

**Development of Novel Analytical Methods to
Study the Metabolism of Coumarin**

by

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A Thesis Submitted for the Degree

of

Doctor of Philosophy

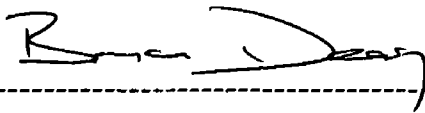
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Declaration

I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of Ph D 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 own work

Signed



Candidate

Date

20/6/96

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*To my family and girlfriend Susan, for all the love
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TABLE OF CONTENTS

	Page
Title Page	i
Declaration	ii
Acknowledgements	iii
Dedication	iv
Table of Contents	v
Abstract	xi

CHAPTER 1

ANALYSIS OF COUMARIN AND 7-HYDROXYCOUMARIN

1.1	HISTORICAL BACKGROUND	2
1.2	PHYSICAL AND CHEMICAL PROPERTIES	3
1.3	INDUSTRIAL USES	3
1.4	METABOLISM	5
1.5	PHARMACOKINETICS	9
1.6	TOXICOLOGY	10
1.7	ANALYSIS OF COUMARIN AND COUMARIN-RELATED COMPOUNDS	10
1 7 1	HIGH PERFORMANCE LIQUID CHROMATOGRAPHY	11
1 7 2	FLUOROMETRIC METHODS	17
1 7 3	OTHER TECHNIQUES	19
1 7 3 1	Paper chromatography	19
1 7 3 2	Thin layer chromatography	20
1 7 3 3	Gas chromatography with mass spectrometric detection	21
1 7 3 4	Capillary electrophoresis	22

1 7 3 5	Radio-labelling methods	24
1 7 3 6	Luminescence	25
1 7 3 7	Polarography and Voltammetry	26
1 7 3 8	Amperometric biosensor detection	26
1.8	REFERENCES	28

CHAPTER 2

	DETERMINATION OF FREE AND TOTAL 7-HYDROXYCOUMARIN IN URINE AND SERUM BY CAPILLARY ELECTROPHORESIS	34
2.1	INTRODUCTION	35
2 1 1	THEORY	37
2 1 2	REVIEW OF CAPILLARY ZONE ELECTROPHORESIS IN BIOMEDICAL ANALYSIS	42
2.2	EXPERIMENTAL	47
2 2 1	CHEMICALS	47
2 2 2	SAMPLE PREPARATION	47
2 2 3	CAPILLARY ELECTROPHORESIS SEPARATION	49
2.3	RESULTS AND DISCUSSION	50
2 3 1	DEVELOPMENT OF THE CE SEPARATION	50
2 3 2	LIMIT OF QUANTIFICATION AND LINEARITY	61
2 3 3	ACCURACY AND PRECISION	61
2 3 4	CLINICAL AND PHARMACOKINETIC STUDIES	64
2.4	CONCLUSION	65
2.5	REFERENCES	66

CHAPTER 3

	STUDY OF COUMARIN METABOLISM BY LIVER MICROSOMES USING CAPILLARY ELECTROPHORESIS AND ELECTROCHEMISTRY	69
3.1	INTRODUCTION	70
3 1 1	NOMENCLATURE	71
3 1 2	SPECIFICITY	73
3 1 3	CYTOCHROME P450 ELECTRON TRANSPORT SYSTEMS	74
3 1 4	PREVIOUS WORK ON COUMARIN METABOLISM USING LIVER MICROSOMES	76
3.2	A STUDY OF COUMARIN METABOLISM BY CAPILLARY ELECTROPHORESIS	81
3 2 1	EXPERIMENTAL	81
3 2 1 1	Chemicals	81
3 2 1 2	Tissue preparation	81
3 2 1 3	Bicinchoninic acid protein assay	83
3 2 1 4	Incubation solution	84
3 2 1 5	NADP ⁺ /NADPH ratio study	85
3 2 1 6	Capillary electrophoresis separation	86
3 2 2	RESULTS AND DISCUSSION	87
3 2 2 1	Overall reaction in the incubation solutions	87
3 2 2 2	Development of the CE separation	87
3 2 2 3	Coumarin metabolism profiles	95
3 2 2 4	NADP ⁺ /NADPH ratio study	102
3 2 2 5	Stability of NADPH	105
3 2 2 6	Interspecies differences in coumarin metabolism	114

3.3	ELECTROCHEMICAL INVESTIGATIONS INTO MICROSOMAL REACTIONS	118
3 3 1	EXPERIMENTAL	118
3 3 1 1	Chemicals	118
3 3 1 2	Tissue preparation	118
3 3 1 3	Apparatus	118
3 3 1 4	Electrochemical methods	119
3 3 2	RESULTS AND DISCUSSION	120
3 3 2 1	Investigations in the anodic region	120
3 3 2 2	Study of incubation reactions using cyclic voltammetry	129
3 3 2 3	Investigations in the cathodic region	132
3.4	CONCLUSIONS	141
3.5	REFERENCES	143

CHAPTER 4

DETERMINATION OF 7-HYDROXYCOUMARIN USING A HORSERADISH PEROXIDASE LABELLED ANTI-7-HYDROXYCOUMARIN ANTIBODY-BASED BIOSENSOR

146

4.1	INTRODUCTION	147
4 1 1	IMMUNOSENSORS BASED ON OPTICAL DEVICES	150
4 1 2	IMMUNOSENSORS BASED ON SURFACE ACOUSTIC WAVE DEVICES	152
4 1 3	IMMUNOSENSORS BASED ON SEMICONDUCTOR DEVICES	153
4 1 4	IMMUNOSENSORS BASED ON ELECTROCHEMICAL TRANSDUCERS	153

4.2	EXPERIMENTAL	155
4 2 1	APPARATUS	155
4 2 2	ELECTRODE PREPARATION	156
4 2 3	PROCEDURES	156
4 2 4	REAGENTS	157
4.3	RESULTS AND DISCUSSION	158
4 3 1	SENSING MECHANISM	158
4 3 2	DEVELOPMENT OF AN OVALBUMIN-7-OH-COUMARIN MODIFIED ELECTRODE	159
4 3 3	DEVELOPMENT OF A THYROGLOBULIN-7-OH-COUMARIN MODIFIED ELECTRODE	161
4 3 4	COMPETITIVE ENZYME LINKED ELECTROCHEMICAL IMMUNOASSAY FOR THE DETERMINATION OF 7-OH-COUMARIN	167
4.4	CONCLUSIONS	170
4.5	REFERENCES	171

CHAPTER 5

5.1	OVERALL CONCLUSIONS	175
5.2	REFERENCES	178

	<u>APPENDIX 1</u>	179
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	List of Publications	179
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ABSTRACT

The research in this thesis revolves around developing analytical methods for the determination of coumarin and 7-hydroxycoumarin for various applications. The techniques used in this work were, capillary electrophoresis, immunosensing and electrochemistry. Chapter 1 serves as general review of the analysis of coumarin and 7-hydroxycoumarin, including the many different types of analytical technique which have been used to analyse this drug. Capillary electrophoresis was used as the basis of a method for the determination of 7-hydroxycoumarin in urine and serum. This method offered advantages over existing technology, due to the speed of analysis compared to existing methods. Capillary electrophoresis was also used to study of the metabolism of coumarin by liver microsomes. This work proved very interesting with a novel method being developed for the determination of the main metabolite of coumarin in humans. This method was also applied to an interspecies study on the metabolism of coumarin in a variety of species, while it also found application in trying to understand some of the different reactions which take place in liver microsomes. Electrochemistry was used to study further some microsome reactions with some success. A biosensor was constructed for the determination of 7-hydroxycoumarin which used electrochemical detection. An immunosensor based on a competitive electrochemical immunoassay was developed. The antibody-based biosensor employed horseradish peroxidase-labelled anti-7-hydroxycoumarin, with the enzyme catalysed reaction involving the reduction of hydrogen peroxide in the presence of a mediator.

CHAPTER 1

ANALYSIS OF COUMARIN AND 7-HYDROXYCOUMARIN

1.1 HISTORICAL BACKGROUND

Coumarin is a naturally occurring constituent of many plants and essential oils, including tonka beans, sweet clover, oil of cassia and lavender. Its name is derived from the plant *Coumarouna odorata*. Coumarin (1,2-benzopyrene) is of significant clinical importance due to its use in the treatment of many diseased states [1,2]. It has been used in clinical practice, in post-thrombotic syndromes for treatment of varicose veins and in post-traumatic oedema. Coumarin has also been used in cancer therapy and has also been found to have an inhibitory effect on the induction of cancer [3,4].

Vogel isolated and purified coumarin from the tonka bean (*Dipteryx odorata*) in 1822. Later it was synthesised in 1868 by Perkin (in Casley-Smith, [5]). By now much information has been generated on its properties and potential uses (Table 1.1). Coumarin is also known as 2H-1-benzopyran-2-one, 1,2-benzopyrone, cis-o-coumarinic acid lactone, coumarinic anhydride, o-hydroxycinnamic acid- δ -lactone, and 2-oxy-1-benzopyran (reviewed by Cohen [6]). Coumarin is a member of a class of compounds called benzopyrones. Benzopyrones consist of fused benzene and α -pyrone rings. Coumarin can exist as an odourless complex conjugated to sugars and acids, which can be broken down by the action of enzymes, acids, or ultraviolet (UV) radiation. Coumarin is also found in hay and its presence gives it a characteristic odour. Coumarin compounds have been used to treat a variety of ailments such as burns, cancer, brucellosis and rheumatic disease.

1.2 PHYSICAL AND CHEMICAL PROPERTIES

The relative molecular mass of coumarin is 146.15 and it exists as a solid with a pleasant and characteristic odour. The melting point of the molecule is 303°C. Coumarin has a UV absorption maximum at 272 nm in chloroform. It is freely soluble in ethanol, chloroform and oils, while only sparingly soluble in boiling water and only slightly soluble in cold water at 20°C.

1.3 INDUSTRIAL USES

Coumarin has many uses in industry, mainly due to its strong fragrant odour. Its uses include that of a sweetener and fixative of perfume, an enhancer of natural oils, such as lavender, a food additive in combination with vanillin, a flavour/odour stabiliser in tobaccos, an odour masker in paints and rubbers, and finally it is also used in electroplating to reduce the porosity and increase the brightness of various deposits, such as nickel (Table 1.1). A number of natural synthetic derivatives of coumarin exist, but only a few are of any commercial importance. These include 3,4-dihydroxycoumarin, mainly used in the perfume industry, 6-methylcoumarin, used as a flavour enhancer, and 7-hydroxycoumarin (7-OHC), used in sun screens and fluorescent brighteners (Table 1.1). Derivatives of 7-OHC are also used as fluorogenic enzyme substrates. 7-amino-4-methylcoumarin, 4-methyl-umbelliferone, and other coumarin derivatives are used as laser dyes. The most important derivative is 4-hydroxycoumarin, which is found

in spoiled hay and is a precursor of dicumarol and warfarin, both of which are vitamin K antagonists

Compound	Toxicity	Applications
Coumarin	LT	Sweetener Fixative Flavour stabiliser Food additive Odour masker Electroplating
4-Hydroxycoumarin	anticoagulant	Production of dicumarol
Warfarin	anti-coagulant	Rodenticide Cancer therapy
3,4-Di-hydroxycoumarin	LT	Perfume industry
6-Methylcoumarin	photo-allergen	Flavour enhancer
7-Hydroxycoumarin	LT	Sun screens Fluorescent brightener
4-Methyl-umbelliferone	LT	Laser dye
Amino-methyl-coumarin acetic acid (AMCA)	LT	Fluorescent label
Flavone-8-acetic acid	LT	Cancer therapy

LT = limited evidence of toxicity

Table 1 1 Properties of coumarin and some of its common derivatives

1.4 METABOLISM

Coumarin is metabolised initially by a specific cytochrome P450 linked mono-oxygenase enzyme (CYP2A6) system in liver microsomes, resulting in hydroxylation at positions 7 and 3. Human liver microsomes contain high levels of this enzyme compared with activities in the livers of various animal species. It has been shown that there exists large inter-individual variability in the activity of this enzyme [7]. The possible polymorphism of this enzyme in man has been studied *in vivo*. Rautio *et al* [8] measured the urinary excretion of 7-OHC following administration of coumarin. The rate of 7-OHC formation during the first two hours appeared to be variable but stable and reproducible for a given individual. Four individuals out of a total of 110 were slow metabolisers of 7-OHC. However, a larger population size would be required to statistically prove that a polymorphism exists. The corresponding enzyme in mouse liver (CYP2a-5) was shown to be strain dependant. The human isoform of the enzyme has been transfected into mammalian cells via a retrovirus-mediated expression system, in order to allow this enzyme to be studied *in vitro* (reviewed by Pelkonen *et al* [9]).

Coumarin, having been metabolised to form 7-OHC, then undergoes phase II conjugation prior to excretion (7-OHCG) [10, 11]. The most common routes of hydroxylation are at positions 7 and 3 to yield 7-OHC and 3-hydroxycoumarin (3-OHC), respectively. 3-OHC can be further metabolised, non-enzymatically, by ring splitting to form two further products, o-Hydroxyphenyllactic acid (O-HPLA) and o-hydroxyphenylacetic acid (O-HPAA). The major phase II metabolite in

man is a glucuronide conjugate, 7-OHCG [12] Moran *et al* [13] showed that on average, 63% of a total dose of 200 mg of coumarin was recovered as 7-OHC in the urine of volunteers over a 24 hour period, with most recovered within the first 10 hours Rautio *et al* [8] showed that between 30-100% of an oral dose of coumarin was recovered as 7-OHC within 4 hours of administration The main pathways of coumarin metabolism are shown in Fig 1 1

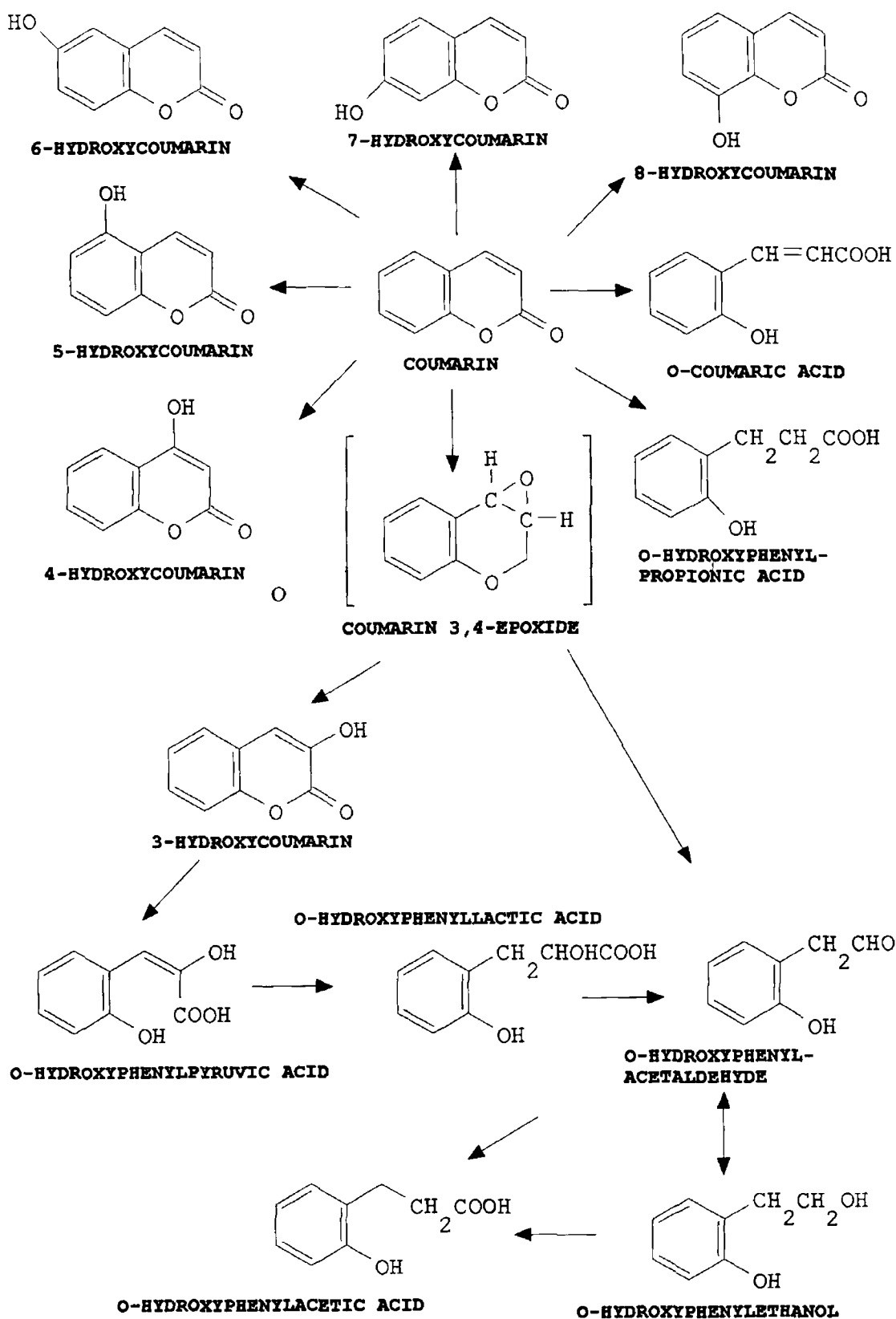


Figure 1.1 Metabolic pathways of coumarin in mammals Reproduced from references [21, 17]

In rats, quite a different metabolic pathway is found, with small amounts of 3-OHC (1-2%) and very little 7-OHC formed. In rabbits, the major metabolites are O-HPAA (18-22%), 3-OHC (18-23%), and 7-OHC (10-16%). In addition, there are trace quantities of 4-, 5-, 6-, and 8-hydroxycoumarin [11]. In the case of the mouse, there exists a quantitative difference between the strains, which is due to differences in isoenzyme activity. Coumarin-7-hydroxylase is much greater in human than rodent microsomes, and conversely, coumarin-3-hydroxylase activity is high in rodents while absent in human microsomes, (reviewed by Cohen [6]). In 1991, Huwer *et al* [14] identified a coumarin mercapturic acid metabolite in the urine of Wistar rats. This metabolite was thought to have arisen from a 3,4-epoxide which was subsequently coupled with glutathione.

In 1983, Ritschel and Hardt [15] showed that the mongolian gerbil metabolised coumarin in a manner similar to man. Unlike most other rodent species, the mongolian gerbil forms a large amount of 7-OHC. This animal has been used as a model for studying the pharmacology and toxicological effects (Dominguez *et al* [16]). A novel metabolite of coumarin, o-hydroxyphenylacetaldehyde (o-HPA), was isolated in rat, gerbil and human hepatic microsomes following incubation with coumarin. It was also shown that this was a major metabolite in all three sources of hepatic microsomes [17]. These authors postulated that o-HPA, formed from coumarin 3,4-epoxide may subsequently give rise to o-HPAA and o-hydroxyphenylethanol. Fentem and Fry [18] showed that treatment of rats with phenobarbitone or β -naphthoflavone increases the amount of o-HPA formed. Metabolism of coumarin by human liver microsomes gave rise to o-HPA, 7-OHC, 3-OHC and trace amounts of 5-, 6- and 8-OHC. Gerbil microsomes metabolised coumarin to give 3-, 5-, 6-, 7-, and 8-OHC, and

3,7-and 6,7-di-hydroxycoumarins, along with o-HPA. They also showed that at low coumarin concentrations (0-10 μ M), hepatic microsomal metabolism in gerbil resembled that in man, with 7-OHC being the major metabolite, but with O-HPA levels being greater in the gerbil than in the human. They concluded that the gerbil was a more appropriate animal model than the rat for comparison with the human.

1.5 PHARMACOKINETICS

It has been demonstrated by Waller and Chasseaud [19], that the pharmacokinetic profile of coumarin in baboons was similar to that in man. The very high levels of 7-OHCG in plasma indicated that all of the coumarin was absorbed from the gut and distributed not only to organs with high blood flow, but also to the extracellular fluid and intracellular compartments of other tissues.

Coumarin is often considered a pro-drug due to its rapid metabolism to form 7-OHC. This theory has been partially substantiated, since coumarin has a short half-life. Sharifi *et al* [20] showed that following large oral or intravenous doses, coumarin was rapidly metabolised to form 7-OHC and 7-OHCG. They found that plasma concentrations of 7-OHC were about ten times lower than 7-OHCG. In urine 7-OHCG was the main metabolite with a recovery of one third of the initial dose. 7-OHC is lipophilic, preventing its renal excretion and leading to its presence in the plasma. They concluded that 7-OHC, and not coumarin, was the pharmacologically active agent.

1.6. TOXICOLOGY

The Food and Drug Administration (FDA) banned the use of coumarin in 1954. Previously it had been used in foodstuffs, and since then there has been an on-going dispute over its toxicity. The data on which the FDA made their decision was derived mainly from animal studies using rats. The preliminary results from these studies indicated that coumarin was a toxin, but it has been shown since that the rat is a poor model to compare with the human for this particular drug metabolism.

1.7 ANALYSIS OF COUMARIN AND COUMARIN-RELATED COMPOUNDS

Many techniques have been used to analyse coumarin and coumarin-related compounds. The two techniques that have been the most widely used are, high performance liquid chromatography (HPLC) [8, 13, 21-23] and spectrofluorimetry [8, 24, 25]. Other techniques that have been used include paper chromatography [26, 27], thin layer chromatography [28, 29], gas chromatography with mass spectrometric detection [30, 31], radio-labelling [10, 15, 32, 33], luminescence [34], polarography and voltammetry [35, 36, 37] and using an amperometric biosensors [38].

These techniques have been developed for the quantitative and qualitative determination of coumarin and coumarin derivatives. They have been used in the analysis of a variety of samples, including urine, plasma, serum,

blood, tissue homogenates, and plant material. Sample preparation varies greatly depending on the method of analysis used. The range of techniques used for the analysis of coumarin and coumarin-related compounds and the samples analysed will be discussed in greater detail below.

1.7.1 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

The UV spectra of coumarin in tris buffer is shown in Fig. 1.2. Many HPLC methods have been developed for the separation and quantitation of coumarin and its metabolites. The first such method was described in 1980 by Walters *et al.* [21]. The HPLC system consisted of a reversed-phase Partisil-10 ODS 2 column, with a flow rate of 2 ml/min, and UV detection at 274 nm. The separation required a two-stage linear gradient using tetrahydrofuran:water:acetic acid. The linear gradient steps were from 9.7% - 12.8% and then to 20% tetrahydrofuran. This gradient run produced a good separation of a range of coumarin derivatives. These included o-hydroxyphenylacetic acid (o-HPAA), 8-OHC, 7-OHC, 6-OHC, 5-OHC, 4-OHC, 3-OHC, coumarin, and o-coumaric acid. One of the drawbacks to the method was the high flow rate, which resulted in high back pressure. The total time required to separate all of these derivatives was approximately 65 min. Walter *et al.* also attempted to extract and quantify coumarin and its metabolites from tissue incubates. However, this was unsuccessful since it was impossible to use a single solvent to extract compounds with such widely varying polarity.

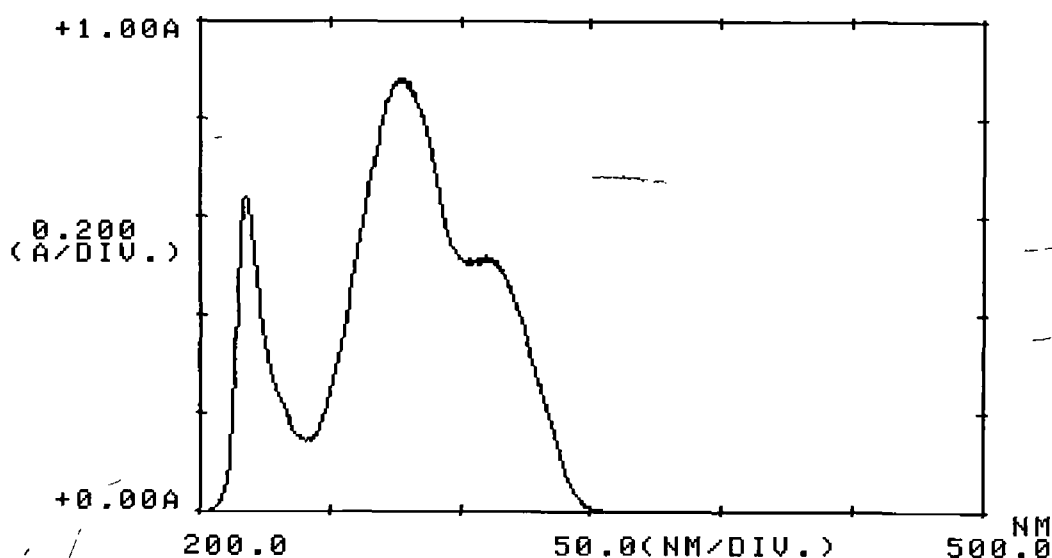


Fig 1 2 UV spectra of coumarin in tris buffer

The method above, developed by Walters *et al* [21], was later used by Lake *et al* [39] for comparing the metabolism and disposition of coumarin in the rat and marmoset. In this study, however Lake *et al* were able to quantify coumarin, o-HPAA, 3-OHC, o-coumaric acid, 7-OHC and 7-OHCG in urine. This method was used to show that both the rat or marmoset were unsuitable as an animal model for studying the metabolism of coumarin in man.

In 1987, a new method was developed by Moran *et al* [13], which was capable of resolving and quantifying coumarin, 7-OHC and its glucuronide conjugate (7-OHCG). This method used a reversed-phase C₁₈ μ Bondapak

column, with a mobile phase of methanol water acetic acid (40 60 0.2), and a flow rate of 2 ml/min. Compounds were detected using a fixed wavelength detector at 280 nm. The use of methanol and the high flow rate again caused high back pressure, however, this method was successfully applied to the analysis of urine samples. Unfortunately, the method of sample preparation was time consuming and cumbersome. The urine samples were freeze dried and later evaporated, leading to an assay time of approximately 24 hours. β -Glucuronidase was used to hydrolyse 7-OHCG, to yield free 7-OHC. This incubation added 16 h to an already long assay time. The retention times of 7-OHC and coumarin were 7 and 10 min, respectively. This assay was successfully used to determine the percentage of coumarin recovered within a 24 hr period, following oral administration. It was also used to determine the urinary excretion profile in man, following oral administration of coumarin.

Egan and O’Kennedy [40] described a HPLC method for the determination of coumarin, 7-OHC and 7-OHCG in urine and plasma. The authors described the use of a Waters μ Bondapak C₁₈ column, a fixed wavelength of 324 nm and a mobile phase of methanol water acetic acid (30 70 0.2), with a flow rate of 2 ml/min. This method was a significant improvement on the method of Moran *et al* [13] above, as the sample preparation time was considerably reduced, although analyte extraction was still required. Figure 1.3 shows the separation of coumarin, 7-OHC and internal standard achieved by the method of Egan and O’Kennedy.

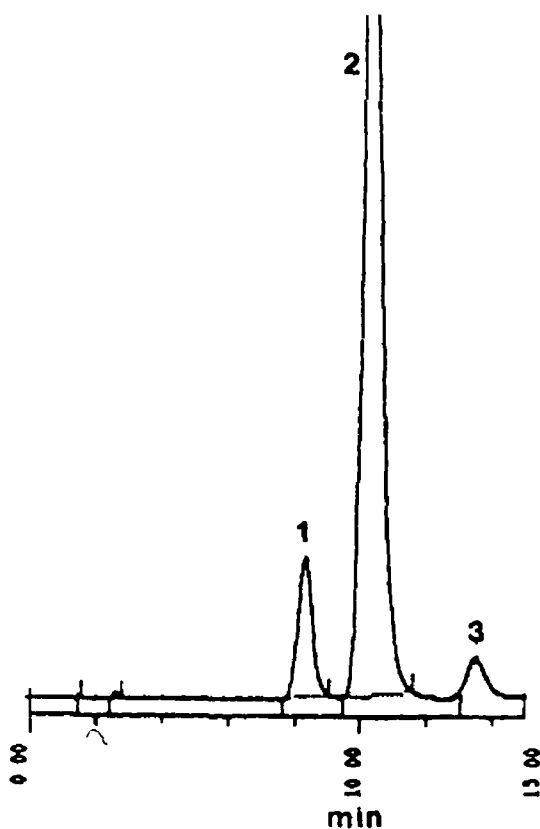


Fig 1 3 Separation of (1) 7-OHC, (2) internal standard and (3) coumarin by HPLC Reproduced from reference [40]

Thompson and Hoffmann [22] showed the presence of coumarin in vanilla flavoring products, using HPLC Separation was carried out using a

μ Bondapak C₁₈ column, with a mobile phase of methanol water (40 60) and a flow rate of 1 ml/min. This was a similar HPLC system to that used by Moran *et al* above, but a lower flow rate was employed. Coumarin and the other derivatives (4-hydroxybenzaldehyde, vanillin and ethyl vanillin) were monitored at 275 nm. No sample extraction was used, but merely dissolution in methanol water (50 50). This HPLC method offered a fast assay for quantifying coumarin in vanilla products.

A HPLC method, able to separate and quantify 7-OHC, was then developed by Rosenberg *et al* [23]. The main objective of this method was to determine the activity of ethoxycoumarin o-deethylase in tissue samples. o-Deethylase breaks down 7-ethoxycoumarin to form 7-OHC, which is fluorescent. Therefore, the method was based on fluorescence of 7-OHC in alkaline solution. Ethoxycoumarin o-deethylase was isolated from cells with low levels of cytochrome P-450, that would otherwise be below detection. The method used a reversed-phase Nova-pak C₈ column, with a mobile phase of methanol 1% acetic acid, (35 65), at a flow rate of 1 ml/min. Samples were extracted with chloroform and were then separated on the HPLC column. 1 N sodium hydroxide was added to the post-column eluate. 7-OHC was detected using a fluorescence detector, with excitation and emission wavelengths of 368 and 456 nm respectively. This method has been improved by Evans and Relling [41]. They automated the system and used 4-methyl-umbelliferone as an internal standard.

In 1991, Huwer *et al* [14] developed a HPLC method for the separation and quantitation of coumarin mercapturic acid. This new derivative was

isolated from rat urine and indicated that an epoxide of coumarin had been formed during metabolism. This method was able to separate and quantify o-hydroxyphenyllactic acid (o-HPLA), o-HPAA, 7-OHC, coumarin-3-mercaptopuric acid, coumarin, 3-OHC and o-coumaric acid. Separation of these compounds was carried out using a reversed-phase Spherisorb ODS 2 column, with a mobile phase of water THF acetonitrile acetic acid (764 100 85 50), at a flow rate of 1 ml/min, and detected at 285 nm. Samples were prepared by washing in acetone methanol water (3 1 1) and concentrated prior to analysis. The total time required for separation and elution of all the coumarin metabolites was 30 min.

Fentem *et al* [17] and Fentem and Fry [18] examined the metabolism of coumarin by rat, gerbil and human liver microsomes. They used a HPLC method based on the method developed by Vande Casteelle *et al* [42]. This method was able to separate a range of coumarin metabolites, including 6,7-di-hydroxycoumarin, 6-OHC, 7-OHC, 8-OHC, 5-OHC, o-coumaric acid, 3-OHC, coumarin and 4-OHC. A new metabolite of coumarin, o-hydroxyphenylacetaldehyde, formed in rat liver microsomes, was also detected. An external standard of 7-methoxy-coumarin was used. Separation was carried out using a reversed-phase Spherisorb 5 ODS 2 column, with 2 stage linear gradient elution. The mobile phases used were formic acid water (5 95) and methanol. Coumarin compounds were detected at 280 nm. All of the metabolites were well separated and eluted within 22 min.

A relatively new HPLC method developed Rautio *et al* [8], based on the method of Fasco *et al* [43], was used to measure 7-OHC and its

glucuronide in human urine. This method was used by Rautio *et al* [8], to show that there is great inter-individual variability in the extent and rate of 7-OH-C formation. Urine was treated with β -glucuronidase, filtered and analysed by HPLC. Separation was carried out using a Nucleosil C₁₈ column. The mobile phase used was 20 % of 1.5 % (v/v) acetic acid, pH 4.85 (Buffer A) and 80 % of buffer B (Buffer A and 50% acetonitrile). A flow rate of 1 ml/min and detection wavelength of 313 nm were used. The assay could be carried out very quickly, since very little sample preparation was required and the retention time of 7-OH-C was 5 min.

1.7.2 FLUOROMETRIC METHODS

Tan *et al* [24] developed a spectrofluorometric method for the determination of coumarin and 7-OH-C. The analysis of coumarin was based on the irradiation of compound in alkaline solution with UV light. This converted coumarin from the *cis*-to the *trans*-isomer, leading to the formation of a fluorophore. The fluorophore showed excitation and emission wavelengths of 361 and 491 nm, respectively. This spectrofluorometric method was used to quantify coumarin and 7-OH-C in whole blood following two solvent extraction's with diethyl ether. The method was shown to be reproducible, sensitive and free from interferences. However, one of the major drawbacks of this method was the substantial time required for sample preparation prior to analysis. Hence, this method had a long assay time. Also, it did not describe a method for the hydrolysis of 7-OHCG.

In 1981, Ritschel *et al* [44] used the method developed by Tan *et al* [24] to determine the apparent partition coefficient, pK_a , and protein and erythrocyte binding of 7-OHC. Ritschel and Hardt [15] again used this method in order to determine the concentration of coumarin, 7-OH-C, and 7-OHCG in blood and brain of gerbils. The tissue was prepared by protein precipitation and homogenisation. This modification of the method of Tan *et al* proved to be very successful in determining the pharmacokinetics of coumarin and its metabolites. The results indicated that the gerbil was a suitable animal model for studying coumarin metabolism.

A modification of the method developed by Tan *et al* was used by Rautio *et al* [8] in order to determine the concentration of 7-OH-C and 7-OHCG, in human urine and serum. Following hydrolysis of 7-OHCG, a sample was extracted with chloroform and diluted with 1 M sodium chloride. 0.01 M sodium hydroxide. The fluorescence in the alkaline phase was measured immediately using a spectrofluorimeter, at excitation and emission wavelengths of 365 and 454 nm, respectively. The method was shown to be both accurate and precise, and having a limit of detection of 0.5 nM.

Egan and O'Kennedy [25] then developed a sensitive spectrofluorimetric assay for the determination of 7-OHC in urine and plasma. Urinary concentrations of free, total and conjugated 7-OHC were determined. Samples were extracted with diethyl ether and a suitable aliquot of reconstituted extract diluted in phosphate buffered saline, and transferred to a 96-well microtitre plate. The fluorescence intensity was determined at excitation and emission wavelengths of 370 and 450 nm, respectively. Linear ranges of 7-OHC in urine and plasma were found to be 0.5-10 and

10-100 µg/ml The method was an improvement on the previously described method of Tan *et al* [24]

1 7 3 OTHER TECHNIQUES

A variety of other methods for the analysis of coumarin and 7-OHC have been developed These include paper chromatography, TLC, gas chromatography with mass spectrometric detection, capillary electrophoresis, radio-labelling, luminescence, polarography and voltammetry and amperometric biosensor detection

1 7 3 1 Paper chromatography

Paper chromatography was used by Booth *et al* [26] as a method for the analysis of coumarin in the urine of rat and rabbit Several metabolites of coumarin, including o-HPAA, o-HPLA, -hydroxyphenylhydrarylic acid, o-coumaric acid, 7-OHC, 4-OHC and 3-OHC were separated o-HPAA, 3-OHC and 7-OHC were extracted from rabbit urine using ethanol and later with carbon tetrachloride followed by chloroform The other metabolites were extracted from urine using diethyl ether and separated using two dimensional paper chromatography Separation was carried out using a solvent system of chloroform acetic acid water (2 1 1) in one dimension and then 20% (w/v) aqueous potassium chloride, in the second Identification was achieved using a set of authentic standards on the basis

of R_f values, behaviour under UV light, and colour after spraying with diazotised sulphanilic acid. This method was used to identify a new metabolite of coumarin, o-HPAA, in both rat and rabbit urine. The method was found to be qualitative but not quantitative.

Another paper chromatographic method was developed by Van Sumere *et al* [27] in 1959. The metabolites of coumarin in urine taken from normal patients and those suffering from various pathological conditions. The samples were examined using circular paper chromatography. The samples were extracted using either diethyl ether or ethyl acetate. The extraction procedure was long and cumbersome and required a great deal of preparation. In order to separate all of the coumarin metabolites, two solvents were used. Using the first system, the extracts were applied to filter paper and separated using petroleum ether and later chloroform, saturated with formamide. The second system used a solvent system of petroleum ether, and later butanol water (4:1). Identification was based on the R_f values, colour reactions and appearance under UV light. The method identified trace amounts of 7-OHC and 6,7-dihydroxycoumarin in normal urine. It also showed large quantities of 7-OHC in the urine of patients receiving prednisone treatment. This method, like the previous one, was unable to quantify the various metabolites detected.

1.7.3.2 Thin layer chromatography

Sherma *et al* [28] developed a method that could determine coumarin in vanilla flavourings using high performance silica gel TLC. This method allowed coumarin to be determined both qualitatively and quantitatively.

Coumarin was separated on pre-treated silica gel plates using a solvent system of toluene methanol (97:3). Coumarin was then detected by spraying the plates with 5-10 % (w/v) sodium or potassium hydroxide and viewed using fluorescence long wave UV light (360 nm). The plates were also sprayed with diazotised sulphanilic acid reagent in order to produce a coloured product. The plates were then scanned for their fluorescence and depth of colour using densitometry. The peak area was calculated and allowed the concentration of coumarin in vanilla samples to be determined. The method had one disadvantage, since both fluorescence and colour changed in intensity with time.

Recently a novel TLC method with fluorescence detection was developed by Cholerton *et al* [29] for the determination of 7-OHC in human urine. 7-OHC was extracted using chloroform and evaporated. A sample of dried extract was applied to a TLC plate and separated using a solvent system of chloroform:water:ethyl acetate:acetic acid (24:12:6:0.5). The plates were then scanned at 313 nm using a mercury lamp. The peak height was determined and used to construct a calibration curve. β -glucuronidase was also used to hydrolyse 7-OHCG. The method proved to be sensitive, accurate, precise and more selective for the analysis of 7-OHC when compared to the spectrofluorometric method of Greenlee and Poland [45].

