USE OF THE LAW OF MASS ACTION FOR PREDICTING FREE LEVELS OF ANTIEPILEPTIC DRUGS IN SERUM

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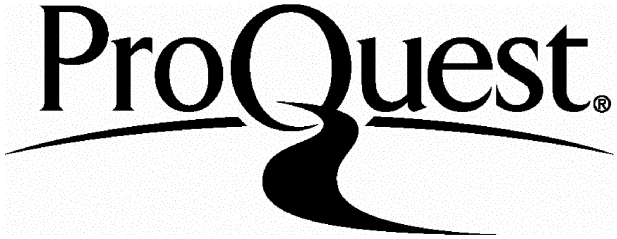


ABSTRACT

The estimation of non-protein-bound levels (free levels) of antiepileptic drugs in serum is considered a useful adjunct in the management of patients with epilepsy. However, present methods for separating free levels e.g., by equilibrium dialysis and ultrafiltration are too laborious to be used routinely except in special centers. Thus this thesis has focused on the development and validation of a set of formulae, based on the Law of Mass Action, for use in predicting free levels of phenytoin, carbamazepine and phenobarbitone in patients on monotherapy and polytherapy from serum total drug and albumin levels.

This work has comprised:-

(i) Establishing a micromethod for the separation of free antiepileptic drugs in serum by ultrafiltration.

(ii) Developing a high performance liquid chromatography micromethod for the determination of free and total antiepileptic drugs in serum.

(iii) Developing a theoretical framework for the prediction of free drug levels in serum for patients on monotherapy and polytherapy.

(iv) Investigating the effect of temperature on the binding of drugs to serum albumin.

(v) Establishing temperature-related therapeutic ranges for free drug levels.

(vi) Evaluating the quality of the predicted versus measured free drug levels at the physiological temperature.

(vii) Comparing the performance of the derived predictive method with that of previous methods based on statistical concepts.

The investigation has demonstrated:-

The feasibility of predicting free drug levels in patients on monotherapy or polytherapy from serum total drug and albumin levels at the physiological temperature.

The methodology developed would allow routine laboratories to provide an estimate of free drug levels in addition to measured total drug levels, thus potentially improving the therapeutic drug monitoring service for the management of patients with epilepsy.

CONTENTS

		Page
TITLE		1
ABSTRACT		2
CONTENTS		4
List of Abbreviations		16
List of Figures		17
List of Tables		20
CHAPTER 1		**22**
INTRODUCTION		
1.1	**Historical Review**	22
1.2	**Classification of Epilepsy**	23
1.3	**Neurophysiological Aspects of Epilepsy**	25
1.4	**Treatment of Epilepsy**	27
1.4.1	Potassium Bromide	28
1.4.2	Phenobarbitone	28
1.4.3	Phenytoin	28
1.4.4	Primidone	31
1.4.5	Ethosuximide	31
1.4.6	Diazepam	31
1.4.7	Carbamazepine	32
1.4.8	Valproic acid	32
1.5	**New Drugs**	33
1.6	**Mechanism of Action of Antiepileptic Drugs**	33

		Page
1.6.1	Phenytoin	34
1.6.2	Carbamazepine	34
1.6.3	Phenobarbitone	34
1.6.4	Primidone	35
1.6.5	Ethosuximide	36
1.6.6	Valproic acid	36
1.6.7	Lamotrigine	37
1.6.8	Vigabatrin	37
1.7	**Clinical Pharmacology of Antiepileptic Drugs**	37
1.8	**Chemistry of Antiepileptic Drugs**	41
1.9	**Plasma Protein Binding of Antiepileptic Drugs**	44
1.10	**Mechanism of Drug-Protein Binding**	44
1.11	**Factors Altering Protein Binding**	46
1.11.1	Protein Concentration	47
1.11.2	Drug Interaction	47
1.11.3	Saturation of Protein-binding Sites	48
1.12	**Methods used in the Measurement of Total levels of Antiepileptic Drugs**	49
1.13	**The Rationale for Therapeutic Drug Monitoring of Total Levels of Antiepileptic Drugs**	52
1.14	**Methods for Separating Free Drug from Serum or Plasma**	53
1.14.1	Equilibrium dialysis	57
1.14.2	Ultracentrifugation	58
1.14.3	Ultrafiltration	59

Page

1.15 The Rationale for Therapeutic Drug Monitoring of Free Antiepileptic Drug Levels 61
1.16 Methods for Predicting Free Levels of Antiepileptic Drugs in Plasma or Serum 63
1.17 Previous Evaluation of the Predictive Methods 70
1.18 Aims of Thesis 72

CHAPTER 2 74

THE DEVELOPMENT OF A MICROMETHOD FOR THE ESTIMATION OF TOTAL AND FREE LEVELS OF ANTIEPILEPTIC DRUGS IN SERUM

2.1 Aims 74
2.2 Introduction 74
2.3 Materials and Methods 75
2.3.1 Apparatus 76
2.3.2 Chemicals and Consumables 76
2.3.3 Calibration Materials for Drug Analysis 78
2.3.4 Calibration Solutions 78
2.3.5 Preparation of HPLC Buffer Solution 78
2.3.6 Quality Control Sera 79
2.3.7 Patients' Samples 79
2.4 Analytical Procedures 80
2.4.1 Fluorescence Polarisation Immunonassay 80

		Page
2.4.2	High Performance Liquid Chromatography	81
2.4.3	Preparation of Calibration Solutions	81
2.4.4	Experimental Procedure for the Estimation of Total Levels of Antiepileptic Drugs by High Performance Liquid Chromatography	82
2.4.5	Experimental Procedure for the Estimation of Free Levels of Antiepileptic Drugs by High Performance Liquid Chromatography	83
2.4.6	Filtration of Samples	83
2.5	**Chromatographic Procedure**	83
2.5.1	Calibration Curves for Total and Free Levels of Antiepileptic Drugs	84
2.5.2	Recovery for Total and Free Levels of Antiepileptic Drugs	84
2.5.3	Accuracy of the Experimental Procedures	85
2.5.4	Imprecision of the Experimental Procedures	85
2.5.5	Correlation between the Fluorescence Polarisation Immunoassay and High Performance Liquid Chromatography Methods	86
2.5.6	Stability of Free Levels during Sample storage	86
2.6	**Results**	86
2.6.1	Chromatograms of Antiepileptic Drugs	86

Page

2.6.2 Calibration Curves for Total and Free Levels of Antiepileptic Drugs 87
2.6.3 Recovery for Total and Free Levels of Antiepileptic Drugs 87
2.6.4 Accuracy of the Fluorescence Polarisation Immunoassay and High Performance Liquid Chromatography Methods 88
2.6.5 Imprecision of the Fluorescence Polarisation Immunoassay and High Performance Liquid Chromatography Methods for Total Levels 88
2.6.6 Imprecision of the Fluorescence Polarisation Immunoassay and High Performance Liquid Chromatography Methods for Free Levels 89
2.6.7 Correlation between the Fluorescence Polarisation Immunoassay and High Performance Liquid Chromatography Methods 89
2.6.8 Stability of Free Levels during Sample Storage 90
2.7 Discussion 107
2.8 Summary 111

CHAPTER 3 112

THEORETICAL CONSIDERATIONS

3.1 The Law of Mass Action Applied to the Binding of a Drug to Plasma Protein(s) 112

Page

3.2 General Equation for Calculating Free levels of Antiepileptic Drugs 114

3.3 Hardware and Software used in Calculations 116

CHAPTER 4 117

TEMPERATURE DEPENDENCE OF THE APPARENT DISSOCIATION CONSTANT AND FREE FRACTION

4.1 Aim 117

4.2 Introduction 117

4.3 Materials and Methods 118

4.3.1 Subjects 118

4.3.2 Blood Samples 118

4.3.3 Albumin Assay 118

4.3.4 Separation of Free Antiepileptic Drugs at Different Temperatures 119

4.3.5 Assay of Total Levels of Antiepileptic Drugs 119

4.3.6 Theoretical Considerations and Calculations 120

4.4 Results 120

4.4.1 Concentration Ratios for Free Phenytoin, Free Carbamazepine and Free Phenobarbitone Related to Temperature 120

Page

4.4.2 The Temperature Dependence of the Apparent Dissociation Constant for Phenytoin, Carbamazepine and Phenobarbitone 120

4.4.3 The Effect of Temperature on Free Fraction 121

4.5 Discussion 128

4.6 Summary 129

CHAPTER 5 130

ESTIMATION OF TARGET RANGES FOR FREE PHENYTOIN, FREE CARBAMAZEPINE AND FREE PHENOBARBITONE AT 25 °C AND 37 °C

5.1 Aim 130

5.2 Introduction 130

5.3 Materials and Methods 131

5.3.1 Subjects 131

5.3.2 Blood Samples 131

5.3.3 Separation of Free Antiepileptic Drugs at 25 °C and 37 °C 131

5.3.4 Assay of Total Levels of Antiepileptic Drugs 132

5.4 Results 132

5.4.1 Concentration Ratios for Free Phenytoin, Free Carbamazepine and Free Phenobarbitone Related to 25 °C and 37 °C 132

Page

5.4.2 The Effect of Temperature on Free Fraction 132
5.4.3 The Effect of Temperature on Target Ranges 133
5.5 Discussion 139
5.6 Summary 141

CHAPTER 6 **142**

COMPARISON OF PREDICTED AND OBSERVED FREE PHENYTOIN AND FREE CARBAMAZEPINE AT 25 ·C AND 37 ·C IN MONOTHERAPY

6.1 Aim 142
6.2 Introduction 142
6.3 Materials and Methods 144
6.3.1 Subjects 144
6.3.2 Blood Samples 144
6.3.3 Analytical Procedures 144
6.3.4 Calculations 145
6.4 Results 146
6.5 Discussion 154
6.6 Summary 155

Page

CHAPTER 7 157

THE EFFECTS OF DRUG DISPLACEMENT BY VALPROIC ACID ON APPARENT DISSOCIATION CONSTANTS, FREE FRACTIONS AND TARGET RANGES FOR PHENYTOIN, CARBAMAZEPINE AND PHENOBARBITONE AT 25 ·C AND 37 ·C

7.1	**Aim**	157
7.2	**Introduction**	157
7.3	**Materials and Methods**	159
7.3.1	Subjects	159
7.3.2	Blood Samples	159
7.3.3	Analytical Procedures	160
7.3.4	Calculations	160
7.4	**Results**	162
7.4.1	Apparent Dissociation Constant, Free Fraction and Target Ranges at 25 °C and 37 °C for Phenytoin in Patients Co-medicated with Valproic Acid	162
7.4.2	Apparent Dissociation Constant, Free Fraction and Target Ranges at 25 °C and 37 °C for Carbamazepine in Patients Co-medicated with Valproic Acid	163

Page

7.4.3 Apparent Dissociation Constant, Free Fraction and Target Ranges at 25 °C and 37 °C for Phenobarbitone in Patients Co-medicated with Valproic Acid 164

7.5 Discussion 171

7.6 Summary 171

CHAPTER 8 173

COMPARISON OF PREDICTED AND OBSERVED FREE PHENYTOIN AND FREE CARBAMAZEPINE AT 37 °C IN PATIENTS CO-MEDICATED WITH VALPROIC ACID

8.1 Aim 173

8.2 Introduction 173

8.3 Materials and Methods 175

8.3.1 Subjects 175

8.3.2 Blood Samples 175

8.3.3 Analytical Procedures 175

8.3.4 Calculations 176

8.4 Results 178

8.5 Discussion 182

8.6 Summary 183

Page

CHAPTER 9 184

COMPARISON OF METHODS FOR PREDICTING FREE PHENYTOIN LEVELS AT 25 °C IN MONOTHERAPY AND POLYTHERAPY PATIENTS

9.1	**Aim**	184
9.2	**Introduction**	184
9.3	**Materials and Methods**	186
9.3.1	Subjects	186
9.3.2	Blood Samples	186
9.3.3	Analytical Procedures	186
9.3.4	Calculations	187
9.4	**Results**	192
9.4.1	Comparison of Method A with the Sheiner-Tozer Method	192
9.4.2	Comparison of Method A with the Dasgupta Method	192
9.4.3	Comparison of Method A with the Haidukewych Method	193
9.5	**Discussion**	197
9.6	**Summary**	198

CHAPTER 10 199

GENERAL DISCUSSION

		Page
10.1	**Methodology for the Determination of Free Levels of Antiepileptic Drugs**	199
10.1.1	Equilibrium Dialysis	199
10.1.2	Ultrafiltration	200
10.1.3	Choice of Methodology for the Measurement of Free Drug Levels	201
10.1.4	Temperature Effects on the Separation of Unbound Antiepileptic Drugs	201
10.1.5	Quality Control of Drug Determinations	203
10.2	**Drug Displacement**	204
10.3	**Empirical Methods for Predicting Free Drug Levels**	205
10.3.1	An Equation Derived from the Law of Mass Action to predict Free Phenytoin or Free Carbamazepine Levels in Monotherapy and Polytherapy Patients	205
10.4	**Clinical Significance of Free Drug Level Monitoring**	206
10.5	**Conclusion**	209
References		210
Acknowledgment		240
Appendices		241
Publications		259

LIST OF ABBREVIATIONS

AED - Antiepileptic drug

Alpha (α) - Free fraction

CBZ - Carbamazepine

CBZ-E - Carbamazepine-10, 11-epoxide

CCI - Ciba-Corning Quality Control Serum I

CCII - Ciba-Corning Quality Control Serum II

CNS - Central nervous system

CV - Coefficient of variation

EEG - Electroencephalography

EMIT - Enzyme Multiplied Immunoassay Technique

F-CBZ - Free Carbamazepine

F-CBZ-E - Free Carbamazepine Epoxide

F-PB - Free Phenobarbitone

F-PHT - Free Phenytoin

FPIA - Fluorescence polarisation immunoassay

GC - Gas chromatography

HPLC - High performance liquid chromatography

K_D - Apparent Dissociation Constant

MPE - Mean prediction error

MPPH - 5-p-tolyl-5-phenylhydantoin

PB - Phenobarbitone

PE - Prediction error

PHT - Phenytoin

r - correlation coefficient

RMSE - Root mean squared error

SD - standard deviation

TDM - Therapeutic drug monitoring

VPA - Valproic acid

LIST OF FIGURES

		Page
Figure 1.1	Chemical structures of commonly used AED's	42
Figure 2.1A	Chromatogram of drugs added to drug-free serum	91
Figure 2.1B	Chromatogram of drugs present in a patient's serum	91
Figure 2.2A	Chromatogram of drugs added to physiological saline	92
Figure 2.2B	Chromatogram of drugs present in an ultrafiltrate from a patient's serum	92
Figure 2.3A	Calibration curves for total levels of AED's	93
Figure 2.3B	Calibration curves for free levels of AED's	93
Figure 2.4A	Correlation between HPLC and FPIA assays for PHT	104
Figure 2.4B	Correlation between HPLC and FPIA assays for CBZ	104
Figure 2.4C	Correlation between HPLC and FPIA assays for PB	104
Figure 2.5A	Correlation between HPLC and FPIA assays for free levels of PHT	105
Figure 2.5B	Correlation between HPLC and FPIA assays for free Levels of CBZ	105
Figure 2.5C	Correlation between HPLC and FPIA assays for free levels of PB	105
Figure 2.6A	Correlation between analyses of patients sera for free levels of PHT immediately after collection and after storage at -20 °C for 84 days	106
Figure 2.6B	Correlation between analyses of patients sera for free levels of CBZ immediately after collection and after storage at -20 °C for 84 days	106
Figure 2.6C	Correlation between analyses of patient sera for free levels of PB immediately after collection and after storage at -20 °C for 84 days	106
Figure 3.1	Model for drug distribution	112

		Page
Figure 4.1A	Concentration ratios for F-PHT at 15 °C, 25 °C and 37 °C	122
Figure 4.1B	Concentration ratios for F-CBZ at 15 °C, 25 °C and 37 °C	122
Figure 4.1C	Concentration ratios for F-PB at 15 °C, 25 °C and 37 °C	122
Figure 4.2	Regression analysis of the apparent dissociation constant (K_D) for PHT, CBZ and PB at 15 °C, 25 °C and 37 °C	124
Figure 5.1A	F-PHT levels (y) at 25 °C versus the total PHT levels (x)	136
Figure 5.1B	F-CBZ levels (y) at 25 °C versus the total CBZ levels (x)	136
Figure 5.1C	F-PB levels (y) at 25 °C versus the total PB levels (x)	136
Figure 5.2A	F-PHT levels (y) at 37 °C versus the total PHT levels (x)	137
Figure 5.2B	F-CBZ levels (y) at 37 °C versus the total CBZ levels (x)	137
Figure 5.2C	F-PB levels (y) at 37 °C versus the total PB levels (x)	137
Figure 6.1A	Observed versus predicted F-PHT levels at 25 °C	148
Figure 6.1B	Observed versus predicted F-PHT levels at 37 °C	148
Figure 6.2A	Observed versus predicted F-CBZ levels at 25 °C	149
Figure 6.2B	Observed versus predicted F-CBZ levels at 37 °C	149
Figure 6.3A	Difference (%) between predicted and observed F-PHT versus observed F-PHT at 25 °C	152
Figure 6.3B	Difference (%) between predicted and observed F-PHT versus observed F-PHT at 37 °C	152
Figure 6.4A	Difference (%) between predicted and observed F-CBZ versus observed F-CBZ at 25 °C	153
Figure 6.4B	Difference (%) between predicted and observed F-CBZ versus observed F-CBZ at 37 °C	153

		Page
Figure 7.1A	F-PHT at 25 °C versus total PHT for patients on PHT and VPA	167
Figure 7.1B	F-PHT at 37 °C versus total PHT for patients on PHT and VPA	167
Figure 7.2A	F-CBZ at 25 °C versus total CBZ for patients on CBZ and VPA	168
Figure 7.2B	F-CBZ at 37 °C versus total CBZ for patients on CBZ and VPA	168
Figure 7.3A	F-PB at 25 °C versus total PB for patients on PB and VPA	169
Figure 7.3B	F-PB at 37 °C versus total PB for patients on PB and VPA	169
Figure 8.1A	Observed versus predicted F-PHT levels at 37 °C	179
Figure 8.1B	Observed versus predicted F-CBZ levels at 37 °C	179
Figure 8.2A	Difference (%) between predicted and observed F-PHT versus observed F-PHT at 37 °C	181
Figure 8.2B	Difference (%) between predicted and observed F-CBZ versus observed F-CBZ at 37 °C	181
Figure 9.1A	Predicted F-PHT levels by Method A versus predicted F-PHT levels by Method B in patients taking PHT	194
Figure 9.1B	Difference (%) between predicted F-PHT levels by Method B and Method A versus predicted F-PHT levels by Method A	194
Figure 9.2A	Predicted F-PHT levels by Method A versus predicted F-PHT levels by Method D in patients taking PHT	195
Figure 9.2B	Difference (%) between predicted F-PHT levels by Method D and Method A versus predicted F-PHT levels by Method A	195
Figure 9.3A	Predicted F-PHT levels by Method A versus predicted F-PHT levels by Method C in patients taking PHT and VPA	196
Figure 9.3B	Difference (%) between predicted F-PHT levels by Method C and Method A versus predicted F-PHT levels by method A	196

LIST OF TABLES

		Page
Table 1.1	AED's Marketed in the United Kingdom and Europe	30
Table 1.2	Pharmacokinetic Parameters of Commonly Prescribed AED's	39
Table 1.3	Pharmacokinetic Parameters of Commonly Prescribed AED's	40
Table 1.4	Physical and Chemical Properties of AED's	43
Table 1.5	Methods for Separating AED's from Serum Protein	56
Table 1.6	Prediction Methods for F-PHT	65
Table 2.1	Recovery of AED's Added to Drug Free Serum	94
Table 2.2	Recovery of AED's Added to Physiological Saline	95
Table 2.3	Within-Batch Imprecision: Analysis of CCI by FPIA and HPLC	96
Table 2.4	Between-Batch Imprecision: Analysis of CCI by FPIA and HPLC	97
Table 2.5	Within-Batch Imprecision: Analysis of CCII by FPIA and HPLC	98
Table 2.6	Between-Batch Imprecision: Analysis of CCII by FPIA and HPLC	99
Table 2.7	Within-Batch Imprecision: Analysis of Pooled Serum Containing AED's by FPIA and HPLC	100
Table 2.8	Between-Batch Imprecision: Analysis of Pooled Serum Containing AED's by FPIA and HPLC	101
Table 2.9	Within-Batch Imprecision: Analysis of Pooled Serum Containing Free AED's by FPIA and HPLC	102
Table 2.10	Between-Batch Imprecision: Analysis of Pooled Serum Containing Free AED's by FPIA and HPLC	103
Table 4.1	Arithmetic Means of Ratios for the Concentration of Free AED's at Different Temperatures	123
Table 4.2	Estimates of K_D at Different Temperatures Derived by Regression Analysis from Figure 4.2	125

		Page
Table 4.3	Arithmetic Mean Values of K_D and CV at Different Temperatures Calculated According to Equation 3.8	126
Table 4.4	The Effect of Temperature on Free Fraction	127
Table 5.1	Arithmetic Means of Ratios for the Concentration of Free AED's at 25 °C and 37 °C	134
Table 5.2	The Effect of Temperature on Free Fraction	135
Table 5.3	Target Ranges for F-PHT, F-CBZ and F-PB at 25 °C and 37 °C	138
Table 6.1	Prediction Error Analysis for F-PHT and F-CBZ levels at 25 °C	150
Table 6.2	Prediction Error Analysis for F-PHT and F-CBZ levels at 37 °C	151
Table 7.1	Drug Regimens of Patients in Monotherapy and Polytherapy Study Groups	161
Table 7.2	The Effect of VPA on K_D at 25 °C and 37 °C for PHT, CBZ and PB	165
Table 7.3	The Effect of VPA on Free Fraction at 25 °C and 37 °C for PHT, CBZ and PB	166
Table 7.4	Target Ranges for F-PHT, F-CBZ and F-PB at 25 °C and 37 °C for Patients Co-Medicated with VPA	170
Table 8.1	Drug Regimens of Patients in PHT and CBZ Study Groups	177
Table 8.2	Prediction Error Analysis for F-PHT and F-CBZ levels	180
Table 9.1	Drug Regimens of Patients in PHT Study Groups	190
Table 9.2	Prediction Methods for F-PHT	191

CHAPTER 1

INTRODUCTION

1.1 Historical Review

Epilepsy is one of the oldest recorded diseases. It was first described by Hippocrates over 2,500 years ago, who indeed recognised it as a condition of the brain and not the result of magical possession:

> " ... this disease seems to me to be no wise more divine than others, but has its nature such as other diseases have, and a cause whence it originates, and its nature and cause are divine only just as much as all others are, and it is curable no less than the others".

