid chromatography can be found in the work of Drake [36] and Kemula [37] in the 1950s. However, it was not until the 1970s that the full potential of the use of thin-layer electrochemistry after liquid chromatographic separation was fully achieved, by further adaptation and refinement of the electrochemical detection technique, by Adams [23] and Kissinger et al. [38].

Electrochemical detectors can be classified into two main categories: (a) voltammetric detectors using solid electrodes such as carbon paste, glassy carbon and platinum and (b) polarographic detectors using the dropping mercury electrode (DME). The latter type of electrode is normally used in the reductive mode as its cathodic range extends up to -1.5 V versus the standard calomel electrode (SCE) in acidic solutions, and extends even further in neutral and acidic media [39]. Some shortcomings associated with the DME is the continous need to deaerate the mobile phase, to prevent reduction of oxygen, and the limited anodic range of the electrode, since mercury is oxidised at approximately + 0.4 V vs. SCE, giving rise to high background currents.

Amperometric detectors are by far the most popular electrochemical detection system in use. The electrode material most frequently employed in commercial amperometric detectors is glassy carbon, as it has good electrocatalytic properties,

lower susceptibility to surface poisoning than that of other materials such as platinum and gold, inertness to chemical attack and small pore size. Other carbon-based electrodes such as carbon paste are not as widely used, as they are limited to aqueous mobile phases only, because organic solvents such as methanol and acetonitrile in the mobile phase dissolve the pasting agent. The working electrode, in amperometric detectors, is maintained at a fixed potential versus the reference electrode and the current or signal results in the oxidation or reduction of a small fraction (usually 1%) of the electroactive species [40]. The analyte concentration in the mobile phase after separation by HPLC is proportional to the current measured.

There are several amperometric cell designs, the most common of which are the tubular [41], wall jet [42] and thin-layer [38] types. The latter design of cell has been the most commercially successful and although there are several variations, it generally consists of a rectangular block with an inlet and an outlet, in which the working electrode is situated in one half of the cell and is parallell to the mobile phase flowing through it [34]. The other half of the cell contains the auxiliary electrode and reference electrode. The auxiliary electrode, which is made of stainless steel, can be at the outlet of the cell [38] or placed opposite the working electrode [43], with the reference electrode downstream of it. A gasket separates the two halves of the thin-layer cell, the thickness of which directly affects the current produced during the detection of the electroactive species.

The selectivity of the amperometric detector can be enhanced by the use of two working electrodes held in either a series or parallel configuration [44]. In the series dual electrode detection system, the electroactive species generated at the upstream electrode can be detected at the downstream

electrode. One of the advantages of this configuration is that reversible or quasi-reversible electroactive species can be detected at a lower background and therefore lower signal to noise ratio than conventional single electrode detection. In the parallel configuration, the electrodes are placed opposite each other, so that the eluent is in contact with the electrodes simultaneously. This configuration permits the simultaneous determination of both oxidisable and reducible species in the same chromatogram.

Although HPLC with electrochemical detection (ED) has been employed in the determination to a wide variety of pharmaceutical agents, several classes of drugs have received more investigative attention than others.

1.4.1. Antimicrobial agents

The antimicrobial agents, nitroimidazoles [45] and erythromycin [46,47] have been determined by HPLC-ED. Eight nitroimidazoles were separated by HPLC with 5% ethanol in 0.1 M acetate buffer, pH 4.5 as the mobile phase. Two compounds with the same capacity ratios could be selectively determined in this procedure by differerential pulse polarography at the HMDE, e.g. 2-nitroimidazole and 4-nitroimidazole both had a capacity ratio of 2.09 but were reduced at the HMDE at -0.392 and -0.511 V versus Ag/AgCl, respectively.

Erythromycin was separated, under isocratic conditions, on a Sepralyte diphenyl column after extraction from alkalinised serum samples with methyl-t-butyl ether. The mobile phase consisted of acetonitrile/sodium perchlorate/ammonium acetate/methanol. Electrochemical detection was by dual coulometric electrodes operated in an oxidative mode. A

detection limit for the assay for the determination of erythromycin in serum was 0.05 ug ml^{-1}. This procedure could detect the agents' gastric degradation products in serum as well as other antibiotics such as erthromycin A, B and D, oleandomycin, tylosin and josamycin. A simple, sensitive HPLC-ED assay using a single electrode cell for the simultaneous determination of erythromycin, its esters and various metabolites in plasma, urine and saliva was described by Croteau et al. [47]. Calibration curves were linear from 0.25 to 10 ug ml^{-1} erythromycin base in plasma and in urine, the standard curves were linear from 0.5 to 15 ug ml^{-1}.

On of the most widely employed groups of antibiotics in medical practice, the penicillins, have not been directly determined by HPLC-ED, owing to the lack of suitable oxidative or reductive properties of most of these compounds [48]. However, Selavka et al. [49] have developed a post column, on-line photolytic derivatisation procedure coupled to electrochemical detection, for the determination of the penicillins. Enhanced selectivity between the different forms of penicillin could be obtained with this detection system as penicillin V, penicillin G and bacampicillin hydrochloride cannot be detected without photolysis, whereas ampicillin and cephoperazone both exhibit some form of inherent electroactive response at +1.1 V and +0.85 V (vs. Ag/AgCl) respectively. Minimum detection limits and linear ranges obtained for the penicillins with this detection technique was in the order of 10 to 40 ppb.

1.4.2. Analgesics

There have been many reports in the literature of the separation of analgesics on HPLC systems with electrochemical

detection. In this group, acetaminophen [50], salicylates [51], codeine [52] and morphine [53] have received particular attention. In the latter procedure, Jordan et al. [53] separated morphine from human serum using a reversed-phase, pH stable, octadecylsilane-modified silica column. The employment of a highly alkaline acetonitrile-phosphate buffer as the mobile phase, facilitated the electrochemical oxidation of morphine at a lower applied potential than is normally used in amperometric detection of this compound. A linear response of 1.2×10^{-12} to 4.0×10^{-10} mol of morphine injected was obtained with this procedure.

The opioid analgesics, ciramadol and dezocine, have been determined in biological fluids by HPLC with oxidative electrochemical detection [54]. Oxidation of the compounds' amine and phenolic groups at the electrochemical detector, permitted levels of 10 ug ml^{-1} ciramadol and dezocine to be detected in plasma.

1.4.3. Chemotherapeutic agents

Many chemotherapeutic agents are electroactive and this permits them to be successfully determined by a variety of electrochemical detection techniques. As most of platinum-based antineoplastic agents possess poor UV spectrophotometric and fluorescence properties, electrochemical detectors provide an excellent method for their determination at trace levels in clinical samples. Cis-platinum complexes have been detected following HPLC at dropping mercury and hanging mercury drop electrodes, glassy carbon electrode, by a halide-catalysed oxidation platinum electrode and thin-layer Au/Hg electrodes [55, 56]. In the case of the latter electrode type, detection limits at the ppb level of cisplatin in plasma was obtained,

when using the electrochemical detector in the reductive mode.

The antimetabolite, methotrexate has being assayed in serum and urine by several HPLC separation procedures with amperometric detection [57]. One procedure used a reversed-phase C18 column with isocratic elution and amperometric detection with a glassy carbon electrode set at +0.95 V (vs. Ag/AgCl) [57]. Sample treatment consisted of a simple deproteinisation step or an extraction procedure on a Sep-Pak C18 cartridge (serum) or an Amberlite column (urine). The detection limit for MTX in serum was 2.2 nmol l^{-1}.

The anthracycline antibiotics, adriamycin and daunomycin, can be detected either by oxidative or reductive electrochemical mode due to the presence of both a quinone and hydroquinone moiety in their molecular structure. Akpofure et al. [58] have detected both these antibiotics and their metabolites in serum and plasma using oxidative ED, at a potential of +0.65 V. According to the authors, the detection limits obtained using electrochemical detection were comparable with those of fluorescence detection i.e. 2 ng on column.

1.4.4. Tranquillising agents

The tranquillising agents, reserpine and rescinnamine have being determined in body fluids using a glassy carbon electrode [59]. Reserpine exhibits a single oxidation peak at +0.65 V (vs. Ag/AgCl), whereas rescinnamine exhibits a single wave at 0.72 V (vs. Ag/AgCl) in the mobile phase of methanol-phosphate buffer, pH 4.5, which contained the sodium salt of heptanesulphonic acid as an ion-pairing agent. Detection limits of 0.9 ng for reserpine and 0.8 ng for rescinnamine were reported with this procedure. Other co-administered drugs with

reserpine such as the cardiovascular drugs, propranolol and pindolol, the antidepressant desipramine or the antibiotic tetracycline did not interfer in the assay. However, rescinnamine due to the similarity of the retention and redox behaviours, would appear as a shoulder on the reserpine peak.

1.4.5. Catecholamines

The catechol nucleus is readily oxidised and therefore anodic amperometric detection has been the electrochemical detection system of choice after separation by HPLC. The well documentated reversed- phase separation of biological fluid extracts of catecholamines, including norepinephrine, epinephrine, dopamine and 3,4-dihydroxy- benzylamine, has been used extensively to explain the merits of dual electrode amperometric detection, in the parallel configuration, by Krull et al. [60]. Picogram levels of plasma catecholamines could be detected with this procedure.

1.5. FLOW INJECTION ANALYSIS WITH ELECTROCHEMICAL DETECTION

The electrochemical detectors used in HPLC-ED can in the most part be used with flow injection analysis (FIA). However, amperometric detectors using solid electrodes are subject more to surface poisoning by adsorption, which results in decreased signal stability. The DME is not affected by this shortcoming, although the drop growth and fall can give rise to fluctuation in current and this appears as noise on the baseline. In order to avoid constant mechanical cleaning and reactivation of the solid electrode after surface poisoning, an

in-situ technique of passing a pulse train to the electrode for 5 seconds is normally used to reactivate the electrode [61].

Although FIA-ED has been used extensively in the determination of pharmaceutical agents, the following examples found in the literature use unmodified solid electrodes in the electrochemical detection systems employed.

1.5.1. Tranquillising agents

Wang and Freiha [62] used adsorptive stripping voltammetry in conjunction with FIA for the selective determination of chlorpromazinein a 100 fold excess of non-adsorbable solution species with similar redox potentials, e.g. ferrocyanide and ascorbic acid. Enhanced sensitivity was obtained as a result of the preconcentration step at +0.3 V vs. Ag/AgCl for 60 s in a flowing phosphate buffer. At a flow rate of 0.3 ml min^{-1}, injection rates of 24 samples an hour and detection limits of a few nanograms were obtained. Reproducible quantitation of chlorpromazine in urine was possible without sample treatment other than a 1:3 dilution.

FIA with oxidative amperometric detection at a carbon fibre electrode was used for the rapid determination of the phenothiazines, namely perphenazine, triflupromazine and fluphenazine, in their formulated state [63]. Calibration curves were linear over 1 to 50 ug ml^{-1} range in aqueous methanol phosphate buffer, pH 7, (1:1) containing 2% sodium acetate solvent mixture. Common tablet excipients such as talc, starch, magnesium stearate and lactose did not interfer in the assay.

1.5.2. Chemotherapeutic agents

Chaney and Baldwin [64] have used the adsorption properties of doxorubicin, to develop a FIA-ED assay. The limit of detection was improved upon by one order of magnitude down to 10^{-9} M in comparison to the batch technique outlined in section 1.3.2.4. [32]. The authors reported that the FIA assay appeared somewhat more effective than the batch technique in the discrimination against adsorbable sample constituents such as uric acid.

1.6. CONCLUSION

The application of electroanalytical techniques to pharmaceutical analysis has being reviewed in the period from the early 1970s to 1990. It is not a complete review of all applications published in this period, but rather a selection of examples, highlighting the diverse range of pharmaceutical agents that can be determined by a variety of electoanalytical techniques. A more complete discussion of this topic, covering the literature published in the years 1983 to 1987 is given by Bersier and Bersier, in their excellent review of the applications of analytical voltammetry to pharmacy [65].

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CHAPTER TWO

COMPARISON OF MERCURY- AND GLASSY CARBON- BASED ELECTROCHEMICAL DETECTION SYSTEMS FOR THE DETERMINATION OF CISPLATIN FOLLOWING HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION

2.1. INTRODUCTION

Antineoplastic agents (also known as cytotoxic agents) are used in the treatment of malignant diseases. Therapy with the cytotoxic agents may cure the disease, cause malignancy to go into remission, or simply palliate the symptoms and prolong life in others.

In an area where so much research is carried out, new anticancer drugs come onto the market each year with increasing frequency. Many of the new therapeutic drugs are simply analogues of the older generation of antineoplastic agents. A case in point is cis-diamminedichloroplatinum(II) (cisplatin), for which, since its cytotoxic properties were first described by Rosenberg et al. [1,2] in 1967, over 2,000 analogues have been evaluated as potential antineoplastic agents [3].

This compound, a co-ordinated complex of divalent platinum, was first described by Peyronne in 1845 [4] and was separated into its cis- and trans-isomers by Werner in 1898 [4]. Interest was reawakened in these compounds by the findings of Rosenberg, Van Camp and Krigas [5] in their investigations on the effects of electrical fields on the growth processes of Escherichia coli. They found that an electric field generated between platinum electrodes seemed to prevent cell division. Further experimentation demonstrated that it was not the electric field, but rather platinum-containing compounds, that were responsible for the observed effects. Small amounts of platinum from the inert platinum electrodes reacted with the ammonia and chloride in the growth medium to produce $(NH_4)_2PtCl_6$ [5]. This compound was then able to react photochemically to form any of several compounds of the general formula $[Pt(NH_3)_nCl_{(6-n)}]^{(2-n)}$, where n=1, 2 or 3 [5]. It was found that only the cis-isomers of $Pt(NH_3)_2Cl_4$ and

the related platinum(II) salt, $Pt(NH_3)_2Cl_2$ (cisplatin), were active [1,2]. Since the compounds inhibited cell division, their potential as cytotoxic agents was then investigated, as cancerous cells are the most rapidly proliferating cells in an organism.

Although cisplatin showed good anticancer activity in humans, its high toxicity and poor stability generated much effort to identify other platinum complexes with better toxicological and pharmaceutical properties. This ongoing synthesis and screening for antineoplastic activity of platinum analogues has identified a number of the following features for anticancer activity [6,7].

(1) Two cis-amine groups or a bidentate amine such as ethylenediamine seem to be necessary for activity.

(2) The complex should be electrically neutral.

(3) For superior activity, platinum should be in the +2 oxidation state.

(4) The compounds should possess "leaving" ligands with intermediate bond strength e.g. chloride.

(5) The activity of the complex appears to be maximised when the "stationary" amine ligands are either in the unsubstituted or primary substituted form.

Initial clinical trials on cisplatin began in 1972, but it was not until 1978 that it was officially approved as a drug by the Food and Drug Administration (FDA) in the United States [6], and in the following year in the United Kingdom [8].

2.2. CHEMICAL AND PHYSIOCHEMICAL PROPERTIES OF CISPLATIN

Structurally, cisplatin is an inorganic complex comprising of a central atom of platinum surrounded by chloride atoms and ammonia groups in a cis-configuration (Figure 2.1.). It has a relative molecular mass of 300.1, with a melting point of 207°C.

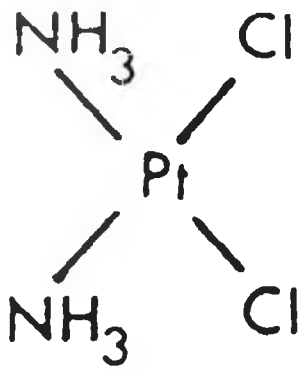

Figure 2.1. Structure of cisplatin (I).

The orange-yellow solid is sparingly soluble at a concentration of 1 mg ml^{-1} in water and at a concentration of 24 mg ml^{-1} in dimethylformamide(pure). In powdered form, it is stable for at least a year when stored in a refrigerator in a dark container.

2.3. HYDROLYSIS REACTIONS OF CISPLATIN IN AQUEOUS MEDIA AND IN VIVO

Cisplatin is usually administered either by an injection or by an infusion into the blood stream. Because of the rather high chloride concentration in the blood plasma (about 100 mM), cisplatin remains largely unaltered in the blood stream [6]. However, a significant proportion of the administered drug, i.e. between 50 to 70%, is lost via excretion [9]. When the neutral complex diffuses through the cell membranes because of the low concentration of chloride in the cells (about 3 mM), cisplatin undergoes hydrolysis reactions, and thus looses its relatively labile chloride ligands. The hydrolysis (aquated) products, i.e. cis-$[Pt(Cl)(NH_3)_2-(H_2O)]^+$ and cis-$[Pt(NH_3)_2(H_2O)_2]^{2+}$, are able to react with a wide variety of cell components such as DNA, RNA and proteins.

The existence of the aquated forms of cisplatin was first establised by chloride ion titrimetry by Martin and co-workers [10,11]. They demonstrated the stepwise replacement of the chloride ligands by water as seen in Figure 2.2.

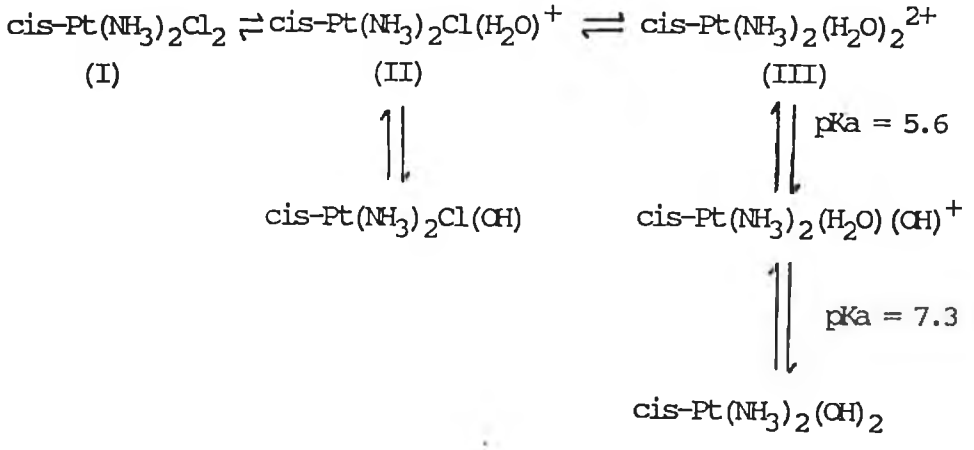

Figure 2.2. Hydrolysis equilibria of cisplatin.

From the studies of the hydrolysis processes of cisplatin in aqueous solutions, the addition of chloride to these solutions would be expected to stabilise cisplatin by shifting the equation equilibrium to the left. Since the equilibrium constant for the displacement of the second chloride ligand by water is small ($K_2 = 1.11 \times 10^{-4}$) [11] relative to that for displacement of the first chloride ligand ($K_1 = 3.63 \times 10^{-3}$) [12], the evaluation of the hydrolysis process can be simplified by consideration of the first hydrolysis step only. The rate of loss of cisplatin at any time is given by:

$$\frac{-d[I]}{dt} = k_1[I] - k_{-1}[II][Cl^-] \qquad 2.1.$$

where $I = [Pt(NH_3)_2Cl_2]$ and
$II = [Pt(NH_3)_2(H_2O)Cl]^+$

Since at equilibrium, the rate of the forward and reverse reactions are equal, then $-d[I]/dt = 0$. The equilibrium constant for the first hydrolysis step may then be defined as:

$$K_1 = \frac{k_1}{k_{-1}} = \frac{[II][Cl^-]}{[I]} \qquad 2.2.$$

From equation 2.2., it is clear that as $[Cl^-]$ is increased, the ratio of [II]/[I] must decrease, since K_1 is constant. Therefore, addition of chloride to such aqueous systems would force the equilibrium position to the left, thereby increasing the fraction present as intact cisplatin.

Greene et al. [12] were the first to systematically study the stabilising effect of the chloride ion on the

hydrolysis reaction, using UV spectrophotometry and chloride ion titrimetry. The data presented was in good agreement with that of Martin et al. [10, 11]. Hincal et al. [13] studied more extensively the rate constant for the forward reaction involving the first hydrolysis step, and their results were in good agreement with those of Greene et al. [12]. Each of these studies [12,13] found substantial stabilisation of cisplatin in aqueous solutions upon the addition of chloride.

In addition, Hincal et al. [13] studied the stability of cisplatin in aqueous solutions containing commonly used components of intravenous additives, such as dextrose, mannitol and sodium bicarbonate in the presence of varying amounts of chloride. Their data, obtained using a high performance liquid chromatographic (HPLC) procedure selective for cisplatin, showed that the presence of dextrose or mannitol does not affect the stability of cisplatin in aqueous solutions, but the addition of sodium bicarbonate adversely affected cisplatin stability, probably due to the raising of the pH and the subsequent formation of mono- and diaquo- species of cisplatin.

2.4. MECHANISM OF ACTION OF CISPLATIN

Due to the reactivity of the aquated forms of cisplatin, much speculation on the mode of action of cisplatin has centered on these forms of the drug. Although the precise mechanism of action of cisplatin is not known, the drug and its hydrolysis products appear to exert their cytotoxic effects by directly binding with DNA, RNA and other cell components. One well established point is that cisplatin causes a primary lesion on cellular DNA [14]. This growth is thought to be caused by inhibiting DNA replication (a prequisite for cell division), while transcriptional activity (RNA synthesis) and the

translational activity (protein synthesis) are relatively unaffected. Roberts and Pascoe [15] have shown that cisplatin is significantly bound to DNA.

However, in the spectrophotometric studies of Horacek and Drobnik [16], it was observed that the affinity of cisplatin for DNA is markedly lowered when the chloride levels are increased, suggesting that the aquated forms of cisplatin are involved in the interaction with DNA constituents. Rosenberg [17] and Lippard [18] have suggested that in the intracellular fluids where the chloride content is low, cisplatin is converted to the aquated platinum species which react with DNA constituents to exert the observed therapeutic action. Various studies have shown that the interaction of cisplatin and its hydrolysis species can take the form of intrastrand crosslinks [15], interstrand crosslinks [19], DNA-protein crosslinks [20] and finally reactions with individual bases, particularly purines [21].

2.5. DRUG METABOLISM AND DISTRIBUTION

In one of the first studies on the biotransformation of cisplatin in mice, Hoeschelle and Van Camp [22] using radiolabelled platinum in tumoured and non - tumoured mice, reported the highest radioactivity in kidneys, liver and spleen, with the lowest uptake in the brain. Litterst and collegues [26] examined the distribution and disposition of cisplatin following administration to beagle dogs. Plasma levels of the drug, as determined by atomic absorption spectrometry, demonstrated a biphasic clearance pattern with a rapid phase $t_{1/2}$ of less than 1 hour, and a slow phase $t_{1/2}$ of nearly five days. Four hours after treatment, plasma levels fell by 90%, whereas 60 to 70% of the administered dose was recovered in the urine. For as

long as 6 days post-treatment, elevated levels of the platinum complex was detected in the kidney, liver, ovary and uterus. This information is of interest in light of the clinical activity of cisplatin in ovarian cancer.

Pharmacokinetic studies in man showed a similar biphasic manner as outlined by Hoechelle and Van Camp [22]. De Conti et al. [24] showed that the initial phase had a plasma half-life of between 25 to 49 minutes and a second phase $t_{1/2}$ of 58 - 73 hours. Urinary excretion of the administered radioactive labelled drug was incomplete, with only 27 to 47% of radioactivity excreted in the first five days, although urinary excretion is the main pathway of elimination of the cytotoxic drug from the body. Piel and co-workers [25] detected platinum in tissue samples for as long as four months after administration.

2.6. CLINICAL APPLICATIONS AND SIDE-EFFECTS OF CISPLATIN

A large number of clinical trials have taken place since initial clinical studies on cisplatin started in 1971. Initially, this platinum complex was not a popular compound because of the severe side effects associated with its use. However, by modifying the administration procedure with diuretics and prehydration, cisplatin was initially approved in 1978 for the treatment of testicular and ovarian cancer.

Indeed, cisplatin has exhibited remarkable activity in non-seminomatous testicular tumours. With an overall response rate of 60%, cisplatin is now recognised as one of the most active drugs in testicular cancer, and therefore has been incorporated into combinations with other chemotherapeutic agents with noteworthy results. The basis for the active

testicular cancer combinations is the vinblastine and bleomycin combination reported by Samuels and co-workers [26,27]. Response rates with vinblastine and bleomycin have been reported as high as 76%. Einhorn et al. [28] and Einhorn and Donahue [29] observed particularly good results when adding cisplatin to vinblastine and bleomycin, and of 20 patients treated, 15 achieved a complete remission and 5 achieved a partial remission.

Although cisplatin alone has been used extensively and successfully for the treatment of advanced ovarian cancer, the response rates varying between 12 to 26% are poor when compared to those obtained in the treatment of testicular tumours. However, early treatments using cisplatin showed significant results compared to previously used drugs [30]. With the known single-agent activity of cisplatin, a number of combination regimens have been studied. However, none have as yet been shown to be superior to cisplatin alone.

Cisplatin has not solely been used in the treatment of testicular and ovarian tumours. Good results have been obtained when cisplatin was used alone or in combination with other neoplastic agents in the treatment of tumours of the head, neck, bladder and lung [21].

As with most types of cancer chemotherapy, the primary disadvantages of cisplatin in the treatment of cancer have been the toxic reactions associated with its use. The toxicity of cisplatin is manifested in various forms, the most severe of which include renal and gastrointestinal problems. Initially the renal toxicity was dose limiting. The severity of the renal toxicity was such that it was sufficient to prevent the use of the drug at therapeutic levels. However, Cvitkovic and co-workers [31,32] demonstrated a substantial reduction in this toxicity by utilising a protocol which included prehydration and

mannitol induced diuresis. This dosage regimen (by reducing the urinary platinum concentration and decreasing the renal residence time of the drug) permitted the continued and expanded use of cisplatin in the treatment of human cancers.

Even though other side effects of the drug, including ototoxicities [33] and allergic reactions, have been noted [34], the primary dose-limiting consideration in the use of cisplatin at the moment is nausea and vomiting [32].

2.7. A REVIEW OF ANALYTICAL PROCEDURES FOR THE DETERMINATION OF CISPLATIN AND ITS HYDROLYSIS PRODUCTS

As has been already outlined in the previous sections, cisplatin is one of the most important drugs available for the treatment of ovarian and testicular cancer. The determination of cisplatin, its hydrolysis products and analogues in biological fluids and tissues presents an interesting challenge to the laboratory analyst. Following administration of the cytotoxic agent into the body, both protein-bound and ultrafilterable (or free) species of platinum are present in plasma [35]. Although the protein-bound platinum, which is considered to be devoid of cytotoxic activity [36, 37], can be removed by centrifugal ultrafiltration, the Pt(II) complexed with low molecular weight proteins of unknown cytotoxicity may pass the commonly applied ultrafilters with molecular weight cut-offs of 25,000 or 50,000. Meaningful pharmacokinetic studies require the measurement of only those species of Pt(II) that retain cytotoxic activity. These include the intact cisplatin, its mono- and diaquo-species, and the fraction that complexes to DNA.

The ideal analytical technique used in these phamacokinetic studies would have a low detection limit (approximately 10 to 50 ng Pt ml^{-1}) for total platinum or the intact drug in ultrafilterable plasma, as this would increase the period that platinum concentrations can be monitored. However, for techniques that are capable of speciating individual platinum compounds, more sensitive techniques would be required, as the concentrations would inevitably be less than the total platinum in the ultrafiltrate.

The analytical methods which have been described in the literature may be conveniently divided into those which rely on non - selective determination of platinum, and those which permit selective determination of the intact molecules. Early phamacokinetic studies which rely on non-selective determination of platinum include graphite furnace atomic absorption spectrometry (GFAAS) [38] or radiochemical analysis using ^{193}Pt-labelled cisplatin [24]. Subsequent studies distinguished between protein-bound and free platinum by ultrafiltration of the plasma and determination by either GFAAS [39] or X-ray fluorescence spectrophometry [40].

Inductively coupled plasma atomic emission spectrometry (ICP-AES) has been used for the determination of total platinum levels in serum and urine [41]. The wide linear dynamic range (four orders of magnitude for ICP-AES compared to and only two for GFAAS), combined with its high precision and accuracy [41], make this technique competitive with the established GFAAS technique. An improvement of detection limits by an order of magnitude to 0.25-0.5 ng ml^{-1}, with the additional advantage that only small samples were required (100 ul per analysis), was reported by Alimonti and co-workers [42] when they introduced the sample into the ICP by electrothermal vaporisation.

Total platinum concentrations in urine have been determined using sodium diethyldithiocarbamate (NaDDTC) as a derivatising agent. The platinum-DDTC complex was extracted into chloroform and determined by HPLC [43]. Similar derivatisation procedures using DDTC as the complexing agent have been reported by Borch et al. [44], Reece et al. [45], Drummer et al. [46] and Andrews et al. [35]. Reece et al. [45], who applied the procedure to plasma ultrafiltrate, reported a detection limit of 2.5 ng ml^{-1}. Drummer et al. [46] applied the DDTC derivatisation to urine as well as plasma ultrafiltrate samples, using nickel(II) as the internal standard. The same procedure was used by Andrews et al. [35] for plasma ultrafiltrate. However, these authors [35] have reported that the percentage of platinum present in the ultrafiltrate (25%) is resistant to DDTC derivatisation, and thus makes this procedure unsuitable for total platinum determinations. Each of the non-selective methods described here share the same disadvantage in that they reflect the total platinum content in biological matrices, and fail to distinguish between the active platinum complexes and the inactive transformation products.

Considerable effort has therefore been directed towards the development of more selective methods for the determination of cisplatin, its hydrolysis products and analogues in biological fluids. Nearly all are based on liquid chromatographic separation techniques, including ion-exchange, ion-pair, normal phase and reversed - phase chromatography in combination with various detection strategies.

The earliest reported method for the determination of intact cisplatin was based on separation on a strong ion-exchange column. The anion exchange system described by Chang et al. [47] is capable of separating cisplatin in the presence of other platinum-containing species. However, 80% of the platinum present in the sample is eluted in the solvent

front [47]. This is probably due to the presence of positively charged species which are unretained in an anion exchange system. Hincal et al. [13], using anion and cation exchange columns in series, separated both cisplatin and its positively charged hydrolysis products. This method was only used, however, as a stability indicating assay for cisplatin in formulations, and not as a bioassay.

Ion chromatography was introduced for the determination of intact platinum drugs by Riley et al. [49-51]. A reversed-phase (C_{18}) column was used with a mobile phase containing hexadecyltrimethylammonium bromide (HTAB). The retention of the cisplatin on this type of solvent generated exchange column was thought to be the result of ion-dipole interactions between the neutral platinum complex and the adsorbed cationic surfactant [50]. The advantage of the latter type of stationary phase is that they may be operated with purely aqueous mobile phases which are compatible with the electrochemical detectors described by Bannister et al. [52], Ding and Krull [53], Richmond et al. [54] and Treskes et al. [55], and the post-column reaction detector described by Marsh et al. [56].

Although the ion-pair chromatographic separation dependent on cationic detergents determines cisplatin in its intact form, it does not separate the important positively charged species, which are unretained in these systems. Therefore ion-pair systems with anionic detergents have been developed for this purpose. Daley-Yates and M^{c}Brien [57] have reported a system using sodium dodecylsulphate (SDS). The disadvantage to using SDS in linear gradient elution is that excessive differences in retention are obtained between the analytes of interest, i.e. intact cisplatin, the platinum-methionine complexes and the hydrated derivatives. The use of gradient elution would significantly reduce analysis time

but elution with acetonitrile-water (9/1: v/v) severely disturbs the adsorption equilibrium of SDS on the stationary phase. Consequently long equilibrium times between successive analyses are required.

Alternative anionic detergents, e.g. alkylsulphonates, have been reported by Parsons et al. [58] when they used heptanesulphonic acid in their ion-pair separation system. The monohydrated derivative of cisplatin was detected less than 1 hour after incubating cisplatin in plasma ultrafiltrate, even in the presence of 100 mM sodium chloride.

As an other option to ion-pair chromatography, Shearan et al. [59] used alumina as the stationary phase material for the separation of the platinum species of interest. By employing a phosphate mobile phase of pH 5.5, the alumina column had an ionic behaviour that is neither strongly anionic nor completely neutral in character [59], and could therefore separate cisplatin and the positively charged mono- and diaquo- species.

Due to the reactivity of cisplatin and its analogues in aqueous and biological matrices and their poor solubility in organic solvents, bioanalysis of these solutes generally involves direct injection of the biological fluid into the chromatographic system. Because of their poor spectroscopic properties and lack of inherent fluorescence, there is a problem to discriminate not only between the analyte and the potentially interfering endogenous compounds, but between the analyte, its hydrolysis and biotransformation products.

Direct injection with UV detection [48] provides limits of detection for cisplatin of about 1 ug ml^{-1} at 280 nm and about 20 ng ml^{-1} at 210 nm. Untreated urine cannot be injected into a mobile phase of greater than 60% aqueous methanol due to precipation of inorganic salts. Cisplatin can be

analysed in urine with direct UV detection at 280 nm using a solvent-generated anion exchange system. The disadvantage to this procedure is that it requires an automated column switching procedure to separate the drug from its endogenous substances [60].

As an alternative to this on-line detection technique, fraction collection and determination of the fractions by GFAAS has been used in combination with HPLC for the determination of cisplatin in plasma ultrafiltrate [61] and urine [60]. Although fraction collection is tedious, sample preparation prior to chromatography is minimal and plasma ultrafiltrate or urine may be analysed by direct injection onto the HPLC column.

The best on-line HPLC detection systems for the determination of intact cisplatin or its analogues seems to be either post-column derivatisation [56] or electrochemical detection [52-55, 58]. Marsh et al. [56] developed a post-column reaction detector, based on the reaction of cisplatin with sodium bisulphite in the presence of potassium dichromate. The method required minimal pretreatment of urine or plasma ultrafiltrate, and provided a detection limit of approximately 50 ng ml^{-1}. A disadvantage to this post-column reaction is its incompatibilty with organic modifiers used in some chromatographic procedures.

Bannister et al. [52] were the first to describe the use of reductive electrochemical detection at mercury drop electrodes. Since cisplatin and its analogues can be detected at 0.0 V vs Ag/AgCl, interferences from plasma and urine constituents are minimal. A detection limit of less than 100 ng ml^{-1} of the intact parent drug in urine was reported with this detection system. The down side to this detection technique was the necessity to rigorously purge the mobile phase with nitrogen to eliminate the interference from dissolved oxygen.

By exploiting the adsorption of cisplatin at the dropping mercury electrode (DME) and its ammonium enhanced catalytic hydrogen reduction, Treskes et al. [55] reported an extremely low detection limit of 3 ng ml^{-1} of cisplatin in human plasma ultrafiltrate. However, the presence of endogenous compounds complicated the quantitation of cisplatin in urine, and the procedure as it stands could not be used in clinical studies.

Parsons et al. [58] have evaluated the use of reductive electrochemical detection with a hanging mercury drop electrode (HMDE) to detect cisplatin, some of its analogues and several degradation products. They concluded that the sensitivity of electrochemical detection for complexed species depends upon the ligands which are co-ordinated to the metal atom. Ligand substitution may produce a large change in electrochemical behaviour, even though there may be no change in the formal oxidation state of the metal.

Solid electrodes made from either platinum [54] or glassy carbon [54] have been used as an alternative to mercury-based electrochemical detection systems. The liquid chromatographic procedure with electrochemical detection reported by Richmond et al. [54] involves chloride-assisted oxidation of cisplatin at platinum electrodes. However, the addition of chloride to the mobile phase should be seriously questioned because of the ligand-exchange potential of chloride ions and the deleterious effects of chloride on the HPLC apparatus. Ding and Krull [53], who used the more convenient glassy carbon electrode-based detection system, reported the enhanced selectivity obtained with the combination of two or more sets of electrodes and a detection limit of approximately 0.5 ng ml^{-1} cisplatin.

This study set out to compare both mercury- and glassy carbon- based electrochemical detection systems for the determination of cisplatin, its hydrolysis products and chloride, following high performance liquid chromatographic separation. The separation reported in this study is based on the work of Parsons and Leroy [62], but has been optimised further with respect to the mobile phase employed.

2.8. EXPERIMENTAL

2.8.1. Materials

All materials used were of analytical grade. Cisplatin was obtained from Sigma, whereas samples of the formulated drug (Neoplatin; containing saline, mannitol and cisplatin) were obtained as a gift from University College Dublin (Pharmacology Department), having previously been donated by the Bristol-Myers Company. All solutions were prepared in water obtained by passing distilled water through a Milli-Q water purification system. Solutions prepared in saline were prepared in 0.15 M NaCl.

The mobile phase composition used throughout the study was 10 mM sodium acetate, pH 4.6, which was made 5 mM in octanesulphonic acid. The mobile phase was first filtered through a 0.45 um membrane disk, degassed using an ultrasonic bath, and finally deoxygenated with helium for 20 min.

2.8.2. Apparatus

The chromatographic system consisted of a Waters

RR/066 solvent delivery system with a Waters U6K injection port (20 ul injection loop) in conjunction with a guard column containing Waters C_{18} Corasil (37-50 um). A Waters analytical column (15 cm x 3.9 mm) packed with Nucleosil 10 C_{18} stationary phase was used in conjunction with the mercury-based electrochemical detection system, and an analytical column (36 cm x 3.9 mm) packed with u -Bondpak C_{18} stationary phase was used in conjunction with the glassy-carbon based electrochemical detection system. The mobile phase was continuously purged with helium to minimise the interference due to dissolved oxygen.

The system employed for detection of the species at mercury was a Princeton Applied Research Corporation (PARC) Model 174A polarographic analyser interfaced with a PARC Model 303A static mercury drop electrode (SMDE). A large mercury drop (area = 0.0261 cm^2) was used routinely throughout this investigation. The cell was modified with a PARC Model 310 flow adapter for liquid chromatographic detection. The system employed for detection of the species at glassy carbon was a PARC Model 400 electrochemical detector.

Chromatograms were recorded on a Philips PM 8251A chart recorder using a chart speed of 10 mm cm^{-1} or 300 mm hr^{-1}. All HPLC injections were performed with a 25 ul Hamilton microsyringe.

2.8.3. Methods

For all HPLC investigations, a 1 mg/ml stock solution of both the formulated and non-formulated cisplatin was prepared by dissolving an appropriate amount of the drug in 0.15 M NaCl or deionised water. From this, standards were prepared by dilutions of the stock with mobile phase or deionised water, and

immediately injected into the chromatographic system. HPLC separation was achieved using a mobile phase of 10 mM sodium acetate buffer, pH 4.6, which was modified with 5 mM octanesulphonic acid, at a flow rate of 1 ml min^{-1}.

2.9. RESULTS AND DISCUSSION

2.9.1. Optimisation of chromatographic conditions

Based on the work of Parsons et al. [62], the use of 10 mM sodium acetate, pH 4.6, containing heptanesulphonic acid as modifier, has been suggested as the optimum mobile phase for the separation of cisplatin and its hydrolysis products. In early studies, the use of similar mobile phases containing both pentanesulphonic acid and octanesulphonic acid as modifiers was investigated. The use of octanesulphonic acid was found to slightly improve both the resolution and the sensitivity obtained for chloride, cisplatin and possible hydrolysis products. If such modifiers were not employed in the mobile phase, only a single broad peak with a shoulder was manifested on the chromatogram.

A typical chromatogram using this system is shown in Figure 2.3. The retention time of cisplatin was not affected by increasing the concentration of the ion-pair reagents investigated. It was found, however, that the concentration of octanesulphonic acid in the mobile phase had to be greater than 4 mM before cisplatin was resolved from the other species. Although the use of such a modifier had no effect on the retention time of the neutral cisplatin complex itself, it affected the retention of hydrolysis products which are positively charged due to the substitution of Cl ions by

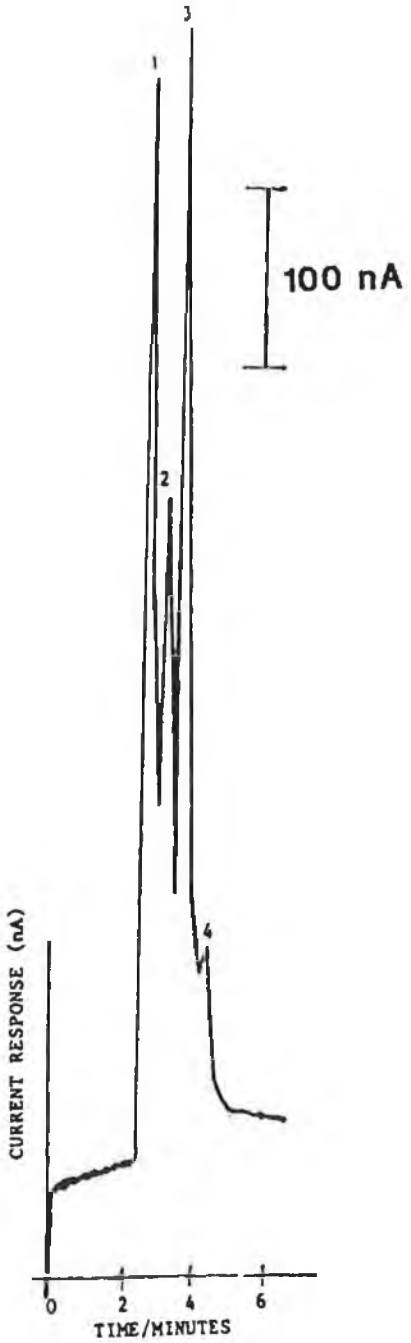

Figure 2.3. HPLC chromatogram of an aged solution of 1 mg ml^{-1} cisplatin, using a HMDE as detector at an applied potential of + 0.05 V vs. Ag/AgCl, with a pulse amplitude of 50 mV. (Peaks: 1 = sodium chloride; 2 = possible hydrolysis product of cisplatin; 3 = cisplatin; 4 = possible hydrolysis product of cisplatin).

uncharged H_2O molecules [62], resulting in improved resolution of the chromatogram. The mechanism of retention of cisplatin could possibly be a hydrophobic interaction of the complex with the reversed-phase C_{18} moieties, rather than the binding of the ion-pairing reagent to the neutral platinum complex [62]. For all subsequent investigations, the mobile phase was made 5 mM in octanesulphonic acid. The optimum flow rate was found to be 1.0 ml min^{-1}. At lower flow rates, the resolution was decreased from that shown in Figure 2.3, whereas at higher flow rates the peaks eluted close to the solvent front.