1.7.3.3 Gas chromatography with mass spectrometric detection

In 1980, a gas chromatographic method with mass spectrometric detection (GC-MS) was used by Legrum and Netter [30] to identify the various dihydroxylated metabolites of coumarin, formed by pre-treating mice with

cobalt Coumarin and 7-OHC were incubated with isolated microsomes from these animals. Diethyl ether was used to extract the samples. The concentrated extract was methylated using diazomethane, which was followed by preparative TLC. This method used two solvent systems, the first being benzene, followed by methanol. The relevant area was removed and analysed by GC-MS. This work showed that pre-treatment with cobalt gave rise to di-hydroxylated metabolites of 7-OHC.

Vries *et al* [31] analysed extracts from *justicia pectoralis* using GC-MS. This plant is used by Indians in the Amazon in tribal medicine. The compounds had first to be derivatised with diazomethane and separated using capillary GC-MS. The retention times and mass spectral data were compared with those of standard compounds. The results suggested the presence of both coumarin and 7-OHC.

1.7.3.4 Capillary Electrophoresis

There has been a number of publications dealing with the analysis of coumarin and its derivatives by capillary electrophoresis [46-50]. Recently, Bogan *et al* [46] have reported on the direct determination of 7-OHC and 7-OHCG in urine without any sample clean-up. Separation was carried out in 90% 100 mM L⁻¹ phosphate buffer, 11 mM L⁻¹ deoxycholic acid and 10% acetonitrile on a 47 cm uncoated silica capillary, at 20 KV, with detection of the analytes at 320 nm. This method has a considerable advantage over existing chromatographic methods in that no sample preparation was necessary. The separation of 7-OHC and 7-OHCG by this method is shown in Figure 1.4.

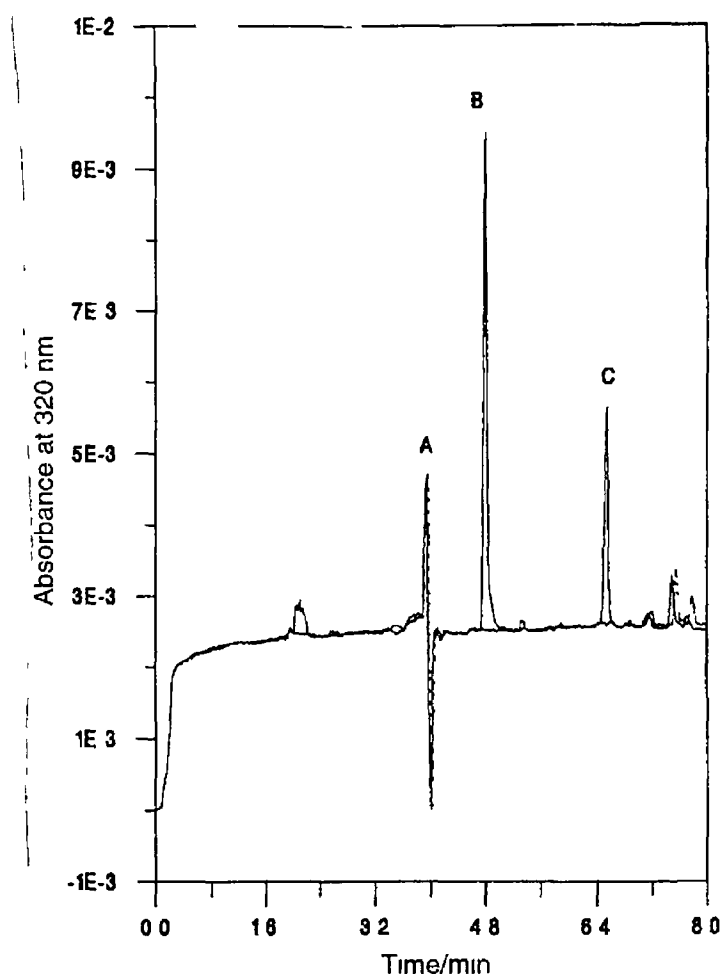


Fig 1 4 Electropherogram showing the separation of (A) solvent front, (B) 7-OHC and (C) 7-OHCG in urine analysis Reproduced from reference [46]

In 1993, Morin and Dreux [47] reported on factors influencing the separation of ionic and non-ionic chemical natural compounds in plant extracts by capillary electrophoresis. Micellar electrokinetic capillary chromatography (MECC) based on sodium dodecylsulfate (SDS) and cetyltrimethylammonium chloride (CTACl) micelles was developed for the separation of uncharged natural compounds (coumarin derivatives) or

charged solutes (phenolic carboxylic acids) The high efficiency separations allowed the resolution of closely related compounds The coumarin derivatives were separated using an applied voltage of 18 kV on a 70 cm x 50 μ m i.d. silica capillary The buffer used was a phosphate-borate buffer /0.1 M SDS pH 7.0 A wavelength of detection of 195 nm was used Upto 7 coumarin derivatives were separated in under 20 mins

Further to the above capillary electrophoresis methods, Chen and Sheu have described another method for the separation of coumarins by MECC [48] Dhulst and Verbeke [49] have described the separation of the enantiomers of coumarinic acid anticoagulant drugs by capillary electrophoresis using maltodextrins as chiral modifiers, and Ochocka *et al* [50] have reported work on the determination of coumarins from *chrysanthemum segetum* by capillary electrophoresis

1.7.3.5 Radio-labelling Methods

Kaighen and Williams [10] and Feuer *et al* [32] used radio-labelled coumarin to study the metabolism and excretion of coumarin in rats The coumarin molecule was labelled at ^{14}C in the third position (3- ^{14}C -coumarin) and administered orally The results indicated that both the urinary and faecal routes were important in the excretion of coumarin in the rat Van Sumere and Teuchy [33] then studied the excretion of coumarin in expired air using radio-labelled coumarin Rats were administered radio-labelled coumarin by intra-peritoneal injection Radio-

labelled CO₂ was detected in the expired air, resulting from decarboxylation of an intermediate preceding the formation of o-HPAA

In 1983, Ritschel and Hardt [15] also used ¹⁴C-labelled coumarin (3-¹⁴C-coumarin) and 7-OHC to determine their distribution in mice. Blood and tissue samples were extracted by solvent extraction using diethyl ether. A beta counter was used to determine the total radioactivity. Analysis was also carried out using TLC and compared to reference standards. From these studies it was concluded that the liver and kidney contained the highest levels of radioactivity following administration with both compounds.

1.7.3.6 Luminescence

Total luminescence spectroscopy has been used by Shanahan *et al* [34] for the detection and identification of coumarin in road fuels. The method, which was used, was similar to that described by Tan *et al* [24]. Coumarin in the fuel was converted from the *cis*- to the *trans*-isomer, following exposure to UV radiation at 366 nm. 3-D spectra were obtained by running successive emission scans from 320 to 600 nm, with excitation wavelength from 300 to 400 nm. The scans that resulted were compared to reference standards. This method was found to be qualitative but not quantitative.

1 7 3 7 Polarography and Voltammetry

A polarographic method for the determination of coumarin in sweet clover herbage was developed by Orlov [35]. Coumarin had to be extracted from boiling ethanol and added to a solution of tetraethylammonium iodide before analysis. The method proved to be both quantitative and selective.

Carrazon *et al* then [36] developed a voltammetric method for the determination of 7-OHC and 7-hydroxy-4-methylcoumarin. A glassy carbon electrode was used for this work in micellar solution and emulsified medium. No solvent purification was required as the medium used was mainly aqueous. The results showed this method to be highly sensitive, which could also be used quantitatively.

Dempsey *et al* [37] then reported on the electrochemical behaviour of 7-OHC at the bare glassy carbon electrode using differential pulse voltammetry. Based on anodic detection of this metabolite at 0.66 V (v's SCE) using DC amperometry, a method was developed for the determination of 7-OHC levels in urine samples, and a pharmacokinetic profile was described.

1 7 3 8 Amperometric Biosensor Detection

Following the previous study using DC amperometry at a bare glassy carbon electrode, Dempsey *et al* [38] developed an amperometric biosensor, based on an anti-7-OHC-antibody immobilised at the surface of

a glassy carbon electrode, and contained behind a cellulose dialysis membrane. The electrochemical behaviour of this metabolite at the bare glassy carbon electrode was found to be well defined using differential-pulse voltammetry, and a 90% decrease in peak height was observed on binding of the antibody to the antigen, which occurred at the electroactive site of the 7-OHC. This system provided a novel method for studying antibody specificity and the kinetics of such antibody-antigen interactions.

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CHAPTER 2

DETERMINATION OF FREE AND TOTAL 7- HYDROXYCOUMARIN IN URINE AND SERUM BY CAPILLARY ELECTROPHORESIS

2.1 INTRODUCTION

Jorgenson and Lukacs [1-5] were the first to perform capillary zone separations. The principle of the method is simple but its realisation encountered a number of difficulties. The separation is carried out in a fused-silica capillary (less than 100 μm I.D.) with a voltage of 10-30 KV, applied over a 50-100 cm capillary [6]. The separation is obtained by differential migration of solutes in an electrical field. In High Performance Capillary Electrophoresis (HPCE), electrophoresis is performed in narrow-bore capillaries, typically 25 to 75 μm inner diameter (i.d.), which are usually filled only with buffer [7]. Most commonly 20-50 mM buffers of both basic and acidic pH can be used, although high pH buffers are preferred in order to eliminate capillary wall charge and prevent e.g. separated protein species from adhering to the capillary wall [6].

Use of the capillary has numerous advantages, particularly with respect to the detrimental effects of Joule heating. The high electrical resistance of the capillary allows the application of high electrical fields (100 to 500 V/cm) with only minimal heat generation. Also, the large surface area-to-volume ratio of the capillary efficiently dissipates the heat that is generated. The use of the high electrical fields results in short analysis times and high efficiency and resolution. Peak efficiency, often in excess of 10^5 theoretical plates, is due in part to the plug profile of the electroosmotic flow, an electrophoretic phenomenon that generates the bulk flow of solution within the capillary [7]. This flow enables the simultaneous analysis of all solutes, regardless of charge. In addition the numerous separation modes, which offer different separation mechanisms

and selectivities, minimal sample volume requirements (1 to 10 nl), on-capillary detection, and the potential for quantitative analysis and automation, HPCE is rapidly becoming a premier separation technique [6,7]

The overall simplicity of the instrumentation is one key feature of HPCE. The early attempts to obtain capillary zone electrophoresis (CZE) separations were invariably done with laboratory-made equipment, however, numerous types of instruments are commercially available now e.g. Microphoretic Systems, Applied Biosystems, Bio-Rad Labs, Beckman Instruments and Dionex [6]. The design and operation of most instruments is very similar, briefly, the ends of a narrow bore fused silica capillary are placed in buffer reservoirs. The content of the reservoirs is identical to that within the capillary. The reservoirs also contain the electrodes used to make electrical contact between the high voltage power supply and capillary. Sample is loaded onto the capillary by replacing one of the reservoirs (usually at the anode) with a sample reservoir and applying either an electric field or an external pressure. After replacing the buffer reservoir, the electric field is applied and the separation performed. Optical detection can be made at the opposite end, directly through the capillary wall [7].

2 1 1 THEORY

Separation by electrophoresis is based on differences in solute velocity in an electric field. When an electric field is applied across a capillary filled with an ionic solution, anions are found to migrate towards the anode, whilst cations move towards the cathode [8]. The velocity of migration, V_{ep} , is related to the potential field strength, E , by the equation

$$V_{ep} = \mu_{ep}E \quad (2.1)$$

where μ_{ep} is the electrophoretic mobility of a solute. This equation can also be expressed as

$$V_{ep} = \mu_{ep}V / L \quad (2.2)$$

where L is the effective length of the capillary. The time taken for a solute to migrate through the capillary under an applied field is given by

$$t = L / V_{ep} = L^2 / \mu_{ep}V \quad (2.3)$$

This equation predicts that short columns and high voltages will lead to fast analysis times [8] As a solute migrates through a capillary, molecular diffusion occurs, and assuming that this is the only source of band broadening taking place, then the dispersion σ^2 , is given by the following equation

$$\sigma^2 = 2Dt = 2DL^2 / \mu_{ep}V \quad (2.4)$$

where D = diffusion coefficient of the solute The number of theoretical plates is now given by

$$N = L^2 / \sigma^2 = \mu_{ep}V / 2D \quad (2.5)$$

Equations 2.2 and 2.4 predict that operation at high voltages will yield highly efficient analyses, within very short run times [8] but there is a limit to the voltage that can be used, due to problems that arise from Joule heating

A fundamental constituent of HPCE operation is electroosmotic, or electroendosmotic flow (EOF) EOF is the bulk flow of liquid in the capillary and is a consequence of the surface charge on the interior capillary wall The EOF results from the effect of the applied electric field

on the solution double-layer at the wall. The EOF controls the amount of time solutes remain in the capillary by superposition of flow on to solute mobility [7]

Under aqueous conditions most solid surfaces possess an excess of negative charges. This can result from ionisation of the surface (that is, acid-base equilibria) and/or from adsorption of ionic species at the surface. For fused silica both processes probably occur, although the EOF is most strongly controlled by the numerous silanol groups (SiOH group that can exist in anionic form SiO^-) on the surface. Although the exact pI of fused silica is difficult to determine, EOF becomes significant above pH 4.0 [7]

Counterions (cations, in most cases), which build up near the surface to maintain charge balance, form the double-layer and create a potential difference very close to the wall. This is known as the zeta potential. When a voltage is applied across the capillary the cations forming the diffuse double-layer are attracted toward the cathode. Because they are solvated, their movement drags the bulk solution in the capillary toward the cathode. This is shown in Figure 2.1

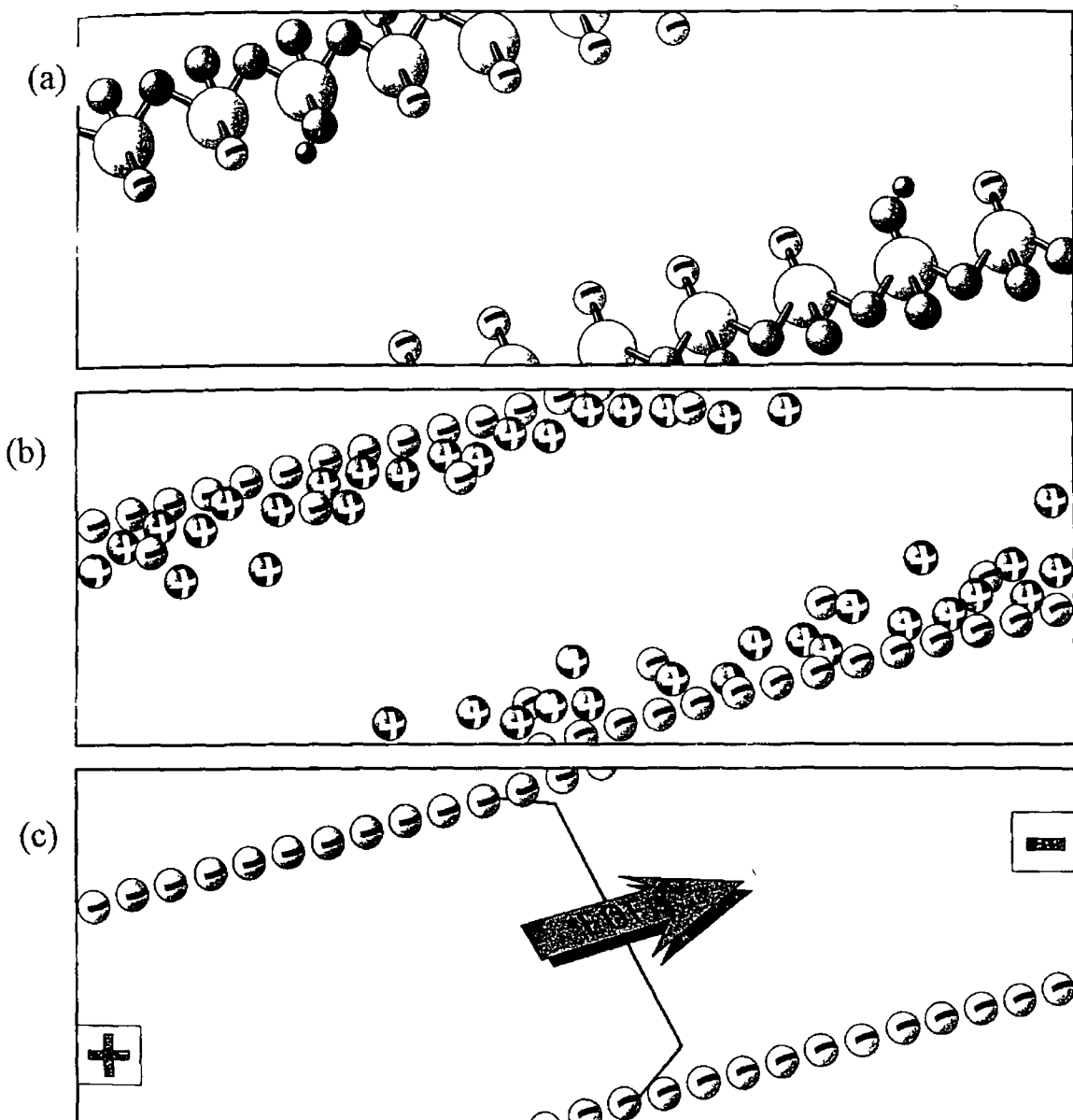


Fig 2 1 Development of the electroosmotic flow (a) negatively charged fused silica surface (Si-O^-), (b) hydrating cations accumulating near the surface, and (c) bulk flow towards the cathode on application of an electrical field (Reproduced from reference [7])

One unique feature of electroosmotic flow in a capillary is that it is plug-like and does not have the characteristic parabolic flow profile associated with pressure driven liquids [8]. The resultant flat flow profile is beneficial since it does not directly contribute to the dispersion of the solute zones.

Another benefit of the EOF is that it causes movement of nearly all species, regardless of the charge, in the same direction. Under normal conditions (that is, negatively charged capillary surface), the flow is from the anode to the cathode. Anions will be forced towards the cathode since the magnitude of the flow can be more than an order of magnitude greater than their electrophoretic mobilities [7]. Thus cations, neutral species, and anions can be electrophoresed in a single run since they all “migrate” in the same direction. This is shown in Fig. 2.2. Here, cations migrate fastest, since both the electrophoretic attraction towards the cathode and the EOF are in the same direction, neutrals are all carried at the velocity of the EOF but are not separated from each other, and anions migrate slowest since they are attracted to the anode, but are still carried by the EOF toward the cathode.

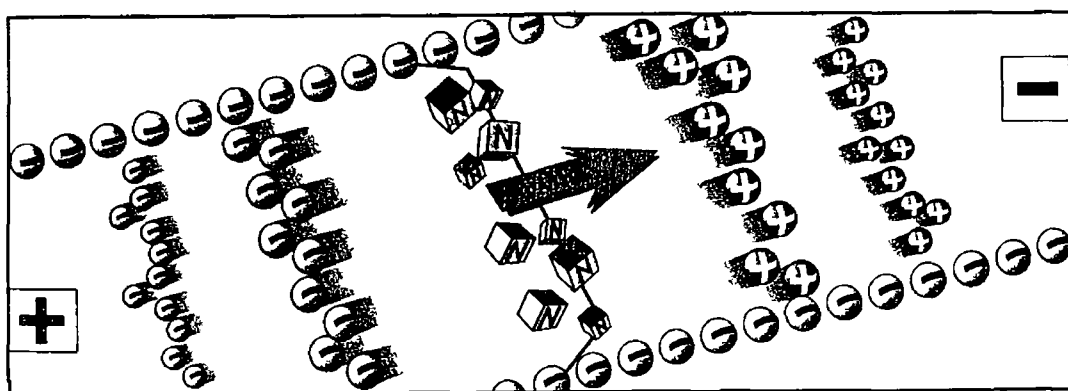


Fig. 2.2 Differential solute migration superimposed on electroosmotic flow in capillary zone electrophoresis (Reproduced from reference [7])

2 1 2 REVIEW OF CAPILLARY ZONE ELECTROPHORESIS IN BIOMEDICAL ANALYSIS

In recent years the number of publications involving capillary electrophoresis has increased steadily, and many reviews have been published in this field [6, 8-12] Capillary Electrophoresis has found application across the full spectrum of pharmaceutical and biomedical analysis

The equation for separation efficiency (N) given in equation 2 5 above takes into account the applied voltage (V), electrophoretic mobility (μ_{ep}) and the diffusion coefficient of the solute (D) With large molecules such as high molecular weight proteins, D is small, which means that very high efficiency separations can be achieved Although classical electrophoresis has been the traditional separation method for large biological molecules, difficulties are encountered because proteins can adsorb onto the surface of fused silica [8] As a consequence, a lot of effort has gone into the quest for ways of overcoming this problem One way of tackling the problem is to use a running buffer at a pH higher than that of the isoelectric point of the protein, whereby both the capillary surface and the protein carry negative charges, so that coulombic repulsion will take place thus minimising adsorption of the protein onto the capillary wall Another approach (and a more popular one) is to deactivate the capillary surface Hjerten [13] reported the bonding of α -methacryloxy-propyltrimethoxysilane to the surface of fused silica, in order to eliminate electroosmosis This in turn sterically prevented analytes from interacting with the capillary wall, reducing adsorption Since the work reported by

Hjerten, there have been many papers describing variants of this approach, for example, Bruin *et al* [14] modified the capillary surface by first coating α -glycidoxypyriltrimethoxysilane and then opening the resulting epoxide group by reacting with polyethyleneglycol-600 to give highly reproducible and stable capillaries. These capillaries were then used to separate mixtures of proteins. A different approach was taken by Widhalm *et al* [15], who described the separation of proteins using a non-cross-linked polyacrylamide gel. It was shown that the test proteins myoglobin, ovalbumin and bovine serum albumin eluted as their SDS complexes in order of increasing molecular mass.

The separation of nucleic acids and their fragments represents another challenging field for CZE. Dolnik *et al* [16] carried out a thorough investigation of various parameters affecting the separation of oligonucleotides. With polycytidines as model compounds, it was demonstrated that variations in pH between 5 and 8 and of ionic strength between 20 and 100 mM had little effect on the separation but if the background electrolyte also contained SDS and spermine, the separation order is considerably affected. Heiger *et al* [17] attempted high performance capillary electrophoretic separations using gel capillary columns of fluorescence-labelled single-stranded oligonucleotides for DNA sequencing. There are indications that HPCE polyacrylamide gel-filled capillary columns are probably better suited to DNA sequencing than CZE separations [18].

Capillary zone electrophoresis plays an important role within the analytical laboratory of most pharmaceutical companies because it is complementary to the most popular analytical technique of all, high performance liquid chromatography. There is an abundance of applications in the literature describing the analysis of small pharmaceutical compounds. Several workers in this area use micellar electrokinetic chromatography (MEKC). This is a hybrid of electrophoresis and chromatography [7], and is one of the most widely used HPCE modes. Its main strength is that it is the only electrophoresis technique that can be used for the separation of neutral solutes as well as charged ones. Fujiwara *et al* [19] were able to resolve seven water soluble vitamins in approximately 25 minutes using 0.02 M phosphate pH 9.0-0.05 M SDS as the electrolyte. Detection was by UV absorption at 254 nm. The MEKC method employed was used to determine the contents of a vitamin injection and was found to be highly accurate and reproducible and was much simpler than the existing gradient HPLC method.

Wainwright [20] reviewed the separation of pharmaceutical compounds using CZE with both coated and uncoated capillaries. Coated capillaries used for these studies were prepared by coating with polyacrylamide. A study of the effect of using coated and uncoated capillaries was undertaken using both positively and negatively charged compounds. This study found that the uncoated capillaries were more suited for work in this area, as it was possible to elute positive and negative analytes in one run.

Perrett and Ross [21] have reported on the rapid determination of drugs in biofluids by capillary electrophoresis, with an example made of the measurement of antipyrine in saliva for pharmacokinetic studies

CZE is also used in the analysis of chiral molecules. Although gas chromatography, and particularly HPLC have been used successfully in the past, CZE is now beginning to play an important role in the analysis of chiral molecules because of the extraordinary efficiencies achievable. Chiral recognition is achieved by the addition of a chiral selector to the electrolyte and examples of the types commonly used are (i) Cyclodextrins (ii) Crown ethers and (iii) Chiral surfactants [8]

Naylor *et al* [11] have discussed the monitoring of drug metabolism by capillary electrophoresis. The authors discuss the use of CE in the study of the reactivity of drug metabolites, analysis of biological samples and the problems associated with analysis in complex biological matrices. The authors point out that the separation by CE of metabolites that are present in complex biological matrices, e.g. microsomal preparations, blood (plasma and serum) and urine, presents a considerable analytical challenge. They suggest that, in part, this is due to the low concentration of the analytes present, as well as the complexity of the biological matrix, which generally contains a multitude of proteins, as well as numerous types of other components. Such proteins have been documented to have caused two problems: (1) drug metabolites adhere to protein surfaces, and hence cannot be separated by CE, and (2) protein introduced onto a capillary coats the wall, leading to analyte loss. Various approaches have been adopted to overcome such problems, including traditional sample

clean-up methods, such as liquid-liquid or solid-phase extraction prior to analysis by CE

The purpose of this study was to develop a CE method for the determination of free and total 7-OHC in urine and serum, with the intention of reducing the separation time needed in comparison to more traditional methods, for example those based on HPLC. The following sections describe how the separation was developed, optimised and applied to metabolic studies involving urine and serum.

2.2 EXPERIMENTAL

2.2.1 CHEMICALS

7-Hydroxycoumarin and the internal standard, 3-(α -acetylbenzyl)-4-hydroxycoumarin, were purchased from Sigma (St Louis, MO, USA). KH_2PO_4 and K_2HPO_4 were obtained from Riedel-de Haen (Hanover, Germany). 7-hydroxycoumarin standards were prepared from a 1 mg/ml stock solution in methanol (HPLC grade, Labscan, Dublin, Ireland)-deionised water (10:90, v/v). Serial dilutions of the 7-hydroxycoumarin (10 to 500 $\mu\text{g/ml}$) were prepared in deionised water. A 1 mg/ml stock solution of the internal standard was prepared in methanol for the analysis of free 7-hydroxycoumarin. The electrolyte solution used was 0.025 M phosphate buffer (pH 7.5), which was prepared fresh daily by preparing 0.02 M K_2HPO_4 and 0.005 M KH_2PO_4 in deionised water.

2.2.2 SAMPLE PREPARATION

Control urine was obtained from a volunteer who had not been treated with coumarin or 7-hydroxycoumarin. Urine from two volunteers, who had been treated with 100 mg coumarin, was obtained at specific time intervals (0, 2, 6, 10, 14 and 24 hr). The urinary volumes were recorded. Control serum was obtained from St James' Hospital, Dublin. It was not possible to analyse 7-hydroxycoumarin in serum or urine without extraction due to the endogenous species present in the matrix which absorb at 210 nm and interfere with the detection of 7-hydroxycoumarin present [11].

A 0.1 ml volume of 7-hydroxycoumarin standard and 0.9 ml of control urine or serum was added into a 10 ml sterile blood tube (Medlabs, Dublin, Ireland), giving a series of calibration standards from 0 to 50 $\mu\text{g/ml}$. A 1 ml volume of a urine sample from a volunteer was also added into a 10 ml tube for analysis. The internal standard (0.05 ml) was added to both standards and unknowns and vortex mixed for 20 seconds. Diethyl ether (3.5 ml) was then added to each tube to extract the 7-hydroxycoumarin and internal standard. The tubes were mixed by rotation for 10 minutes and centrifuged at 600 g for 10 minutes. A 1.8 ml volume of the diethyl ether layer was removed into a 75 \times 12 mm glass tube (M S C, Dublin, Ireland) and evaporated to dryness at 60 $^{\circ}\text{C}$. The sample was reconstituted into 0.1 ml of 0.025 M phosphate buffer (pH 7.5). Samples were not filtered before injection. Since 7-hydroxycoumarin is found predominantly in the 7-hydroxycoumarin-glucuronide form in urine, it must be treated with β -glucuronidase to liberate it to the free (non-conjugated) form for analysis. Therefore, the urine was treated with 1 ml of β -glucuronidase (Sigma) at 5000 units/ml in 1 M sodium acetate (Sigma) buffer, pH 5.0. The mixture was gently mixed and incubated at 37 $^{\circ}\text{C}$ for 30 min. There was no internal standard used in the determination of total 7-hydroxycoumarin. The sample was then analysed on a Beckman Capillary Electrophoresis instrument (P/ACE System 2050).

2 2 3 CAPILLARY ELECTROPHORESIS SEPARATION

The capillary used was a 27 cm x 50 μ m I D fused silica column (Beckman Instruments), with a capillary to detector distance of 19.3 cm. The preparation step for priming of the capillary was a 1 min rinse with 0.1 M sodium hydroxide, and then a 1 min rinse with electrolyte solution (0.025 M phosphate buffer, pH 7.5). The sample was applied to the capillary by a 3 second pressurized injection (0.5 psi) and separation achieved with an applied voltage of 20 kV (rise time 0.2 min) at 25 °C. Typical running current was 100 μ A. The resultant electropherogram was monitored at 210 nm with a fixed wavelength detector using Beckman System Gold software. Migration times for all components varied within ± 0.1 min due to slight differences in operating conditions e.g. capillary conditioning. The precision of migration time values could perhaps be improved by longer conditioning of the capillary with electrolyte solution. Concentrations for total and free 7-hydroxycoumarin in the volunteer's urine was calculated from the respective standard curves prepared.

For the analysis of free 7-hydroxycoumarin, plots of absorbance ratio versus concentration (μ g/ml) were made. Absorbance ratios for the analysis of free 7-hydroxycoumarin were calculated from the equation (absorbance of 7-hydroxycoumarin / absorbance of internal standard), and for total 7-hydroxycoumarin the concentration was calculated from a plot of 7-hydroxycoumarin absorbance versus standard concentration (μ g/ml). Inter-assay and intra-assay variation in absorbances, and absorbance ratios were assessed (n=5 (free), n=6 (total)) by analysing calibration standards and calculating the mean absorbances and absorbance ratios and their related

standard deviations Percentage relative standard deviations were also calculated

2.3 RESULTS AND DISCUSSION

2.3.1 DEVELOPMENT OF THE CE SEPARATION

Various buffers were first investigated in an attempt to separate coumarin and 7-OHC. Although several of the buffers showed some separation, phosphate buffer was chosen, as it gave rise to the best resolution between coumarin and 7-hydroxycoumarin. The pH of the phosphate buffer electrolyte (using 25 mM) was then investigated between pH 5.6 and pH 7.5, and the best separation between coumarin and 7-OHC was achieved at pH 7.5 (Fig. 2.3). The ionic strength of the buffer was then assessed. At 0.1 M, a very high running current was exhibited, but use of 0.025 M phosphate buffer concentration was found to decrease the current to 100 μ A. In the analysis of free 7-hydroxycoumarin, an internal standard, 3-(α -acetylbenzyl)-4-hydroxycoumarin, was utilised. However, the internal standard was not used in the determination of total 7-hydroxycoumarin.

A CE separation of a mixture of coumarin, 7-hydroxycoumarin and the internal standard is shown in Figure 2.4. From this it can be seen that the three compounds are well separated with baseline resolution within 1.2 minutes. Coumarin has a net zero charge at pH 7.5 and migrates with the solvent front, i.e. at the same migration time as a neutral marker, benzamide.

This means that the method described above cannot be used for the quantification of coumarin in biological fluids

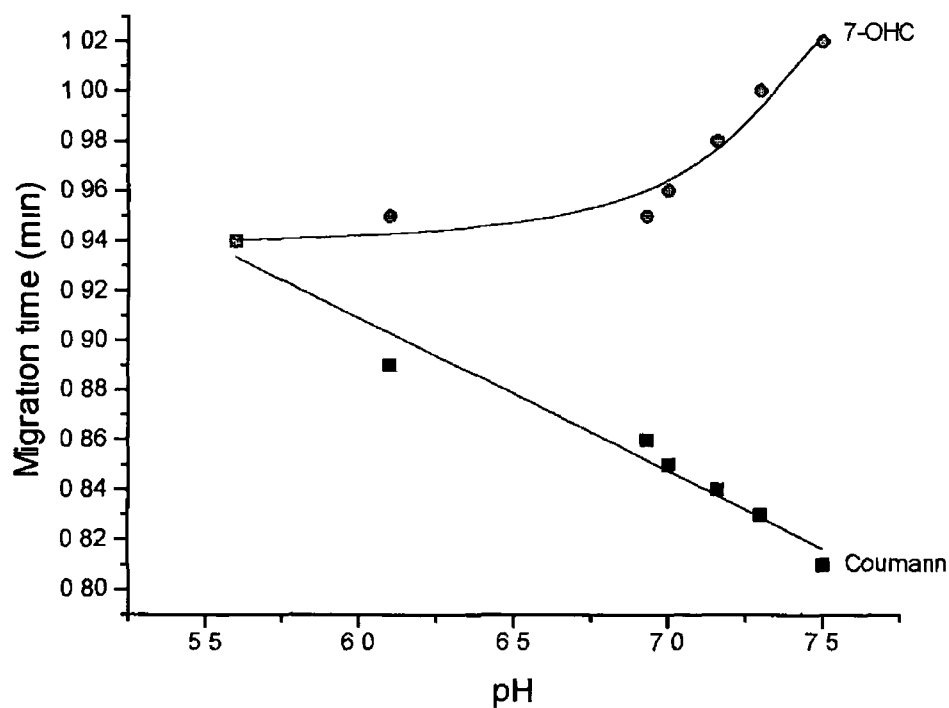


Fig 2.3 Plot showing separation of coumarin and 7-OHC between pH 5.6 and pH 7.5 in 25 mM phosphate buffer electrolyte

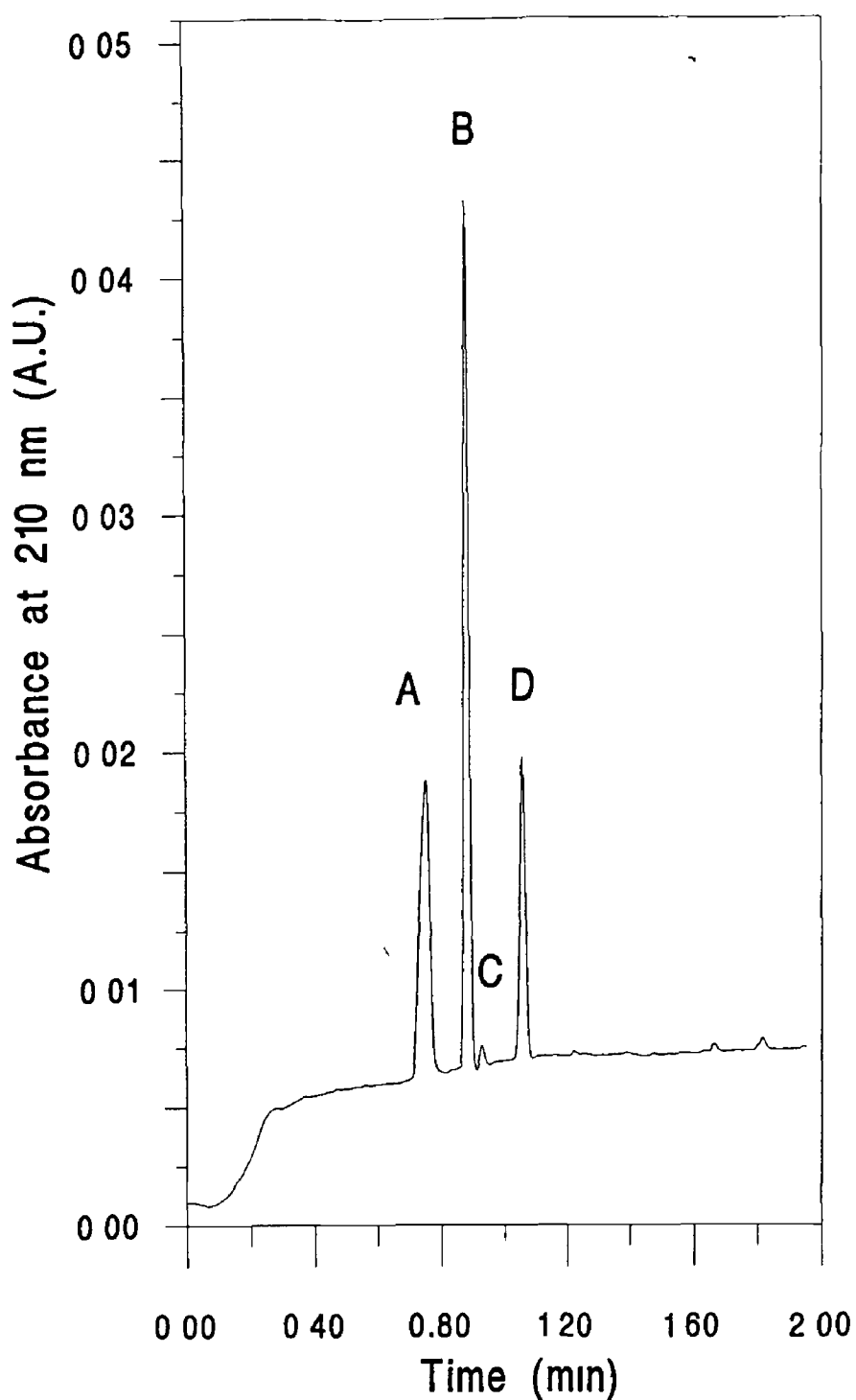


Fig 2 4 Capillary electrophoresis separation of (A) coumarin, (B) 7-OHC, (C) contaminant of 7-OHC standard, and (D) internal standard They were prepared in 0 025M phosphate buffer, pH 7 5 and analysis was carried out as in Experimental

At the pH chosen, the separation of coumarin from 7-hydroxycoumarin is achieved by virtue of the charge difference between the two compounds (7-hydroxycoumarin is partially negatively charged at pH 7.5). The separation was then applied to the analysis of urine and serum samples as outlined under the Experimental section. The separation of the compounds in the urine extract is shown in Figure 2.5, where it can be seen that there are only minimal interferences to the analysis of 7-hydroxycoumarin from co-extracted endogenous species in the urine. The CE analysis of total 7-hydroxycoumarin in a urine sample taken from a volunteer 6 hours after administration of coumarin is shown in Figure 2.6. It indicates that most of the interferences have longer migration times compared with those of the drug and its metabolite. This figure also shows that the internal standard cannot be used in the analysis of total 7-hydroxycoumarin owing to the greater number of interferences occurring at its migration time. There was also irreproducible extraction of the internal standard after the treatment of the urine sample with β -glucuronidase. Efforts to find an alternative internal standard which would elute later in the electropherogram and extract reproducibly, proved fruitless.