Such wisdom, though, was seldom shared, and throughout history epilepsy associated as it was with mystical or demonic powers, was viewed with fear, dread and suspicion. Epilepsy is the second most prevalent neurological condition that affects man, yet no disorder can claim to be as misrepresented, and epilepsy still carries a considerable social stigma.

The word "epilepsy" is commonly used to describe a group of conditions characterised by a sudden loss, or alteration in consciousness, perception or sensation resulting from excessive and synchronous electrical discharges in the brain. The unpredictability of the condition and often the absence of any permanent physi-

cal symptoms encouraged our "superstitious" ancestors in their beliefs that angry devils or evil spirits had entered the body. Many an unfortunate epileptic may have come round from an attack to find himself or herself being "exorcised" by concerned friends or relatives who were busily boring an "escape exit" in the back of his or her skull to release the spirit. Sir Victor Horsley (Horsley, 1887) documented these primitive surgical skills from his examinations of photographs of trephines in human skulls in the Broca museum, Paris.

The ancient Greeks were the first to seek a rational cause for epilepsy; the term epilepsy itself is derived from a Greek word that means "to take hold of, to seize, or to possess". The British neurologist Hughlings Jackson (Hughlings Jackson, 1931) gave direction to the understanding of epilepsy in the late nineteenth century by carefully analysing individual cases. From his observations, he formulated the modern definition of epilepsy;

> "an occasional, an excessive, and a disorderly discharge of nerve tissue".

1.2 Classification of Epilepsy

Throughout history, epilepsy has been classified in many different ways, none of which have been either comprehensive or completely satisfactory. Although the Greeks had differentiated idiopathic from sympathetic epilepsy no major advances in the classification or

treatment of epilepsy were made for many centuries. Epilepsy was considered one of the eight cardinal contagious diseases (Lennox, 1960) along with bubonic plague, tuberculosis, scabies, erysipelas, anthrax, trachoma and leprosy. The introduction of electroencephalography (EEG), however, allowed classification according to observed changes in electrical activity of the brain during a seizure. The first recorded report of an EEG was made by Richard Caton in 1875, who recorded electrical signals in the brain following sensory input from peripheral stimuli and realised that this baseline activity oscillated continually for as long as the animals were alive (Caton, 1875).

In more recent times, the International League against Epilepsy Commission on Terminology and Classification has made various attempts at classification (Gastaut, 1969; 1983). Classification was based on a combination of five main criteria: (i) Clinical seizure type, (ii) EEG seizure type, (iii) EEG interictal expression, (iv) anatomical substrate and (v) aetiology and age. The epilepsies are broadly divided into the following types (From Parsonage, 1983): (i) Partial seizures (beginning locally with simple or complex symptomatology), (ii) Generalised seizures (bilaterally symmetrical or seizures with a local onset), (iii) Unilateral (or predominantly unilateral) and (iv) Unclassified epileptic seizures (Parsonage, 1983). Classification of the epilepsies is continually updated, refined and revised by the Epilepsy Commission (International League Against Epilepsy, 1989).

1.3 Neurophysiological Aspects of Epilepsy

The simplest form of epileptic process recognizable by the EEG is a local disturbance generating random spikes or sharp waves. This interictal activity is sometimes transformed into a repetitive discharge, which may remain local or may become generalized and involve other portions of the brain to a varying extent, with the accompanying clinical manifestations we call a seizure. In many examples there is widespread and diffuse neuronal involvement such as diffuse degenerative disease, metabolic disturbance or toxic state (Hunter and Jasper, 1949). In these cases there may be a diffuse increase in excitability of neurones and explosively rapid involvement of cortex and subcortex, but such diffuse disturbance is not essential, and generalized seizures have been elicited by focal electrical stimulation of mesial thalamus or midbrain (Hunter and Jasper, 1949).

The neuronal circuitry of the brain is exceedingly complex. Neurones of the brain transact much of their activity by generating action potentials which, delivered to other neurones, serve to excite or inhibit them. The neurone is a polarized unit, the inside being at a potential of approximately 70 mV negative to the outside (Hunter and Jasper, 1949). Excitatory or inhibitory influence at a synapse is accomplished through the release of chemical transmitters from the endings of the pre-synaptic fibres. An excitatory transmitter

induces a small depolarization in the subsynaptic membrane, the excitatory post-synaptic potential (EPSP), which rises rapidly and declines almost exponentially over a period of some milliseconds. The small depolarizations produced by a number of synapses may sum, and if a critical level is reached an action potential is generated, usually at the initial segment of the axon which is the region of lowest threshold. Inhibitory synaptic transmitters induce a comparable potential of opposite polarity, a hyperpolarization, the inhibitory post-synaptic potential (IPSP). EPSP's and IPSP's interact and the net effect on a neurone at any given moment depends on the magnitude, duration and temporal relationship of the depolarizing and hyperpolarizing influences (Harris and Mawdsley, 1974).

With such a complex circuitry, it is apparent that synaptic enhancement of inhibition is a important mechanism for regulation of central nervous system excitability. Inhibition of the nervous system is mediated by a number of neurotransmitters and their corresponding receptors, for example gamma-aminobutyric acid (GABA) and glycine acting on post-synaptic GABA and glycine receptors respectively (Glaser et al, 1980). This enhancement of inhibition of central nervous system excitability has led research activity toward the design of drugs for the treatment of epilepsy.

1.4 Treatment of Epilepsy

The early treatment of epilepsy emerged from ignorance, superstition, and religious beliefs, and this is illustrated from the record of treatment given to King Charles II at the time of his death.

> "While being shaved one morning in 1685, the king fell backward and had a violent convulsion. Treatment was immediately begun by a dozen or more physicians who were called in. He was bled to the extent of 1 pint from his right arm. Next, his shoulder was incised and 'cupped', depriving him of another 80 oz. of blood. After an emetic and 2 purgatives, he was given an enema containing antimony, sacred bitter, rock salt, mallow leaves, violets, beet root, camomile flowers, fennel seed, linseed, cinnamon, cardamom seed, saffron, and aloes. The enema was repeated in 2 hours and another purgative given. After continued bleeding and purging, the king's condition did not improve and, as an emergency measure, 40 drops of human skull extract were given to allay convulsions. A rallying dose of Raleigh's antidote was also given. This contained an enormous amount of herbs and animal extracts. Finally bezoar stone was given. As the king's condition grew increasingly worse, the grand finale of Raleigh's antidote, pearl julep, and ammonia water were forced down the dying King's throat" (Gilman et al, 1980).

Early Greek philosophers stressed the importance of a healthy lifestyle. This consists of moderate exercise, sufficient sleep, healthy diet, abstinence from sexual intercourse and masturbation, and regular bowel movements (Temkin, 1945). Consequently, castration or circumcision (Tissot, 1870) was a treatment approach which was adopted throughout the 19th century (Gowers, 1885).

1.4.1 Potassium Bromide

The first antiepileptic drug (AED) (demonstrated in 1857 to be useful in the management of epilepsy) was potassium bromide (Woodbury and Pippenger, 1982). However, potassium bromide was found to be particularly toxic as it induced at low doses impaired thinking, drowsiness, irritability and emotional difficulties, and at high doses delirium and coma (Woodbury and Pippenger, 1982). Due to these extreme side-effects the drug was withdrawn in 1921, but since then many AED's have been introduced for the management of patients with epilepsy. Some of these drugs e.g., phethenylate and benzchlorpropamide were later withdrawn due to unacceptable side effects; the currently used AED's are listed in Table 1.1.

1.4.2 Phenobarbitone

Phenobarbitone (PB), a barbiturate, was introduced for the management of epilepsy in 1912. PB is the drug of choice for febrile seizures and for treatment of neonatal seizures. However, dose-related side-effects include sedation and unsteadiness (Eadie and Tyrer, 1989).

1.4.3 Phenytoin

The introduction in 1938 of phenytoin (PHT), a hydantoin derivative with a ring structure analogous to that of the barbiturates, heralded a major advance in the

management of epilepsy because it was the first "designer" AED (Merritt and Putnum, 1938). PHT is effective in the treatment of tonic-clonic, simple and complex partial seizures (Eadie and Tyrer, 1989). Dose-related side-effects include drowsiness, unsteadiness, slurred speech and occasionally abnormal movement disorders (Eadie and Tyrer, 1989).

TABLE 1.1 AED'S MARKETED IN THE UNITED KINGDOM AND EUROPE

Non-proprietary name	Year introduced	Trade name	Company
Phenobarbitone	1933	Prominal	Winthrop
Phenytoin	1938	Epanutin	Parke Davis
Primidone	1952	Mysoline	ICI
Ethosuximide	1960	Zarontin	Parke Davis
Diazepam	1973	Valium	Roche
Carbamazepine	1963	Tegretol	Ciba-Geigy
Valproic acid	1974	Epilim	Sanofi
Vigabatrin	1989	Sabril	Merrell-Dow
Lamotrigine	1991	Lamictal	Wellcome

1.4.4 Primidone

Primidone (PRM) has a structure similar to PB, except that the carbonyl group at the 2-position of the PB ring is reduced in PRM. PRM was introduced in 1953 and found to be useful in the treatment of all seizure types with the exception of absence seizures (Gilman et al, 1980). PRM is metabolised to PB and phenylethylmalonamide (PEMA) in the liver (Baumel et al, 1972). Dose-related side effects include sedation and unsteadiness (Eadie and Tyrer, 1989).

1.4.5 Ethosuximide

Ethosuximide (ESM), a succinimide, was introduced in 1960, and it has become the drug of choice in the management of absence seizures (Gilman et al, 1980). Dose related side-effects include nausea, drowsiness, dizziness and unsteadiness (Eadie and Tyrer, 1989).

1.4.6 Diazepam

Diazepam, a benzodiazepine introduced in 1968, has become the most potent agent available for the emergency treatment of status epilepticus (Gilman et al, 1980). In addition, clonazepam, a chlorinated derivative of the sedative nitrazepam which was introduced in 1975, has been found to be effective in the control of absence seizures (Eadie and Tyrer, 1989). Dose-related side-effects for diazepam and other benzodiazepines in-

clude sedation and drowsiness. For this reason non-sedative alternatives are usually recommended in the routine management of epilepsy. The development of tolerance to the antiepileptic action of the benzodiazepine group of drugs also greatly limits their usefulness (Meldrum and Porter, 1986).

1.4.7 Carbamazepine

Carbamazepine (CBZ), a dibenzazepine derivative, was introduced in 1953 (Gilman et al, 1980). CBZ has been effective against complex partial seizures and tonic-clonic seizures. Dose-related side effects include dizziness, double vision, unsteadiness, nausea and vomiting (Eadie and Tyrer, 1989). In addition, CBZ exhibits antidiuretic properties which may lead to water intoxication, hyponatraemia and low plasma osmolality (Perucca et al, 1978; Perucca and Richens, 1980). The major metabolite of CBZ, carbamazepine-10, 11-epoxide (CBZ-E), which also has antiepileptic activity, may contribute to the reported dose-related side-effects of CBZ (Patsalos et al, 1987; Eadie and Tyrer, 1989).

1.4.8 Valproic Acid

Valproic acid (VPA), a short branch chain carboxylic acid, was introduced in 1963 (Gilman et al, 1980). VPA is most effective in treating absence and myoclonic seizures of generalised epilepsy (Hassen et al, 1976; Convanis et al, 1982), photosensitive epilepsy (Jeavons et al, 1977), febrile convulsions (Cavazzuti, 1975) and

all varieties of partial seizures (Simon and Penry, 1975; Mattson et al, 1978). Dose-related toxicity includes tremor, irritability, restlessness and occasionally confusion. VPA intake also tends to cause raised plasma ammonia levels (Coulter and Allen, 1981; Haidukewych et al, 1985), particularly if other AED's are also taken concurrently (Zaccara et al, 1987).

1.5 New Drugs

Although currently available AED's (e.g., PB, PHT, PRM, CBZ and VPA) are effective in the management of more than half the total number of patients with epilepsy, there remain a sizable number who continue to suffer seizures and who are exposed to unacceptable acute or chronic side-effects. Thus there is a need for new drugs which are more potent but less toxic. At present, more than 17 AED's are under evaluation (Meldrum and Porter, 1986) and two of these, lamotrigine (LTG) and vigabatrin (GVG), have recently been licensed as add-on AED's in the treatment of intractable epilepsy.

1.6 Mechanism of Action of AED's

Despite widespread clinical use and intensive investigation, the principal mechanism of action of AED's remains elusive. Their development has been essentially empirical, with their antiepileptic activity being determined in animal models of epilepsy and with them subsequently being used in patients.

1.6.1 Phenytoin

Non-synaptic effects of PHT include reduction in post-tetanic potentiation (Macdonald, 1983). Post-synaptic effects include enhancement of GABA-mediated inhibition and reduction of excitatory synaptic transmission. The drug also has pre-synaptic effects on calcium entry into neurones and may block neurotransmitter release. However, many of these actions on neuronal excitability and synaptic transmission do not occur at therapeutically relevant drug concentrations and their relevance to the drug's antiepileptic action in man is not clear. The reduction of repetitive firing of neurones due to slowing of the recovery of sodium channels from inactivation however does occur at low concentrations of PHT, and may turn out to be an important factor in the drug's antiepileptic action (Woodbury, 1955).

1.6.2 Carbamazepine

The most likely mode of action of CBZ is to limit high frequency, sustained, repetitive firing, an action similar to that of PHT (Macdonald et al, 1985).

1.6.3 Phenobarbitone

PB has been shown to have numerous different actions on CNS neurones, such as modification of post-synaptic neurotransmitter responses, enhancement of GABA-mediated inhibition and the diminishing of glutaminergic and cholinergic excitation (Eadie and Tyrer, 1989);

PB at a therapeutically relevant concentration inhibits calcium influx into depolarised synaptosomes from rabbit cerebral cortex (Sohn and Ferendelli, 1976). Non-synaptically, the drug may reduce voltage dependent sodium and potassium conductances and block repetitive firing (Sohn and Ferendelli, 1976).

Augmentation of GABA responses and antagonism of glutamate excitation occurs at clinically relevant PB concentrations and thus may be the underlying mechanism of antiepileptic activity (Schultz and MacDonald, 1981). Other actions, for example direct increase in chloride conductance, may be more important for the sedative and anaesthetic properties of PB. Enhancement of GABAergic responses at low PB concentration appears to be effected by the facilitating of GABA binding to GABA receptors alone, as PB does not directly alter chloride conductance under these conditions (Eadie and Tyrer, 1989).

1.6.4 Primidone

It is considered that a large part of the antiepileptic effect of PRM may be due to the major metabolite PB (Rimmer and Richens, 1988). In experimental animal models of epilepsy however, the PRM metabolite PEMA has been shown to have antiepileptic properties (Eadie and Tyrer, 1989). The presence of active metabolites makes it difficult to assess the mode of action of PRM in epileptic patients.

1.6.5 Ethosuximide

The high degree of therapeutic specificity of ESM in human seizure disorders is reflected by its selective antiepileptic action against pentylenetetrazole seizures in animals. The drug has no effect on maximal electroshock seizures (Glaser et al, 1980). Despite its widespread use in the treatment of absence seizures, the exact mode of action of ESM is unknown. It has been reported to inhibit cortical Na^+K^+-ATPase activity; to reduce slightly the activities of succinate dehydrogenase and aldehyde reductase; to alter cerebral neurotransmitter levels; and to have several actions on synaptic transmission (Glaser et al, 1980). It is ineffective against repetitive neurone firing and there is no evidence for enhancement of GABA inhibition (Patsalos and Lascelles, 1981). Insufficient information is available to allow one to explain the antiepileptic action of ESM in terms of altered synaptic transmitter activity.

1.6.6 Valproic Acid

VPA has been shown to be effective in a variety of experimental animal models of epilepsy. Although a number of hypotheses have been advanced to account for the antiepileptic activity of the drug, the mechanism of action is far from clear (Chapman et al, 1982). Suggested mechanisms include increasing the brain concentrations of the inhibitory neurotransmitter GABA; limiting sustained repetitive firing; and reducing ex-

citatory neurotransmission. The drug has been shown to interact with several enzymes involved in the synthesis and degradation of GABA, but these enzymatic effects only occur at relatively high drug concentrations (Glaser et al, 1980).

1.6.7 Lamotrigine

The mode of action of LTG has been shown to involve diminishing the release of excitatory amino acid glutamate (Meldrum and Porter, 1986).

1.6.8 Vigabatrin

The mode of action of GVG has been shown to involve inhibition of GABA aminotransferase (Meldrum and Porter, 1986).

1.7 Clinical Pharmacology of AED's

Pharmacokinetics relates to the variation with time of drug concentration, particularly in serum or plasma, as a result of absorption, distribution and elimination. Pharmacokinetic principles applied to the monitoring of AED levels aid in selection and adjustment of drug dosage, and facilitate interpretation of measured plasma or serum drug levels. A wealth of literature providing information on the absorption, distribution and elimination parameters of individual AED's is available (Eadie and Tyrer, 1989). A summary of the

most important pharmacokinetic parameters of AED's is given in Tables 1.2 and 1.3.