2.9.2. Optimisation of parameters affecting electrochemical detection at a mercury electrode

In this study, the use of three different current sampling schemes, involving sampled DC, normal pulse and differential pulse modes, was investigated. In the sampled DC mode, only the applied potential has to be considered. After setting the initial potential, the current is measured (sampled) for a short period of time at the end of the "drop time" set on the instrument. In both the normal pulse and differential pulse modes of operation, two potentials must be considered, i.e. the initial applied potential and the potential reached on application of the pulse. By careful selection of these potential values, the half-wave potentials of the reactions of interest can be positioned between the two potential settings. The difference between the latter two detection techniques is that in normal pulse detection, the current is measured at the end of the pulse, while in the differential pulse mode, the current is measured before and at the end of the applied pulse, and the difference recorded. The improvement in selectivity over the sampled DC mode, coupled with lower limits of detection available compared to the normal pulse mode, made the

differential pulse mode, the mode of choice for this study.

Depending on the concentration of cisplatin used and the applied potential, a maximum of three peaks and a shoulder to the third peak were detected following HPLC separation of the formulated drug (Figure 2.3). A similar study using pure cisplatin dissolved in saline produced a similar response. When chloride ions were injected separately onto the column, only the first peak (t_R = 3.0 min) appeared on the chromatogram. When a solution of cisplatin made up in water was prepared and injected immediately onto the column, only the peak at t_R = 4.0 min (peak 3) appeared on the chromatogram. The second peak at t_R = 3.6 min and the shoulder to the third peak only appeared at high concentrations of cisplatin (> 100 ug ml^{-1}) and in solutions which had been left to stand for a few days. It would appear, therefore, that these are both due to hydrolysis products, as proposed by Parsons and LeRoy [62]. At concentrations < 100 ug ml^{-1}, the chromatogram was reduced to two well resolved peaks with retention time of 3.0 and 4.0 minutes, corresponding to chloride and cisplatin respectively.

In order to determine the optimum applied potential that would give the highest sensitivity for determination of cisplatin, as well as for chloride and possible hydrolysis products, chromatovoltammetric curves were constructed by plotting the current for each peak as a function of the initial applied potential in the range 0.00 to + 0.16 V (vs Ag/AgCl). The applied potentials were increased in a stepwise manner (20 mV) over a series of injections. The results of this study are shown in Figure 2.4, where it can be seen that the optimum potential for the detection of the platinum species at mercury is at + 0.05 V (vs Ag/AgCl).

Using an initial applied potential of + 0.05 V (vs Ag/AgCl), linear calibration curves were obtained for the three

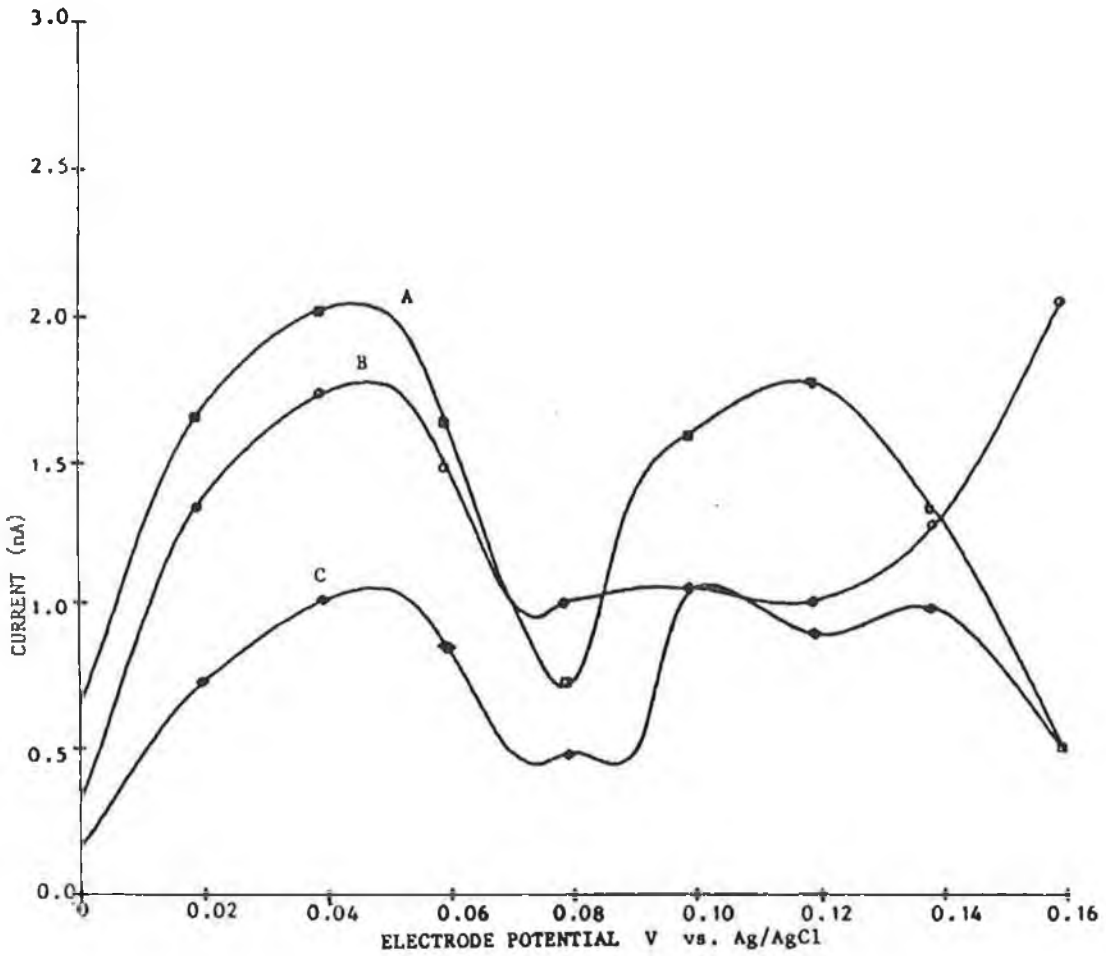

Figure 2.4. Chronatovoltammetric curves obtained for an aged solution of 1 mg ml^{-1} cisplatin at a HMDE over the potential range 0.00 to + 0.16 V vs. Ag/AgCl. (Curves: A = cisplatin; B = sodium chloride; C = possible degradation product of cisplatin).

peaks. Linear regression analysis of the data obtained for peak 3 showed that the current (I/nA) was related to concentration (C/ug ml^{-1}) through $I = 0.470\ C + 0.084$ ($r = 0.99$). The limit of detection of cisplatin at the HMDE, based on a signal-to-noise ratio of 3:1, was found to be approximately 5 ug ml^{-1}.

2.9.3. Optimisation of operating parameters for the detection of cisplatin at a glassy carbon electrode

Cisplatin gives rise to a well defined oxidation wave as shown by cyclic voltammetry in Figure 2.5. The oxidative behaviour of cisplatin and related platinum-containing drugs has previously been reported by Kauffmann et al. [63] at platinum and carbon paste electrodes.

A chromatovoltammetric curve was then constructed to optimise the applied oxidative potential to determine cisplatin (Figure 2.6). No platinum species were detected in the range 0.0 to + 1.0 V. The optimum potential was selected at + 1.2 V, because of the good sensitivity and reproducibilty obtained for cisplatin at this potential. Increasing the applied potential to more positive values increased the sensitivity of the electrode towards cisplatin but at the expense of poor reproducibility.

Both cisplatin and a hydrolysis degradation product were detected under these conditions. A typical chromatogram using oxidative detection is shown in Figure 2.7. Three peaks, with a shoulder on the first eluting peak, were obtained on the injection of 25 ul of an aged 50 ug ml^{-1} cisplatin standard dissolved in water. The peak at $t_R = 3.3$ min was identified as residual chloride ions. The shoulder ($t_R = 3.5$ min) to the aforementioned peak and the negative peak prior to the elution of cisplatin were due to solvent peaks. The peak at $t_R = 4.5$

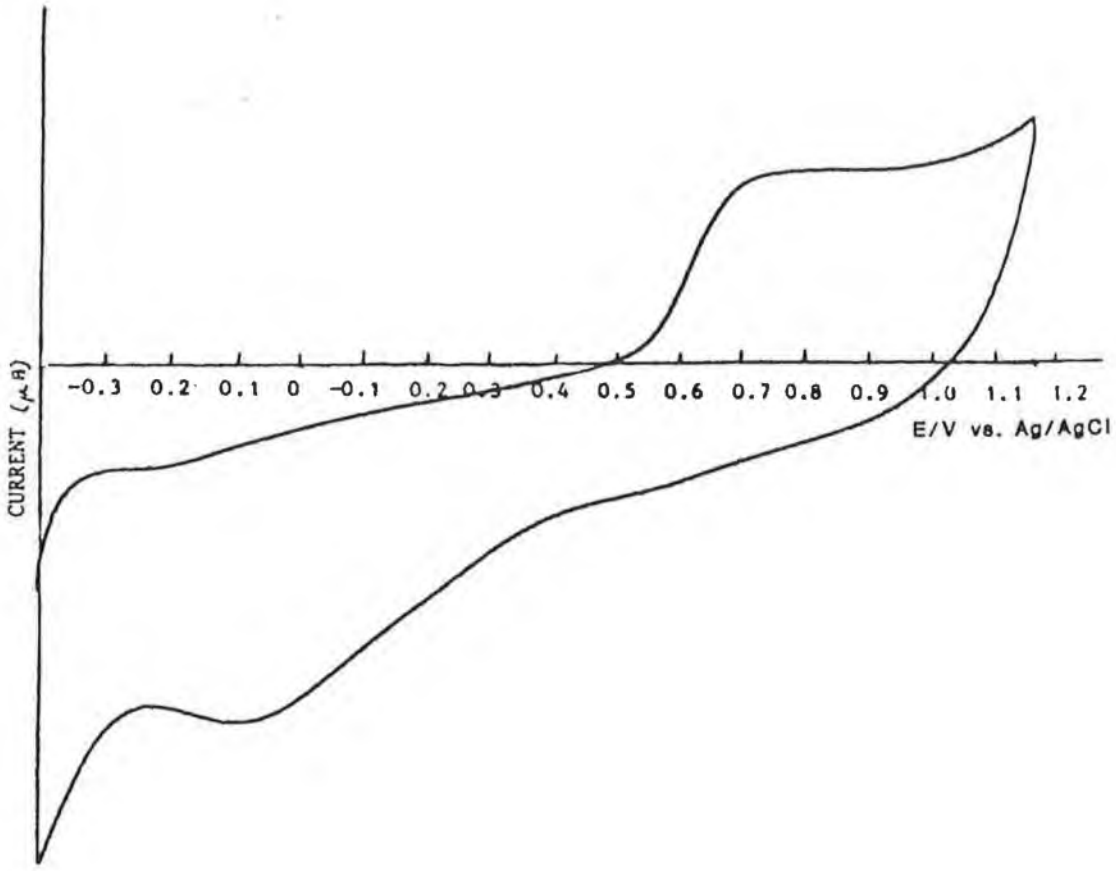

Figure 2.5. A cyclic voltammogram obtained for cisplatin at a glassy carbon electrode in a solution of 10 mM sodium acetate, pH 4.6, containing 5 mM octanesulphonic acid. (Scan rate 200 mV s^{-1}).

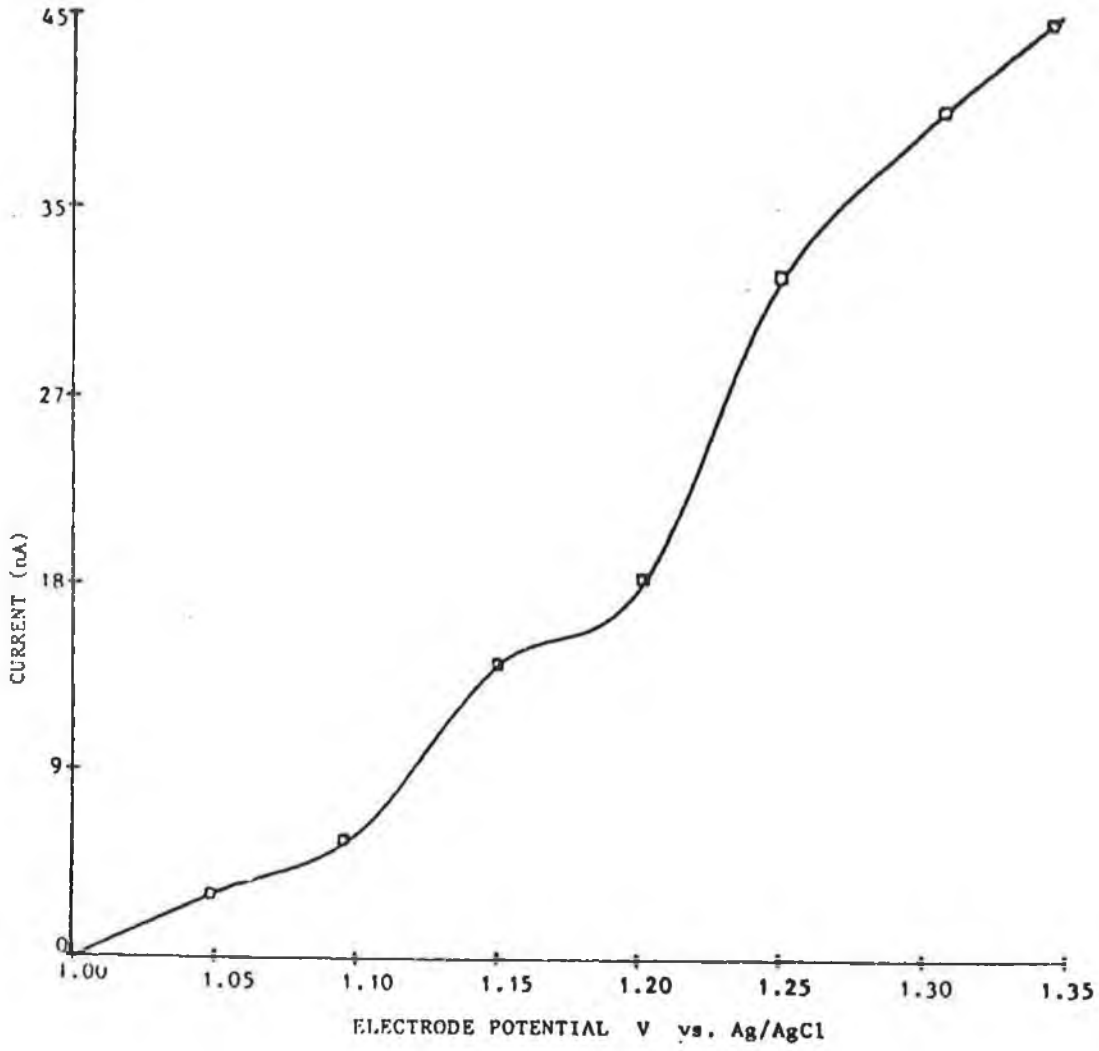

Figure 2.6. A chromatovoltammetric curve obtained for a fresh solution of 50 ug ml^{-1} cisplatin at a single glassy carbon electrode over the potential range +1.00 to + 1.35 V vs. Ag/AgCl.

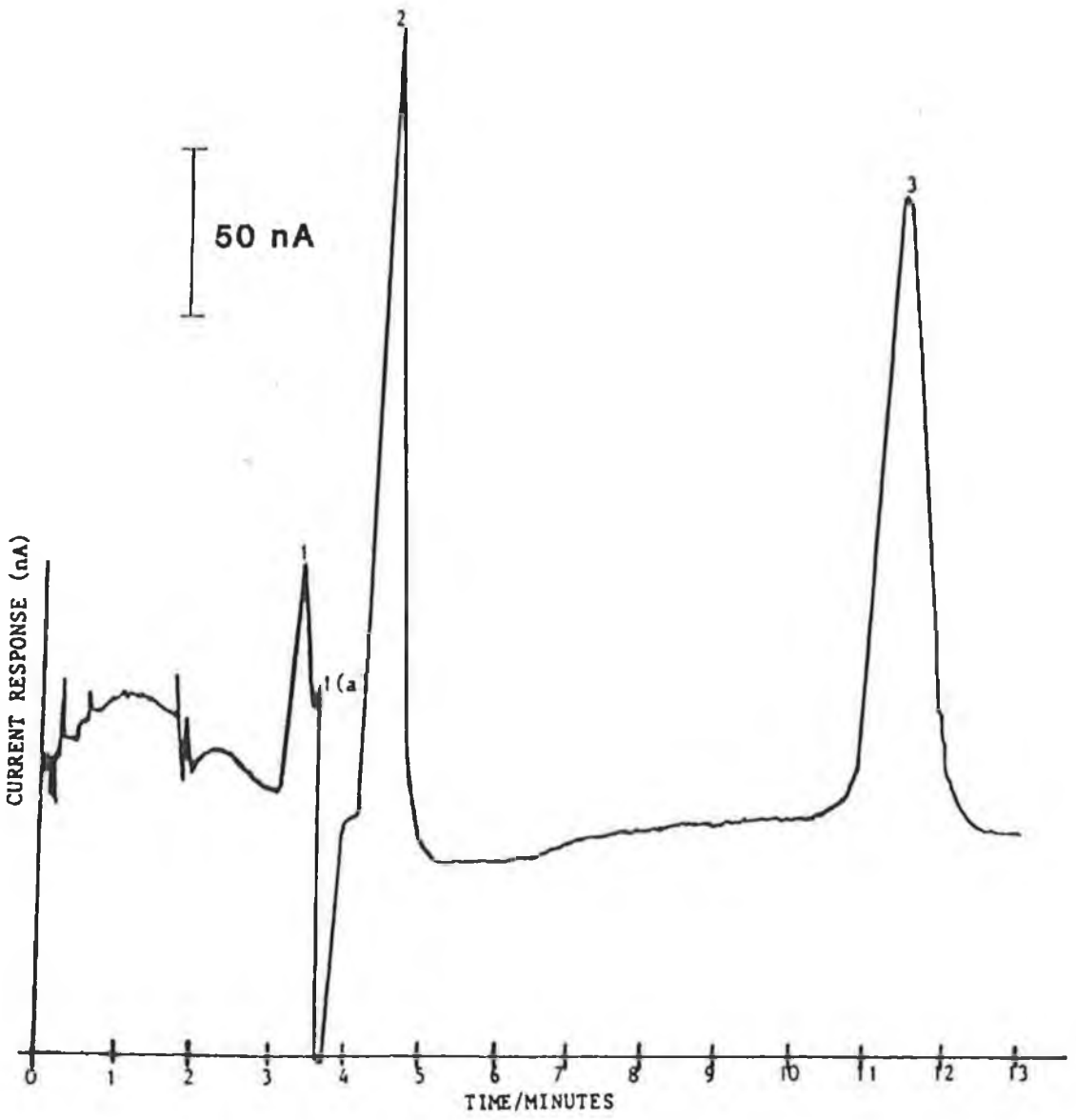

Figure 2.7. HPLC chromatogram of a fresh solution of 50 ug ml^{-1} cisplatin, using a single glassy carbon electrode at an applied potential of + 1.2 V vs. Ag/AgCl. (Peaks: 1 = sodium chloride; 1(a) = solvent peak; 2 = cisplatin; 3 = possible hydrolysis product of cisplatin).

min was identified as due to cisplatin. The remaining peak (t_R = 11.3 min) was provisionally identified as a hydrolysis degradation product of cisplatin.

A cisplatin standard prepared in water was analysed over a 6 hour period. The peak at t_R = 11.3 min was not detected initially. However, within an hour of preparation, this peak was present in chromatograms of the standard. On further standing, the size of the peak due to cisplatin decreased with a corresponding increase in the peak due to the hydrolysis product.

Using an applied potential of + 1.2 V, a linear calibration curve was obtained for cisplatin. The current response of cisplatin was related to concentration through I(nA) = 0.346 C(ug ml^{-1}) + 1.650 (r = 0.99). Under these particular conditions, a detection limit of approximately 370 ng ml^{-1} cisplatin was obtained.

2.9.4. Application of a Dual Electrode Detection System for Cisplatin Determination.

Experience with the use of a single glassy carbon electrode for cisplatin determinations highlighted particularly the problem of fouling. Fouling of the carbon surface is probably due to the high oxidative potential (+ 1.2 V) employed, and deposition of some form of platinum on the surface. The application of such a detection system to the determination of cisplatin in biological fluids would further increase electrode fouling. In addition, the detection limit of 370 ng ml^{-1} cisplatin was not considered satisfactory to determine the analyte in biological fluids. A glassy carbon dual electrode was therefore investigated to lower the detection limit, so that

this method could be applied at a future stage to the analysis of cisplatin in biological matrices.

In order to enhance selectivity for determinations in biological matrices, the dual electrode was used in the parallel orientation with a differential mode of detection. In this mode of detection, the two electrodes are set at potentials encompassing the region in which cisplatin is detected [64]. The current response at the lower potential is then substracted from the current response at the higher potential. This results in an improvement in the signal-to-noise ratio, resulting in a decrease in the limit of detection to approximately 25 ng ml^{-1}.

2.10. CONCLUSION

This study has shown that limits of detection of approximately 5 ug ml^{-1} and 370 ng ml^{-1} cisplatin can be obtained using a HMDE and a single glassy carbon electrode respectively. The use of a parallel dual glassy carbon electrode in the differential mode enhances the oxidative current response of cisplatin so that the detection limit can be decreased to 25 ng ml^{-1}.

The major disadvantage to the use of glassy carbon in the detection of cisplatin is that electrode fouling necessitates the polishing of either the single or dual carbon electrodes at least once a day. The HMDE has the advantage of providing a new electrode surface for each injection into the chromatoghraphic system and thus overcomes the problem of stationary electrode fouling. However, in comparison to the dual glassy carbon electrode, the sensitivity of the HMDE at oxidative potentials is much poorer.

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CHAPTER THREE

ELECTROANALYTICAL STUDIES OF CIPROFLOXACIN

3.1. INTRODUCTION

A large number of antimicrobial compounds, collectively known as the 4-quinolones and structurally related to nalidixic acid, have been developed in the last decade. They have rapidly become a significant class of drugs, currently holding nearly 10% of the world antibiotic market, and it is predicted by the pharmaceutical industry that this will increase by at least a further 50% [1]. Although nalidixic acid was introduced into medical use in the early 1960s, it was not used clinically to any great extent because of the rapid development of resistance to the compound [2]. In the 1970s, pipemidic acid was synthesised in Japan; however, this quinolone analogue, similar to nalidixic acid, showed a narrow spectrum of antimicobial activity, and the low concentrations obtained in serum limited its use essentially against gram-negative enteric bacilli encountered in urinary tract infections [3]. Among the group of recently synthesised quinolones, which show a broad spectrum of antimicrobial activity and low incidence of resistance, ciprofloxacin is the most potent agent against both gram-positive and gram-negative pathogens. Initially developed by Bayer Pharmaceuticals for oral and intravenous use in 1983, it was introduced into general medical practice in Great Britain in 1987, and in Germany and other parts of Europe somewhat earlier.

Although the 4-quinolones were originally used in the treatment of urinary tract infections, it soon became apparent that the clinical applications of the newer members of this group were very much wider. These included infections affecting virtually all organ systems. They are now the antimicrobial compounds of choice in the treatment of gastro-intestinal infections, urinary tract infections and most recently in the eradication of sexually transmitted bacterial infections.

3.2. MODE OF ACTION OF CIPROFLOXACIN AND POSSIBLE MECHANISMS OF RESISTANCE

The 4-quinolone antimicrobial agents (including ciprofloxacin) are thought to interact with a bacterial enzyme, DNA gyrase. Supercoiling of bacterial DNA, mediated by DNA gyrase, is essential to enable bacteria to accomodate their very long chromosomes within the cell envelope. Bacterial DNA gyrase, unlike the comparable mammalian enzyme, is susceptible to 4-quinolone antimicrobial agents.

The DNA gyrase is composed of four subunits, two A monomers of MW 105 KD and two B monomers of MW 95 KD [5]. It is believed that the A subunits first introduce a nick into each strand of the double-stranded chromosomal DNA. The DNA is then thought to be supercoiled by the B subunits which utilise a molecule of ATP for each twist that is removed. Finally, the resultant supercoils are locked into the chromosome by the A subunits which are thought to seal the nicks that they first introduced [6]. It was proposed by Gellert et al. [6] that the 4-quinolones somehow prevented the A subunits of DNA gyrase from finally sealing the staggered nicks they first introduced into chromosomal DNA. However, according to Smith [7], the mutations that affect the B subunit of DNA gyrase change bacterial sensitivity to the 4-quinolone antibiotic agents and hence the 4-quinolones would seem to affect both the B and A subunits of DNA gyrase.

Ciprofloxacin, norfloxacin and ofloxacin are thought to possess a second mechanism which vastly increases their potency against gram-negative and gram-positive pathogens. At high concentrations of the 4-quinolones, RNA synthesis is inhibited which in turn prevents their bactericidal effect from taking place [7]. However, according to Smith, in the case of

ciprofloxacin, norfloxacin and ofloxacin, it was found that the bactericidal effects of these antimicrobial agents are not as susceptible to antagonism by RNA synthesis inhibition. The occurence of this second mechanism may explain the lower frequencies of mutants resistant to therapeutic levels of ciprofloxacin, norfloxacin and ofloxacin relative to the parental 4-quinolone, nalidixic acid.

The probability of the development of resistance to the new generation of 4-quinolone antibacterial agents has been minimised in comparison with other unrelated agents through (i) reliance upon the apparent absence of plasmid-mediated resistance to the 4-quinolones, and (ii) the far greater potency per mole of agents like ciprofloxacin when compared with the parental 4-quinolone, nalidixic acid. Although resistance encoded mutations in several bacterial species to the new generation fluoroquinolones, have been described and mapped, the overall mechanisms behind the observed reduction in cellular accumulation of the fluoroquinolones in all the mutants are not clearly understood. None of these mutations results in resistance to therapeutic levels of the 4-quinolones. However, the presence of these mutations may well result in an increased probability of the selection of "second-step" mutations which do effect resistance to high levels of the drugs [8].

The best described mechanisms of resistance to the 4-quinolones involve mutations in either the gyrA or gyrB structural genes for the sub-units of DNA gyrase, which result in the production of an enzyme resistant to the toxic effects of the drugs <u>in vitro</u>. It is still to be determined if the resistance mechanism involves either (a) the provision of a new target molecule which has the reduced probability of a physico-chemical interaction with the quinolone, or (b) the provision of a modified target molecule which still interacts with the drug, but this interaction either fails to elicit a

reaction or elicits a novel and relatively inconsequential reaction. This knowledge is important as it would assist in the rational development of future generations of 4-quinolone based agents, since it would indicate which reactive components of the core molecule need to be modified in order to provide agents capable of countering the available defensive measures of the bacteria.

Two specific mutations in the gyrB structural gene for the subunit of DNA gyrase have been identified and characterised to the point of identifying the specific nucleotide and amino acid changes responsible for the occurrence of resistance [9]. The resistance mechanisms of these mutations called nalC and nalD remain obscure, as it is generally understood that the principal target of the 4-quinolones is the nicking/sealing function encoded in the A subunit of DNA gyrase. It is possible that some peripheral part of the 4-quinolone molecule reacts with the B subunit in conjunction with the principal reaction with the A subunit and that the nalC and nalD mutations affect this secondary component of the interaction with DNA gyrase. Alternatively, the changes in the B subunit resulting from these mutations might affect the resistance indirectly by altering the conformation of the gyrase A subunit which is thought to be the main site of 4-quinolone binding [1].

Mutations arising in the gyrA structural gene for the A sub-unit of DNA gyrase are more common than gyrB located mutations. Although gyrA mutations have been characterised extensively in E. coli, these type of mutations have also been identified in other gram-negative and gram-positive bacteria [1]. In all cases of mutations in the gyrA gene conferring resistance to the 4-quinolones, no mechanism of resistance has been described as there is still uncertainity over the exact mechanism of action of these agents.

Laboratory studies have clearly indicated that the new generations of the fluoroquinolones are much less likely to select resistant mutants than the older agents, such as nalidixic acid. However, some studies show that in certain instances some species of bacteria appear to be far more prone to develop resistance than might be anticipated. Results indicate that the nature of the bacterial environment operating at the site of an infection might play a significant role in determining the probability of the selection of 4-quinolone-resistant mutants. Roberts et al. [10] reported that the frequency of ciprofloxacin resistance in Ps. aeruginosa in cystic fibrosis patients was as high as 37%. Under laboratory conditions the mutation frequency to ciprofloxacin resistance in clinical isolates of Ps. aeruginosa from cystic fibrosis patients are acceptably low [11]. Such data might well confirm the above hypothesis that the likelihood of resistant pathogens being selected may be significantly influenced by the location of the site of infection.

3.3. PHARMACOKINETICS AND METABOLISM OF CIPROFLOXACIN

Ciprofloxacin is rapidly absorbed after oral administration, with the time to reach the maximum concentration level in serum (T_{max}) occurring at between 30 to 70 minutes depending on the oral doses administered, i.e. 50-, 100- and 750- mg of ciprofloxacin. Peak serum levels (C_{max}) vary from 0.28 mg/liter (after a 50 mg oral dose) to 2.65 (after a 750 mg oral dose). After the rapid distribution of the antibiotic agent in the serum, ciprofloxacin is slowly eliminated from this biological routeway, with the terminal serum elimination half-life reported at approximately 206 and 285 minutes for 50- and 750-mg doses respectively [12].

The main pathway for the elimination of ciprofloxacin from the body is through the passing of urine. Studies have shown that up to 76% of intact ciprofloxacin is recovered in the urine collected from the time of intravenously administering the agent to 24 hours after the event. However, a significant reduction in the percentage recovery of ciprofloxacin to 31% over the same time period is observed if the agent is administered orally [13]. According to Hoffken et al. [12], this discrepancy could be explained by the significant increase in the proportion of ciprofloxacin excreted in the form of its metabolites after oral administration in comparison to intravenous administration. The authors suggest that the higher degree of metabolism of the drug after oral dosing indicates a first-pass effect of the liver and underlines the complexity of the pharmokinetics of ciprofloxacin [12].

3.4. CLINICAL USES OF CIPROFLOXACIN

The newer generation of quinolones, of which ciprofloxacin is a member, are the drugs of first consideration in three areas today; namely gastro-intestinal infections, urinary tract infections and sexually transmitted bacterial infections.

In gastro-intestinal infections, the quinolones appear to out perform other antimicrobial agents with respect to the shortening of the clinical illness and the eradicating the bacteria from the stool, and hence prevent or limit the spread of infection. Trials have demonstrated their effectiveness in the treatment of infections by Salmonella, Shigella and Campylobacter spp., and travellers' diarrhoea [1].

The quinolones are very active agents against

causative bacteria in urinary tract infections especially in hospital-acquired infections. The secret to their success may lie with their ability to eradicate some bacteria from important carrier sites, such as the vagina or faecal flora, and preventing early reinfection by the same strain [1].

The third area for possible primary use of the quinolones is in the treatment of sexually transmitted bacterial infections. In some trials, a 100% success has been obtained with a single-dose treatment for gonorrhoea, and according to Croker et al. [14] they are more effective than other agents in eradicating gonococci from the oropharynx.

3.5. TOXICITY AND SIDE EFFECTS OF CIPROFLOXACIN

In a review of data collected from 6500 patients of European, Japanese and North American origin, Ball [15] has reported that the overall incidence of adverse reactions amongst patients receiving ciprofloxacin is 3.0% and 6.5% in Europe and Japan respectively. An increased incidence (13.4%) which has been reported from the U.S.A. may be related to the higher level of dosages used in that country. Most of the adverse experiences observed with ciprofloxacin relate to gastro-intestinal or central nervous system disturbances.

The reactions affecting the alimentary tract occur in between 2 and 8% of patients, usually comprising of nausea and/or vomiting, abdominal discomfort, or diarrhoea. Although nausea affects only 1-2% of European patients, some studies in the U.S.A. have reported incidences of 15-18% and single dose therapy of gonorrhoea has been associated with nausea in 2.5-10% of subjects [15]. The increased incidence of nausea could in some cases be related to theophylline toxicity (which is

coadministered with ciprofloxacin on occasions) but, in the majority, the cause of the transatlantic difference remains unclear. However, nausea has rarely necessitated interruption of therapy and, in many cases, has been controlled by a reduction in dosage levels [15].

According to Ball, the analysis of European and Japanese data on ciprofloxacin indicates that central nervous system effects, usually dizziness or headache, have occurred in 0.4-1.1% of patients. A higher figure is quoted from American sources (4.4%), possibly due to the use of larger dosages.

With the exception of the interaction with theophylline, which has already been referred to above, only one other potentially disadvantageous reaction has been reported. Pronounced reductions in mean peak serum ciprofloxacin concentrations and urinary recoveries have been reported after coadministration of "Maalox", possibly as a result of formation of magnesium or aluminium chelates [15].

In conclusion, serious adverse experiences with ciprofloaxcin are rare. Most reactions are trivial and therapy has to be discontinued in less than 2% of patients. There appear to be no major forms of adverse reaction or toxicity so far encountered which limit general clinical use in adults. With regard to children, where animal trials have indicated possible adverse effects on growing joints, ciprofloxacin therapy is limited to cases where potential benefit entirely outweighs the possible side effects.

3.6. REVIEW OF ANALYTICAL PROCEDURES TO DETERMINE CIPROFLOXACIN AND ITS METABOLITES IN BIOLOGICAL FLUIDS

Since ciprofloxacin was initially synthesised in the early 1980's and its therapeutic importance as an antimicrobial agent with high *in vivo* activity was discovered, there has been an increasing demand from a variety of areas of pharmaceutical research to develop analytical procedures for the selective and sensitive determination of this drug in biological fluids. Although microbiological and spectrophotometric procedures have been reported in the literature to determine ciprofloxacin and related new generation quinolones, up to now the most common method to investigate the metabolism, pharmacokinetics and analytical control of ciprofloxacin has been high performance liquid chromatography (HPLC) with fluorescence or ultra violet (UV) spectrophotometric detection.

3.6.1. Microbiological assays

Up to 1984, the microbiological assay was the routine procedure used to investigate the pharmacokinetic profile and metabolism of ciprofloxacin in human volunteers [3,12,16]. All assays were based on the agar diffusion technique with some variation in both the agar and the bacterial reference strains used. Isotonic sensitest agar (Oxoid) was the selected medium to perform the microbiological assay by two research groups [16,3], while antibiotic medium, pH 7.4 [12] and a mixture of antibiotic medium, pH 6.5 and granular agar [17] were the preferred nutrient media by other groups. Antimicrobial activity was estimated using the test organisms Escherichia coli [3,17] or Kiebsiella pneumoniae [16,12].

Both Joos et al. [17] and Jehl et al. [3] compared the bioassay with HPLC procedures and concluded that although good correlation was found, this depended on whether antimicrobially active metabolites were present in the matrix where an overestimation of the intact drug concentration was obtained. This factor, which can cause confusion and bias in the experimental results in addition to the inherently poor precision when applied to biological matrices and overnight incubation periods, make microbiological assays less than ideal as the procedure of first choice in biopharmaceutical studies of ciprofloxacin.

3.6.2. High performance liquid chromatography

Most published HPLC assays for ciprofloxacin and its metabolites in biological fluids have up to the present made use of reversed-phase chromatography based mainly on employing Spherisorb ODS, uBondapak C_{18} or Nucleosil C_{18} columns as the stationary phase. The eluents have consisted of phosphate buffer adjusted to a low pH of between 2 and 3.5, methanol [18-21] or acetonitrile [3,4,22-28] or a mixture of both [29-31] as the organic modifier, and ion-pairing agents such as tetrabutylammonium salts [3,19,22,24,27,30,31] or sodium dodecylsulphate [21].

In the majority of published procedures, liquid-liquid extraction procedures have been employed to isolate ciprofloxacin from the biological matrix. These clean-up procedures, which can be complicated and time consuming to perform, include initial extraction with chloroform followed by back extraction into phosphate buffer, pH 2.0 [24], or deproteinisation with acetonitrile, centrifugation, extraction of the supernatant with a mixture of chloroform and propan-2-ol,

followed by centrifugation. Finally, the organic layer is evaporated to dryness and the residue dissolved in the mobile phase [20]. Better recoveries were reported with less rigorous clean-up procedures, including extraction with chloroform only [19,31], or a single step deproteinisation with acetonitrile [18,25], perchloric acid [29] or trichloroacetic acid [17]. A liquid-solid extraction procedure using a Chrom-Prep. PRP-1 cartridge was reported by Krol et al. [32], while Gau et al. [22] and Nilsson-Ehle et al. [28] simply filtered the biological samples prior to the chromatographic step.

Spectrofluorimetric detection was the most common detection system employed in HPLC assays to determine ciprofloxacin. Many procedures take advantage of the intrinsic fluorescence of ciprofloxacin, enhancing its specificity and sensitivity over ultraviolet spectrophotometric detection methods.

HPLC assays of ciprofloxacin are almost entirely restricted to the quantitation of ciprofloxacin. Only five published papers have been concerned with the separation and detection of its metabolites. The quantitation of these metabolites are of some importance, as only 30% of an orally administered dose is excreted in the urine as the intact drug and the biodisposition of ciprofloaxcin produces products with some antimicrobial activity [3]. Hoffken et al. [12] were one of the first research groups to separate and to identify the M1 and M2 metabolites of ciprofloxacin in human urine with fluorescence detection. In subsequent research by Gau et al. [22], Borner et al. [25] and Krol et al. [32], three metabolites were determined in serum and urine using either flouresence or UV detection.

It was not until 1987 that Scholl et al. [27] published a HPLC procedure in which all four known metabolites

could be determined with the same sensitivity and selectivity as ciprofloxacin. Although fluorescence detection is very sensitive for ciprofloxacin and metabolite M1, it is much less sensitive for M3 and M4. The fluorescence of M2 is so weak that UV detection, which for the other metabolites is less selective and sensitive, is normally used to detect this metabolite. To overcome this problem, Scholl et al. employed a post-column reaction system permitting both photolysis and thermolysis to convert metabolites M2, M3 and M4 into secondary products with fluorescence comparable to that of ciprofloxacin. Detection limits for ciprofloxacin and its metabolites after post-column derivatisation were in the range of 0.23 to 2.2 ng ml^{-1}. Chromatography was performed with two different solvent systems comprising 4% aqueous tetrabutylammonium bisulphate solution with 7-10% acetonitrile for M1 and ciprofloxacin and 25-30% acetonitrile for M2, M3 and M4. According to Scholl et al., this procedure is quicker and more reliable than the separation of all five substances in only one run, which would require gradient elution and reconditioning of the column every 45 minutes. The differences in polarity between the metabolites are too large for a single isocratic solvent system.

Although Myers and Blumer [4] determined ciprofloxacin in routine pharmacokinetic studies on an isocratic system comprising of 19% acetonitrile and phosphate buffer, pH 2.5, gradient elution with the acetonitrile component of the mobile phase varying from 15 to 35% was employed to determine the metabolites of ciprofloxacin. A dual spectrophotometric detection system was used to detect four fluorescent metabolites in serum, sputum and urine, and two additional ultraviolet-absorbing metabolites found in urine only. The first two eluting metabolites were provisionally identified as M1 and a product resulting from the further metabolism of M3. The remaining two fluorescent metabolites were thought to be products of the cleavage of the piperazinyl ring in ciprofloxacin. In the case

of the UV-absorbing metabolites, Myers and Blumer identified them as metabolites M2 and M3. In the opinion of this group, the UV-absorbing metabolites are the most important and can account after oral administration for about 12% of the dose in urine, while the identified fluorescent metabolites account for less than 2% of the dose.