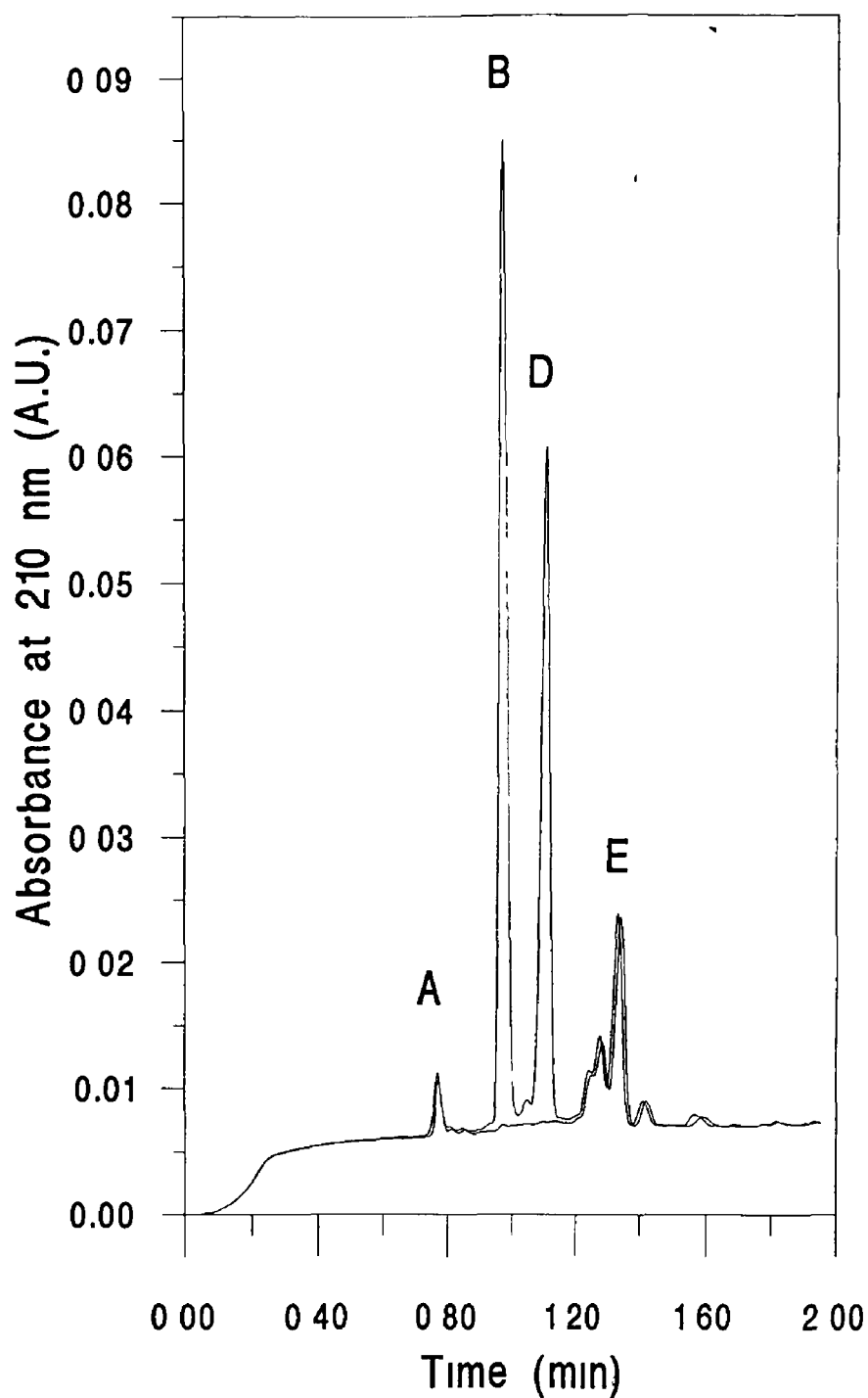


Fig 2.5 Electropherograms of blank urine and 50 $\mu\text{g/ml}$ 7-hydroxycoumarin standard and internal standard prepared in urine (A) solvent front, (B) 7-hydroxycoumarin, (D) internal standard and (E) co-extracted endogenous species. The samples were prepared and analysed as outlined in Experimental. The figure shows an overlay of the two electropherograms.

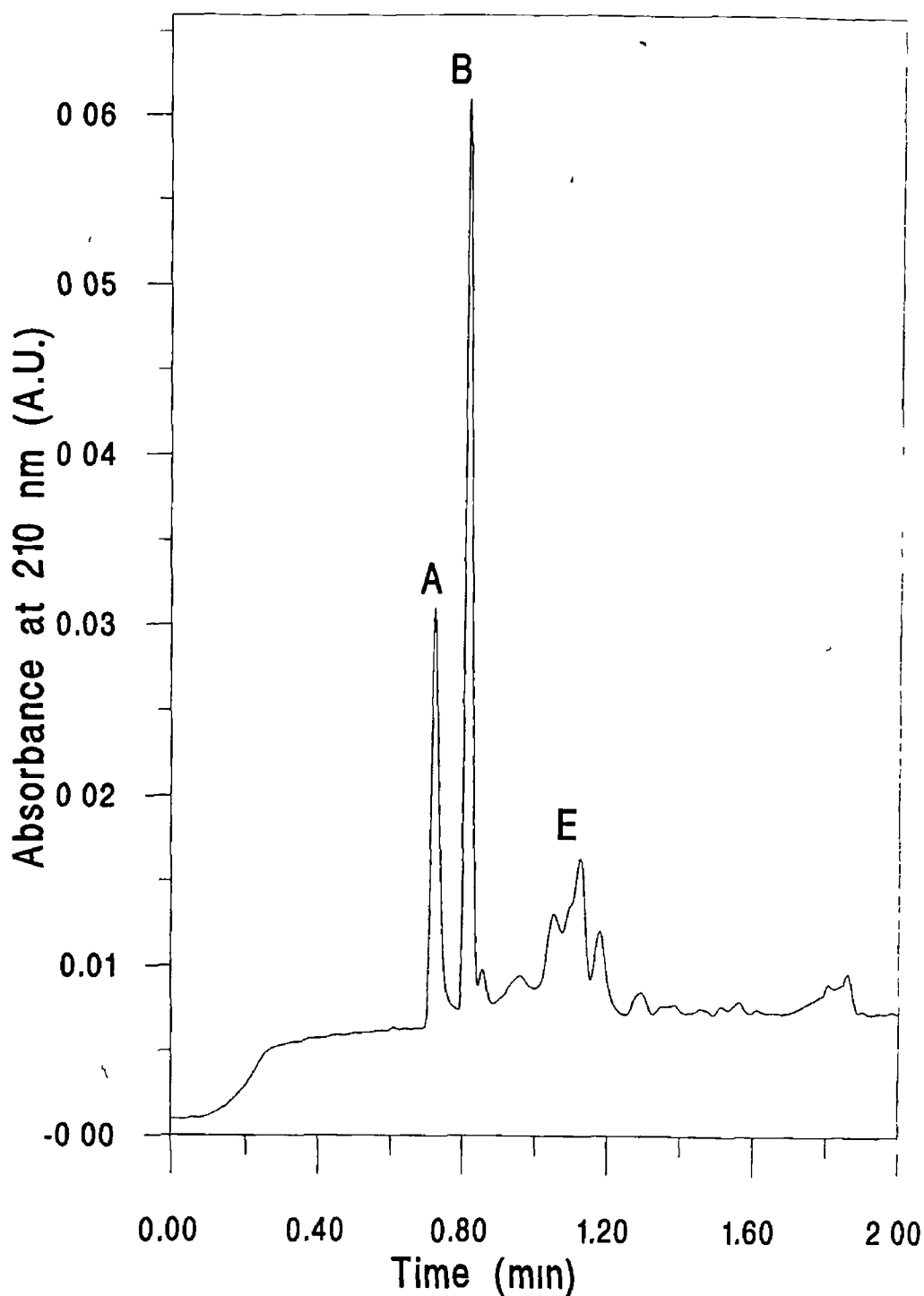


Fig 2 6 CE analysis of total 7-hydroxycoumarin in a urine sample from volunteer 2, 6 hours after administration of coumarin (A) Solvent front containing any compounds which would be liberated by the addition of β -glucuronidase, thus explaining the increase in absorbance at the solvent front as compared to untreated urine, (B) 7-hydroxycoumarin and (E) co-extracted endogenous species in urine

The CE analysis of a serum extract is shown in Figure 2.7. From this it can be seen that there is good separation between the metabolite, the internal standard and the majority of the co-extracted interfering substances.

The method was then compared to the previously developed HPLC method [22] for the quantification of 7-hydroxycoumarin in the urine of volunteers who had been administered coumarin. Tables 2.1 and 2.2 compare the results obtained from each of the methods. Figure 2.8 shows the comparison of CE analysis versus HPLC analysis of total 7-OHC excretion for volunteer 2. This plot shows that the two methods of analysis give the same profile for excretion of 7-OHC by a given volunteer. When, however, one compares the run times for the samples, one immediately realises the diminution in the time for the determination step (at least eight fold) between the two techniques. The method also has a fast regeneration step to recondition the capillary column between every analysis. There are no carryover problems, less solvents are used, and there is minimal organic solvent waste as compared with the reversed-phase HPLC method. To improve the sensitivity of the method, 324 nm could be used as the detection wavelength, as in the HPLC [22] method. However, as with the HPLC method, a clean-up procedure is needed for the biological samples prior to analysis [11].

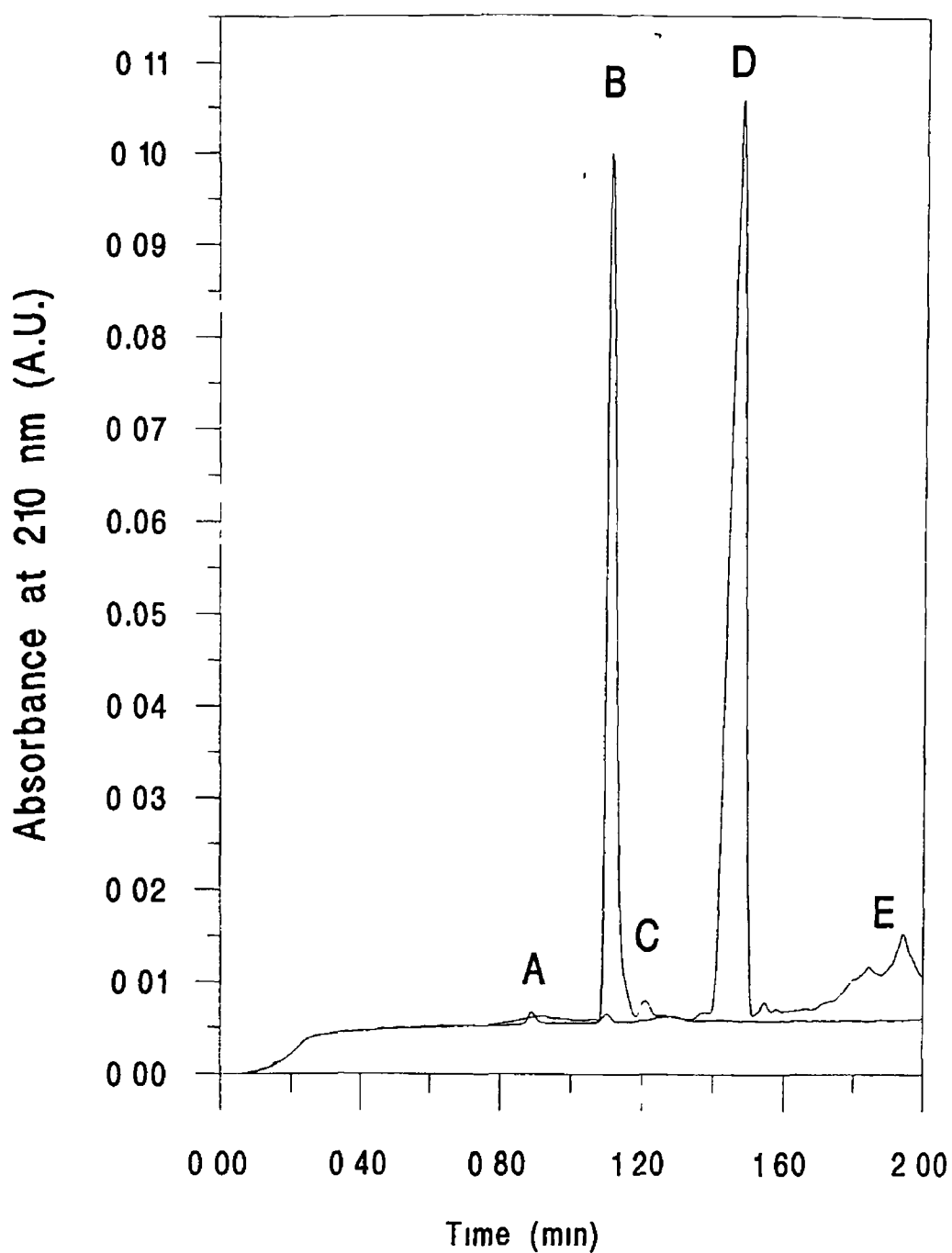


Fig 2.7 Electropherograms of blank serum and 50 $\mu\text{g/ml}$ 7-hydroxycoumarin standard and internal standard in serum (A) Solvent front, (B) 7-hydroxycoumarin, (C) contaminant in standard, (D) internal standard, and (E) co-extracted endogenous species in serum

7-OHC concentration (µg / ml)						
	Volunteer 1			Volunteer 2		
Time (hr)	Free (CE)	Total (CE)	Total (HPLC)	Free (CE)	Total (CE)	Total (HPLC)
0	1.4	0.0	0.0	0.0	1.0	1.2
2	5.9	429.0	479.0	9.0	735.0	759.0
6	0.0	77.2	73.3	0.0	42.7	35.6
10	1.0	25.8	23.6	0.0	3.8	4.0
14	1.7	11.3	9.3	0.0	0.0	0.0
24	0.0	0.0	1.0	0.0	0.0	1.0

Table 2.1 Comparison of HPLC and CE analysis of urinary concentration of 7-OHC excreted over 24 hours following oral administration of 100 mg of coumarin

7-OHC excreted (mg)				
	Volunteer 1		Volunteer 2	
Time (hr)	CE	HPLC	CE	HPLC
0	0 0	0 0	0 50	0 6
2	64 35	71 85	73 50	75 90
6	23 16	21 99	25 62	21 36
10	3 61	3 30	2 09	2 20
14	1 13	0 93	0 0	0 0
24	0 0	0 30	0 0	0 4
Total	92 25	98 37	101 71	100 46
Coumarin excreted*(%)	83 1	88 7	91 7	90 5

* % Coumarin excreted calculated from molecular mass ratios (0 9012), i e
(mol wt coumarin)/(mol wt 7-OHC)

Table 2 2 Comparison of HPLC and CE analysis for determination of the amount of 7-hydroxycoumarin, in milligrams, excreted and % coumarin excreted over a 24 hour period

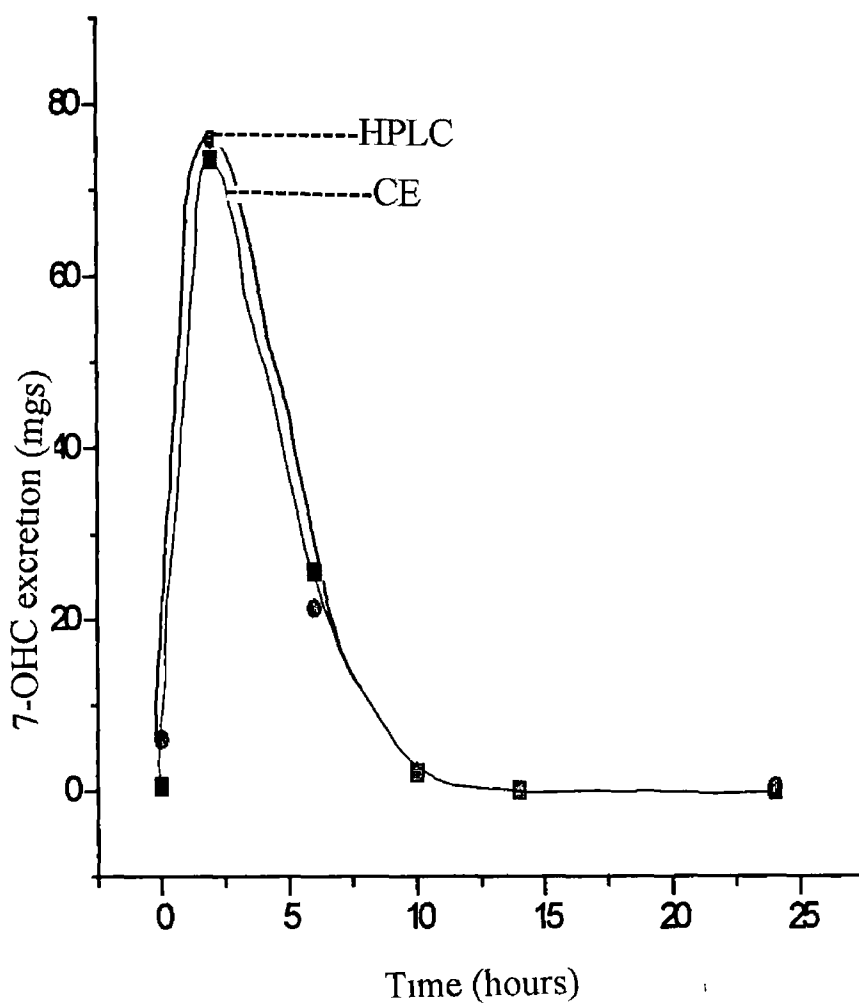


Fig 2 8 Plot of CE analysis versus HPLC analysis of total 7-OHC excreted over 24 hours for volunteer 2

2 3 2 LIMIT OF QUANTIFICATION AND LINEARITY

In both urine and serum the limit of quantification of 7-hydroxycoumarin was found to approximately 1 0 µg/ml and the linear detection range for the analysis of the drug was from 1 to 50 µg/ml Correlation coefficients were always better than 0 990

2 3 3 ACCURACY AND PRECISION

Inter- and intra- assays for the determination of 7-hydroxycoumarin were carried out and the respective mean absorbances and mean absorbance ratios, and standard deviations calculated (Tables 2 3 and 2 4) The percentage relative standard deviation was found to be within 0 8% to 7 3% The inter-assay accuracy and precision for free 7-hydroxycoumarin was determined over a five day period The intra-assay accuracy and precision for free drug was determined over five calibration sets on one specific day The inter-assay analysis for total drug was determined over six days The use of the internal standard was not of significant benefit for the determination of free 7-hydroxycoumarin

Concentration ($\mu\text{g} / \text{ml}$)	Absorbance ratio (mean \pm S.D.) Free 7-OHC	R.S.D. (%)	Absorbance (mean \pm S.D.) Total 7-OHC	R.S.D. (%)
0	0.0	0.0	0.0	0.0
1	4.0E-2 \pm 2.8E-3	7.0	2.35E-3 \pm 1.59E-4	6.8
5	0.1541 \pm 1.13E-2	7.3	8.58E-3 \pm 5.30E-4	6.2
10	0.2951 \pm 9.90E-3	3.4	1.70E-2 \pm 9.25E-4	5.4
20	0.5812 \pm 2.20E-2	3.8	3.09E-2 \pm 1.68E-3	5.4
50	1.278 \pm 3.63E-2	2.8	7.13E-2 \pm 3.25E-3	4.6

Table 2.3 Inter-assay precision and accuracy for free and total 7-hydroxycoumarin analysis following extraction from urine (free n = 5, total n = 6)

Concentration ($\mu\text{g/ml}$)	Absorbance ratio (mean \pm S D)	R S D (%)
0	0	0 0
1	3 52E-2 \pm 1 90E-3	5 4
5	0 1509 \pm 7 20E-3	4 8
10	0 2809 \pm 6 36E-3	2 3
20	0 5221 \pm 2 35E-2	4 5
50	1 1750 \pm 8 90E-3	0 8

Table 2 4 Intra-assay precision and accuracy for free 7-hydroxycoumarin analysis following extraction from urine (n=5)

2 3 4 CLINICAL AND PHARMACOKINETIC STUDIES

The method developed above was applied to urine samples obtained from two volunteers who had been administered coumarin. Their urine was analysed for free and total 7-hydroxycoumarin. During the analysis no free or conjugated coumarin was observed in the volunteer's urine (this was determined from HPLC analysis). All of the coumarin appears in either of the 7-hydroxylated forms. The concentrations of free and total 7-hydroxycoumarin present were calculated from standard curves prepared on the day of analysis and the results obtained were related to the urinary volumes excreted giving the total amount of 7-hydroxycoumarin excreted in milligrams. Percentage of administered coumarin, excreted as 7-hydroxycoumarin was then calculated from molecular weight ratios (Table 2 2)

The results showed that up to 70 % of the coumarin is excreted in the first two hours and that approximately 90 % is excreted within 24 hours. These results are in accordance with those of Egan *et al* [22] and Rautio *et al* [23]. In the comparison of the results from CE and HPLC analysis of the volunteer's urine samples there was no statistical difference (Tables 2 1 and 2 2)

2.4 CONCLUSION

The use of capillary electrophoresis for the determination of 7-hydroxycoumarin in urine and serum was found to be straight forward and reliable, and in comparison to more established methods (e.g. HPLC), the separation step was much more rapid. To determine concentrations of 7-hydroxycoumarin in a pharmacokinetic study or clinical trial [24], where the coumarin dosage is from 100 mg upwards, the use of CE would be ideal due to the availability of autosamplers and the speed at which the data is obtained. Thus the analysis of multiple samples can be made faster. The total preparation time of the samples does not vary from the other methods available [22], but the analysis time is greatly decreased. To analyse 7-hydroxycoumarin at lower concentrations, other detection methods could be utilised [25,26]. The availability of photodiode array detectors, computer controlled systems, the minimal solvent waste involved, and the ease of method development makes CE a very useful and rapid analytical tool in the analysis of biological samples.

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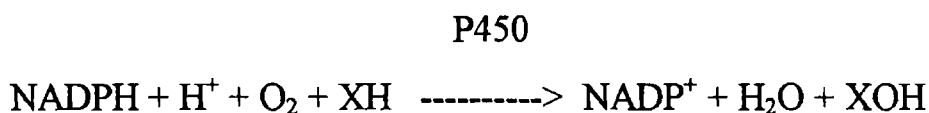
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CHAPTER 3

STUDY OF COUMARIN METABOLISM BY LIVER MICROSOMES USING CAPILLARY ELECTROPHORESIS AND ELECTROCHEMISTRY

3.1 INTRODUCTION

The metabolism of coumarin or any compound in the human body can be described by the general reaction shown below



where P450 is a cytochrome P450 enzyme and XOH is a metabolite which in the case of coumarin is mainly 7-OHC (in humans). NADPH (nicotinamide adenine dinucleotide phosphate) is needed to donate two electrons to the heme iron of cytochrome P450 [1]. The above reaction is referred to as a monooxygenation and the enzyme as a monooxygenase because only one of the two oxygen atoms is incorporated into the substrate. Microsomal preparations from liver contain cytochrome P450 isoforms and have been used to study the metabolic pathways of drugs [2-5].

The cytochrome P450's are a family of heme proteins present in all mammalian cell types, with the exception of mature red blood cells and skeletal muscle cells. These enzymes catalyse the oxidation of a wide variety of structurally diverse compounds. Substrates for these enzyme systems include endogenously synthesised compounds such as steroids, and compounds such as drugs, food additives or industrial by products that enter the body through food sources, ingestion, inhalation from the air or absorption through the skin. The cytochrome P450 has many effects in medicine. It has roles in (a) inactivation or activation of therapeutic

agents, (b) metabolism of chemicals to highly reactive molecules, which may produce unwanted cellular damage, cell death or mutations, (c) participation in several steps in steroid hormone biosynthesis, and (d) metabolism of fatty acids and their derivatives [1]

3 1 1 NOMENCLATURE

The original designation of a protein as cytochrome P450 originated from its spectral properties. The cytochrome P450 enzymes have a unique absorbance spectrum which is obtained by adding a reducing agent (e.g. sodium dithionite), to a suspension of endoplasmic reticulum vesicles (microsomes), this is followed by the addition (through bubbling) of carbon monoxide gas into the solution. The carbon monoxide becomes bound to the reduced heme protein and this produces an absorbance spectrum with a peak at 450 nm. Specific forms of cytochrome P450 can differ in their maximum absorbance wavelength, and the range varies between 446 and 452 nm [1]

It is clear that in mammalian cells, cytochrome P450's serve as terminal electron acceptors and monooxygenases in electron transport systems, which are contained either in the endoplasmic reticulum or the inner mitochondrial membrane. The cytochrome P450 structure has present a single iron group, and the resulting heme protein has binding sites for both an oxygen molecule and the particular substrate. The heme iron of all known cytochrome P450's is bound to the four pyrrole nitrogen atoms of the porphyrin ring and two axial ligands. The heme iron can exist in two

different spin states (1) a hexa-coordinated low spin iron, or (2) penta-coordinated high-spin state. The low and high spin states are descriptions of the electronic shells around the iron atom. When a substrate binds to cytochrome P450, there is a change in these electronic shells and the heme iron atom changes from the hexa-coordinated to the penta-coordinated state. The substrate-bound, penta-coordinated state has a more positive reduction potential than the hexa-coordinated unbound state. This accelerates the rate at which cytochrome P450 may be reduced by electrons donated from NADPH (Fig 3.1). In order for the hydroxylation (monooxygenation) reaction to occur, the heme iron must be reduced from the Fe(III) to the Fe(II) state, so that oxygen may bind to the heme iron. A total of two electrons are required for the monooxygenation reaction [1].

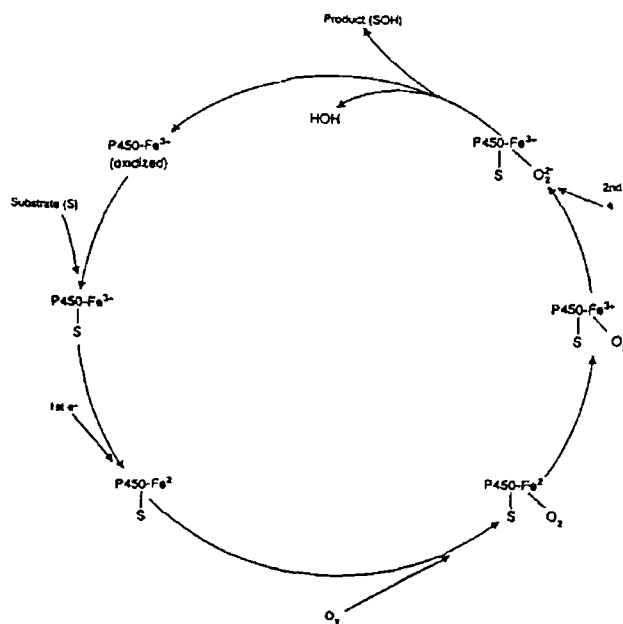


Fig 3.1 The sequence of reactions at cytochrome P450. The diagram demonstrates the binding of substrate, transfer of the first and second electrons, and binding of molecular oxygen. Reproduced from Ref [1].

3 1 2 SPECIFICITY

It has been known since the mid-1950's that it is possible to insert one of the atoms of molecular O_2 into a substrate being metabolised. This process of monooxygenation is also performed by other specialised proteins such as hydroxylases but none of these proteins display the versatility of the members of the cytochrome P450 family. It is now clear that many forms of cytochromes P450 have emerged due to gene duplication events occurring in the last 5-50 million years. The different forms of cytochrome P450, which are now known among the various animal species, have most likely arisen from the selective pressure of environmental influences, such as dietary habits or exposure to environmental agents [1]

By 1990, over 150 cytochrome P450 genes, coding for different proteins catalysing the oxidation of a variety of endogenous substrates had been characterised. One of the ways of characterising these enzymes is by substrate specificity. While this has been possible with many of the members of this family, the similarity of molecular weights and other molecular properties has made purification of individual cytochrome P450's from the same organ very difficult.

The induction of various cytochromes P450 by both endogenous and exogenous compounds has been known since the mid 1960's. The mechanism of the induction of cytochrome P450 has been demonstrated to be at the transcriptional or post-transcriptional level and it is not possible to predict the mode of induction based on the inducing compound. For some time it was thought that understanding the control of the intracellular

level of a protein was primarily a question of knowing what controlled the synthesis of that protein i.e. a question of genetic regulation. More recently, it has been learned that intracellular protein degradation is also important in determining the enzyme level. Clearly the intracellular concentration of an enzyme molecule is determined by both its rate of synthesis and its rate of degradation [6]

3.1.3 CYTOCHROME P450 ELECTRON TRANSPORT SYSTEMS

It is well known that the cytochrome P450-catalysed reaction requires $2e^-$ to accomplish its task of heme iron reduction, oxygen binding and oxygen cleavage, but a basic mechanistic problem is the direct and simultaneous transfer of electrons from NADPH to cytochrome P450. Pyridine nucleotides are $2e^-$ donors, but cytochrome P450, with its single heme prosthetic group, may only accept $1e^-$ at a time. As a result of this, a protein that serves to transfer electrons from NADPH to the cytochrome P450 molecule must have the capacity to accept $2e^-$ but operate as a $1e^-$ donor. This problem is overcome by the presence of a NADPH-dependent flavoprotein reductase, which accepts $2e^-$ from NADPH simultaneously, but transfers the electrons individually to cytochrome P450. The transfer of electrons from NADPH to cytochrome P450 is accomplished by two distinct electron transport systems that reside almost exclusively in either mitochondria or endoplasmic reticulum [1]

NADPH donates electrons to a flavoprotein called NADPH-cytochrome P450 reductase, in the endoplasmic reticulum. This reductase has a mass of approximately 78,000 and contains both flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) as prosthetic groups. A significant number of residues at the amino end of the molecule are hydrophobic, and this portion of the molecule is embedded in the endoplasmic reticulum (Fig 3.2).

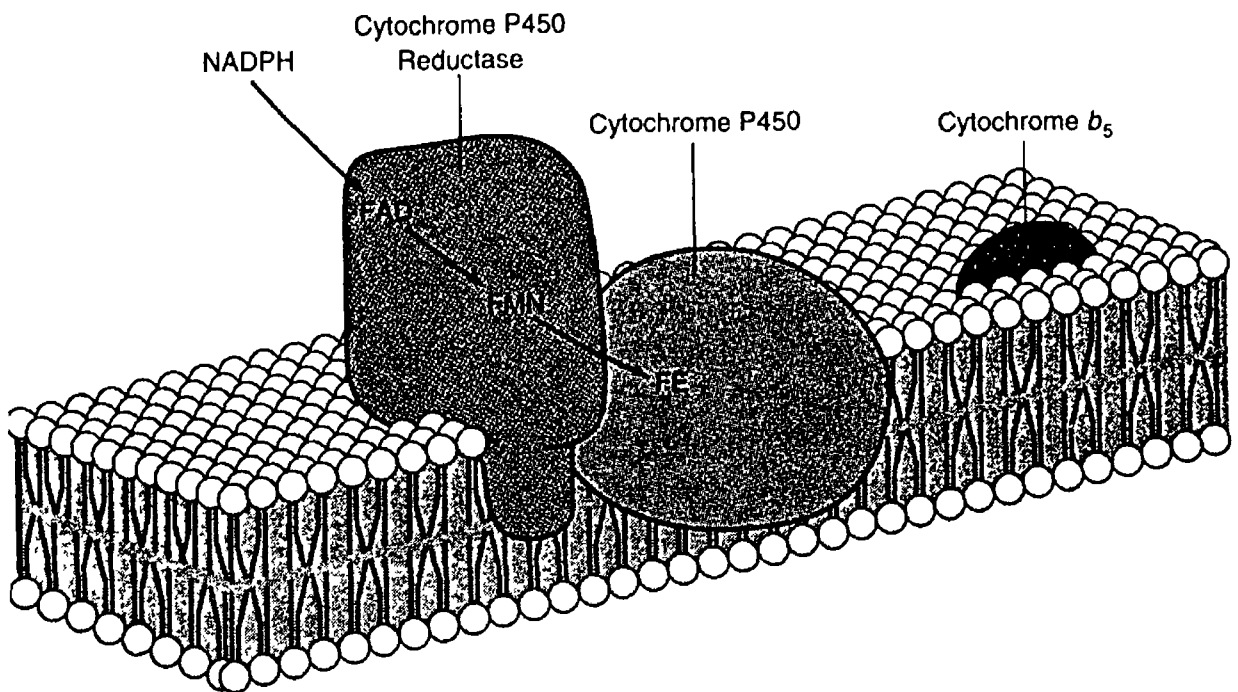


Fig 3.2 The sequence of reactions at cytochrome P450. The diagram demonstrates the binding of substrate, transfer of the first and second electrons, and binding of molecular oxygen. Reproduced from Ref [1]

The FAD serves as the entry point for electrons from NADPH, and FMN serves as the exit point, transferring electrons individually to cytochrome P450. Because the flavin molecule may exist as 1 or 2e⁻ reduced forms and two flavin molecules are bound per reductase molecule, the enzyme may receive electrons from NADPH and store them between the two flavin molecules before transferring them individually to the heme iron for oxygen binding (first electron) and cleavage of the oxygen molecule (second electron), see Fig 3.1

3.1.4 PREVIOUS WORK ON COUMARIN METABOLISM USING LIVER MICROSOMES

Fentem and Fry [7-10] have done significant work on monitoring coumarin metabolism in liver microsomes. Most of this work has been done with the aid of HPLC. In 1990, Fentem *et al* [7] reported on the effect of various inducers of cytochrome P450 on the metabolism of coumarin by rat liver microsomes. Liver microsomes were prepared from rats given sodium phenobarbitone, β -naphthoflavone, isoniazid or pregnenolone-16- α -carboxitrile. The incubation mixture contained the microsomes, phosphate buffer, MgSO₄, NADP⁺, glucose-6-phosphate, glucose-6-phosphate dehydrogenase and coumarin. The mixture was then incubated for 10 minutes followed by the addition of trichloroacetic acid (TCA) to stop the reaction; the TCA had the effect of precipitating out protein. The precipitated protein was then removed by centrifugation, and the supernatant assayed by HPLC. The HPLC system consisted of a Spherisorb 5 ODS-2 column, UV detection at 280 nm and a flow rate of 2

ml/min, with the column being maintained at 40°C. Gradient elution was used for the separation with mobile phase A = formic acid/water (5/95) and B = methanol. The gradient program was 2 stage, 0-2 min, 10-20% B, 2-25 min, 20-65% B. This produced a good separation of coumarin from 10 of its reported metabolites. One of the major drawbacks of the HPLC method was that the protein from the incubation mixture had to be removed prior to injection onto the HPLC system. This is a common problem with standard reversed-phase columns as the protein would rapidly degrade the column.

Fentem and Fry [8] again used this HPLC method to study the effect of cimetidine on the metabolism of coumarin by rat, gerbil and human liver microsomes. Fentem *et al* [9] also reported on a novel metabolite of coumarin (o-hydroxyphenylacetaldehyde) formed by rat, gerbil and human liver microsomes. The above HPLC method was used to follow metabolism along with isolating and partially purifying the new metabolite by collecting appropriate fractions of the column eluent. The new metabolite was identified by mass-spectral data.

Metabolism of coumarin by rat, gerbil and human liver microsomes was then reported by Fentem and Fry in 1992 [10]. This above HPLC method was central to the analysis of the metabolites. This publication shows the separations achieved after the incubations (the protein had been precipitated out by TCA and centrifugation). The separation, however, exhibited a poor baseline (even after protein precipitation), underlining the problems which can be encountered when trying to separate components of biological reactions. The matrix can be extremely complex, leading to problems in achieving a good baseline throughout a separation.

Lake *et al* [11-17] have also done a lot of work on monitoring coumarin metabolism in liver microsomes. In 1980, Walters *et al* [11] reported on their investigations which were undertaken in order to develop a HPLC procedure capable of resolving and quantitating the known metabolites of coumarin which would be applicable to the determination of metabolites in biological samples and tissue incubates. A number of different stationary phases were evaluated in this work, with the best results being achieved with a Partisil-10 ODS-2 column, along with a two stage linear gradient. Reservoir A contained THF:water:acetic acid (10:90:0.1), reservoir B contained THF:water:acetic acid (20:80:0.1). Elution was with 46% B for 16 min followed by a linear increase of 1% per min in B up to 62% B (i.e. over 16 min). This was held for 8 min, then 5% increase in B up to 100% B. This method was able to separate O-HPLA, 8-OHC, 6-OHC, 7-OHC, C, 5-OHC, 3-OHC, 4-OHC and o-coumaric acid. Difficulty was encountered when working with real biological samples and ultracentrifugation of incubation mixtures was needed.

The effect of inducers of cytochrome P450 on the metabolism of [3-¹⁴C]coumarin by rat hepatic microsomes was reported by Peters *et al* [12] in 1991. Metabolic studies on radio-labelled coumarin were carried out using HPLC as the analytical tool. The metabolites were separated by using reversed-phase liquid chromatography employing a Partisil 10 ODS-2 column and a mobile phase containing 25% THF in water (A), water (B) and 1% (w/w) formic acid in water (C). Elution was achieved by starting with 22% A, 58% B, 20% C and after 20 minutes changing to 36% A, 44% B, 20% C using a concave gradient over 15 minutes and then to 80% A, 0% B, 20% C over 7 minutes and held for a further 30 minutes before re-equilibration of the column. The eluent was monitored at 273 nm.

The metabolism of [3-¹⁴C]coumarin to polar and covalently bound products by hepatic microsomes from the rat, hamster, gerbil and human was reported on by Lake *et al* [13] in 1992. The metabolism of the radio-labelled coumarin was studied by HPLC, with the metabolites being determined by comparison with retention times of known standards and the concentrations being quantified by measurement of the radioactivity present in the appropriate HPLC fractions. Incubations were terminated with ice cold methanol, followed by centrifugation prior to injection onto the HPLC system. The HPLC system (same as was used in [12] above) could separate coumarin and 13 of its metabolites, but it suffered from the necessity to centrifuge the samples prior to analysis.

Lake *et al* [14] also used this method to identify o-hydroxyphenylacetaldehyde as a major metabolite of coumarin in rat hepatic microsomes. The metabolism of [3-¹⁴C]coumarin by human liver microsomes was then studied in 1994 by Van Iersel *et al* [15] using this HPLC method and Steensma *et al* [16] also used it to study the metabolism of coumarin and 7-ethoxycoumarin by rat, mouse, guinea pig, monkey and human liver slices. Very recently Lake *et al* [17] again used the method to study the metabolism of coumarin by calf liver microsomes.