TABLE 1.2 PHARMACOKINETIC PARAMETERS OF COMMONLY PRESCRIBED AED'S*

Drug	Bioavailability	Protein binding (% bound)	Volume of distribution (L/Kg)	Total body clearance (ml/hr/kg)
PHT	(85-95%)	88-90	0.6 - 0.7	21.6 - 22.4
CBZ	(75-85%)	70-78	1.0 - 2.0	50 - 100
PB	(95-100%)	48-54	0.7 - 1.0	2.1 - 4.1
PRM	(90-100%)	20-30	0.4 - 1.1	29 - 31
VPA	(100%)	88-92	0.1 - 0.5	7 - 8
ESM	(90-95%)	0	0.6 - 0.9	10 - 15

* References:- Taylor and Caviness, 1986; Eadie and Tyrer, 1989

TABLE 1.3 PHARMACOKINETIC PARAMETERS OF COMMONLY PRESCRIBED AED'S*

Drug	Elimination half-life (Hours)	Route(s) of elimination	Specific comments
PHT	15 - 20	Saturable hepatic metabolism	Enzyme inducer; Elimination half-life concentration-dependent
CBZ	25 - 50	Hepatic metabolism; CBZ-E active metabolite	Enzyme inducer; Autoinduction of metabolism
PB	70 - 100	Hepatic metabolism; 25% excreted unchanged	Enzyme inducer; Sedative
PRM	6 - 12	Hepatic metabolism; Active metabolites; 40% excreted unchanged	PB a major metabolite
VPA	8 - 12	Hepatic metabolism	Minor enzyme inhibitor; Concentration-dependent protein binding
ESM	30 - 70	Hepatic metabolism; 25% excreted unchanged	More rapid clearance in children

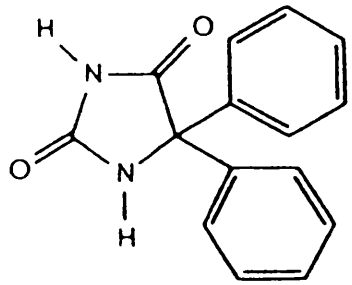
* References:- Taylor and Caviness, 1986; Eadie and Tyrer, 1989

1.8 Chemistry of AED's

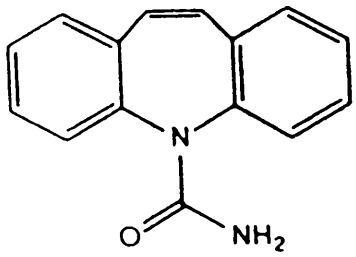
With the exception of VPA, most commonly used AED's have heterocyclic ring structures (Figure 1). The physical and chemical properties of AED's are presented in Table 1.4.

PHT is a white, crystalline, bitter tasting powder. It is sparingly soluble in water and almost insoluble in acid, but will dissolve in aqueous bases and in organic solvents such as ethanol, acetone and chloroform. CBZ is a white powder and is soluble in polar organic solvents such as propylene glycol, ethanol and acetone. PB is a white, crystalline powder, poorly soluble in water but soluble in organic solvents such as ethanol and chloroform. PRM is a white, crystalline powder, poorly soluble in water and most organic solvents. ESM is a white crystalline powder, which is readily soluble in water and in organic solvents e.g., ethanol. VPA is a hygroscopic white powder which is soluble in ethanol and methanol as well as water. It is usually prescribed as its sodium salt, which has a molecular mass of 166.20 daltons (Eadie and Tyrer, 1989).

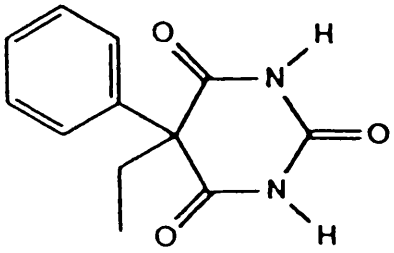
Figure 1.1 Chemical structures of commonly used AED's



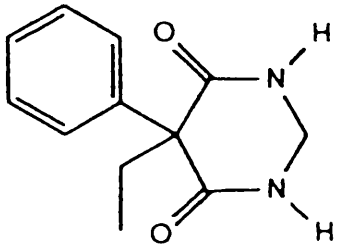
Phenytoin



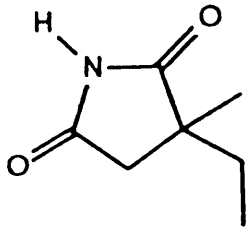
Carbamazepine



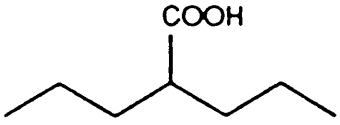
Phenobarbital



Primidone



Ethosuximide



Valproic Acid

TABLE 1.4 PHYSICAL AND CHEMICAL PROPERTIES OF AED'S

Drug	Chemical name	Molecular mass (Daltons)	pKa
PHT	5,5-Diphenyhydantoin	252.26	8.06
CBZ	5-Carbamyl-5H-dibenz[b,f]-azepine	236.26	neutral in solution
PB	5-Ethyl-5-phenylbarbituric acid	232.23	7.2
PRM	5-Ethyldihydro-5-phenyl-4,6-pyrimidinedione	218.25	neutral in solution
ESM	2-Ethyl-2-methysuccinimide	141.20	9.3
VPA	2-Propylpentanoic acid	144.20	4.95
LTG	3,5-Diamino-6-(2,3-dichlorophenyl)-1,2,4-triazine	256.09	5.5
GVG	DL-4-Aminohex-5-enoic acid	129.16	4.02, 9.74

1.9 Plasma Protein Binding of AED's

Most drugs in the systemic circulation are bound to plasma proteins. AED's are bound to varying degrees, with some being over 85% protein-bound (PHT and VPA) and others showing negligible binding (PRM, ESM) (Table 1.2). The binding of drugs to plasma proteins has several important implications. Firstly, it reduces the amount of drug available for transfer to the site of action, since only free drug can diffuse out of the capillaries. Secondly, it provides a reservoir of drug in the circulation, replenishing that lost by metabolism. Thirdly, if the drug is loosely bound to albumin, displacement can occur when a second drug with higher affinity is co-administered. Furthermore, in disease characterized by hypoalbuminaemia (e.g., hepatic or renal failure), concentration of free drug will increase relative to the total concentration (free + bound).

1.10 Mechanism of Drug-Protein Binding

Although drugs bind to numerous proteins, including the gamma-globulins and lipoprotein, the major drug-binding proteins are albumin and α-1-acid glycoprotein (Eadie and Tyrer, 1989). Albumin is quantitatively the most important binding protein. It exhibits a high affinity for acidic drugs such as salicylic acid, warfarin, phenylbutazone, penicillins and sulphonamides,

and a low affinity for basic drugs such as propranolol (Piafsky and Borga, 1977) and neutral drugs like digitoxin. At physiological pH, basic groups of the amino acids arginine, histidine and lysine are responsible for binding acidic drugs and the acidic groups of the amino acids aspartic acid, glutamic acid and tyrosine are responsible for binding basic drugs. At blood pH of 7.4, their acidic carboxyl groups are protonized to positive ions. Acidic drugs are strongly bound to albumin. Basic, positively-charged drugs are weakly bound to a larger number of sites on the albumin molecule.

Endogenous compounds such as bilirubin and fatty acids can compete with drugs for binding to albumin. There are two main drug binding sites:- one of these (Site I) binds a range of structurally unrelated drugs e.g., warfarin, PHT and sulphonamides, whereas the other site (Site II) binds a smaller number of drugs (e.g., diazepam, phenylbutazone and ibuprofen). Some of the drugs binding to site II may also have an affinity for Site I (Muller and Wollert, 1979; Fehske et al, 1981).

The forces involved in drug binding are primarily hydrophobic interactions and hydrogen bonding, although ionic bonds and van der Waals forces also contribute (Koch-Weser and Sellers, 1976). The interaction between proteins and drugs is governed by the Law of Mass Action, i.e., the proportion of bound drug remains constant provided the binding sites are not saturated; at equilibrium the affinity between the drug and protein

is expressed as the equilibrium constant. For the majority of drugs saturation is not observed within the therapeutic range, although saturable binding within the therapeutic concentration range has been reported for VPA (Bowdle et al, 1980) and disopyramide (Meffin et al, 1979).

Only the free (non-protein-bound) drug is capable of crossing the various lipoprotein membranes that surround the receptor sites.

1.11 Factors Altering Protein Binding

There are three factors that can affect the extent to which plasma proteins bind a given drug:-

(a) Protein concentration.

(b) Drug interactions.

(c) Saturation of protein-binding sites.

When any one of the above occurs, the drug's pharmacological effect can be expected to be altered. Thus pharmacological response cannot be predicted from measurement of total drug concentration (Pippenger, 1984; Rowland and Tozer, 1989).

1.11.1 Protein Concentration

Hypoalbuminaemia, commonly observed in geriatric patients, results in a reduction in the amount of bound drug. With some drugs, this reduction in binding will result in a transitory increase in the free drug level in serum, followed by a compensatory decrease in the total drug level owing to increased metabolism of free drug. This occurs because the rate of metabolism of these drugs is proportional to the free drug level. In patients with hepatic dysfunction, however, the free level may rise and total level may remain the same because of the patient's reduced capacity to metabolise the increased free drug. In either case, measurement of the total drug level provides an inaccurate assessment of the drug's clinical effect (Pippenger, 1984).

1.11.2 Drug Interaction

Drug interaction can be either direct competition by two drugs for the same binding sites, thus resulting in the displacement of the one with weaker binding, or it can be the situation of one drug affecting the metabolism and clearance of another drug. In order for displacement to occur, the displacing compound must have a higher affinity for the same binding site than the displaced drug, and it must be present in high molar concentrations relative to the concentration of the binding protein. For example, doses of VPA cause displacement of PHT from serum albumin, resulting in increase

in the PHT free levels (Kober et al, 1980). Thus the effect on the pharmacokinetics of a drug is greatly affected by protein binding, since only the free drug can leave the blood compartment. A decrease in binding capacity of 1% for a drug that is normally 99% bound to plasma protein would have the effect of doubling the free drug available for distribution (Kwong, 1985).

Another factor that may perturb the drug-protein binding equilibrium is a change in the affinity for the drug. This occurs when the binding of a second drug or endogenous substance to protein causes an alteration in the affinity for the first drug (Rowland, 1980). For example, when salicylate binds to albumin it modifies the albumin structure by means of acetylation. Acetylalbumin, the product of this binding interaction, has a decreased affinity for various other drugs (Pippenger, 1984).

1.11.3 Saturation of Protein-Binding Sites

At therapeutic concentrations, most drugs do not saturate the binding capacity of the binding proteins. However, saturation of available binding sites occurs with certain drugs at concentrations within their therapeutic ranges e.g., VPA (Bowdle et al, 1980) and salicylate (Rowland, 1980).

1.12 Methods Used in the Measurement of Total Levels of AED's

The measurement of drug levels in a biological fluid usually involves the separation of the drug from other substances present in the fluid and the measurement of the quantity of drug that has been isolated. The techniques most commonly employed in the measurement of AED's at present are high performance liquid chromatography (HPLC), gas chromatography (GC) and various types of immunoassay (Reviewed by Goldberg et al, 1983).

HPLC and GC are both column chromatographic techniques, but while the mobile phase in which the substances to be analysed are dissolved is liquid for HPLC, they are dispersed in a gaseous phase for GC. Compared to GC, HPLC does not require high temperatures for the separation of components, which might cause decomposition, and columns packed with the stationary phase usually are kept at ambient temperature. Thus the HPLC technique is becoming the method of choice for the measurement of AED's in serum. In HPLC, substances are separated according to their solubility in aqueous or organic solvents. Highly polar compounds will be more soluble in highly polar solvents like water, whereas less polar drugs will dissolve better in organic solvents such as chloroform. As the mobile liquid phase and the stationary phase come into contact in a column, separation occurs in a fashion similar to other chromatographic methods. The use of "reverse phase"

chromatography (i.e., non-polar column packing and polar mobile phase as against the silica column and non-polar solvents of "normal phase" chromatography) has allowed the direct determination of number of AED's including PHT, CBZ, PB, PRM and ESM. After separation on the column, substances are passed through flow cell detectors which are based on the techniques of ultraviolet (UV) absorbance, fluorescence or refractive index. The detectors are coupled with computerized printer-plotters for the detection and recording of drug peaks and the quantitation of results.

The most commonly used immunoassay methods for the measurement of AED's in biological fluids are the enzyme multiplied immunoassay technique (EMIT) and fluorescence polarization immunoassay (FPIA). Both immunoassay techniques have good sensitivity and are simple to perform, and both are homogeneous assays, i.e., no separation stages are necessary prior to the assay being carried out.

In the EMIT method, a known quantity of drug labelled with an enzyme (glucose-6-phosphate dehydrogenase) competes with the drug from a patient's sample for antibody binding sites. At equilibrium, the amount of unbound enzyme-labelled drug will be directly proportional to the concentration of drug. Substrate added to the sample catalyzes an enzymatic reaction that can be measured spectrophotometrically in the UV range.

The FPIA method involves fluorescence detection coupled with polarized light emission. Fluorescein-tagged drug and drug in a patient's serum are incubated with antibody and then excited with light passed through a polarizing lens. With high drug levels, there is an increase in unbound fluorescent-labelled molecules that move freely in solution, causing the light to be depolarized upon emission. At low drug levels, the labelled antigen-antibody complex rotates more slowly and polarization of the emitted light is maintained. Rapid turnaround time, good sensitivity and ease of operation make the FPIA method appropriate for routine purposes. Also recent work suggests that the calibration curves are particularly stable in the FPIA technology by comparison with the EMIT technique (Ratnaraj et al, 1986).

The specificity, stability, reliability, ease, speed and expense of existing methods for assaying AED's have been extensively evaluated (Berlin et al, 1972; Kumps et al, 1980; Couri et al, 1980; Rovei et al, 1980; Furlanut et al, 1981; Ratnaraj et al, 1986). To ensure comparability of results from different techniques for the purpose of therapeutic drug monitoring (TDM), national and international quality assessment schemes have been established both in Europe (Richens, 1975) and in the United States and Canada (Pippenger et al, 1976; 1978). Such schemes have significantly reduced inter-laboratory assay variability (Griffiths et al, 1980) thus improving the clinical management of

patients with epilepsy (Reynolds, 1975; Richens and Warrington, 1979; Koch-Weser, 1981).

1.13 The Rationale for TDM of Total Levels of AED's

The criteria for the TDM of total levels of AED's are:-

1. That a direct relationship should exist between serum or plasma drug levels and therapeutic or toxic effects.

2. That therapeutic response should be measurable.

3. That a narrow therapeutic index should exist, i.e., the difference between therapeutic and toxic serum or plasma levels should be relatively small.

4. That serum or plasma drug levels achieved on standardised doses should exhibit wide interpatient variability, i.e., poor predictability of an individual's level from the dose.

5. That therapeutic tolerance should not develop, e.g., in the case of PB, tolerance develops to its sedative effect but not to its AED effect.

6. Polytherapy drug interactions, which commonly occur in epilepsy, are a strong indication for drug monitoring, particularly if there is inhibition of metabolism, e.g., sulthiame inhibiting PHT metabolism.

7. That non-compliance is suspected. Up to 50% of epileptic outpatients do not take what is prescribed.

1.14 Methods for Separating Free Drug from Serum or Plasma

A variety of methods have been developed to separate non-protein-bound drug from the protein-bound-component. Table 1.5 lists the methods most commonly employed. The separation of protein-bound from unbound AED's in serum or plasma is usually performed by equilibrium dialysis, ultracentrifugation or ultrafiltration techniques (Troupin and Friel, 1975; Johannessen et al, 1976; Rowland, 1980; Mattson et al, 1982; Riva et al, 1982; Levy and Moreland, 1984; Bock and Ben-Ezra, 1985; Levy and Schmidt, 1985; Jack et al, 1986; Theodore, 1987; Taylor and Ackerman, 1987). In all three methods a physical separation of non-protein-bound (free) drug from the binding macromolecules is required.

In order to determine the concentration of the drug separated from protein these procedures may be carried out in the presence of trace quantities of labelled drug, so that estimation of the labelled drugs present in the exudate or supernatant gives a measure of the level of free drug (Cramer and Mattson 1979; Kilpatrick et al, 1984; Barre et al, 1985; Verbeeck and Cardinal

1985; Zysset and Hegel, 1987). Alternatively the estimation of free drug level can be carried out using the same types of technique as for the measurement of total drug levels, namely GC, HPLC or immunoassay techniques (Reidenberg et al, 1971; Booker and Darcey, 1973; Troupin and Friel, 1975; Cramer and Mattson, 1979; Mattson et al, 1982; Riva et al, 1982; Kulpmann et al, 1984; Levy et al, 1984; Bock and Ben-Ezra, 1985; Miller and Pinkerton, 1985; Agbato et al, 1986; Elyas et al, 1986; Jack et al, 1986, Taylor and Ackermann, 1987).

These methods of separation and estimation of free drugs usually yield comparable results (Cramer and Mattson, 1979; Levy et al, 1984; Zysset and Hegel, 1987), but all have associated problems which may render it difficult to determine the correct unbound drug levels (Cramer and Mattson, 1979; Rowland, 1980; Riva et al, 1982; Levy and Moreland, 1984; Levy et al, 1984; Taylor and Ackerman, 1987), and no one method has so far gained acceptance as being entirely suitable for routine use.

AED's also distribute into saliva, and saliva levels are considered to be in equilibrium with free plasma concentrations (Knott and Reynolds, 1983). The major advantage of saliva monitoring is that sampling is non-invasive. However use of this technique has been restricted because of some continuing controversy about the equivalence of results, oral contamination and reports of decomposition of drug metabolites in saliva

under certain conditions (Perucca, 1980). Though measurement of free drug levels in serum or plasma requires a separation step, it is the more commonly used biological fluid in laboratories for drug measurement.

TABLE 1.5 METHODS FOR SEPARATING AED'S FROM SERUM PROTEIN

Method	Sample size* (ml)	Time*	Comment
Equilibrium dialysis	0.25 - 1.0	3 - 6 hr	Some dilution; initial equilibrium disturbed; membrane binding and instability can be problems.
Ultracentrifugation	1 - 10	4 - 24 hr	No dilution; binding to tube and low-density lipoproteins can be problems
Ultrafiltration	1 - 10	10 - 20 min	No dilution; serious problem if binding to membrane occurs

* Commonly reported values

1.14.1 Equilibrium Dialysis

Equilibrium dialysis can be regarded as the "classical" method for determining free levels of drugs in plasma or serum. In this method the plasma sample and a drug-free buffer solution are placed either side of a semi-permeable membrane. The unbound drug in plasma passes through the membrane until, at equilibrium, the level of unbound drug in the buffer solution and unbound drug in plasma are equal. By measuring the drug concentration on both sides of the membrane, the free level or free fraction of drug in plasma can be calculated. Most studies on drug protein binding have been performed by this method, which offers a number of advantages. For example both free and total concentrations can be simultaneously measured and the experimental procedure can be carried out at physiological temperature (Riva et al, 1982; Verbeeck and Cardinal, 1985).

Equilibrium dialysis is however too time-consuming to be practicable as a routine analytical procedure (Table 1.5) (Troupin and Friel, 1975; Rowland, 1980, Bock and Ben-Ezra, 1985; Theodore, 1987; Taylor and Ackerman, 1987). Dialysis time and buffer concentration must be carefully selected and controlled, and highly ionized drugs may be adsorbed onto the dialysis membrane (Rowland, 1980; Kwong, 1985; Theodore, 1987; Taylor and Ackerman, 1987). Difficulties in interpretation can

arise when the level of drug in the plasma at equilibrium is lower than that initially present, partly due to the diffusion of drug into the buffer solution and partly due to the dilution of plasma by water moving from the buffer solution into the protein solution, as a consequence of osmotic gradients (Rowland, 1980; Theodore, 1987; Barre et al, 1988). In addition, a formula has to be applied in each case to correct for the fall in concentration resulting from the redistribution of the drug between plasma and buffer compartments in the dialysis cell (Rowland, 1980; Kwong, 1985; Theodore, 1987; Taylor and Ackerman, 1987). Stringent precautions must also be taken to avoid bacterial contamination during the lengthy processes of incubation and dialysis. All these factors limit the application of equilibrium dialysis to free drug monitoring in a routine laboratory setting.