3.6.3. UV Spectrophotometry

Ultraviolet spectrophotometry has been applied to the determination of ciprofloxacin in pharmaceutical preparations [33-37]. In general these assays are unselective and tedious to perform, and in comparison to HPLC procedures with fluorescence detection are a hundred times less sensitive. The initial spectrophotometric procedures were based on the reaction of acidified ferric chloride diluted in water [33,34] or in methanol [35] with ciprofloxacin dissolved in acid [33], water [34] or a mixture of DMSO and methanol [35]. The latest published assays have reacted an aliquot of ciprofloxacin with either ethanolic benzoquinone, pH ajusted to 7.8 with phosphate buffer [36], or an ethanolic solution of methyl orange or bromothymol blue mixed with phthalate buffer, pH 4.0, [37]. In the latter procedure, the organic complex was extracted into chloroform and the absorbance measured at 425 nm and 410 nm for the methyl orange- and bromothymol blue- based complexes respectively. A detection wavelength of 495 nm was employed in the former assay [36].

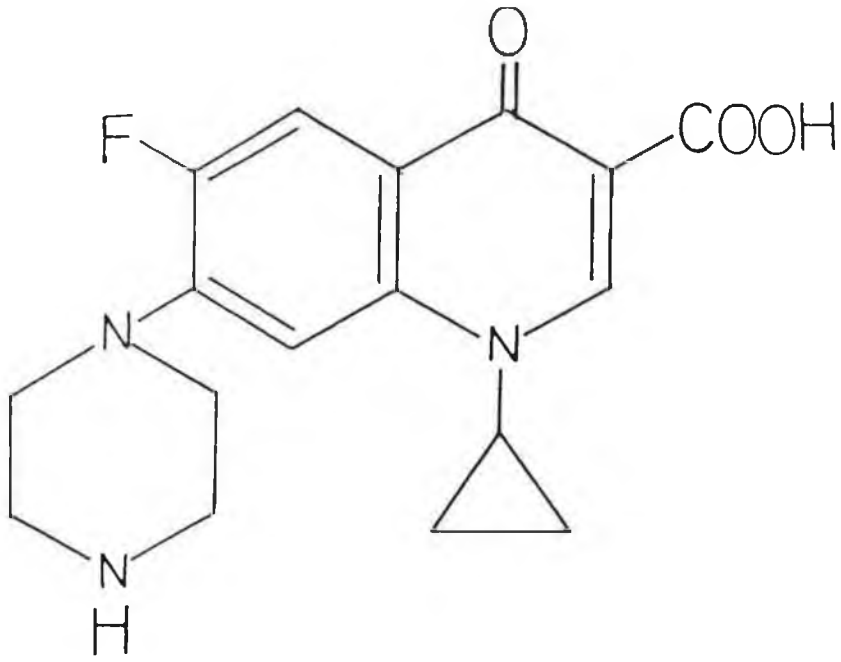
3.7. DETERMINATION OF CIPROFLOXACIN BY DIFFERENTIAL PULSE POLAROGRAPHY

Up to now, there have been no reports in the literature of the application of electrochemical techniques to the determination of ciprofloxacin (1-cyclopropyl-6-fluoro-1, 4-dihydro-4-oxo-7-(1-piperazinyl)-3- quinolinecarboxylic acid, [I]). A study of this antibiotic employing such techniques not only increases the information about the physicochemical properties of ciprofloxacin, but provides the basis for the development of an electroanalytical assay. Polarography, in particular differential pulse polarography (DPP), is considered a convenient method for the quantitation of the therapeutic drugs present in high concentrations in formulated tablets. Among the advantages of this particular electroanalytical technique are the wide linear range, excellent reproducibility (<1%), and low experimental cost. This section describes the development of a rapid and sensitive polarographic assay for the determination of ciprofloxacin.

3.7.1. Experimental

3.7.1.1. Apparatus and reagents

All chemicals were of analytical reagent grade. Samples of pure ciprofloxacin and the formulated drug were kindly donated by Laboratorios Dr. Esteve S.A. All solutions were prepared in deionised water. Perchloric acid (0.1 M) and Britton-Robinson (BR) buffers (with an ionic strength of 0.12 M) were employed as the background electrolytes.



[I]

Linear sweep voltammetry was performed with a Metrohm VA detector (E-611) connected to a Graphtec WX 4221 X-Y recorder. A Metrohm E-506 polarograph coupled to a Metrohm stand was employed for differential pulse polarography (DPP) and direct current (DC) polarographic measurements. A three-electrode system was used for all voltammetric measurements. In DPP, the working electrode was a static mercury drop electrode (SMDE) with a drop size of 0.5668 mm^2, whereas either a hanging mercury drop electrode (HMDE) with a drop area of 1.82 mm^2 or a carbon paste electrode of surface area 4.52 mm^2, was used as the working electrode in adsorptive stripping voltammetry (AdSV). In all cases the reference electrode was Ag/AgCl, and a platinum wire served as the auxiliary electrode. Sample solutions were magnetically stirred at constant speed (500 rpm) in AdSV, using a 1 cm magnetic follower. The DPP technique was performed using a controlled drop time of 0.4 seconds with a scan rate of 10 $mV\ s^{-1}$ and a pulse amplitude of 20 mV.

The carbon paste electrodes were prepared by adding the required amount of paraffin oil (Uvasol, Merck) to spectroscopic grade graphite powder (30% by mass) and grinding this with a pestle and mortar for 30 minutes. A teflon barrel-type electrode with a screwed stainless steel contact was filled with a 3 mm thick layer of carbon paste and used as the working electrode. The surface was smoothed on a sheet of plain white paper placed on a flat glass stand.

3.7.1.2. Procedures

3.7.1.2.1. Determination of ciprofloxacin in formulated tablets by DPP

A number of formulated tablets of ciprofloxacin were ground to a fine powder using a pestle and mortar. The powder was then dissolved in a small volume of water. The solution was filtered and made up to a fixed volume (producing a solution that was approximately 0.01 M). Both the 0.01 M ciprofloxacin standard and the samples were diluted (1:2) with deionised water prior to the voltammetric step. The determination was carried out by first recording a polarogram of the supporting electrolyte (20 mL BR buffer, pH 8.5) following deaeration for about 8 minutes in a stream of oxygen-free nitrogen. To this solution a 30 ul aliquot of the diluted sample was added; after dearation and stirring for 1 minute and a rest period of 15 seconds, a polarogram of the sample was recorded in the DPP mode.

Three successive 30 ul aliquots of standard ciprofloxacin were then added to the cell, and the DPP curves were recorded after each addition. The concentration of ciprofloxacin in the sample was estimated from the resulting standard addition plot.

3.7.1.2.2. Electrode activation and preconcentration step with the carbon paste electrode

The following *in situ* pretreatment was employed to activate the surface of the electrode. The electrode was kept in

a stirred sample solution (20 mL) for 35 seconds at a potential of 1.39 V. The applied potential was switched off and the accumulation proceeded at open circuit for 1 minute. The stirring was then stopped, and after a 15 second rest period, the stripping voltammogram was recorded in the anodic direction using a sweep rate of 40 mV/s.

3.7.1.2.3. Determination of ciprofloxacin in urine by Adsorptive Stripping Voltammetry at carbon paste electrodes

An aliquot of human urine (2 mL) was spiked with standard ciprofloxacin to give a final concentration in urine of 1.2×10^{-6} M. The spiked urine was then diluted with 8 mL of 0.05 M phosphate buffer pH 2.0 and passed through a C_{18} Sep Pak cartridge. This in turn was washed with 10 mL of phosphate buffer (pH 2.0) followed by 10 mL of phosphate buffer (pH 8.0). Ciprofloxacin was eluted from the cartridge with 3 x 1 mL aliquots of methanol. The organic solvent was evaporated to dryness at 50°C under a nitrogen stream. Prior to analysis, the residue was reconstituted in 2 mL of the supporting electrolyte (BR buffer, pH 6.3) and a 1 mL aliquot was transferred into the cell (containing 20 mL of background electrolyte). After the first adsorptive stripping voltammogram had been recorded, three successive aliquots of standard ciprofloaxcin solution were added to the cell and the voltammograms were recorded after each addition. The concentration of ciprofloxacin in the sample was estimated from a standard addition plot of peak height versus concentration of added standard.

3.7.1.2.4. Determination of ciprofloxacin by Adsorptive Stripping Voltammetry at a hanging mercury drop electrode.

Electrolyte solutions (10 mL of BR buffer, pH 8.5) were initially purged with a stream of nitrogen for 10 minutes. An aliquot of standard ciprofloxacin was then added to the cell. For a given period, accumulation proceeded at open circuit under a nitrogen atmosphere and without stirring.

In subsequent voltammetric studies of ciprofloxacin in urine, an aliquot (600 uL) of human urine was directly transfered to 9.4 mL of the supporting electrolyte in the cell. To this solution, aliquots of standard ciprofloxacin were added and voltammograms were recorded. Unless otherwise stated, linear sweep voltammetry was performed at a scan rate of 30 mV s^{-1}.

3.8. RESULTS AND DISCUSSION

3.8.1. Cyclic Voltammetry

A study of the effect of the pH on the cyclic voltammetric (CV) behaviour of ciprofloxacin (at a concentartion of 4 X 10^{-5} M), is shown in Figures 3.1a and b. At pH values less than 5.0, ciprofloxacin yields a single, nearly reversible cathodic process (as shown in Figure 3.2), the half-peak potential and current of which are virtually independent of pH (Figure 3.1). In neutral or alkaline media, six cathodic peaks (B-G) were observed; the third, peak D, also exhibited reversible characteristics. This is illustrated in Figure 3.3 for ciprofloxacin in BR buffer, pH 8.5. The current of each peak

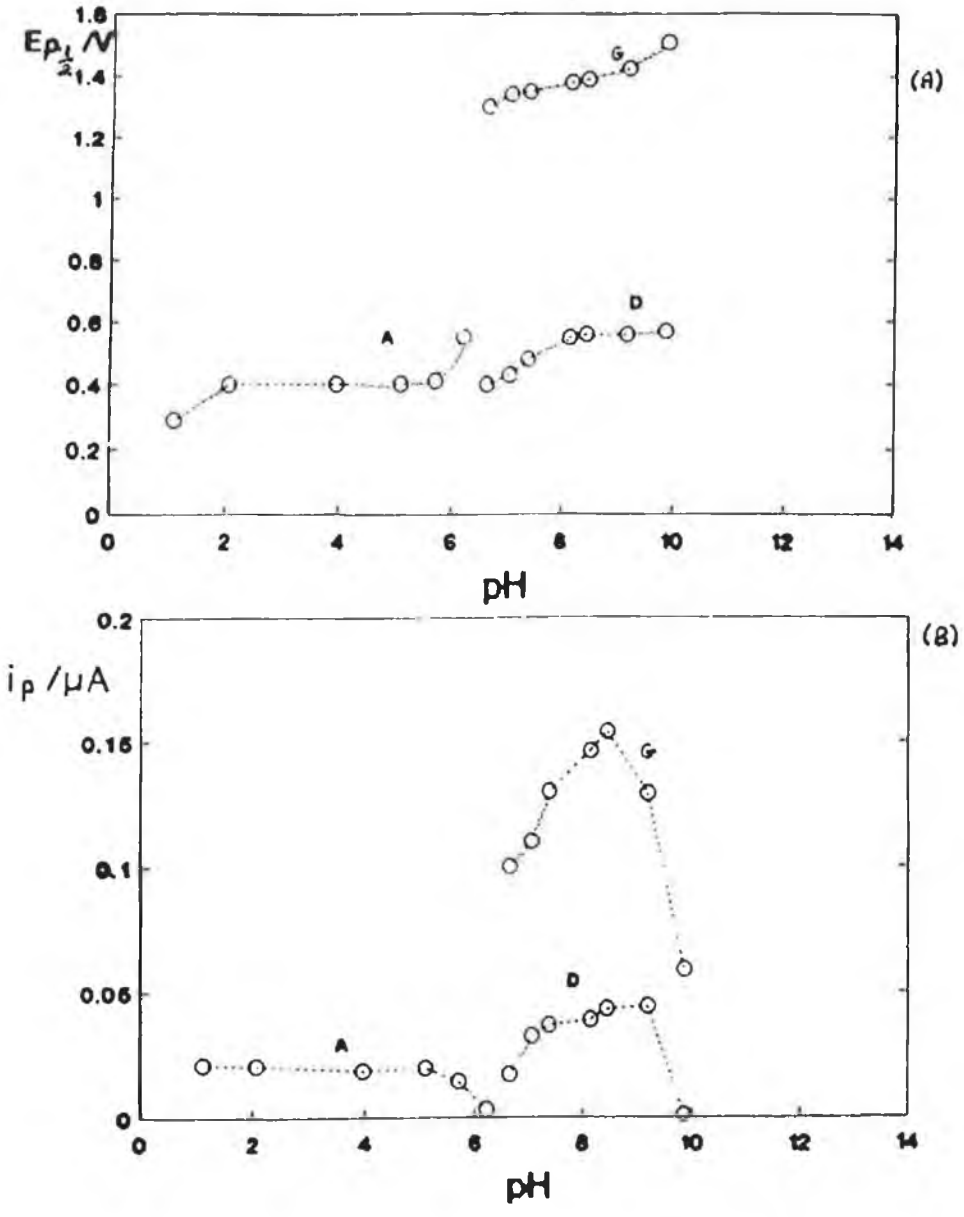


Figure 3.1. Dependence of the cyclic voltammetric (A) half-peak potentials and (B) peak currents of 4 x 10^{-5} M ciprofloxacin on pH. Scan rate, 100 mV/s.

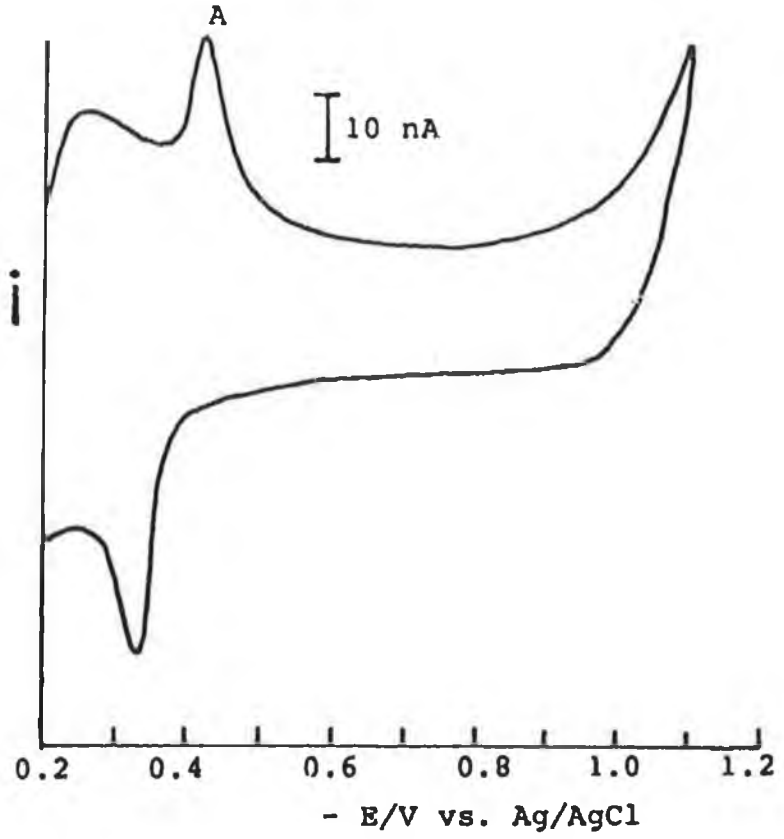


Figure 3.2. Cyclic voltammogram of 4×10^{-5} M ciprofloxacin in BR buffer, pH 2.0 at a carbon paste electrode. Scan rate, 100 mV/s.

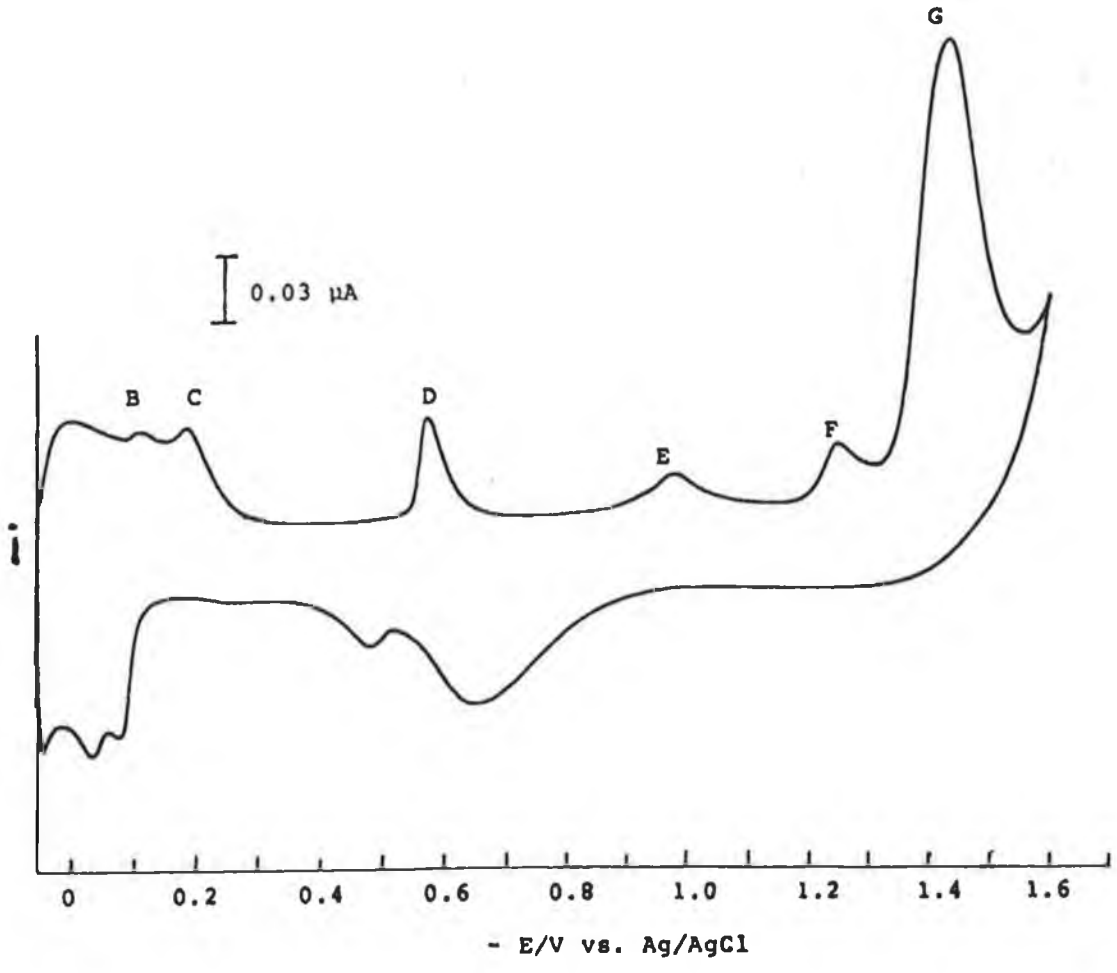


Figure 3.3. Cyclic voltammogram of 4×10^{-5} M ciprofloxacin in BR buffer, pH 8.5. Scan rate, 100 mV/s.

decreased in highly alkaline media, and all voltammetric responses disappeared completely at pH values greater than 11.0. Only peaks D and G, which are the largest and best defined of the processes obtained in basic media, are shown in Figure 3.1a and b.

Although the half-peak potential of peak D was found to be virtually independent from pH 8.0 to 9.0, the main process (i.e. peak G) showed a linear relationship between its half-peak potentials and pH between pH 7.0 and 9.2, according to the equation $E_{p1/2} = -0.967 - 0.051pH$. In addition, although the peak current for peak D was virtually constant within the pH range, the current of peak G was found to be markedly dependent on pH, reaching a maximum value at pH 8.5. Beyond this pH, the peak current decreased until it overlapped with the background electrolyte decay at pH 10.5.

The complex electrochemical behaviour of ciprofloxacin is further complicated by the dissociation of the carboxylic group with increasing pH. A similar situation has been reported by Van Oort et al. [38] in their detailed work on the polarographic reduction of nalidixic acid, a quinolone carboxylic acid derivative. According to Takacs-Novak et al. [39], who carried out a systematic study of the protonation equilibria of quinolone antibacterial agents, the pK_{COOH} for norfloxacin (structurally very similar to ciprofloxacin) occurs at 6.22. This dissociation may be reflected in the point of inflection observed in Figure 3.1b at approximately 6.5. Furthermore, the shift of the half-peak potentials of peak G with a reciprocal slope of $E_{p1/2}/ pH = 0.051$ V/pH possibly indicates a protonation step prior to the reduction of the carboxyl group.

To further elucidate the nature of the process that gives rise to peak G, the influence of the scan rate (v_s) on

the peak height was studied at different concentrations. A linear relationship between peak height and $v^{1/2}_s$, through the centre of origin was observed at a concentration of 1 x 10^{-4} M and at scan rates lower than 30 mV s^{-1}. At concentrations less than 1 X 10^{-4} M, a linear dependence with v_s was seen. This means that, depending on the concentration of ciprofloxacin in the cell, the process can be diffusion or adsorption controlled.

A similar scan rate study was performed on peak A in perchloric acid electrolyte. At a concentration of 1 x 10^{-4}M, and at a scan rate of greater than 20 mV, peak height was independent of both v_s and $v_s^{1/2}$. Another remarkable observation was that although the acidic process that contributed to peak A was observed from approximately pH 1.0 to pH 5.0 using CV, it was not detected using DPP. Finally, in order to determine the nature of this process, a surfactant was added to the background electrolyte. The addition of 0.5 ppm Triton-X produced a 60% reduction in the CV current response, and at a concentration of 1.5 ppm, no observable peak was detected. These investigations implied that the acidic process was nothing more than a surface effect of ciprofloxacin at high concentrations.

3.8.2. Direct Current and Differential Pulse Polarographic behaviour

Depending on the specific pH in alkaline media, ciprofloxacin gave rise to between one and three reduction peaks using DPP. Between pH 7.0 and 8.0, only the DPP peak relating to peak H (peak G in the cyclic voltammogram illustrated in Figure 3.3) was observed at -1.44 V, but this was closely followed by the electrolyte decay. At pH values of 8.0-10.0, an additional

peak (peak I) was seen at -1.64 V (Figure 3.4), which was not seen using CV because of the electrolyte decay curve. At pH values greater than 10.0, a third DPP peak was seen at -1.74 V (peak J). In very alkaline media, however, this was the only remaining process that was seen.

Peak I, the potential of which was pH independent, could be due to the reduction of the ethylenic bond in the quinolone ring of ciprofloxacin. A similar process was reported by Van Oort et al. [38] in their study of nalidixic acid. The peak at -1.74 V (peak J), which was not observed for nalidixic acid, could be due to the reduction of a degradation product of ciprofloxacin in alkaline media.

A buffer solution of pH 8.5 was chosen for analytical purposes because it gave rise to the highest peak currents for peaks H and I and gave the best discrimation against the background electrolyte decay (Figure 3.4). It was therefore hoped that both peaks could be employed for the determination of the antibiotic in pharmaceutical preparations.

Both processes corresponding to peak H and I were found by DC polarography to be diffusion controlled, since both diffusion currents were dependent on temperature, the square root of the mercury height, and the concentration, and were thus in good agreement with the Ikovic equation.

A linear relationship between pulse amplitude and current was obtained for both peaks between 10 and 50 mV. As the pulse height increased further, the peak height reached a limiting value at around 70 mV (Figure 3.5). A pulse amplitude of 20 mV was selected in order to improve the resolution between the two peaks. The initial potential at which the differential pulse scan began was optimised at -1.10 V.

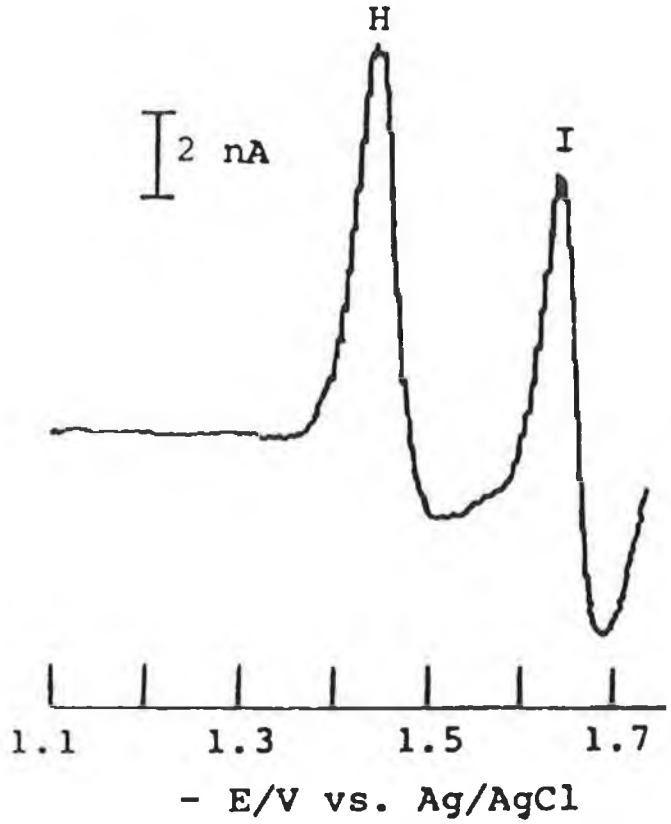


Figure 3.4. DPP behaviour of 1 x 10^{-5}M ciprofloxacin in BR buffer, pH 8.5.

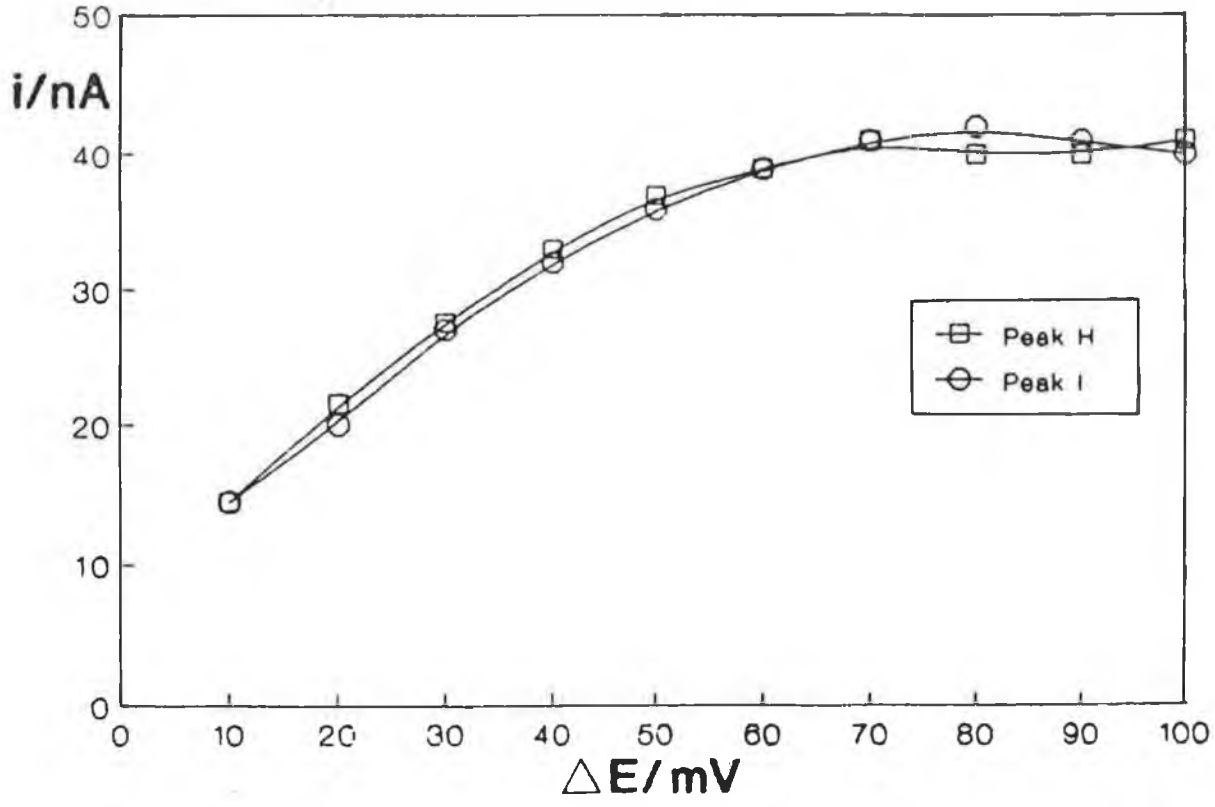


Figure 3.5. Variation of peak current as a function of the pulse amplitude.

Using the polarographic conditions described previously, linear calibration plots were obtained for both peaks from 5×10^{-7} to 3×10^{-5} M and 5×10^{-7} to 5×10^{-5} M with correlation coefficients of 0.9999 and 0.9989 for peaks H and I, respectively. Although a wider linear range was detected for peak I, the slope of the first peak was greater and, hence gave rise to a more sensitive signal. A detection limit of 2×10^{-7} M was obtained under these conditions.

3.8.3. Determination of ciprofloxacin in formulated tablets by DPP

Ciprofloxacin was subsequently determined in formulated tablets by DPP following the procedure indicated in the Experimental Section and employing both DPP reduction peaks. The results obtained are summarised in Tables 3.1 and 3.2. Excellent reproducibilty was observed with a relative standard deviation (RSD) of 0.34% when peak H was employed in the determination of this antibiotic. A significant decrease in the reproducibilty, demonstrated in the corresponding increase in the RSD to 7.83%, occured when the determination was based on peak I. The proximity of the background at -1.70 V to this peak and hence the difficulties of measuring it accurately was the major factor in the poor reproducibility.

TABLE 3.1. Standard addition data for the determination of ciprofloxacin in pharmaceutical preparations using peak H[a]

Sample	Slope ($Amol^{-1}$ L)	r[b]	Sample Concentration (M)[c]
1	1.728×10^{-3}	0.9997	7.64×10^{-6}
2	1.728×10^{-3}	0.9980	7.64×10^{-6}
3	1.736×10^{-3}	0.9995	7.60×10^{-6}
4	1.732×10^{-3}	0.9994	7.62×10^{-6}
5	1.736×10^{-3}	0.9999	7.60×10^{-6}

a Average ciprofloxacin concentration 7.62×10^{-6} M; standard deviation, 1.57×10^{-8} M; relative standard deviation, 0.34%.

b Calculated for n = 4.

c Sample was diluted to a concentration of approximately 7.5×10^{-6} M ciprofloxacin.

Table 3.2. Standard addition data for the determination of ciprofloxacin in pharmaceutical preparations using peak I[a]

Sample	Slope ($Amol^{-1}$ L)	r[b]	Sample Concentration (M)[c]
1	1.761×10^{-3}	0.9998	7.16×10^{-6}
2	1.732×10^{-3}	0.9993	7.97×10^{-6}
3	1.816×10^{-3}	0.9992	6.61×10^{-6}
4	1.844×10^{-3}	0.9940	6.51×10^{-6}
5	1.808×10^{-3}	0.9982	6.64×10^{-6}

a Average ciprofloxacin, 6.98×10^{-6} M; standard deviation, 5.45×10^{-7} M; relative standard deviation, 7.81%.

b Calculated for n = 4.

c Sample was diluted to a concentration of approximately 7.5×10^{-6} M ciprofloxacin.

3.8.4. Comparison of Adsorptive Stripping Voltammetry at mercury and carbon paste electrodes for the determination of ciprofloxacin in urine

The electrolytic deposition of metals and organics at an electrode surface is routinely used as a selective preconcentration step for anodic and cathodic stripping analysis on mercury electrodes [40]. Recent work has focused on the controlled preconcentration of analytes based on spontaneous adsorption at the electrode surface at open circuit prior to voltammetric measurement. The selectivity of measurements for organic compounds such as chlorpromazine [41], cocaine [42], and adriamycin [43] has been enhanced by such a procedure.

This section reports the development of an adsorptive stripping voltammetric (AdSV) assay for the determination of trace levels of ciprofloxacin in urine using a spontaneous preconcentration step at both a HMDE and a carbon paste electrode (CPE).

3.8.4.1. Optimisation of conditions for Adsorptive Stripping Voltammetry at the HMDE

As reported in section 3.8.1., ciprofloxacin (at a concentration of 4×10^{-5} M) gave rise to six cathodic peaks in alkaline media. Initial studies had shown that at lower concentrations, the main reduction process (at a potential of -1.44 V) exhibited adsorptive properties at the HMDE. For analytical purposes, a pH of 8.5 was chosen, since it gave rise to the highest peak current together with good discrimination against the background electrolyte decay.

The effect on the accumulation of ciprofloxacin at the electrode surface in quiescent and stirred solutions was investigated. Although accumulation in quiescent solutions resulted in reduced sensitivity (Figure 3.6.) in comparison to stirred solutions, there was better discrimation between the reduction process and the background electrolyte decay. Consequently, for subsequent accumulation studies, preconcentration of ciprofloaxacin at the HMDE took place both in a quiescent solution and under open circuit conditions.

When the peak current was plotted versus scan rate, a linear behaviour for the reduction process was observed between 5 to 40 mV s^{-1} with a correlation coefficient r of 0.999. As the scan rate increased further, the growth in peak current tailed off. Although the line did not pass through the point of origin, it did confirm that the process was mainly adsorption controlled.

To determine ciprofloxacin in urine, a preconcentration time of 2 minutes was selected to increase the sensitivity of the stripping signal. No difference in analyte response was obtained if urine samples containing 5×10^{-6} M or 1×10^{-5} M ciprofloxacin were directly transferred to the cell or separated first using the C_{18} Sep Pak cartridge/cleanup procedure described in the Experimental Section. Therefore the direct dilution of human urine in the supporting electrolyte was used in all subsequent studies. The background discharge occurred at less negative potientals when urine was present in the cell electrolyte. By diluting the urine by a factor of at least 1 in 17, the matrix effect on the background discharge potential was less pronounced, and this led to improved detection limits.

The presence of urine in the supporting electrolyte affected the adsorption behaviour of ciprofloxacin at the

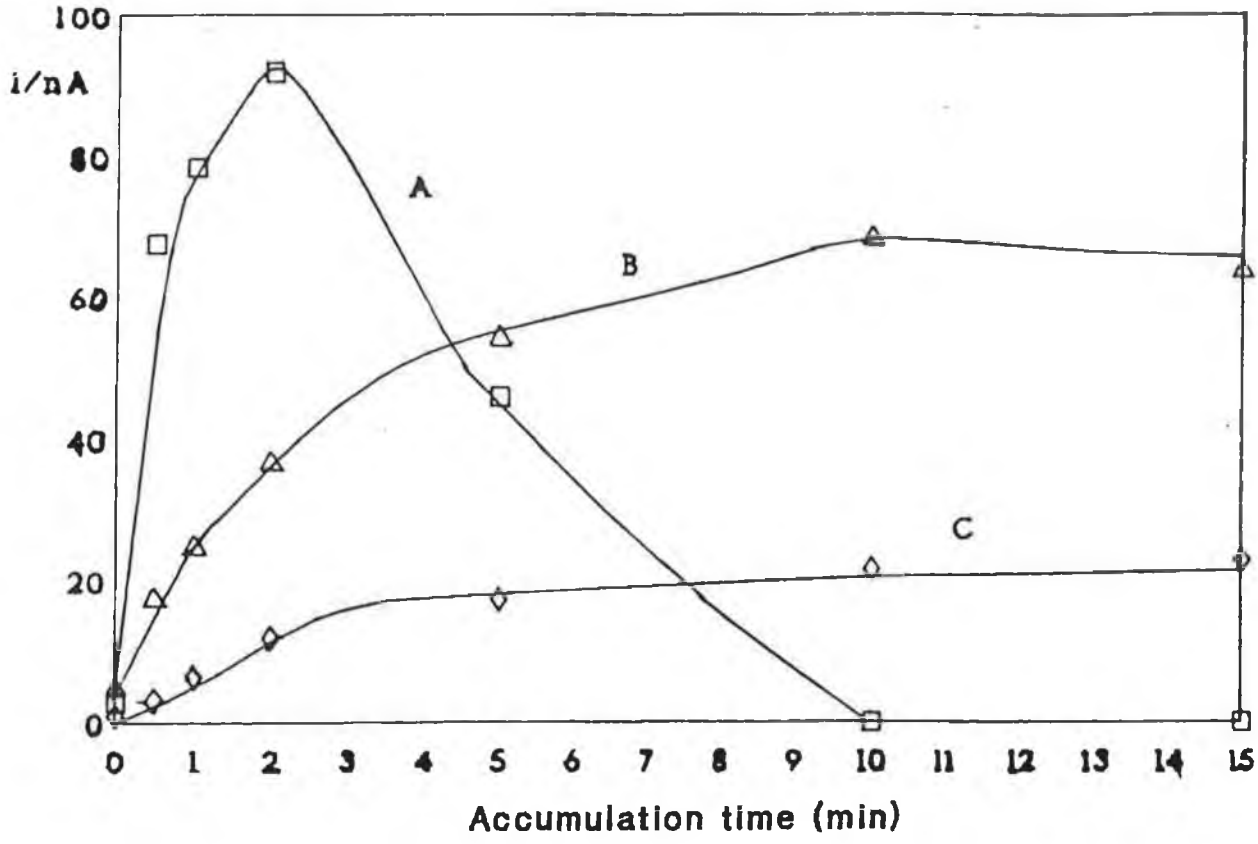


Figure 3.6. Dependence of peak current on the preconcentration time for ciprofloxacin in (A) stirred 1 X 10^{-6}M solution, (B) quiescent 1 X 10^{-6}M solution, and (C) quiescent 5 X 10^{-7} M solution.

mercury electrode. A significant reduction in the analyte signal was more than likely due to the competition for surface sites on the electrode between natural surfactants present in urine and ciprofloxacin. Comparison of the enhancement factors (i.e. the maximum peak current obtained divided by the current peak obtained at zero preconcentration time), determined from the accumulation curves of both standard ciprofloxacin and ciprofloxacin in urine, gave an approximate measure of the interfering matrix effect. At a concentration of 5×10^{-7} M ciprofloxacin in the supporting electrolyte, the peak current increased with the accumulation of ciprofloxacin at the electrode surface by a factor of 22.7. The presence of urine in the supporting electrolyte reduced this enhancement factor to 2.7.

At accumulation times greater than 30 seconds for concentrations at or less than 1×10^{-6} M ciprofloxacin, a significant increase in peak current was observed and the analyte signal became characteristically thin and sharp. To reduce this probable tensammetric process at low concentrations of the antibiotic compound, an accumulation time of 15 seconds was selected, because the analyte peak obtained without preconcentration was not as well defined as that after accumulation for 15 seconds.

Using the voltammetric conditions described above, linear calibration plots were obtained for the peak in the presence of pure ciprofloxacin standard and for blank urine spiked with the antibiotic compound in the supporting electrolyte between 4×10^{-7} to 1×10^{-6} M and 4×10^{-7} M to 8×10^{-7} M, respectively (correlation coefficients, 0.999).

Because of the extremely narrow linear range of the electroactive process in urine, in addition to the poor sensitivity obtained, it was concluded that the adsorptive

stripping assay developed could not be readily applied to the determination of ciprofloxacin in human urine.

3.8.4.2. Optimisation of conditions for Adsorptive Stripping Voltammetry at carbon paste electrodes

A study of the effect of pH on the cyclic voltammetric behaviour of ciprofloxacin at carbon paste electrodes (at a concentration of 5×10^{-5} M) is shown in Figure 3.7. Depending on pH, ciprofloxacin gave rise to one to two irreversible oxidation peaks. The main process (peak A) was observed throughout the pH range, the half-peak potential of which was significantly dependent on pH. In neutral or slightly alkaline media, a second anodic process (peak B) was seen. Nevertheless at pH values greater than pH 8.5, peak A was the only process observed.

Although the half-peak potential of peak B was found to be somewhat dependent on pH, the main process (i.e. peak A) exhibited a linear relationship between half-peak potentials ($E_{p,1/2}$) and pH values between 2.17 and 12.21; the linear equation was $E_{p,1/2} = 1.348 - 0.063$ pH. At pH values less than 7.0, the peak current of peak A was independent of pH; however, in more alkaline media the currents of both peak A and B decreased. Even though the peak current of peak A increased once again at pH values greater than 8.5, the voltammetric signal decreased again at pH values greater than 10.0.