HPLC was also used by Goeger and Anderson [18] in the biochemical characterisation of coumarin 7-hydroxylase activity in chick embryo liver microsomes. After incubation, the reactions were terminated by the addition of methanol followed by centrifugation. The HPLC system consisted of a μ Bondapak C₁₈ column with an isocratic mobile phase of methanol-water-acetic acid, at a flow of 2 ml/min. The 7-OHC produced was monitored using a fluorescence detector.

Other methods have also been used to study the metabolism of coumarin by liver microsomes. In 1980, Legrum and Netter [19] used gas chromatography-mass spectroscopy (GC-MS) to study the characteristics of coumarin metabolism by liver microsomes from cobalt-pretreated mice. Reactions were stopped by the addition of TCA followed by centrifugation and the supernatant was extracted with diethyl ether. Derivatisation was by methylation, which was carried out by passing diazomethane into the extract. Isolation of the metabolites was by TLC followed by GC-MS.

The purpose of this present study was to develop a capillary electrophoresis method to study the metabolism of coumarin without any sample preparation. The separations are also carried out in much shorter times. The following sections will describe how the separation was developed, optimised and applied to metabolic studies using human liver microsomes, with a separate study on interspecies differences in coumarin metabolism. Electrochemical methods were also used in an effort to follow the metabolism of coumarin.

3.2 A STUDY OF COUMARIN METABOLISM BY CAPILLARY ELECTROPHORESIS

3 2 1 EXPERIMENTAL

3 2 1 1 Chemicals

7-hydroxycoumarin, NADPH, NADP⁺, D-glucose-6-phosphate and glucose-6-phosphate dehydrogenase were purchased from Sigma (St Louis, MO) KH₂PO₄ and K₂HPO₄ were obtained from Riedel-de Haen (Hanover) 7-OHC standards were prepared from a 1 mg/ml stock in methanol (HPLC grade, Labscan, Dublin) deionised water (10 90, v/v) by dilution in deionised water The electrolyte solution used when monitoring metabolite formation was 0 025 M phosphate buffer, pH 7 0 The pH was adjusted to pH 7 5 when monitoring NADPH and NADP⁺ migration The electrolyte was prepared daily by dissolving 0 02 M K₂HPO₄ and 0 005M KH₂PO₄ in deionised water Coumarin (2.05×10^{-4} M in 0 1% methanol 99 9% ultra pure water) was donated by Schaper and Brummer

For the interspecies investigations the electrolyte used was 50 mM phosphate buffer, pH 6 8

3 2 1 2 Tissue preparation

The five donors of human microsomes were patients either with carcinoma of the colon or small intestine and a single liver metastasis, or patients with primary liver tumours, who all underwent partial hepatectomy Portions of macroscopically healthy liver, taken from the excised lob, were chilled immediately in 1 15% KCl (0°C) and were then frozen in liquid nitrogen

within a few minutes. Microsomes were obtained by differential centrifugation using the sucrose method [20]. The characteristics of the donors are summarised in Table 3.1. The protein concentrations of these microsome preparations were calculated using the bicinchoninic acid (BCA) assay (Pierce).

Table 3.1 Details of liver donors

Liver	Donor sex	Donor age (years)	Smoking status
P13	F	64	NS
P14	F	48	NS
P17	F	54	S
P18	F	44	NS
R1	M	54	NS

For the interspecies investigations, a kit of microsomes of different species (beagle dog, cynomolgus monkey, New Zealand white rabbit, female rat SD-OFA) was purchased from IFFA Credo, L'Arbresle Cedex, France. Values for protein concentrations in the microsomal preparations were included for each species. Livers from other species (gerbil, mouse, (Schofield, CD1), bovine, porcine) were obtained from freshly sacrificed animals. Microsomal preparations from these species were prepared by the

sucrose method of Wolff *et al* [19] Protein concentrations were determined by the Bicinchoninic Acid (BCA) Assay (Pierce, Illinois, USA)

3.2.1.3 Bicinchoninic Acid (BCA) Protein Assay

The protein concentration of the microsomes were determined by this assay method. In this assay Cu(II) combines with protein, under alkaline conditions to form Cu(I), which reacts with BCA to give a coloured product. Protein was detected quantitatively using BCA (Pierce). Two separate reagents are supplied: reagent A, an alkaline bicarbonate buffer, and reagent B, 4 % (w/v) copper(II) sulphate solution. The working solution required the addition of 50 parts of reagent A to 1 part of reagent B. A range of protein standards (0-1 mg/ml) were prepared in 0.1 M PBS, pH 7.4. 10 µl of standard or sample was added to 190 µl of working solution in a well of a 96 well microtitre plate. For control wells, 10 µl of diluent was used. The plate was incubated at 37°C for 30 min. The absorbance was recorded at 562 nm on a Titretek Twinplus reader. All samples and standards were prepared in duplicate. The protein concentration of the unknown samples were determined from a standard curve.

3 2 1 4 Incubation solution

The concentrations of the NADPH-generating system in all human liver microsome incubations was 4U/ml glucose-6-phosphate dehydrogenase, 2 mg/ml D-glucose-6-phosphate, and 1.2 mM NADP⁺. This was higher than that required to maintain maximum metabolite rates under the chosen *in vitro* conditions. The coumarin (substrate) concentration in the incubation solutions was always 1.6×10^{-4} M.

For the incubations 195 μ l of a coumarin standard (30 μ g/ml, prepared in phosphate buffer, pH 7.5, 25 mM), 10 μ l of D-glucose-6-phosphate (50 mg/ml), 15 μ l of NADP⁺ (20 mM) and 5 μ l of glucose-6-phosphate dehydrogenase were mixed together in an eppendorf tube to give final concentrations as above. As can be seen, the reaction was carried out in phosphate buffer, pH 7.5, as this is the optimum pH for the P450 reaction [2]. The reaction was started by the addition of 25 μ l of microsomal suspension (the microsomal preparations were called P13, P14, P17, P18 and R1), resulting in a final protein concentration of about 2 mg/ml. Following mixing, the open eppendorf tube was transferred immediately into the incubation bath (37°C), and a 20 μ l sample taken and analysed. The metabolite formation was monitored over a two hour period, taking samples at 0, 7, 14, 20, 30, 45, 60, 90 and 120 minutes, respectively. These samples were analysed using a Beckman capillary electrophoresis instrument (P/ACE System 2050). In the case of analysis by LC, the incubation was first stopped by the addition of 50 μ l of ice-cold trichloroacetic acid (20% w/v) and centrifuged (low speed) before injection. The LC analysis was carried out according to the HPLC method of Egan and O'Kennedy [21]. The HPLC system used was a reversed-

phase chromatographic system, with a degassed mobile phase of methanol-water-acetic acid (30:70:0.2). The column used was a Waters C₁₈ μ Bondapak (30 cm x 3.9 mm i.d.) at ambient temperature, with a 10 μ m particle size. The flow rate was 2 ml/min and the wavelength of detection was 324 nm. Quantitation of 7-OHC was performed by measuring peak heights relative to a standard curve.

The incubation solution for the interspecies investigations was very similar to that used above for the human liver microsome work. The incubation solution consisted of 195 μ l of 30 μ g/ml coumarin, 10 μ l of 50 mg/ml glucose-6-phosphate, 5 μ l of 200 units/ml glucose-6-phosphate dehydrogenase, 15 μ l of 20 mM NADP⁺ and 25 μ l of the microsomal preparation. For these investigations, the incubations were carried out in 1.5 ml eppendorf tubes at 37°C. The reaction was again commenced with the addition of the microsomes. 10 μ l samples were taken and analysed immediately with minimal time delay between sampling and analysis. The concentration of 7-OHC was determined at predefined times i.e. 1, 6, 12, 20, 30, 45, 60, 90 and 120 minutes, respectively. The analysis was carried out in triplicate.

3.2.1.5 NADP⁺/NADPH ratio study

For this study, the concentration of glucose-6-phosphate dehydrogenase was reduced to 0.1 U/ml to allow a slower turn-over of NADP⁺ to NADPH. The remainder of the incubation mixture was as above. The

study was carried out in the presence and in the absence of coumarin, or the microsomal preparation

3.2.1.6 Capillary electrophoresis separation

Separations were carried out on a Beckman Capillary Electrophoresis instrument (P/ACE system 2050). The capillary used was a 27 cm x 50 μ m fused silica column (Beckman), with a capillary to detector distance of 19.3 cm. The preparation step for priming of the capillary was a 1 minute rinse with 0.1 M sodium hydroxide, followed by a 1 minute rinse with electrolyte solution (25 mM phosphate buffer, pH 7.0 or 7.5, depending on the separation required). The sample was applied to the capillary by a 3 second pressurised injection (0.5 psi) and separation achieved with an applied voltage of 20 KV (rise time 0.2 minutes) at 25 °C. Typical running current was 100 μ A. The resultant separation was monitored at 210 nm with a fixed wavelength detector using Beckman System GoldTM software. For the interspecies investigational work, the same capillary was used as above. This time the capillary was purged for 0.75 minutes with 0.1M sodium hydroxide. Conditioning of the capillary was then achieved by a 1 min rinse with the 50 mM phosphate buffer electrolyte solution. The sample was again applied by a three second pressurised injection (0.5 psi) and the separation was carried out at 15 KV (rise time 0.2 minutes) at 25°C, with UV detection at 214 nm.

3.2.2 RESULTS AND DISCUSSION

3.2.2.1 Overall reaction in the incubation solutions

The overall reactions in the incubation solutions are as follows:

Gl-6-ph-dehydrogenase



P450



When the incubation solution reaction is initiated NADP^+ is converted to NADPH, after which time the P450 can begin to catalyse the reaction between the NADPH and coumarin (in the presence of hydrogen and oxygen). As the reaction proceeds, NADP^+ is produced and it can then react again with glucose-6-phosphate, and NADPH is recycled in this way. Any chromatographic method to follow this reaction must be able to separate coumarin and 7-OHC from the co-factors and interference from the microsomal protein absorbances.

3.2.2.2 Development of the CE separation

The CE method developed for monitoring 7-OHC in plasma and urine as discussed in chapter 2 was adapted for studying the metabolism of coumarin by human liver microsomes. When interested in NADP^+ and

NADPH, a pH of 7.5 was required, as these compounds exhibit a poor peak shape in pH 7.0 electrolyte (phosphate buffer, 25 mM). The absorbance of coumarin and 7-OHC could be monitored with good sensitivity at 210 nm. Fig 3.3 shows the separation of coumarin and 7-OHC (same conditions as in the experimental section above, except using 15 KV). The electropherogram shows that the compounds can be separated well by this capillary electrophoresis system.

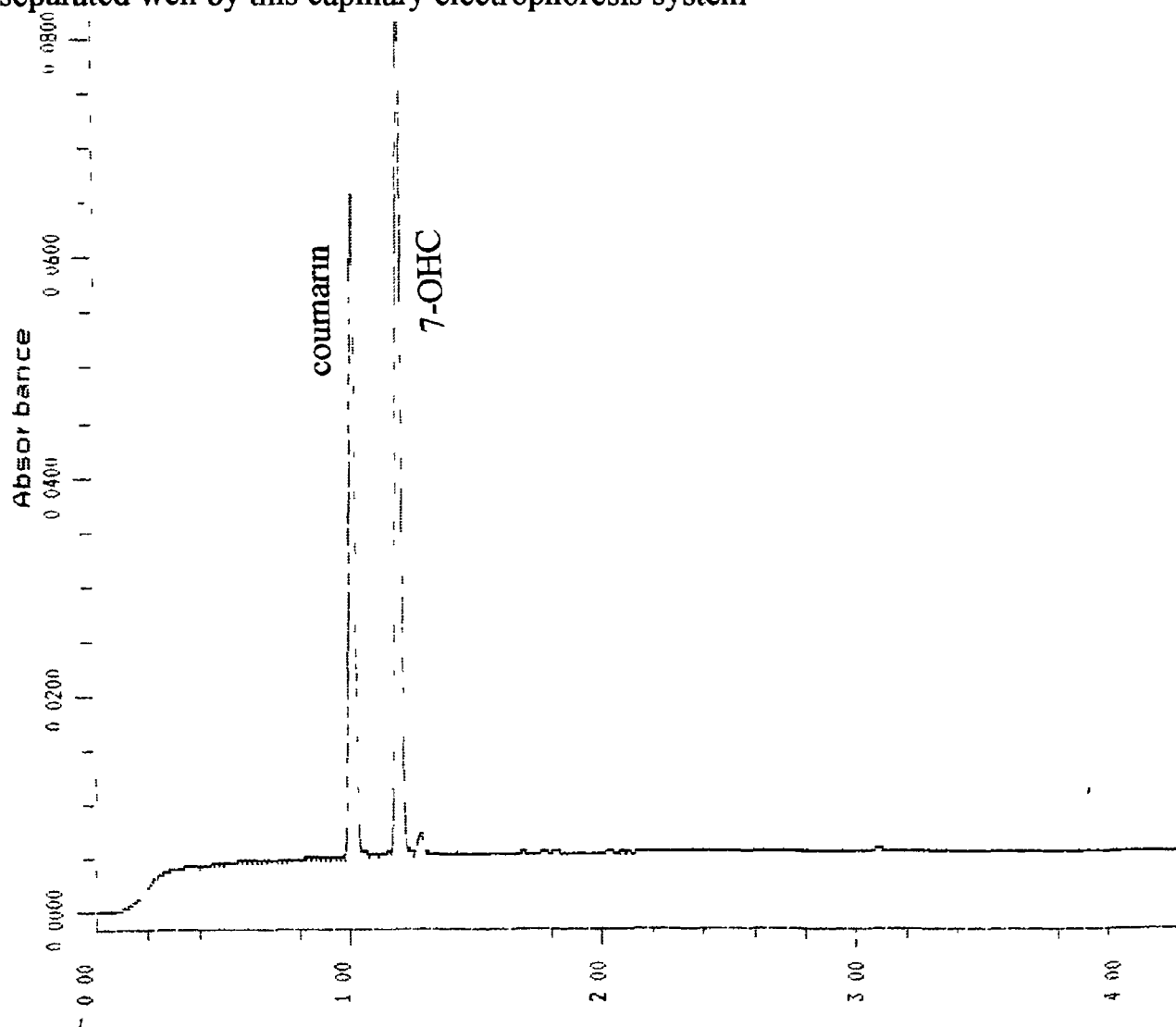


Fig 3.3 Separation of coumarin and 7-OHC by the capillary electrophoresis system, using a 25 mM phosphate buffer electrolyte, pH 7.5, at 210 nm

Fig 3 4 shows the typical electropherograms at 0 min and after 45 minutes for the metabolic incubation of the P18 microsomal preparation. This figure shows that NADP^+ and NADPH are well separated and quantifiable at an electrolyte pH of 7.5. The metabolite, 7-OHC, migrates at 1.0 minutes, and its increase in concentration can be followed under the conditions outlined in the Experimental section above. However, it is not well separated from the broad peak of the microsomal preparations at pH 7.5, and it was found that monitoring at an electrolyte pH of 7.0 (Fig 3 5) gave better resolution between the compounds. This figure shows how 7-OHC can be monitored after 0, 13 and 45 minutes of the incubation. A number of different phosphate electrolyte pH values between 4.8 and 7.5 were investigated to optimise the separation, and pH 7.0 produced the best results. Other electrolyte buffers were also tried, and borate gave promising results at pH 7.0, but the background absorbance of the buffer was very high, thus decreasing the limit of detection. Hepes buffer (pH 7.0) was also investigated, but on mixing with the phosphate buffer from the incubations a large dip in the baseline resulted halfway through the run. It was therefore felt that phosphate buffer would be the most appropriate, as the electrolyte, because phosphate buffer was being used in the incubations and this would alleviate any such problems as seen with Hepes buffer above. The use of micellar-based electrolyte solution was also assessed, but there was no benefit found in utilising such a system for the determination of 7-OHC, especially when phosphate buffer is relatively simple to prepare.

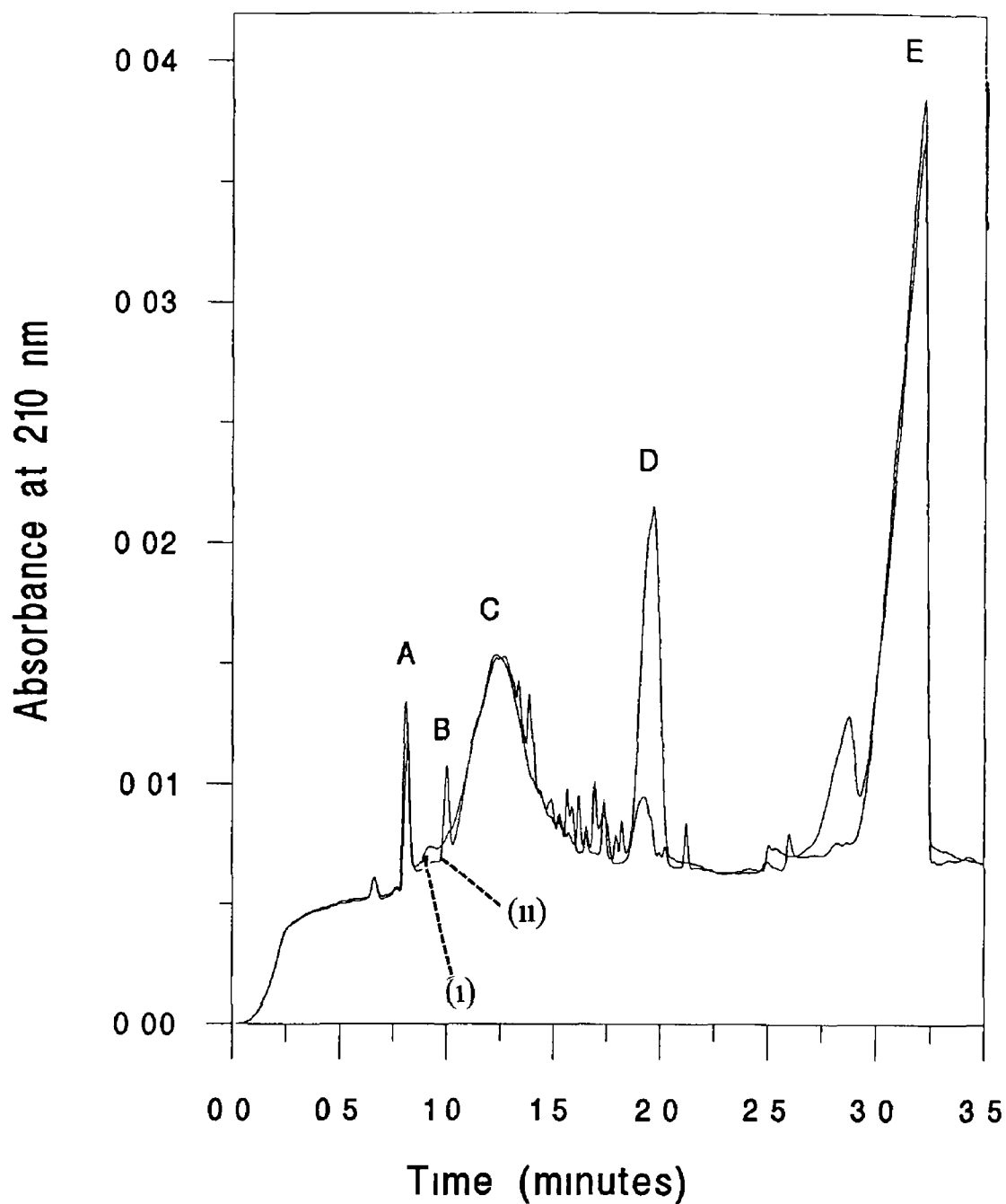


Fig 3 4 Electropherograms showing separation of (A) all neutral compounds including coumarin, (B) 7-OHC, (C) microsomes, (D) NADP^+ , and (E) NADPH, at (i) 0 minutes and (ii) at 45 minutes monitored at 210 nm using 25 mM phosphate buffer, pH 7.5

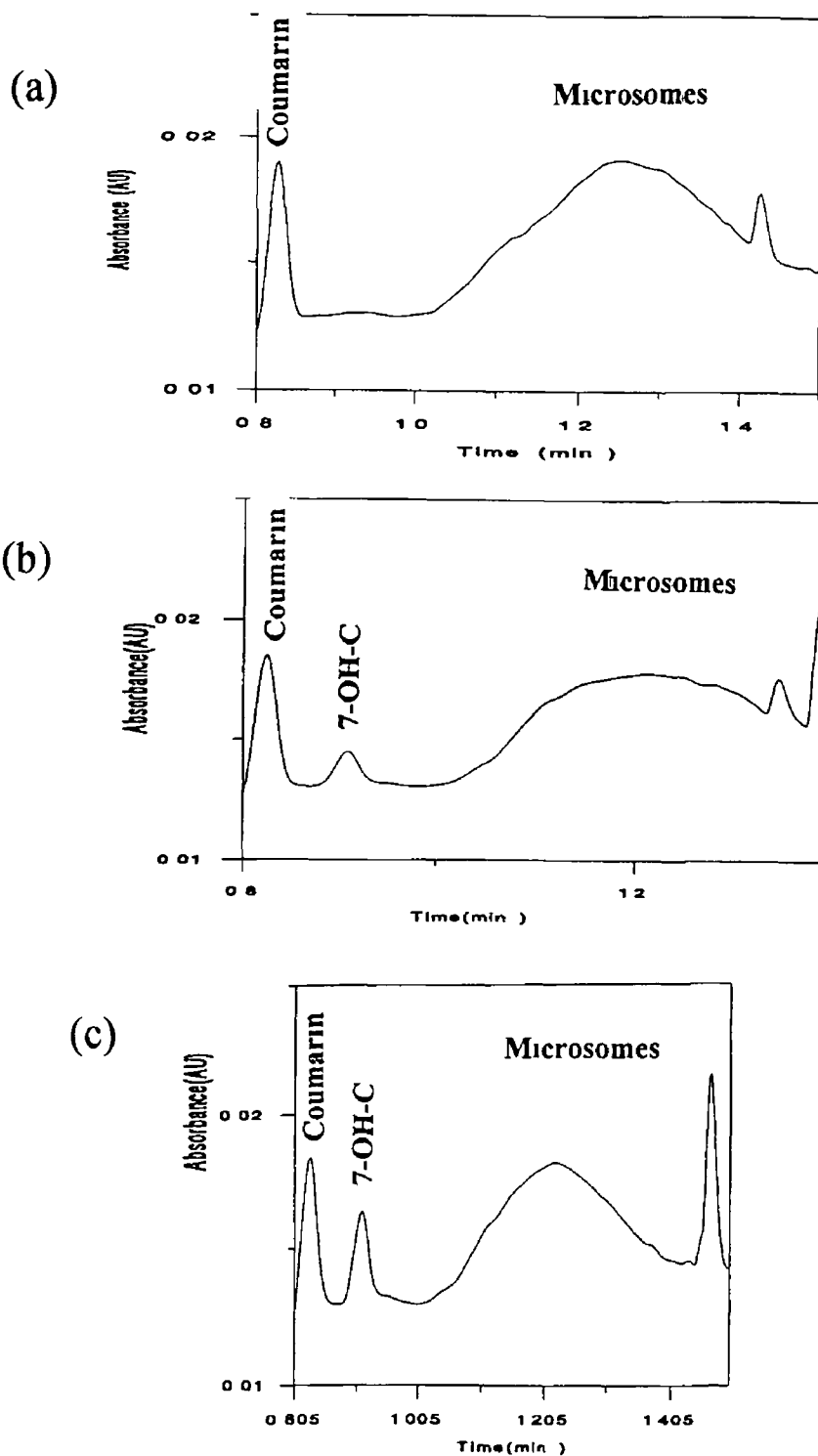


Fig 3 5 Separation of coumarin, 7-OHC and microsomes at (a) 0, (b) 13 and (c) 45 minutes of incubation respectively The incubation solution was monitored at 210 nm using 25 mM phosphate buffer electrolyte, pH 7.0

A neutral marker (benzamide) was run under the conditions above, and it found to co-migrate with coumarin, thus suggesting that coumarin is behaving as a neutral molecule at this pH. This seems logical when the structure is considered.

By knowing the migration time of the neutral marker it is possible to say that all the compounds that have longer migration times than coumarin are negatively charged and hence migrate more slowly than coumarin. 7-OHC is negatively charged at medium pH values due to dissociation of the hydroxyl group at the 7-position. The components of the microsomes migrate more slowly than the 7-OHC and are seen as a broad peak due to the different types of proteins in the microsomal suspensions. NADP⁺ has a structure with four negative charges and a positively charged nitrogen associated with it (Fig. 3.6). This leads to an overall charge of 3 negative charges. This high overall negative charge explains the slower migration of NADP⁺. The NADPH molecule has a similar structure to NADP⁺, but the positive charge on the nitrogen is no longer present (Fig. 3.6). As a result, this highly negatively charged compound (4 negative charges) has the slowest migration rate of all the compounds.

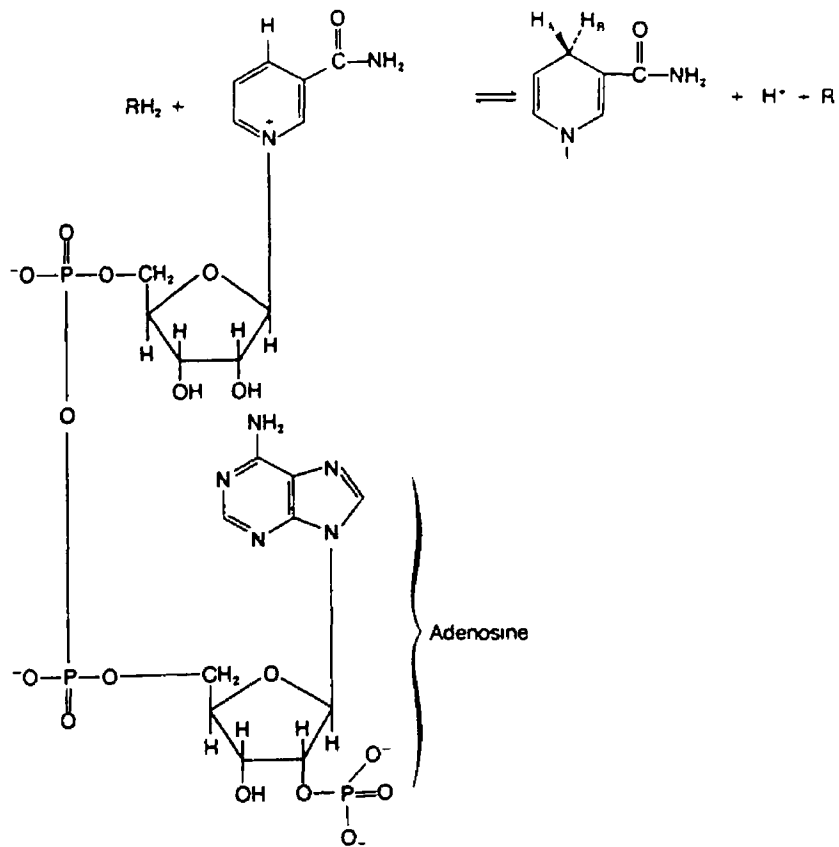
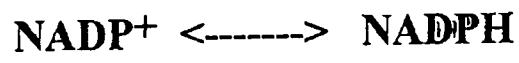


Fig 3 6 Structures of NADP⁺ and NADPH

A number of control experiments were carried out to ensure that the cytochrome P450 enzymes were responsible for the new compound detected at the migration time of 7-OHC. Initially a control incubation was carried out which had all the components except the NADPH generating system. No 7-OHC was seen after 130 minutes of incubation, also, the coumarin peak remained at a constant height through this study. A control experiment was also carried out which had only microsomes present in the incubation to investigate if any compounds were produced on incubating the P450 enzymes at 37°C. No compounds were detected near the coumarin/7-OHC migration time region. Finally, a control experiment was run which had all the components in it except coumarin. In this case, a compound was produced in significant amounts which had the same migration time as coumarin. This compound needed the presence of the NADPH generating system to allow its formation, so it may possibly be the product of another enzyme reaction which uses NADPH as its co-factor. The compound formed is likely to be a neutral compound (since coumarin is considered neutral relative to the benzamide neutral marker) which may suggest that it was not a product of another P450 metabolic reaction, as most of these would form relatively polar products. As a result of the formation of this compound throughout these investigations, it was unfortunately not possible to monitor the disappearance of the coumarin as the reaction proceeded.

The conclusion to these control experiments was that only in the presence of all of the incubation components (i.e. NADPH generating system, coumarin, and the microsomes) that 7-OHC was produced, giving rise to the new peak around 0.9 min.

3 2 2 3 Coumarin metabolism profiles

Concentrations of 7-OHC in the human liver microsomes were calculated from the mean of three standard curves prepared by spiking standards of 7-OHC into three different microsome preparations Fig 3 7 below shows the (A) 10 and (B) 20 µg/ml spikes of 7-OHC in the P14 microsome preparation

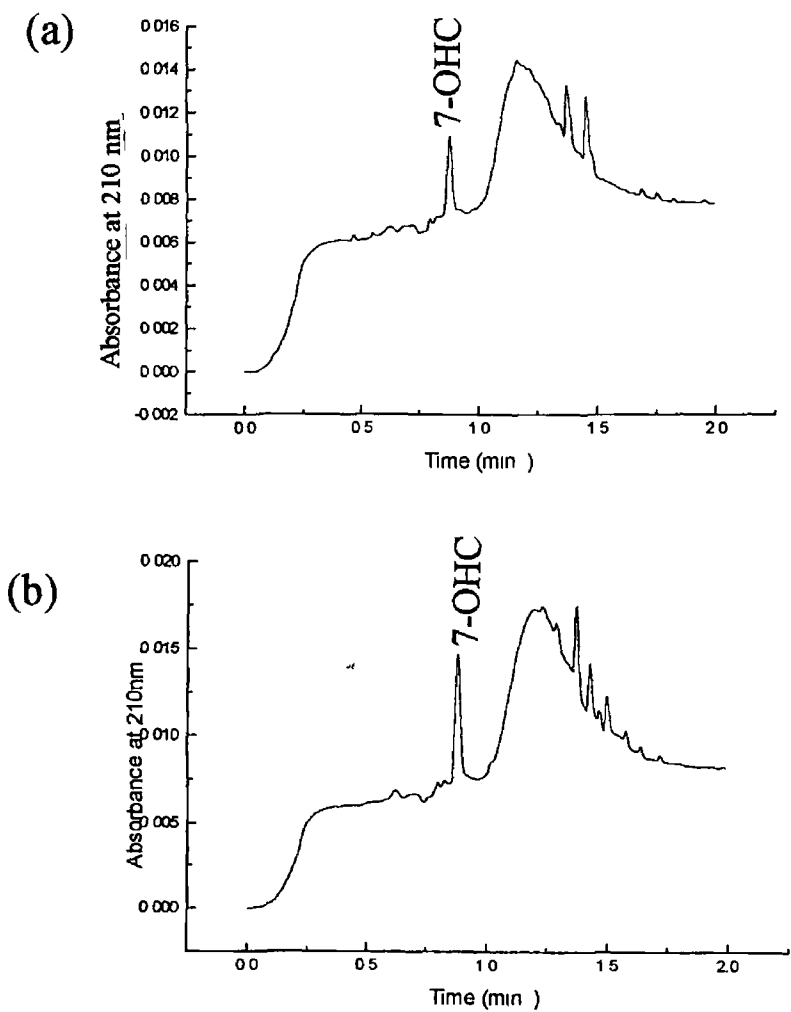


Fig 3 7 Electropherograms showing the spiking of (a) 10 and (b) 20 µg/ml of 7-OHC into P14 microsome preparation

The concentration of 7-OHC in the microsomes was calculated from a plot of absorbance versus standard concentration ($\mu\text{g/ml}$). A linear calibration curve was obtained for 7-OHC between 2-50 $\mu\text{g/ml}$ which passed through the origin (see Fig 3.8). The limit of detection ($3 \times \text{S.D. background}$) was found to be around 1 $\mu\text{g/ml}$.

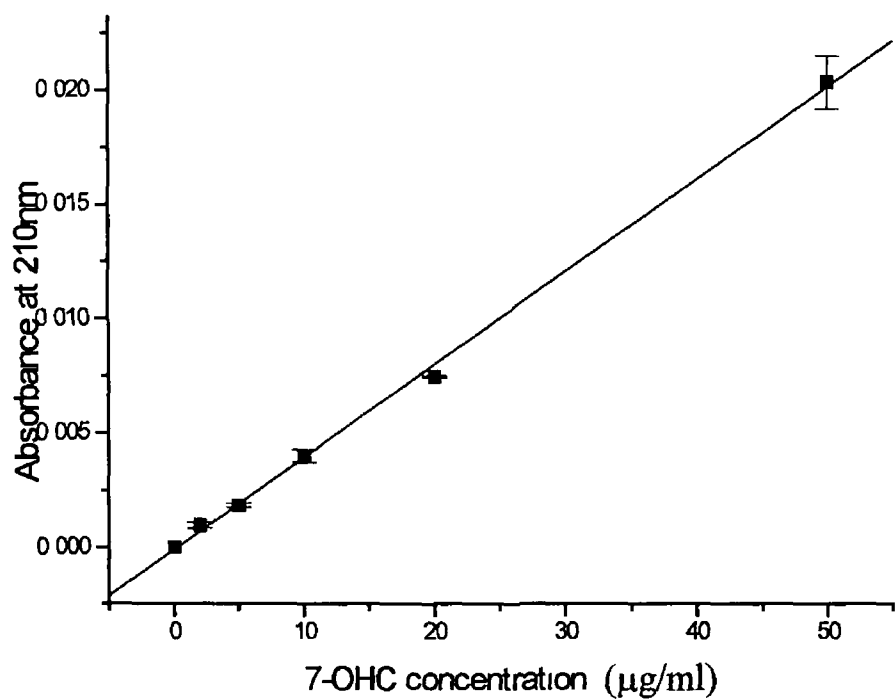


Fig 3.8 Calibration curve for 7-OHC in the microsomal preparations

Linear regression analysis of this data gave an equation

$$y = a + bx \quad \text{where } a = -0.00011$$

$$b = 0.0004$$

$$R = 0.99931$$

For the interspecies investigations, standards were prepared by spiking 7-OHC standards (2 μ l) and microsomes (4 μ l) into buffer (34 μ l). The linear range was 2-50 μ g/ml with a limit of detection of approximately 1 μ g/ml. The concentration of 7-OHC produced was calculated from a plot of absorbance versus concentration of 7-OHC. The standard curves were prepared in each of the microsomal preparations on the day on which the assay was carried out to allow for any day-to-day variations.

The protein concentration of the human liver microsomes was determined by the BCA assay as described in the Experimental section above. The protein concentrations of the different human liver microsomes as determined by this method is summarised in Table 3.2.

Human Liver Microsome Preparation	Average Protein Concentration (mg/ml) n = 2
P13	15.97
P14	15.00
P17	14.42
P18	13.58
R1	18.91

Table 3.2 Protein concentrations of the human liver microsomes

Microsome preparations from five different patients were used in the metabolism studies. Incubations were carried out in triplicate. Fig. 3.9 shows a plot of concentration of 7-OHC (nmol/ml/mg protein \pm S.D.), versus time for each of the five metabolic studies. It shows that each of the microsomal preparations has a different coumarin metabolism profile, this is in agreement with results from many other groups [10,22]. In fact, the inter-individual variability of human coumarin 7-hydroxylase activity in liver microsomes is very large, and can be up to 70 fold [22]. Although the errors in concentration calculations are relatively high, the errors correspond to errors in microsomal metabolism results from other researchers [2]. It must be considered that these microsomal preparations are extremely complex protein solutions of which total cytochrome P450 is less than 1% and 7-hydroxylase being but a small fraction of this.

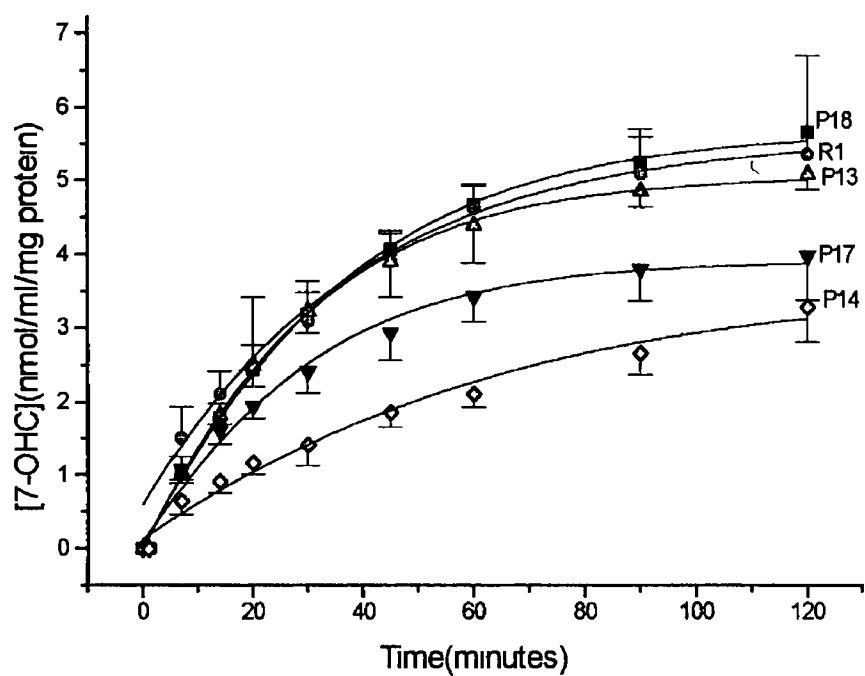


Fig 3 9 Plot of concentration of 7-OHC (nmol/ml/mg protein) \pm S D versus time for five microsome preparations

Fig 3 10 below shows the comparison of using liquid chromatography (LC) against capillary electrophoresis for the analysis of the metabolite formation. It can be seen that there are no significant differences between the results from both methods. LC is the common method used for this analysis of metabolites in cytochrome P450 studies [2-5, 7-17] and also for the quantitation of 7-OHC. The LC methods involve stopping the enzyme reactions by precipitating out the protein, followed by centrifugation to minimise degradation of the HPLC system. The protein would have a negative effect both on the HPLC column and also the detector cell. The cell can become coated with protein and this can affect both its sensitivity and the system's ability to run gradients with relatively flat baselines (a dirty detector cell can give rise to an interfering absorbance in gradient runs). The column would also have a shortened life if protein solution are allowed to pass through them as the protein can become coated on the C₁₈ packing material. The column frits could also become blocked, resulting in high back pressures, which is generally detrimental to pump performance.