1.14.2 Ultracentrifugation

Ultracentrifugation, which involves separating the small unbound drug molecule from the high molecular weight drug-protein complex by centrifugation, neither disturbs the equilibrium nor alters the unbound drug concentration. Theoretically it is a preferred method, but it is not practicable in a routine setting because it is time-consuming and requires costly equipment. In addition problems do exist if the drug binds to low-density lipoproteins, which rise to the top of the tube on centrifugation (Rowland, 1980; Verbeeck and Cardinal, 1985; Barre et al, 1985; Jack et al, 1986; Zys-

set and Hegel, 1987). Other possible sources of error are back diffusion of drug from the pellet into the supernatant (Barre et al, 1985) or sedimentation of small amounts of unbound drug from the supernatant (Oellerich and Muller-Vahl, 1984), and temperature needs to be carefully controlled to avoid alterations in the binding characteristics of drugs (Rowland, 1980; Verbeeck and Cardinal, 1985; Jack et al, 1986; Zysset and Hegel, 1987).

1.14.3 Ultrafiltration

Ultrafiltration involves filtration under high speed centrifugation through a semi-permeable membrane which allows small molecules through while retaining large protein molecules. When this technique was first introduced there were problems of protein leakage from the devices, which gave spuriously high values for unbound drug fraction (Ruprah et al, 1981). Another problem associated with ultrafiltration was significant non-specific adsorption onto the membrane, which led to an underestimation of free drug level (Rowland, 1980).

In the past four years, considerable progress has been made in this field, and for an increasing number of drugs the filtration devices that are available on the market allow reliable determinations of free drug level (Cramer et al, 1983). It is still incorrectly stated that the disturbance of equilibrium during ultrafiltration partition affects the free drug level. Several reports (Sophianopoulos et al, 1978; Whitlam and Brown,

1981; Bowers et al, 1984) have clearly shown that the free drug level measured by ultrafiltration remains unaltered during the filtration procedure and that it is independent of the plasma or serum volume used.

Ultrafiltration uses a fine-mesh membrane filter to separate unbound from protein-bound drug. Its primary advantages are speed, simplicity, and the absence of dilutional effects seen in equilibrium dialysis (Wandell and Wilcox-Thole, 1983). Speed is important as, for example, concentrations of free fatty acids produced by lipolysis of triglycerides increase during storage (Nilsen et al, 1977) and dialysis (Rudman et al, 1971), and may alter the original equilibrium between the free and bound components.

A combination of ultrafiltration and HPLC can have the advantages of flexibility, speed and accuracy which are essential elements for the provision of a therapeutic drug monitoring service for free levels of AED's (Cramer et al, 1983; Levy et al, 1984; Bock and Ben-Ezra, 1985; Barre et al, 1985; March and Blanke, 1985; Taylor and Ackerman, 1987). Such an approach could also be used to undertake studies on theoretical applications of protein binding. In practical terms it is a simple method, but temperature-controlled ultracentrifuges are expensive and not widely available in routine laboratories.

1.15 The Rationale for TDM of Free AED Levels

AED's are assumed to interact with specific receptors within the central nervous system, and are believed to prevent seizures by binding to synaptic receptors or altering neurotransmitter release. This effect is proportional to the level of drug not bound to protein. Since it is impracticable to monitor receptor site drug levels in the routine situation, the measurement of free drug levels in serum is considered to be a measure of the effects of the drug on the receptor sites.

The criteria for monitoring free levels of an AED's are:-

1. If a direct relationship should exist between serum or plasma free drug levels and therapeutic or toxic effects.

2. If the drug has a narrow therapeutic range.

3. If the drug concerned is highly protein bound. (free fraction less than 0.4).

4. If the free fraction is variable (free and total drug levels are dissociated).

It is now recognised that the estimation of free levels of AED's in plasma can be a useful adjunct in the management of patients with epilepsy under circumstances likely to be associated with altered binding capacity (Booker and Darcey, 1973; Perucca, 1984; Levy and Schmidt, 1985; Taylor and Ackerman, 1987). This occurs in many situations including renal failure (Reidenberg et al, 1971; Odar-Cederlof and Borga, 1974; Reynolds et al, 1976; Tiula et al, 1987), nephrotic syndrome (Gugler and Azarnoff, 1976), hepatic disease (Hooper et al, 1974; Blashke et al, 1975; Olsen et al, 1975), pregnancy and the puerperium (Dean et al, 1980; Ruprah et al, 1980; Perucca et al, 1981; Perucca, 1984), infancy (Rane and Wilson, 1976; Hamar and Levy, 1980), old age (Crooks et al, 1976; Wallace et al, 1976; Patterson et al, 1982) and hypoalbuminaemia from any cause (Lunde et al, 1970; Porter and Layzer, 1975; Koch-Weser and Sellers, 1976), such as occurs post-operatively (Fremstad et al, 1976; Elfstrom, 1977) or following severe burns (Bowdle et al, 1980a). Free levels of AED's are also influenced by polypharmacy with antipyretic analgesics (Shoeman and Azaroff, 1975; Ehrnebo and Odar-Cederlof, 1977; Neuvonen et al, 1979; Paxton, 1980; Frazer et al, 1980), diazoxide (Roe et al, 1975), tolbutamide (Wesseling and Mols-Thurkow, 1975) and cimetidine (Hetzel et al, 1981).

It is known that the extent to which a particular AED is bound to plasma proteins may change when the AED regimen is altered, e.g., when PHT is co-administered with VPA (Perucca, 1984; Levy and Moreland, 1984; Levy

and Schmidt, 1985). The addition or withdrawal of one AED may have a marked effect on protein binding characteristics when several AED's are competing for binding sites on plasma protein, for example albumin (Patsalos and Lascelles, 1977; Dahlqvist et al, 1979; Levy and Koch, 1982; Knott et al, 1982; Theodore, 1987). In these situations the interpretation of total drug levels can be difficult if not impossible.

1.16 Methods for Predicting Free Levels of AED's in Plasma or Serum

Measurement of free levels of AED's is expensive and time consuming, and therefore not available in every laboratory but only in specialised centres. Thus, a procedure for predicting free AED levels from measurement of total drug level and albumin concentration in plasma or serum could be an invaluable analytical tool and could be of clinical importance in the management of patients with epilepsy. Four such predictive methods have been developed so far based on **statistical** correlations between data for the concentrations of total and free drug and albumin in serum (Table 1.6). All four methods have involved prediction of free PHT (F-PHT) levels.

1. Gugler Method

This method (Gugler et al, 1975) was developed for prediction of F-PHT in patients with nephrotic syndrome, i.e., where plasma albumin level is commonly low. Six

patients and six healthy subjects received a PHT daily dose of 300 mg for 14 days. Steady state levels of PHT and F-PHT in plasma were measured by radioimmunoassay (See Table 1.6 for description). Albumin concentration were assayed by protein electrophoresis. Based on the data, an inverse correlation was established between plasma albumin and the PHT free fraction (α) (the fraction of total drug that is not bound to albumin, i.e., free fraction (α) = F-PHT / PHT). It was found that for a decrease of the albumin level of 10 g/L in the range of 20 - 30 g/L, the free fraction increased on the average by 10% (Gugler et al, 1975). Using a graphical approach, α for PHT at an individual albumin concentration could be found and used to calculate F-PHT (i.e, F-PHT = α * PHT).

Because of the small number of subjects investigated, no attempt was made to validate this statistically based method either in terms of the precision of the predicted F-PHT or for use in subjects with higher (normal) plasma levels of albumin.

TABLE 1.6 PREDICTION METHODS FOR F-PHT

Method	Separation procedure	Experimental temperature	Method of analysis	Epileptic patient population
Gugler method	Equilibrium dialysis	37 °C	RIA	Hypoalbuminaemic patients receiving PHT
Sheiner-Tozer equation	Theoretically derived equation from nomogram	not stated	--	Hypoalbuminaemic patients receiving PHT
Haidukewych equation	Ultrafiltration	25 °C	FPIA	Normal polytherapy population receiving PHT and VPA
Dasqupta's equation	Ultrafiltration	25 °C	FPIA	Normal monotherapy population receiving PHT

2. Sheiner-Tozer Method

This method (Winter and Tozer, 1986) also aims at predicting F-PHT in patients with hypoalbuminaemia. It has been developed as follows:-

In 1978 Sheiner and Tozer published a graph correlating the free fraction (α) and k*p, where k is the relative affinity constant for PHT for serum albumin (i.e., k = K'/K, where K' is altered affinity in the particular patient and K is normal affinity of PHT for albumin) and p is the relative albumin concentration in serum (i.e., p = P'/P, where P'is the particular patient's albumin concentration in serum and P is normal albumin) (Sheiner and Tozer 1978, page 99). The graph is presumably based on experimental data, though no details have been found about its origin. The equation for the regression lines is given as:-

$$\alpha'/\alpha = 1 / (k * p - \alpha * k * p + \alpha)$$

This equation can be simplified as follows:

$$\alpha'/\alpha = 1 / [k * p (1 - \alpha) + \alpha]$$

and inverted to obtain

$$\alpha / \alpha' = k * p (1 - \alpha) + \alpha$$

The free fraction (α) can be defined as

$$\alpha = \text{F-PHT}_{observed} / \text{PHT}_{observed}$$

α' can be deduced from the Sheiner-Tozer graph as

$$\alpha' = \text{F-PHT}_{observed} / \text{PHT}_{adjusted}$$

where $\text{PHT}_{adjusted}$ would represent the PHT level corrected to normal albumin concentration (though the experimental basis for this assumption does not seem to have been published by Sheiner and Tozer).

If the ratios for α and α' are substituted in the simplified equation above, the following expression is obtained;

$$(\text{F-PHT}/\text{PHT}_{obs})/(\text{F-PHT}/\text{PHT}_{adj}) = P'/ P\ (1-0.1)+0.1$$

If it is assumed (i) that affinity for albumin remains normal, k is equal to unity as defined by Sheiner-Tozer (see above), (ii) that normal PHT free fraction (α) is 0.1, and (iii) that P = 4.4 g/dL (647 μmol/L), then the equation above takes the form;

$$\text{PHT}_{adj}/\text{PHT}_{obs} = (0.9/4.4) * P' + 0.1$$

or

$$\text{PHT}_{adjusted} = \text{PHT}_{observed}/(0.205 * \text{albumin}) + 0.1$$

This equation, the so called Winter-Tozer equation (Winter and Tozer, 1986), was originally intended for predicting level of PHT in hypoalbunaemic patients.

Assuming a normal free fraction of 0.1 for PHT, one can predict F-PHT level from the adjusted PHT as follows:-

$$F\text{-}PHT = PHT_{adjusted} * 0.1$$

(In renal disease the Sheiner and Tozer method assumed the relative affinity of serum albumin for PHT to be decreased by about 50%, i.e., K'/K = 0.5).

The Winter-Tozer equation is thus modified

$$PHT_{adj} = PHT_{obs} / (0.1 * Albumin) + 0.1)$$

3. Haidukewych's Method

This method (Haidukewych et al, 1989) was developed for predicting F-PHT in plasma in patients receiving PHT and VPA, and is based on the observation that the PHT free fraction (α) varies as a function of VPA concentration. Forty-three healthy adult subjects were selected for this study. F-PHT, PHT and VPA in serum were measured by the FPIA method (see Table 1.6 for details). A correlation (r = 0.82) could be established between serum PHT free fraction (α) and VPA concentration with the following regression equation;

$$PHT \text{ free fraction } (\alpha) = (0.095 + 0.001) * (VPA)$$

By definition α = F-PHT / PHT

Substituting for α in the regression equation will give the following equation that can be used to predict the F-PHT level in serum.

$$F\text{-}PHT = (0.095 + 0.001\ [VPA])\ [PHT]$$

where [VPA] and [PHT] represent total levels of drugs in plasma. The method is only applicable to patients with normal albumin concentration.

4. Dasgupta's Method

Dasgupta's method (Dasgupta et al, 1991) is the most recent method for prediction of F-PHT levels to be published and is based on the calculation of [PHT]/[ALBUMIN] ratios. Fifty-six healthy adult subjects were selected for this study. F-PHT and PHT levels in serum were measured by the FPIA method (see Table 1.6 for details). A good correlation (r = 0.98) could be established between F-PHT levels and [PHT]/[ALBUMIN] ratios in patients on AED monotherapy, whose albumin levels were greater than 480 μmol/L (33 g/L). The equation for the regression line was calculated as;

$$F\text{-}PHT = (83.1 * [PHT]/[ALBUMIN]) - 0.6)$$

The authors claim that their method is useful in predicting F-PHT levels for patients in the absence of hypoalbuminaemia but that it cannot be used for patients on polytherapy. Consequently they recommend direct measurements of F-PHT level in the situation of polytherapy, e.g., patients receiving PHT with VPA or PHT with an antibiotic.

1.17 Previous Evaluation of the Predictive Methods

Beck and his co-workers have recently evaluated the accuracy and precision of the Gugler and Sheiner-Tozer predictive methods (Beck et al, 1987). They found that both of these methods lacked precision for predicting F-PHT levels, and exhibited temperature-dependent bias. These observations have recently been confirmed by Mauro and his co-workers (Mauro et al, 1989). That temperature will introduce bias in the prediction of F-PHT level under these circumstances is comprehensible, as the prediction methods assume the physiological temperature of 37 °C and ultrafiltration for the determination of F-PHT has usually been carried out at a temperature of 25 °C. The Haidukewych method also does not take account of the effect of temperature in the separation of F-PHT and thus the predicted F-PHT values are biased because the experimental data supporting the predictive equation have been established at 25 °C. May and his co-workers have recently confirmed that the Haidukewych method is reproducible at 25 °C and constructed a nomogram for predicting F-PHT in patients co-medicated with VPA (May et al, 1991).

This would indicate that the Haidukewych method can be used for comparative purposes using a reference range for predicting F-PHT at 25 °C. However it is important to realise that the predicted values will not necessarily reflect F-PHT level at 37 °C. There has been no previous attempt to evaluate the Dasgupta method.

Temperature can be assumed to effect all equilibrium reactions, including the binding of AED's to albumin. Experimental evidence for a temperature effect in the binding of PHT to albumin was provided as early as 1970 by Lunde and his co-workers, and these data have recently been confirmed by Allison and Comstock (Lunde et al, 1970; Allison and Comstock, 1988). No systematic study has been carried out on the effect of temperature on free AED levels in serum in vitro. Such information would be of fundamental importance for evaluating the accuracy of any method attempting to predict free AED levels in vivo.

In this thesis an alternative predictive method for free levels of AED's based on the Law of Mass Action and taking into account a number of factors, including the effect of temperature, the nature of drug-binding to albumin and medication with more than one drug, is described, as is outlined under "Aims of Thesis".

1.18 Aims of Thesis

1 To develop a high performance liquid chromatography (HPLC) micromethod for the measurement of total and free levels of PHT, CBZ and PB in serum and to compare the HPLC method with the routinely used fluorescence polarisation immunoassay (FPIA) method.

2 To evaluate the stability of free levels of PHT, CBZ and PB during sample storage.

3 To study the effects of temperature on the estimation of F-PHT, F-CBZ and F-PB, and to use the Law of Mass Action to determine temperature-dependent apparent dissociation constants (K_D) for each drug.

4 To establish target ranges (therapeutic ranges) for F-PHT, F-CBZ and F-PB at 25 °C and 37 °C.

5 To evaluate the precision of the model equation derived from the Law of Mass Action in predicting F-PHT and F-CBZ at 25 °C and 37 °C in monotherapy.

6 To determine the effect of drug displacement on apparent dissociation constant (K_D), free fraction (α) and target ranges for PHT, CBZ and PB at 25 °C and 37 °C.

7 To evaluate the precision of the model equations in predicting F-PHT or F-CBZ at 37 °C in patients co-medicated with VPA.

8 To compare the model equation derived from the Law of Mass Action for predicting F-PHT with other existing methods.

CHAPTER 2

THE DEVELOPMENT OF A MICROMETHOD FOR THE ESTIMATION OF TOTAL AND FREE LEVELS OF AED'S IN SERUM

2.1 Aims

To develop a high performance liquid chromatography (HPLC) micromethod for the measurement of total and free levels of PHT, CBZ and PB, and compare the HPLC method and the routinely used fluorescence polarisation immunoassay (FPIA) method for the measurement of total and free levels of PHT, CBZ and PB. To evaluate the stability of free levels of PHT, CBZ and PB during sample storage.

2.2 Introduction

When work for this thesis was started in 1982 no methodology for free levels of AED's with documented data for inaccuracy and imprecision was found in the literature. It was, however, considered important that experimental data used in patient care should be of a high quality and at the same time practicable in the routine situation. The same considerations apply with equal force to data used to establish predictive equa-

tions for free levels of AED's in serum from estimations of total levels and albumin.

Thus it was decided to develop micromethods for obtaining ultrafiltrate from serum and for determination of PHT, CBZ and PB in serum and ultrafiltrates by HPLC. It was realised that such methods could be of value both in TDM and for future pharmacokinetic study of such drugs.

Since an immunological method of drug analysis had been used for the predictive method proposed before 1980 (Gugler et al, 1975), an FPIA method was chosen for comparative purposes.

A limited study of the stability of AED's in serum during storage of sample was also undertaken in order to determine a suitable storage procedure for the samples used in the main study in this thesis.

2.3 Materials and Methods

All the nomenclature used in this chapter is in accordance with the recommendations published by the International Union of Pure and Applied Chemistry (Guilbault and Hjelm, 1989).

2.3.1 Apparatus

Spectra-Physics SP8789 Liquid chromatographic system with an SP 8780 XR autosampler - **(Spectra-Physics, Maidenhead, UK)**

Spectroflow 873 uv detector - **(Spectra-Physics, Maidenhead, UK)**

SP 4270 integrator/printer plotter - **(Spectra-Physics, Maidenhead, UK)**

LABNET data system - **(Spectra-Physics, Maidenhead, UK)**

Vectra ES/12 microcomputer - **(Hewlett-Packard, Bracknell, UK)**

HP 7440 A graphics plotter - **(Hewlett-Packard, Bracknell, UK)**

STATPAK computer program - **(Hewlett-Packard, Bracknell, UK)**

SIGMA PLOT scientific graphic computer program - **(Jandel Corporation, USA)**

Sorvall RC-5B refrigerated centrifuge - **(Dupont, Stevenage, UK)**

SM24 rotor - **(Dupont, Stevenage, UK)**

Vibrax-IKA Shaker - **(Sartorius-IKA, Epsom, UK)**

TDX analyser - **(Abbott, Maidenhead, UK)**

Abbott microcentrifuge - **(Abbott, Maidenhead, UK)**

Finnpipette liquid dispensers - **(Labsystems, Basingstoke, UK)**

2.3.2 Chemicals and Consumables

Acetonitrile, HPLC-grade - **(FSA, Loughborough, UK)**

Water, HPLC-grade - **(FSA, Loughborough, UK)**

Methanol, "Hypersolv" grade - **(BDH, Poole, UK)**

Dihydrogen potassium phosphate (anhydrous), "Analar" grade -**(BDH, Poole, UK)**

Dipotassium hydrogen phosphate (anhydrous), "Analar" grade - **(BDH, Poole, UK)**

5-p-Tolyl-5-phenylhydantoin (MPPH) - **(Aldrich, Gillingham, Dorset, UK)**

Ciba-Corning Anticonv/Asth I,II quality control sera (CCI and CCII) - **(Corning Medical, Halstead, UK)**

Drug-free serum - **(Blood bank, National Hospital, London, UK)**

Physiological saline - **(Pharmacy, National Hospital, London, UK)**

Amicon centrifree micropartion units - **(Amicon, Stonehouse, UK)**

Tips for finnpipettes - **(Labsystems, Basingstoke, UK)**

Cartridges and cuvettes for TDX analyser - **(Abbott, Maidenhead, UK)**

Total level phenytoin, carbamazepine and phenobarbital II kits and free level phenytoin and carbamazepine kits for TDX analyser - **(Abbott, Maidenhead, UK)**

Calibrators for TDX analyser - **(Abbott, Maidenhead, UK)**

Polypropylene centrifuge tubes with cap (1.5 ml size) - **(Sarstedt, Leicester, UK)**

Glass vials (1 ml size) - **(Chrompak, London, UK)**

Glass inserts (0.1 ml size) - **(Chrompak, London, UK)**

Merck HI-Bar HPLC column, size 250 x 4mm, packed with Lichrosorb RP select B, 5μm - **(BDH, Poole, UK)**

2.3.3 Calibration Materials for Drug Analysis

All the materials used in this study were of pure analytical grade.