For analytical purposes, a pH of 4.0 was selected, since only peak A was observed at this potential and there was no risk of interference from the second oxidative process. A linear relationship between scan rate and peak current was obtained from 5 to 60 mV s^{-1}, at a concentration of 3×10^{-7}

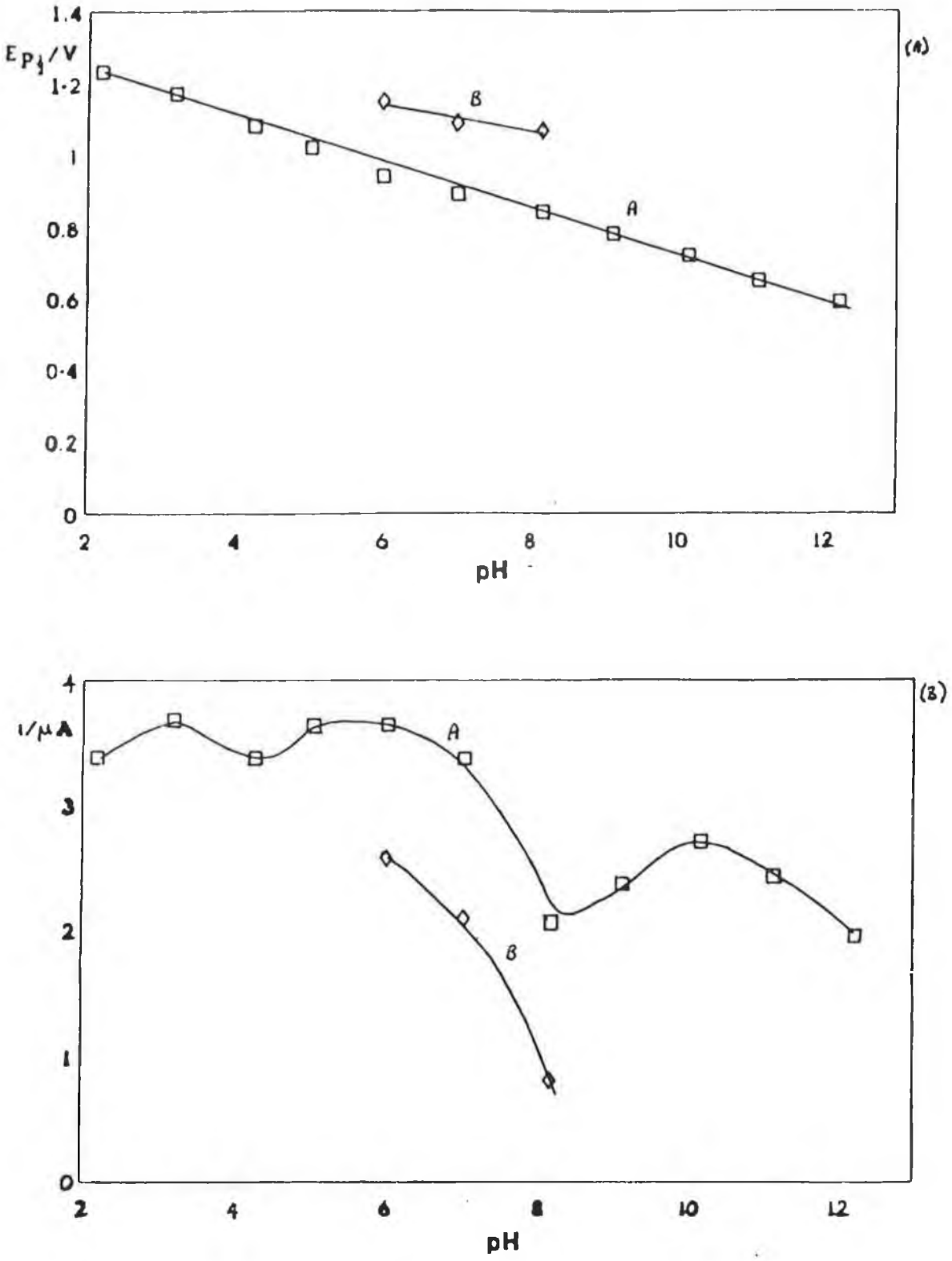


Figure 3.7. Dependence on pH of the cyclic voltammetric (A) half-peak potential and (B) peak current of ciprofloxacin (5×10^{-5} M). Scan rate, 100 mV/s.

M ciprofloxacin, indicating an adsorption-controlled process. The deviation from linearity obtained at higher scan rates may be due to adsorption of the oxidised product at the surface of the electrode, thus reducing the analyte signal. For all subsequent studies, a scan rate of 40 mV s^{-1} was selected because it gave rise to a well-defined current signal.

The optimum accumulation of ciprofloxacin at carbon paste occurred when the electrolyte solution was stirred under open circuit conditions. As illustrated in Figure 3.8, at high concentrations of ciprofloxacin (i.e. 5 x 10^{-7} M), the electrode surface became saturated after accumulation times of more than 2 minutes. At lower concentrations (i.e. 1 x 10^{-7} M), the peak current was directly dependent on the accumulation times between 10 to 60 seconds. To balance a sensitive analyte signal with a short accumulation time, hence to reduce the possible adsorption of interfering compounds from the cell electrolyte, a deposition time of 60 seconds was selected.

A major problem associated with carbon paste electrodes is the gradual fouling of their surfaces. Several methods for activating these surfaces have been proposed [44, 45]. After activation, the electrodes exhibit excellent reproducibilty of the analyte signal and can be used for analytical purposes.

The use of high anodic potentials for a short period of time has proved effective as an activation step because the electrode transfer characteristics are similar to those shown by "dry" graphite immersed in an aqueous solution [46].

Using the adsorptive stripping voltammetric conditions described above, the reproducibilty of the assay was investigated. Adequate precision in the voltammetric signal could not be obtained unless either the carbon paste was renewed

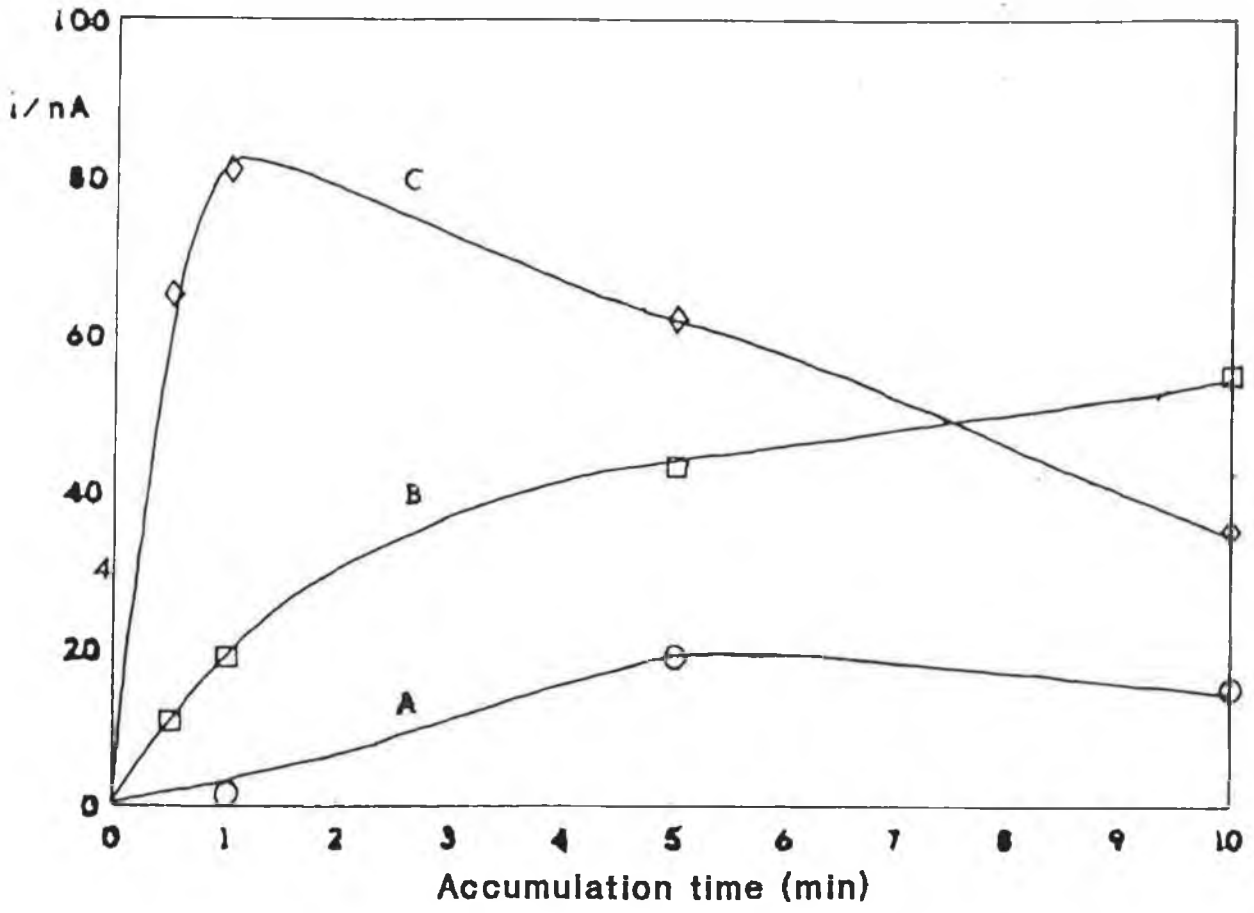


Figure 3.8. Dependence of peak current on the preconcentration time for ciprofloxacin at (A) 5×10^{-8} M, (B) 1×10^{-7} M, and (C) 5×10^{-7} M ciprofloxacin in a non-quiescent electrolyte.

after every voltammetric measurement, or the electrode surface was pretreated by an *in situ* activation procedure before the voltammogram was recorded. The latter procedure was preferred because it reduced analysis time. Determination of the optimum activation procedure involved the anodisation of a new electrode surface in the range 1.0-1.5 V for selected periods of time.

In a buffer solution of pH 4.0, however, reproducibilty was not improved by activating the electrode in this manner. The voltammetric peaks were not well defined or the background as "clean" as the voltammetric response obtained without activation. Although no significant improvement was obtained when the cell electrolyte was changed to pH 5, at pH 6.25 good reproducibilty was obtained after anodic activation, and the voltammogram stopped after the first of the two oxidative processes that are observed at this pH. Upon activation of the electrode surface, the second oxidative process merged with the background decay and the continuation of the voltammetric scan at potentials greater than 1,10 V reduced the overall precision of the pretreatment procedure. Reproducible voltammetric signals were obtained with a relative standard deviation of 0.7% for n = 10 (Figure 3.9), when the carbon paste electrode was activated at 1.39 V for 35 seconds in a stirred solution prior to accumulation of the ciprofloxacin at the electrode surface at open circuit; a stripping voltammogram was recorded between 0.4 and 1.10 V.

Linear calibration plots were then obtained using the first oxidative process between 2×10^{-8} to 3×10^{-7} M (correlation coefficient, 0.999), and the adsorptive stripping voltammetric assay was considered to be suitable for investigations of human urine samples.

It had been reported that selectivity of voltammetric procedures utilised in the determination of the analyte in a

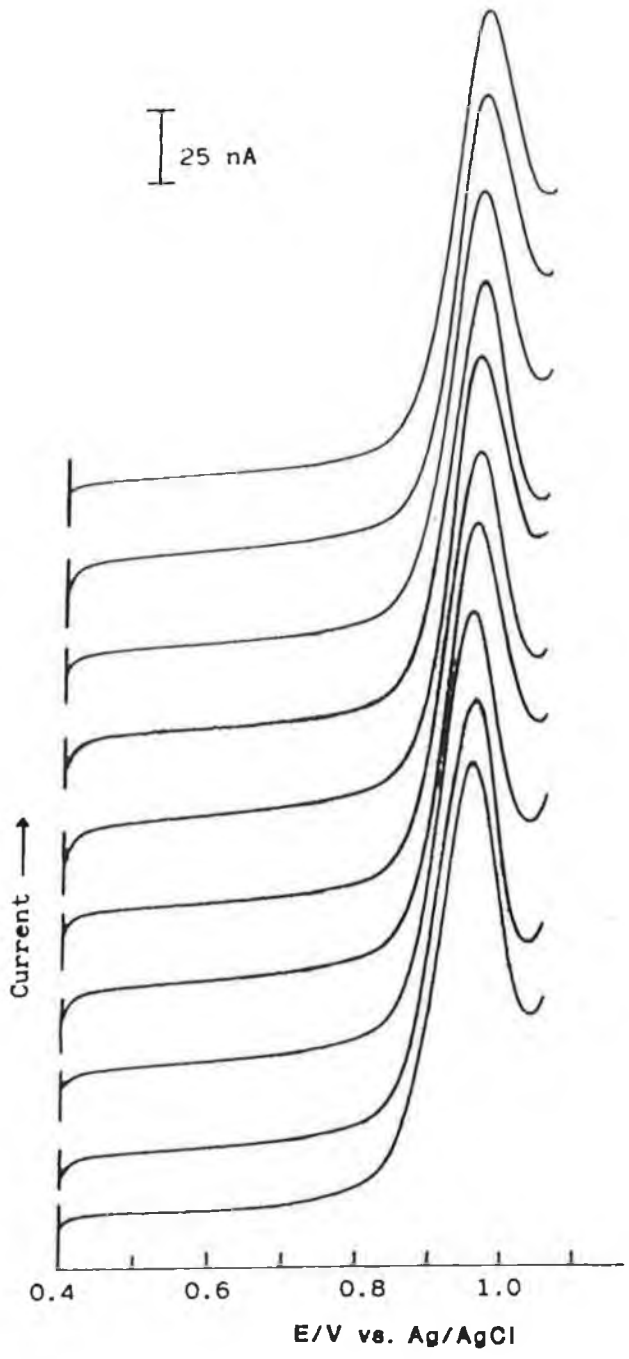


Figure 3.9. Reproducibility of the linear sweep voltammetric signal for ciprofloxacin at a CPE. Ten successive voltammograms of 2 X 10^{-7} M ciprofloxacin were recorded with a high anodic potential activation of the electrode surface before each measurement.

complex matrix can be enhanced by preconcentrating the analyte in one solution and transfering the electrode into a different solution to record the voltammogram [47]. This "medium exchange" procedure would avoid the need for a lengthy cleanup pretreatment of the urine and would keep analysis time to a minimum. It was found that a hundred-fold dilution of urine in supporting electrolyte was necessary to reduce matrix interferences. Although an improved voltammogram of the blank urine was obtained by activating the electrode in pure electrolyte rather than in the presence of urine diluted with electrolyte, the presence of a broad interfering peak at a potential of approximately 0.8 V, in addition to a large background current, made it necessary to develop a more rigorous cleanup procedure for urine.

The adsorptive stripping voltammograms obtained for urine residues dissolved in electrolyte following separation on a C_{18} Sep Pak cartridge exhibited a well-defined peaks at a potential of 0.95 V, as shown in Figure 3.10. The washing of the cartridge containing the concentrated urine sample with pH 2.0 and pH 8.0 phosphate buffers removed interferences from urine compounds, hence reduced the background current. In addition, the evaporation of the elution solvent (i.e. methanol) was found to be necessary because the presence of the organic solvent in the cell electrolyte significantly reduced the peak response.

The calibration plots obtained by spiking the cell electrolyte containing blank urine extract with standard ciprofloxacin were found to be linear in the concentration range of 3×10^{-8} to 2×10^{-7} M in the supporting electrolyte. The mean recovery of the antibiotic compound based on the slope ratio (i.e. the slope of the line obtained by standard additions to spiked urine divided by the slope of the line obtained by standard additions to blank urine and multiplied by 100) was 81%. The coefficient of variation of the procedure at a

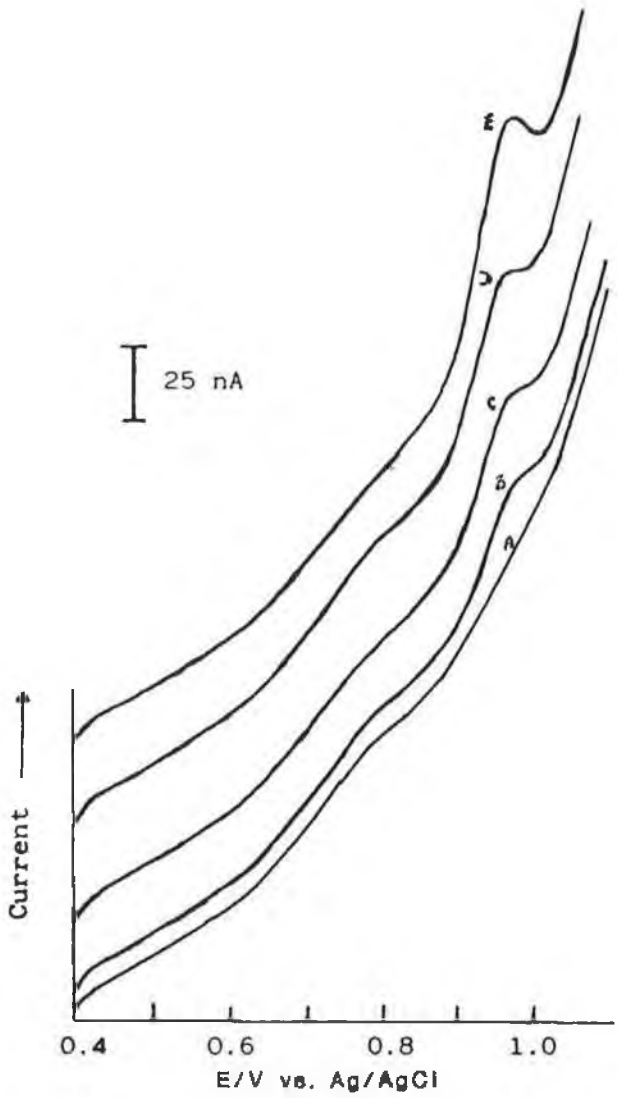


Figure 3.10. Determination of ciprofloxacin in spiked urine by the standard addition method: (A) urine blank, (B) spiked urine, (C and D) successive additions of 40 uL of 1 x 10^{-5} M ciprofloxacin, and (E) 200 uL of 1 x 10^{-5} M ciprofloxacin standard.

concentration of 1.2×10^{-6} M ciprofloxacin in urine was 4.1% (n=5). The detection limit in spiked urine samples was approximately 6×10^{-7} M.

It is envisaged that this new analytical procedure could be applied to urine samples from patients who have been given oral or intravenous doses of ciprofloxacin. The major excretion route of the antibiotic and its metabolites is through urine (i.e., 55 and 75% of the administered oral or intravenous dose, respectively) [48]. Although 10-20% of oral or intravenous doses of ciprofloxacin is eliminated in the form of metabolites (the majority of which are excreted in the urine), this procedure would give a good estimation of the concentration of ciprofloxacin in pooled urine samples after administration of doses of 100 mg or greater.

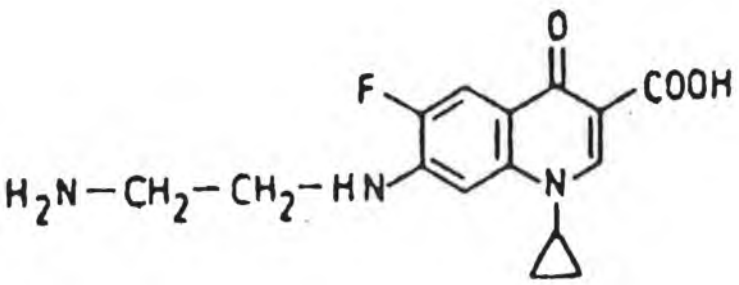
3.9. AN INVESTIGATION OF GLASSY CARBON-BASED ELECTROCHEMICAL DETECTION IN FLOWING SOLUTIONS FOR THE DETERMINATION OF CIPROFLOXACIN AND SOME METABOLITES

Ciprofloxacin is metabolised *in vivo*, to give six metabolites, some of which are microbiologically active compounds. To date, four of the six metabolites have been positively identified (Figure 3.11).

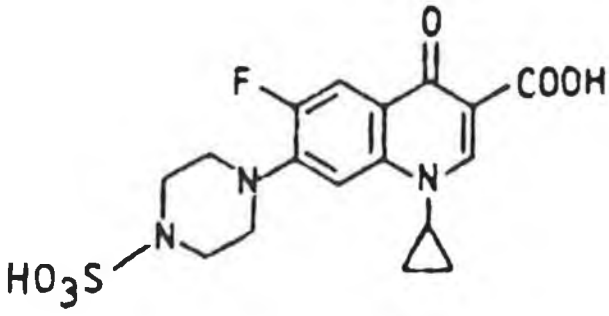
As has been already discussed in section 3.6.2., the most common method for the determination of ciprofloxacin and its metabolites in biological fluids has been HPLC with fluorescence or UV spectrophotometric detection. All methods, with the exception of the method of Scholl et al. [27], suffer from the same problem whereby the metabolites cannot be determined with the same sensitivity and selectivity as the parent pharmaceutical agent.

Another approach could be to use electrochemical detection, which is noted for its sensitivity, controllable selectivity, good precision and accuracy, and linear response over a wide concentration range. The excellent current signal response of ciprofloxacin at carbon paste electrodes (as reported in section 3.8.4.1.) suggested the possibility of determining ciprofloxacin in a flowing solution with glassy carbon-based electrochemical detection.

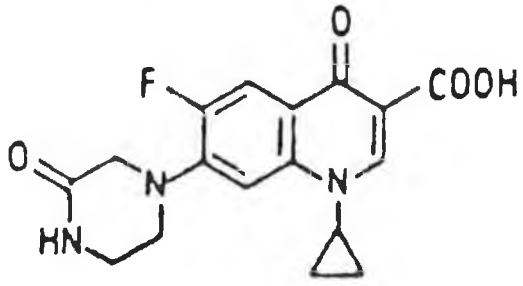
In this section, therefore, the development of a flow injection analysis (FIA) assay for the determination of ciprofloxacin in formulated tablets is reported. For the purposes of determining the optimum background electrolyte pH to detect the metabolites of ciprofloxacin in further applications to flowing systems with electrochemical detection, the changes of the voltammetric behaviour of four metabolites with pH at a



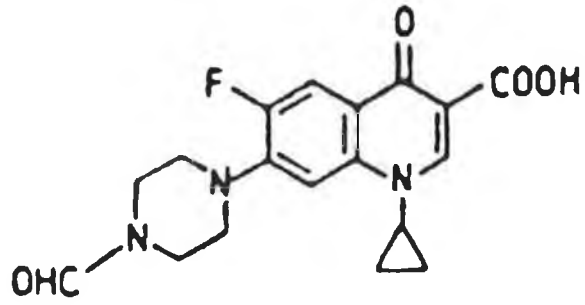
M1 (BAY R 3964)



M2 (BAY S 9435)



M3 (BAY Q 3542)



M4 (BAY P 9357)

Figure 3.11. Four known metabolites of ciprofloxacin.

carbon paste electrode is also reported.

3.9.1. Experimental

3.9.1.1. Reagents

All chemicals were of analytical-reagent grade. All solutions were prepared using deionised water which was obtained by passing distilled water through a Millipore Milli-Q water purification system. Samples of pure ciprofloxacin and the formulated drug were kindly donated by Laboratorios Dr. Esteve S.A. The metabolite standards M1 (Bay Q 3964.HCL), M2 (Bay S 9435.NH_3), M3 (Bay Q 3542) and M4 (Bay P 9357) were kindly supplied by Bayer AG, Wuppertal, FRG. Stock solutions of 1 x 10^{-2} M (or 3.858 mg/mL) ciprofloxacin, 1 x 10^{-2} M M1 and 1 x 10^{-3} M M2 were made up in deionised water and 1 x 10^{-3} M M3 and 1 x 10^{-3} M M4 were dissolved in 0.01 M and 0.05 M sodium hydroxide respectively. The stock solutions were stored at 4^{o}C and used within 48 hours of preparation. Working standards were prepared daily by dilution of the stock standard with de-ionised water. Britton Robinson (BR) buffers (with an ionic strength of 0.12 M) and phosphate buffer pH 6.0 with 45% methanol were employed as background electrolytes in the voltammetric and FIA procedures respectively.

3.9.1.2. Instrumentation

3.9.1.2.1. Cyclic Voltammetry

Cyclic voltammetry was performed with a Metrohm VA-E611 scanner in conjunction with a Metrohm VA- E611 potentiostat connected to a Graphtec WX 4421 X-Y recorder. The working electrode was a carbon paste electrode (area = 4.52 mm^2). The reference electrode was a Ag-AgCl electrode and a platinum wire served as the auxilary electrode. Alternatively, hydrodynamic cyclic voltammetry was performed with a Metrohm 663-VA voltammetric stand equipped with a glassy carbon electrode (area = 3 mm^2) and a Metrohm VA E612 scanner connected in series to a Metrohm VA-E611 detector to Graphtec WX 4221 X-Y recorder. The reference and auxillary electrode were the same as above.

The carbon paste electrode was prepared according to the procedure outlined in section 3.7.1.1.

3.9.1.2.2. Differential Pulse Polarography

The same apparatus as described in section 3.7.1.1. was employed for all DPP measurements.

3.9.1.2.3. Flow Injection Analysis

The flow injection system consisted of a Waters Model 510 solvent delivery system with a Waters U6K variable injection port. A 140 cm long stainless steel tubing (0.5 mm i.d.), which

was coiled to a 10 cm diameter connected the injection port to the inlet of the electrochemical detector.

The electrochemical detection system consisted of a Princeton Applied Research (PAR) glassy carbon cell block which was connected to a BAS Bioanalytical Systems liquid chromatography Model 4A amperometric potentiostat. Both the reference ("in house" built Ag-AgCl) electrode and auxillary (a short piece of stainless steel tubing 0.5 mm i.d.) electrode were placed in the outlet of the glassy carbon electrode cell block. All signals were recorded on a Houston Omniscribe strip-chart recorder.

3.9.1.3. Procedures

3.9.1.3.1. Cyclic Voltammetry

An aliquot of standard ciprofloxacin was transferred to 20 mL of background electrolyte to give a final concentration of 5×10^{-5} M. The standard solution, in which a carbon paste electrode was placed, was stirred for 15 seconds, and after a 15 second rest period the cyclic voltammogram was recorded using a sweep rate of 50 mV s^{-1}. A blank voltammogram was obtained prior to the addition of the aliquot of standard ciprofloxacin.

Alternatively, hydrodynamic cyclic voltammetry was performed with a glassy carbon electrode (area = 3 mm^2) at a rotation speed of 500 rpm.

3.9.1.3.2. Differential Pulse Polarography

The polarographic assay for the determination of ciprofloxacin in formulated tablets as set out in section 3.7.1.2.1. was followed.

3.9.1.3.3. Flow Injection Analysis

The glassy carbon electrode was polished gently with 0.1 um alumina slurry until a mirror-like surface was obtained, (after ca. 1 minute polishing). After rinsing the electrode with de-ionised water and methanol, the electrochemical detector was re-assembled. The background electrolyte (or carrier solution) was de-aerated with helium gas for 20 minutes prior to using the flow system. After equilibrating the system by allowing the electrolyte carrier of phosphate buffer, pH 6.0:methanol (55:45) for 25 minutes with the cell kept at a constant potential of 1.10 V, flow injection measurements could then be made.

The surface of the glassy carbon electrode was cleaned daily by passing de-ionised water and methanol consecutively through the FIA system for 10 minutes each. On average, the glassy carbon cell block was dismantled and polished every two to three weeks.

3.10. RESULTS AND DISCUSSION

3.10.1. Optimisation of electrolyte pH for the electrochemical detection of the four known metabolites of ciprofloxacin

Due to the simplicity and speed of measurements, the conventional voltammetric batch measurement technique was selected to study the effect of pH on the cyclic voltammetric behaviour of the metabolites of ciprofloxacin at carbon paste electrodes (at a concentration of 5×10^{-5} M). Although the electrochemical behaviour of the metabolites would be expected to be comparable at both glassy carbon and at carbon paste electrode surfaces, the use of the latter electrode in this study was preferred because of its lower residual current and the ease of obtaining reproducible voltammograms.

As already observed with ciprofloxacin in section 3.8.4.2., metabolite M1 gave rise to one or two irreversible oxidation peaks depending on the pH (Figure 3.12). The main process (peak A) was observed throughout the pH range, the half peak potential of which was significantly dependent on pH. In acidic media, a second anodic process (peak B) was observed. However at pH values greater than 5.1, this process merged with peak A.

Although peak potentials for both processes were dependent on pH, both exhibited different linear relationships between half peak potentials ($E_{p1/2}$) and pH with slopes of 67 mV $(pH)^{-1}$ and 46 mV $(pH)^{-1}$ observed for peak A and peak B respectively.

The shift of $E_{p1/2}$ of peak A was nearly equivalent

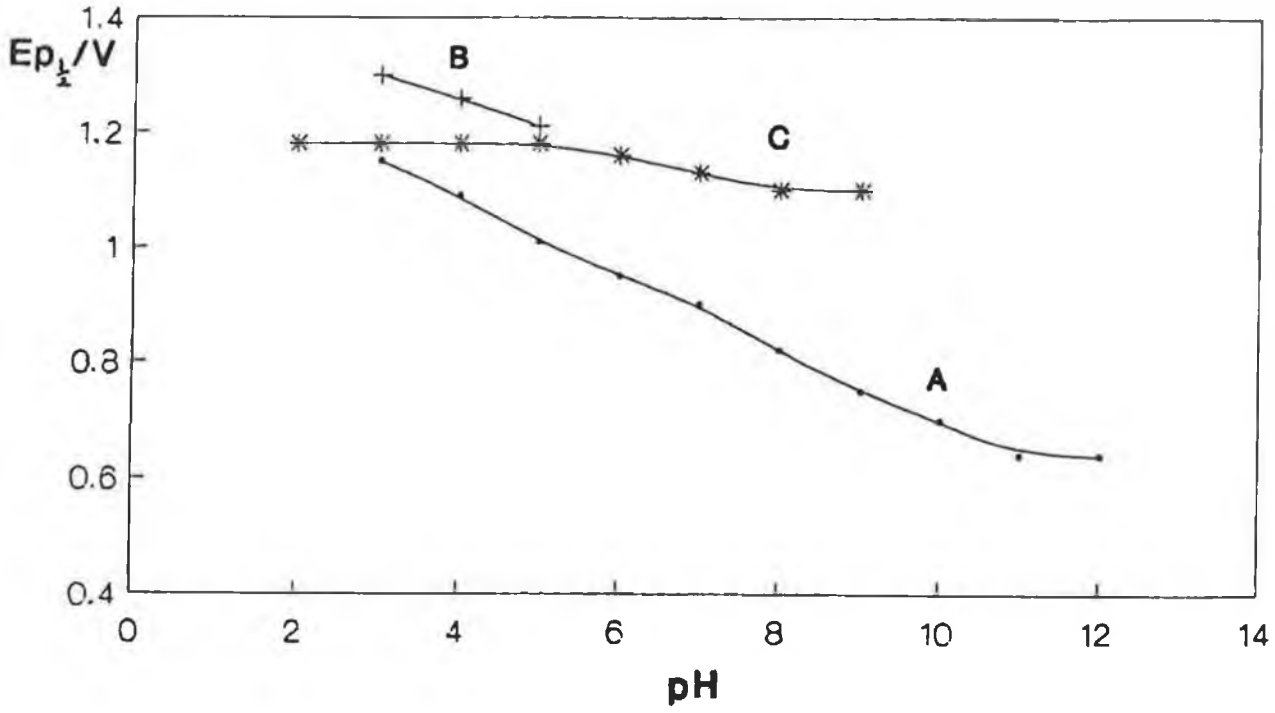


Figure 3.12. Dependence on pH of the cyclic voltammetric half-peak potentials of metabolites M1 and M2 of ciprofloxacin (5×10^{-5} M). Scan rate, 100 mV/s.

to that of the main oxidation process of ciprofloxacin where a slope of 63 mV $(pH)^{-1}$ was obtained in the same potential range. These preliminary results suggests that the oxidation of both M1 and the parent antibiotic could share similar electrochemical mechanisms and that the site of oxidation was at the amino group on the piperazinyl ring. Nevertheless, in relation to ciprofloxacin, the proposed mechanism would need to encorporate the cleavage of the intact ring to form the N-ethyleneamine side chain prior to possibly following the complex dual mechanism suggested by Barnes and Mann [49] for the oxidation of primary amines at carbon paste electrode in acetonitrile forming a cation radical ($RCH_2\overset{\bullet}{N}H_2^{+}$), which almost entirely decomposes to a carbonium ion ($R\overset{+}{C}H_2$) and a amidogen radical ($^{\bullet}NH_2$) or the formation of an iminum salt ($RCH=NH_2^{+}$), depending on the oxidation pathway followed.

The significant proton shift for both M1 and ciprofloxacin suggests that in aqueous media the former pathway,

$$RCH_2NH_2 \longrightarrow RCH_2\overset{\bullet}{N}H_2^{+} + e$$

$$RCH_2\overset{\bullet}{N}H_2^{+} \longrightarrow R\overset{+}{C}H_2 + NH_2$$

$$R\overset{+}{C}H_2 \longrightarrow H^{+} + \text{hydrocarbons}$$

which involves the loss of one electron and the decomposition of the carbonium ion with the loss of a proton may be the dominant mechanism.

Differing structurally from ciprofloxacin through the presence of a carbonyl group on the piperazinyl ring, M3 exhibited a complex electrochemical behaviour at the carbon paste surface. Three irreversible oxidation peaks were observed throughout the pH range (Figure 3.13). The $E_{p1/2}$ values of the the three anodic processes were independent of pH in acidic

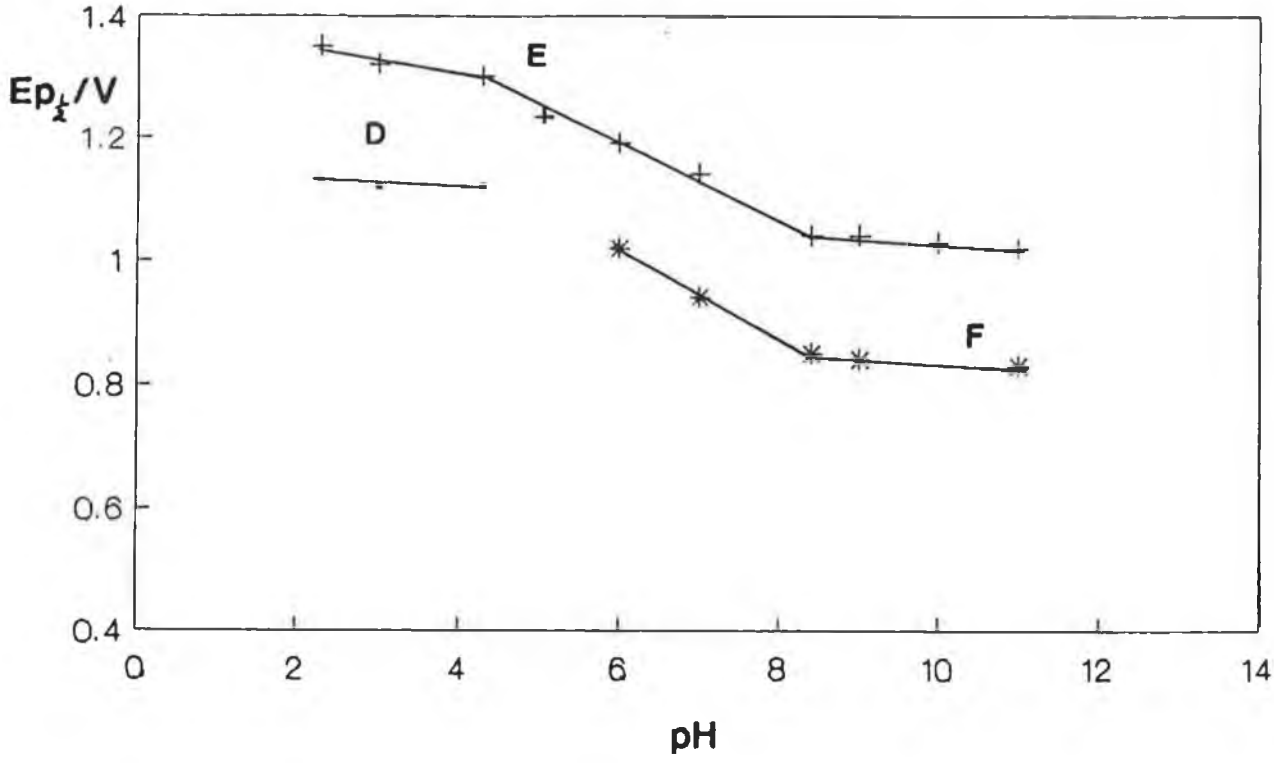


Figure 3.13. Dependence on pH of the cyclic voltammetric half-peak potential of metabolite M3 of ciprofloxacin (5×10^{-5} M). Scan rate, 100 mV/s.

and alkaline media. Nevertheless between pH 4.3 to pH 8.4 there was a linear dependence between $E_{p1/2}$ and pH with an equation of the line of $E_{p1/2}$ = 1.400 - 0.066 pH and $E_{p1/2}$ = 1.577 - 0.063 pH for processes E and F respectively. The intersections of the lines relating the half peak potentials with pH of the two processes clearly shows that in alkaline media, metabolite M3 demonstrates a pK value at approximately 8.4. This is in agreement with the findings of Takacs-Novak et al. [38], who found that flouroquinolones with similar structure to M3 and which also possessed a secondary amine functional group on the piperazinyl ring, had pK values between 8.39 and 8.78.

The addition of an electron withdrawing substituent such as the carbonyl group on the piperazinyl ring appeared to significantly alter the oxidative mechanism of M3 in comparison to ciprofloxacin and M1. Instead of a significant proton shift for the main anodic processes of the latter compounds throughout the entire pH range, the pH independence of half peak potentials of processes D, E and F in acidic and alkaline media, indicated the oxidation of stable species which predominated in such media.

The effect of pH on the cyclic voltammetric behaviour of M4 at carbon paste electrodes held certain similarities to that of M3. Depending on pH, M4 gave rise to one to two irreversible peaks (Figure 3.14). The first process (peak G) was observed only in acidic and neutral media, the half peak potentials of which were slightly dependent on pH. In neutral and alkaline media, the second process (peak H) was seen. Both the initial shift of $E_{p1/2}$ with pH of 71 mV between 5.7 to 8.4 and its subsequent independence of pH at values greater than 8.4 was similar to the anodic characteristics of peak F of M3. The pK value of 8.4 obtained in alkaline media was in agreement to that obtained for M3.

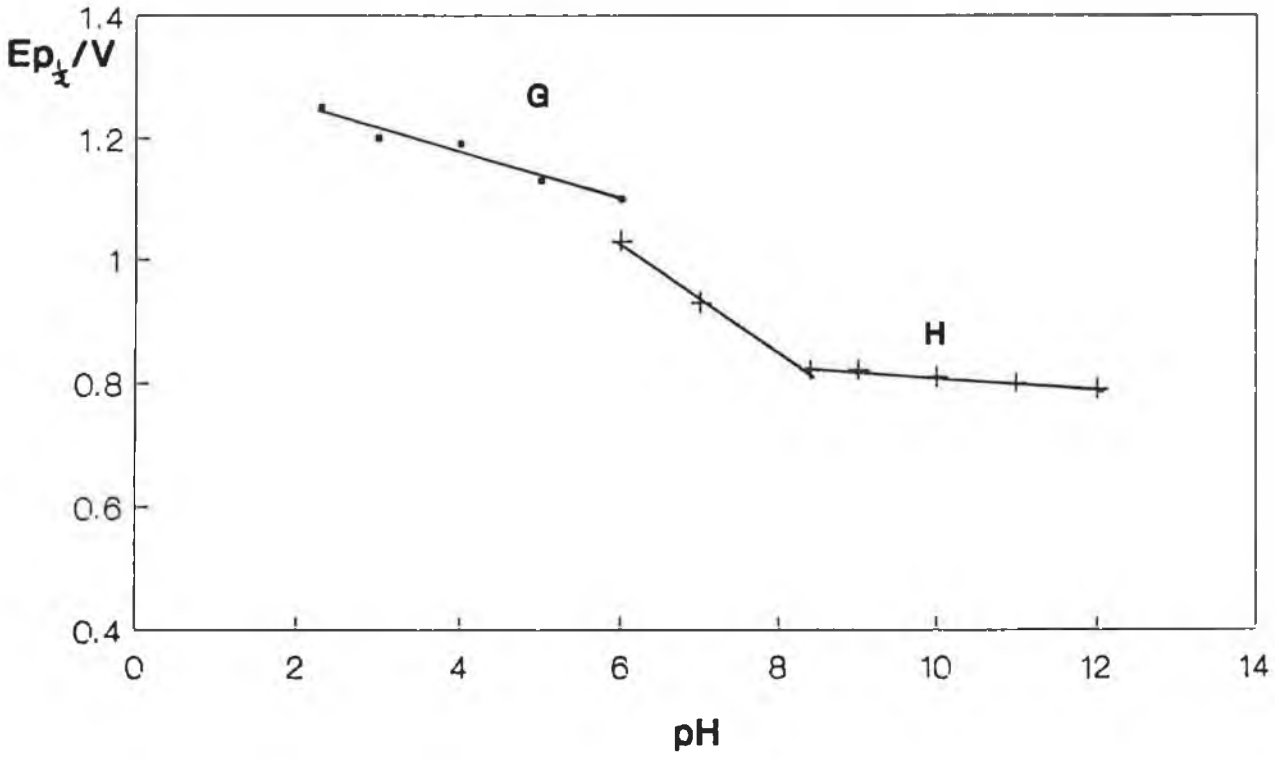
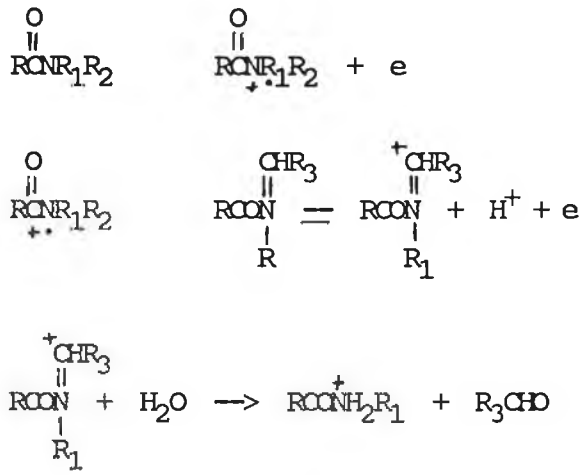


Figure 3.14. Dependence on pH of the cyclic voltammetric half-peak potential of metabolite M4 of ciprofloxacin (5×10^{-5} M). Scan rate, 100 mV/s.