It was not necessary to precipitate out the protein in the CE method used as it involved injecting the incubation mixture directly onto the capillary. This saves a lot of time in sample preparation and is the main advantage of the method. The reaction is stopped by the separation of the individual components in the capillary in a very short time, and once separated, they are no longer in contact with each other and are able to react. Also the application of 20 KV would terminate an enzyme reaction very quickly. 7-OHC is detected after 0.9 min using the CE method (Fig 3 5), but it takes up to 12 minutes using the LC method of Egan and O'Kennedy [21], this is also an important advantage of the method, as it means that many

more samples can be run in a shorter time in comparison with HPLC. The speed of the CE separation is mainly due to the high efficiency that can be achieved by CE [23]. Furthermore, up to 500 injections were made on to the CE capillary during these studies without any visible degradation of capillary performance. If one compares the LOD of the available LC system [21] with the LOD of the CE system developed, there is little difference observed.

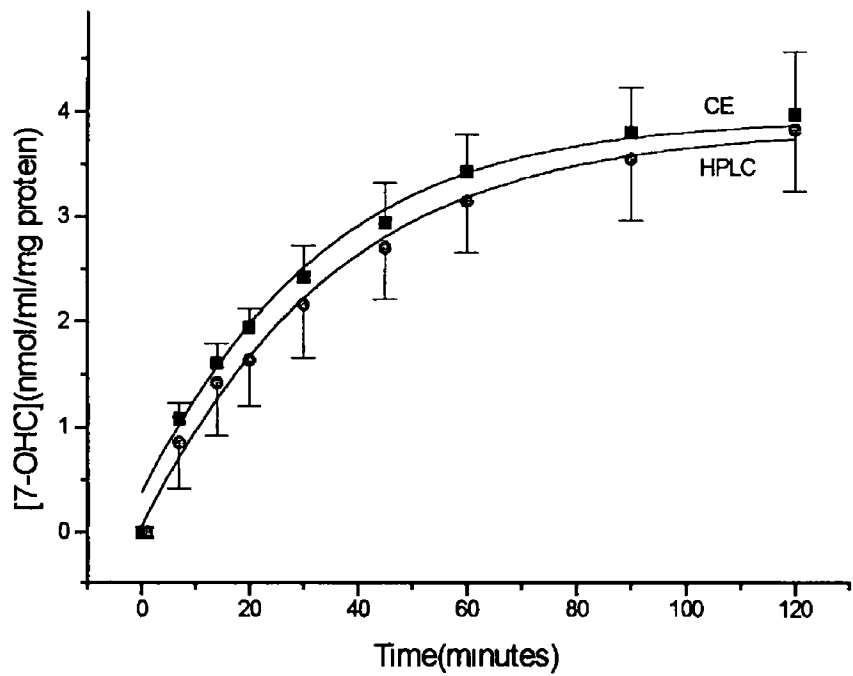


Fig 3 10 Comparison of HPLC vs CE for the monitoring of 7-OHC formation by P17 microsomes

3 2 2 4 NADP⁺/NADPH ratio study

During this study, it was also discovered that it was possible to monitor an integral part of the monooxygenase reaction, i.e. the NADPH-dependent coumarin metabolism reaction. Using the capillary electrophoresis separation technique, one could follow the effect on both the NADP⁺ and the NADPH present during drug metabolism, as a result of the NADPH regeneration system in the incubation mixture. To slow down the production of the NADPH from NADP⁺, the concentration of the glucose-6-phosphate dehydrogenase was reduced to 0.1 U/ml as compared to 4 U/ml for the metabolism study (otherwise the NADPH is formed too rapidly), all other conditions were as for the metabolism study. P18 microsomes were used for this ratio study. The electrolyte for this work was 25 mM phosphate buffer, pH 7.5, to achieve a good peak shape for both NADP⁺ and NADPH.

Fig. 3.11 shows the electropherograms after 40 minutes of incubation, of incubation mixtures in (a) the absence and (b) the presence of coumarin. The absorbance ratio for NADP⁺/NADPH can be determined from this separation, and one can see the difference of having coumarin present in the incubation mixture. With coumarin present, more NADP⁺ is produced and hence NADPH is slower to be turned over by the NADPH regeneration system.

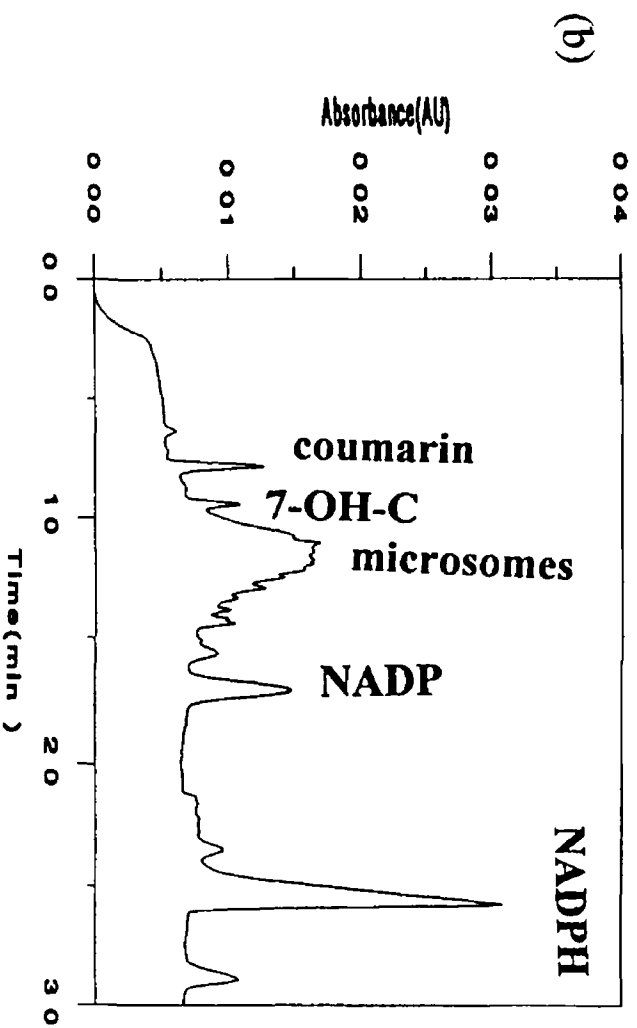


Fig 3 11 Changes in the $\text{NADP}^+/\text{NADPH}$ ratio in (a) the absence and (b) presence of $24 \mu\text{g/ml}$ of coumarin after 40 minutes of incubation

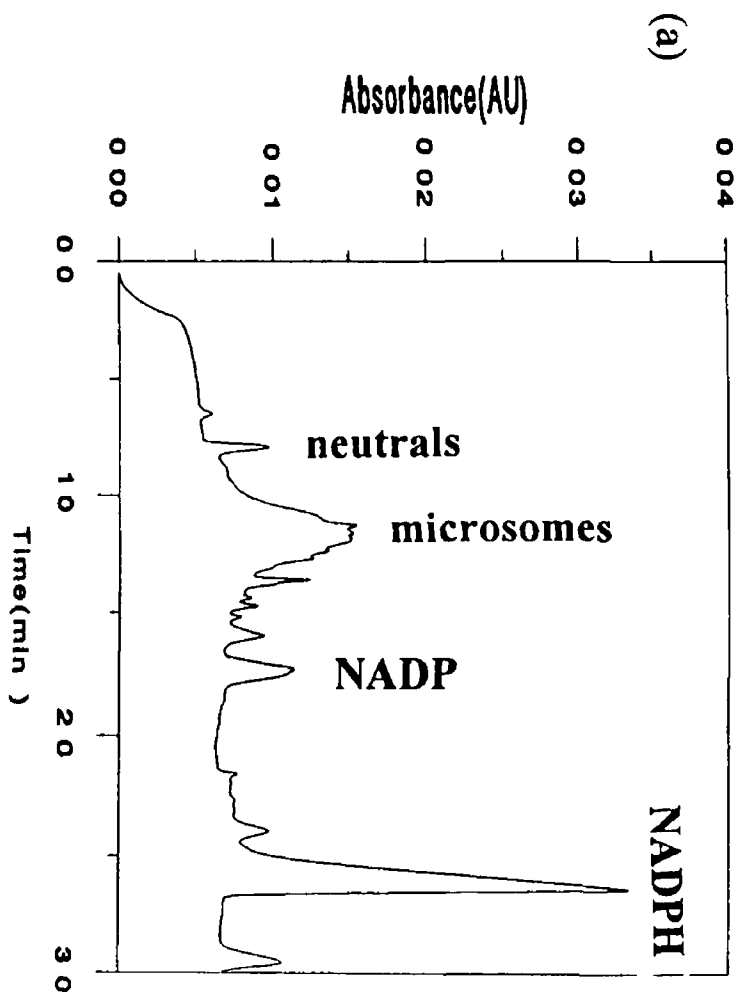


Fig 3 12 below shows a plot of $\text{NADP}^+/\text{NADPH}$ absorbance ratio versus time for the incubation mixture (a) and also for incubations in the absence of coumarin (b), or microsomes (c) As can be seen from this figure, the ratio starts off high (>1) since NADPH is only just being formed through the regeneration system As time progresses, more NADPH than NADP^+ is present and the ratio decreases

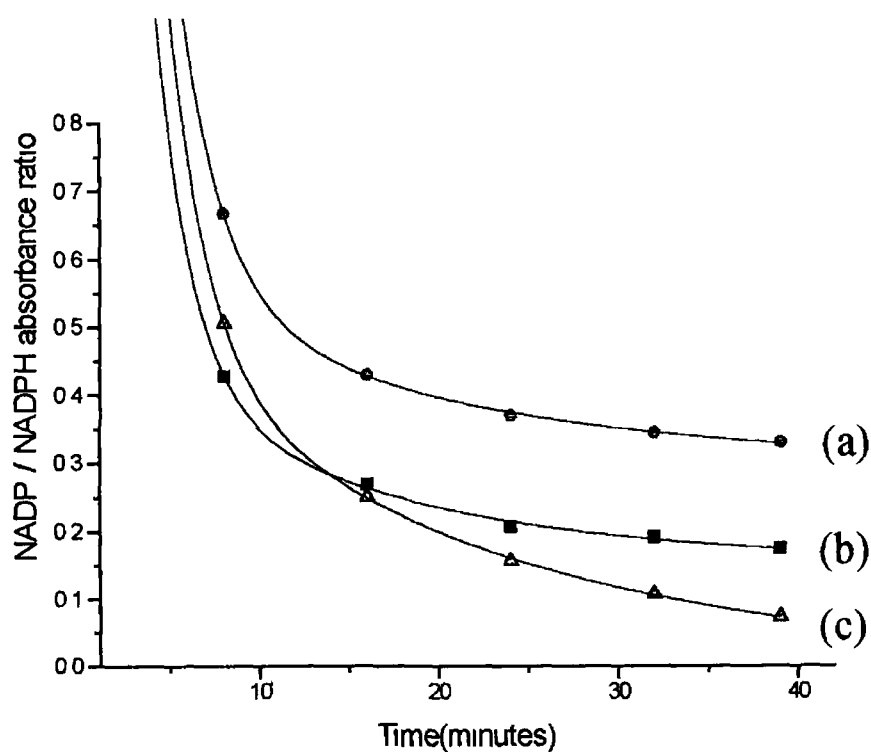


Fig 3 12 Plot of $\text{NADP}^+/\text{NADPH}$ absorbance ratio versus time, for (a) the incubation mixture (i e coumarin present), (b) absence of coumarin and (c) absence of microsomes

As seen already from the control experiments (section 3 2 2 2), NADPH is utilised by the microsomes for other than the coumarin metabolism, possibly producing a neutral compound by other biological processes. This is also seen from plot B in Figure 3 12. Plot C shows how NADP⁺ is converted to NADPH in the absence of any draw on the NADPH. Fig 3 12 is useful in that one can see clearly that when coumarin is being metabolised, the NADP⁺/NADPH ratio is quite different to when it is not present for metabolism, this may be of use when investigating whether a given drug is metabolised using the NADPH dependent cytochrome P450 system or not.

3 2 2 5 Stability of NADPH

In the course of this work, it was also noticed that as incubations were progressing, a compound was being produced with a slightly faster migration time than NADPH, and the concentration of this compound became progressively larger as the incubation neared its completion (Fig 3 13). Further investigations were therefore carried out to determine how this compound was produced. In one experiment, an incubation was performed with all the elements present (as in Experimental section) except the microsomes, with phosphate buffer taking their place to give the correct final volume. Figures 3 14 (A-D) show how this compound is slowly formed over time. Figure 3 15 shows the profile of peak height ratio of NADPH/unknown over 140 minutes of incubation. Fig 3 16 shows a plot of peak height ratio of NADPH/coumarin vs time, with the coumarin acting as an internal standard in the incubation solution. Controls showed coumarin to be free from interference in these incubation mixtures. The

results show the NADPH peak to increase up to 40 minutes followed by a decrease in its concentration. These results show NADPH to be falling in concentration as time progresses and seems to be possibly degrading. Since all that is in the incubation solution is coumarin, NADP^+ , NADPH, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase, it should be easier to determine its origins in comparison to having the microsomes also present. The products of the glucose-6-phosphate dehydrogenase enzyme reaction (reaction 1, section 3.2.2.1) are NADPH and ribose-6-phosphate. The ribose-6-phosphate does not have a significant UV absorbance, and also the unknown in question is not produced at the same rate as NADPH, so it is clearly not a product of the enzyme reaction. It is clear from the electropherograms where NADPH migrates, and this unknown has a migration time slightly faster than this. This gives us the information that the unknown is slightly less negative in charge compared to NADPH. It is conceivable that this unknown is a degradation product of NADPH, with NADPH possibly not being stable either in light, phosphate buffer or at 37°C . Throughout this investigation, Sigma (from whom these chemicals were purchased) were contacted. They suggested that the unknown may have been either adenosine-diphosphate, β -NADP or adenosine-monophosphate. It is obvious that the unknown is not NADP^+ from migration times. Figure 3.17 shows the structure of adenosine diphosphate (ADP). From a comparison of this structure with that of NADP^+ (Fig. 3.6), it seems more probable that the unknown is adenosine diphosphate, rather than adenosine monophosphate (one less phosphate group), since in theory AMP should migrate much faster than NADPH, being one phosphate group less negative and a much smaller molecule in size. It is postulated that the reason ADP would migrate faster than

NADPH is because of its smaller molecular weight, along with the extra negative charge associated with the hydroxyl groups of the ribose structure on the upper part of the molecule. It may be possible that NADPH slowly degrades to ADP in phosphate buffer solution at 37°C. A concentrated standard (10 mM) of NADPH was run under the electrophoretic conditions described above, but with a longer 50 cm capillary (other conditions as above). This is shown in Figure 3 18, which shows that NADP^+ and an unknown are present in the Sigma standard. This may be the same unknown as above, and this shows the value of capillary electrophoresis in running impurity profiles for quality control purposes.

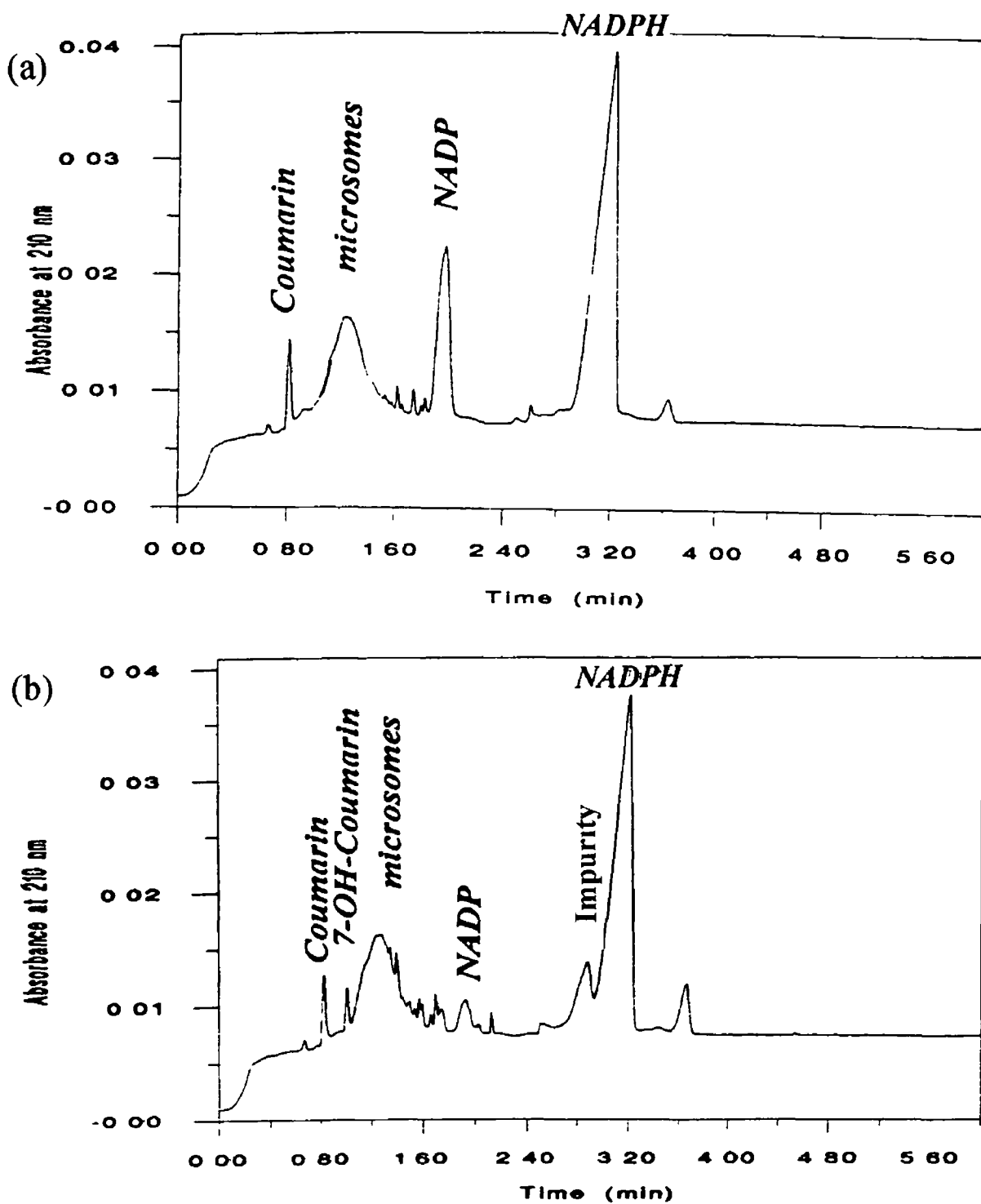


Fig 3.13 Electropherograms showing how impurity near NADPH migration time increases as incubation proceeds. The figure shows impurity at the start of incubation (a) and after 45 minutes (b).

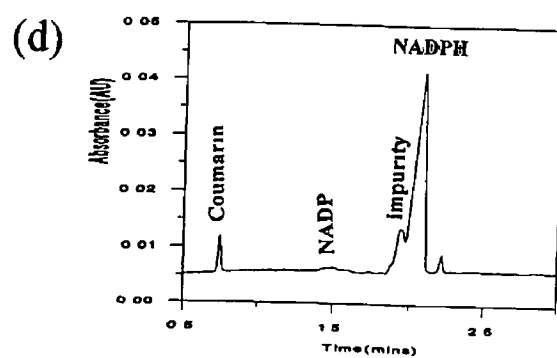
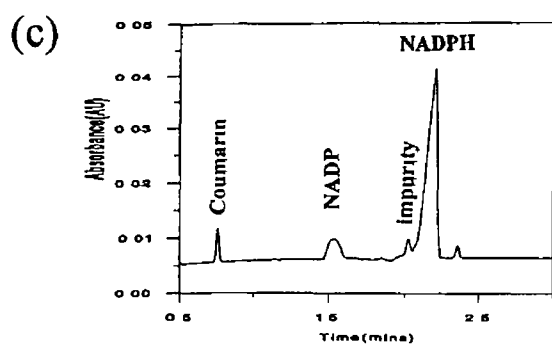
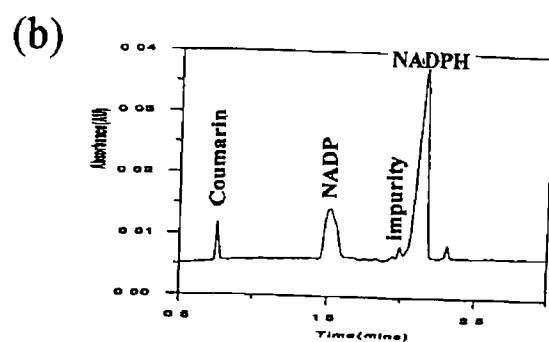
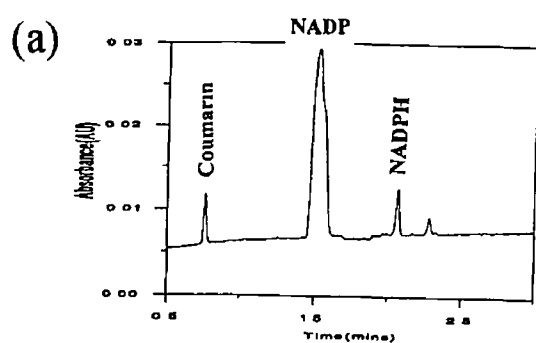


Fig 3 14 Conversion of NADP^+ to NADPH after (a) 1, (b) 15, (c) 30 and (d) 80 minutes of incubation using the NADPH generation system

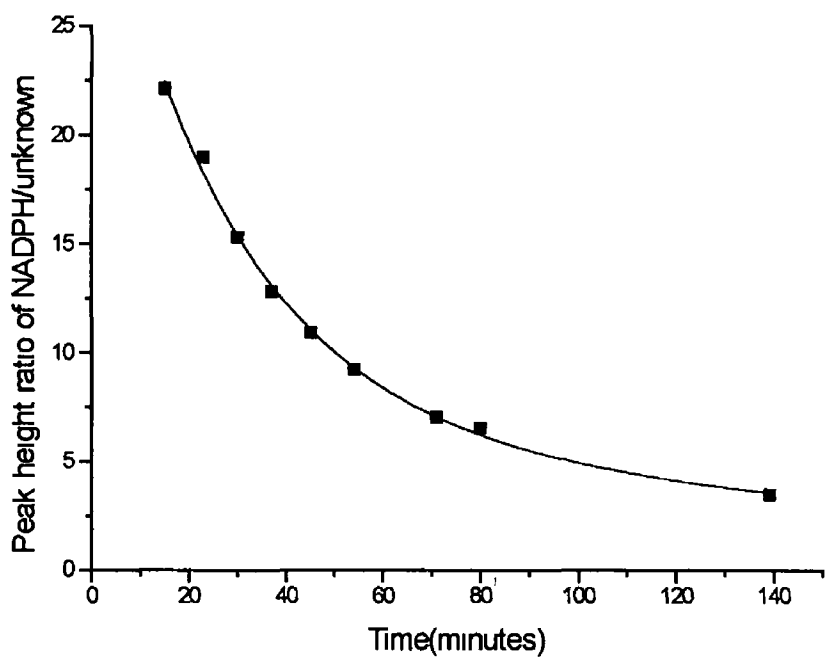


Fig 3 15 Plot showing the peak height ratio of NADPH/unknown over 140 minutes of incubation (same incubation as in Figure 3 14 above)

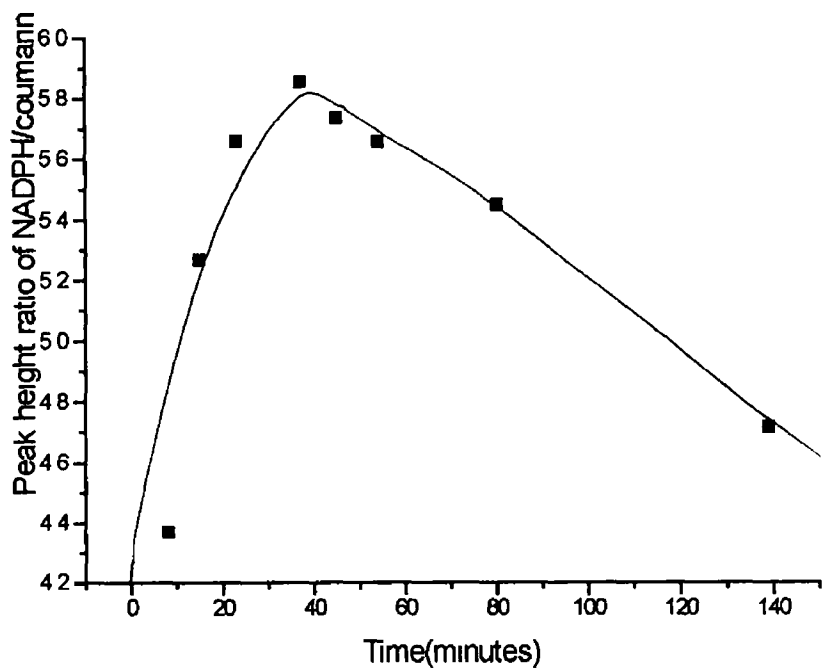
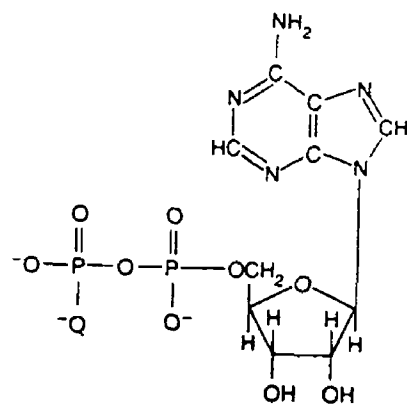


Fig 3 16 Plot of peak height ratio of NADPH/coumarin vs time, with the coumarin acting as an internal standard in the incubation This data is taken from the same incubation as in Fig 3 14 above



Adenosine diphosphate

Fig 3 17 Structure of adenosine diphosphate

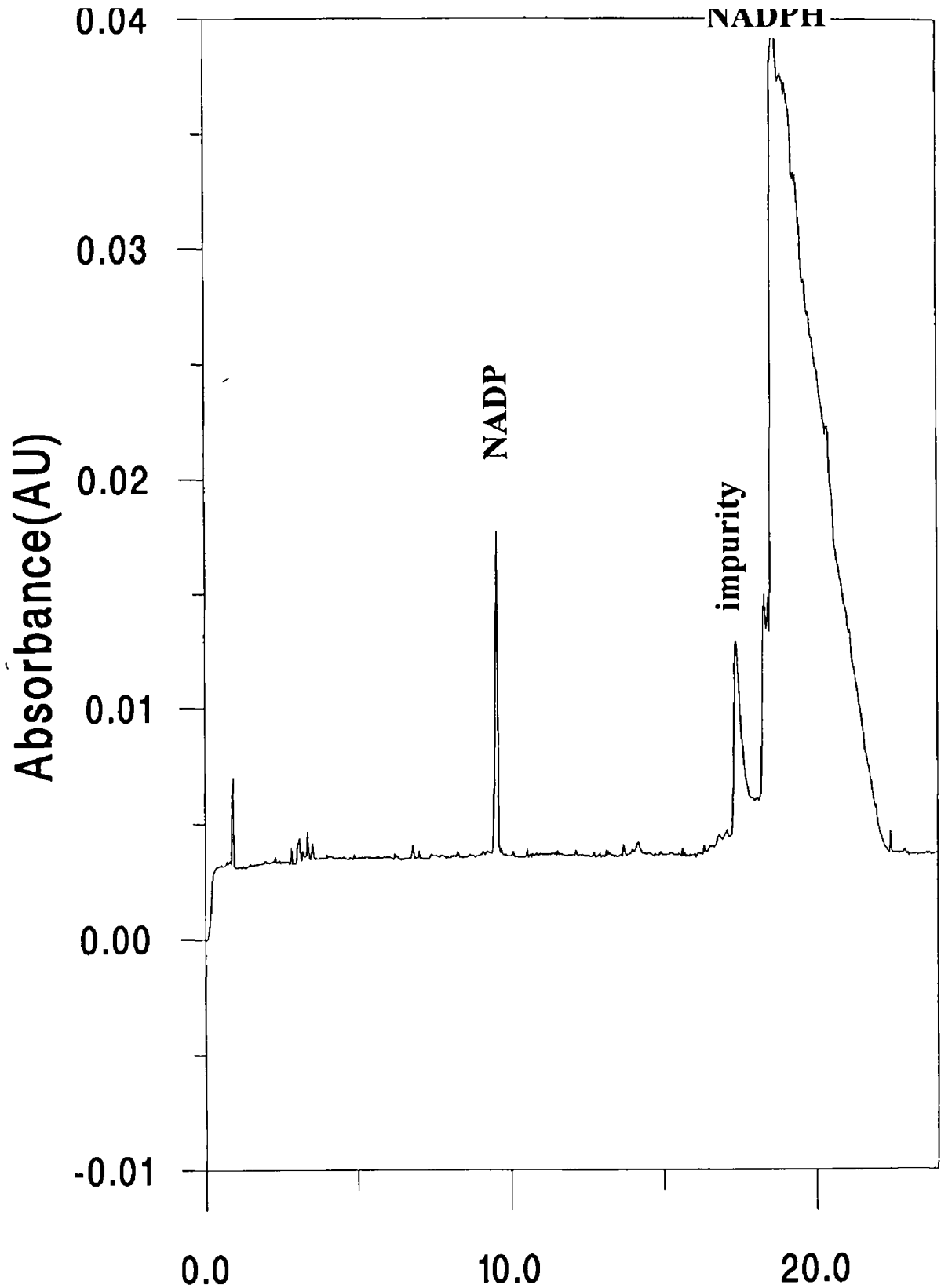


Fig 3 18 Electropherogram of a 10 mM NADPH standard under the conditions as outlined in the experimental section, except using a longer 50 cm capillary

3 2 2 6 Interspecies differences in coumarin metabolism

When carrying out the interspecies investigations, the electrolyte used was 50 mM phosphate buffer, pH 6.8. This proved to be the best electrolyte concentration and pH for the separation of components in the incubation mixture.

For this work a series of controls were carried out similar to those carried out in section 3 2 2 2 above, to show that the peak seen at the 7-OHC migration time was in fact 7-OHC and not some other compound. The series of controls included the withdrawal of the various members of the NADPH regeneration system, the absence of the liver microsomes and the absence of coumarin. Again it was not necessary to stop the reaction prior to the analysis, as the application of the high voltage separated the individual components in the incubation mixture, preventing further reaction. A 10 μ l aliquot was removed at the specified time and added into a microvial, from which it was analysed by capillary electrophoresis. Again there was no need for the addition of the trichloroacetic acid to precipitate the protein, as the method developed allowed the separation of the endogenous species in the sample from the analytes of interest (Fig 3 19).

The regeneration of the surface between every run also aided in maintaining the reliability of the method. There was some inter-day variability in the migration times for the different components, however, this was attributed to the day to day slight variations in preparing the electrolyte buffer and was always less than $\pm 10\%$.

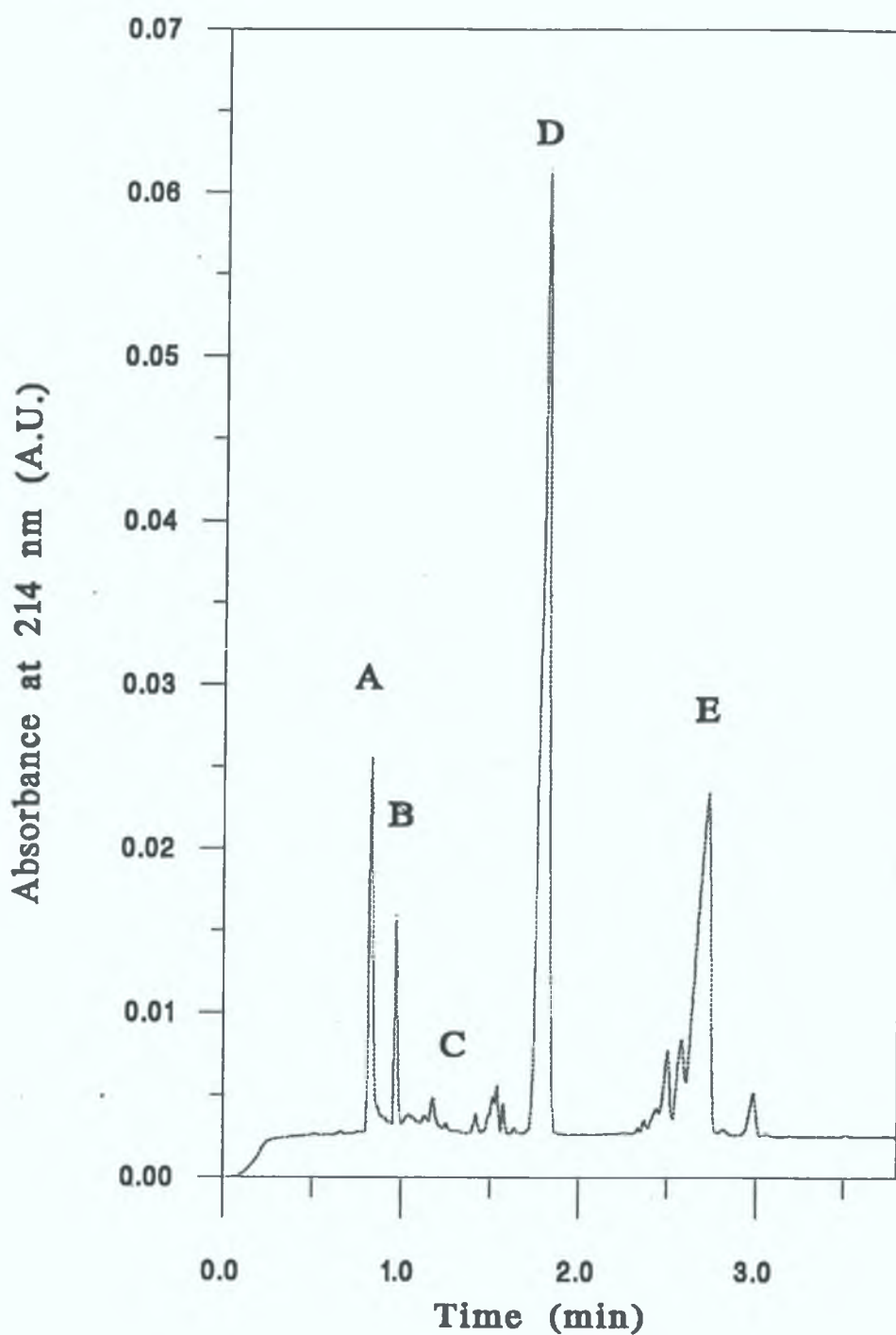


Fig. 3.19 Electropherogram showing the separation of (A) coumarin, (B) 7-OHC, (C) microsomes, (D) NADP and (E) NADPH. The separation was carried out on a 27 cm untreated silica capillary at 25 °C, using 50 mM phosphate buffer, pH 7.4, and UV detection at 214 nm.

For some species (e.g. the cynomolgous monkey) the metabolism of coumarin to 7-OHC proceeded linearly for the first 45 minutes. The metabolism plateaus after 45 minutes, as the activity of the microsomes (at 37°C) decreases. All activity disappears if the microsomes are not stored correctly or mishandled during the *in vitro* assay. When a comparison was made between the different species, the human liver microsomes demonstrated the highest coumarin 7-hydroxylase activity (Table 3.3). However, the activity of the microsomes from the cynomolgous monkey are analogous with the human microsomal preparations studied. The other species, however, have at least a ten-fold diminution in activity as compared with the human samples. This work does show that different species do not predominantly metabolise coumarin to 7-OHC, this is in agreement with other work in this area [22].

Species (source)	Microsomal protein concentration ¹ (mg/ml)	7-hydroxycoumarin concentration ² (nmol/ml)	Coumarin 7-hydroxylase activity ³ (pmol/mg protein/min)
Rabbit (IFFA Credo)	13.7	13.0	211.0
Monkey (IFFA Credo)	14.5	45.1	691.0
Bovine (self prepared)	26.9	14.0	116.0
Porcine (self prepared)	11.2	0	0
Rat (IFFA Credo)	18.4	0	0
Mouse (CD1, self prepared)	7.5	0	0
Mouse (Sch self prepared)	16.5	0	0
Gerbil (self prepared)	12.6	11.4	201.0
Dog (IFFA Credo)	11.2	0	0
Human results			
P13	16.0	62.37	866.3
P14	15.0	27.91	413.5
P17	14.4	42.35	653.5
P18	13.6	55.27	903.1
R1	18.9	73.78	867.5

Table 3.3 Comparison of species for Coumarin 7-hydroxylase activity

¹ Protein concentrations were determined by the BCA assay (see Experimental section)

² The 7-OHC concentration was calculated at 45 minutes, which was defined as the time when the optimal activity of the microsomes decreased significantly, above which the metabolism profile was no longer linear

³ The coumarin 7-hydroxylase activity was determined at 45 minutes

3.3 ELECTROCHEMICAL INVESTIGATIONS INTO MICROSOMAL REACTIONS

3 3 1 EXPERIMENTAL

3 3 1 1 Chemicals

NADPH, flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) were purchased from Sigma (St Louis, MO) Coumarin was donated by Schaper and Brummer Sodium dithionite was purchased from Sigma Phosphate buffer (0.1M, pH 7.5) was prepared daily by dissolving 0.08M K_2HPO_4 and 0.02M KH_2PO_4 in deionised water

3 3 1 2 Tissue preparation

The same microsome preparations were used as already described in section 3.2.1.2 Genetically engineered homogenate from V79 cells expressing rat CYP 1A2 with a protein concentration of 4 mg/ml was donated by Prof Johannes Doehmer, Department of Toxicology, University of Mainz, Germany

3 3 1 3 Apparatus

The instrumentation used for this work involved a conventional three electrode system with glassy carbon, silver/silver chloride and platinum wire as working, reference and counter electrodes respectively These were connected to a BAS-50W voltammetric analyser Unless otherwise

stated, all electrochemical investigations were carried out in a small cell containing 10 ml of 0.1 M, phosphate buffer, pH 7.5

3.3.1.4 Electrochemical methods

Cyclic voltammetry was predominantly used in the electrochemical investigations. Scan rates, and initial and final potentials were optimised during the studies in both the positive and negative potential regions. Prior to use the glassy carbon electrodes were always electrochemically pretreated by multi-cycling in phosphate buffer to reduce background currents. Incubation studies were carried out on solutions containing 2 mM coumarin, 1 mM NADPH and 2 mg/ml protein concentration of human microsomal suspension. This was made up by adding 34 μ l of 10^{-2} M coumarin, 136 ml of 0.1 M, phosphate buffer, pH 7.5, 10 μ l of 20 mM NADPH and finally 20 μ l of human microsomal suspension (giving approximately 2 mg/ml protein concentration). Investigations were also carried out in the absence of coumarin and/or microsomes. Studies were carried out to optimise the electrochemical response from the microsomes by investigating how best pre-concentration of the microsomes could be achieved. For the electrochemical work, the studies were carried out between 0 and +1.0 V (vs Ag/AgCl) in the anodic region, and between 0 and -1.0 V (vs Ag/AgCl) in the cathodic region. As well as cyclic voltammetry, differential pulse voltammetry was also used to provide better sensitivity in some studies. All potentials in this work were with respect to a Ag/AgCl reference electrode.