Phenytoin (PHT) - **(Warner-Lambert, Eastleigh, UK)**
Carbamazepine (CBZ) and Carbamazepine -10, 11-epoxide (CBZ-E) - **(Ciba Geigy Pharmaceuticals, Horsham, UK)**
Phenobarbitone (PB) - **(BDH, Poole, UK)**

2.3.4 Calibration Solutions

Stock solutions of PHT, CBZ, CBZ-E and PB and the internal standard MPPH were made up individually to a concentration of 10 mmol/L in methanol. These solutions were stable for three months when stored at 4 °C.

Working solutions of PHT, CBZ, CBZ-E and PB were prepared every week by diluting the stock solutions 1 in 10 with methanol, to give a final concentration of 1 mmol/L. A working solution of the internal standard (MPPH) was prepared by diluting the stock solution 1 in 10 with acetonitrile to give a final concentration of 1 mmol/L.

2.3.5 Preparation of HPLC Buffer Solution

A solution of 50 mmol/L phosphate buffer was prepared by dissolving 6.8 g of anhydrous potassium dihydrogen phosphate and 7.1 g of anhydrous disodium hydrogen

phosphate in 1 L of HPLC-grade water, and then mixing 950 ml of the 50 mmol/L potassium dihydrogen phosphate solution with 50 ml of the 50 mmol/L disodium hydrogen phosphate solution to give a pH of 5.6.

2.3.6 Quality Control Sera

Quality control sera were prepared from pooled patients sera. Serum samples known by previous analysis to contain PHT, CBZ and PB were pooled and filtered with thorough mixing, aliquoted into bottles and stored at -20 °C until required for analysis. In addition to these quality control sera, Ciba-Corning Anticonv/Asth I (CCI) and II (CCII) were used. They contained known amounts of commonly prescribed AED's, including PHT, CBZ and PB as analysed by Ciba-Corning.

2.3.7 Patients Samples

Non-heparinised blood samples for routine measurement of AED's were collected from patients, and centrifuged within two hours. The sera thus obtained were stored at -20 °C until required for analysis, and then allowed to equilibrate at room temperature for 60 minutes before the analysis was carried out. In addition, a number of serum samples chosen at random from patients on AED monotherapy were filtered and analysed for free levels of PHT, CBZ and PB immediately after collection and again after storage at -20 °C for 84 days to evaluate the stability of free drug levels in serum during storage.

2.4 Analytical Procedures

2.4.1 Fluorescence Polarisation Immunoassay

Determinations of total (free+bound) levels of PHT, CBZ or PB in patients sera were carried out on the TDX analyser using phenytoin, carbamazepine and phenobarbital II assay kits. Determinations of free levels of PHT (F-PHT) and CBZ (F-CBZ) were carried out using free phenytoin and free carbamazepine assay kits. The free levels of PB (F-PB) in serum are usually high since the drug is only 50% bound to protein (Perucca, 1984), therefore levels of F-PB were determined using phenobarbital II assay kits.

An aliquot (50μl) of serum or filtrates to be analysed was placed in each of the sample wells of a TDX sample cartridge, held in a 20-place carousel which was locked into position in the TDX analyser. All the stages of the assay, including dilution of the samples with TDX buffer, addition of reagents, and transfer of the mixtures into individual cuvettes for analysis at a temperature of 35 °C were completely automated. The light source was a tungsten halogen lamp, excitation peak 485 nm, emission band 525-550 nm. The analysis of a full carousel took 15 minutes.

Calibration curves were prepared using the appropriate calibration kits for total or free levels supplied for use with the TDX analyser, and stored in memory. The

curves were stable for at least one month (Ratnaraj et al,1986).

2.4.2 High Performance Liquid Chromatography (HPLC)

2.4.3 Preparation of Calibration Solutions

Calibration solutions for the estimation of total PHT, CBZ, CBZ-E and PB were prepared by diluting the appropriate working solutions with drug-free serum to give a range of concentrations as follows:-

PHT; 8, 16, 24, 40 and 80 μmol/L.
CBZ; 8, 17, 25, 42 and 85 μmol/L
CBZ-E; 4, 8, 12, 20 and 40 μmol/L
PB; 17, 34, 52, 86, and 172 μmol/L

The working solution of MPPH was diluted 1 in 200 with acetonitrile to give a final concentration of 50 μmol/L.

Calibration solutions for the estimation of free levels of F-PHT, F-CBZ, free CBZ-E (F-CBZ-E) and F-PB were prepared by diluting the appropriate working solutions with physiological saline to give a range of concentrations as follows:-

PHT; 4, 8, 12, 20, and 40 μmol/L
CBZ; 4, 9, 13, 21, and 42 μmol/L
CBZ-E; 2, 4, 6, 10, and 20 μmol/L
PB; 4, 9, 13, 22, and 43 μmol/L.

The working solution of MPPH was diluted 1 in 1000 with acetonitrile to give a final concentration of 10 μmol/L.

2.4.4 Experimental Procedure for the Estimation of Total Levels of AED's by HPLC

Concentrations of PHT, CBZ and PB in patients' sera were determined as follows:- An aliquot (30 μl) of serum was pipetted into a 1.5 ml polypropylene centrifuge tube with cap and 75 μl of internal standard solution (50 μmol/L of MPPH in acetonitrile) added. The tube was shaken for 60 seconds on a Vibrax-IKA shaker, centrifuged for three minutes at 9500 g in an Abbott centrifuge, and 90 μl of the solution pipetted into a 0.1 ml glass insert held in a 1 mL glass vial and 20 μl injected into the chromatograph by the automatic injection procedure.

Chromatograms were run at ambient temperature on a Merck HI-BAR column (250 x 4.0mm) packed with Lichrosorb RP select B, 5μm. A mobile phase of acetonitrile : 50 mmol/L phosphate buffer, pH 5.6 (32:68) with a flow rate of 1.4 mL/min at 175 bar was used. The column effluent was monitored at 215 nm with a sensitivity range of 0.04 absorption units full scale and a chart speed of 0.5 cm/min.

2.4.5 Experimental Procedure for the Estimation of Free Levels of AED's by HPLC

2.4.6 Filtration of Samples

Serum (250 µl) was pipetted into the sample reservoir of an Amicon Centrifree micropartition system, and centrifuged for 20 minutes at 1000 g at a temperature setting of 25 °C in a Sorvall RC-5B superspeed centrifuge fitted with an SM24 rotor. Temperature control to within ± 1 °C was achieved. (At a later stage in this study, samples were also filtered at 15 °C and 37 °C; see Chapter 4).

2.5 Chromatographic Procedure

A 30 µl volume of the filtrate in the micropartition system filtrate cup was pipetted into a 1.5 ml polypropylene centrifuge tube with cap and 75 µl of the diluted internal standard solution (10 µmol/L in acetonitrile) added. The tube was shaken for 60 seconds on a Vibrax-IKA shaker, and 90 µl of the solution was pipetted in to a 0.1 ml glass insert held in a 1 ml glass vial and 20 µl injected into the chromatograph by the automatic injection procedure. The chromatographic conditions were as described above, but the range of the detector was adjusted to 0.02 absorption units full scale to increase sensitivity.

2.5.1 Calibration Curves for Total and Free Levels of AED's

An aliquot (30 μL) of the calibration solutions of PHT, CBZ, CBZ-E and PB, diluted with drug-free serum or physiological saline as required, were analysed as above in order to establish the calibration curves. The calibration curves were plotted by the ratio of the peak areas of each drug to the peak area of internal standard against concentrations of the drug. The AED concentrations were determined from the calibration curves.

2.5.2 Recovery for Total and Free Levels of AED's

Experiments to determine the recovery of total levels of PHT, CBZ and PB were carried out by analysing drug standards made up in drug-free serum, using the HPLC procedure described above.

Similarly, experiments to determine the recovery of free levels of PHT, CBZ, PB or CBZ-E were carried out by analysing drug standards made up in physiological saline, using ultrafiltration and the HPLC procedure. This experimental procedure determined whether there was binding of PHT, CBZ, CBZ-E or PB onto the filtration membranes.

2.5.3 Accuracy of the Experimental Procedures

The accuracy of the FPIA and HPLC procedures for the analysis of total levels of PHT, CBZ or PB was determined by analysing the Ciba-Corning quality control sera (CCI and CCII).

No commercial quality control materials were available for determining the accuracy of the procedure for the analysis of free levels of PHT, CBZ or PB.

2.5.4 Imprecision of the Experimental Procedures

Both the within-batch and between-batch imprecision of the FPIA and HPLC assays for total levels of AED's were determined from analyses made on pooled patients sera, prepared as described in Section 2.3.6. Within-batch imprecision was determined from 36 analyses of this material carried out on one day, and between-batch imprecision from three analyses on 12 separate occasions.

The imprecision of the FPIA and HPLC assays for free levels of AED's was also determined from analyses made on the pooled patients sera. Within-batch imprecision was determined from 24 analyses of this material carried out on one day, and between-batch imprecision from three analyses on 11 separate occasions.

2.5.5 Correlation between the FPIA and HPLC Methods

Correlation between the FPIA and HPLC methods for the analysis of total and free levels of PHT, CBZ and PB was examined by analysing randomly chosen patients' samples for the three AED's by the above mentioned methods. Regression analyses for PHT, CBZ and PB for FPIA and HPLC were carried out using the STATPAK and SIGMA PLOT computer programs.

2.5.6 Stability of Free Levels during Sample Storage

A number of serum samples chosen at random from patients on PHT, CBZ or PB monotherapy were divided into two sets immediately after centrifugation. One part was filtered and analysed by HPLC as described above. The other part was kept frozen at -20°C for 84 days, then allowed to assume room temperature, filtered and analysed by HPLC.

2.6 Results

2.6.1 Chromatograms of AED's

Figures 2.1A and 2.1B show chromatograms of a standard extract from spiked serum and of a patient's serum respectively. A chromatogram of drugs in low concentration extracted from spiked physiological saline is shown in Figure 2.2A and a chromatogram of a patient's sample after ultrafiltration by the standard procedure

is shown in Figure 2.2B. It can be seen that the the peaks are well separated using the chromatographic conditions described. Several extraneous peaks are seen close to the solvent front, but there is no interference with the drug peaks either from materials normally present in patients sera or from other drugs commonly prescribed in neurological or paediatric hospitals. The limit of detection for drugs in patients samples is 0.1 μmol/L with the detector range setting maintained at 0.04 or 0.02 absorption units full scale for analysis of total or free levels of AED's respectively. The complete chromatographic run takes 15 minutes.

2.6.2 Calibration Curves for Total and Free Levels of AED's

Figure 2.3A shows calibration curves for PHT, CBZ, CBZ-E and PB obtained by the analysis of the drugs added to drug free sera. The curves are linear over a wide range of concentrations.

Figure 2.3B shows calibration curves for F-PHT, F-CBZ, F-CBZ-E and F-PB obtained by the HPLC analysis of the drugs added to saline. The curves are linear over a wide range of concentrations.

2.6.3 Recovery for Total and Free Levels of AED's

Recoveries of drugs added to drug-free sera and analysed by the HPLC procedure are shown in Table 2.1,

and vary from 98.77% to 101.26% for CBZ, PB, PHT and CBZ-E.

Recovery of non-protein-bound AED's was determined by analysing drug standards made up in physiological saline, using the standard procedure for filtration and HPLC analysis. The results presented in Table 2.2 show recoveries approaching 100% for F-PB, F-PHT, F-CBZ and F-CBZ-E, indicating that there is little or no binding of the non-protein-bound drugs onto the membranes of the micropartition systems.

2.6.4 Accuracy of the FPIA and HPLC Methods

Tables 2.3 to 2.6 respectively show the results obtained by analysing the quality control sera CCI and CCII by FPIA and HPLC to determine the within-batch and between-batch accuracy of the assays. The mean results for PB, PHT, and CBZ fall within the quoted ranges for CCI and CCII for both the FPIA and HPLC methods. The standard deviation (SD) is less than 2.3 μmol/L and the coefficient of variation (CV) 4% or less for the CCI samples, while the corresponding figures for the CCII quality control serum are less than 3.7 μmol/L and 5% or less (Tables 2.3 to 2.6).

2.6.5 Imprecision of the FPIA and HPLC Methods for Total Levels

Results obtained by analysing samples of pooled patients' sera to determine the within-batch and

between-batch imprecision of the FPIA and HPLC methods for total levels of AED's are shown in Tables 2.7 and 2.8. The mean results demonstrate that there is close agreement between both methods, with SD less than 2.0 μmol/L and CV 9% or less in all instances.

2.6.6 Imprecision of the FPIA and HPLC Methods for Free Levels

Results of analyses of pooled patients' sera to determine the within-batch and between-batch imprecision of the FPIA and HPLC methods for free levels of AED's are shown in Tables 2.9 and 2.10. The mean results demonstrate that there is close agreement between the FPIA and HPLC methods, with SD less than 1.4 μmol/L and CV 13% or less in all instances, which is acceptable for the analysis of AED's in low concentration (Wilson et al, 1989).

2.6.7 Correlation between the FPIA and HPLC Methods

Figures 2.4A to 2.4C show a comparison between the FPIA method and the HPLC method for PHT, CBZ and PB respectively. A good correlation between the two methods for PHT (r=0.997), CBZ (r=0.988) and PB (r=0.998) over a wide range of concentrations is observed.

The comparison between the HPLC and FPIA procedures for the estimation of levels of F-PHT, F-CBZ and F-PB is presented in Figures 2.5A to 2.5C respectively. Again

a good correlation between the two methods for F-PHT (r=0.980), F-CBZ (r=0.975) and F-PB (r=0.999) over a wide range of concentrations is observed.

2.6.8 Stability of Free Levels during Sample Storage

Figures 2.6A to 2.6C show the effects of storage of serum samples for 84 days on levels of F-PHT, F-CBZ and F-PB. The correlation between the two sets of analytical results is extremely good for each drug, with correlation coefficients (r) ranging from 0.989 to 0.999. These results indicate that serum samples can be stored frozen for a considerable length of time without prejudicing the accuracy of the assays for F-PHT, F-CBZ and F-PB.

Figure 2.1A Chromatogram of drugs added to drug-free serum.

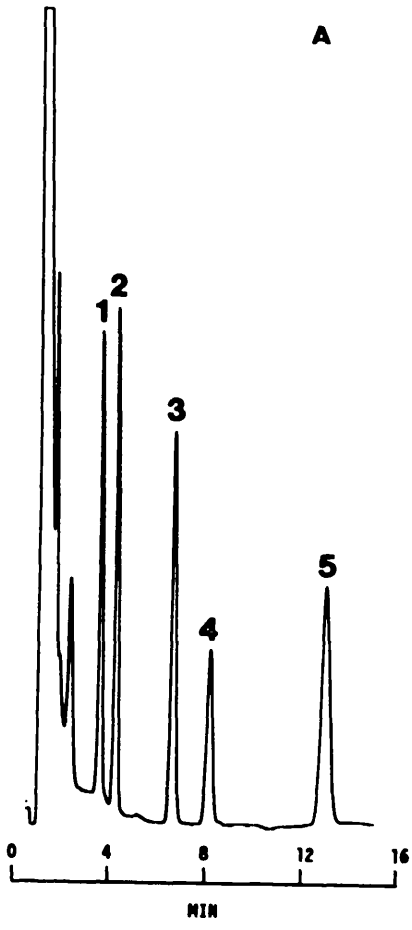
Peaks:

1. CBZ-E
2. PB
3. CBZ
4. PHT
5. MPPH (internal standard).

Figure 2.1B Chromatogram of drugs present in a patient's serum.

Peaks:

1. CBZ-E
2. PB
3. CBZ
4. PHT
5. MPPH (internal standard).


A
1
2
3
4
5
0
4
8
12
16
MIN

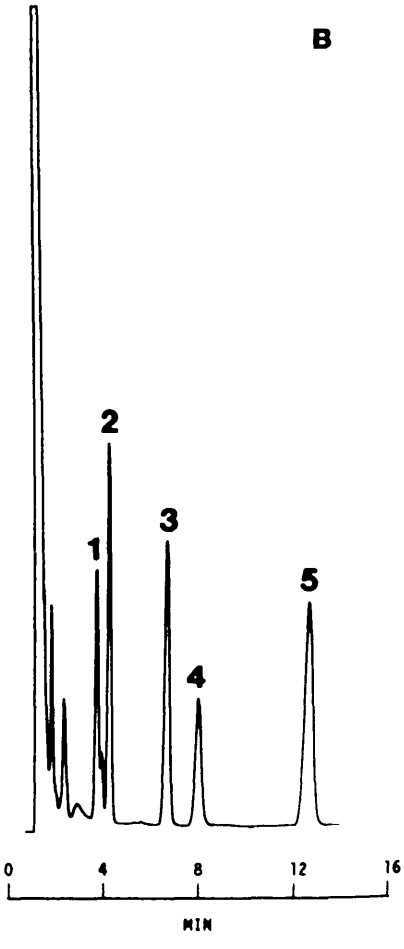

B
1
2
3
4
5
0
4
8
12
16
MIN

Figure 2.2A Chromatogram of drugs added to physiological saline.

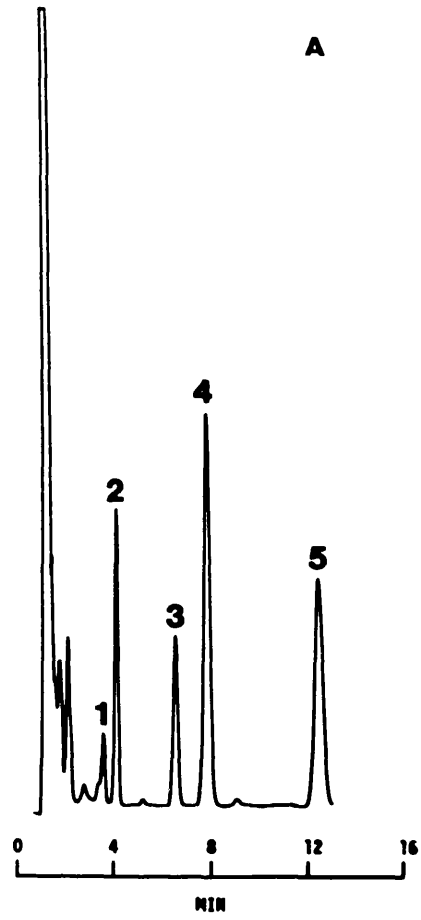
Peaks:

1. CBZ-E
2. PB
3. CBZ
4. PHT
5. MPPH (internal standard).

Figure 2.2B Chromatogram of drugs present in an ultrafiltrate from a patient's serum.