The similarities and difference of the oxidative electrochemical behaviour of metabolites 3 and 4 is more than likely due to the common feature of a carbonyl group on the piperazinyl ring and the different position it occupies in the respective ring structures (i.e. forming secondary and tertiary amides respectively). According to O'Donnell and Mann [50], secondary and tertiary amides are oxidised to produce dealkylated amide and the corresponding aldehyde in the presence of water.



The initial proton shift of processes F and H, followed by independence of the half peak potential with pH, with a clear pK_a value at 8.4, suggests that these anodic processes could be due to a amide cation radical, which was generated at the surface of the working electrode, underwent a loss of a proton and electron to provide a cation which reacted with water to give rise to a dealkylated amide and aldehyde. In the case of tertiary amides, such as M4, the dealkylated amide is protonated and protected from further oxidation. The parallel anodic characteristics of processes E and F of metabolite M3 suggests that the former process could be due to further dealkylation of the oxidised product of process F.

Finally, M2 gave rise to the simplest oxidative

behaviour of the four metabolites at the carbon paste electrode. Only one anodic process (peak C) was observed, the half peak potential of which was pH independent from pH 2.28 to pH 8.98 corresponding to the oxidation of a species which predominates in the media solution throughout the entire pH range of its existence (Figure 3.12).

M2, with an average half peak potential of 1.15 V, was not readily oxidisable and this was attributed to the presence of the strong electron withdrawing sulphonyl group on the tertiary amine of the piperazinyl ring situated furthest away from the carboxylic acid quinolone ring structure.

At pH values less than 7, the peak current of the main anodic processes of M1 and M2 (ie. peak A and peak C) were independent of pH (Figure 3.15 and 3.16). However in more alkaline media the peak currents decreased with pH. For M3, the current of the peak D increased somewhat in acidic media to reach a maximum at pH 4.3 prior to its disappearance at pH values greater than 5.0 (Figure 3.17). The peak current of peak F in alkaline media was independent of pH. On the other hand, the voltammetric signal of peak E varied significantly with pH reaching a current maximum at pH 5.37 in slightly acidic media and a second lower maximum at pH 7.25 in neutral media. The peak current of the first anodic process (i.e. peak G) of M4 decreased significantly from a maximum in highly acidic media before its eventual disappearance at pH values greater than 9.0 (Figure 3.18). Peak H exhibited a similar decrease in current until at a pH value greater than 9.0 the voltammetric response became independent of pH.

For amperometric detection purposes the background electrolyte pH of 7.0 would be considered suitable to detect ciprofloxacin and the four metabolites. Even though a major increase in signal sensitivity could be obtained for such

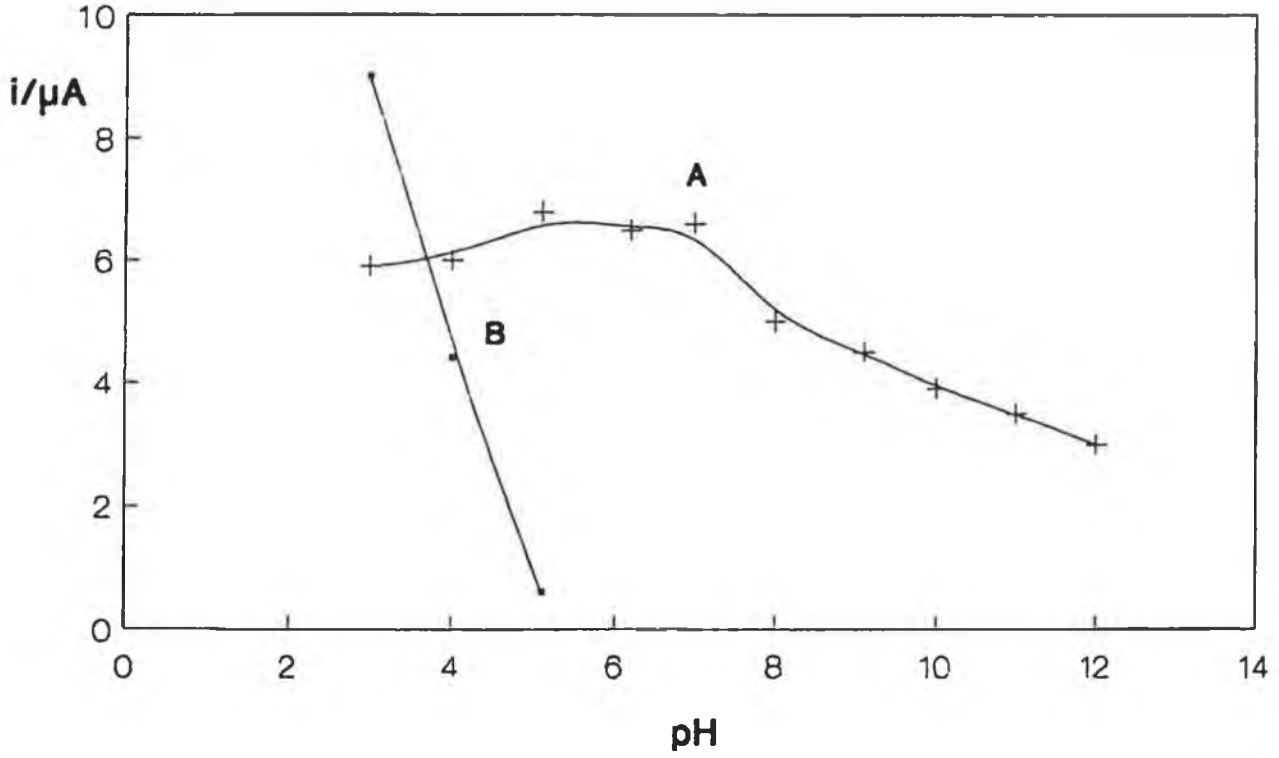


Figure 3.15. Dependence on pH of the cyclic voltammetric peak current of metabolite M1 of ciprofloxacin (5×10^{-5} M). Scan rate, 100 mV/s.

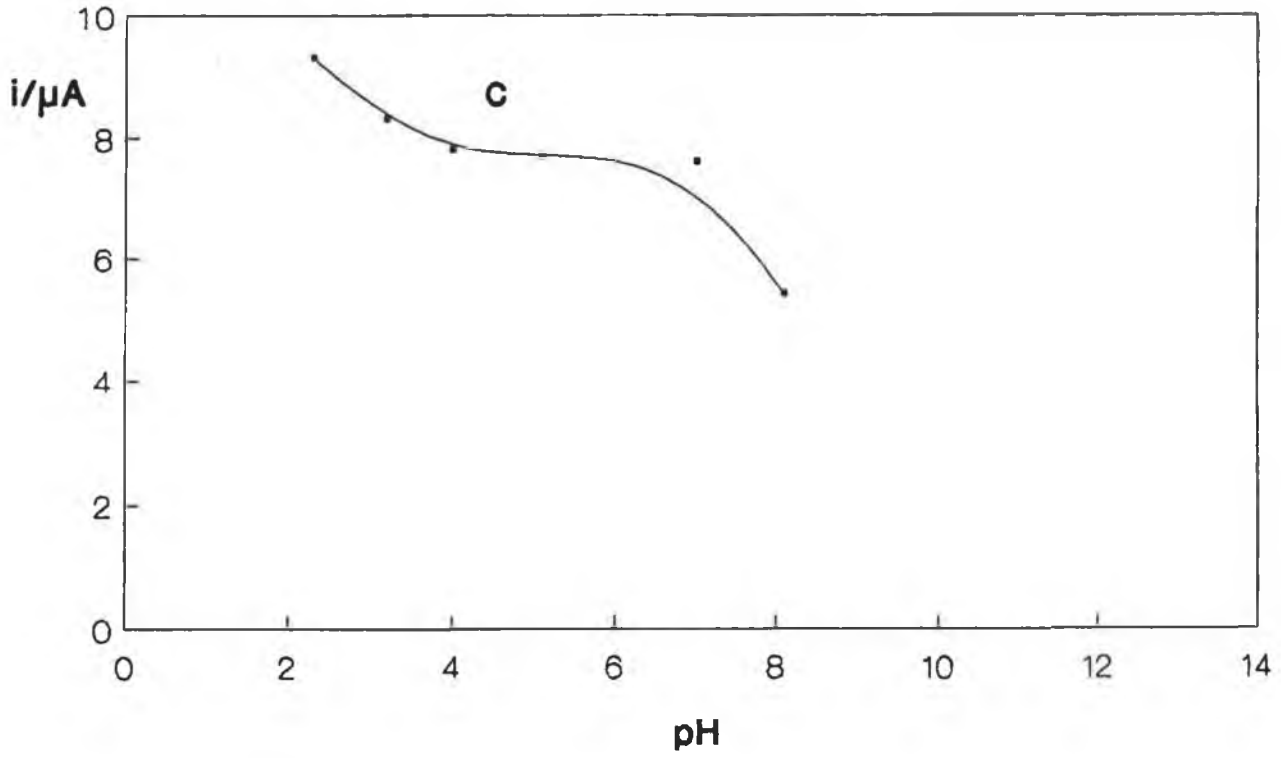


Figure 3.16. Dependence on pH of the cyclic voltammetric peak current of metabolite M2 of ciprofloxacin (5 x 10^{-5} M). Scan rate, 100 mV/s.

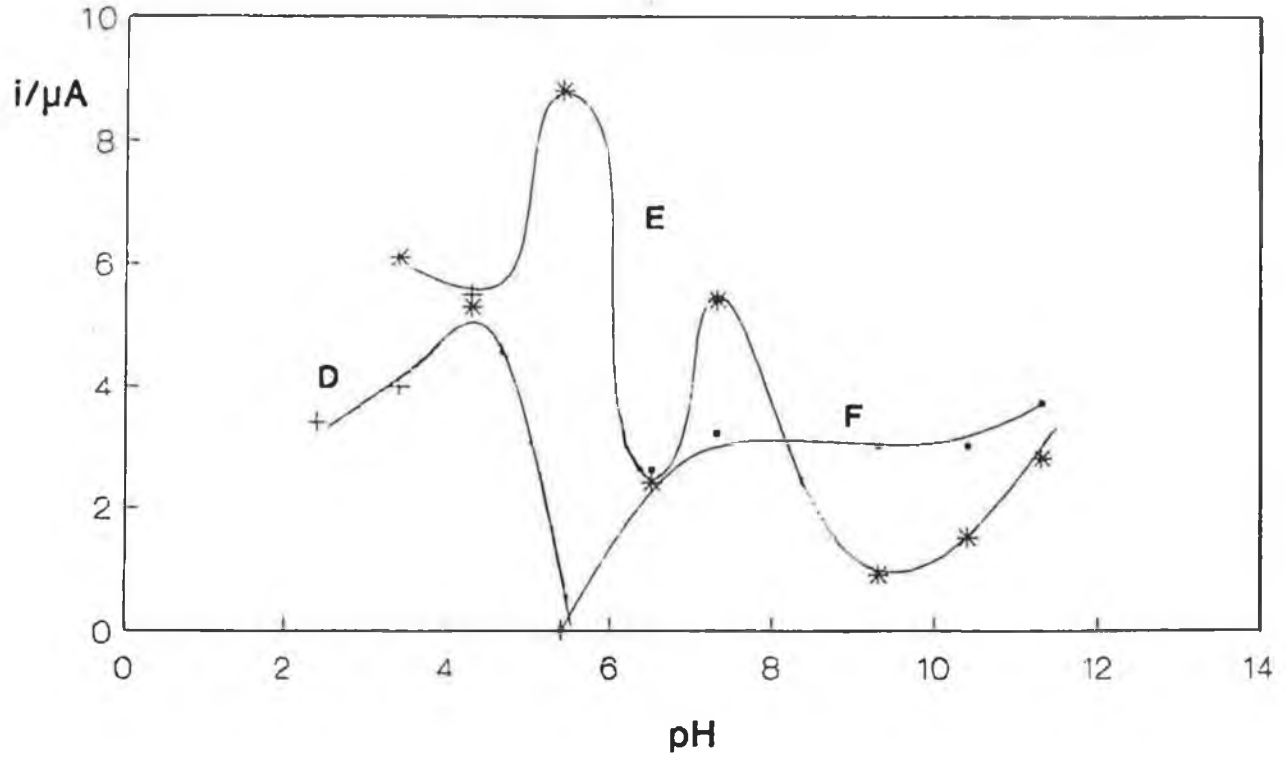


Figure 3.17. Dependence on pH of the cyclic voltammetric peak current of metabolite M3 of ciprofloxacin (5 x 10^{-5} M). Scan rate, 100 mV/s.

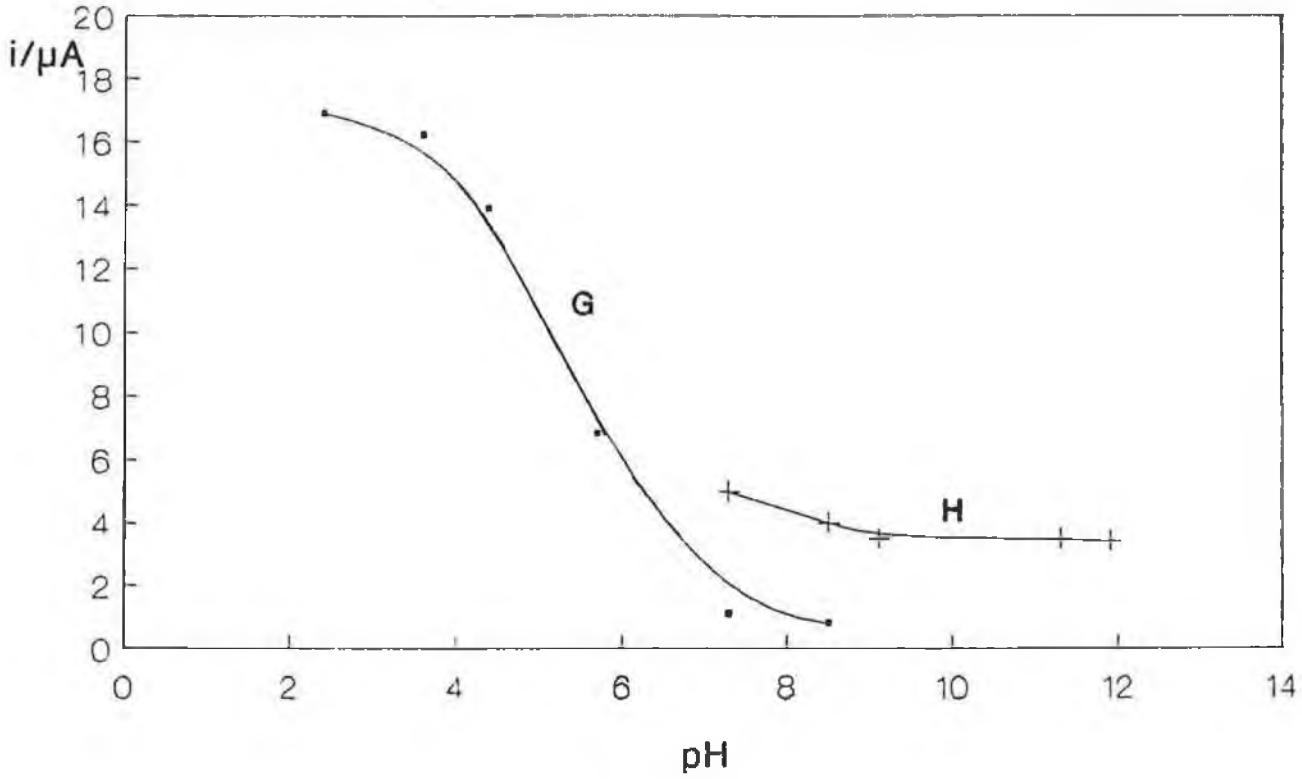


Figure 3.18. Dependence on pH of the cyclic voltammetric peak current of metabolite M4 of ciprofloxacin (5×10^{-5} M). Scan rate, 100 mV/s.

metabolites as M1 and M4 in more acidic media, the high applied potentials necessary to detect the metabolites could not be used on a routine basis in conjunction with a glassy carbon based detection system.

By opting for a neutral electrolyte the selectivity of the electrochemical detector is further enhanced. The significant difference in half peak potentials (i.e. 200 to 250 mV) between ciprofloxacin, M1, M3 and M4 on the one hand and M2 on the other at pH 7.0, indicates that by carefully selecting the applied potential the former compounds can be selectively detected in the presence of M2. This is in addition to the normal reduction in matrix interferences that can be expected when lower applied potentials at the glassy carbon surface are employed.

3.10.2. Optimisation of conditions for Flow Injection Analysis

As has been already reported in section 3.8.4.2., ciprofloxacin gave rise to two irreversible oxidation peaks in neutral to slightly alkaline media. A background electrolyte of pH 6 was chosen for the adsorptive stripping procedure, since it gave rise to reproducible peaks once the carbon paste electrode was activated "in situ" prior to each accumulation and stripping voltammetric measurement step.

A phosphate buffer of pH 6.0 was therefore initially selected as the background electrolyte in the FIA system. In order to ascertain if there was a change in the voltammetric behaviour when the working electrode surface was changed from carbon paste to glassy carbon, a cyclic voltammogram was obtained of ciprofloxacin (at a concentration of 5×10^{-5} M)

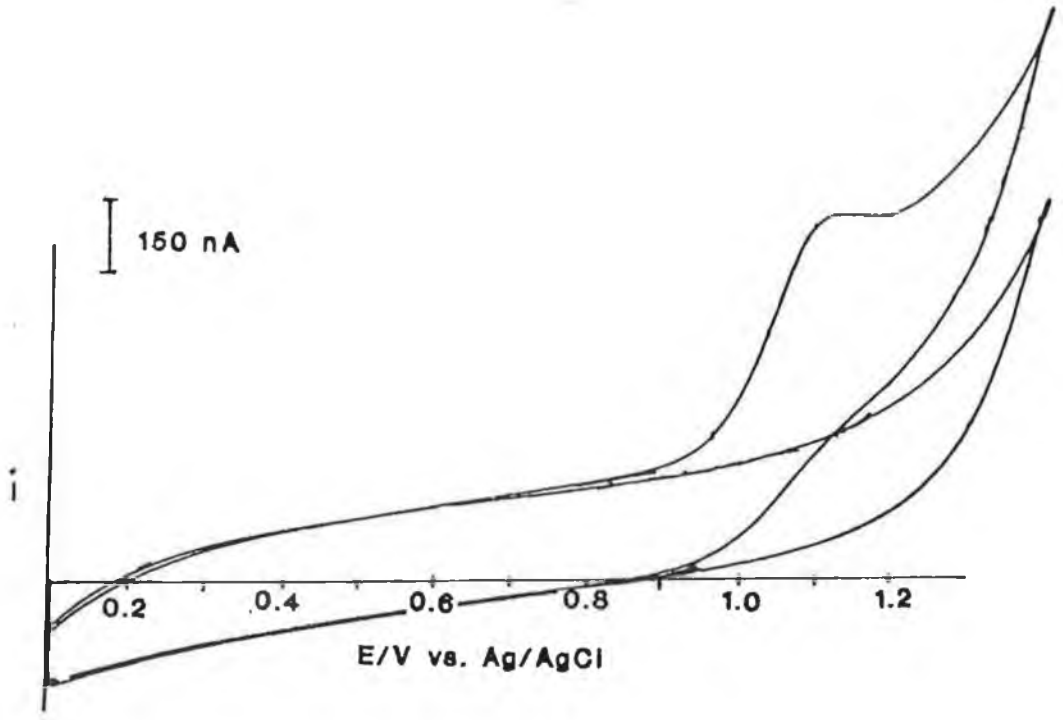


Figure 3.19. Cyclic voltammogram of 5 x 10^{-5} M of ciprofloxacin in phosphate buffer, pH 6.0. Scan rate, 100 mV/s.

at a rotating glassy carbon electrode (Figure 3.19). Only one anodic process was observed with a half peak potential 40 mV more anodic than that obtained on the carbon paste electrode. The absence of the second anodic process on this type of electrode suggested that this second peak could be due to the oxidation of an absorbed product on the carbon paste electrode. The size of the anodic shift was thought to be large even when one bore in mind the change in electrode surface, the change of background electrolyte from Britton Robinson buffer, to phosphate buffer and that the voltammetric measurement was made on a rotating electrode rather than a stationary electrode. It was thought that the concentration employed may have saturated the electrode surface thus partially inactivating the electrode surface with the consequent anodic shift of half peak potential.

It was immediately apparent that the reproducibility of the peak current was a considerable problem under the experimental conditions employed. The amperometric response steadily increased with repeated injections until reaching a steady response after five to seven injections. Neither increasing the flow rate, nor the time between injections (and thus increasing the efficiency of the buffer "to wash" the surface of the electrode) significantly improved the variability in peak current under fixed conditions.

The addition of organic solvents such as acetonitrile and methanol to the electrolyte carrier were investigated to reduce the obvious fouling of the electrode. Although peak reproducibilty improved slightly with the presence of acetonitrile in the buffer, this was at the expense of an unsteady baseline. The more polar methanol was more effective in reducing the adsorption of ciprofloxacin at the electrode surface. After modifying the electrolyte carrier of phosphate buffer pH 6.0 with 45% methanol, the relative standard deviation

(RSD), after seven repeat injections at a fixed potential of 1.10 V, was less than 2.5%. Peak current sensitivity which had decreased by 70% with the addition of 45% methanol to the carrier, declined further when the percentage of organic solvent was increased and was not compensated by a concurrent improvement in the reproducibilty of the peak signal.

In order to determine the optimum applied potential that would give the highest sensitivity for the determination of ciprofloxacin, hydrodynamic voltammetric curves were constructed by plotting the average current of three repeated peaks as a function of the initial applied potential in the range of 0.70 to 1.20 V. The applied potentials were increased in a stepwise manner (50 mV) over a series of injections. However, in the case of constructing the hydrodynamic voltammetric curve of ciprofloxacin in phosphate buffer pH 6.0, five to seven repeat injections were made and the peak current of the last three reproducible voltammetric responses were plotted. The results obtained are shown in Figure 3.20.

As would be expected, the half peak potential of ciprofloxacin in the carrier containing an organic solvent was shifted ca. 25 mV more positive in comparison to the result obtained in a carrier containing phosphate buffer pH 6.0 only. The former half peak potential was in good agreement with the half peak potential of 0.95 V observed at the rotating glassy carbon electrode for ciprofloxacin in a background electrolyte of phosphate buffer pH 6 modified with 45% methanol. For subsequent studies, an applied potential of 1.10 V was selected because not only was peak current independent of applied potential at this value, but good sensitivity and reproducibility was obtained for the determination of ciprofloxacin.

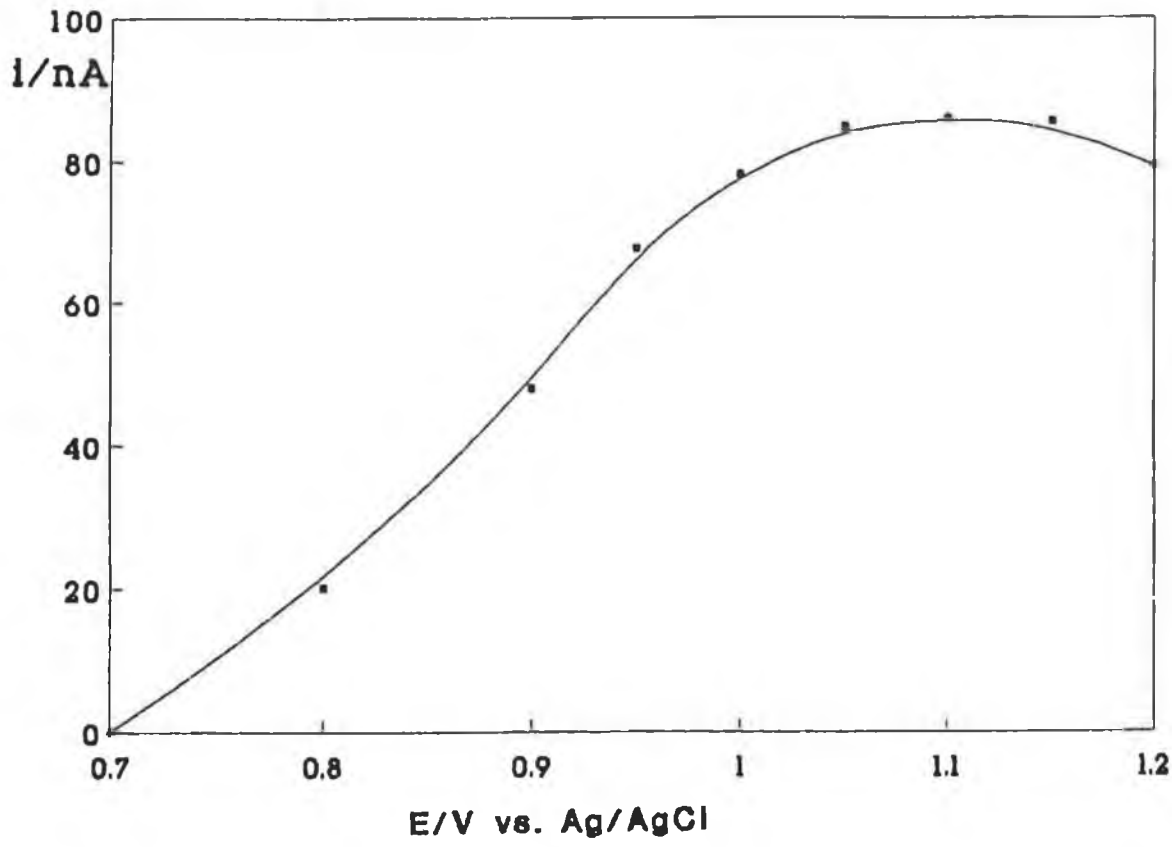


Figure 3.20. A hydrodynamic voltammetric curve obtained for a solution of 1 x 10^{-5} M ciprofloxacin at a single glassy carbon electrode over the potential range of +0.5 to 1.3 V vs. Ag/AgCl.

By increasing the length of tubing between the injection port to the inlet of the electrochemical detector, one increases the dispersion of the injected plug leading to a increase in analyte travel time (t_a) and baseline to baseline time (Δt). Consequently, a tube length of 140 cm was selected as the t_a was sufficient to provide a good separation between the injection event and the ciprofloxacin peak. Coiling the tubing, which decreases the dilution of the injected sample (i.e. dispersion) improved peak shape and at a coil diameter of 10 cm increased the voltammetric response by 35%.

Flow rates through the FIA system were varied from 0.5 to 3.0 mL/min. At low flow rates, the eluting peaks were ill-defined and at 0.5 mL/min a peak maximum occurred at 42 seconds. Increasing the flow rate not only improved peak shape, but the time for the peak maximum to appear and peak current decreased to 18 seconds and increased by 14% respectively when the flow rate was increased from 1.0 mL/min to 2.0 mL/min. Although further improvements in peak response were obtained when the flow rate was increased to 3.0 mL/min, at flows greater than 2.0 mL/min a significant reduction in peak reproducibilty was observed. This could possibly be due to turbulence at high flow rates at the reference and auxilary electrode situated at the outlet of the glassy carbon electrode block.

Using a flow rate of 2.0 mL/min, the injection volume was varied from 10 uL to 150 uL of 1.93 mg/mL ciprofloxacin and gave rise to a linear relationship with a correlation co-efficient (r) of 0.9894. As the volumes injected increased, t correspondingly became larger and increased from 25 seconds at an injected volume of 10 uL to 75 seconds after an injection of a 150 uL aliquot. For all subsequent studies an injection volume of 10 uL was selected.

In order to maintain reproducibility of the amperometric response of ciprofloxacin, the glassy carbon surface requires adequate "cleaning" of surface bound ciprofloxacin or oxidised product after each measurement to minimise carry-over effects. Depending on the nature of interaction of the analyte to the electrode surface, the "cleaning" conditions and their duration differ from analyte to analyte. In this case, the electrode surface was simply flushed with carrier electrolyte for ca. 60 seconds prior to each injection. Allowing for 25 seconds for peak measurement and 60 seconds for electrode "cleaning", the overall sample throughput was estimated for this procedure at 42 injections per hour.

Under the experimental conditions described above, linear calibration curves were obtained for standard ciprofloxacin between 0.77 mg/mL to 38.58 mg/mL with an equation of the line of i(nA) = 0.6947 + 11.968 mg/mL and a correlation co-efficient (r) of 0.9999. The upper limit of the linear range could not be determined due to the inability to decrease further the current sensitivity of the amperometric detection system employed to permit the recording of the signal output. A limit of detection of ca. 0.4 mg/mL was obtained with this procedure.

3.10.3. Determination of ciprofloxacin in formulated tablets by FIA

Ciprofloxacin was subsequently determined in formulated tablets by FIA with glassy carbon based electrochemical detection (FIA/ED) and the procedure compared to the DPP assay outlined in section 3.7.1.2.1. The results obtained are summarised in Table 3.3.

TABLE 3.3. A comparison of results between FIA/ED and DPP assay for the determination of ciprofloxacin in pharmaceutical preparations.

SAMPLE	SAMPLE CONCENTRATION (mg/mL)	EXPERIMENTAL DETERMINATION OF CIPROFLOXACIN CONCENTRATION (mg/mL)			
		DPP	%DEVIATION[a]	FIA	%DEVIATION[a]
1	3.8927	4.0636	+ 4.4	3.7706	- 4.7
2	3.7033	3.9104	+ 5.6	3.4719	- 6.2
3	3.8811	4.0945	+ 5.5	3.6852	- 5.1
4	3.8619	4.0278	+ 4.3	3.6299	- 6.0
5	3.8727	4.0509	+ 4.6	3.6489	- 5.8

[a] Calculated for n = 3.

As can be seen from the results, the DPP assay over estimated the content of pharmaceutical preparations by between 4.3% to 5.6%. This consistency in the results was in agreement with the relative standard deviation (RSD) of 1.4% for the assay which was determined from the slopes of the linear standard addition equations. In comparison to the DPP assay, the FIA procedure underestimated the ciprofloxacin concentration in the formulated tablets by between 4.7 to 6.2% and the RSD for this assay was estimated at 2.4 %. Although both procedures showed excellent precision, the consistent differences in the estimation of ciprofloxacin concentration in the pharmaceutical preparations assayed highlighted the need that both the DPP and FIA procedures would need to be compared with a more routine

method for the determination of ciprofloxacin.

Although the presented results would suggest that glassy carbon-based electrochemical detection is not the most suitable detector for the high sample throughput required by FIA, this mode of detection could find applicability in conjunction with liquid chromatography. Reported isocratic chromatography [4] with two different solvent systems for the separation of ciprofloxacin and the four metabolites was completed within 15 minutes, which is ample time to "clean" the electrode of surface bound ciprofloxacin and electrochemically oxidised product. Further work is required to see if some or all the metabolites share similar adsorption characteristics for the glassy carbon surface as shown by the parent antibiotic. The addition of a low concentration of surfactant to the liquid chromatographic mobile phase, in order to minimise analyte adsorption at the working electrode, may be more compatible with this technique rather than the high concentrations of organic solvent required in the FIA assay.

Even though an initial comparison between the detection limit of ciprofloxacin as determined by the glassy carbon-based detection system at 400 ng/mL and the detection limits of 140 ng/mL and 1.8 ng/mL reported for UV and flourescence spectrophometry respectively [4] is unfavourable, no final conclusions on the potential applications of this mode of detection can be made until the expected improvement in selectivity and detection limit for ciprofloxacin using a dual glassy carbon-based detection system has been determined.

3.11. REFERENCES

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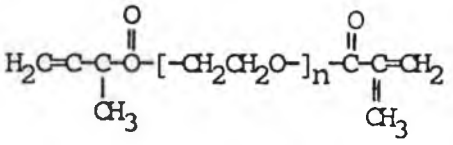
CHAPTER FOUR

ANALYSIS OF ANAEROBIC SEALANTS AND ADHESIVES

4.1. INTRODUCTION

Anaerobic sealants are basically designed to remain stable in liquid form in air, but once confined between two closely fitting metal parts, thereby excluding oxygen, the monomeric constituents in the liquid polymerise to form a tough heat and solvent resistant solid material. Therefore, anaerobic sealants are predominately used in the mechanical engineering industry, finding applications in the sealing, locking and retaining of metal objects.

The initial steps in developing these type of sealants began in the early 1950s, when Burnett and Nordlander of the General Electric Company in the United States [1] patented the use of anaerobic monomers based essentially on polyethyleneglycol dimethacrylates (the general structure of which is given below; I) in a product called "Permafil".



Where n > 2

Polyethyleneglycol dimethacrylates (I)

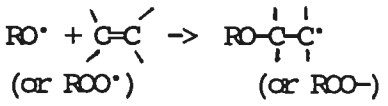
Triethyleneglycol dimethacrylate (TEGDMA) is a typical example of an anaerobic monomer derived from polyethyleneglycol dimethacrylates.

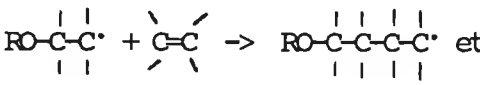
Burnett and Nordlander discovered that TEGDMA, oxygenated by heating at 60-80°C in the presence of bubbled air, remained liquid when cooled as long as aeration was maintained. However, when the air bubbling was discontinued or when the liquid was pressed as a thin film between glass microscope slides, rapid crosslinking occurred forming a solid polmeric material.

This early polyethyleneglycol dimethacrylate-based sealant was commercially limited, not only because the curing system required the continuous passage of oxygen or air prior to initiation, but because of its limited stability. However, many of the anaerobic products available today are based on the monomers disclosed by Burnett and Nordlander in the original General Electric Company's patent form.

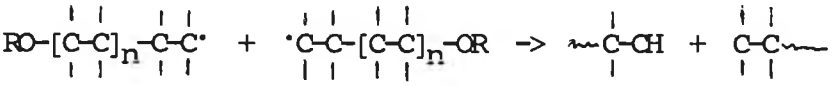
For instance, Krieble [2] in the late 1950s successfully developed the first anaerobic sealants composed of methacrylate monomers containing, small amounts of an organic hydroperoxide. This combination of polyethyleneglycol dimethacrylate (typically TEGDMA) and organic hydroperoxide catalyst (typically cumene hydroperoxide, CHP) continues to form the basis for many of the more than 250 U.S. patents issued since then [3]. Other components are added to the formulations to improve both the curing rate and increase the shelf life of the products at ambient temperature.

The mechanism by which anaerobic systems cure to thermoset polymers is based on a metal catalysed redox-based cure process. The simplified reaction scheme given below explains the chemistry behind what happens when the anaerobic sealant is confined between two closely fitting metal parts and the methacrylate monomeric constituents in the liquid polymerise in the absence of air.





The metal surface functions as an intrinsic component of the cure mechanism by providing metal ions which can react with a peroxide initiator to form free radicals. The free radicals RO· and ROO· react with one of the terminal double bonds of the monomer to form a methacrylate free radical which in turn will react with the terminal double bond of another monomeric molecule in a chain propagation sequence. This process will proceed in this manner until termination occurs most probably by disproportionation [5].



The linear polymer chains undergo subsequent reaction with the propagating polymer radicals to produce a three-dimensional cross-linked thermoset resin, possessing good heat stability and solvent resistance.

In the presence of atmospheric oxygen, the propagating free radicals can react readily with oxygen to produce peroxy free radicals. However, these radicals react very slowly with

methacrylate monomers, and thus oxygen strongly inhibits rather than catalyses such polymerisation reactions.

An anaerobic sealant containing only a polymethacrylate monomer and organic peroxide is therefore strictly limited by its slow rate of polymerisation, the sensitivity of the curing process on the type of metal surface (or substrate) that the sealant is applied to, and the stability of the product. Therefore, modern research has concentrated on the need to produce an adhesive or sealant formulation that contains a prudent balance between rapid cure speed and a stable packaged product with a long shelf life.

In order to increase the cure rate at room temperature, cure promoters or accelerators, such as organic acids or bases, are normally present in an anaerobic sealant formulation. A variety of organic acids are used, including p-tolune-sulphonic acid and maleic acid, while organic reducing agents are usually substituted aromatic amines such as N,N-dimethyl-p-toluidine and tetrahydroquinoline. A successful accelator combination that has been disclosed in the literature [7] consists of N,N-dimethyl-p-toluidine and an organic sulphimide, o-benzoic sulphimide (also referred to as saccharin). These compounds act as accelators in their own right, but together they produce a synergistic increase in cure speed. No mechanistic details explaining the role of each substance in the formulation has been published, and so the precise part that each plays in the curing mechanism remains open to conjecture.

For commercial purposes, the presence of atmospheric oxygen alone is no longer sufficient to ensure adequate stabilty either during manufacture or subsequent storage in the packaged state prior to use. Free radical inhibitors may typically include compounds such as hydroquinone, p-methoxyphenol,

pyrogallol and other substituted phenolic compounds. However, these stabilisers may achieve stability at the expense of performance on non-ferrous metallic surfaces such as cadmium and zinc plate [5]. One way to overcome this problem is to fully oxygenate the formulation containing the methacrylate monomer and saccharin/N,N-dimethyl-p-toluidine at 25°C and then add the organic peroxide such as CHP prior to use [8], or alternatively by treating the less active metallic surfaces with a suitable accelerating primer. Such primers consist of dilute solutions of transition metal salts in a volatile organic solvent.

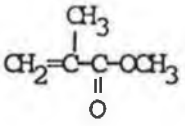
Sequestering (or chelating) agents are frequently included in the formulation to avoid premature polymerisation initiated by trace levels of contaminating transition metal ions arising from raw materials, the manufacturing process or final packaging. The preferred chelating agents possess a combination of oxygen and nitrogen ligands such as sodium salts of ethylenediamine tetraacetic acid (EDTA) [9]. On the other hand, sequestering agents containing only nitrogen ligand atoms (eg. dipyridyl) reduce significantly the polymerisation process on the low active metal surfaces. It is thought that the dipyridyl complexes so rapidly with the trace transition metal ions present on the surface that the metal ions are unable to contribute to the curing system [5].

Finally, in order to obtain the desired physical form and appearance, viscosity modifiers, such as plasticisers and thickeners, inorganic fillers, silica-based thixotropic agents, pigments and dyestuffs are also incorporated into the formulation.

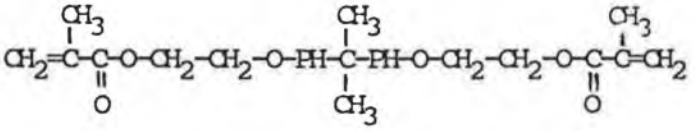
4.2. THE ANALYSIS OF ANAEROBIC SEALANTS AND ADHESIVES

4.2.1. Monomers

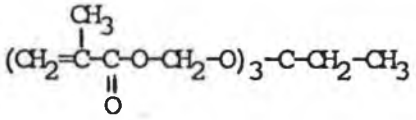
The polymerisable methacrylate monomer is the major constituent in a commercial anaerobic sealant formulas. Although polyethyleneglycol dimethacrylate continues to be the most widely used monomer because of its low cost, availabilty, low toxicity, high reactivity and the good environmental resistance of the cured polymer, other methacrylate esters shown below have been used [10].



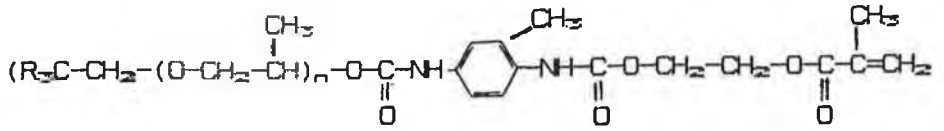
Methyl methacrylate (II)



Ethoxylated bisphenol a dimethacrylate (III)



Trimethylolpropane trimethacrylate (IV)



Urethane methacrylate resin (V)

The presence and identity of these monomeric constituents in an anaerobic sealant formulation is normally established by nuclear magnetic resonance (NMR) spectroscopy. Their identities and concentrations can be verified and quantified by either gas chromatography (GC) or high performance liquid chromatography (HPLC) with an internal standard.