3 3 2 RESULTS AND DISCUSSION

The initial aim of this work was to develop a biosensor capable of monitoring the cytochrome P450-catalysed metabolism of specific compounds. This effort was unsuccessful for a number of reasons, as will be discussed, however, some interesting observations were made from these investigations and these will be discussed below. The electrochemical work is divided into work in the anodic region, and work in the cathodic region.

3 3 2 1 Investigations in the anodic region

Initial studies involved direct electrochemistry of the microsome suspensions to investigate if any electrochemical responses could be detected. Figure 3 20 shows a cyclic voltammogram of a solution which contains 20 μl of human microsomal preparation (from patient R6) and 170 μl of 0.1 M, phosphate buffer, pH 7.5, at a bare glassy carbon surface. The scan was between 0 and +1.0 V at a scan rate of 15 mV/s. This figure shows two oxidation peaks at approximately +0.40 and +0.75 V respectively. The peak at +0.75 V is very pronounced while the peak at +0.40 V is quite faint. These two oxidation peaks were found in all the human liver microsome preparations. Scans were also undertaken at more positive potentials, but no extra peaks were detected. A study was undertaken to try to detect these oxidation processes with greater sensitivity. These efforts included trying to immobilise the microsomal preparation at the glassy carbon surface with Nafion and glutaraldehyde, but it seemed that these two immobilisation techniques retarded the flow of

electrons and very small currents were detected. It was found that dropping 5 μ l of microsomal suspension onto the glassy carbon electrode surface and allowing this to dry for 1 hour before running the cyclic voltammogram (under the same conditions, in 10 ml of 0.1 M phosphate buffer, pH 7.5) proved the most successful. The cyclic voltammogram (CV) for such a system is shown in Figure 3.21. Unfortunately the scan had to be taken very quickly as the microsome preparation was seen to dislodge from the electrode quickly, and only one scan was possible with this procedure.

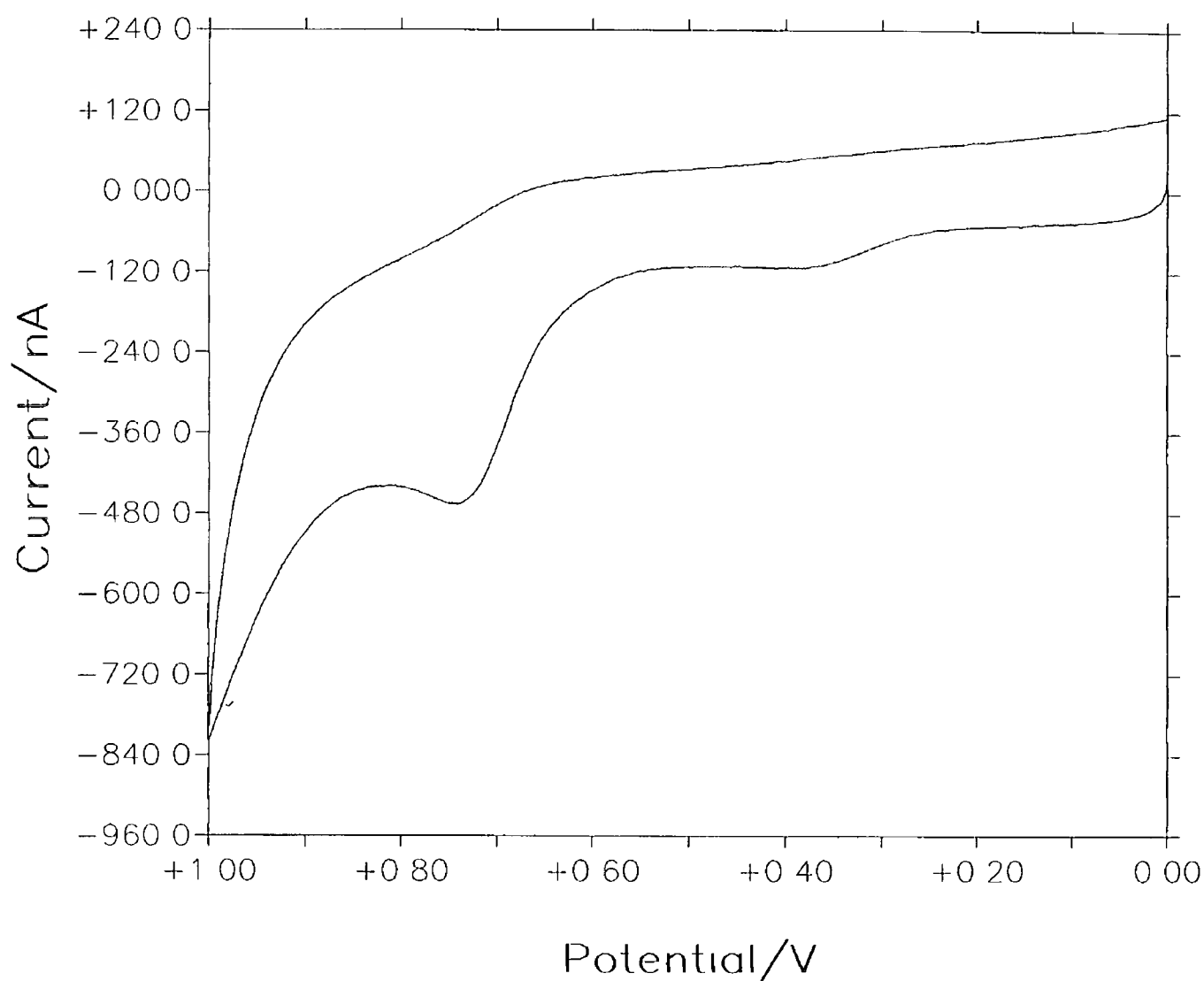


Figure 3 20 Cyclic voltammogram of a solution which contains 20 μl of human microsomal preparation (from patient R6) and 170 μl of 0.1M phosphate buffer, pH 7.5, at a bare glassy carbon surface. The scan was between 0 and +1.0 V at a scan rate of 15 mV/s

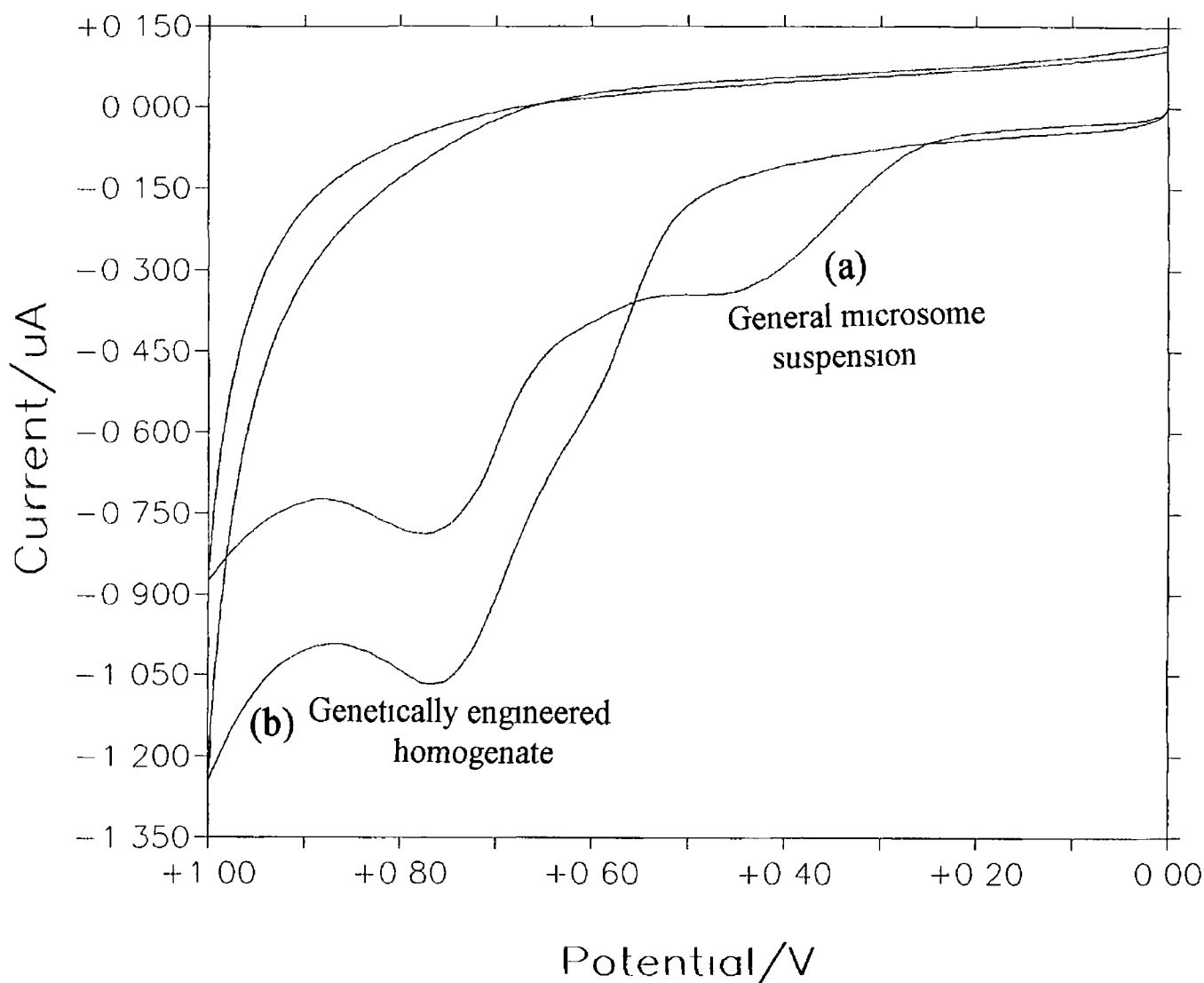


Fig 3 21 Cyclic voltammograms of (a) human microsomal suspension after 5 μ l of microsomal suspension was dropped onto a glassy carbon electrode surface and (b) genetically engineered cells, after 5 μ l of genetically engineered homogenate was dropped onto a glassy carbon electrode surface and each allowed to dry for 1 hour

Figure 3 21 shows that the oxidation peaks are seen more clearly now at approximately +0.40 and +0.75 V. An experiment was also carried out by dropping 5 μ l of homogenate from genetically engineered V79 cells (expressing CYP 1A2) onto a clean glassy carbon electrode, allowing this to air dry for 1 hour and running a cyclic voltammogram as above. This CV is also shown in Fig. 3 21. It shows that one of the oxidation peaks (+0.75 V) is also seen in the genetically engineered fraction. Differential pulse voltammetry was used to try to detect these oxidative peaks with more sensitivity, and this was performed by dropping 5 μ l of R6 microsomal preparation onto the clean glassy carbon electrode, allowing to dry for 1 hour and followed by a differential pulse scan between 0.0 and 1.0 V, at 15 mV/s. This is shown in Figure 3 22 below. It shows more definitely that two oxidation peaks are present in the general microsome preparations. Work was then performed to try to determine the origin of these oxidation peaks. It was initially thought that these peaks were due to electrochemical reactions of some proteins in the microsome suspensions, but this was discounted when after boiling a solution of the microsome suspension (50 μ l of microsomes + 100 μ l phosphate buffer) for 30 minutes, the oxidation peaks were still present. Any protein should have been denatured after this treatment.

After some study into what the peak at +0.75 V was due to, NADPH was thought to be a possibility, and Fig. 3 23 shows 3 voltammograms of 3 solutions which contains (a) 170 μ l of pH 7.5, 0.1M phosphate buffer and 20 μ l of R6 microsome suspension, (b) same as (a) with 0.2 mM of NADPH spiked into it, and (c) a CV which was performed with a glassy carbon electrode which had 5 μ l of the genetically engineered cells dried on it for 1 hour prior to scanning in phosphate buffer, 0.1M, pH 7.5. The

CVs were scanned between 0.0 and +1.0 V, at 15 mV/s. NADPH is oxidised in this region as will be seen later

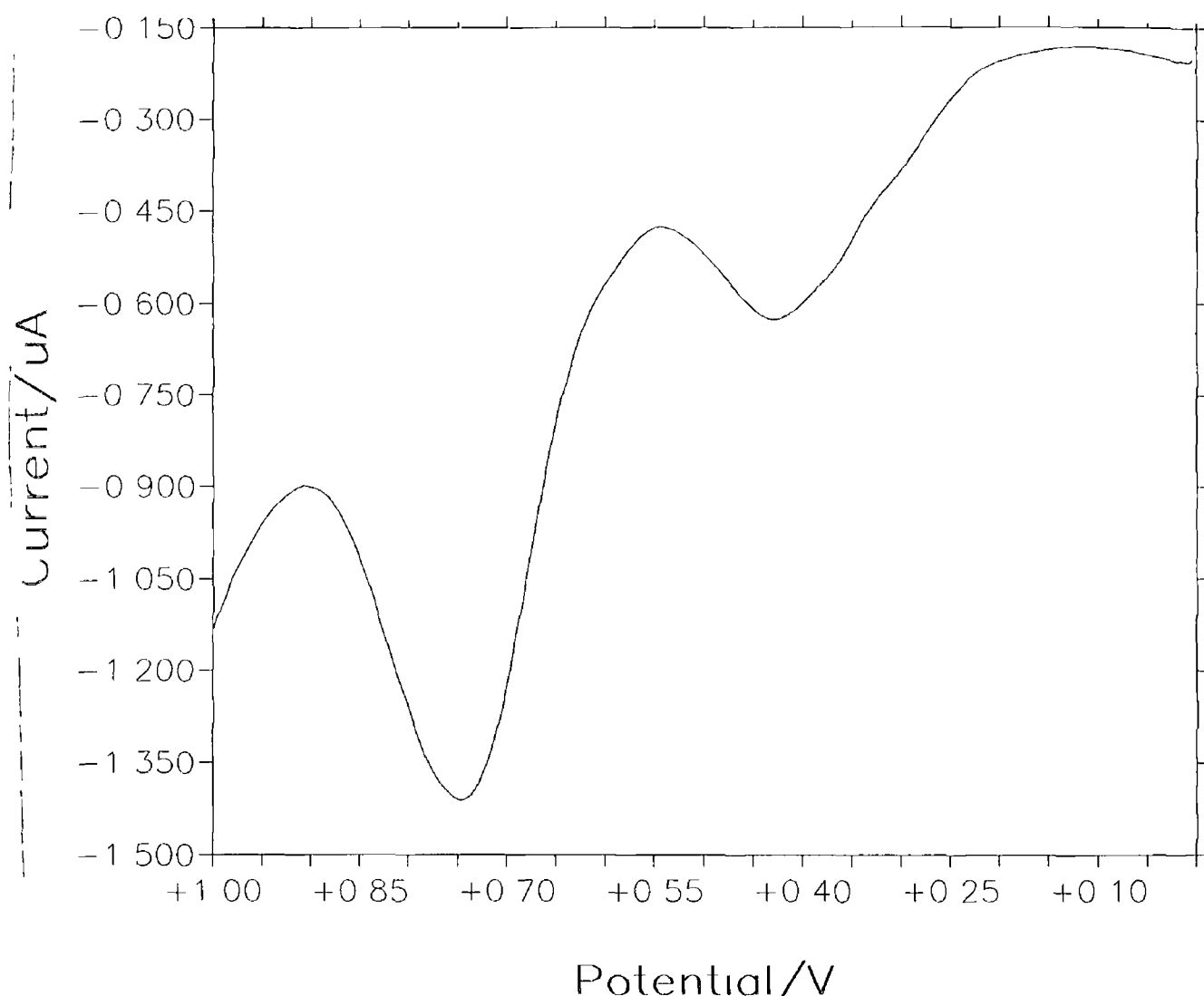


Fig 3.22 Differential pulse voltammogram of a glassy carbon electrode which had 5 μ l of R6 microsome suspension dropped onto and dried for 1 hour, prior to scanning. It was scanned from 0.0 and +1.0 V at 15 mV/s in 10 ml of 0.1 M, phosphate buffer, pH 7.5

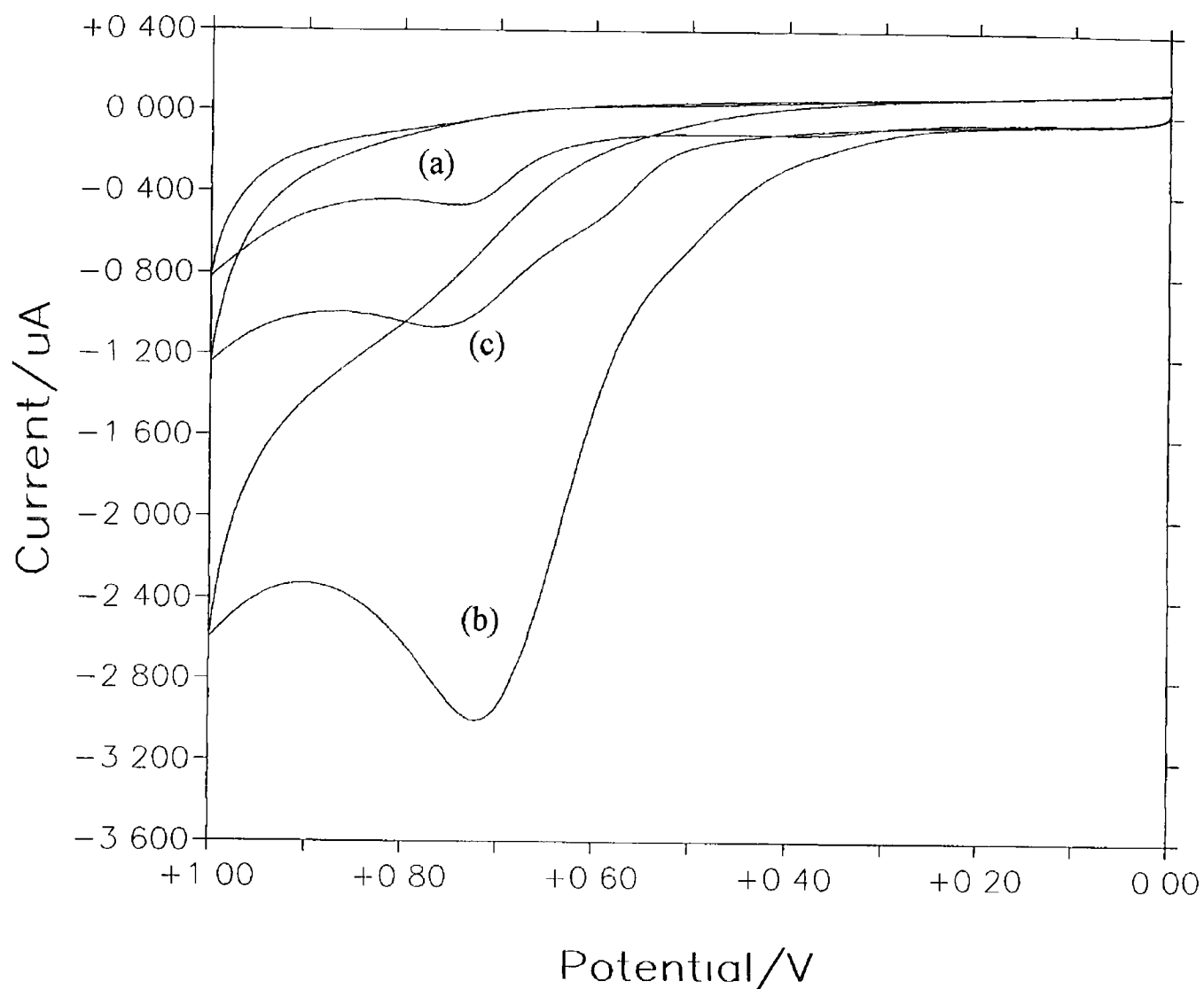


Fig 3 23 Voltammograms of 3 solutions which contain (a) 170 μ l of 0.1 M phosphate buffer, pH 7.5, and 20 μ l of R6 microsome suspension, (b) same as (a) with 0.2 mM of NADPH spiked into it, and (c) a CV which was run with a glassy carbon electrode which had 5 μ l of the genetically engineered cells dried on it for 1 hour prior to scanning in 0.1 M phosphate buffer, pH 7.5. The CVs were scanned between 0.0 and +1.0 V, at 15 mV/s.

This figure shows that the oxidation peak at +0.75V could be due to native NADPH in the microsomes and genetically engineered cells. More evidence towards this theory was obtained when an experiment was carried out, when after recording CVs (under the same conditions as above) of a human liver microsome suspension (50 μ l of R1 + 100 μ l 0.1M, phosphate buffer, pH 7.5) at time 0 and after 120 minutes at room temperature, the oxidation peak at 0.75V was seen to disappear. This is shown in Figure 3.24. This has already been seen to happen when NADPH was incubated in the presence of microsomes (see sections 3.2.2.2 and 3.2.2.4). It is used up in natural biological processes in the microsomes. This was seen to happen here over 120 minutes even at room temperature (20°C). It must also be remembered that the peak did not disappear when the microsome solution was rapidly boiled for 30 minutes. This would have destroyed any protein, hence NADPH would not be used up by protein related reactions in the microsomes. This result seems to suggest that the peak at +0.75V may be due to native NADPH in the microsomes but further proof would be needed for this. No experiments were able to suggest what the peak at +0.40 V was due to.

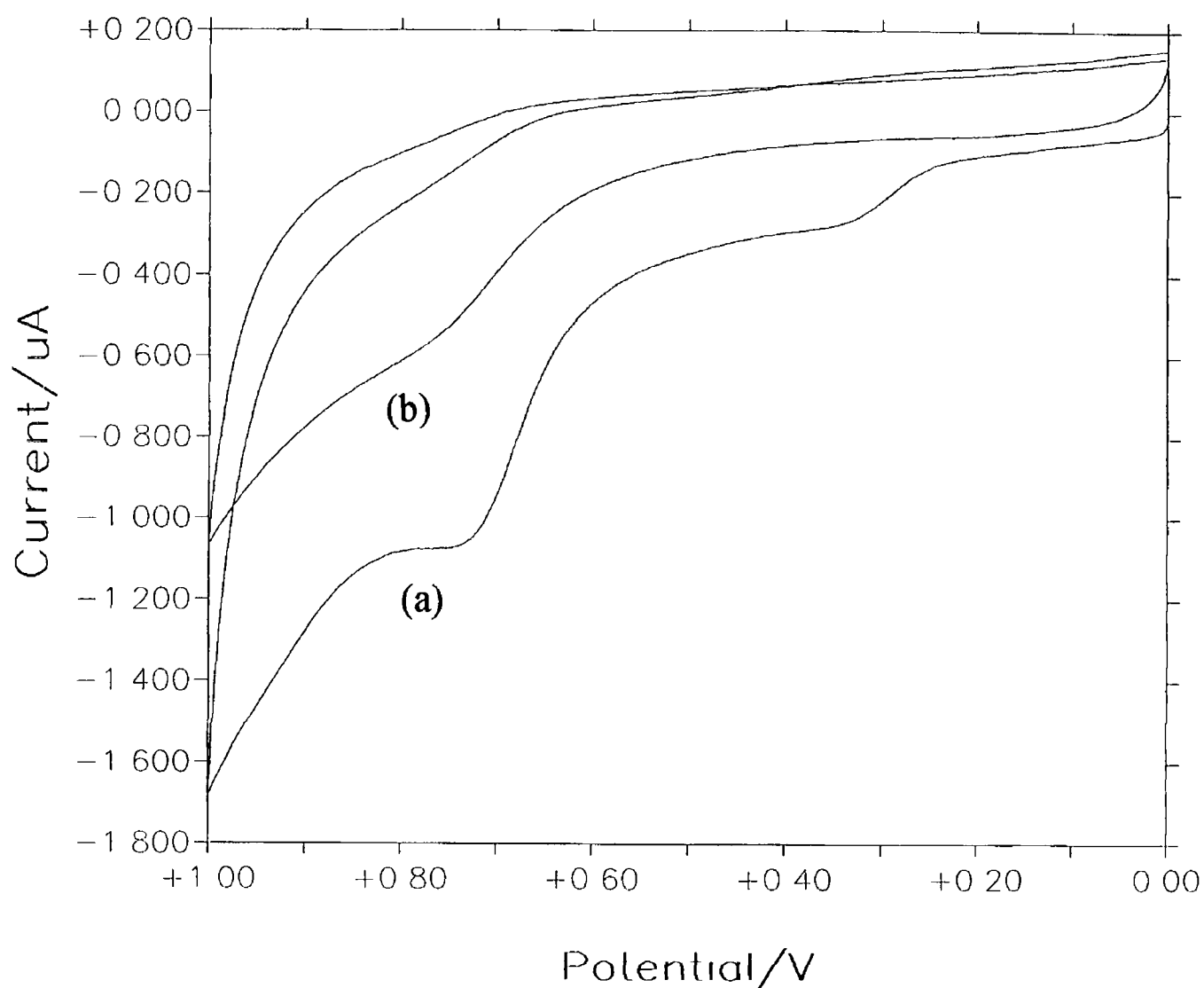


Fig 3 24 Cyclic voltammograms of a human liver microsome suspension (50 μ l of R1 + 100 μ l 0.1M, phosphate buffer, pH 7.5) at (a) time 0 and (b) after 120 minutes at room temperature. Scan taken between 0.0 and +1.0 V at 15 mV/s

3 3 2 2 Study of incubation reactions using cyclic voltammetry

By monitoring the NADPH present in an microsomal incubation solution using cyclic voltammetry, it was possible to follow the disappearance of this cofactor, just as it was by using capillary electrophoresis. A series of experiments were designed and the results found were very similar to that found using CE.

An interesting experiment carried out was the incubation of 20 μ l of human liver microsome preparation R6 (in 170 μ l of 0.1 M, phosphate buffer, pH 7.5) with 1 mM NADPH at 37 °C. It is worth noting that there is no substrate to cytochrome P450 present in this solution. This incubation was monitored by running a cyclic voltammetric scan between 0.0 and +1.0 V at 15 mV/s at the start of the incubation and after 20 minutes of incubation (see Fig. 3.25). This voltammogram shows that NADPH is used up by cellular processes in the microsomes even in the absence of any substrate to cytochrome P450. This is similar to the result that we have also seen using capillary electrophoresis (see sections 3.2.2.2 and 3.2.2.4). Fig. 3.26 shows a CV (under the same conditions as above) of 1 mM NADPH in 0.1 M, phosphate buffer, pH 7.5, before and after 20 minutes of incubation at 37 °C. This shows that the above observation is not decomposition of NADPH but the utilisation of the cofactor in cellular processes. The reason for the shift in potential between Figs 3.25 and 3.26 is probably due to the NADPH being in significantly different matrices (microsomes versus buffer).

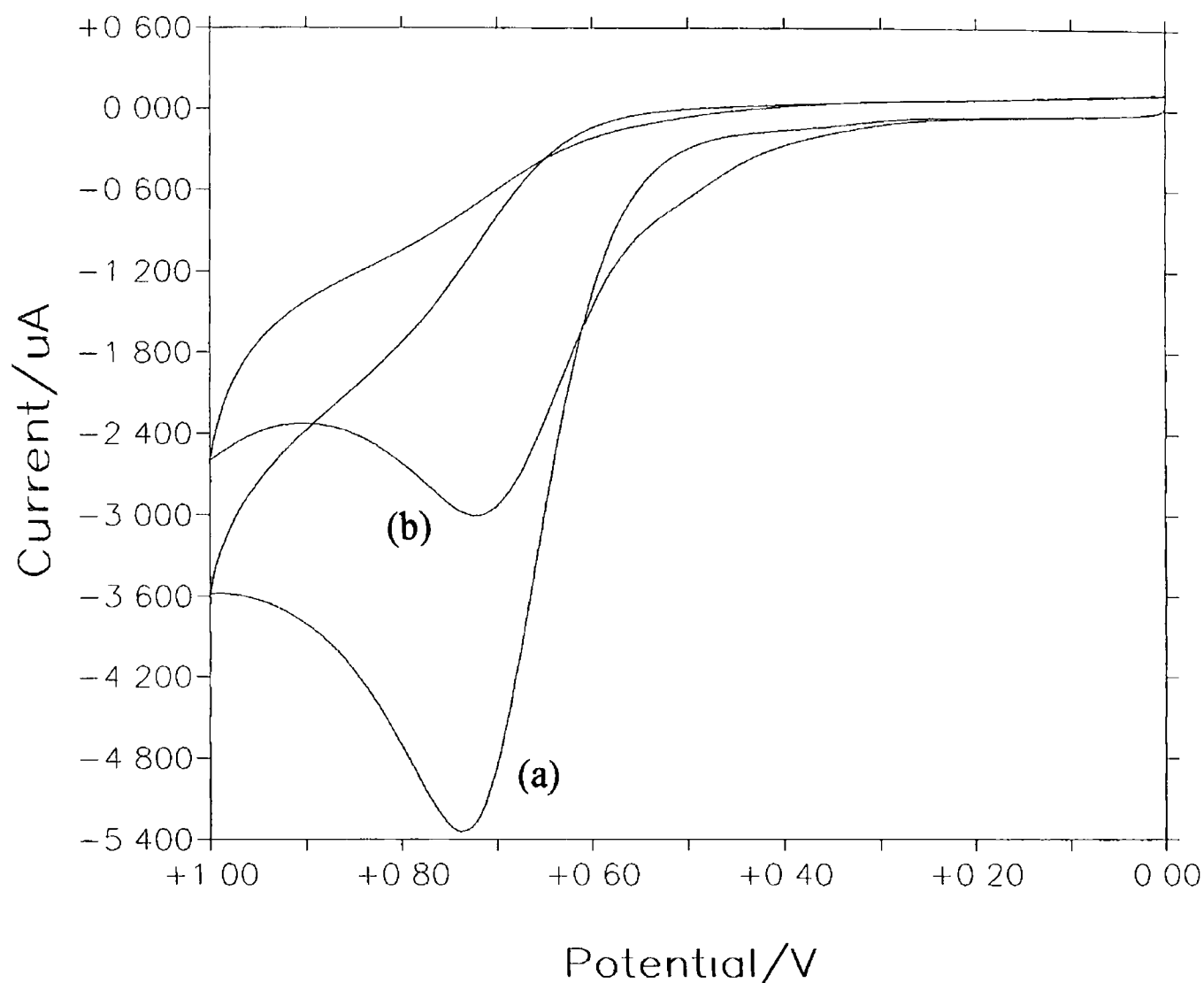


Fig 3 25 Incubation of 20 μ l of human liver microsome preparation R6 (in 170 μ l of 0.1M, phosphate buffer, pH 7.5) with 1 mM NADPH at 37 $^{\circ}$ C. This incubation was monitored by running a cyclic voltammetric scan between 0.0 and +1.0 V at 15 mV/s at (a) the start of the incubation and (b) after 20 minutes of incubation.

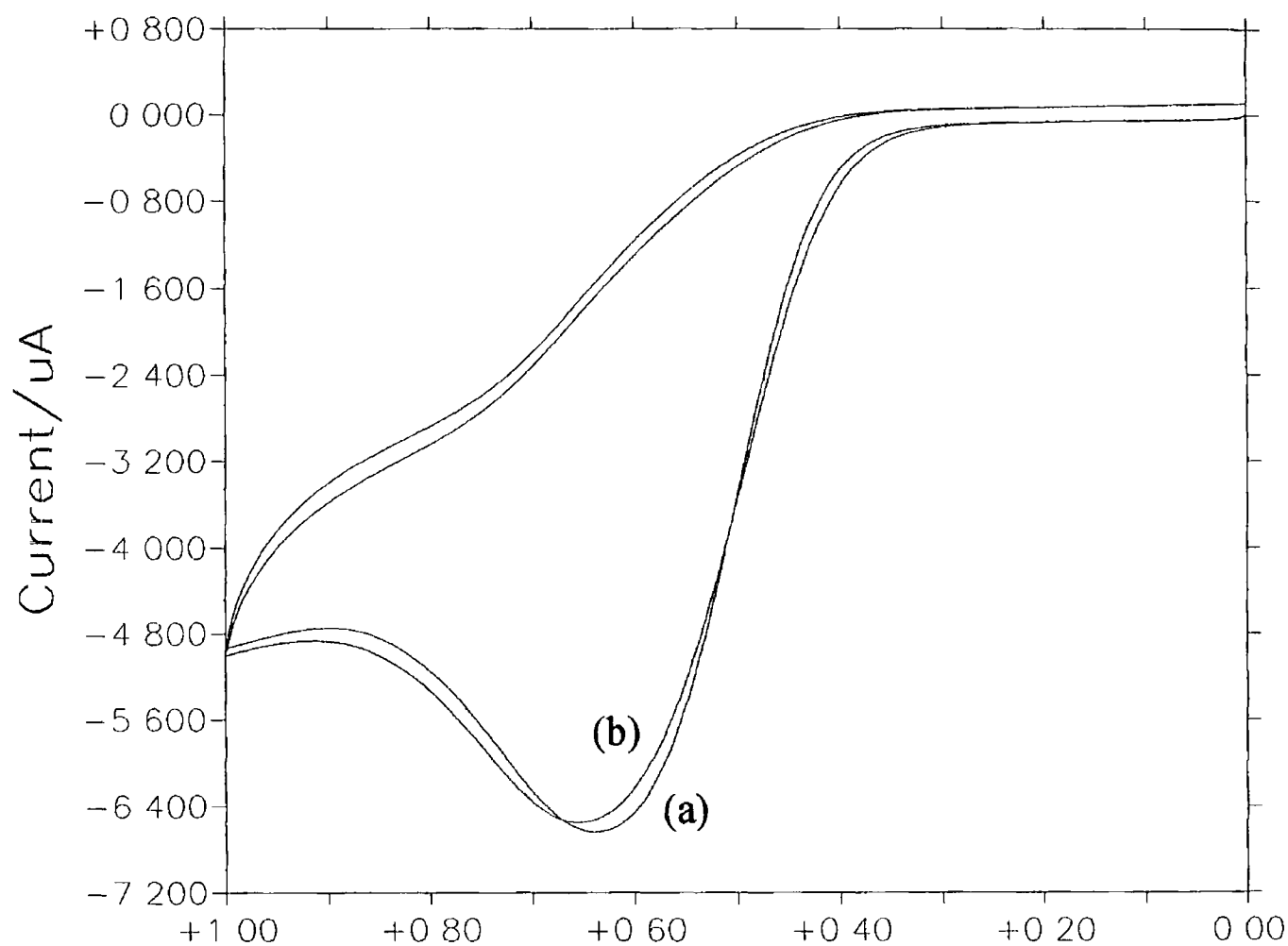
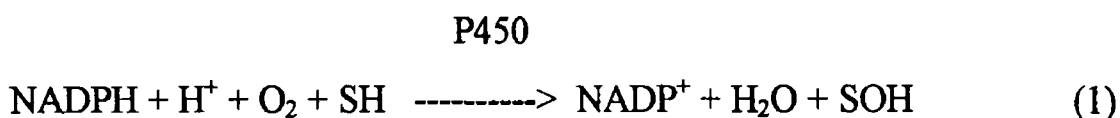


Fig 3 26 Cyclic voltammogram (under the same conditions as Fig 3 25) of 1 mM NADPH in 0.1 M, phosphate buffer, pH 7.5, (a) before and (b) after 20 minutes of incubation at 37 °C

As outlined already, the initial aim was to develop a biosensor capable of monitoring cytochrome P450 enzyme reactions. With reference to the equation



any attempt to design a biosensor with a view to monitoring cytochrome P450 enzyme reactions would fail if the sensor were to utilise the appearance or disappearance of NADPH, for the reason that NADPH is used by many cell processes. Also, oxygen or hydrogen ion consumption could not be made use of in constructing a sensor, since these are also used in numerous cell reactions. The best approach to constructing a biosensor to follow cytochrome P450 would be to directly monitor the change in redox states of the heme group in the P450 enzyme.

3.3.2.3 Investigations in the cathodic region

Investigations were also carried out in the cathodic region to study if any electrochemical responses could be detected in this region. Electrolytes in this region were deaerated with nitrogen prior to CV, as otherwise the reduction of oxygen would mask any small electrochemical response. Figure 3.27 shows the cyclic voltammogram of a solution which contains 200 μl of human liver microsomes (R6) + 100 μl of 0.1M, phosphate buffer, pH 7.5. This solution was de-aerated for 15 minutes by gently bubbling nitrogen through it, followed by scanning between 0.0 and -1.0 V.