Peaks:

1. F-CBZ-E
2. F-PB
3. F-CBZ
4. F-PHT
5. MPPH (internal standard).


A
1
2
3
4
5
0
4
8
12
16
MIN

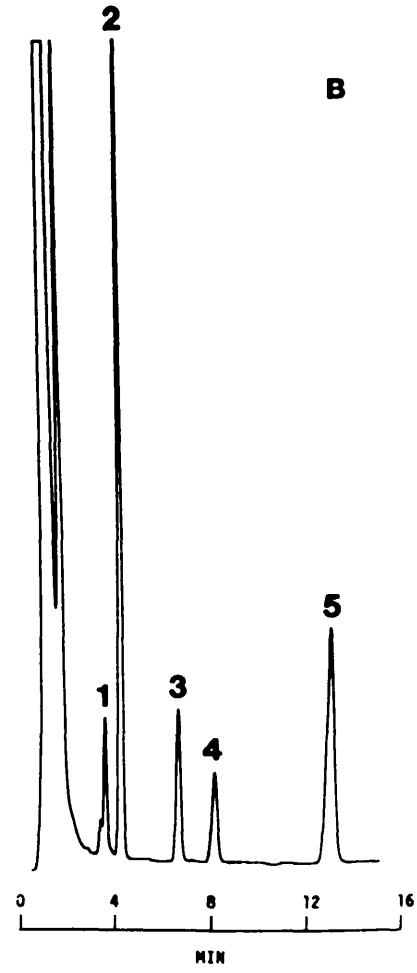
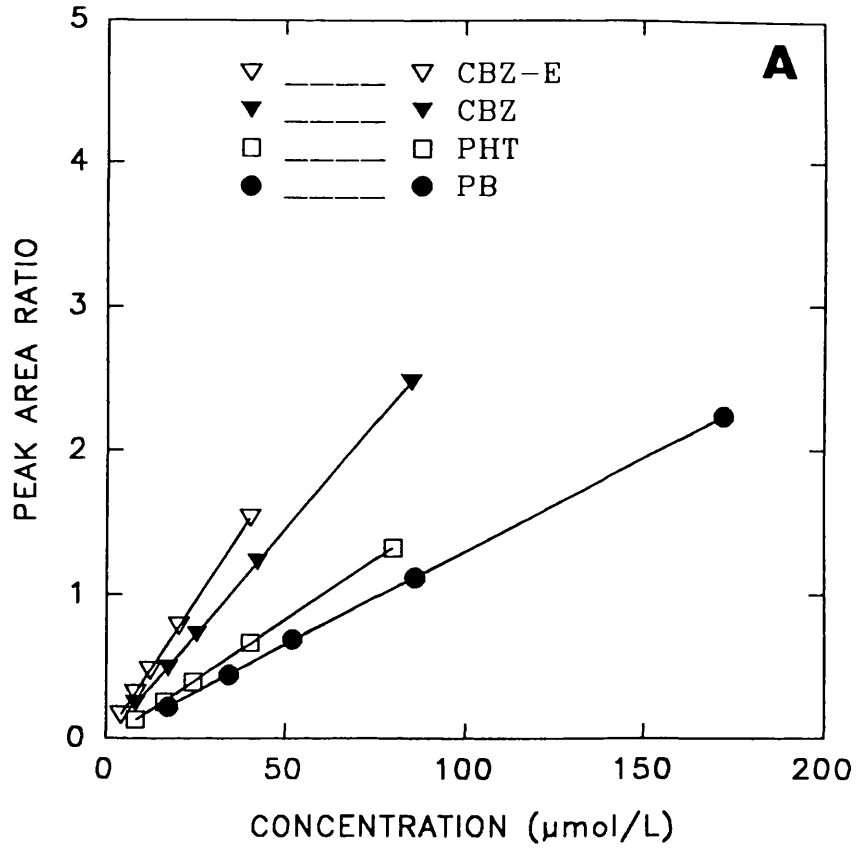

B
1
2
3
4
5
0
4
8
12
16
MIN

Figure 2.3A Calibration curves for total levels of AED's.

Figure 2.3B Calibration curves for free levels of AED's.


A
CBZ-E
CBZ
PHT
PB
PEAK AREA RATIO
CONCENTRATION (µmol/L)
0
1
2
3
4
5
0
50
100
150
200

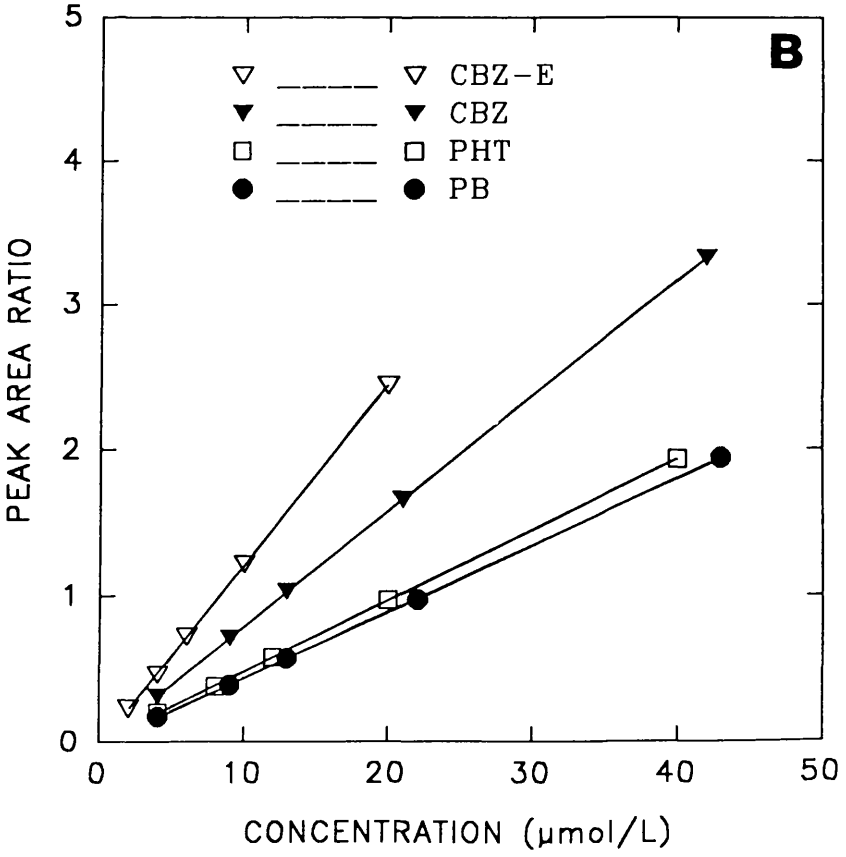

B
CBZ-E
CBZ
PHT
PB
PEAK AREA RATIO
CONCENTRATION (µmol/L)
0
1
2
3
4
5
0
10
20
30
40
50

TABLE 2.1 RECOVERY OF AED'S ADDED TO DRUG FREE SERUM

Drug	Calculated concentration (μmol/L)	Measured concentration (Mean ± SD; n=30) (μmol/L)	CV %	% Recovery
PB	86.00	85.24 ± 1.24	2	99.12
PHT	39.64	40.14 ± 0.76	2	101.26
CBZ	25.20	24.89 ± 1.10	2	98.77
CBZ-E	8.00	7.92 ± 0.89	6	99.00

TABLE 2.2 RECOVERY OF AED'S ADDED TO PHYSIOLOGICAL SALINE

Drug	Calculated concentration (μmol/L)	Measured concentration (Mean ± SD; n=30) (μmol/L)	CV %	% Recovery
PB	21.53	21.43 ± 0.61	3	99.5
PHT	3.96	3.90 ± 0.34	9	98.5
CBZ	4.23	4.18 ± 0.37	9	98.8
CBZ-E	3.96	4.01 ± 0.14	3	101.3

TABLE 2.3 WITHIN-BATCH IMPRECISION: ANALYSIS OF CCI BY FPIA AND HPLC

Drug	Method	Assigned concentration ± SD (μmol/L)	Observed concentration (Mean ± SD; n=36) (μmol/L)	CV %
PB	FPIA	67 ± 13	66.29 ± 2.22	3
	HPLC	66 ± 17	66.14 ± 1.26	2
PHT	FPIA	25 ± 5	28.00 ± 1.08	4
	HPLC	25 ± 5	28.29 ± 0.71	3
CBZ	FPIA	18 ± 3	19.51 ± 0.51	3
	HPLC	18 ± 3	17.17 ± 0.38	2

TABLE 2.4 BETWEEN-BATCH IMPRECISION: ANALYSIS OF CCI BY FPIA AND HPLC

Drug	Method	Assigned concentration ± SD (μmol/L)	Observed concentration (Mean ± SD; n=36) (μmol/L)	CV %
PB	FPIA	67 ± 13	66.08 ± 2.17	3
	HPLC	66 ± 17	66.92 ± 1.96	3
PHT	FPIA	25 ± 5	28.25 ± 1.11	4
	HPLC	25 ± 5	28.50 ± 0.88	3
CBZ	FPIA	18 ± 3	19.19 ± 0.71	4
	HPLC	18 ± 3	17.28 ± 0.45	3

TABLE 2.5 WITHIN-BATCH IMPRECISION: ANALYSIS OF CCII BY FPIA AND HPLC

Drug	Method	Assigned concentration ± SD (μmol/L)	Observed concentration (Mean ± SD; n=36) (μmol/L)	CV %
PB	FPIA	169 ± 31	170.42 ± 3.25	2
	HPLC	156 ± 32	174.00 ± 1.71	1
PHT	FPIA	67 ± 13	78.86 ± 3.36	4
	HPLC	67 ± 13	79.86 ± 0.85	1
CBZ	FPIA	35 ± 6	34.69 ± 0.76	2
	HPLC	36 ± 6	33.31 ± 0.41	1

TABLE 2.6 BETWEEN-BATCH IMPRECISION: ANALYSIS OF CCII BY FPIA AND HPLC

Drug	Method	Assigned concentration ± SD (μmol/L)	Observed concentration (Mean ± SD; n=36) (μmol/L)	CV %
PB	FPIA	169 ± 31	170.17 ± 2.74	2
	HPLC	156 ± 32	173.83 ± 1.89	1
PHT	FPIA	67 ± 13	80.33 ± 3.64	5
	HPLC	67 ± 13	80.42 ± 2.13	3
CBZ	FPIA	35 ± 6	34.31 ± 1.45	4
	HPLC	36 ± 6	33.17 ± 0.56	2

TABLE 2.7 WITHIN-BATCH IMPRECISION: ANALYSIS OF POOLED SERUM CONTAINING AED'S BY FPIA AND HPLC

Drug	Method	Measured concentration (Mean ± SD; n=36) (μmol/L)	CV %
PB	FPIA	54.86 ± 0.85	2
	HPLC	53.85 ± 1.00	2
PHT	FPIA	34.43 ± 1.70	5
	HPLC	37.14 ± 0.65	2
CBZ	FPIA	41.28 ± 0.89	2
	HPLC	40.00 ± 1.08	3
CBZ-E	FPIA	- -	-
	HPLC	7.54 ± 0.55	7

TABLE 2.8 BETWEEN-BATCH IMPRECISION: ANALYSIS OF POOLED SERUM CONTAINING AED'S BY FPIA AND HPLC

Drug	Method	Measured concentration (Mean ± SD; n=36) (μmol/L)	CV %
PB	FPIA	55.19 ± 0.98	2
	HPLC	53.92 ± 1.57	3
PHT	FPIA	35.50 ± 1.96	6
	HPLC	38.16 ± 1.84	5
CBZ	FPIA	41.25 ± 0.94	2
	HPLC	40.58 ± 1.63	4
CBZ-E	FPIA	- -	-
	HPLC	8.90 ± 1.48	9

TABLE 2.9 WITHIN-BATCH IMPRECISION: ANALYSIS OF POOLED SERUM CONTAINING FREE AED'S BY FPIA AND HPLC

Drug	Method	Measured concentration (Mean ± SD; n=24) (μmol/L)	CV %
PB	FPIA	45.90 ± 0.69	2
	HPLC	47.23 ± 1.32	3
PHT	FPIA	4.23 ± 0.31	3
	HPLC	4.47 ± 0.40	9
CBZ	FPIA	10.42 ± 0.39	4
	HPLC	11.14 ± 0.69	6
CBZ-E	FPIA	- -	-
	HPLC	3.68 ± 0.17	5

TABLE 2.10 BETWEEN-BATCH IMPRECISION: ANALYSIS OF POOLED SERUM CONTAINING FREE AED'S BY FPIA AND HPLC

Drug	Method	Measured concentration (Mean ± SD; n=33) (μmol/L)	CV %
PB	FPIA	47.13 ± 1.03	2
	HPLC	46.40 ± 0.75	2
PHT	FPIA	4.14 ± 0.31	7
	HPLC	4.13 ± 0.53	12
CBZ	FPIA	9.97 ± 0.48	5
	HPLC	10.02 ± 1.14	11
CBZ-E	FPIA	- -	-
	HPLC	3.39 ± 0.52	13

Figure 2.4A Correlation between HPLC and FPIA assays for PHT.

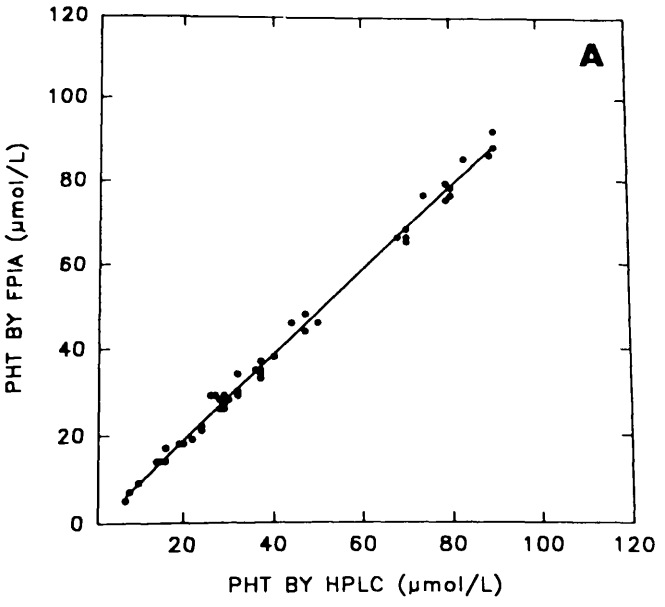
$y = -1.069 + 0.993x$; $r=0.997$; $p<0.001$; $n=52$.

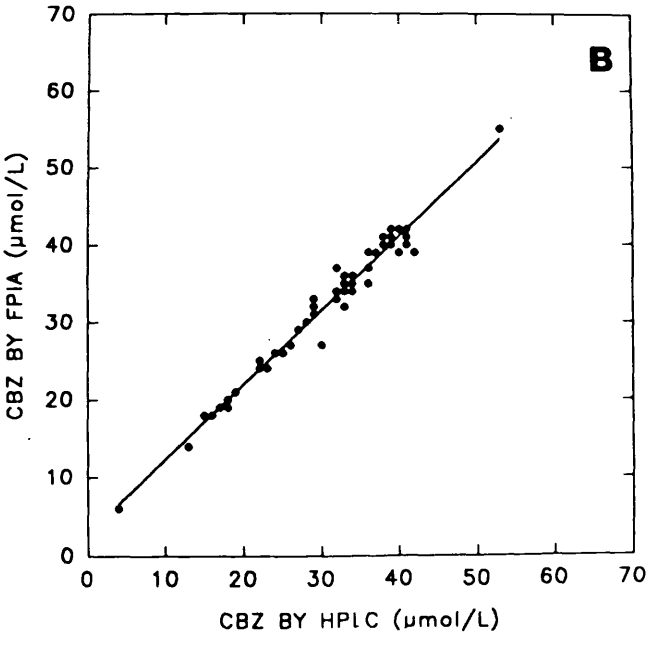
Figure 2.4B Correlation between HPLC and FPIA assays for CBZ.

$y = 2.733 + 0.960x$; $r=0.988$; $p<0.001$; $n=60$.

Figure 2.4C Correlation between HPLC and FPIA assays for PB.

$y = 2.909 + 0.965x$; $r=0.998$; $p<0.001$; $n=38$.


A
PHT BY FPIA (µmol/L)
PHT BY HPLC (µmol/L)
0
20
40
60
80
100
120


B
CBZ BY FPIA (µmol/L)
CBZ BY HPLC (µmol/L)
0
10
20
30
40
50
60
70

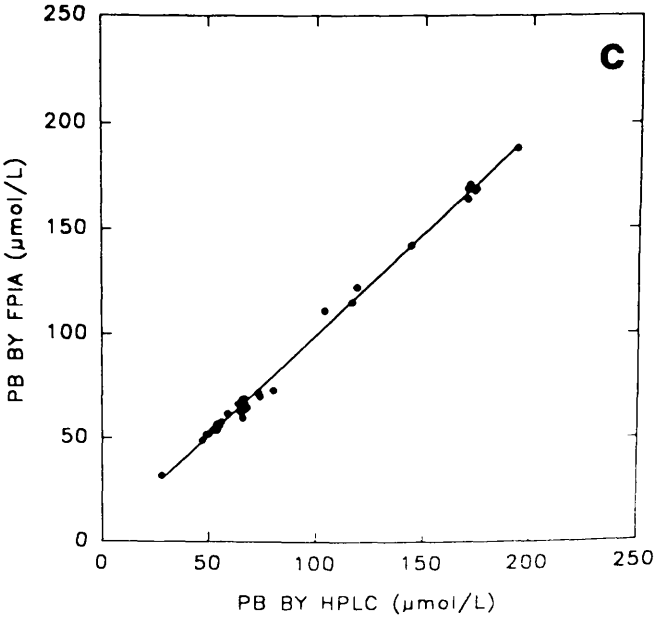

C
PB BY FPIA (µmol/L)
PB BY HPLC (µmol/L)
0
50
100
150
200
250

Figure 2.5A Correlation between HPLC and FPIA assays for free levels of PHT.

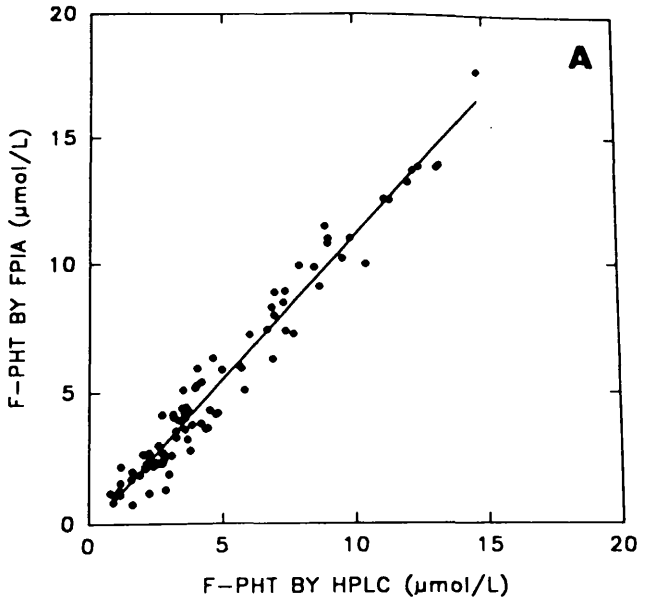
$y = -0.192 + 1.129x$; $r = 0.980$; $p < 0.001$; $n = 94$.

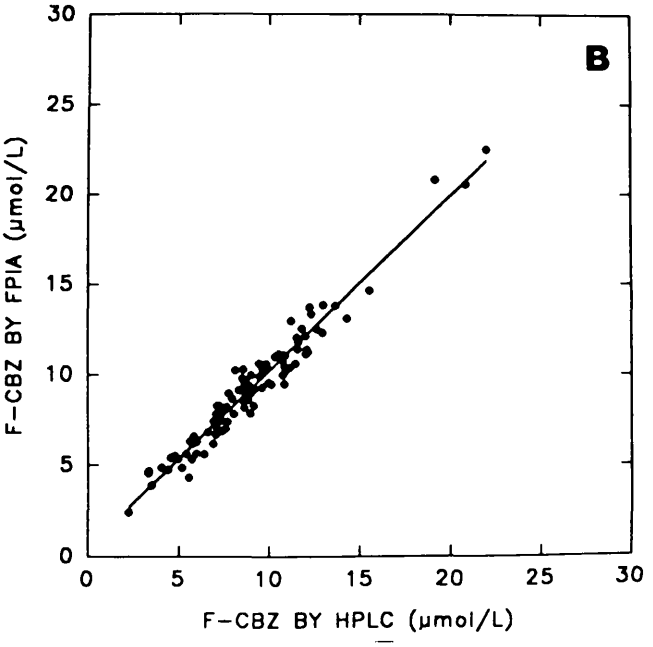
Figure 2.5B Correlation between HPLC and FPIA assays for free levels of CBZ.

$y = 0.478 + 0.974x$; $r = 0.975$; $p < 0.001$; $n = 97$.

Figure 2.5C Correlation between HPLC and FPIA assays for free levels of PB.

$y = -0.067 + 1.026x$; $r = 0.999$; $p < 0.001$; $n = 40$.