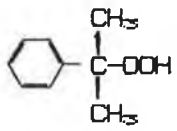
In GC analysis, OV1 (polydimethylsiloxane) or OV 101 (polyphenylmethylsiloxane) are the preferrred stationary phases packed in either narrow or wide bore capillary columns. The normal mode of detection is flame ionisation detection (FID) but when unambiguous confirmation of peak identity is required the FID can be substituted for a bench top mass spectrometer (MS) [10].

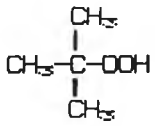
HPLC is the chromatographic mode of choice in the case of ultra-violet cure initiated formulations which use the higher molecular weight urethane-methacrylate resins. The relatively involatile monomers are separated on a reversed-phase C_{18} column using a tetrahydrofuran/water mixture as mobile phase with ultra-violet/visible detection [10].

4.2.2. Cure accelerators

The cure system normally includes an organic peroxide, an organic acid and an organic reducing agent. Such additives

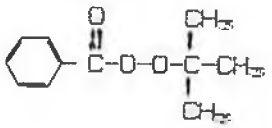
not only increase the rate of curing but the percentage of monomer polymerised. Typical peroxides used are alkyl hydroperoxides such as cumene hydroperoxide (VI) and tertiary butylhydroperoxide (VII) [10].

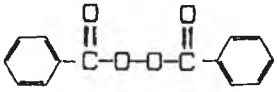




Cumene hydroperoxide (VI) tertiary butylhydroperoxide (VII)

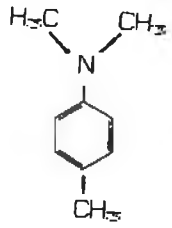
Peresters and diacylperoxides are also used occasionally, of which the following are an example [10].

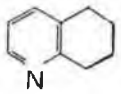




Tertiary-butylperbenzoate (VIII) benzoylperoxide (IX)

Typical organic acids used in formulations are p-toluene-sulphonic acid and maleic acid. Aromatic amines such as N,N-dimethyl-p-toluidine (X) and tetrahydroquinoline (XI) are usually the organic reducing agents used in anaerobic curative systems.





N,N-dimethyl-p-toluidine (X) tetrahydroquinoline (XI)

Finally, no commercial anaerobic sealant formulation is complete without free radical stabilisers such as hydroquinone, p-benzoquinone, p-toluquinone, chloranil and 1,4-naphtoquinone.

Thin layer chromatography (TLC) has been found to be the most convenient procedure to confirm the presence of organic peroxides, organic acids and organic reducing agents in a formulation. As an analytical technique it has the combined advantages of being simple, inexpensive and rapid, and possesses the ability to separate the curative agents from their complex matrices and from each other. The presence and identity of the separated promoting agents can be made by the comparision of the specific R_F values of the various analytes on the TLC plate (with specific detection with a spray reagent) with the corresponding standard solutions run on the same plate. In the case of peroxy initiators, after their separation on a precoated silica gel plate with an appropriate solvent, (generally toluene mixtures with carbon tetrachloride, methanol or acetic acid), the TLC plate is treated with an aqueous methanolic solution of N,N-dimethyl-p-phenylenediamine dihydrochloride and heated gently [11]. The peroxide will give a typical purple response against a blue background. Other developing reagents used in the detection of peroxides and hydroperoxides are acidified potassium iodide and p-aminodimethylaniline hydrochloride [12]. A dilute aqueous solution of 2,6-dichlorophenolindophenol sodium

salt is used in the detection of organic acids on TLC plates [13]. Similarily, after TLC separation, the aromatic amines are normally detected using a dilute aqueous solution of ferric chloride and potassium hexacyanoferrate [14] and the free radical stabilisers are detected with rhodanine and ammonia to give a coloured complex [15].

The peroxides present in sealants/adhesives react stoichiometrically with the iodide ion in acidic solution, producing iodine, which can be titrated with a standard solution of sodium thiosulphate [16,17] or estimated colorimetrically as tetra-n-butylammonium iodide [18]. The end point of the titration can be poteniometrically determined using a platinum-reference electrode combination. The acidic components of the formulation are similarily quantified by titration using dilute caustic or tetrabutylammonium hydroxide as titrants in aqueous or non-aqueous media respectively, and potentiometric detection with a glass/reference electrode combination [10]. Aromatic amine content is normally established by non-aqueous titration with perchloric acid, using acetic acid as a solvent. The end point is determined potentiometrically with a glass/reference electrode combination [10].

The standard iodometric titration for the determination of organic peroxides in raw materials is relatively time consuming, and alternative methods using either GC (using a OV 1 column with FID detection) or HPLC (using a C_{18} column with a mobile phase of acetonitrile and water with ultra-violet detection at 254 nm) have been investigated. In general, chromatographic procedures possess a relatively short analysis time (5-6 minutes) as compared to the titrimetric assay (15 minutes) [10]. Peroxides are normally reduced to alcohols prior to gas chromatographic analysis. The direct analysis of peroxides is somewhat more rare owing to their sensitivity to heat and surfaces [18]. However, this approach is favoured in

the Loctite laboratories, with the thermal decomposition of the peroxide in the injection port prevented by the use of an on-column injection technique [10]. From the studies carried out in these laboratories it was concluded (i) that either GC or HPLC analysis may be substituted for iodometric analysis in the case of benzoyl peroxide and CHP, without any apparent loss in either precision or accuracy; (ii) that GC analysis may be substituted for iodometric analysis in the case of t-butyl hydroperoxide without any apparent loss in precision or accuracy (whilst there was some loss of accuracy in the GC analysis of di-t-butylperoxide, the precision of the assay was acceptable and thus preferred, in this case, to the tedious and problematic iodometric titration); (iii) the HPLC analysis may be subsituted for iodometric analysis in the case of t-butylperbenzoate without any apparent loss in either precision or accuracy.

A much less popular, but nevertheless excellent method for the quantitation of peroxides is polarography. The method has been described in detail by Martin [20] who has provided tables of half-wave potentials for hydroperoxides and other peroxide compounds. The disadvantage to this technique is that it cannot differentiate properly between individual peroxides in mixtures. The polarographic reduction waves tend to be long and drawn out due to the irreversibilty of the reduction process; a separation of about 0.4 V in half-wave potentials is necessary before discrete steps become apparent [20]. The diffusion current is proportional to peroxide concentration over a 10^{-2}-10^{-6} M range, but since the proportionality constant varies with structure, individual calibration is necessary for accurate work [20].

4.2.3. Other additives

Viscosity modifiers or thickeners are used in varying concentrations throughout the range of sealants and adhesives. Their presence in combination with fillers and thixotropic agents are necessary to maintain a reasonable thickness of sealant prior to initiation of polymerisation. The more commonly encountered thickeners are listed below.

Poly(alkyl methacrylates) - homo and copolymers
Poly(alkyl acrylates) - homo and copolymers
Polystyrene
Acrylonitrile-butadiene-styrene copolymers
Polyvinyl acetate
Fumaric and maleic acid based polyesters

Typical examples of a thixotrope and filler are fumed silica and calcium carbonate respectively.

The normal procedure for the separation of the thickener from the formulation matrix involves the precipitation of the thickener with a polar solvent (such as methanol) from an adhesive sample initially diluted with chloroform. After making certain all traces of solvent are removed, the chemical identity of the dried sample can be confirmed by IR spectroscopy [10]. Another technique useful in the characterisation of polymer thickeners used in the formulation of sealants is pyrolysis gas chromatography. In this analytical technique, complex involatile materials are broken down into smaller volatile constituent molecules by the use of high temperatures. A fingerprint characteristic of the sample injected is obtained. Normally a mass spectrometric detector is used in conjunction with pyrolysis GC to facilitate identification of the pyrolysis products [21].

Gel permeation chromatography (GPC) can also be used to determine the molecular weight range/distribution of the polymeric thickener. GPC separates sample molecules on the basis of their size and shape. Using a column packed with a highly crosslinked spherical polystyrene/divinyl benzene matrix with a tightly controlled pore diameter, the sample molecules will be eluted in order of decreasing molecular size. The larger molecules cannot penetrate the gel particles and therefore will pass straight through the column through the spaces between the individual particles. The smaller molecules however will penetrate the gel particles depending on their size and their progress through the column will be retarded proportionally.

In order to characterise the molecular weight distribution (MWD) of an unknown polymer, the GPC column can be calibrated with a series of narrow MWD standards of known molecular weights by plotting a graph of $\log_{10}$ molecular weight versus elution volume or time. The number of polymers for which suitable standards are available is limited. Polystyrene calibration standards are the most widely used and many polymers of different chemical structure and molecular weights are reported relative to polystyrene [23].

The stability of many sealants is critically dependent on both the type and concentration of the free metal ion chelator. EDTA is one of the most frequently used chelators and can be quantified in formulations using differential pulse voltammetry (DPV). Certain metal EDTA complexes are reduced at more negative potentials than the metal ion itself. Therefore, if an excess of the metal ion such as copper (II) is added to a suitable supporting electrolyte (eg. 0.1 M acetate buffer, pH = 5.0) containing a known amount of dissolved sealant, then the voltammogram will exhibit two peaks [10]. The peaks represent the free metal ion and the complexed ion respectively. The chelator in the sealant can be quantified, using the standard

addition procedure, by the addition of aliquots of standard chelator of known concentration to the electrolytic cell and measuring the subsequent current response.

Due to the ability of trace metals to initiate the polymerisation process, it is necessary to monitor both the raw materials and finished formulations for trace metal ion content. This is normally carried out by atomic absorption spectrometry (AAS) or direct current plasma spectrometry [24]. Both of these techniques determine total metal ion concentration and only allow for single element determination. Therefore, to meet the need of a multielement approach, and to distinguish between "free" and "chelated" metal ions, the feasibilty of using ion chromatography (IC) has been recently explored [25,26].

A very promising approach appears to be the use of "dynamically coated" reversed-phase HPLC [27], combined with post-column derivatisation using 4-(2-pyridylazo)-2-resorcinol (PAR) reagent and visible detection at 500 nm. This method has been applied to the simultaneous determination of six divalent metal ions in some anaerobic sealant formulations, with limits of detection as low as 30 ppb for certain metals [27].

As this technique is essentially restricted to the analysis of divalent metal ions, polarography, either in the differential pulse or direct current mode, is the preferred technique in the determination of multivalent metals such as chromium, which as a contaminant can occur as chromium (III) or chromium (VI), or the speciation between Fe(II) and Fe(III).

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CHAPTER FIVE

INVESTIGATION OF A CATION EXCHANGE SEPARATION METHOD FOR THE DETERMINATION OF TRANSITION METAL IONS IN ANAEROBIC SEALANTS

5.1. INTRODUCTION

As has been previously outlined in chapter four, the determination of trace metal ions in anaerobic sealants is of importance due to their ability to initiate the polymerisation process, resulting in premature setting of the products and thus reduced commercial shelf life. While atomic absorption spectrometry (AAS) and inductively coupled plasma (ICP) spectroscopy are the usual analytical techniques employed in their analysis, the need has arisen in recent years for a complementary instrumental method to speciate between different valence states of a particular metal and to distinguish between free and chelated metal ions. This has lead to the investigation of using high performance liquid chromatography (HPLC) or ion chromatography (IC) as an alternative technique, which permits a multi-element approach and can distinguish between the different metal species (either free or complexed) present in the anaerobic sealant matrix. The application of HPLC and IC to inorganic analysis has gained in popularity during the past twenty years, and a large number of papers are now published annually on the subject today. In general, the HPLC separation of metal ions have been obtained via the formation, off-line or on-line, of metal chelates which can be directly determined by UV-Vis spectrophotometric or fluorescence detection, or after separation, by post-column derivatisation using a suitable complexing agent with a good chromophore.

Although ion chromatography was initially developed in 1975 by Small and co-workers [33] at Dow Chemicals, several years passed before the development of suitable strategies for transition metal ions. The advent of low exchange capacity columns with an eluent which could complex polyvalent metal ions overcame the initial problems of precipitation of metal hydroxides on the separation column or in the detection system.

Although conductimetric detection has often been used to detect transition metals, better sensitivity has been obtained if the separated cations are derivatised in a post-column detection system prior to spectrophotometric detection.

The following section will briefly review HPLC- and IC-based procedures for the separation of trace metal ions, which have provided the basis for several recently published papers for the determination of selected trace metals in anaerobic adhesive formulations [1,2,3].

5.2. TRACE METAL SEPARATION BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

5.2.1. β-Diketonates

Huber et al. [4] reported the first HPLC separation and determination of metal co-ordination complexes in 1972. They showed that liquid-liquid partition HPLC with a ternary two-phase system consisting of water-2,2,4-trimethyl pentane-ethanol could be used for isocratic separation of several metal acetylacetonates. The water-rich or polar phase served as the stationary liquid phase and was supported on silica particles (5-10 and 10-20 um). The hydrocarbon-rich or non-polar phase served as the eluent. Spectrophotometric detection was achieved at 310 nm. Six metal acetylacetonates, namely those of Be(II), Cu(II), Al(III), Cr(III), Ru(III) and Co(III), could be separated within 30 minutes. However, due to hydrolytic reactions of Ni(II), Al(III) and Fe(III) complexes with the water-rich stationary phase, asymmetric peaks were produced for these complexes. The Al(III)-acetylacetonate was found to undergo hydrolysis in the stationary phase producing several

hydroxoacetylacetonato-aluminum(III) species which could be separated on the column.

An extensive study of the elution behaviour of a series of metal β-diketonates on HPLC columns which included bonded phases, alumina and silica gel has been carried out by Tollinche and Risby [5]. It was found that the best separations were obtained on normal phase silica gel columns with non-polar mobile phases and UV spectrophotometric detection at 280 nm.

5.2.2. β-Ketoamines

Normal phase HPLC (liquid-solid) was employed by Uden and Walters [6] for the isocratic separation of neutral copper and nickel chelates of N,N'-ethylenebis(acetylacetoneimine) [$H_2(enAA_2)$] and N,N'-ethylenebis(salicylaldimine) [H_2-$(enSal_2)$] on 10-um diameter silica with UV spectrophotometric detection at 254 nm.

Gaetani et al. [7] reported on the use of reversed-phase and normal phase HPLC for the separation of several β-ketoamine metal chelates. This study included several chelates of $H_2(enAA_2)$, N,N'-trimethylenebis(acetylacetoneimine) [$H_2(tmAA_2)$] and N,N'-ethylenebis(benzoylacetoneimine) [$H_2(enBA_2)$] with Co(II), Ni(II), Cu(II) and Pb(II). Separation of $CoenAA_2$, $NienAA_2$ and $CuenAA_2$ in less than five minutes was obtained on a reversed-phase C_{18} column with methanol-phosphate buffer (pH 7.8) as eluent.

5.2.3. Hydrazones and semicarbazones

Heizmann and Ballschmiter [8] in 1973 reported the first successful liquid-solid HPLC separation of Hg(II), Cu(II), Pb(II) and Zn(II) chelates of bisacetylbisthiobenzoylhydrazone. In a paper that represents one of the important early breakthroughs in the field of HPLC of metal chelates, satisfactory correlation with TLC data and detection limits in the nanogram range for Hg(II) and Cu(II) were reported.

These same authors in a later paper [9] demonstrated the possibility of separating 1,2-diketobisthiosemicarbazone and 1,2-diketobisthiobenzhydrazone chelates of Cd(II), Hg(II), Cu(II), Ni(II) and Co(III) isocratically on a silica column. Binary mixtures containing benzene, n-hexane, chloroform, acetonitrile, cyclohexane and n-heptane were investigated as mobile phases. It was shown that elution characteristics of the chelates were influenced by varying the substituents on the ligand molecules. Detection of the separated metal chelates at the nanogram level was performed by monitoring the UV absorbance at 360 nm.

The low solubility of metal chelates of 1,2-diketobisthiosemicarbazone in organic solvents has limited its analytical use as a ligand in trace metal analysis. Heizmann and Ballschmiter [9] reported on the separation of Hg(II), Cu(II), and Ni(II) chelates of glyoxybis(2,2,3,3,-tetramethylbutyl) thiosemicarbazones on a normal phase HPLC system with Alox T as the stationary phase and benzene as the eluent.

5.2.4. Dithiocarbamates

The use of dithiocarbamates (DTC) as metal chelating agents have been the subject of much interest. Heizmann and Ballschmiter [9], in their paper dealing with the separation of semicarbazone and hydrazone metal complexes, also included a study of the separation of the dithiocarbamates of Cd(II), Hg(II), Cu(II), Ni(II) and Co(II) using normal phase HPLC and UV spectrophotometric detection at 360 nm.

Uden and Bigley [10] reported a method for the separation of Cu(II), Ni(II), and Co(III) as their diethyldithiocarbamate complexes using sodium diethyldithiocarbamate (NaDEDTC) as the ligand on an isocratic normal phase HPLC system consisting of a silica column (8 um particles) with an eluent of 5% acetonitrile, 15% diethyl ether and 80% light petroleum ether. Detection of the chelates was accomplished by means of UV absorption at 254 nm.

A normal phase separation of mixtures of dithiocarbamate complexes of Zn(II), Cu(II), Mn(II), Ni(II), Pb(II), Co(II), Cd(II) and Fe(II) on LiChrosorb SI 60 (10 um) with chloroform-hexane as the mobile phase and UV spectrophometric detection at 360 nm was described by Liska et al. [11].

Edward-Inatimi and Dalziel [12] have reported a multi-element analysis scheme using solvent extraction and normal phase HPLC with UV spectrophotometric detection at 280 nm. They were able to detect the separated mixtures of DTC complexes of Cu(II), Ni(II), Hg(II), Pb(II), Co(II), Mn(II) and Bi(II) in the ppb range.

In a series of three papers, Schwedt [13] initially described a reversed-phase HPLC system for the determination of Cu(II)-, Ni(II)-, and Pb(II)-DTC complexes using acetonitrile-water mixtures as eluent and UV spectrophotometric detection at 254 nm. He also reported on the separation of Cr(III), Ni(II) and Co(II) chelates by reversed-phase HPLC [14]. In 1979, Schwedt [15] then investigated the possibilities of reversed-phase HPLC separation of tetramethylenedithiocarbamates of Cd(II), Pb(II), Ni(II), Co(II), Zn(II), Cu(II) and Hg(II) on LiChrosorb RP-8 using methanol-water (70:30) as the mobile phase.

In a further development to the approach adopted by Schwedt [13], Bond and Wallace [16] formed the metal-dithiocarbamate complexes in situ. This was achieved by incorporating NaDEDTC into the mobile phase, and thus permitted the direct injection of the metal ions onto the column. Although this approach was successful for some metal ions eg. Cu(II), in the case of Cr(III) and Cr(VI), complexation was found to take up to 2-3 h, rendering this procedure inappropriate for these ions. After separation, the metal complexes were determined by electrochemical detection which was found to be both selective and sensitive with a limit of detection of 1 ng of copper(II) reported. In order to determine Cr(III) and Cr(VI), the same authors developed a procedure which involved the formation of the DEDTC complexes of the metal ions externally prior to injection in to a reversed-phase HPLC system with a mobile phase that contained NaDEDTC. In this way, Cu(II), Ni(II), Co(III), Cr(III) and Cr(VI) could be simultaneously separated in a single run [17].

5.2.5. Dithizonates

Dithizone (DZ) has been used extensively as an effective analytical solvent extraction reagent in the past. Its ability to form neutral chelates with a large number of divalent metal ions, as well as high chelate molar absorptivities in the visible region of the spectrum (500-530 nm) makes it an ideal potential ligand for multi-element HPLC analysis with spectrophotometric detection.

The first successful separation of DZ chelates including those of Pb(II), Zn(II), Cd(II), Hg(II), Cu(II) and Co(II) was reported by Lohmuller et al. [18] using normal-phase HPLC on 30 um silica particles with a variety of organic solvents (including benzene, chloroform, tetrahydrofuran and toluene) as the mobile phase and visible spectrophotometric detection at 525 nm. At approximately the same time, O'Laughlin and O'Brien published their paper which included an investigation of the behaviour of Ni(II), Co(II), Cu(II), Zn(II), Hg(II) and Pb(II) dithizonates on 37 to 50 um u-Corasil and 10-um u-Porasil with a variety of mobile phases ranging in polarity from heptane to isopropanol. The best separations however, were obtained on a u-Porasil column with toluene as the mobile phase and UV detection at 275 nm. Detection limits for the separated metals ranged from 10 to 100 ng.

As an alternative to the use of organic solvents as the eluent in normal phase HPLC for the separation of divalent metal dithizonates, Henderson et al. [20] investigated a variety of binary and ternary mobile phases containing acidic or basic modifiers such as acetic acid and various amines. Detection was accomplished by visible absorption at 475-525 nm.

5.2.6. 8-Hydroxyquinolates

In a similar fashion to dithizone, 8-hydroxyquinoline possesses the ability to complex with many ions to produce neutral chelates and is therefore a potentially an ideal ligand to use for the separation of multi-element mixtures by HPLC.

Berthod et al.[21] were the first to report a HPLC separation of metal 8-hydroxyquinolates. Using reversed-phase HPLC in combination with three modes of detection; namely UV absorption, electrochemical and atomic absorption spectroscopy, they were able to separate the 8-hydroxyquinolates of Cu(II), Co(II), Ni(II), Hg(II) and Fe(II).

In 1980, Hambali and Haddad [22] described the separation of the chelates of Al(III) and Co(III) using a silica column and a mobile phase of 5% methanol-chloroform with UV detection at 254 nm. Detection limits in the nanogram range were reported for both metals.

More recently, the determination of Cu(II) and Fe(III) in some anaerobic adhesive formulations was reported by Mooney et al. [1]. After a liquid-liquid extraction of the metal ions into dilute hydrochloric acid from a typical anaerobic adhesive formulation, the extract was passed through a C_{18} Sep-Pak cartridge and the eluate mixed with 8-hydroxyquinoline to form the corresponding metal chelates of Cu(II) and Fe(III). As a futher step in the sample clean-up scheme, the complexes were extracted into dichloromethane and the organic phase passed through a silica Sep-Pak cartridge. The metal chelates were eluted with methanol, separated on a C_{18} reversed-phase column with an acetonitrile/acetate mobile phase, pH 6.0, containing 8-hydroxyquinoline and subsequently detected spectrophotometrically at 400 nm. Limits of detection of the order of

100 to 250 ppb of Cu(II) and 250 to 600 ppb Fe (III) could typically be achieved with this procedure.

In a futher application of the above method, Meaney et al. [23] separated the 8-hydroxyquinolates of Fe(III) and Al(III) after the extraction of the respective metal ions from soil and clay samples.

5.2.7. 1,10-Phenanthrolines and ethylenediamines

O'Laughlin and Hanson [24] reported the successful use of paired-ion reversed phase HPLC for the separation of 1,10-phenanthroline (phen) chelates of Fe(II), Ni(II) and Ru(II). The separations were performed on a uBondapak-CN column with methanol-(0.5%) aqueous acetic acid (20:80) as the mobile phase which contained 0.015 M methanesulphonate as the counter ion. Detection was achieved by monitoring the UV absorption signal at 265 nm. A graphite furnace atomic absorption spectrophotometer was used to confirm the elution of the phen chelates of Ni(II) and Cu(II), while in the case of the phen chelate of Ru(II), fluorescence detection was employed.
In a more recent paper, O'Laughlin [25] described the separation of the phen complexes of Ni(II), Ru(II) and Fe(II) by paired-ion HPLC on polystyrene-divinylbenzene polymer with acetonitrile-water-perchloric acid mixtures as the mobile phase, and UV spectrophotometric detection at 265 nm.

5.2.8. 1-(2-pyridylazo)-2-naphthol (PAN) and 4-(2-pyridylazo) resorcinol (PAR)

The chemical derivatisation of metal ions with PAN and

PAR has been reported to be carried out in a pre-column or post-column mode. The formation of metal-chelate complexes with these ligands prior to chromatographic separation will be discussed here, and the application of such ligands in post-column derivatisation detection systems will be discussed at a later stage in this chapter.

Schwedt and Rudde [26] investigated the separation of metal-Pan chelate complexes on a reversed-phase HPLC system. Only mixtures of complexes of Cu(II), Co(II), and Ni(II) or Cu(II), Fe(II) and Co(II) could be separated. All other metal complexes showed instability in reversed-phase systems. Optimum separation conditions were obtained on a RP-2 type column with a ternary mobile phase of acetonitrile-water-citrate buffer pH 5.0 (80:18:2) containing 0.01 M ammonium thiocyanate.

The determination of the PAR chelates of Cu(II), Co(II), Ni(II) and Fe(II) at the ppb level was reported by Roston [27]. Isocratic elution carried out with 65% 0.1 M $NH_4H_2PO_4$-$(NH_4)_2HPO_4$ buffer pH 6.5, 35% distilled methanol on a 5-um Biophase ODS column. Fixed wavelength UV absorption (254 nm) and oxidative thin-layer amperometric detection were employed for the detection of the PAR chelates. Typical detection limits, expressed as amount of metal ion injected onto the column in ng (for both UV and amperometry) were Cu (0.34, 0.74); Co (0.06, 0.02); Ni (1.2, 0.83) and Fe (1.2, 0.93) respectively.

As an alternative to the formation of the metal-chelate complex prior to reversed-phase HPLC separation, Dinunzio et al. [28] investigated on-column chelation of metal ions with PAR. The change of elution and efficiency with the use of different non-polar stationary phases with an acidic mobile phase of methanol - 0.01M bisulphate buffer, pH 2.54, (52:48 v/v) on the metal-chelates was reported. Detection limits of 20

ng Zn(II), 1 ng Fe(II), 2 ng Ni(II) and 8 ng Co(II) were obtained with visible spectrophotometric detection at 525 nm.

Another ligand similar in chemical structure to PAR, but based on the thiazolyl system, i.e. 4-(2-thiazolylazo)-resorcinol (TAR), has been reported recently which may have better properties than PAR as a pre-column derivatising agent [29]. Although the two reagents have the same chelating system with metal ions, both TAR and its chelates are insoluble in water. However they are soluble in solvents that are miscible with water and can be extracted into solvents that are immiscible with water. These properties permit the separation of most TAR metal chelates by HPLC and to be detected spectrophotometrically. Lin et al. [29] determined Fe(II), Co(II) and Ni(II) as chelates with TAR by reversed-phase chromatography on a Zorbax ODS column (6 um) with a mobile phase of methanol-water (40:60 v/v) containing 0.01M KH_2PO_4-Na_2HPO_4 buffer (pH 7.0) and spectrophotometric detection of the chelates at 580 nm.

5.2.9. Separation on dynamically coated columns

As an alternative to buying expensive polymer ion exchange columns, a reversed-phase C_{18} column can be modified with alkyl sulphonic acids to yield columns with cation exchange characteristics. Reversed-phase HPLC columns generally exhibit higher column efficiency than cation exchange resins. This increased efficiency is exhibited by sharper and narrower peaks. Cassidy and Elchuk [30] were the first to use such "dynamically coated" columns to separate the transition metals Co(II), Ni(II), Cu(II), Zn(II), Pb(II) and Mg(II). After separation, the metals were determined by post-column reaction with PAR followed by spectrophotometric detection at 570 nm. Tartaric acid was

incorporated in the mobile phase to act as a chelate for the transition metals to provide selectivity and peak sharpness between the eluting metals and reduce the elution time of the separated metals.

This approach reported by Cassidy and Elchuk was further optimised with regard to the volume of sample injected and the size and temperature of the post-column reaction coil, by Deacon et al. [2] in their determination of seven divalent metal cations in some anaerobic sealant formulations.

5.3. TRACE METAL SEPARATION BY ION CHROMATOGRAPHY

According to the literature, dating back approximately 5,000 years, Moses was one of the first to recognise the capabilities of ion exchange, when he threw a decomposed log (an ion-exchange resin), into a pool of brackish water at Marah. Apparently, the exchange of ions made the water potable,(Exodus, chapter 15, verses 24 and 25). More recently the word "chromatography" originated with the Russian botanist, Tswett, who in 1903, produced coloured bands by separating concentrated plant extract on a column of adsorbent material [31]. The application of ion-exchange chromatography to the separation of transition metals can be traced back to Samuelson in 1939 [32], who used a conventional ion exchange resin and quantified the separated metals by manual spectrophotometric analysis of the individual fractions.

It was not until 1975, however, that Small and co-workers at Dow Chemical Corporation, USA, proposed an automated multi-ion procedure which overcame the drawbacks of the background electrolyte used as the eluent and permitted conductimetric detection of the separated ionic species [33].

The cations, in this case alkali and alkaline earth metals, were separated on a low capacity cation exchange resin used in conjunction with a second "suppressor" column. The function of this "suppressor" column was to greatly reduce the background conductivity of the eluent, and thus leave only the separated cations as the remaining conducting species in the column effluent. Hydrochloric acid, which acted as the mobile phase on the first separator column,

$$HCl + Resin\text{-}OH^- \Leftrightarrow Resin\text{-}Cl^- + H_2O$$

was removed by strong basic resins in the suppressor column

$$M^+Cl^- + Resin\text{-}OH^- \Leftrightarrow Resin\text{-}Cl^- + M^+OH^-$$

and converted the separated alkali and alkaline earth metal chlorides to the more highly conducting hydroxides.

As the cation suppression column relies on the conversion of a chloride to a hydroxide, this method precludes transition metal determinations because metal hydroxides would precipitate out of solution prior to reaching the conductivity detector.

5.3.1. Dual-column procedure

To avoid the formation of metal hydroxides, Nordmeyer et al. [34] employed a suppressor column filled with an anion-exchange resin in the sulphate form. They reported the separation of Mn(II), Fe(II), Co(II), Ni(II), Cu(II), Zn(II) and Cd(II) using barium and lead salts as the mobile phase. The selectivity for the separation of the transition metals was poor and detection limits of 0.05 ug ml^{-1} were reported with the

conductimetric detection.

An alternative approach to the precipitation of metal hydroxides in the strongly basic anion exchange suppressor column could be the addition of complex-forming chelates to the eluent. An ethylenediamine tartrate solution has been used for the rapid and selective determination of Cu(II), Ni(II), Cd(II), Zn(II), Mn(II) and Pb(II) [35]. The first three cations were detected conductimetrically on the basis of positive peaks, and the others on the basis of negative peaks (the eluent in the case of the latter was more conducting than the sample cations). Detection limits were of similar order of magnitudes to those reported by Nordmeyer et al. [34].

5.3.2. Single column procedure

Ion chromatographic analysis of anions and cations can be performed without the use of a suppressor column if the ion exchange resin used in the separation column has a sufficiently low exchange capacity and if a very dilute eluent, with low conductivity is used [36].

5.3.2.1. Conductimetric detection

For the separation of alkali and alkaline earth metals, a cation exchange column of low capacity (0.017 mequiv/g) was used in conjunction with a conductivity detector. Mixtures of dilute nitric acid and an ammonium salt solution of ethylenediamine were used as mobile phases for the separation of monovalent and divalent cations respectively [36]. Because both of these eluents are more highly conducting than the sample

cations, the sample peaks are negative relative to the background. Detection limits of 0.1 ug ml^{-1} for Na^{+} and 0.5 ug ml^{-1} for Ca^{2+} were reported with this procedure.

5.3.2.2. Electrochemical detection

Takata and Fujita [37] developed a coulometric detector to monitor the rapid separation of heavy metal ions by single column cation exchange chromatography using sodium tartrate solution as eluent. The separation of a six component mixture of metal ions (Cu(II), Zn(II), Ni(II), Pb(II), Co(II) and Cd(II)) was completed within 2 min under optimum conditions. Girard [38] modified the detection system by using primary and secondary-controlled potential coulometry. After the separation of Zn(II), Cu(II), Ni(II) and Co(II) on a Aminex A4 column with sodium tartrate/tartaric acid as the eluent, a dilute solution of copper diethylenetriaminepenta acetate was injected into the eluent and the cations were detected by coulometry of the copper displaced from the complex.

Hojabri et al. [39] reported on the indirect amperometric detection of Cd(II), Pb(II) and Ni(II) in sea-water at concentrations of up to 0.5 ug ml^{-1} and Cu(II) and Fe(II) up to 2.0 ug ml^{-1}. The electrochemical detection system was based on a decrease in the oxidation current of pyrrolidine-thiocarbamate when it formed a stable complex with the eluting sample cation. Separation of the transition metal ions was obtained on a Dionex CS-2 column, with a mixture of tartaric acid and citric acid (pH 4.3) as the eluent.

5.3.2.3. Spectrophotometric detection in conjunction with post-column derivatisition

The single-column procedure for the determination of transition meatls is most often used in conjunction with a spectrophotometric detector. After separation, the cations of the transition metals are complexed with organic chelates in a post-column reaction to form highly coloured compounds which are detected in the visible spectral region.

Erichrome Black T (EBT) was used as the post-column chelating agent by Jones et al. [40]. The spectrophotometric detector was set at the $_{max}$ of EBT, i.e. 610 nm and a decrease in absorbance was monitored as the metal-EBT complex passed through the detector. Mn(II), Fe(II), Co(II), Ni(II) and Zn(II) were detected in such a manner after separation on a Aminex A9 column with a tartrate eluent.

Fe(II) and Al(III) in drinking water and food products were determined on a Dionex CS-2 column with a mobile phase of sulphosalicyclic acid and ethylenediamine (pH 5.0) [41]. After reaction with a post-column mixture of Chromazaurol S, cetyltrimethylammonium and Triton X 100 (pH 5.9), the ions were detected at a wavelength of 610 nm. Although good separation was obtained between Fe(II) and Al(III), the retention times of Fe(III) and Al(III) were the same with the separation procedure used.

5.3.2.4. Spectrophotometric detection in conjunction with post-column derivatisation with PAR

Chemical derivatisation of trace metals can be carried

out prior to chromatographic separation, or on-column (whereby the ligand is added to the mobile phase) or via a post-column reaction prior to spectrophotometric detection. The main advantages of post-column reactions over the former modes of derivatisation are:

- the metals are separated in their original form, which often permits the adoption of published materials

- artefact formation plays a minor role, which is in distinct contrast with derivatisation prior to separation

- the reaction does not need to be complete and the reaction products need not be stable; the only requirement is reproducibility

- the technique well suited to automation, in contrast to pre-column derivatisation techniques which are normally carried out off-line to the chromatographic process.

There are however, some disadvantages to post-column reaction detection; namely: (1) the requirement of an additional pump; (2) band broadening in the post-column reaction coil prior to detection; and finally (3) the optimum eluent for the separation is often not the optimun reaction medium for the chelating ligand.

The success of PAR as a post-column chelating reagent in comparison to other ligands rests with its superior chemical/chelating characteristics. PAR was first prepared in 1915, by Chichabin, by coupling resorcinol with sodium 2-pyridyldiazote [42]. More recently the dye is synthesised as the sodium salt as it is more soluble in water than the free dye and therefore broadens its potential applications in the analytical field [43]. As the sodium salt, it is soluble in acid

and alkaline solutions and to a lesser extent in alcohol. It is insoluble in ether.

It was not until the early sixties that two research groups lead by Sommer [44] and Pollard [45] investigated the spectrophometric and metal-chelating properties of this dye. In aqueous media, depending on pH, PAR has four chromophoric forms, i.e. H_3R^+ (at pH < 2.5), H_2R (at pH 3 to 5.5), H_1R^- (at pH 6 to 12.5), and R^{2-} (at pH > 12.5). The azo dye reacts with metals to give red or red-violet complexes. PAR does not react with alkali metals, chromium (VI), antimony(III), molybdenum (VI), tungsten(VI) and arsenic(III) or (V). From the investigative results from both groups, Pollard et al. [45] concluded that PAR chelates with metals through the nitrogen atom, the azo-nitrogen atom furthest from the heterocyclic ring, and the o-hydroxyl group. PAR thus acts as a tridentate ligand forming two stable 5-membered chelate rings, and the commonest chelates are of the type M(PAR) and $M(PAR)_2$.

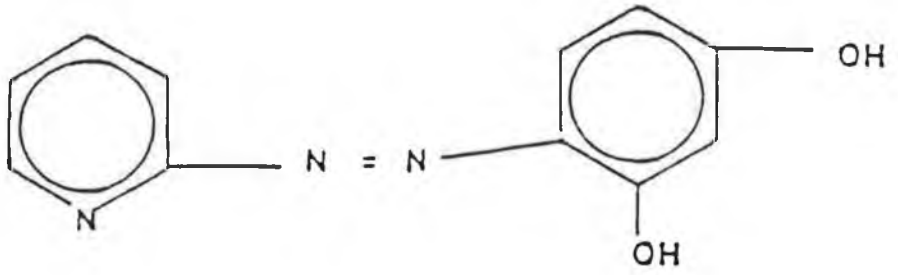

Figure 5.1. Chemical structure of 4-(2-pyridylazo)resorcinol (PAR).

Among the characteristics that make PAR suitable as a post-column detection reagent is its ability to form water soluble complexes with a larger number of metal ions than any other commonly available indicator. These complexes have large molar absorptivities (approximately 10^4) at about 420 nm to 502 nm [45], depending on pH, which makes it possible to determine them at very low concentrations. The chelation of the metals by PAR is rapid and the stability constants are large [45].

Elchuk and Cassidy were one of the first to report the use of PAR as a post-column chelating reagent [46] after separation of the lanthanides on either high-efficiency bonded phases or conventional ion-exchange resins. Most of the metal ions could be determined with spectrophotometric detection at 540 nm in the ng ml^{-1} and pg ml^{-1} ranges.

A later paper reported on the application of the ligand to the detection of transition metals after gradient separation of the cations on a 10 um Nucleosil SA column with a lactic acid eluent [47]. The absorbance of the post-column derivatised metal chelates was monitored at 498 nm, and detection limits of the order of 0.1 ug ml^{-1} could be obtained with good precision (i.e. RSD < 2%).

5.4. INVESTIGATION OF A CATION EXCHANGE SEPARATION METHOD FOR THE DETERMINATION OF TRANSITION METAL IONS IN ANAEROBIC SEALANTS

The aim of this project was to develop a separation method for the simultaneous determination of eight metal ions, namely Fe(II), Fe(III), Cu(II), Co(II), Mn(II), Ni(II), Zn(II) and Cr(III), in typical anaerobic sealant formulations.

Based on the literature, it was decided to investigate the use of a low capacity ion-exchange column operating via a "push-pull" mechanism, where the "pushing effect" is provided by the ethylenediammonium (EDA) cation, and the "pulling effect" by a weakly complexing organic acid anion [47]. This system was expected to offer not only the capability of multi-element separation, but the ability to speciate between Fe(II) and Fe(III), and the flexibility to be coupled to the detection system involving post-column derivatisation with PAR.

5.4.1. Experimental

5.4.1.1. Materials

All chemicals used were of analytical reagent grade. Raw materials used in adhesive formulations were supplied by Loctite (Ireland) Ltd. Deionised water, which was obtained by

passing distilled water through a Milli-Q water purification system, was used in the preparation of all aqueous solutions.

Solutions of Cu(II), Co(II), Ni(II), Cr(III) and Zn(II) were prepared from chloride salts, while solutions of Mn(II), Fe(II) and Fe(III) were prepared from sulphate, ammonium sulphate and nitrate salts respectively. Both Fe(II) and Fe(III) stock solutions were stable for a period of a week if made up in sulphuric acid (10% w/v).

The colour-forming post-column reagent used for the detection of the metal ions was the monosodium salt of 4-(2-pyridylazo)-resorcinol (PAR) which was supplied by Sigma Ltd. Silica and C_{18} sample preparation cartridges (Sep-Pak) were obtained from Waters Associates. The Interaction^R Ion-210 metals column, supplied by Technicol Ltd., was a 0.32 cm x 10 cm i.d. stainless steel column containing Macronex^R MC-210 cation-exchange polymer with a capacity of approximately 500 ueqv/g. The pulse dampener consisted of a stainless steel column containing polystyrene beads and was supplied by Pye-Unicam Ltd. An Interaction^R Ion-Guard GC-200 was employed to protect the analytical column.

5.4.1.2. Apparatus

Chromatography was performed using a Waters Associates M-45 pump connected to 20 ul Waters U6K injection port. The post-column reagent was delivered by a Waters Associates 6000 dual piston pump to the mixing T piece, situated between the end of the column and the reaction coil, at the same flow rate as was used for the elution of the metal ions from the ion-exchange column. Detection was achieved using an Applied Chromatography Systems (ACS) Ltd. fixed wavelength (520 nm) detector in

conjunction with a Philips 8251 single pen recorder. All pH measurements were made on a Corning 240 pH meter.

5.4.2. Methods

5.4.2.1. Extraction Procedure (1)

Extractions were performed by dissolving 5 ml of plasticiser (polyethylene glycol di(2-ethylhexanoate)), which had been spiked at the 10 ppm level with Fe(II), Cu(II), Ni(II), Mn(II), Co(II) and Zn(II), in 5 ml of chloroform and extracting the metal ions with an equal volume of 0.1M hydrochloric acid. The acid extract was passed through a C_{18} Sep Pak column prior to being injected on to the ion-exchange separation system.