(vs Ag/AgCl) at 200 mV/s It shows that a redox couple is detected at approximately -0.40 V (vs Ag/AgCl) This redox couple was detected in all of the human liver microsomes, with the currents observed varying between microsomes from different patients Investigations were carried out to try to identify the origin of the redox couple

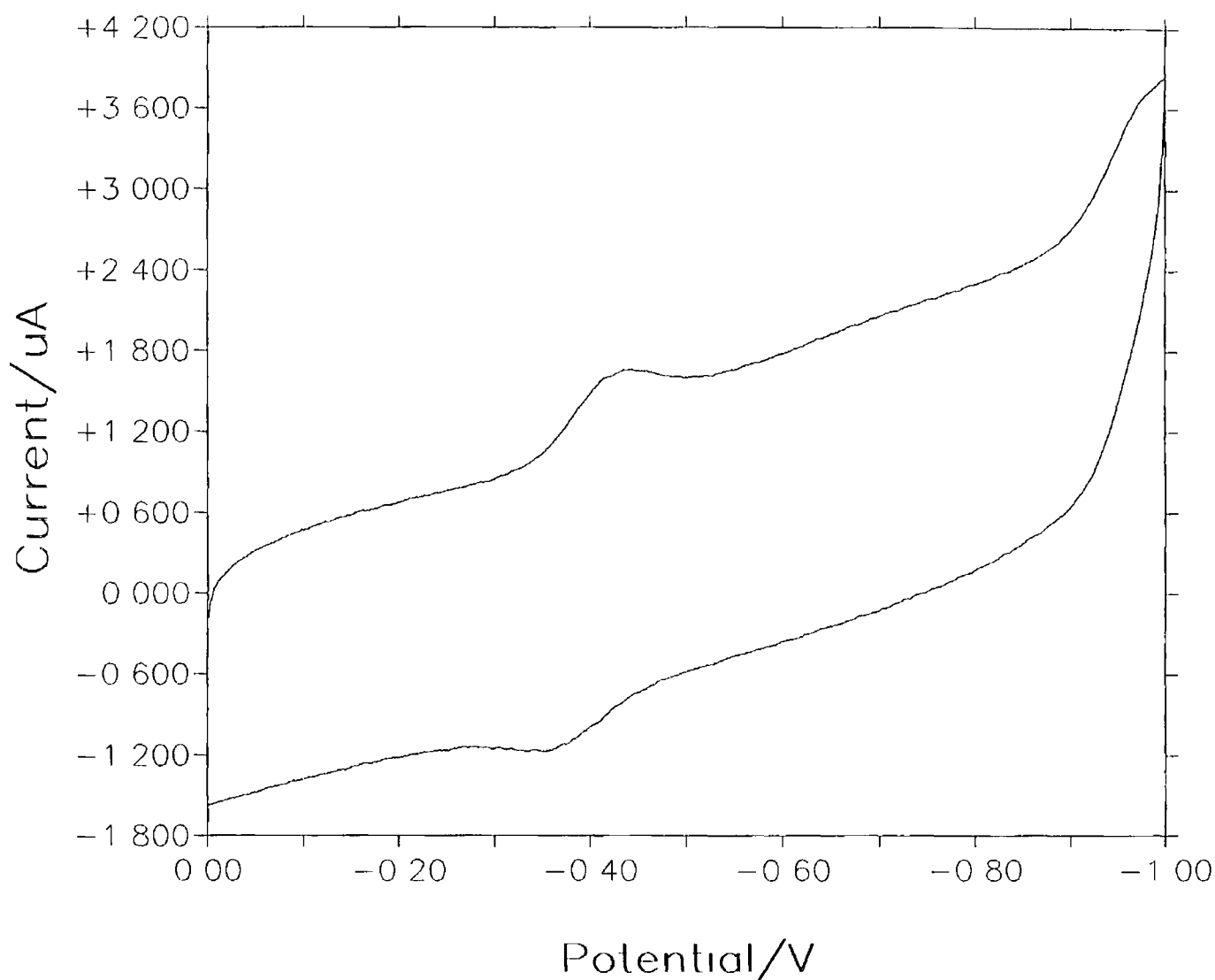


Figure 3.27 Cyclic voltammogram in the cathodic region of a solution which contains 200 μl of human liver microsomes (R6) + 100 μl of 0.1M, phosphate buffer, pH 7.5. This solution was deaerated for 15 minutes by gently bubbling nitrogen through it, followed by scanning between 0.0 and -1.0 V (vs Ag/AgCl) at 200 mV/s.

An interesting experiment was carried out by incubating 50 μ l of human liver microsomes (R6 preparation) + 100 μ l 0.1M, phosphate buffer, pH 7.5, with 2 mM coumarin and 1 mM NADPH. A cyclic voltammetric scan was run after 45 minutes of incubation at 37 $^{\circ}$ C, after de-aeration. Figure 3.28 shows the CV obtained. It also shows a CV of the R6 microsomal suspension (50 μ l + 100 μ l of 0.1M, phosphate buffer, pH 7.5) in the absence of substrate and cofactor, under the same conditions. The figure shows that the redox couple is affected by the incubation with substrate and cofactor, and this redox couple effectively disappears after incubation for 45 minutes. The redox couple is also affected by the presence of the reducing agent sodium dithionite (SDT). When 1 mM of SDT was added to the microsomal suspension (50 μ l of preparation R1 + 100 μ l of 0.1M, phosphate buffer, pH 7.5), the currents from the redox couple were considerably reduced (Fig. 3.29). The CVs were run between -0.20 and -0.80 V at 600 mV/s. This observation was interesting because SDT is used in cytochrome P450 investigations to reduce the heme group from Fe(III) to Fe(II).

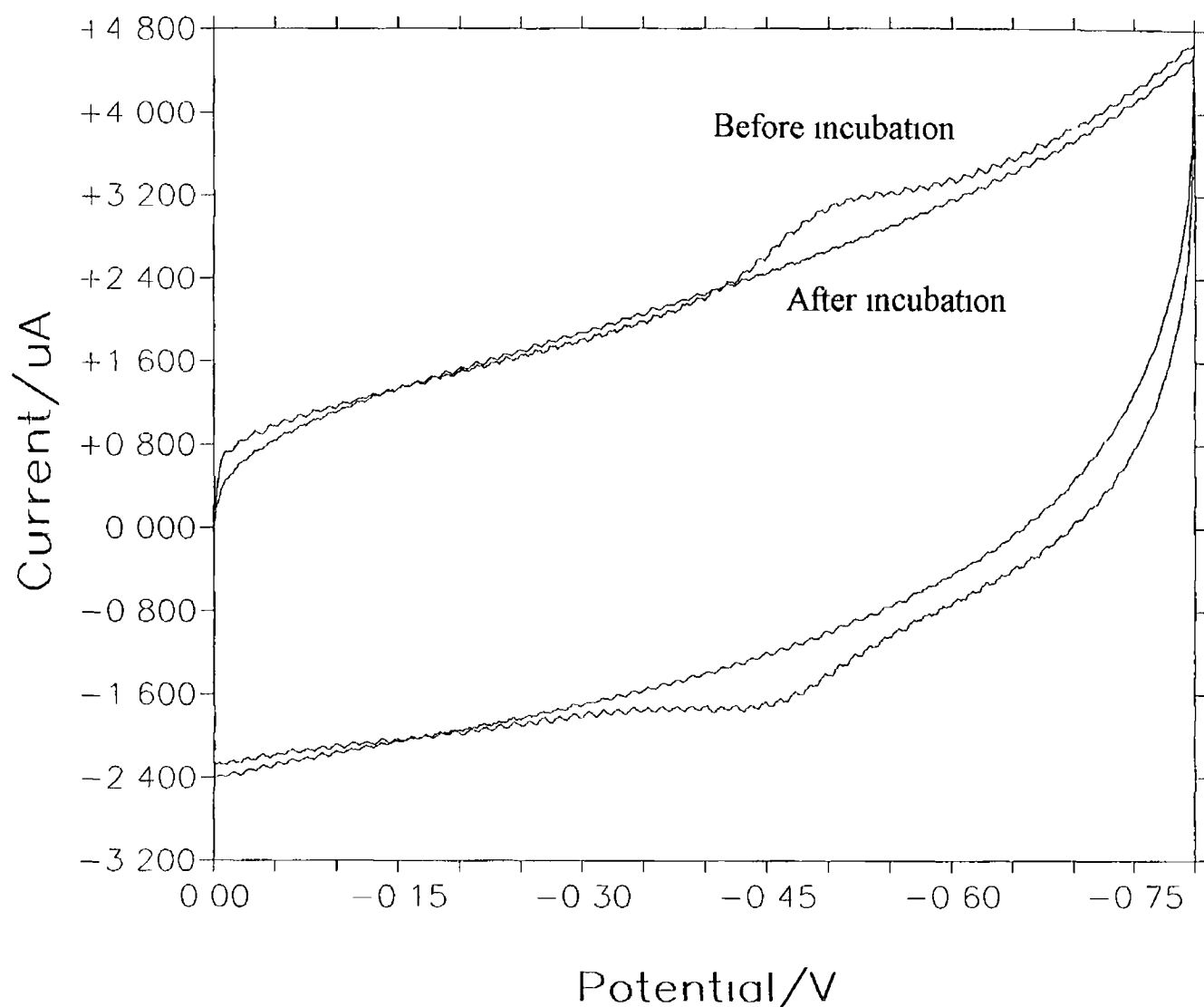


Fig 3 28 Incubation of 50 μ l of human liver microsomes (R6 preparation) + 100 μ l 0.1M, phosphate buffer, pH 7.5, with 2 mM coumarin and 1 mM NADPH. Cyclic voltammetric scan was run after 45 minutes of incubation at 37 °C, after deaeration. This figure also shows a CV of the R6 microsomal suspension (50 μ l + 100 μ l of 0.1M, phosphate buffer, pH 7.5) in the absence of substrate and cofactor, under the same conditions, these conditions being a CV scan between 0.0 and -1.0 V at a scan rate of 600 mV/s.

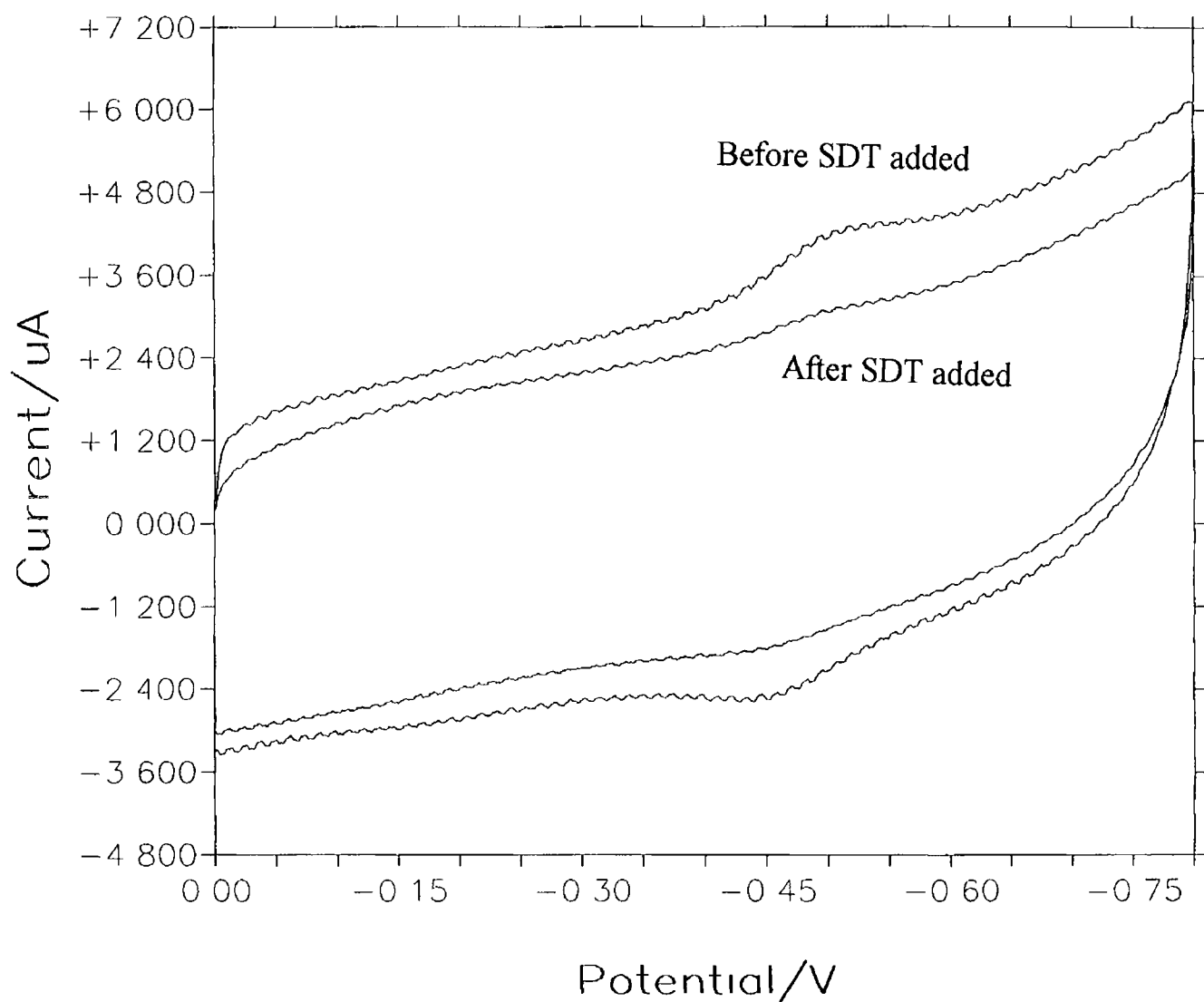


Fig 3 29 Cyclic voltammogram before and after 1 mM of SDT was added to the microsomal suspension (50 μ l of preparation R1 + 100 μ l of 0.1M, phosphate buffer, pH 7.5),

It may be possible that the heme group of the cytochrome P450 is being detected in this experiment, but it is also possible (and probably more likely) that the redox couple in the above cyclic voltammograms is due to the presence of cytochrome P450 reductase in the endoplasmic reticulum (microsomal) cytochrome P450 system. Cytochrome P450 reductase is bound by its hydrophobic tail to the membrane whereas cytochrome P450 is deeply embedded in the membrane [1]. Hence the heme group is extremely difficult to access and it is more likely that electrons can be transferred more easily to the reductase. As mentioned already (section 3.1.3), cytochrome P450 reductase contains both flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) groups. The FAD serves as an entry point for electrons from NADPH, and FMN serves as the exit point, transferring electrons individually to cytochrome P450.

Figure 3.30 shows a CV of 5 mM FMN (prepared in 0.1M pH 7.5 phosphate buffer) between 0 and -1V at 100 mV/s. FMN has a redox couple centred at -0.44V. Figure 3.31 shows a CV of 10 mM FAD (prepared in 0.1M phosphate buffer, pH 7.5) between 0.0 and -1.0 V at 100 mV/s. It is possible that if the cytochrome P450 reductase is accessible then we could be able to detect electron transfer between the reductase and the electrode surface, however much more work would have to be done to conclusively prove this. Along with these investigations other working electrodes, e.g. platinum and gold, were investigated in an effort to obtain more sensitivity in detecting the redox couple, but these were less successful than glassy carbon. Also surface modifiers on these working electrodes, e.g. L-lysine, were utilised, but again with no success.

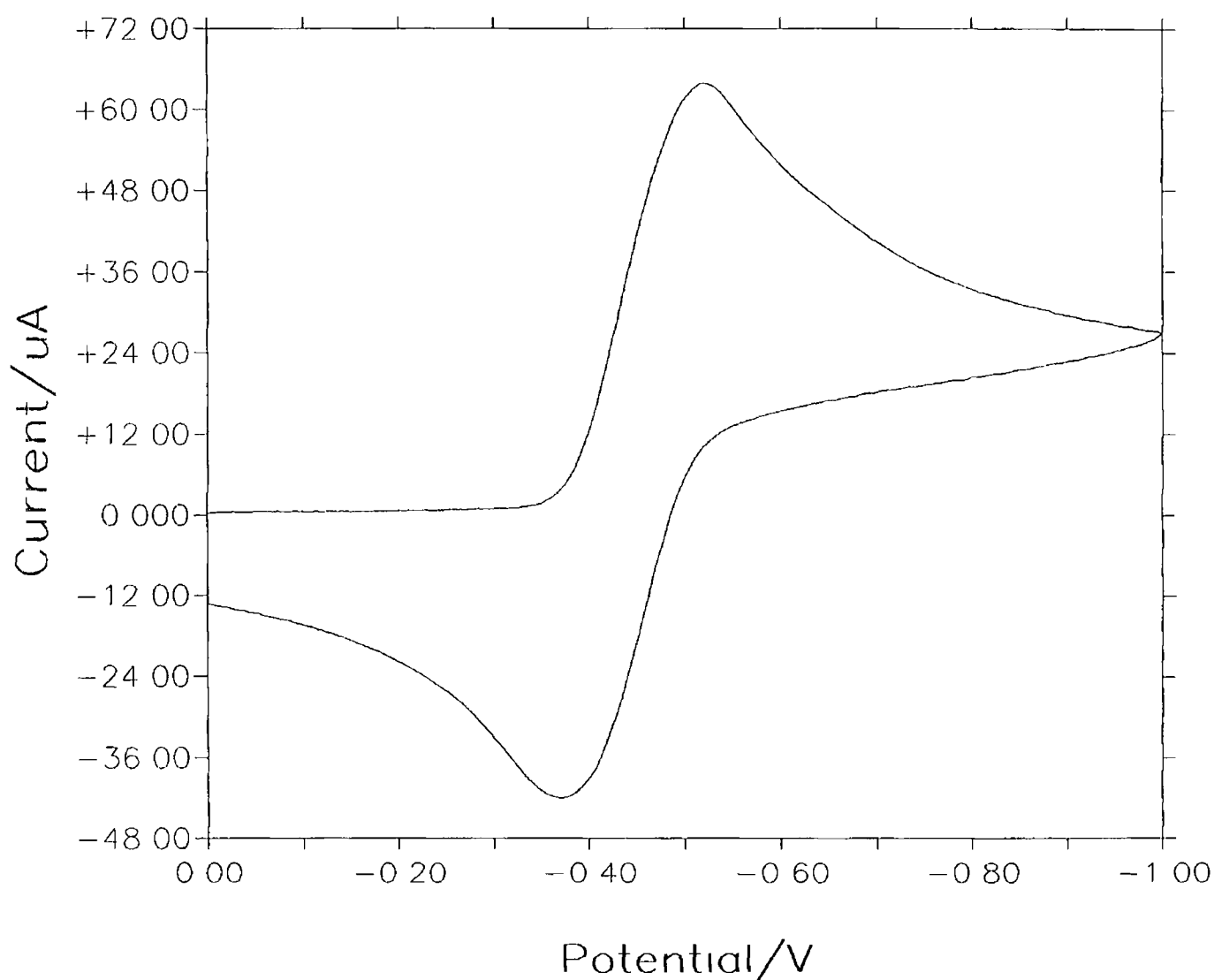


Figure 3.30 Cyclic voltammogram of 5 mM FMN (prepared in 0.1M phosphate buffer, pH 7.5) between 0.0 and -1.0 V at 100 mV/s. FMN has a redox couple centred at -0.44V.

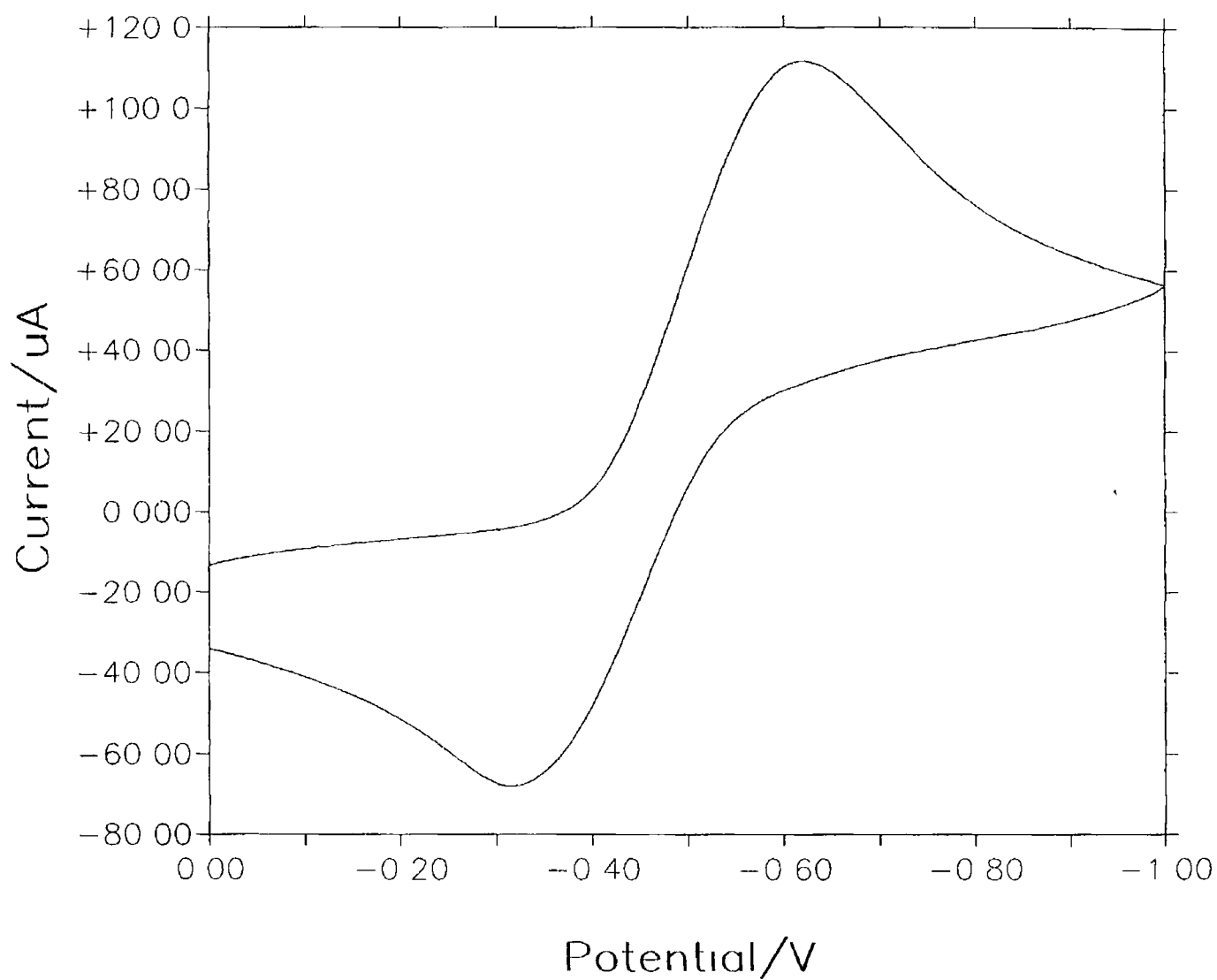


Figure 3.31 Cyclic voltammogram of 10 mM FAD (prepared in 0.1M phosphate buffer, pH 7.5) between 0.0 and -1.0 V at 100 mV/s

3.4 CONCLUSIONS

The above chapter has utilised two analytical techniques, capillary electrophoresis and cyclic voltammetry, to study metabolism reactions involving liver microsomes. Capillary electrophoresis was utilised to monitor the formation of 7-OHC by human liver microsomes without the need to stop the cytochrome P450 enzyme reaction. Significant advances were also made due to the fact that it was not necessary to add trichloroacetic acid to precipitate the protein prior to analysis. This is a considerable advantage compared to the HPLC methods used to date. It was shown that CE proved a useful technique and an alternative to HPLC in the monitoring of cytochrome P450 reactions.

What was also noticed throughout the work was that the cofactor for the cytochrome P450 reaction (NADPH) could also be monitored as the incubation progressed. This is also an advantage compared to existing technology. Some interesting observations were made about the cofactor, and it became clear that the cofactor was used for a number of processes in the liver microsomes. It was also shown from the NADP/NADPH ratio studies, that it was possible to use this ratio to determine if a compound's metabolism used NADPH as the cofactor. The stability of the NADPH was also studied and it was postulated that the cofactor is not stable in phosphate buffer at 37°C for long periods.

Capillary electrophoresis was also used for the study of interspecies differences of coumarin metabolism, and the findings from the work agreed with other work in the area, i.e. that the cynomolgous monkey proved to be the species most closely resembling the human metabolism of coumarin.

Electrochemistry also proved useful in the study of liver microsomal reactions. Investigations in the anodic region showed the presence of oxidation peaks, one of which may have been native NADPH in the microsome suspension. However, further work would be needed to conclusively prove this. In the cathodic region a redox couple was detected, and although again it was not possible to explain its origins it could be due to the presence of cytochrome P450 reductase in the microsome suspensions. Again much more work needs to be done to be sure of any explanation. The electrochemical work with the microsomes is made very difficult due to the fact that signals are very faint. This is primarily due to microsomes containing all the components of cells which will mask any signal.

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CHAPTER 4

DETERMINATION OF 7 - HYDROXYCOUMARIN USING A HORSE RADISH PEROXIDASE LABELLED ANTI -7 - HYDROXYCOUMARIN ANTIBODY-BASED BIOSENSOR

4.1 INTRODUCTION

Immunological techniques are particularly suited to the detection of low level concentrations of analytes in biological matrices. In the determination of low levels of analytes there are two main requirements (1) a sensitive assay, and (2) high selectivity. Immunosensors take advantage of the high selectivity provided by the molecular recognition of an antigen by its specific antibody. In principle, antibodies of high affinity may be produced against any antigen. Immunosensors are thus characterised by high selectivity, sensitivity and versatility. In the design of immunosensors (or any molecular sensor) two phenomena must be combined

(i) the selective molecular recognition of the target molecule. An important factor is the intrinsic selectivity of the biological material involved in the recognition process, as problems of interference may occur in complex samples,

(ii) the occurrence of a physical or chemical signal consecutive to the molecular recognition event, where the transduction mode can be either electrochemical, thermal or optical in nature or based on a change in mass

High sensitivity, as well as high selectivity and reproducibility, are essential requisites for immunosensors. Antibody properties for biosensor applications have been reviewed [1], together with future trends and future aspects of the research and development of immunosensors [2]

Along with these there have been a number of other reviews concerning antibody-based biosensors [3-7]. The selective reaction between an analyte and the corresponding antibody ensures the high specificity of the immunosensor in biological fluid analysis. The specificities of immunosensors are determined in part by the affinity constants of the antibody-antigen interactions [8]. While a high affinity constant will give rise to high specificity, too high an affinity constant will result in an irreversible reaction, unless protein denaturing conditions are used.

The major processes involved in any biosensor system in Fig 4.1 [9]. There are a number of ways in which the binding event can be transduced into a measurable signal. Typical transducers include electrochemical, optical, acoustic and semiconductor devices.

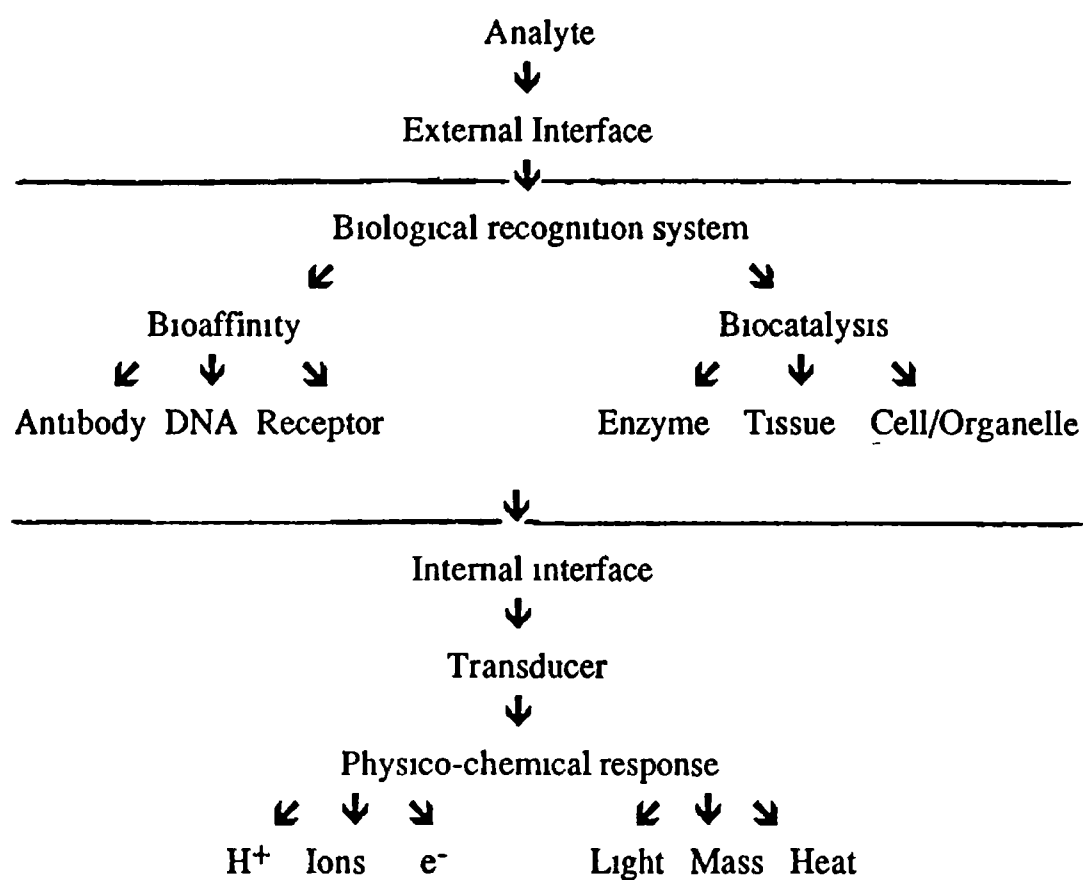


Fig 4 1 Scheme representing the processes taking place in a typical biosensor system (Reproduced from reference [9])

4 1 1 IMMUNOSENSORS BASED ON OPTICAL DEVICES

In a surface plasmon resonance (SPR) immunosensor system, when the antigen binds, minute changes in the refractive index of the SPR device are detected as a shift in the angle of total absorption of light incident on a metal layer containing immobilised antibodies. The SPR device can consist of a prism or a glass slide carrying the thin metal layer. Recently, Morgan and Taylor [10] have developed a surface plasmon resonance immunosensor based on the streptavidin-biotin complex. Nice *et al* [11] have published work on the mapping of the antibody and receptor-binding domains of granulocyte colony-stimulating factor using an optical biosensor. The data was obtained using a BIAcore biosensor, with surface plasmon resonance detection.

A BIAcore biosensor was also developed for the analysis of 7-OHC in biological samples [12]. The BIAcore sensor system utilises a combination of optical and continuous flow technologies to facilitate interaction analysis. Biomolecular binding can be studied by immobilising one of the components on the surface of a specialised sensor chip within the biosensor, and allowing the other to flow over the surface in solution. Mass changes at the biosensor chip surface due to the binding of two or more components causes a change in the resonance angle directly proportional to the mass change. In the 7-OHC BIAcore biosensor, 7-OHC-bovine serum albumin was first immobilised on the sensor chip surface. A 20 ml volume of antibody-sample mixture was then injected into the system. The 7-OHC-BSA conjugate immobilised on the sensor surface and the 7-OHC in the sample compete for the antibody binding. The resulting signal due to the unbound antibody binding to the drug

protein conjugate immobilised on the sensor chip is inversely proportional to the amount of 7-OHC present in the sample. The range of the assay was quoted to be from 0.5 - 80 $\mu\text{g/ml}$.

Optical immunosensors can also be based on internal reflections in a waveguide with antibodies immobilised on the surface of the guide which is in contact with the analyte. Use can then be made of the evanescent wave penetrating a fraction of a wave-length into the optically rarer medium when light coming from a denser medium is incident on the interface with an angle above the critical angle. Fluorescent techniques can be used to good advantage with these devices. Bright [13] has reported on probing biosensor interfaces, by multi-frequency phase and modulation total-internal reflection fluorescence. There are quite a number of immunosensors in the recent literature using the fiber optic approach [14,15]. For example, Bier *et al* [16] have made use of a fiber optic immunosensor for the detection of certain pesticides. Aminoethyltriazine was immobilised on one end of a silica fiber and the fiber was inserted into a flow-through glass cell. Triazine antibodies (conjugated with fluorescein isothiocyanate) were detected after binding to the fiber surface based on the decrease in the fluorescence signal caused by the presence of triazines due to the inhibition of antibody binding to the fiber.

4 1 2 IMMUNOSENSORS BASED ON SURFACE ACOUSTIC WAVE DEVICES

Surface acoustic wave (SAW) devices, which can be sensitive to changes in the elasticity, density or electrical conductivity of a surface on which a surface acoustic wave propagates, can also be used as immunosensors. The device usually consists of a piezoelectric crystal, such as quartz carrying thin-film interdigitated electrode arrays. Radiofrequency excitation of the electrode pair creates a synchronous mechanical surface wave, which is propagated on the surface of the piezoelectric substrate and received by another electrode pair. Koenig and Graetzel [17] have used a piezoelectric immunosensor for the detection of human T-lymphocytes. The frequency difference between the coated crystal (coated with antibody) and a reference crystal was first measured, then the coated biosensor was placed in a solution of human T-lymphocytes and a second frequency difference was measured.

Prusak-Sochaczewski and Luong [18] have also used a piezoelectric crystal immunosensor for the determination of human serum albumin. The sensor was prepared by coating the crystal with protein A and reacting this with anti-human serum albumin antibody. This surface could then be used in the determination of human serum albumin.

4 1 3 IMMUNOSENSORS BASED ON SEMICONDUCTOR DEVICES

In immuno field effect transistors (ImmunoFETs), the gate of an ion selective field effect transistor is covered with an antibody-containing membrane. They can be constructed for the detection of the minute potential changes associated with the formation of the antibody-antigen complex. An example of an immunoFET was developed by Schasfoort *et al* [19], who reported on an immunosensor to monitor the immunological reaction between human serum albumin and its antibody.

4 1 4 IMMUNOSENSORS BASED ON ELECTROCHEMICAL TRANSDUCERS

Electrochemical measurements for immunosensing fall into two broad classes, (i) amperometric devices and (ii) potentiometric devices. Amperometric devices involve the measurement of the current flowing between two electrodes in response to the application of a fixed potential. In contrast, potentiometric devices require the derived voltage to be determined with reference to a second electrode under conditions of zero current flow.

In terms of potentiometric immunoelectrodes, Blackburn *et al* [20] have recently described a biosensor employing catalytic antibodies as the molecular recognition element. In their system, a pH microelectrode was modified with a monoclonal antibody that catalysed the hydrolysis of

phenyl acetate to produce protons which would then be sensed at the potentiometric electrode

Some recently developed amperometric immunosensors include a sensor for the determination of cholinesterase [21] and immunosensors based on electropolymerised poly(tyramine)-modified electrodes [22] Recently, Sadik and Wallace have used pulsed amperometric detection for the determination of human serum albumin using an antibody-containing conducting polymer [23]

Dempsey *et al* [24] developed an immunosensor based on the use of anti-7-OHC antibodies immobilised at the surface of a glassy carbon electrode behind a permeable cellulose dialysis membrane When the antibody was present at the electrode surface, there was a significant decrease in the 7-OHC electrochemical response The binding of the antigen to the antibody was believed to occur at the electroactive site of the drug Anodic detection of the oxidised product was achieved at 0.60 V using DC amperometry

In work carried out by the candidate as part of his Ph D studies, but not reported in detail in this thesis, a novel amperometric immunosensor was developed for the quantitation of Factor VIII, a glycoprotein whose concentration is important in the diagnosis of haemophilia [25] The method was based on the decrease in current observed when free Factor VIII competes with Factor VIII immobilised on a glassy carbon electrode for specific anti-Factor VIII antibody labelled with horseradish peroxidase Current was found to be inversely proportional to the concentration of Factor VIII in solution The enzyme horseradish

peroxidase (HRP) catalyses the reaction between hydrogen peroxide and hydroquinone, and electrochemical biosensing was based on the amperometric monitoring of the electrochemical reduction of the oxidised mediator, benzoquinone

The purpose of this present study was to develop an amperometric immunosensor for the determination of 7-hydroxycoumarin using horseradish peroxidase labelled anti-7-hydroxycoumarin antibody, which would be easier to prepare in comparison to the immunosensor of Dempsey *et al* [24] and would function with a similar principle to that of the Factor VIII immunosensor described above [25]

4.2 EXPERIMENTAL

4.2.1 APPARATUS

A conventional 3 electrode system, with glassy carbon, saturated calomel and platinum gauze as working, reference and counter electrodes respectively, was connected to an EG&G PAR (Princeton Applied Research, Princeton, NJ, USA) Model 264A polarographic analyser with output currents being measured by a WPA (Linton, Cambridge, UK) Model CQ95 recorder. A batch amperometric mode of operation was used throughout, with a magnetic stirrer and bar providing the convective transport. A heat gun was used in the electrode preparation and an incubator (Mettler, West Germany) was used in the temperature studies.

4 2 2 ELECTRODE PREPARATION

Before use, the bare glassy carbon electrode was first polished with alumina slurry, after which it was sonicated in distilled water, rinsed with distilled water and allowed to dry in air. Modification of the electrode was achieved via drop coating of a 10 μ l aliquot of the appropriate amount of conjugated protein (ovalbumin-7-OH-coumarin or thyroglobulin-7-OH-coumarin) in 5% Nafion, onto the electrode surface. The modified electrode was then dried for 1 hour using the heat gun.

In the course of the optimisation studies the modified electrode was incubated in the appropriate concentration of HRP-labelled anti-7-OH-coumarin antibody (in 0.1 M phosphate buffer, pH 7.3) at the required temperature for 60 minutes. During the competitive assay, the modified electrode was incubated in a solution of anti-7-OH-coumarin antibody [made up in 0.1M phosphate buffer, pH 7.3] and the appropriate concentration of free 7-OH-coumarin (10 mM stock solution made up in 90:10 water-methanol daily).

4 2 3 PROCEDURES

The modified electrode was then rinsed in 0.1 M phosphate buffer, pH 7.3, and introduced into the electrochemical cell. DC amperometry was carried

out at a potential of -0.03 V Vs SCE after purging the phosphate buffer electrolyte solution (pH 7.3) with oxygen-free nitrogen for 20 minutes. The hydroquinone mediator (0.1 M stock solution made up in 0.1 M phosphate buffer immediately prior to use) was injected into the cell to reach a final concentration of 2 mM and the hydrogen peroxide substrate was added to give a final concentration of 1 mM in the cell. The reduction current of the oxidised mediator was then measured.

4.2.4 REAGENTS

Batch experiments were conducted at room temperature in 0.1 M phosphate buffer (pH 7.3), and all solutions were prepared in deionised water obtained by passing distilled water through a Milli-Q water purification system (Millipore, Milford, MA, USA). 7-hydroxycoumarin (Umbelliferone) was purchased from Sigma (St. Louis, MO, USA). The anti-7-OH-coumarin antibodies were raised in rabbits [26] and the HRP-labelling was carried out according to Tijssen [26]. A 5 mg ml⁻¹ solution of the HRP-labelled antibody was diluted with 0.1 M phosphate buffer, pH 7.3. Diazotisation and conjugation of 7-OH-coumarin to BSA, ovalbumin and thyroglobulin was performed using a modification of methods reported by Morgan et al. [27] and Baker et al. [28].