A
F-PHT BY FPIA (µmol/L)
F-PHT BY HPLC (µmol/L)
0
5
10
15
20


B
F-CBZ BY FPIA (µmol/L)
F-CBZ BY HPLC (µmol/L)
0
5
10
15
20
25
30

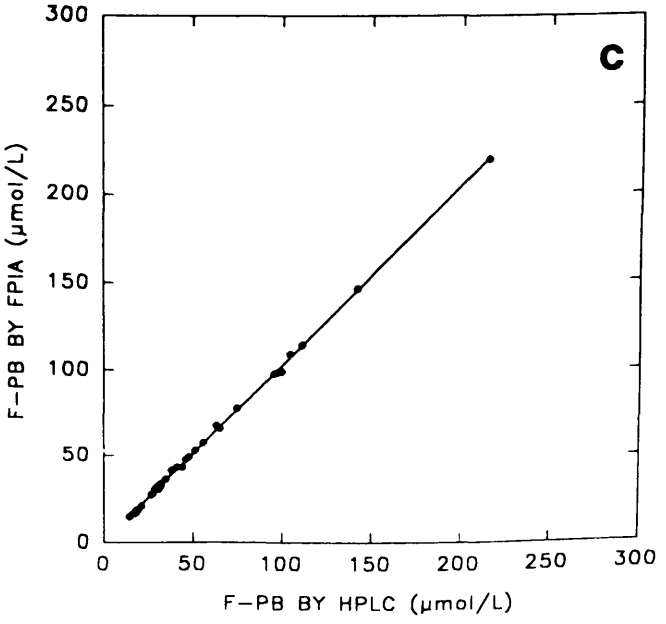

C
F-PB BY FPIA (µmol/L)
F-PB BY HPLC (µmol/L)
0
50
100
150
200
250
300

Figure 2.6A Correlation between analyses of patients sera for free levels of PHT immediately after collection and after storage at -20 °C for 84 days.

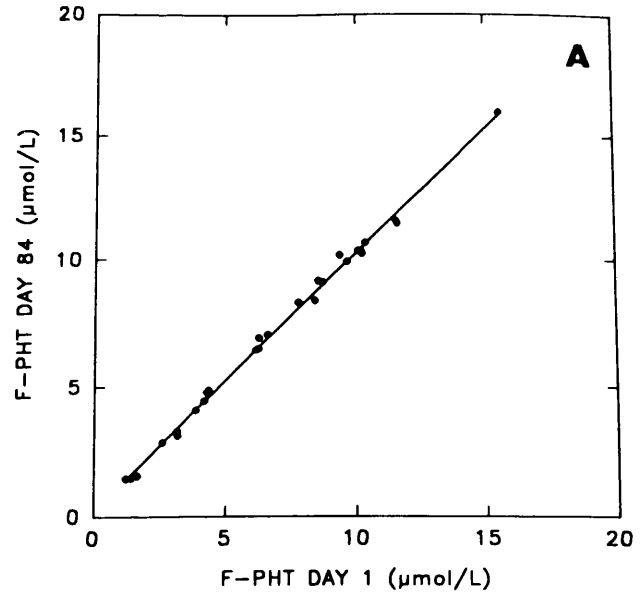
$y = 0.201 + 1.006x$; $r = 0.998$; $p < 0.001$; $n = 28$.

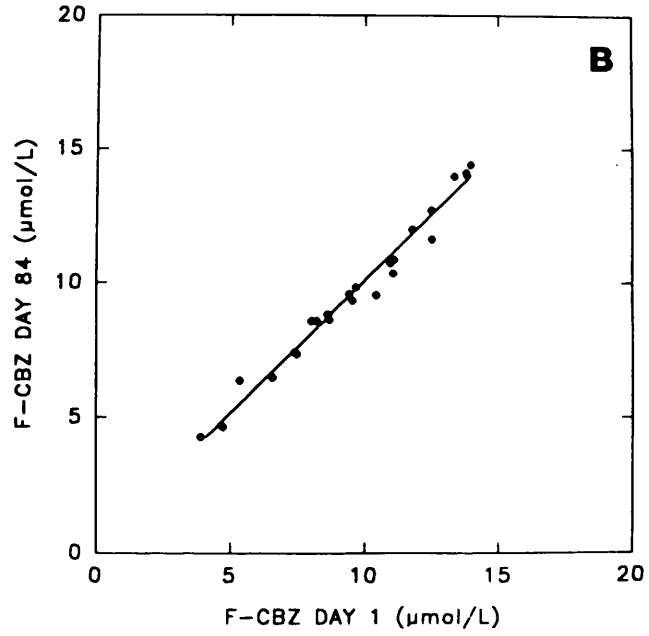
Figure 2.6B Correlation between analyses of patients sera for free levels of CBZ immediately after collection and after storage at -20 °C for 84 days.

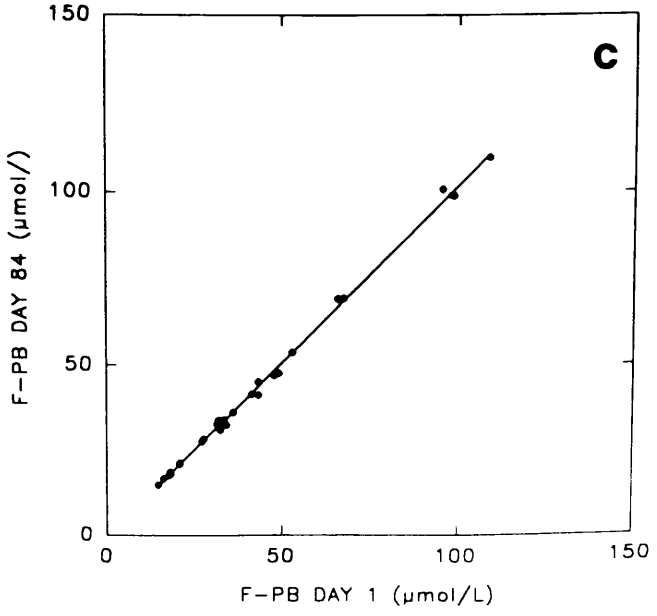
$y = 0.208 + 0.987x$; $r = 0.989$; $p < 0.001$; $n = 28$.

Figure 2.6C Correlation between analyses of patients sera for free levels of PB immediately after collection and after storage at -20 °C for 84 days.

$y = -0.213 + 1.018x$; $r = 0.999$; $p < 0.001$; $n = 28$.


A
F-PHT DAY 84 (µmol/L)
F-PHT DAY 1 (µmol/L)
0
5
10
15
20


B
F-CBZ DAY 84 (µmol/L)
F-CBZ DAY 1 (µmol/L)
0
5
10
15
20


C
F-PB DAY 84 (µmol/)
F-PB DAY 1 (µmol/L)
0
50
100
150

2.7 Discussion

Much of the controversy over the use of free levels of AED's in patient management has centred on doubts about the accuracy of the results obtained using the various separation techniques (Rimmer et al, 1984; Taylor and Ackerman, 1987; Theodore, 1987).

With its qualities of speed and simplicity, the technique of ultrafiltration seems to have the greatest potential for use in the separation of free drug from its protein-bound component (Levy and Moreland, 1984; Bock and Ben-Ezra, 1985; Melten et al, 1985; Taylor and Ackerman, 1987), although binding of drugs onto the filters or leakage of protein into the ultrafiltrate were reported in early experiments (Joern, 1981; Ruprah et al, 1981; Levy et al, 1984; Melten et al, 1985; Taylor and Ackerman, 1987).

The experiments carried out in this study show that these problems can be overcome. The excellent results obtained in recovery experiments using spiked samples show that there is no significant binding of drugs onto the filters used (Table 2.2), which is in agreement with recent findings for a number of drugs including AED's (Bock and Ben-Ezra, 1985; Zysset and Hegel, 1987). The method is simple to operate, and filtration followed by preparation of 50 samples for HPLC takes only 90 minutes, by contrast with three to six hours reported for equilibrium dialysis and four to 24

hours for ultracentrifugation techniques (Rowland, 1980; Oellerich and Muller-Vahl, 1984; Kwong, 1985; Verbeeck and Cardinal, 1985; Taylor and Ackerman, 1987; Zysset and Hegel, 1987). Temperature control to within ± 1°C is consistently achieved with the Sorvall refrigerated centrifuge at a setting of 25 °C.

An advantage of the HPLC method described in this chapter is that it can be used for the analysis of both total and free levels of AED's. Each HPLC run for a sample containing PB, PHT, CBZ and CBZ-E takes 15 minutes, but the use of the automatic injection procedure allows samples to be chromatographed overnight in a programmed run. In addition, the simple clean-up process for the samples developed in this study allows several hundred samples to be chromatographed before there is any sign of peak trailing which is an indication of column deterioration. The inclusion of a pre-column, which in itself would be a cause of peak spreading and increased retention time, is therefore unnecessary. Several hundred patients' serum samples for total and free levels of PHT, CBZ, or PB alone or in combination have been analysed in this study without encountering any problems of interference from co-prescribed drugs or other substances usually present in patients' sera.

The low levels of drug present after the separation process require that the analytical procedures used for free levels of AED's should be considerably more sensitive than those used in the analysis of total levels,

which comprise both free and protein-bound elements. In the case of PHT and CBZ, free levels higher than 20 μmol/L are very rarely seen except in overdose situations. On the other hand, free levels of PB may exceed 42 μmol/L, (which was the top standard concentration used for the calibration curve shown in Figure 2.3B), but this does not cause any problem from a methodological point of view since the calibration curve for this drug is linear up to 200 μmol/L. The validity of this HPLC procedure for the analysis of total and free levels is confirmed by the good correlation between this method and the FPIA (TDX) procedure. (Figures 2.4A to 2.4C and 2.5A to 2.5C).

An important advantage of the ultrafiltration/HPLC procedure is the small size of the serum samples required, 250 μl compared with the 400-500 μl previously reported as the minimum volume necessary (Oellerich and Muller-Vahl, 1984; Bock and Ben-Ezra, 1985; Levy and Schmidt, 1985) allowing its use in routine paediatric practice. Moreover, in cases where the amount of serum available is less than 250 μl it has been found that the method can be scaled down to 100 μl, without the need to modify the filtration apparatus such as is required with other methods (Wittfoht et al, 1984). In this context, the analysis of levels of F-PB, F-PHT and F-CBZ in a single sample is very important, since patients with intractable epilepsy, both children or adults, are frequently on polytherapy. Also the small volume of serum sample required makes this method ideal

for pharmacokinetic studies in experimental animal models.

The fact that the HPLC method can be used for the analysis of CBZ-E and F-CBZ-E is a further advantage, particularly for pharmacokinetic studies and in cases where CBZ toxicity is suspected even though the levels of CBZ determined are well within the therapeutic range for CBZ (Agbato et al, 1986; Elyas et al, 1986; Patsalos et al, 1987).

Stability of free AED's during sample storage is not affected. Therefore serum samples can be stored frozen for a considerable length of time without affecting the accuracy of the assays for free AED's in serum. This is a considerable advantage in research projects and pharmacokinetic studies.

Both HPLC and FPIA are convenient methods for the routine analysis of total levels of AED's and for free levels when combined with ultrafiltration. Both methods yield reproducible results (Tables 2.7 to 2.10). The major disadvantage of the FPIA method for analysis of samples from patients on polytherapy is that each drug must be analysed separately. This means that FPIA is less cost-effective and requires more sample than the HPLC method described in this chapter. In addition, no FPIA kit is available for the analysis either of CBZ-E or F-CBZ-E at present.

The monitoring of free levels of AED's will probably not replace the need for analysis of total levels. However, free level monitoring will continue to be required for selected patients (Rowland, 1980; Rimmer et al, 1984; Jack et al, 1986; Theodore, 1987; Barre et al, 1988; Johno et al, 1988). In these cases, results obtained by analysing free levels as well as total levels provide additional clinical information necessary for patient care.

2.8 Summary

A micromethod for estimating free and total levels of AED's in patients' sera is described. The ultrafiltration method for the separation of free levels of AED's and the HPLC method for estimating both total and free levels are simple, accurate and reproducible. Mean recovery for AED's after ultrafiltration exceeds 98% showing that there is no significant binding of drug to the membrane used in the filters. Furthermore serum samples can be stored for several months before ultrafiltration without a measurable effect on the binding characteristics of the drugs. There is no interference in the HPLC method from other substances normally present in patients' sera and there is good correlation between results obtained by this method and an FPIA method for both free and total levels of AED's.

CHAPTER 3

THEORETICAL CONSIDERATIONS

3.1 The Law of Mass Action Applied to the Binding of a Drug to Plasma Protein(s)

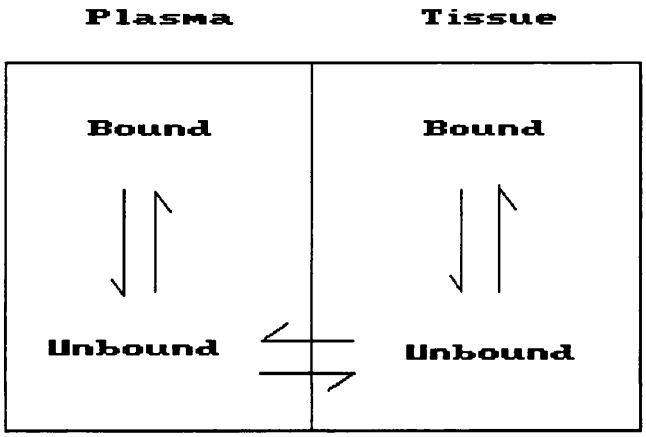


Figure 3.1 Model for drug distribution

At equilibrium, the distribution of a drug within the body depends on binding to both plasma proteins and tissue components as shown in Figure 3.1. In the model, only the unbound drug is capable of entering and leaving the plasma and tissue compartments (Rowland and Tozer, 1989).

The binding of a drug to protein in plasma, mainly albumin, is a reversible phenomenon, where the concentration of protein [P], and concentration of unbound drug [D] are in equilibrium with a drug-protein complex [DP]

(Koch-Weser and Sellers, 1976). This situation can be expressed as;

$$[D] + [P] \underset{k_2}{\overset{k_1}{<\!\!-\!\!-\!\!-\!\!-\!\!-\!\!-\!\!-\!\!-\!\!>}} [DP] \qquad \text{Equation} \quad (3.1)$$

where k_1 and k_2 are rate constants.

Application of the Law of Mass Action to Equation (3.1) results in the following expression;

$$[DP]/[D]*[P] = K_2/K_1 = K_D \qquad \text{Equation} \quad (3.2)$$

where K_D is the equilibrium constant.

As for any reversible chemical reaction, the value of K_D is dependent for example on temperature, pH and the presence of interfering compounds. In this situation ligands to albumin, changing its affinity for a particular AED, represent an important class of interfering compounds which include other AED's. Thus K_D in Equation (3.2) cannot be uniquely determined and consequently should be defined as the **Apparent Dissociation Constant.**

3.2 General Equation for Calculating Free Levels of AED's

If the fraction of the total concentration of a drug that is unbound (i.e., the free fraction) is represented by α, then the unbound concentration (i.e., the free concentration) can be expressed by [α C], where C represents the total concentration of drug, and the bound concentration [DP] is represented by [(1 - α) C]. Substitution into Equation (3.2), results in;

$$K_D\,[P] = (1 - \alpha)\ C/\alpha * C$$

which can simplified to

$$K_D[P] = (1 - \alpha)/\alpha \qquad \text{Equation} \quad (3.3)$$

By rearranging Equation (3.3) the free fraction α can be expressed in terms of the apparent dissociation constant (K_D) and protein concentration [P] resulting in Equation (3.4) as follows:-

$$\alpha\ K_D[P] = 1 - \alpha$$

$$\alpha + \alpha K_D[P] = 1$$

$$\alpha(1 + K_D[P]) = 1$$

or

$$\alpha = 1/(1 + K_D * [P]) \qquad \text{Equation} \quad (3.4)$$

By definition, the free fraction (α) is also given by:-

$$\alpha = [D_F]/[D_T] \qquad \text{Equation} \quad (3.5)$$

where $[D_F]$ is the free (unbound) level and $[D_T]$ is the total (free + bound) drug level.

Combining Equations (3.4) and (3.5) results in Equation (3.6);

$$[D_F]/[D_T] = 1/(1+K_D*[P]) \qquad \text{Equation} \quad (3.6)$$

and by rearrangement Equation (3.7) is obtained;

$$[D_F] = [D_T]/(1+K_D*[P]) \qquad \text{Equation} \quad (3.7)$$

Thus the free concentration of a drug in serum can be calculated from Equation (3.7), given the total concentration of the drug, the albumin concentration and K_D.

For the purpose of this thesis it is assumed, (i) that there is only one binding site on albumin and (ii) that only a small fraction of the sites available for binding are occupied. The normal average albumin concentration in plasma is 40 g/L, which is equivalent to 588

μmol/L (molecular mass of albumin = 68000 daltons). As a consequence Equation (3.7) can only be used for calculating D_F in situations where the drug concentration is well below this level. This condition can be assumed to be fulfilled in patients whose AED's concentrations are within the therapeutic range.

Equation (3.7) can be rearranged to calculate individual values for K_D as follows;

$$K_D = ([D_T] - [D_F])/([D_F]*[P]) \quad \text{Equation} \quad (3.8)$$

3.3 Hardware and Software used in Calculations

A Hewlett Packard Vectra ES/12 microcomputer using STATPAK and SIGMA PLOT programs with graphs plotted on a Hewlett Packard Color Pro 7440A graphics plotter were used for all calculations involving the formulae derived in this chapter and for subsequent chapters.

CHAPTER 4

TEMPERATURE DEPENDENCE OF THE APPARENT DISSOCIATION CONSTANT (K_D) AND FREE FRACTION (α)

4.1 Aim

To study the effects of temperature on the estimation of apparent dissociation constants (K_D) and free fraction (α) for PHT, CBZ and PB.

4.2 Introduction

Several of the commonly prescribed AED's are bound to plasma protein to a varying extent, with albumin quantitatively by far the most important protein. PHT is more than 90% bound, CBZ is approximately 70% bound, and PB is approximately 50% bound (Perucca, 1984; Levy and Schmidt, 1985; Theodore, 1987; Eadie and Tyrer, 1989). Drug binding tends to decrease with increasing temperature (Hooper et al, 1973; Schottelius, 1984; Allison and Comstock, 1988). This may have implications for patient care as analytical results for the levels of free drug separated at a temperature of e.g., 25 °C, may not accurately reflect the free levels in vivo (Levy et al, 1984). Most of the published studies have concentrated on the effect of temperature on F-PHT levels. In this study the effect of temperature on the

free levels of CBZ and PB as well as PHT has been investigated.

4.3 Materials and Methods

4.3.1 Subjects

Epileptic patients on PHT, CBZ or PB monotherapy (n = 81; female = 39, male = 42) between the ages of 24 and 58 years with normal biochemical profiles, renal function and liver function as determined by routine assays comprised the study group.

4.3.2 Blood Samples

Non-heparinised blood samples were collected, centrifuged and stored as described in Chapter 2.

4.3.3 Albumin Assay

The concentrations of serum albumin (molecular mass 68000 daltons) were assayed on a Technicon RA-XT analyser, using Technicon albumin reagent kits based on the principle that Bromocresol Green (BCG) dye combines with albumin at pH 4.2 to form a stable coloured complex with a maximum absorbance at 600 nm. The reference range for albumin in healthy subjects is 34 - 52 g/L (500 - 765 μmol/L). The precision of the analysis of albumin for the pooled patients sera and control sera was as follows:-

	N	Within-day Albumin (g/L)	CV%	Between-day Albumin (g/L)	CV%
Pooled sera	25	44 ± 0.46	1.0	44 ± 0.68	2.0
Control sera	25	33 ± 0.51	2.0	33 ± 0.67	2.0

4.3.4 Separation of Free AED's at Different Temperatures

Serum (250μl) was pipetted into the sample reservoir of an Amicon centrifree micropartition system, and centrifuged as described in Chapter 2 at temperature settings of 15 °C, 25 °C or 37 °C. The temperature settings were accurate within ± 1 °C.

The concentrations of AED's present in the ultrafiltrates were determined by HPLC using the method described in Chapter 2.

4.3.5 Assay of Total Levels of AED's

Concentrations of PHT, CBZ and PB in the sera were determined by the HPLC method described in Chapter 2.

4.3.6 Theoretical Considerations and Calculations

The theoretical considerations and equations used in this chapter were presented in Chapter 3.

4.4 Results

4.4.1 Concentration Ratios for F-PHT, F-CBZ and F-PB Related to Temperature

Figures 4.1A to 4.1C show graphically the concentration ratios for F-PHT, F-CBZ and F-PB in patients' sera at 15 °C, 25 °C and 37 °C, with the values for 25 °C taken as the unit concentrations. Table 4.1 gives the arithmetic means and SD of the concentration ratios for the three drugs at 15 °C and 37 °C. As would be expected, the effect of temperature on the level of free drug is most pronounced for PHT, intermediary for CBZ, and least pronounced for PB.