5.4.2.2. Extraction Procedure (2)

As in the case of extraction procedure (1), 5 ml of plasticiser containing spiked metal ions was dissolved in chloroform. This was then passed through a silica Sep Pak column. The column was washed with 20 ml of chloroform and the retained metal ions were eluted with 2 ml of 3.9 mM EDA and 17 mM citric acid, i.e. the mobile phase employed in the separation of the transition metal ions on the ion exchange column.

5.5. RESULTS AND DISCUSSION

5.5.1. Optimisation of post-column derivatisation reaction

Based on the work of Wang et al. [47], it had been suggested that the optimum post-column reagent concentration was $2x10^{-4}M$ PAR dissolved in 2 M ammonia hydroxide and 1 M ammonium acetate, the pH being approximately 11.

The success of a post-column reaction detection system depends on minimising loss of resolution due to dead volume effects, rate of reaction between reagent and metal ions, background absorbance and noise and efficiency of the mixing chamber. A T-type mixing chamber with a dead volume of about 1 ul was selected in preference to the Y-type chosen by Wang et al., as the former mixing chamber showed better mixing efficiency which produced an increase in detector response to the chelated metal ions [47].

The length of the reaction coil from the mixing T-piece to the detector determines the reaction time of the PAR reagent with the metal ion. Although it would be expected that the longer the reaction time the better the colour development, the length of the post-column reaction tubing was fixed at approximately 80 cm, as this tubing length produced no back pressure in the post-column reactant delivery pump, and hence minimised detection noise. The post-column tubing was loosely coiled as it effected a relative decrease in band broadening due to secondary flow phenomena [48].

In this study, the PAR concentration was varied between $1x10^{-4}M$ to $8x10^{-4}M$, keeping the pH constant at approximately 10.2, and the optimum concentration was found to

be $3x10^{-4}$M. Although an improved detector response (at 520 nM) was obtained at higher concentrations of PAR, the baseline noise increased significantly at concentrations greater than $3x10^{-4}$M PAR. As the PAR concentration increased, so did the difference in absorbance between the post-column reagent and the column eluent. Short term fluctuations in the flow of both streams caused short term changes in absorbance, which in turn resulted in increased detector noise.

Using a PAR concentration of $3x10^{-4}$ M, the pH of the reagent was varied from pH 8.01 to pH 10.90 by adjusting the volume of concentrated ammonium hydroxide in 1 M ammonium acetate. The actual pH at which the metal ions were complexed with the post-column reagent was determined by monitoring the pH of the eluent passing through the HPLC detector. The optimum pH was found to be 10.14 for the reagent being introduced into the system. The pH of the eluent resulting from this was found to be 10.02.

PAR is a tridentate ligand usually forming chelates with a ligand to metal ion ratio of 1:1 in acid solution and 1:2 in alkaline solution [44]. In the visible region the 1:2 complexes in water exhibit a characteristic single absorbance maximum, (at approximately 495-510 nm), which has been confirmed in this study by obtaining UV-Visible spectra after separation by ion chromatography and derivatisation with PAR on all the eluting metal-PAR complexes, with the exception of Fe(III) and Cr(III). Although these results are in agreement with Hnilickova and Sommer [44], they are at variance with a recent report which suggests that the ligand to metal ion ratio for the Cu(II) is 1:1 in alkaline solutions [49].

5.5.2. Optimisation of mobile phase composition

In the optimisation of the separation of the transition metal ions on the low capacity ion-exchange column, it was decided to adopt the following procedures. The choice of the ethylenediammonium (EDA) cation was based on the work of Sevenich and Fritz [50], and this compound was used throughout this study to provide the mass action "pushing" effect in the separation process, i.e. the EDA cation competes with the metal ions for sites on the cation exchange column. A variety of organic acids were then investigated for their "pulling" effect. These included citric acid, tartaric acid, lactic acid, oxalic acid, malonic acid, sulphosalicyclic acid, fumaric acid and malic acid. The organic acids contribute to the "pulling effect," by interacting with the metal ions to form weak anionic complexes in the eluent with a subsequent reduction in the concentration of metal ions competing for the ion exchange sites. This type of interaction not only decreases the effective charge of the metal cations and hence their retention time, but also increases the selectivity of the separation process. For closely eluting metal ions bearing a charge of similar magnitude, i.e. Cu(II), Zn(II), Ni(II), Mn(II), Fe(II) and Co(II), the distribution coefficients without the use of complexing acid differs only slightly. If a weakly complexing organic acid is present in the eluent, the variation in retention times between the metal ions can be attributed to the large differences in the values of the equilibrium constants between the metal-organic acid complexes formed [51].

In each case the concentration of the EDA cation was first kept at 3.5 mM and the concentration of the organic acid varied between 5 and 20 mM. When an optimum separation was reached in such a system, the concentration of the organic acid was kept constant and the concentration of the EDA cation varied

between 2 and 4.5 mM.

The organic acid which gave rise to the best chromatographic separation was found to be citric acid, a result which is in agreement with the finding of the manufacturers of the Interaction^{R} column used in this study [52]. This was at some variance to the work of Sevenich and Fritz [50] who advocated the use of tartaric acid. It was found, however, that the use of tartaric acid as the complexing agent resulted in the co-elution of Ni(II) and Zn(II), and that Co(II) and Fe(II) were not adequately resolved. Increasing the tartaric acid concentration, while keeping the EDA concentration constant at 3.5 mM, improved the resolution between Co(II) and Fe(III). However, the separation of Ni(II) from Zn(II) never improved beyond forming a shoulder peak to the latter eluting cation.

Neither lactic acid or oxalic acid resulted in a good separation of the metal ions, and the adsorption of the metal ions onto the ion-exchange column became a significant problem when these complexing agents were employed in the mobile phase. Varying the ratio of the organic acid to EDA and reducing the concentration of the injected metal ion standards did not improve matters.

Although good sensitivity and resolution was obtained between Fe(III) and Cu(II) when malonic acid was used as the complexing agent, the remaining metal ions were not separated and eluted as one broad peak. Sulphosalicylic acid and fumaric acid exhibited no selectivity between the transition metal ions. However, malic acid (the cis-isomeric form of fumaric acid) showed reasonable selectivity between the metal ions and permitted the separation of five of the seven metal ions injected into the ion- exchange separation system. As in the case of tartaric acid, Ni(II) co- eluted with Zn(II), but Fe(II) and Mn(II) were not separated and eluted as one peak.

The optimum concentration of citric acid was found to be 17 mM citric acid (in the presence of 3.9 mM EDA). During this study, the effect of pH was monitored by measuring the pH of the mobile phase. The optimum pH was found to be approximately 3.0, i.e. just below the first pKa value of citric acid (pK_{a1} = 3.13; pK_{a2} = 4.76; pK_{a3} = 6.40 at 25°C). The reason for this is that one only wants partial dissociation of the organic acid in order for it to perform the "pulling effect". At higher pH, the citric acid complexed more strongly with the transition metal ions and the retention times decreased with the consequent reduction in the resolution between the eluting ions. The best separation obtained for Cu(II), Ni(II), Zn(II), Co(II), Fe(II) and Mn(II) can be seen under these conditions in Figure 5.2.

The preceeding divalent metal ions belong to the Irving-Williams series: Mn, Fe, Co, Ni, Cu and Zn [53]. In this series the ionic radii decreases and the ionisation potential increases to copper. Thus from Mn to Cu the charge density increases progressively with a parallel increase in the complexation ability. This should be reflected in the elution order of the metal cations after separation, whereby Cu(II) with the largest complexation abilty should elute prior to Ni(II), Co(II), Fe(II), and Mn(II), in that order. Table 5.1 contains the stability constants for the metal-citrate complexes investigated [53]. As can be seen in Figure 5.2, the metal cations have eluted according to the predictions of the Irving-Williams series and with the exception of Fe(II) and Mn(II), the elution order has been determined by the decrease in the stabilty of the complexes formed.

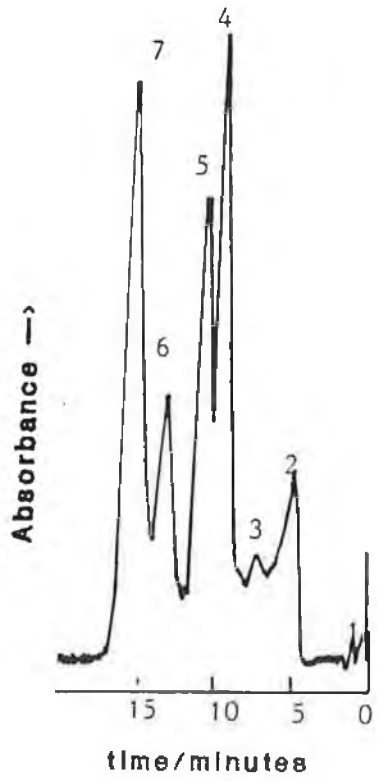

Figure 5.2 Separation of (1) 110 ppm Fe(III); (2) 10 ppm Cu(II); (3) 10 ppm Ni(II); (4) 10 ppm Zn(II); (5) 10 ppm Co(II); (6) 10 ppm Fe(II); and (7) 10 ppm Mn(II) using a mobile phase of 3.9 mmol dm^{-3} EDA and 17 mmol dm^{-3} citric acid.

TABLE 5.1

Stability constants for the metal-citrate complexes.

Metal	Log K_1	Log K_2	Log K_3
Fe(III)	12.2	10.9	25.0
Cu(II)	12.0	6.1	18.0
Ni(II)	9.0	4.8	14.3
Zn(II)	8.7	4.5	11.4
Co(II)	—	—	—
Fe(II)	7.3	3.1	15.5
Mn(II)	8.0	3.4	—

Cr(III) was not detected, because it does not form a complex with PAR readily under ambient temperature. In addition, highly alkaline media the extinction coefficient of the complex is not only very low but there is a strong competing reaction to form the hydroxide of the Cr(III) ion [54, 55]. The optimised isocratic separation system was not suitable for the speciation of Fe(II) and Fe(III). The elution of Fe(III) within one minute of the injection suggests that a gradient separation system would be more appropriate for the separation of Fe(III) from the other transition metal ions. The poor detector response for Fe(III), which could only be detected at the 110 ppm level, was in part due to the presence of this metal ion in the deionised water employed in this study.

Linear calibration curves were obtained for Zn(II), Co(II), Fe(II), and Mn(II) over the concentration range of 1.0 to 10 ppm and

for Cu(II) from 2 ppm to 9 ppm. The limit of detection based on the peak height being greater than 3 times the baseline noise was approximately 0.5 ppm for Zn(II), Co(II), Fe(II) and Mn(II) and 1 ppm for Cu (II).

5.5.3. Development of extraction procedures

A typical plasticiser used in an anaerobic sealant formulation manufactured by Loctite (Ireland) Ltd. is polyethylene glycol di(2-ethylhexanoate). Studies were therefore directed to the efficient extraction of trace metal ions from this matrix. Extractions performed using extraction scheme (1) gave rise to recoveries from 88.4 to 99.8% for all the metal ions, except for Fe(II) where only 35.3% recovery was obtained. The loss of Fe(II) in the procedure was thought to be due to the partial retention of the metal ion in the chloroform.

Using extraction scheme (2), similar extraction recoveries were obtained for the metal ions, although there was a complete loss of Fe(II). Further investigation of the procedure showed that Fe(II) was retained in both the chloroform layer and on the silica Sep Pak column. More work is therefore required to find an alternative organic solvent to dissolve the plasticiser, and investigate the retention of Fe(II) on the silica Sep Pak column.

However, with regard to the other transition metal ions, the second extraction method was an improvement on the first procedure, as it permitted a preconcentration of the metal ions present in the plasticiser. This extraction and preconcentration procedure was only limited by the availabilty of a large volume of sample and the capability of the silica Sep Pak column to preconcentrate trace metal ions from in large volumes of plasticiser.

5.6. CONCLUSION

This preliminary investigation has shown the possible application of ion-exchange chromatography with detection by post-column derivatisation for the determination of transition metal ions in anaerobic sealants. Further investigation would be required on the optimisation of the extraction of the trace metal ions from plasticisers and other more important constituents in anaerobic adhesives such as the monomers polyethylene glycol dimethacrylate (PEGMA) and triethylene glycol dimethacrylate (TRI-EGMA).

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CHAPTER SIX

A POLAROGRAPHIC STUDY OF REACTIONS OF ELEMENTAL COPPER AND IRON IN THE PRESENCE OF 1-ACETYL-2-PHENYLHYDRAZINE AND 1-ACETYL-2-PHENYLDIAZENE BASED CURE SYSTEMS

6.1. INTRODUCTION

6.1.1. Anaerobic sealant cure mechanisms

Until recently, there have been few reports in the literature which have attempted to postulate the cure mechanism of anaerobic adhesives. One of the first published studies was that of methyl methacrylate (MMA) polymerisation using various amine salts of benzoic sulphimide (BS) [1]. The highest polymer conversion was obtained from the N,N-dimethyl-p-toluidine (DMPT) salts of BS. The rate of the polymerisation was reported to be dependent on the square root of the amine salt concentration, and the activation energy was determined to be 79 KJ/mol [1]. On the basis of these results, the authors speculated that the polymerisation for this cure system proceeded by a radical polymerisation mechanism.

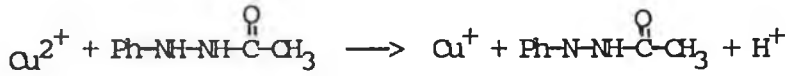
Okamoto of Loctite Corporation [2] extended the above study by investigating the addition of a peroxide, namely cumene hydroperoxide (CHP), to this curing system. He found that although polymerisation took place without CHP, the polymerisation was approximately 20-50% faster in the presence of CHP and that the rate of polymerisation was independent of the CHP concentration. Although Okamoto was uncertain as to the exact role that CHP played in the polymerisation process, he was in agreement with the previous study [1] that polymerisation proceeded by a redox radical polymerisation based on both the low apparent activation energy, 43 KJ/mole, and on a study of the curing system's structure-curing rate relationship.

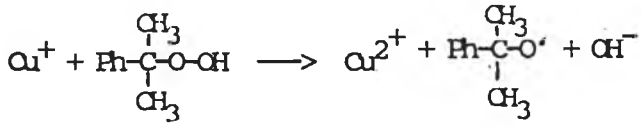
In order to broaden his studies on anaerobic adhesive curing systems, Okamoto [3] examined the polymerisation mechanism of the acetylphenylhydrazine-BS-CHP (APH-BS-CHP)

curing system, including Cu(II), with MMA. A slightly lower activation energy with this curing system than that obtained with the DMT-BS-CHP system was attributed by Okamoto to the presence of Cu(II) as catalyst. The low activation energy indicated that polymerisation proceeded most likely by a redox radical polymerisation.

The role that each organic cure component played in the polymerisation process was investigated by a series of kinetic studies on MMA polymerisation using the APH-BS-CHP cure system. A simple APH-BS-CHP system produced a slow cure and a long induction period, or time lag between mixing of the cure components and initiation of polymerisation, of over 5 hr. However, the addition of Cu(II) reduced this induction period to 10-15 min. An examination of the effect of BS on the cure system indicated that it behaved as a catalyst to shorten the induction period from 30-40 min to 10 min. Although an interaction between CHP and APH was detected, the nature of the reaction was uncertain. Finally, a kinetic study monitoring the effect of MMA concentration on the rate of polymerisation suggested that the monomer does not participate in the initiation step of redox polymerisation.

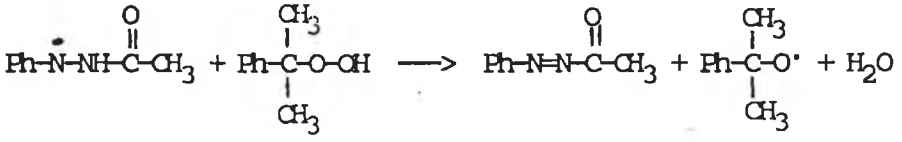
From these studies, Okamoto postulated the following initiation mechanism to explain APH-BS-CHP metal-catalysed redox radical polymerisation:

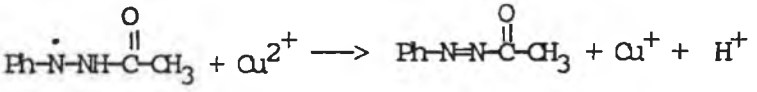




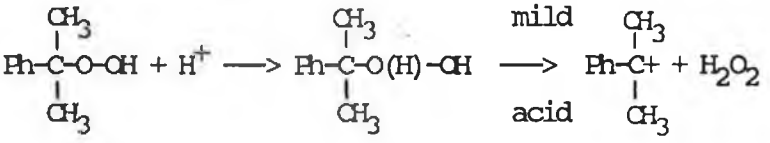
In the above mechanistic sequence, the postulated cumyloxy radical which is generated initiates the MMA polymerisation, APH and CHP behave as a reducing and oxidising agent respectively, and copper acts as the catalyst.

Further studies at Loctite (Irl.) Ltd. have indicated that additional reactions between APH, CHP and copper in the initiation step of a redox polymerisation may take the following form [4]:





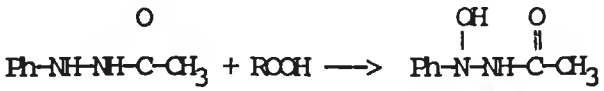
The behaviour of BS as a mild catalyst may be explained in the following reaction:



1

An acid may attack the oxygen of CHP to form the intermediate 1. Under mildly acidic conditions, this equilibrium reaction may be shifted towards the formation of hydrogen peroxide [3].

The long induction period encountered with the APH-BS-CHP systems (when copper is not present in the cure formulation) could be due to the formation of compound 2 by the following reaction:



2

Compound 2, which has a hydroxyamine structure, could, if in contact with a radical source generate, a nitroxide radical, a paramagnetic species of exceptional inherent stability, and therefore behave as a good radical inhibitor [3].

In order to complement and extend the investigation into the metal-catalysed APH-BS-CHP cure system, attention has recently focused on the reaction routes adopted by APH in the presence of maleic acid and the aforementioned organic cure components [4]. Gas chromatography-mass spectrometry (GC-MS) and reversed-phase high performance liquid chromatography (RP-HPLC) (using a C_{18} column and a mobile phase of 40% THF. and 60% water with UV detection at 254 nm) were employed to monitor the reaction routes and to identify the products originating from APH in these reactions.

The findings from these studies supported the postulated mechanism of Okamoto [3] and Leonard [4] for the reaction of APH in the presence of a peroxide, acid component and active metal. The acetylphenyldiazene (APD) which forms from the reaction between the APH radical and the peroxide was found to undergo further metal catalysed homolytic decomposition.

One of the striking features of this study was the different reaction rates and routes catalysed by copper in comparison to those catalysed by iron. Another important observation was the difference between acid components, BS and maleic acid in their contribution to overall reaction rates and pathways.

In order to confirm these results, and as a continuation of this study, it was decided to concentrate on the behaviour of the metal in the presence of the various combinations of the organic cure components and to pay particular attention to the valence state of copper and iron in the reactions.

6.1.2. Polarographic determination of Fe(II) and Fe(III)

Polarography was selected as the analytical technique of choice to monitor the production of the metal species in the presence of a variety of organic cure components. Among the reasons for this selection was the ability of polarography to speciate between the different valence states of the metals of interest in the presence of a complex matrix, good sensitivity and very importantly, the ease in setting up the instrumentation required, and the rapidity in carrying out the determination of the metal species after sampling the reaction system. Although there are numerous polarographic procedures for the determination of Fe(II), Fe(III) and total iron, there are few papers that simultaneously speciate the valence states of the iron in the one experiment.

Initial investigations on the electrochemical behaviour of Fe(II) date back to 1931, when Prajzler [5] found that the half-wave potential of the ferrous ion is 1.3 V vs. SCE in barium chloride. In 1 M ammonium perchlorate as supporting electrolyte, where complexation of the ferrous ion is minimal, Kern et al. [6] reported that the half-wave potential is even more negative at 1.46 V vs. SCE

The hydrogen ion discharge easily masks the ferrous ion wave, since the Fe(II) wave is virtually coincident with that of

the hydrogen ion. Therefore the supporting electrolyte pH must be above 5 and below approximately 7, at which point precipitation of ferrous hydroxide occurs. The pH of the electrolyte cannot be regulated with buffers, because the acid constituents of buffers can show a hydrogen wave.

Lingane [7] studied systematically the polarography of Fe(III) and Fe(II) ion in tartrate, citrate and oxalate media. The reduction of Fe(III) complexes to Fe(II) complexes in these electrolytes produces well formed waves at pH values smaller than about 6. In all three media, the subsequent reduction of Fe(II) complexes to the metal is masked by the hydrogen ion or alkali metal ion discharge, with the exception of strongly alkaline citrate and ammonical tartrate supporting electrolytes where the reduction wave with a half-wave potential of about 1.53 V vs. SCE is clearly seen.

The Fe(III)-Fe(II) complex couples behave reversibly at pH values less than about 7 in all three media, i.e. the half-wave potentials of the anodic waves of the ferrous complexes are identical with the cathodic half-wave potentials of the ferric complexes. With the exception of oxalate media, the Fe(III)-Fe(II) complexes behave irreversibly in alkaline tartrate and citrate electrolytes; the anodic ferrous waves being at a much more positive potential than the cathodic ferric waves. At pH values greater than 7.9 in oxalate media, the hydrous ferric oxide precipitates.

The optimum pH for the determination of Fe(III) in 0.5 M sodium citrate is about 10 with a half-wave potential of 0.85 V vs SCE, whereas a pH of 6 to 7 is more appropriate for the determination of Fe(II) complexes [7]. In tartrate media, pH 6.0 containing 0.005% gelatin, the half-wave potential of the cathodic ferric to ferrous wave occurs at -0.19 V vs. SCE and the ferrous to metal wave at -1.52 V vs. SCE. A significant

shift in the half-wave potential of the Fe(III) to Fe(II) couple, to -1.21 V vs. SCE is observed when the pH of the tartrate media is adjusted to 9.0 and the half wave potential of the second reduction process is seen at -1.73 V vs. SCE. Lingane felt that the considerably more positive half wave potentials observed in the oxalate medium for the reversible ferric-ferrous complex couples as compared with citrate and tartrate media was due to the oxalate complex of the ferric ion being weaker or that the oxalate complex of ferrous ion is stronger, or both, than the corresponding citrate and tartrate complexes [7].

Voriskova [8] reported a well-defined reduction wave for the ferrous ammonia complex, with a half-wave potential of -1.48 V vs. SCE in a supporting electrolyte containing 1 M ammonia-ammonium chloride with 0.005% gelatin.

Iron yields two waves in a supporting electrolyte of 3 M potassium hydroxide that contains 3% mannitol, with which Fe(II) and Fe(III) ions form soluble complexes. The first wave, with a half-wave potential of -1.12 V vs. SCE corresponds to the reduction of the ferri-mannitol complex to the ferrous state, and the second wave, with a half wave potential of about -1.74 V vs. SCE is due to the reduction of the ferro-mannitol complex to the metal [9].

Contradictory results were obtained by different research groups when the polarography of iron in fluoride media was investigated. Von Stackelberg and von Freyhold [10] reported that the ferric iron produces a reduction wave in potassium fluoride with a half wave potential of about 1.36 V vs. SCE. According to Heyrovsky [11], the Fe(II)-Fe(III) couple does not behave reversibly in fluoride media, the anodic wave corresponding to the oxidation of the ferrous ion to the ferric-flouride complex (which was not observed by von Stackelberg and von Freyhold) is at a considerably more positive

potential than the reduction wave of the ferric-fluoride complex. However, West and Dean [12], using a 1 M sodium fluoride supporting electrolyte of pH about 5.8 containing 0.004% gelatin, claimed that they did not observe the reduction wave for the ferric-fluoride complex in this media as reported by von Stackelberg and von Freyhold and by Heyrovsky. The reason behind this contradictory result is not immediately evident from the literature, but gelatin may have obliterated the wave, or the pH of the supporting electrolytes (which was not reported by the latter two research groups but was probabily acidic) was significantly different from that used by West and Dean.

Fe(III)-Fe(II) mixtures have been determined in oxalate media by direct current polarography while total iron in the same electrolyte was determined by alternating current polarography [13]. Detection limits of approximately 100 uM were reported with this procedure. As the redox process is reversible in this media, the half-wave potentials for the reduction of Fe(III) and the oxidation of Fe(II) are the same. Consequently this procedure could not be adapted to differential-pulse polarography as it is not possible to differentiate between the two oxidation states of iron with this polarographic mode.

In pyrophosphate media at a pH 8, the Fe(III)-Fe(II) redox couple is not reversible. According to Parry and Anderson [14], this characteristic is one of the most important requirements in determining polarographically a redox couple in solution. Among other factors that they listed as important in selecting a suitable electrolyte included: (1) all waves observed in the electrolyte should be cathodic of mercury oxidation; (2) the waves should be sufficiently separated to allow for individual measurement; (3) the waves should be well-defined and it would be desirable that the limiting currents be proportional to the respective concentrations; and (4) there should be no interaction of a given ion on the other

waves. If there is such interaction, it should be small and easily correctable. Parry and Anderson, using normal-pulse polarography, reported the half-wave potentials of Fe(II) and Fe(III) at -0.38 V vs. SCE and -1.03 V vs. SCE respectively in pyrophosphate media (pH 8).

Kennedy [15] investigated the possibilty of determining Fe(III)-Fe(II) mixtures by differential-pulse polarography using a supporting electrolyte based on ammonium tartrate buffered by a suitable buffer e.g. 2-(N-morpholino)ethanesulphonic acid (MES), 3-(N-morpholino)-1-propanesulphonic acid (MOPS), or N - (2-hydroxyethyl) piperazine - N'- (2-ethanesulphonic acid) (HEPES). He reported that at low pH a high proportion of Fe(III) was reduced to Fe(II), while at high pH, trace amounts of oxidants converted the Fe(II) into Fe(III). However, by buffering the electrolyte with such a buffer at a pH of 7.4, reasonably stable, well separated peaks corresponding to the Fe(II) oxidation and Fe(III) reduction were produced.

6.1.3. Polarographic determination of Cu(I) and Cu(II)

Due to the instability of the lower valence state of copper, it is difficult to determine Cu(I) polarographically. There are a limited number of supporting electrolytes which form more stable complexes with Cu(I) than with Cu(II), thereby stabilising Cu(I) sufficiently to permit the stepwise reduction of Cu(II) complexes. Thanheiser and Maasen [16] were the first to report that in the presence of electrolytes containing high concentrations of ammonia or chloride ions, a polarogram of consisting of a double wave, whose two parts were of equal height, was obtained. This corresponded to the anodic wave resulting from the oxidation of the cuprous complex to the cupric state and a cathodic wave resulting from the reduction of

the cuprous complex to the metal. The stepwise reduction of Cu(II) also occurs in thiocyanate or pyridine supporting electrolytes. Lingane and Kerlinger [17] reported that in 0.1 M potassium thiocyanate the half-wave potentals were -0.02 V and -0.39 V, and in 0.1 M pyridine-0.1 M pyridinium chloride +0.05 V and -0.25 V vs. SCE. Because the first wave starts so close to the oxidation of mercury, a blank experiment with the supporting electrolyte is normally required to determine the conversion of Cu(II) to Cu(I). Such background correction is not necessary with a supporting electrolyte of 1 M ammonia-ammonium chloride, as the half-wave potentials for the two processes are -0.22 V and -0.50 V vs. SCE respectively [16].

Meites investigated thoroughly the influence of pH, the effect of total tartrate concentration, and the effect of gelatin on the polarographic characteristics of copper in tartrate media [18]. At pH values less than 6, only a single wave was observed whose half-wave potential varies linearly with pH. From a pH of about 6.0 to 13.5, the wave divides into two parts accompanied by a significant negative shift of the half-wave potentials. According to Meites, this doublet arises from the reduction of separate tartrate complexes, the identity of which was not confirmed. Meites also found that gelatin causes distortion of the waves when present in concentrations greater than 0.001%.

In addition to his investigations with tartrate media, Meites studied the polarography of copper in citrate, oxalate and carbonate supporting electrolytes. The polarographic behaviour of copper in citrate is similar to that in tartrate media, with a comparable significant effect of pH on the half-wave potential being observed [19]. In 0.5 M potassium citrate, the half-wave potential shifts from -0.02 V at pH = 3.0 to -0.22 V vs. S.C.E. at pH = 7.5. Between pH 7.5 and 8.5, an abrupt shift of half-wave potential to -0.38 V vs. SCE was seen

which remains constant up to a pH of about 12.0, where it then increased up to -0.50 V vs. SCE at pH = 14.0. Meites found that, in his studies with copper in 0.1 M potassium oxalate, the half-wave potential shifts from -0.03 V vs. SCE at pH = 1.0 to -0.20 V at pH = 5.7, and remains constant at the latter value up to a pH of about 10 [20].

Although the polarography of pyrophosphate complexes of copper had been briefly examined in the past, Laitinen and Onstott [21] were the first to carry out a more systematic investigation. Two waves were observed in solutions containing excess pyrophosphate in the pH range from 5.0 to 12.5, which were attributed by Laitinen and Onstott to the reduction to the metal in two different Cu(II) complex species. Only a single wave was observed in pyrophosphate solutions having a pH smaller than about 5.0 and greater than about 12.5. With 0.1 M sodium pyrophosphate and 0.2 M sodium acetate, the half wave potential of the single wave was -0.085 V vs. SCE at pH = 4.5, and the slope of the log plot of the wave corresponded to a reversible 2-electron reduction [21]. In 0.05 M pyrophosphate (pH = 12.5) the half-wave potential was -0.3 V.

A single well-formed wave corresponding to the direct reduction of the Cu(II) complex to the metal was reported by West et al. [22] in a supporting electrolyte of 0.5 M sodium fluoride containing 0.01% gelatin. According to these authors, the half-wave potential at -0.003 V vs. SCE indicated only a slight complexation with the fluoride ion.

Laitinen et al. [23] then reported a single reversible wave due to the direct reduction of the complexes of copper with ethylenediamine, propylenediamine, diethylenetriamine and glycine to the metal [23]. The half-wave potentials vs. SCE obtained in the following supporting electrolytes containing 0.1 M potassium nitrate, 0.01% gelatin and 1 M amine were

respectively: ethylendiamine, -0.57 V; propylenediamine, -0.58 V; diethylenetriamine, -0.60 V. In a supporting electrolyte composed of 0.05 M potassium dihydrogen phosphate and 1 M glycine pH 6.3, the half wave potential was -0.24 V vs. SCE. In the authors opinion, two molecules of the amine or of glycine are coordinated to the Cu(II) ion when a large excess of the complexing agent was present.

At a pH of about 7.0, and from 9.0 to 11.3, a single reversible process with a half-wave potential of -0.41 V and -0.47 V vs. SCE respectively was observed in a supporting electrolyte of 0.25 M EDTA [24].

6.2. EXPERIMENTAL

6.2.1. Reagents

All chemicals used were of analytical reagent grade. Both the raw materials used in adhesive sealant formulations and the iron and copper filings were supplied by Loctite (Ireland) Ltd. Deionised water, which was obtained by passing distilled water through a Milli-Q water purification system, was used in the preparation of all aqueous solutions.

Solutions of Cu(I) and Cu(II) were made up from chloride salts, while solutions of Fe(II) and Fe(III) were prepared from ammonium sulphate and sulphate salts respectively.

During the polarographic study, the following supporting electrolytes were used: (1) 1 M ammonia - 0.1 M ammonium chloride in the determination of Cu(I), (2) 2 M ammonium acetate, pH 3.0 in the determination of Cu(II), and (3)

0.1 M sodium pyrophosphate, pH 8.0, in the determination of Fe(II) and Fe(III).

6.2.2. Apparatus

Polarographic measurements were obtained using a Princeton Applied Corporation (PARC) Model 264A polarographic analyser in conjunction with a PARC Model 303A static mercury drop electrode (SMDE) and a Houston Omniscribe chart recorder. A small mercury drop size was used routinely throughout this investigation with a drop time of 0.5s, pulse height of 50 mV and an equilibrium time of 15 s prior to commencing the polarographic scan.

6.2.3. Procedures

The reaction mixtures which contained the catalyst 1-acetyl-2-phenylhydrazine (APH) or acetylphenyldiazene (APD) in the presence of iron or copper filings and other constituents found in anaerobic sealant formulations were prepared and labelled in the following manner.

All reactions took place in covered 100 ml acid-washed conical flasks. In a step by step approach which initially starts with a reaction mixture of metal filings in a solvent containing the dissolved catalyst, further reaction mixtures were prepared which contain various combinations of organic cure components at concentrations at which they are normally present in anaerobic adhesives.

Reaction vessel
Code

A1	1% APH	+	2% iron filings (w/v in methanol).
A2[1]	As A1	+	2% saccharin.
A3	As A1	+	1% maleic acid.
A4[1]	As A1	+	1% cumene hydroperoxide.
A5	As A2	+	1% cumene hydroperoxide.
A6	As A3	+	1% cumene hydroperoxide.
A7	As A6	+	2% saccharin.

The above experimental sequence was repeated and APD was used rather than APH. Finally, this entire procedure was followed again and copper filings were substituted for iron filings.

[1] In the case of the reaction mixtures prepared for the monitoring of the production of Cu(II) in the presence of APH and copper filings:

A2	As A1	+	1% cumene hydroperoxide.
A4	As A1	+	2% saccharin.

The concentrations of Fe(II) and Fe(III) produced in the presence of iron filings were monitored with time by sampled direct current (DC) polarography. This determination was carried out by initially recording a sampled DC polarogram of the supporting electrolyte (5 mL of 0.1 M sodium pyrophosphate, pH 8.0) following deaeration for about 6 minutes in a stream of oxygen-free nitrogen. Depending on the concentration of Fe(II)/Fe(III) generated, an aliquot of 50 uL or 10 uL of the reaction mixture was added; after deaeration and stirring for one minute and an equilibrium step of 15 seconds, a sampled DC polarogram of the sample was recorded. By means of a standard

addition procedure, the concentrations of Fe(II) and Fe(III) were quantified by 3 successive aliquots each of 25 uL and 20 uL of 1000 ug ml^{-1} Fe(II) and Fe(III) respectively, and a sampled DC polarogram obtained after each addition.

Differential pulse polarography (DPP) was used to monitor the Cu(I) and Cu(II) concentrations produced with time in the reaction mixtures containing copper filings. Supporting electrolytes of 1 M ammonia-ammonium chloride and 2 M ammonium acetate, pH 3.0, were used in the DPP determinations of Cu(I) and Cu(II) respectively. Similar to the procedure outlined above for the Fe(II)/Fe(III), the concentrations of Cu(I) and Cu(II) were quantified by a standard addition method; the only variation was that three successive 50 uL aliquots of 50 ug ml^{-1} of standard Cu(I) or Cu(II) were added to the cell.

6.3. RESULTS AND DISCUSSION

6.3.1. Polarographic determination of Fe(II)/Fe(III) and Cu(I)/Cu(II) in APH- and APD- based catalytic systems

The polarographic procedure for the determination of Fe(II) and Fe(III) in reactions containing iron filings and various combinations of organic cure components of anaerobic adhesives was based on the work of Parry and Anderson [14], except that sampled direct current polarography was used rather than normal pulse polarography for detection purposes. This mode of current sampling was selected in preference to that of normal or differential pulse polarography because the polarographic traces of Fe(II) and Fe(III) obtained in the presence of the adhesive cure components gave two well defined waves that could

be easily measured. Good stability of the Fe(II) and Fe(III) responses were detected, and contrary to the report of Parry and Anderson [14], no significant reduction of Fe(III) to Fe(II) in the pyrophosphate supporting electrolyte was detected. This stability was attributed in part to using a less sensitive polarographic detection mode and a shorter analysis time to that used by Parry and Anderson.

A recently published procedure which reported the simultaneous determination of Fe(II) and Fe(III) by DPP was also investigated [15]. As an alternative to the use of pyrophosphate, Kennedy [15] examined the possibility of employing a supporting electrolyte based on ammonium tartrate. Ammonium tartrate was selected for the following reasons: (1) it forms soluble chelates with both Fe(II) and Fe(III); (2) it is frequently used in the polarographic determination of Fe(III) in solution at pH 9.0; and (3) its pKa value lies well below the electrolyte of pH 7.4 used in the determination of Fe(II) and Fe(III), ensuring that the tartrate ion is fully ionised.

However, when the aforementioned procedure was repeated in this study, it was found that not only was Fe(III) not fully resolved from the background current using either DPP or sampled DC polarography, but the peak current for Fe(II) also decreased with time, suggesting an interconversion of Fe(II) to Fe(III) in this supporting electrolyte. Therefore, for the remainder of this study, Fe(II) and Fe(III) were simultaneously determined using sampled DC polarography with 0.1 M sodium pyrophosphate as the electrolyte.

An investigation on the effect of the organic cure components (used in the series of experimental catalytic reactions) on the polarographic determination of Fe(II) and Fe(III) was initially made. No competing electrochemical process was observed with APH, BS and maleic acid in the pyrophosphate

supporting medium in the potential range of -0.1 to -1.3 V. However, both CHP and APD interfered in the polarographic determination of the iron species. The potential range, size, and duration of the interfering processes depended upon the combination of cure components present with the peroxide and the diazene in the reaction system.

In the case of an iron catalysed reaction system consisting only of either APH or APD with CHP, no iron of either valence state was detected due to an excessively high polarographic background produced by the peroxide in the supporting electrolyte. As CHP reacts poorly with APH or APD in such catalytic systems, little decomposition of the peroxide occurred during the experimental period (i.e. 70 h) with the result that the interfering process did not decrease with time. Although interference from the peroxide was found in APH based solutions containing CHP/maleic acid and CHP/BS/maleic acid, sufficient CHP had reacted within an hour of starting the reactions to decrease the electrolyte background polarographic response, thus permitting the estimation of both iron species in the respective reaction systems.

APD, in the pyrophosphate supporting electrolyte, yields two reduction waves, with a half wave potential of -0.17 and -0.31 V vs Ag/AgCl respectively. As the Fe(II) reduction wave has a half-wave potential of -0.37, the Fe(II) ion cannot be determined in the presence of a significant concentration of unreacted APD in a reaction solution. Therefore, the time period at which the Fe(II) could be detected varied from 6 to 44 hours, depending on the reaction system. The exception to this was the experimental solutions of APD/maleic acid and APD/maleic acid/CHP where detection of the Fe(II) ion was near instantaneous. Maleic acid, which was the stronger of the two acids investigated, could catalyse, when present as a single acidic component in a reaction solution, the lethargic homolytic

decomposition of APD in iron-based reaction systems.

Both Cu(I) and Cu(II) were determined by DPP using ammonia-ammonium chloride and ammonium acetate (pH 3.0) as supporting electrolytes respectively [16,25,26]. In the presence of weak chelating agents such as acetate, Cu(II) forms more stable complexes than the Cu(I) ion, and the polarogram consists of a single peak corresponding to the direct reduction of Cu(II) to the metal. On the other hand, supporting electrolytes containing high concentrations of ammonia or chloride, stabilise the Cu(I) ion sufficiently so that the step-wise reduction to copper(0) occurs. The DC polarogram obtained in such electrolytes consists of a double wave, where the first wave corresponds to the reduction of Cu(II) to Cu(I) and the second wave to Cu(I) to Cu(0). As the separation of the two half-wave potentials of the two waves is about 0.27 V [16], the two distinct electrochemical processes are not resolved in DPP and only one broad peak is obtained. In order to estimate the concentration of Cu(I) generated in the reaction mixtures of copper filings and organic cure constituents, a difference calculation was made whereby the Cu(II) concentration is subtracted from the Cu(I) and (II) concentration obtained by DPP in the ammonia-ammonium chloride supporting electrolyte.

The polarographic determinations of Cu(I) and Cu(II) produced in the series of experimental reactions containing organic cure components was not subject to the degree of matrix interference encountered in the Fe(II) and Fe(III) polarographic assay. A large broad peak due to the peroxide was detected initially with the APD or APH/CHP reaction solution, in the ammonia-ammmonium chloride and ammonium acetate (pH 3.0) supporting electrolytes, but after a reaction time of approximately 5 h, this interfering process decreased to a minimum permitting the detection of the copper species in the reaction solution. Although APD produces a potentially large

broad peak in the same potential range as Cu(I) in the ammonia-ammonium chloride supporting electrolyte, this catalyst was quickly converted to its oxidised (non interfering form) in the highly reactive copper catalysed APD system, and thus the interfering process disappeared within 10 minutes from the start of the reaction.

6.3.2. Behaviour of the metal catalyst in APH-based reactions

Significantly higher concentrations of Cu(II) are generated with APH/maleic acid compared to APH/BS when in contact with elemental copper as is shown in Figure 6.1. Similar behaviour is observed in iron catalysed systems where APH/maleic acid produces a higher level of Fe(III) with time than systems based on APH/BS only (Figure 6.2). However this situation is reversed in the case of the lower valence state of the metals, where relatively insignificant levels of Cu(I) are detected with APH/maleic acid, in contrast to APH/BS where significant levels of Cu(I) are produced (Figure 6.3). This trend is also reflected in Figure 6.4, where APH/BS generates higher levels of Fe(II) with time than APH/maleic acid.