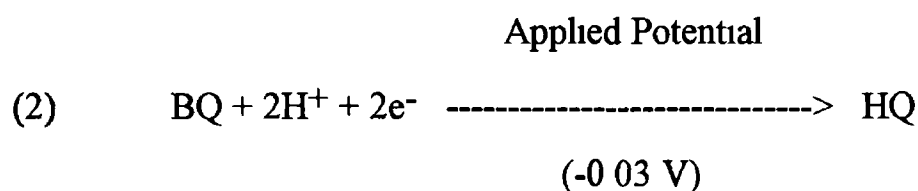
Potassium dihydrogen phosphate and dipotassium hydrogen phosphate were obtained from Riedel-de-Haen (Hannover, Germany). Methanol (HPLC grade) was obtained from Labscan (Dublin, Ireland). Hydroquinone was purchased from the Aldrich (Dorset, UK). Nafion

(perfluorinated ion-exchange powder, 5% solution in lower aliphatic alcohol's and 10% water) was purchased from the Aldrich (Dorset, UK) Hydrogen peroxide was obtained from Riedel-de-Haen (Hannover, Germany) A 0.1 M solution of hydrogen peroxide was made up daily in 0.1 M phosphate buffer, pH 7.3

4.3 RESULTS AND DISCUSSION

4.3.1 SENSING MECHANISM

The enzyme horseradish peroxidase (HRP) catalyses the reaction between hydrogen peroxide and hydroquinone (HQ), and electrochemical biosensing is based on the amperometric monitoring of the electrochemical reduction of the oxidised mediator benzoquinone (BQ) [29]



4 3 2 DEVELOPMENT OF AN OVALBUMIN-7-OH-COUMARIN MODIFIED ELECTRODE

A 15% (w/v) ovalbumin-7-OH-coumarin modified electrode was prepared as described in the Experimental section. This modified electrode was then incubated in a 0.33 mg ml^{-1} anti-7-OH-coumarin antibody solution at 37°C for 1 hour, after which it was rinsed with 0.1 M phosphate buffer (pH 7.3). The steady state response of this modified electrode to successive additions of $1 \text{ mM H}_2\text{O}_2$, in the presence of 2 mM hydroquinone as mediator, is shown in Fig 4.2 (a).

The steady state response for the control electrode (ovalbumin only on surface) is shown in Fig 4.2 (b). This demonstrates that there is a significant interference at this protein modified electrode. This was not entirely unexpected as the rabbits were initially immunised with a bovine serum albumin (BSA) -7-OH-coumarin conjugate [26] and some of the antibodies produced have an affinity for BSA itself. Ovalbumin is a very similar protein to BSA [30] (both being albumins), and this would be likely to explain the high response for the control electrode. Globulins are significantly different proteins compared to albumins [30], and therefore, a thyroglobulin-7-OH-coumarin conjugate was synthesised [27,28], and a thyroglobulin modified electrode was compared to the ovalbumin modified electrode.

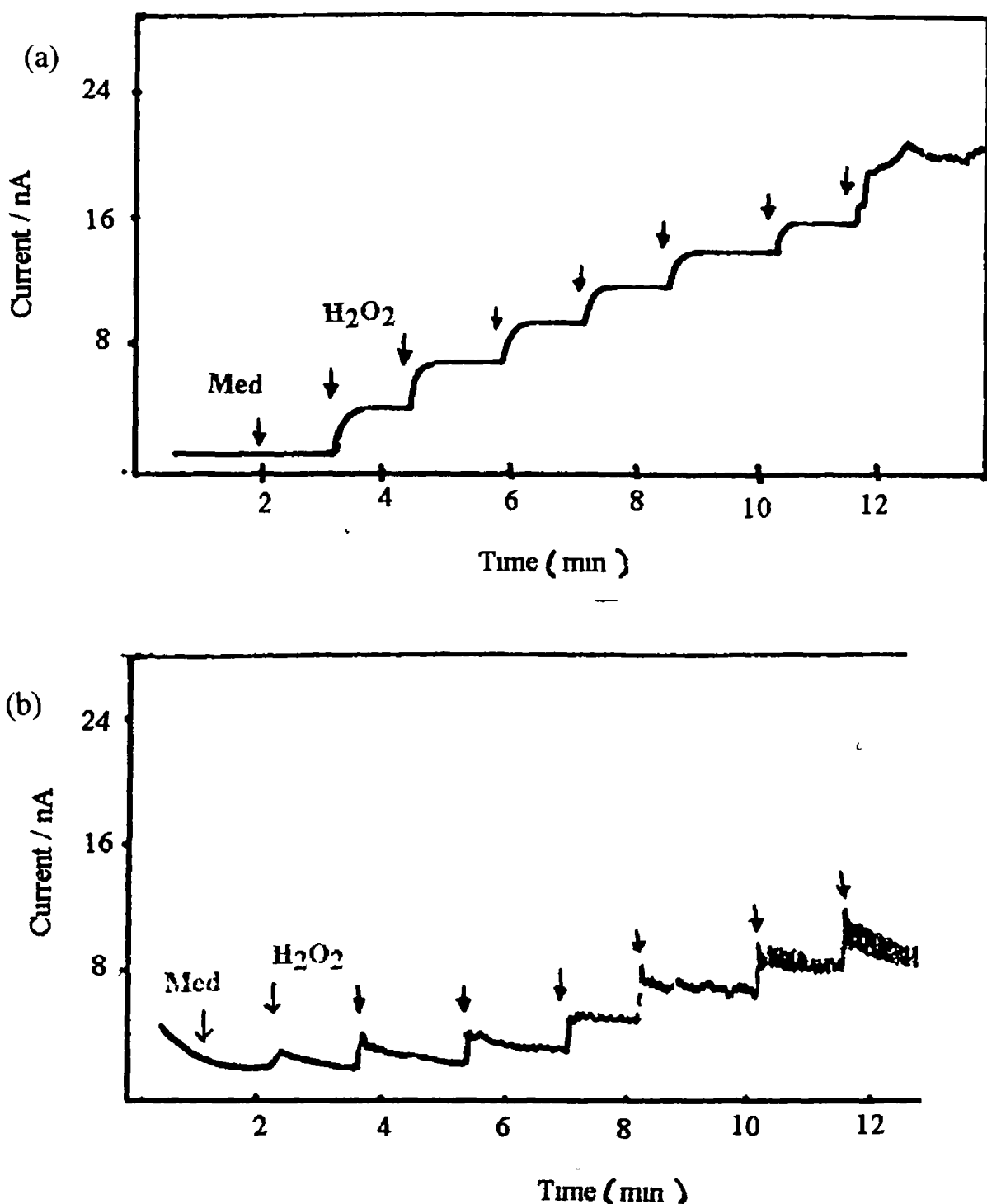


Fig 4.2 Current-time response for successive 1 mM injections of H_2O_2 at (a) ovalbumin-7-OH-coumarin modified electrode and (b) ovalbumin-only modified electrode, in a solution containing 2 mM hydroquinone Operating potential -0.03 V with 450 rpm stirring rate Electrolyte, 0.1 M phosphate buffer, pH 7.3 The modified electrodes had first been incubated in a 0.33 mg ml^{-1} anti-7-OH-coumarin antibody solution for 1 hour at 37°C

4 3 3 DEVELOPMENT OF A THYROGLOBULIN-7-OH-COUMARIN MODIFIED ELECTRODE

These modified electrodes were prepared in a similar way to those using ovalbumin. A 15% (w/v) thyroglobulin-7-OH-coumarin modified electrode was prepared and incubated in a 0.33 mg ml⁻¹ solution of anti-7-OH-coumarin antibody for 1 hour at 37 °C. Fig. 4.3 (a) shows the steady state current response of this modified electrode to successive additions of 1 mM H₂O₂ (in the presence of 2 mM of mediator). It can also be seen from this figure that introduction of the mediator into the electrochemical cell produces no response. Fig. 4.3 (b) shows the response of a 15% (w/v) thyroglobulin modified control electrode. The resulting calibration plot for the thyroglobulin-7-OH-coumarin modified electrode is shown in Fig. 4.4. These results suggest that the anti-7-OH-coumarin antibodies have much less affinity for the thyroglobulin protein compared to ovalbumin and for the remainder of the work the thyroglobulin conjugate was used in the development of an immunosensor for 7-OH-coumarin.

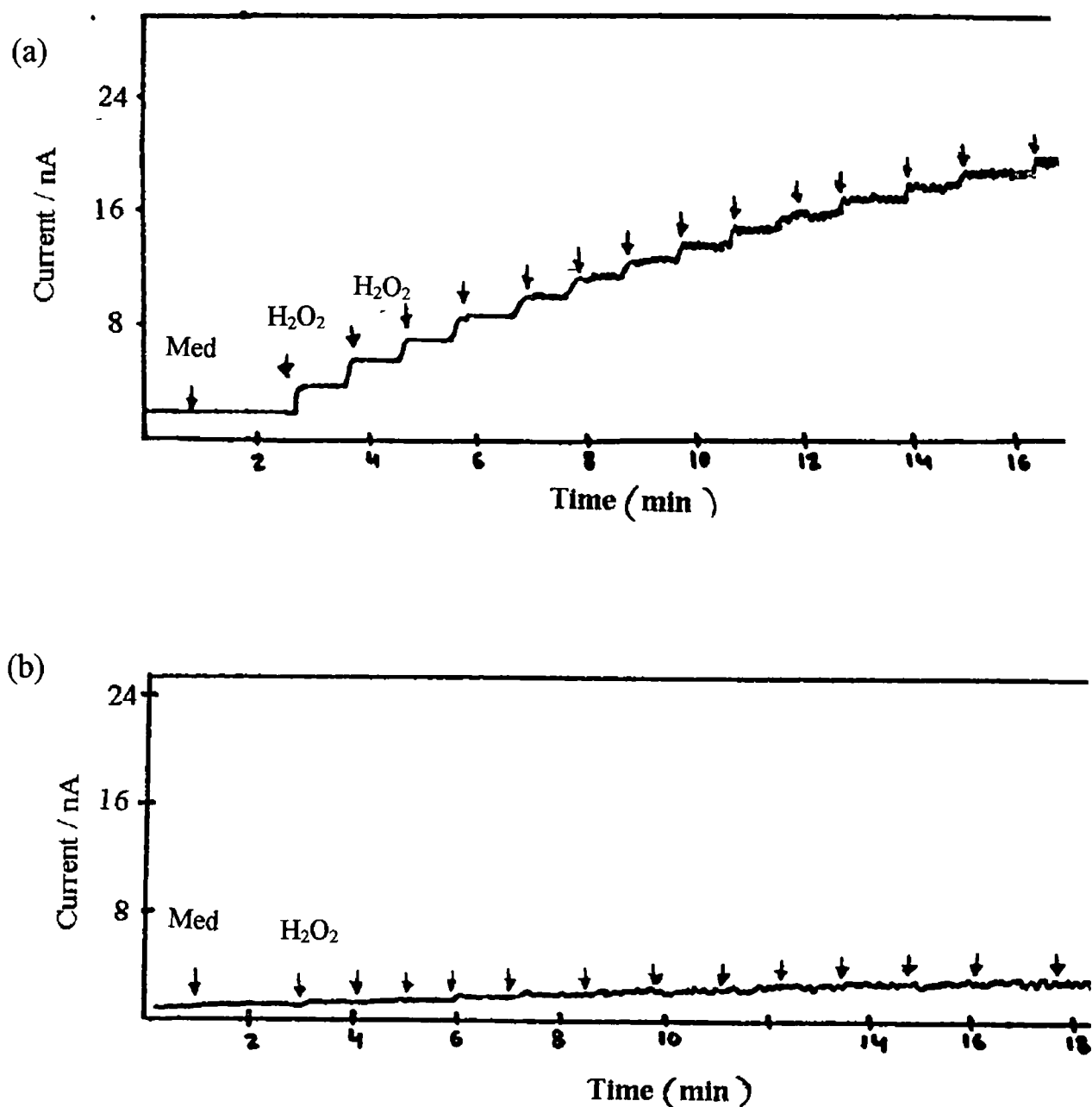


Fig 4.3 Current-time response for successive 1 mM injections of H_2O_2 at (a) thyroglobulin-7-OH-coumarin-modified electrode and (b) thyroglobulin-modified electrode, in a solution containing 2 mM hydroquinone. Operating potential -0.03 V with 450 rpm stirring rate. Electrolyte 0.1 M phosphate buffer, pH 7.3. The modified electrodes had first been incubated in a 0.33 mg ml^{-1} anti-7-OH-coumarin antibody solution, for 1 hour at 37°C .

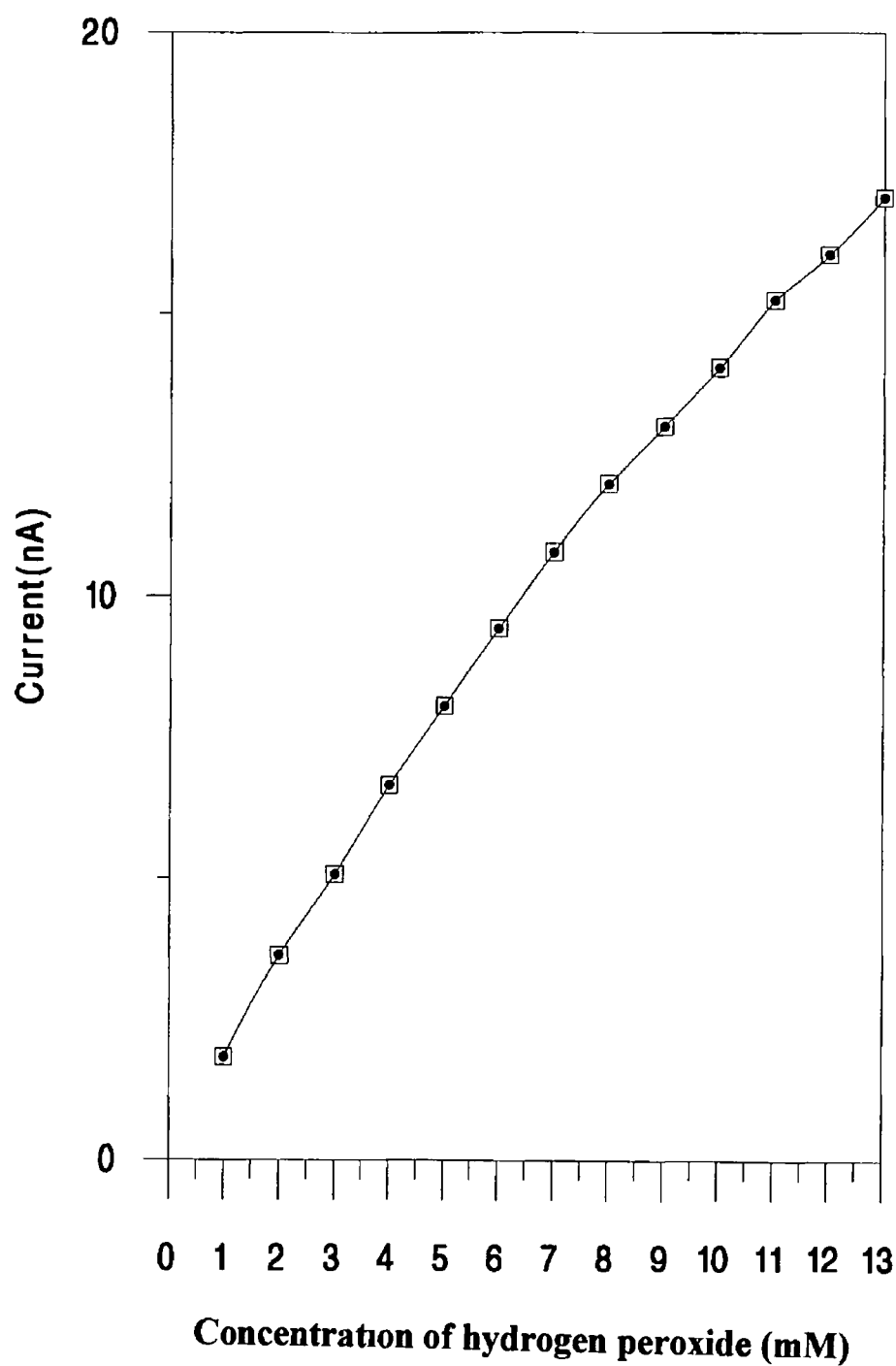


Fig 4 4 Calibration plot for 15% (w/v) thyroglobulin-7-OH-coumarin-modified electrode Conditions as in Fig 4 3

The effects of many experimental variables were then investigated in order to optimise analytical performance. The optimum reaction of 7-OH-coumarin with its specific antibody takes place at pH 7.3, as determined in a previous study [24]. Nafion was used in the modified electrode preparation as it forms an insoluble film and is capable of keeping the protein conjugate immobilised within it. Also, the $-\text{SO}_3\text{H}$ groups of Nafion can dissociate to form $-\text{SO}_3^-$ in aqueous solution and this can repel negatively charged species [34]. Various concentrations of Nafion were investigated and a 5% Nafion film was shown to be optimum since lower concentrations of it either cracked on drying or resulted in the protein conjugate leaching out. The effect of the protein conjugate loading (thyroglobulin-7-OH-coumarin) in the Nafion film is shown in Fig. 4.5. In this case antibody concentration and temperature of incubation were kept constant. The optimum response was achieved using a 15% (w/v) loading. When higher loadings were investigated, the response time of the electrode became very slow.

The effect of various antibody concentration is shown in Fig. 4.6. In this case protein loading in the Nafion film and the temperature of incubation were kept constant. An optimum current response was observed for a concentration of 0.33 mg ml^{-1} .

The effect of temperature of incubation on antibody activity was also studied with an optimum being found near 37°C , i.e. the temperature where most antibodies are shown to bind to the antigen on the modified electrode surface. Therefore this temperature was taken as that providing maximum antibody activity for the operation of this immunosensor.

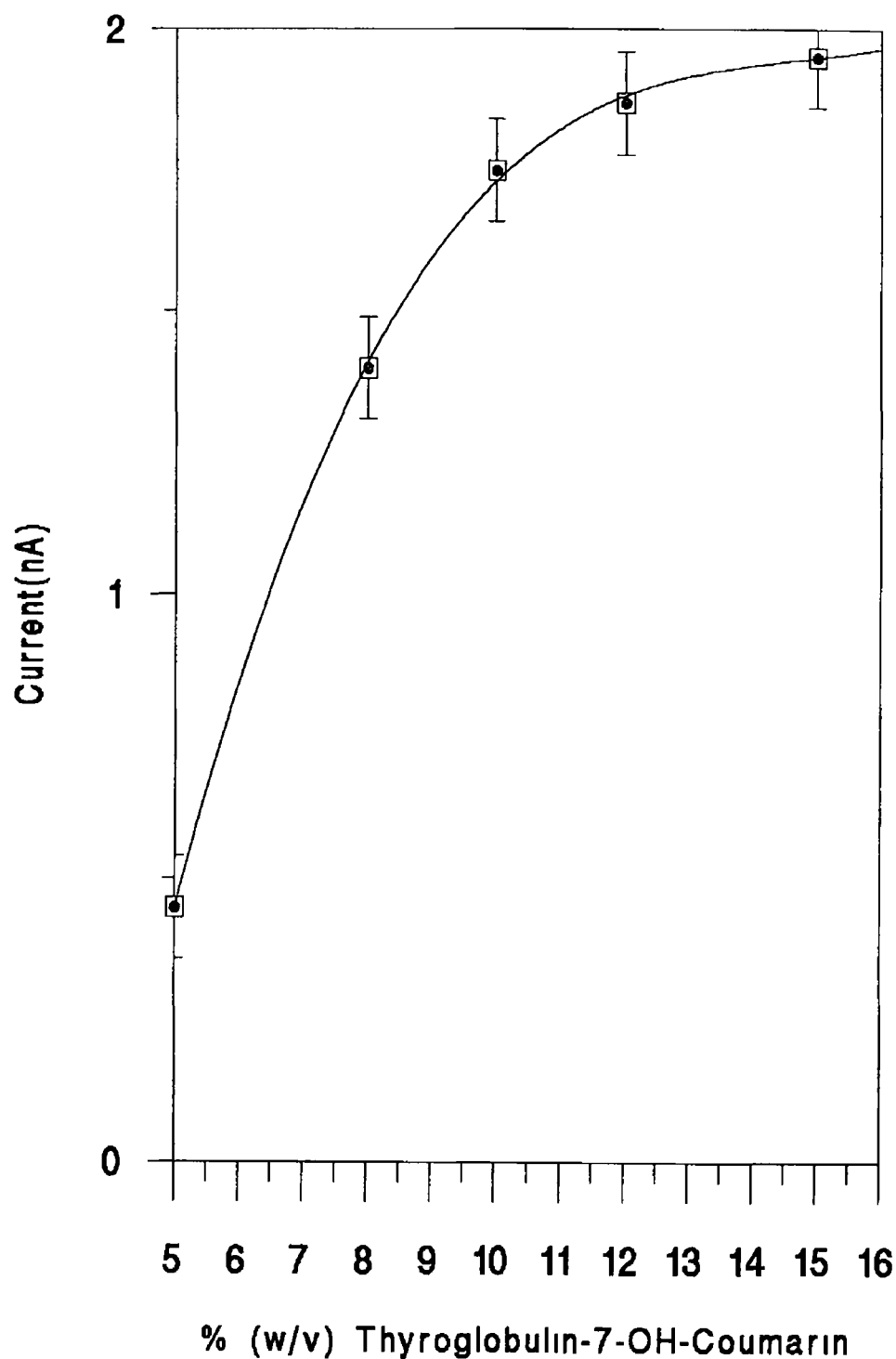


Fig 4 5 Current dependence on thyroglobulin-7-OH-coumarin loading in the Nafion film upon injection of 1 mM hydrogen peroxide (in the presence of 2 mM hydroquinone) The antibody concentration was kept constant at 0.33 mg ml^{-1} and the temperature was 37°C throughout the investigation

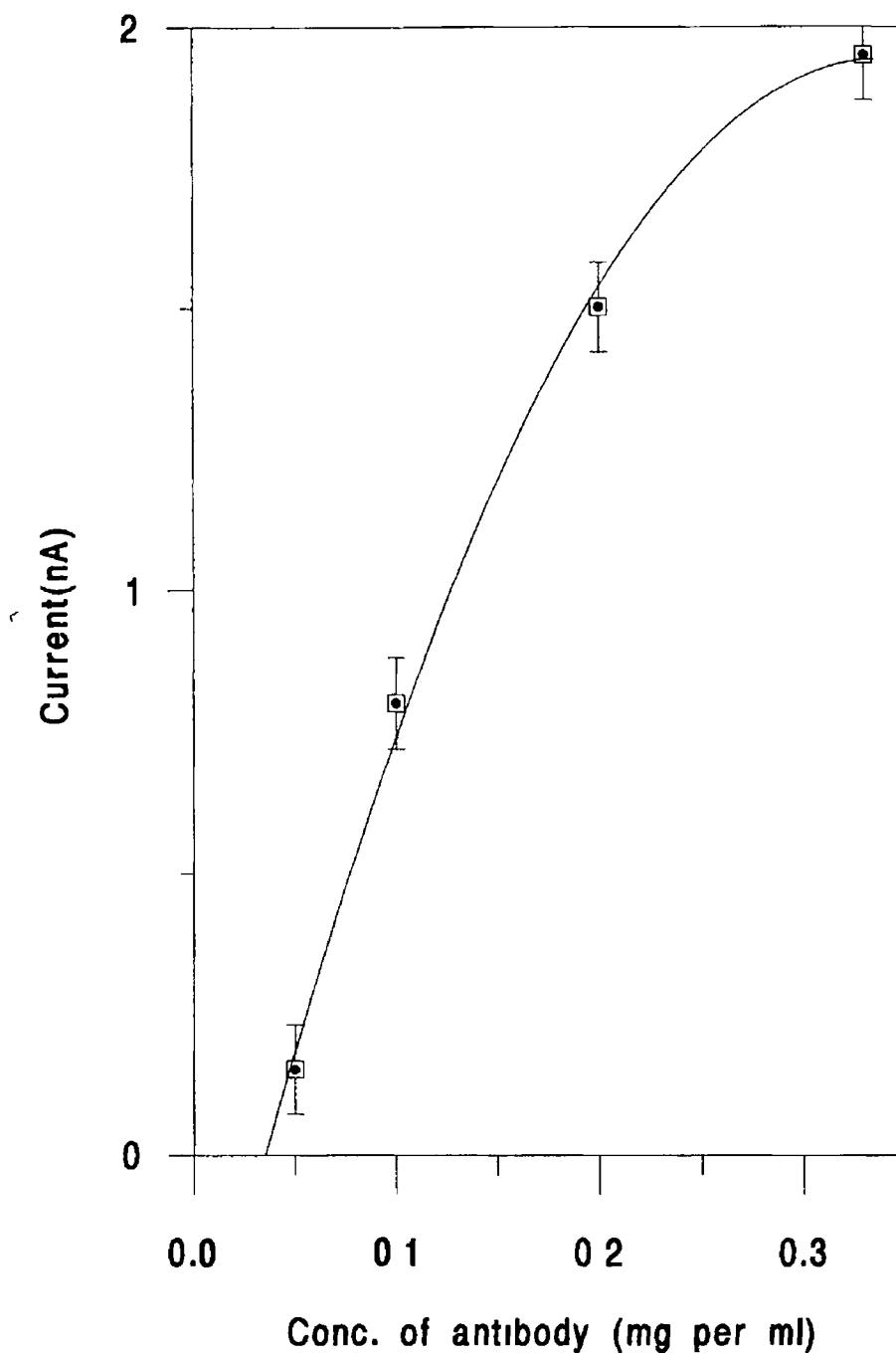


Fig 4 6 Effect of anti-7-OH-coumarin antibody concentration on response to a 1 mM injection of hydrogen peroxide (in the presence of 2 mM hydroquinone) Thyroglobulin-7-OH-coumarin loading was kept constant at 15 % (w/v) and temperature of incubation was 37°C throughout study

4 3 4 COMPETITIVE ENZYME LINKED ELECTROCHEMICAL IMMUNOASSAY FOR THE DETERMINATION OF 7-OH- COUMARIN

With these experimental variables investigated, a calibration curve of 7-OH-coumarin was prepared for the immunosensor. A competitive enzyme-linked electrochemical assay was carried out where both free and solid-phase antigens competitively react for the HRP-labelled anti-7-OH-coumarin antibody (Fig 4 7). After rinsing with phosphate buffer, the enzyme attached to the electrode surface was determined by measuring the current after the addition of 1 mM hydrogen peroxide, in the presence of 2 mM of mediator.

The calibration curve of current Vs concentration of 7-OH-coumarin is shown in Fig 4 8. The response decreases exponentially, as less antibody binds to the immobilised antigen when the concentration of free 7-OH-coumarin in solution increases. Each point on the calibration curve represents the average of three determinations, at a particular concentration of free 7-OHC, with its associated error bars. From this plot 7-OH-coumarin can be determined between 0 and 1 mM in solution and has a limit of detection of 24 μ M. This range has been shown to be physiologically relevant [31,32].

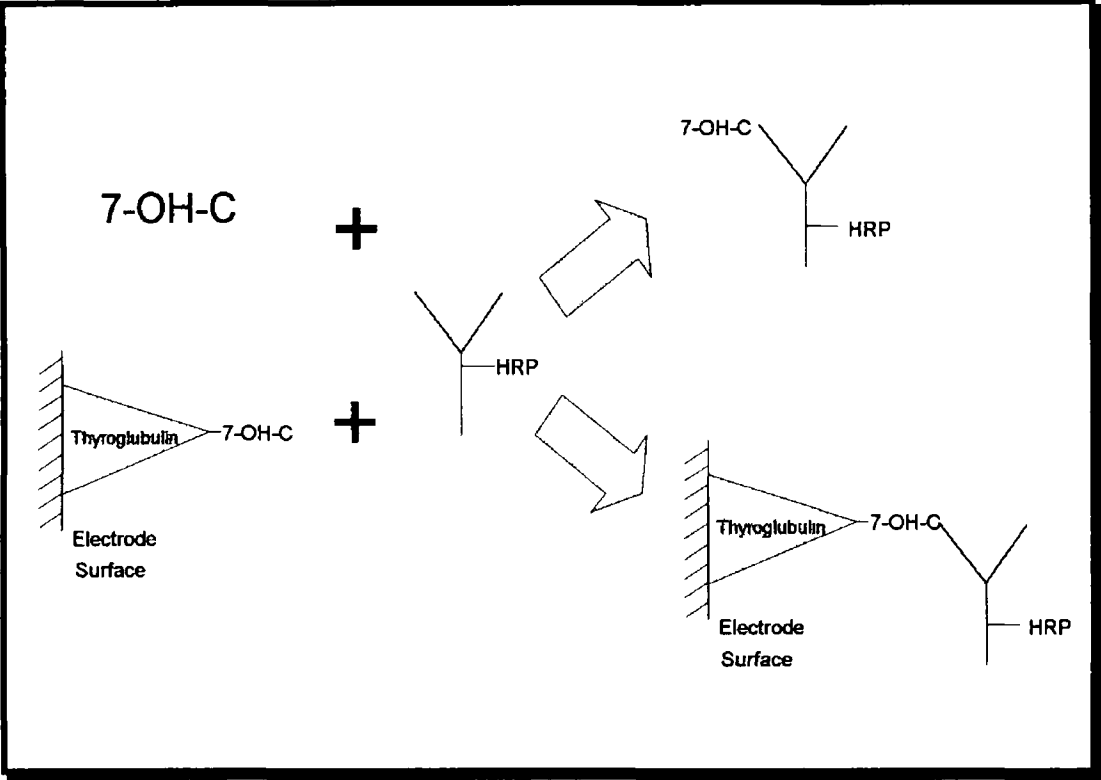


Fig 4 7 Diagram of competitive enzyme-linked immunosorbent assay

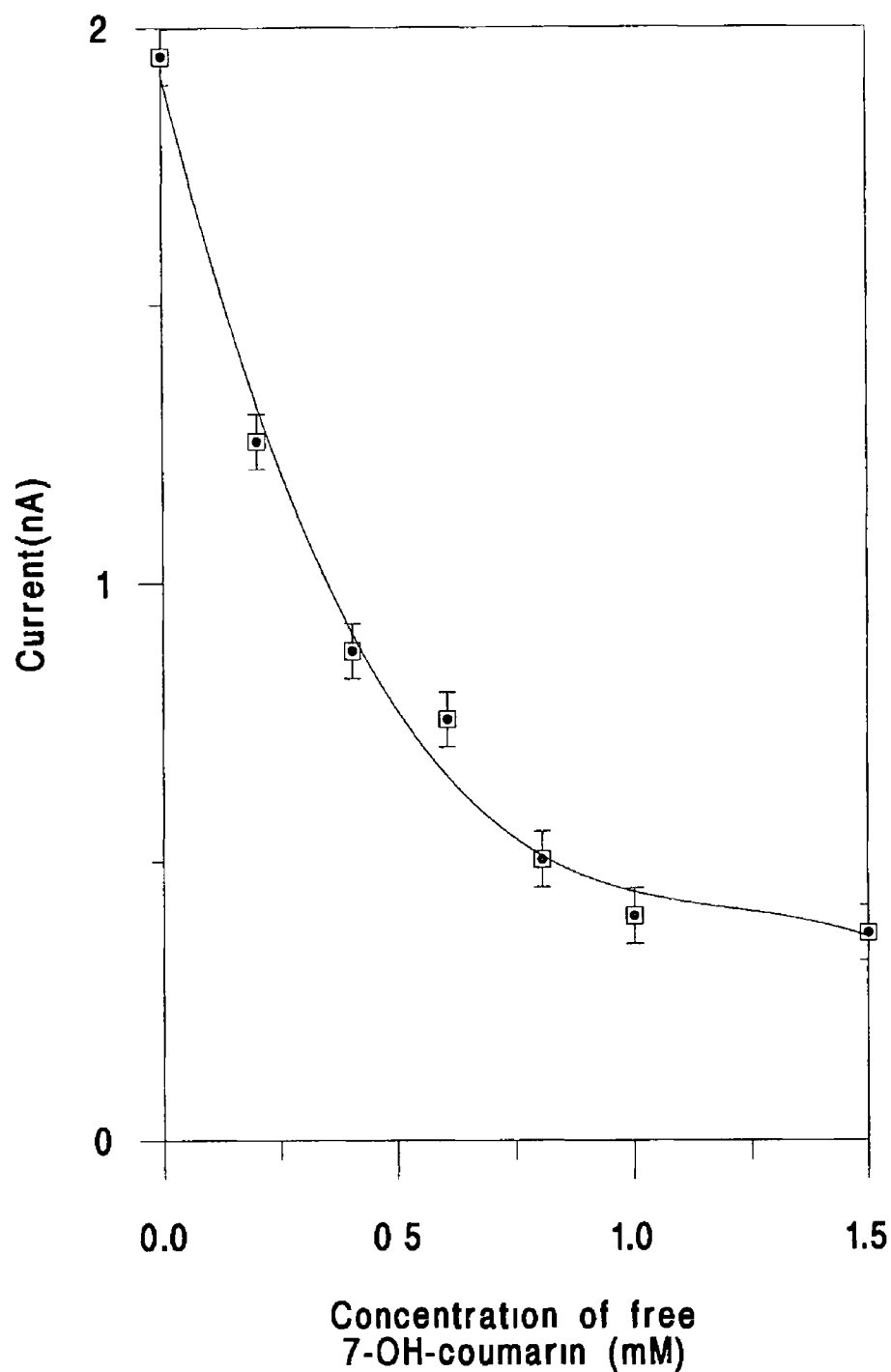


Fig 4 8 Calibration curve for the determination of 7-OH-coumarin by electrochemical immunoassay Thyroglobulin-7-OH-coumarin loading was 15% (w/v), antibody concentration was 0.33 mg ml⁻¹ and the temperature of incubation was 37°C for each point on the calibration curve

4.4 CONCLUSIONS

The system described in this paper clearly demonstrates a novel electrochemical approach for studying antibody-antigen binding and provides a further method for the determination of 7-OH-coumarin in solution. The metabolite can be determined between 0 and 1 mM in solution and has a limit of detection of 24 μM . In the preparation of the modified electrodes it is important to use a protein conjugate which is significantly different from the protein conjugate used in the initial immunisation of the rabbits. Work has since been done to investigate how the antibody-antigen interaction on the electrode surface can be reversed after a single determination, to produce a modified electrode capable of repeated use, for example in flow injection analysis or high performance liquid chromatography. This was achieved by rinsing the modified electrode at the end of the determination in a buffer of low pH, which is seen to cleave the antibody-antigen interaction on the electrode surface [33]. Pulsed amperometric detection can also be used to reverse the antibody-antigen interaction, as has been shown by Sadik and Wallace [23].

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CHAPTER 5

OVERALL CONCLUSIONS

5.1 OVERALL CONCLUSIONS

The determination of 7-hydroxycoumarin in urine and serum by capillary electrophoresis was shown to have a number of advantages over existing methods for the analysis of this coumarin metabolite. The capillary electrophoresis method was found to be relatively straight forward to use and shown to be reliable. There was a big advantage gained over the existing HPLC methods in that the separation is much more rapid to run, which in the case of analysing numerous samples would prove a worthwhile advance.

However, there still exists a long sample preparation which takes from the advances made here in the separation step. This is one area where further investigations would need to be done and since this project was completed, a capillary electrophoresis method has been developed which can analyse 7-hydroxycoumarin and 7-hydroxycoumarin-glucuronide without any sample preparation [1]. This now combines a fast chromatographic separation with almost no sample preparation. One of the major drawbacks to working with HPLC is that careful sample preparation is required if column degradation is to be avoided especially when working with protein solutions. This is an area where capillary electrophoresis can be very useful in terms of the ruggedness of the technique.

Considerable advances were made in the monitoring of 7-hydroxycoumarin formation in liver microsomes by capillary electrophoresis. This relatively new analytical technique (in comparison to HPLC) was utilised to monitor the formation of 7-OHC by human liver

microsomes without the need to precipitate the microsomal proteins before analysis. What was worth noting from this metabolism study was that the same capillary was used during the entire investigation without a noticeable drop in capillary performance. The ability to monitor the cofactor as the metabolism progressed proved to be interesting and was also an advantage over present HPLC technology. This study showed that the cofactor is involved in a number of cellular processes within the microsomes and this ruled out the possibility of designing a biosensor which would use NADPH variation as a means of following cytochrome P450 reactions. The $\text{NADP}^+/\text{NADPH}$ ratio study also showed that it might be possible to use this ratio to determine if a compound's metabolism used NADPH as the cofactor. The stability of NADPH in solution was discussed in the metabolism investigations and for future work it would have been interesting to identify the impurity near the NADPH migration time. The use of a diode array detector would help in this regard.

The study did show how 7-hydroxycoumarin formation could be monitored for a number of different human liver microsomes and the results were comparable to those in the literature. An interspecies investigation of coumarin metabolism was also carried out and the findings from the work agreed with those of other workers in the area. The cynomolgous monkey proved to be the species most closely resembling the human metabolism of coumarin.

Electrochemistry also proved useful in the study of liver microsomal reactions. The investigations in the anodic region showed the presence of oxidation peaks one of which may have been native NADPH in the microsomal suspension. Further work is obviously needed to conclusively

show this The work with NADPH did show that this cofactor is needed in many cellular processes as was shown in the capillary electrophoresis investigations It was difficult to work with the microsomes electrochemically since the signals were very faint and future work into trying to amplify these responses would be advantageous In the cathodic region a redox couple was detected, without again being able to prove its origins What would be of use for electrochemical investigations is the availability of purified cytochrome P450 This would obviously be considerably more pure than working with liver microsomes but although possible, not many researchers in this area have access to these enzymes Another way of possibly amplifying these faint signals is the use of mediators which would help in transferring electrons to and from electrode surfaces

The immunosensor which was developed for the determination of 7-hydroxycoumarin demonstrates a novel electrochemical approach for studying antibody antigen binding and provides another method for the determination of 7-hydroxycoumarin in solution It was possible to determine the metabolite upto 1 mM and the method had a limit of detection of 24 μ M One of the disadvantages of the method was that it was not possible to use the modified electrode for a number of determinations by cleaving antibody-antigen binding after a determination This would have been advantageous and has since been achieved by rinsing the modified electrode at the end of the determination with a buffer of low pH, which is seen to cleave the antibody-antigen interaction on the electrode surface [2]

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APPENDIX 1

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