The addition of CHP to the APH/acid solution confirmed the differences between copper and iron reaction rates and pathways observed in a previous study [4]. In the copper catalysed system, the concentration of Cu(II) declined in the presence of the organic peroxide, while the level of Cu(I) rose slightly relative to APH/acid systems. The reduction of detectable levels of Cu(II) supports the hypothesis that copper becomes an intrinsic part of the catalytic cycle, whereby, as Cu(II) is produced, it is immediately reduced by the hydrazine to Cu(I), which in turn catalyses the homolysis of the peroxide.

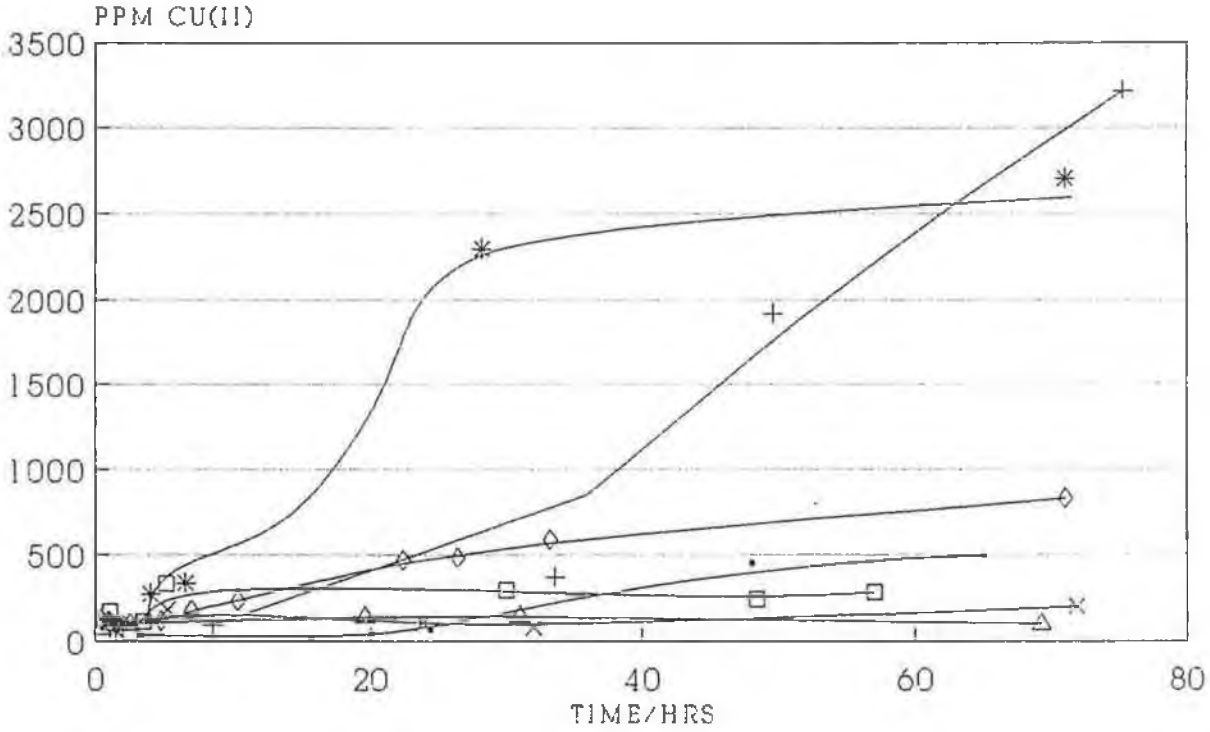


Figure 6.1. A plot of Cu(II) produced with time in the following copper catalysed reaction systems: —●— A1, (APH); —+— A2, (APH, CHP); —✳— A3, (APH, maleic acid); —□— A4, (APH, BS); —×— A5, (APH, BS, CHP); —◇— A6, (APH, CHP, maleic acid); —△— A7, (APH, CHP, BS, maleic acid).

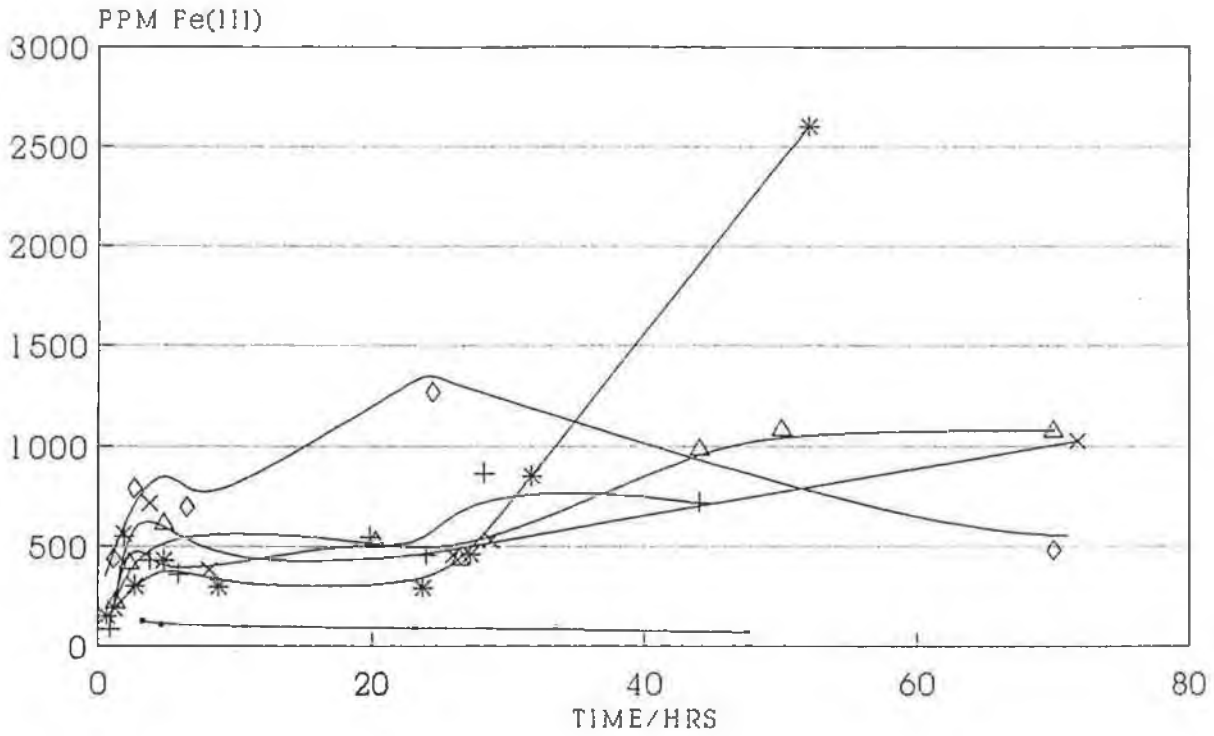


Figure 6.2. A plot of Fe(III) produced with time in the following iron catalysed reaction systems: —●— B1, (APH); —+— B2, (APH, BS); —✳— B3, (APH, maleic acid); —×— B5, (APH, BS, CHP); —◇— B6, (APH, CHP, maleic acid); —△— B7, (APH, CHP, BS, maleic acid).

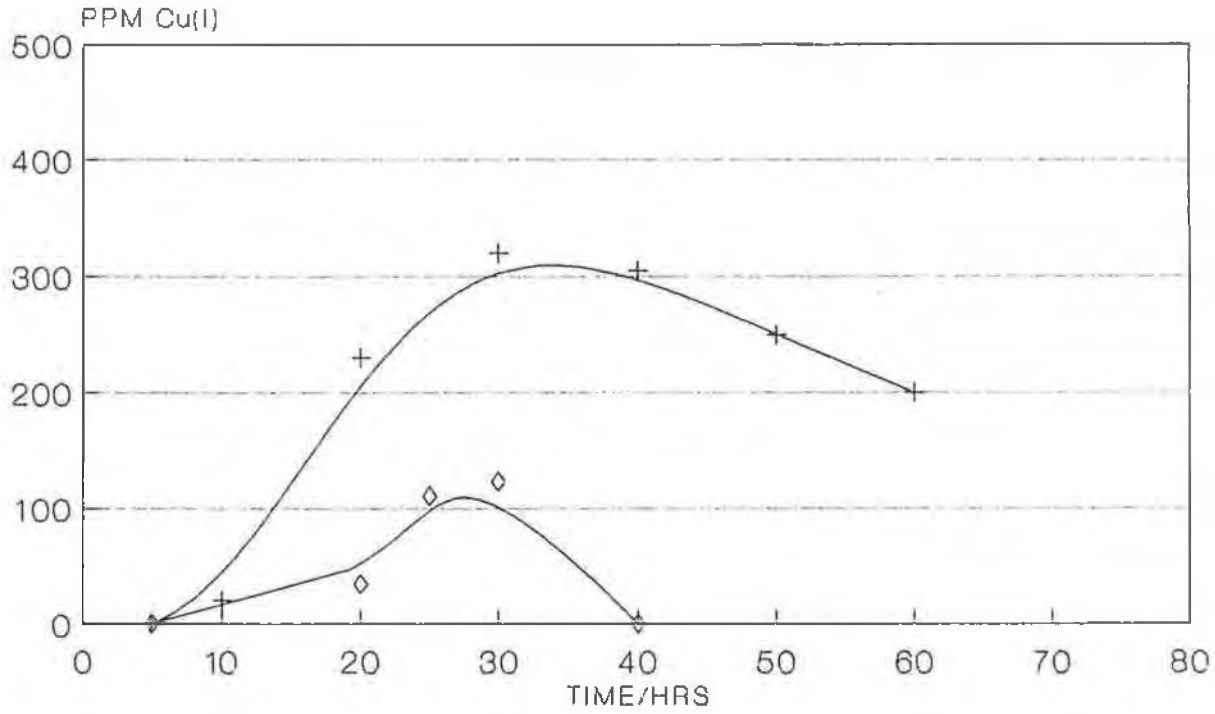


Figure 6.3. A plot of Cu(I) produced with time in the following copper catalysed reaction systems: —+— C2, (APH, BS); —◇— C6, (APH, CHP, maleic acid).

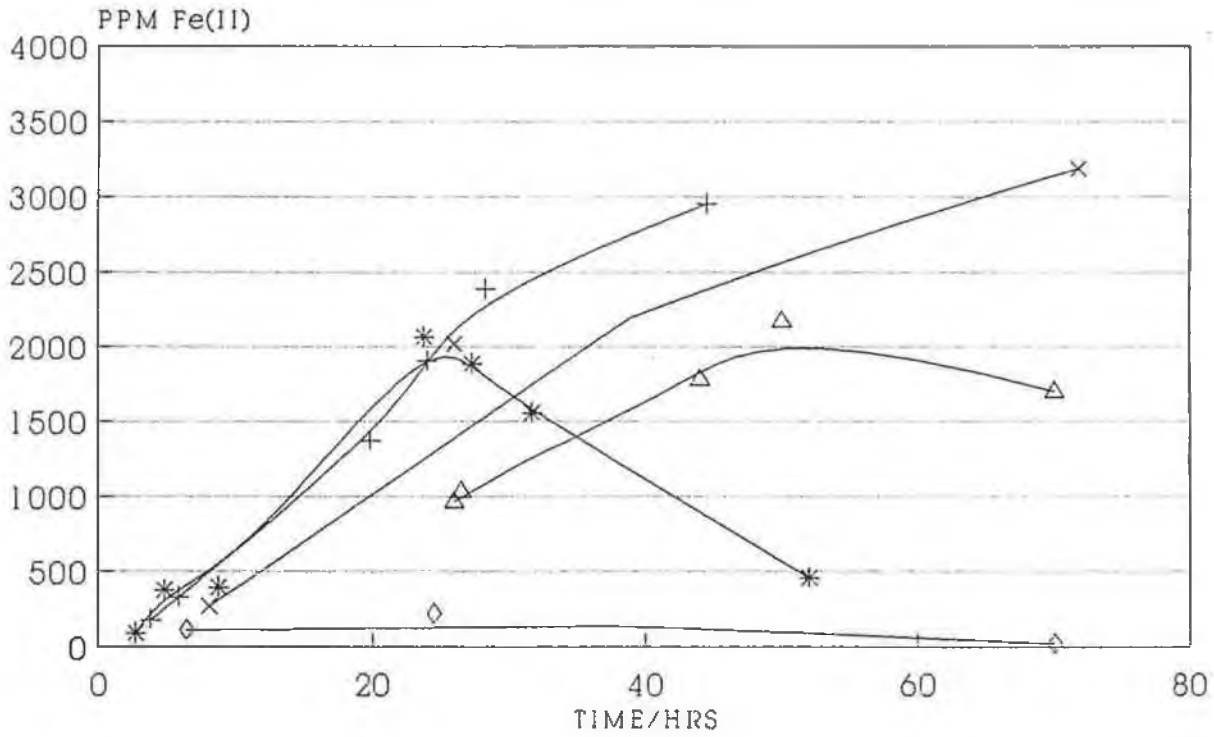


Figure 6.4. A plot of Fe(II) produced with time in the following iron catalysed reaction systems: —+— B2, (APH, BS); —*— B3, (APH, maleic acid); —x— B5, (APH, BS, CHP); —◇— B6, (APH, CHP, maleic acid); —△— B7, (APH, CHP, BS, maleic acid).

However, the slight increase of Cu(I) concentration detected in the presence of CHP suggests that the peroxide increases the reactivity of a system which is already highly reactive.

These findings are in contrast with the results of the iron catalysed system, where both the concentration of Fe(II) and Fe(III) declined in the presence of CHP. The relatively higher level of Fe(II) present in the APH/BS system suggests a more rapid formation of the cumyloxy radical with this system as compared to the APH/maleic acid system. This observation has already been noted in previous studies [4]. According to Leonard [4] a possible explanation for this is that maleic acid, being the stronger acid, has a greater tendency to protonate APH, and therefore effectively diminish the reducing power of the hydrazine. BS, on the other hand has a lesser tendency to produce the protonated hydrazine and to interfere with its reducing ability.

This hypothesis would suggest that the addition of maleic acid to the iron catalysed APH/CHP/BS system should reduce its reactivity and consequently increase its inherent stability. Closer examination of Figure 6.4 seems to support this argument where a significant decrease in the production of Fe(II) is observed when maleic acid is present in the solution compared to a system of APH/CHP/BS only.

However, in the case of highly reactive copper catalysed systems, a large increase in the production of the lower valence state of the metal is seen when maleic acid is added to the cocktail of APH/CHP/BS in comparison to all other combinations of the cure components (Figure 6.5). Although higher levels of Cu(I) are observed in the APH/CHP/BS compared to APH/CHP/maleic acid systems, which is in the agreement with the results of the iron catalysed reactions, the combination of BS and maleic acid in the curative mixture containing copper

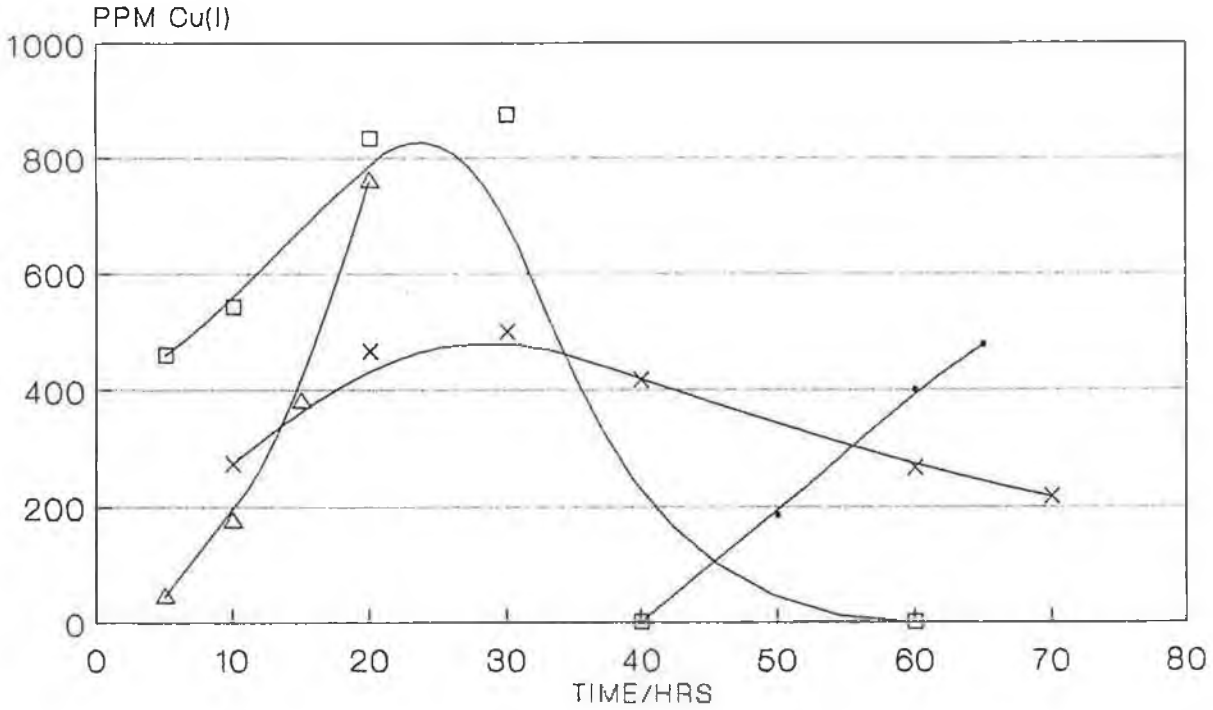


Figure 6.5. A plot of Cu(I) produced with time in the following copper catalysed reaction systems: —●— C1, (APH); —□— C4, (APH, CHP); —×— C5, (APH, BS, CHP); —△— C7, (APH, CHP, BS, maleic acid).

appears to result in a significant increase in the system's reactivity at the cost of its overall stability, which is contrary to what was expected.

These provisional findings suggest that anaerobic sealant cure formulations should take into account the type of metal expected to catalyse the redox radical polymerisation; one formulation that possesses good reactivity and stability properties when catalysed by a particular metal may not be appropriate when it is catalysed by another metal.

6.3.3 Behaviour of the metal catalyst in APD-based reactions

As can be seen from Figures 6.6 and 6.7, no significant differences in the levels of Cu(II) produced in APD/maleic acid and APD/BS systems were detected. However, some variation was observed with the lower valence state of the metal, where slightly more Cu(I) was present in APD/BS systems compared to APD/maleic acid systems (Figures 6.8 and 6.9). When CHP was added to the respective systems, there was an expected decrease in the levels of Cu(I) found (Figures 6.8 and 6.9), as Cu(I) was used up as soon as it is produced in the catalytic degradation of the peroxide.

In the case of iron catalysed systems, significantly higher levels of Fe(III) were generated in the APD/maleic acid solution in comparison to the APD/BS system (Figure 6.10). However, relatively little difference between the Fe(II) concentration levels were found with these solutions (Figure 6.11). The addition of CHP to the APD/BS system unexpectedly increased the relative levels of Fe(II) and Fe(III) produced (Figures 6.10 and 6.11). This suggested that iron is a

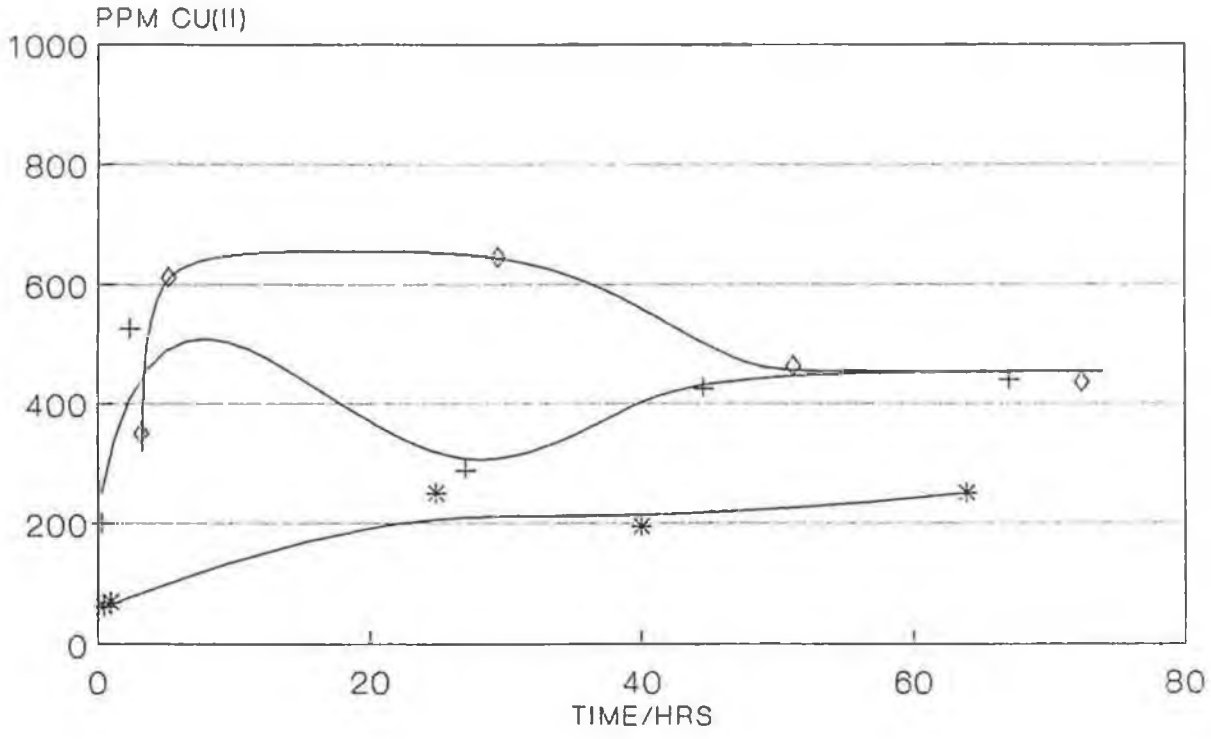


Figure 6.6. A plot of Cu(II) produced with time in the following copper catalysed reaction systems: —+— D2, (APD, BS); —*— D3, (APD, maleic acid); —◇— D6, (APD, maleic acid, CHP).

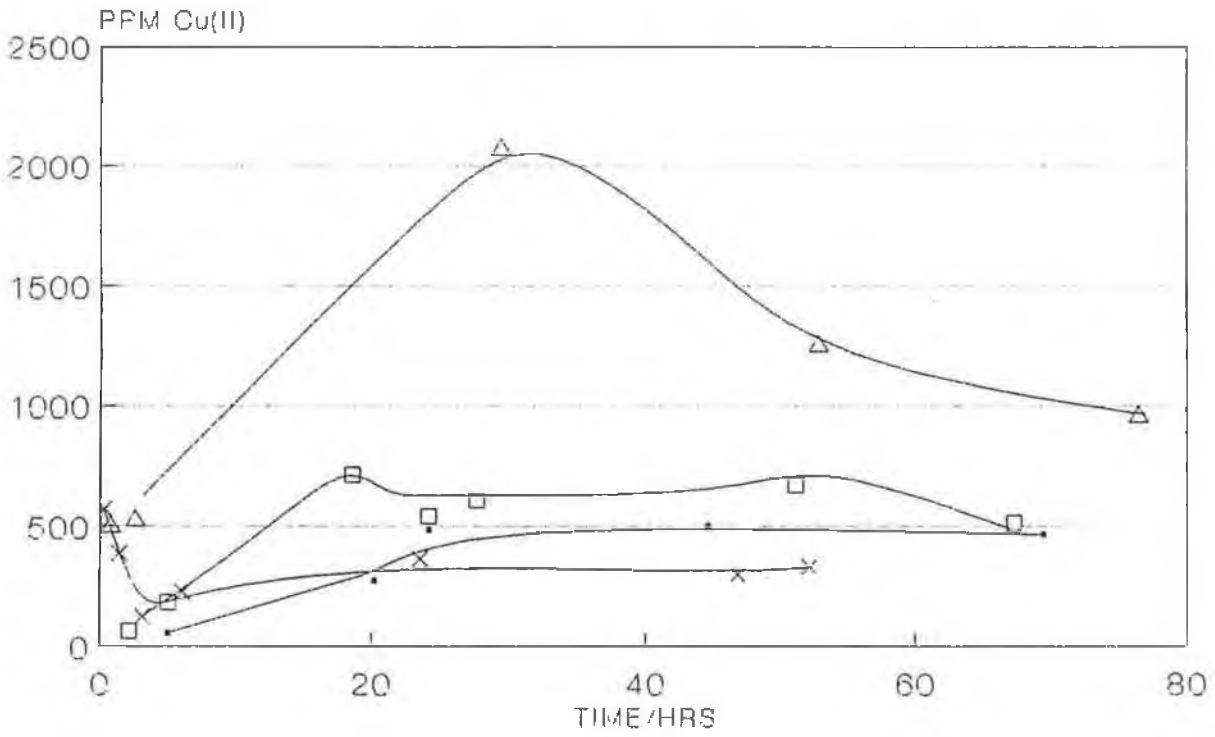


Figure 6.7. A plot of Cu(II) produced with time in the following copper catalysed reaction systems: —•— D1, (APD); —□— D4, (APD, CHP); —×— D5, (APD, BS, CHP); —△— D7, (APD, CHP, BS, maleic acid).

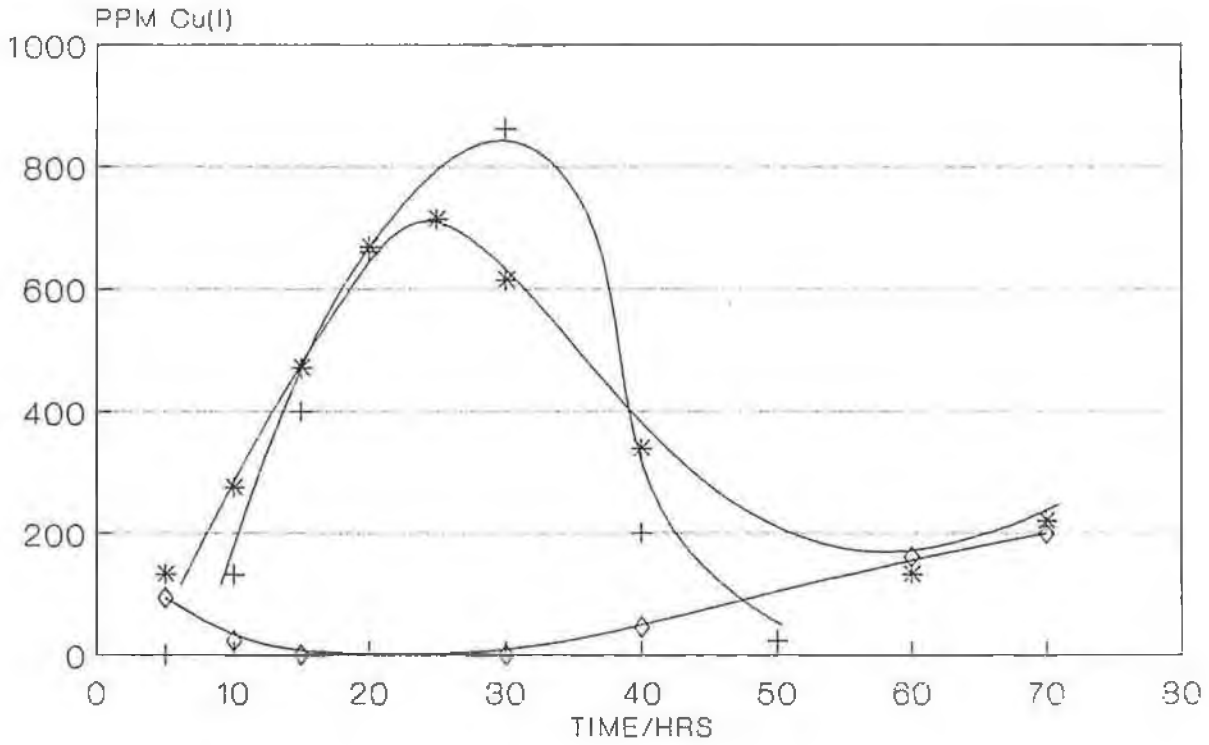


Figure 6.8. A plot of Cu(I) produced with time in the following copper catalysed reaction systems: —+— E2, (APD, BS); —✳— E3, (APD, maleic acid); —◇— E6, (APD, maleic acid, CHP).

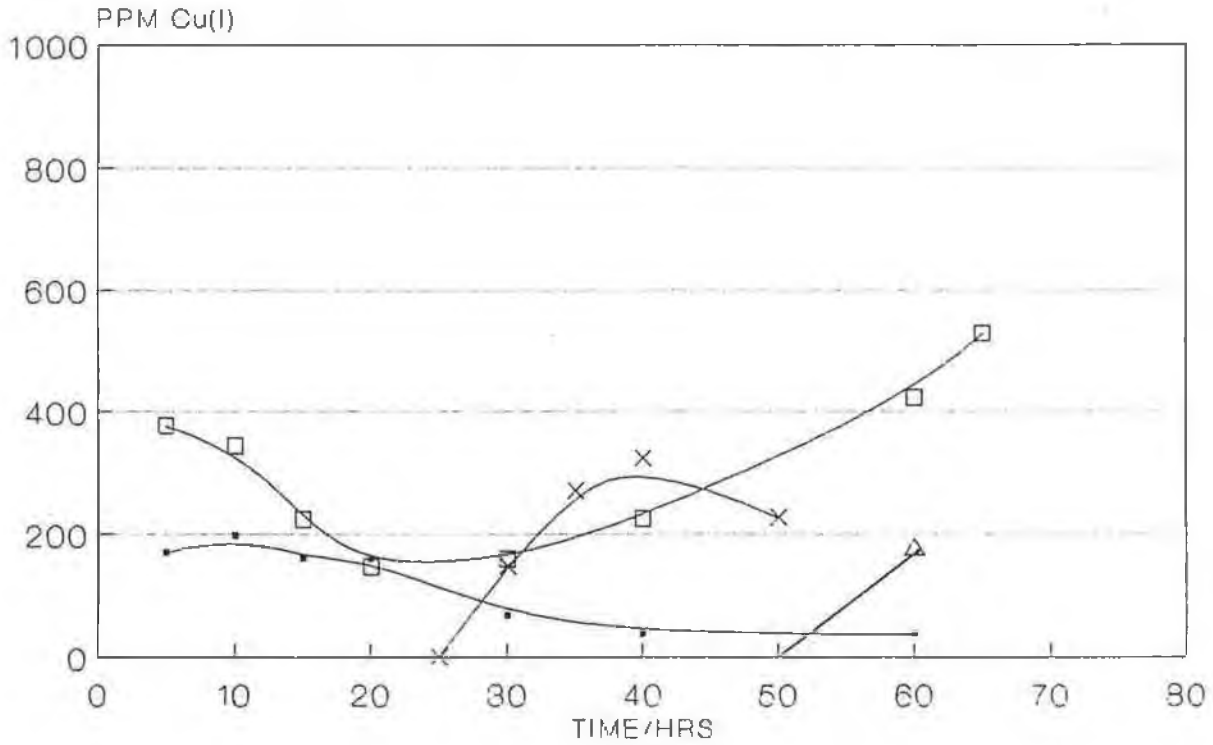


Figure 6.9. A plot of Cu(I) produced with time in the following copper catalysed reaction systems: —•— E1, (APD); —□— E4, (APD, CHP); —×— E5, (APD, BS, CHP); —△— E7, (APD, CHP, BS, maleic acid).

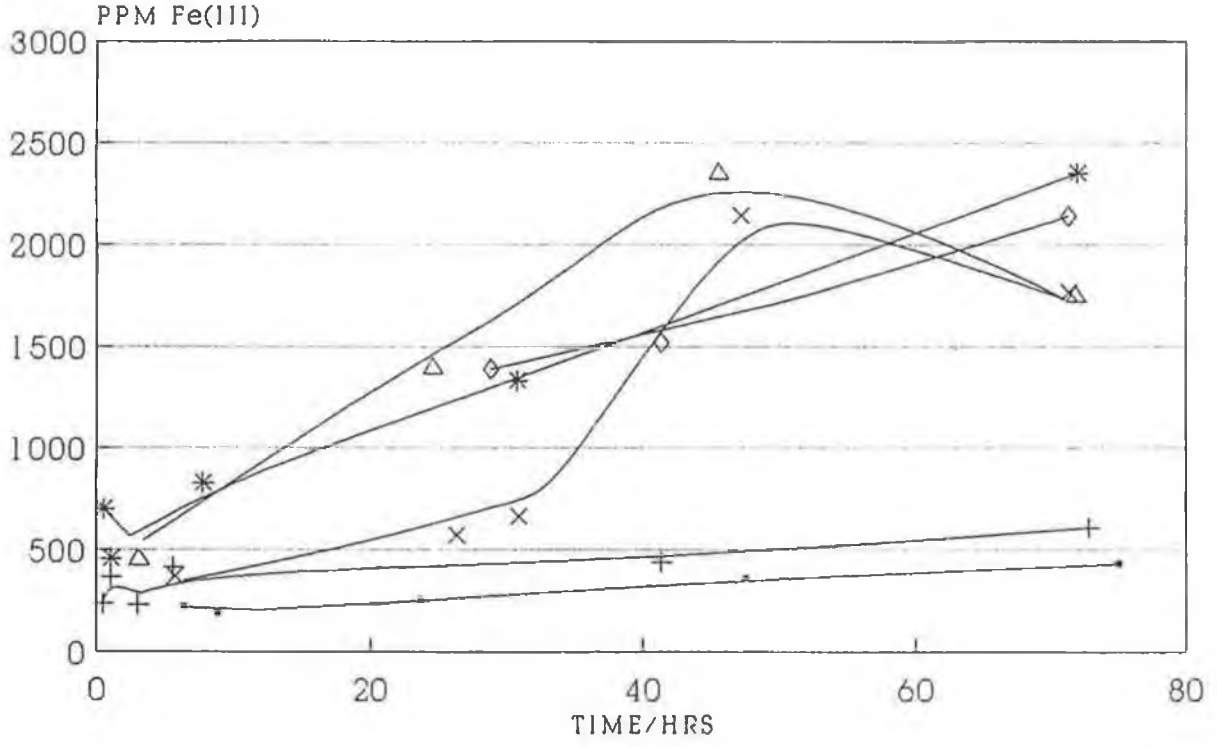


Figure 6.10. A plot of Fe(III) produced with time in the following iron catalysed reaction systems: —●— F1, (APD); —+— F2, (APD, BS); —✱— F3, (APD, maleic acid); —✕— F5, (APD, BS, CHP); —◇— F6, (APD, CHP, maleic acid); —△— F7, (APD, CHP, BS, maleic acid).

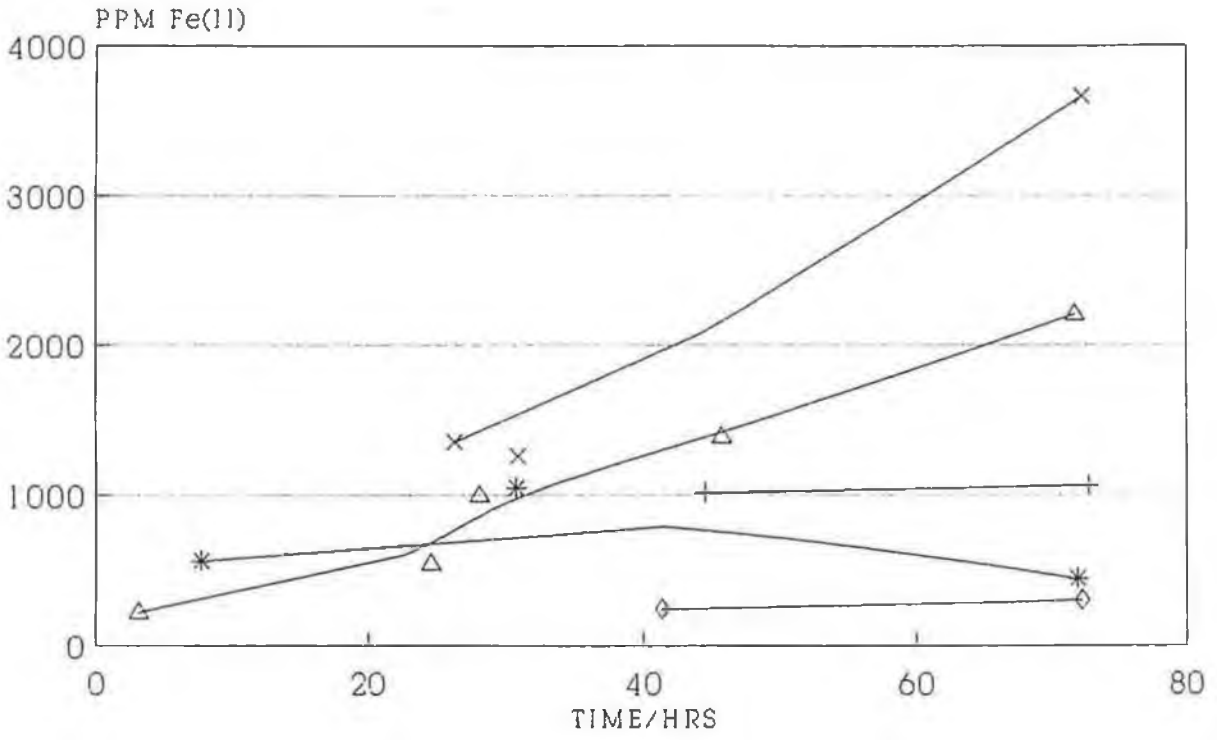


Figure 6.11. A plot of Fe(II) produced with time in the following iron catalysed reaction systems: —+— G2, (APD, BS); —✳— G3, (APD, maleic acid); —✕— G5, (APD, BS, CHP); —◇— G6, (APD, CHP, maleic acid); —△— G7, (APD, CHP, BS, maleic acid).

relatively lethargic catalyst in this solution, i.e. the production of both iron species occurs at a higher rate than the rate of utilisation in the homolysis of CHP. By substituting BS for a stronger acid such as maleic acid in this system, a slight decrease of Fe(II) levels is detected compared to a system containing APD/maleic acid only (Figure 6.11), which indicates that maleic acid may be the better acid component for iron catalysed systems that contain CHP as the peroxide. Examination of Figures 6.10 and 6.11 show that the presence of the two acids, maleic acid and BS together, in the APD/CHP system produces relatively elevated levels of Fe(II) and Fe(III) in comparison to all other systems, with the exception of APD/CHP/BS, thereby implying that such a system would be the most reactive and produce the fastest cure. In the case of the copper species, the reverse situation is seen. A decrease in reactivity, signified by the minimal production of Cu(I) in Figure 6.9, was observed when maleic acid was added to the APD/CHP/BS system.

Due to the different reaction pathways and reaction rates adopted by copper and iron in the presence of APD, the rational behind the observed results may at times appear contradictory. In highly reactive copper catalysed APD-based systems, the strength of the acid component does not seem to influence the production of Cu(II), and to a lesser extent this holds true for the production of Cu(I) as well. These findings would complement the hypothesis made in APH systems, whereby the protonated version of APH retarded the conversion of the higher valence state of the metal to the lower state. Since this situation does not arise with APD, the Cu(II) that is generated is readily reduced to Cu(I). However an exception arises in relatively acidic conditions such as an APD/CHP/BS/maleic acid system, where the production of Cu(I) falls significantly.

In the less reactive iron catalysed systems, the

APH/maleic acid does generate significant levels of Fe(III) by comparison to APH/BS, but this is offset by the relatively slow reaction between Fe(III) and APD resulting in a similar degree of conversion to Fe(II) in the case of both BS and maleic acid solutions. The presence of two acid components, i.e. maleic acid and BS, significantly increases the reactivity of the system. This suggests that in the case of iron catalysed APD cure systems, these acid components would be essential in the formulation if a rapid cure is to be obtained.

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Appendix A: PUBLICATIONS

1. Comparison of mercury- and glassy carbon- based electrochemical detection systems for the determination of cisplatin following high-performance liquid chromatographic separation, O'Dea, P., Shearan, P., Dunne, S., and Smyth, M. R., Analyst, 1988, 113, 1791.

2. Determination of ciprofloxacin by differential-pulse polarography, O'Dea, P., Costa Garcia, A., Miranda Ordieres, A. J., Tunon Blanco, P. and Smyth, M. R., Electroanalysis, 1990, 2, 637.

3. Comparison of adsorptive stripping voltammetry at mercury and carbon paste electrodes for the determination of ciprofloxacin in urine, O'Dea, P., Costa Garcia, A., Miranda Ordieres, A. J., Tunon Blanco, P., and Smyth, M. R., Electroanalysis, 1991, 3, 337.

4. Investigation of a cation exchange separation method for the determination of transition metals in anaerobic sealants, O'Dea, P., Deacon, M., Smyth, M. R. and Leonard, R. G., Analytical Proceedings, 1991, 28, 82.