4.4.2 The Temperature Dependence of the K_D for PHT, CBZ and PB

In Figure 4.2, individual values for K_D (calculated from Equation (3.8), page 116) are correlated with the temperature at which ultrafiltration of the samples was carried out (15 °C, 25 °C and 37 °C). K_D is inversely correlated with temperature for all drugs investigated. Values for K_D calculated from the regression equations in Figure 4.2 are given in Table 4.2. A marked change

with temperature is found for K_D(PHT) and a minimal change for K_D(PB).

The arithmetic mean values and CV for K_D(PHT), K_D(CBZ) and K_D(PB) at 15 °C, 25 °C and 37 °C, based on individual values calculated according to Equation (3.8) (Chapter 3) are presented in Table 4.3. The CV's for these estimates are of comparable order and vary between 12 and 19 %.

4.4.3 The Effect of Temperature on Free Fraction (α)

Observed mean values of α for all three drugs at 15 °C, 25 °C and 37 °C, (calculated using Equation (3.5), page 115) are presented in Table 4.4. The difference in the mean values of α between 25 °C and 15 °C or 37 °C and 25 °C are statistically significant for all three drugs (Table 4.4). The CV's for these estimates vary between 7 and 16 %.

Figure 4.1A Concentration ratios for F-PHT at 15 °C, 25 °C and 37 °C. n=28.

Figure 4.1B Concentration ratios for F-CBZ at 15 °C, 25 °C and 37 °C. n=28.

Figure 4.1C Concentration ratios for F-PB at 15 °C, 25 °C and 37 °C. n=25.

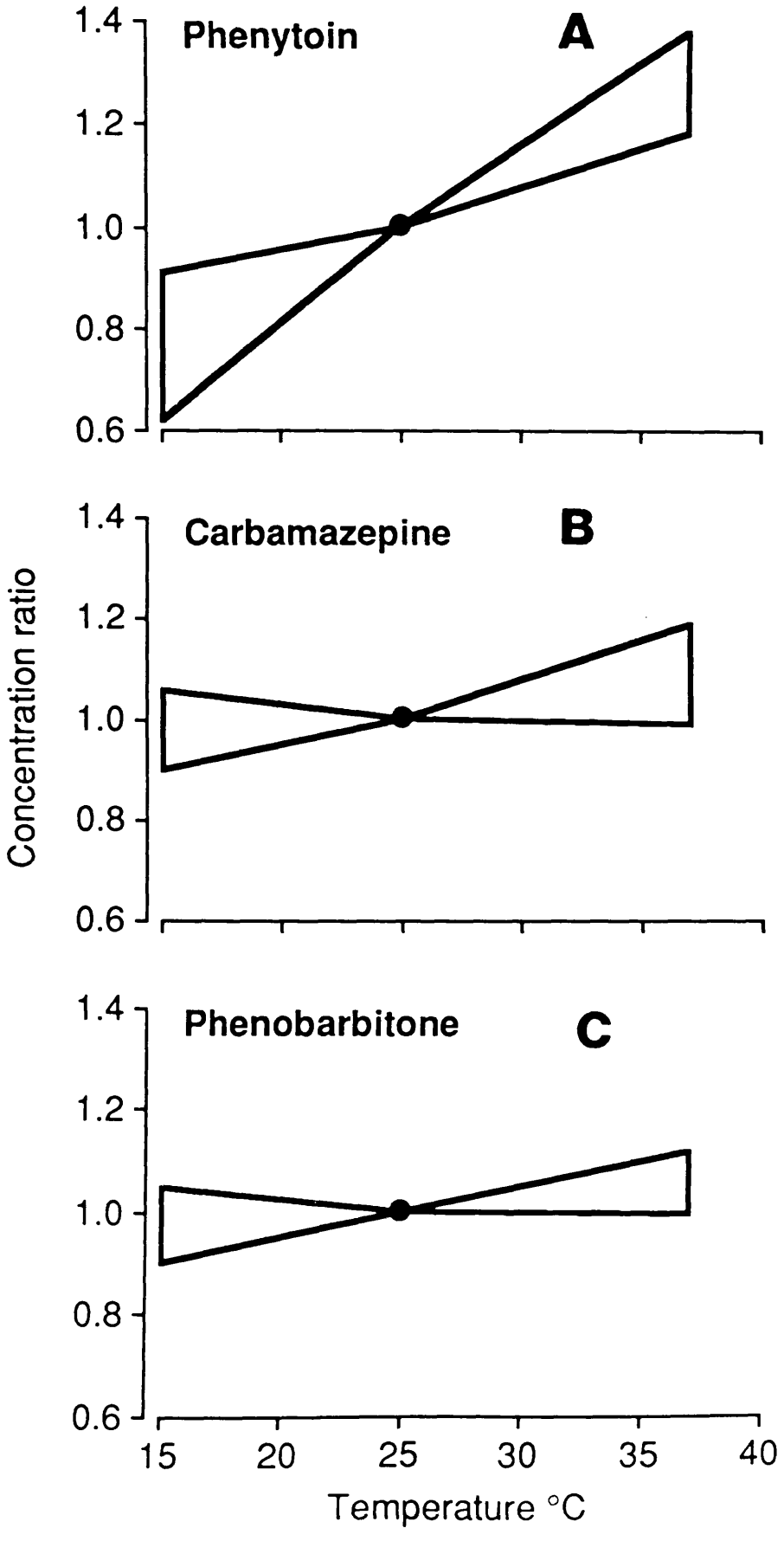

Phenytoin
A
Carbamazepine
B
Phenobarbitone
C
Concentration ratio
1.4
1.2
1.0
0.8
0.6
15
20
25
30
35
40
Temperature °C

TABLE 4.1 ARITHMETIC MEANS OF RATIOS FOR THE CONCENTRATION OF FREE AED'S AT DIFFERENT TEMPERATURES

	N	15°C / 25°C	CV %	25°C / 25°C	37°C / 25°C	CV %
F-PHT	28	0.78 ± 0.07	9	1.00	1.26 ± 0.04	3
F-CBZ	28	0.98 ± 0.04	4	1.00	1.12 ± 0.06	6
F-PB	25	0.98 ±0.03	3	1.00	1.06 ± 0.03	3

Figure 4.2 Regression analysis of the apparent dissociation constant (K_D) for PHT, CBZ, and PB at 15 °C, 25 °C and 37 °C.

$K_{D(PHT)} = 2.16 \times 10^{-2} + -3.26 \times 10^{-4}$ Temp.
r= - 0.8035; p < 0.001; n=28.

$K_{D(CBZ)} = 4.72 \times 10^{-3} + -3.56 \times 10^{-5}$ Temp.
r= - 0.5544; p < 0.002; n=28.

$K_{D(PB)} = 1.34 \times 10^{-3} + -9.65 \times 10^{-6}$ Temp.
r= - 0.4562; p < 0.02; n=25.

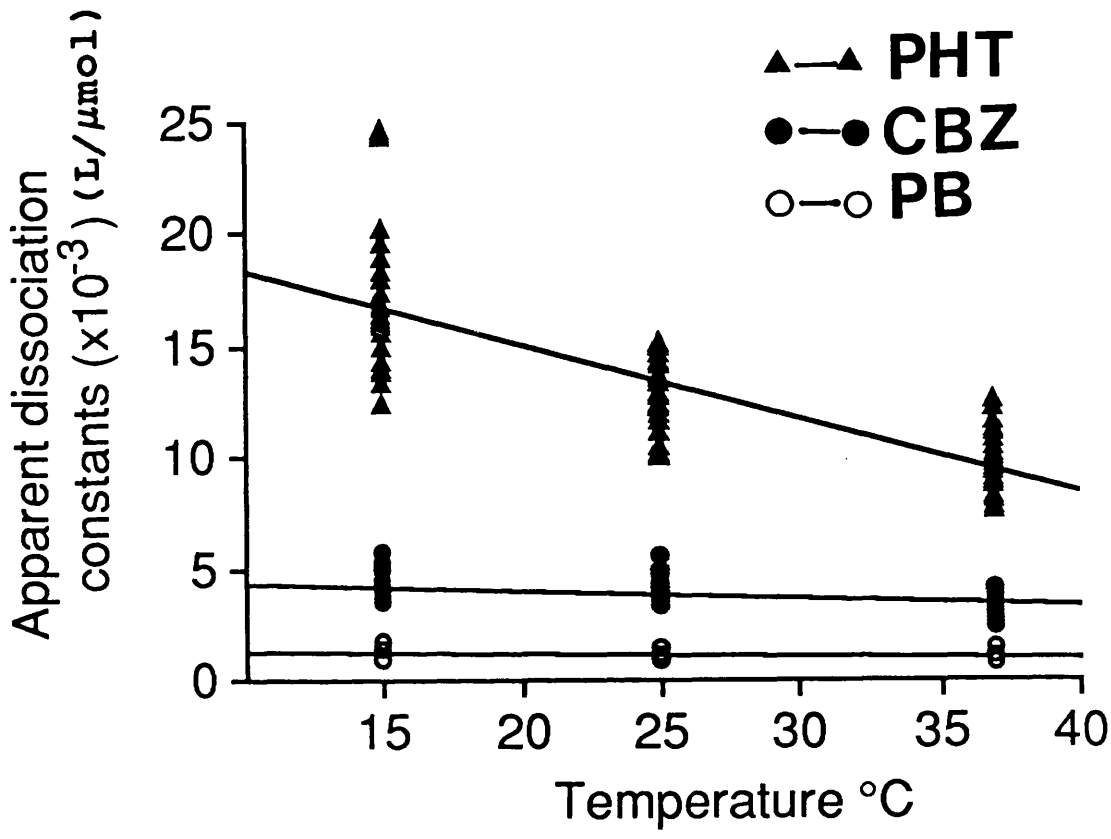

PHT
CBZ
PB
Apparent dissociation constants (x10-3) (L/µmol)
25
20
15
10
5
0
15
20
25
30
35
40
Temperature °C

TABLE 4.2 ESTIMATES OF K_D AT DIFFERENT TEMPERATURES DERIVED BY REGRESSION ANALYSIS FROM FIGURE 4.2

Drug	$K_D \times 10^{-3}$ (L/μmol)		
	15 °C	25 °C	37 °C
PHT	16.67	13.41	9.50
CBZ	4.18	3.83	3.14
PB	1.20	1.10	0.98

TABLE 4.3 ARITHMETIC MEAN VALUES OF K_D AND CV AT DIFFERENT TEMPERATURES CALCULATED ACCORDING TO EQUATION 3.8

	N	$K_D \times 10^{-3}$ (L/μmol) 15 °C	CV %	$K_D \times 10^{-3}$ (L/μmol) 25 °C	CV %	$K_D \times 10^{-3}$ (L/μmol) 37 °C	CV %
PHT	28	17.04 ± 3.21	19	12.72 ± 1.43	11	9.81 ± 1.26	13
CBZ	28	4.11 ± 0.50	12	3.96 ± 0.46	12	3.34 ± 0.47	14
PB	25	1.19 ± 0.22	18	1.12 ± 0.15	13	0.98 ± 0.14	14

TABLE 4.4 THE EFFECT OF TEMPERATURE ON FREE FRACTION (α)

	N	Observed (α) ± SD	CV%
PHT			
15 °C	28	0.08 ± 0.01	16
25 °C		0.10 ± 0.01	13
37 °C		0.14 ± 0.02	14
Δ α (25 °C - 15 °C)		0.02	
Δ α (37 °C - 25 °C)		0.04	
CBZ			
15 °C	28	0.25 ± 0.02	9
25 °C		0.26 ± 0.02	9
37 °C		0.30 ± 0.03	10
Δ α (25 °C - 15 °C)		0.01	
Δ α (37 °C - 25 °C)		0.04	
PB			
15 °C	25	0.56 ± 0.04	9
25 °C		0.57 ± 0.04	7
37 °C		0.60 ± 0.04	7
Δ α (25 °C - 15 °C)		0.01	
Δ α (37 °C - 25 °C)		0.03	

$p < 0.001$ for differences (Δ α) between mean (α) at 25 °C and 15 °C

$p < 0.001$ for differences (Δ α) between mean (α) at 37 °C and 25 °C

4.5 Discussion

The binding of AED's to albumin in vivo is a complex process. Therefore in this study only healthy epileptic patients on AED monotherapy were selected to study the effect of temperature on binding of AED's to albumin. The binding of PHT, CBZ and PB to albumin was found to be inversely related to temperature with the largest effect seen in the case of PHT (Table 4.1), and these findings are in accordance with previously published data for PHT (Hooper et al, 1973; Schottelius, 1984; Levy et al, 1984; Theodore, 1987; Allison et al, 1988). The depiction of concentration ratios of the free levels of the drugs at 15 °C and 37 °C compared with values at 25 °C shows that with increasing temperature there is a marked decrease in binding of PHT compared with the very much smaller temperature effect observed for CBZ and PB (Figures 4.1A to 4.1C). As would be expected, K_D for PHT decreased sharply and in parallel with the decreased binding at increased temperature (see Table 4.2), but this effect was small for both CBZ and PB (Figure 4.2). The values of K_D for PHT, CBZ and PB at 15 °C, 25 °C and 37 °C derived from Figure 4.2 by regression analysis equations correspond very closely to the calculated arithmetic means for K_D (Tables 4.2 and 4.3). These results also show that K_D decreases with increasing temperature as the level of free drug increases.

The mean values of K_D seem statistically sufficiently robust for calculating free levels in patients samples using Equation (3.7). It may be possible, having established mean values for K_D at a given temperature for a particular population on AED monotherapy, to use these values to predict free levels accurately for other patients in similar circumstances, knowing total levels of drug and albumin only. This aspect will be discussed further in a later chapter (Chapter 6). The fact that values for K_D remain reasonably stable at physiological temperature for a particular population of patients on PHT, CBZ or PB monotherapy forms the basis for investigations of changes in binding in polytherapy (Chapter 7). The effect of temperature on the binding of AED's to albumin is also illustrated by the increased value of the free fraction (α) with increasing temperature (Table 4.4).

4.6 Summary

Serum samples from patients on PHT, CBZ or PB monotherapy were filtered at 15 °C, 25 °C and 37 °C and the free levels of the AED's measured by HPLC. The mean values of K_D at each temperature were calculated using Equation 3.8. K_D was inversely correlated with temperature for all three AED's. A marked change with temperature was found for K_D(PHT) and a minimal change for K_D(PB). The differences in the mean values of α for all three AEDs' at 15 °C, 25 °C and 37 °C are statistically significant ($p<0.001$).

CHAPTER 5

ESTIMATION OF TARGET RANGES FOR F-PHT, F-CBZ AND F-PB AT 25 °C AND 37 °C

5.1 Aim

To establish target ranges (therapeutic ranges) for F-PHT, F-CBZ and F-PB at 25 ° and 37 °C.

5.2 Introduction

As has been discussed in Chapter 1, it is the free level of a drug that is pharmacologically active and also related to side effects (Booker and Darcey, 1973; Shoeman and Azarnoff, 1975). The free level of an AED in a blood sample is among other factors dependent on temperature, which influences the binding of the drug to albumin as has been demonstrated in Chapter 4. The free levels of an AED estimated after separation at a different temperature from the body temperature may provide information that is biased both in pharmacological and toxicological terms.

In this chapter, free levels of PHT, CBZ and PB determined after ultrafiltration at 25 °C and 37 °C have been correlated with total levels of the drugs. Target values for free levels (which would be analogous to the therapeutic ranges quoted for total drug levels) have been derived from these data by two different methods.

Comparisons are then made between these target ranges, which were calculated from the therapeutic ranges for total drug levels (i) using free fraction (α) and (ii) using regression equations derived from the relationship between free drug levels and total drug levels. Ratios for the free levels at 25 °C and 37 °C have also been calculated, in order to provide estimates of relative changes in free levels at the two temperatures.

5.3 Materials and Methods

5.3.1 Subjects

Epileptic patients on PHT, CBZ or PB monotherapy (n = 369; female = 173, male = 196) between the ages of 20 and 55 years with normal biochemical profiles, renal function and liver function, as determined by routine assays, comprised the study groups.

5.3.2 Blood Samples

Non-heparinised blood samples were collected, centrifuged and stored as described in Chapter 2.

5.3.3 Separation of Free AED's at 25 °C and 37 °C

Serum (250μl) was pipetted into the sample reservoir of an Amicon centrifree micropartition system, and centrifuged as described in Chapter 2 at temperature settings of 25 °C and 37 °C. The temperature settings were accurate within ± 1 °C.

The concentrations of AED's present in the ultrafiltrates were determined by HPLC using the method described in Chapter 2.

5.3.4 Assay of Total Levels of AED's

Concentrations of PHT, CBZ and PB in the sera were determined by the HPLC method described in Chapter 2.

5.4 Results

5.4.1 Concentration Ratios for F-PHT, F-CBZ and F-PB Related to 25 °C and 37 °C

Table 5.1 gives the mean and SD for the concentration ratios for PHT, CBZ and PB at 25 °C and 37 °C. The effect of temperature on the level of free drug was most pronounced for PHT (34% lower at 25 °C), intermediary for CBZ and least pronounced for PB (6% lower at 25 °C). The CV's for the ratios vary between 3 and 15 %. The ratio for the concentrations at 25 °C and 37 °C are similar to those obtained in a different group of patients in Chapter 4.

5.4.2 The Effect of Temperature on Free Fraction (α)

The mean observed values of α for all three drugs at 25 °C and 37 °C, (calculated using Equation (3.5), page 115) are presented in Table 5.2. The difference in the mean values of α between 25 °C and 37 °C is statisti-

cally significant for all three drugs (Table 5.2). The CV's for these estimates vary between 9 and 17 %.

5.4.3 The Effect of Temperature on Target Ranges

In Figures 5.1A to 5.1C, values for F-PHT, F-CBZ or F-PB following ultrafiltration at 25 °C have been correlated with the total drug levels. Similarly Figures 5.2A to 5.2C show the results for F-PHT, F-CBZ and F-PB obtained at 37 °C in the same group of patients. Target values for the free drug levels of the three AED's are then calculated at 25 °C and 37 °C from the established therapeutic ranges for total drug levels (i) using mean free fraction (α) and (ii) using regression equations derived from the relationship between free drug levels and total drug levels. A comparison between the target values for F-PHT, F-CBZ and F-PB at 25 °C and 37 °C calculated using the two methods is presented in Table 5.3.

TABLE 5.1 ARITHMETIC MEANS OF RATIOS FOR THE CONCENTRATION OF FREE AED'S AT 25 °C AND 37 °C

	N	25 °C / 37 °C	CV %
F-PHT	131	0.66 ± 0.10	15
F-CBZ	121	0.86 ± 0.05	7
F-PB	36	0.94 ± 0.03	3

TABLE 5.2 THE EFFECT OF TEMPERATURE ON FREE FRACTION (α)

	N	Observed (α) ± SD	CV%
PHT			
25 °C	131	0.10 ± 0.02	17
37 °C	131	0.15 ± 0.02	14
$\triangle \alpha$		0.05	
CBZ			
25 °C	121	0.26 ± 0.02	9
37 °C	121	0.30 ± 0.03	10
$\triangle \alpha$		0.04	
PB			
25 °C	36	0.60 ± 0.09	14
37 °C	36	0.63 ± 0.08	13
$\triangle \alpha$		0.03	

$p < 0.001$ for differences ($\triangle \alpha$) between mean (α) at 25 °C and 37 °C.

Figure 5.1A F-PHT levels (y) at 25 °C versus the total PHT levels (x).

F-PHT = -0.442 + 0.112 PHT, r = 0.975; p < 0.001; n = 131.

Figure 5.1B F-CBZ levels (y) at 25 °C versus the total CBZ levels (x).

F-CBZ = -0.188 + 0.267 CBZ, r = 0.969; p < 0.001; n = 121.

Figure 5.1C F-PB levels (y) at 25 °C versus the total PB levels (x).

F-PB = -1.323 + 0.621 PB, r = 0.967; p < 0.001; n = 36.

The squares in the figure indicate the locally used therapeutic ranges for total levels **(a)** and theoretical target ranges for free levels **(b)**.

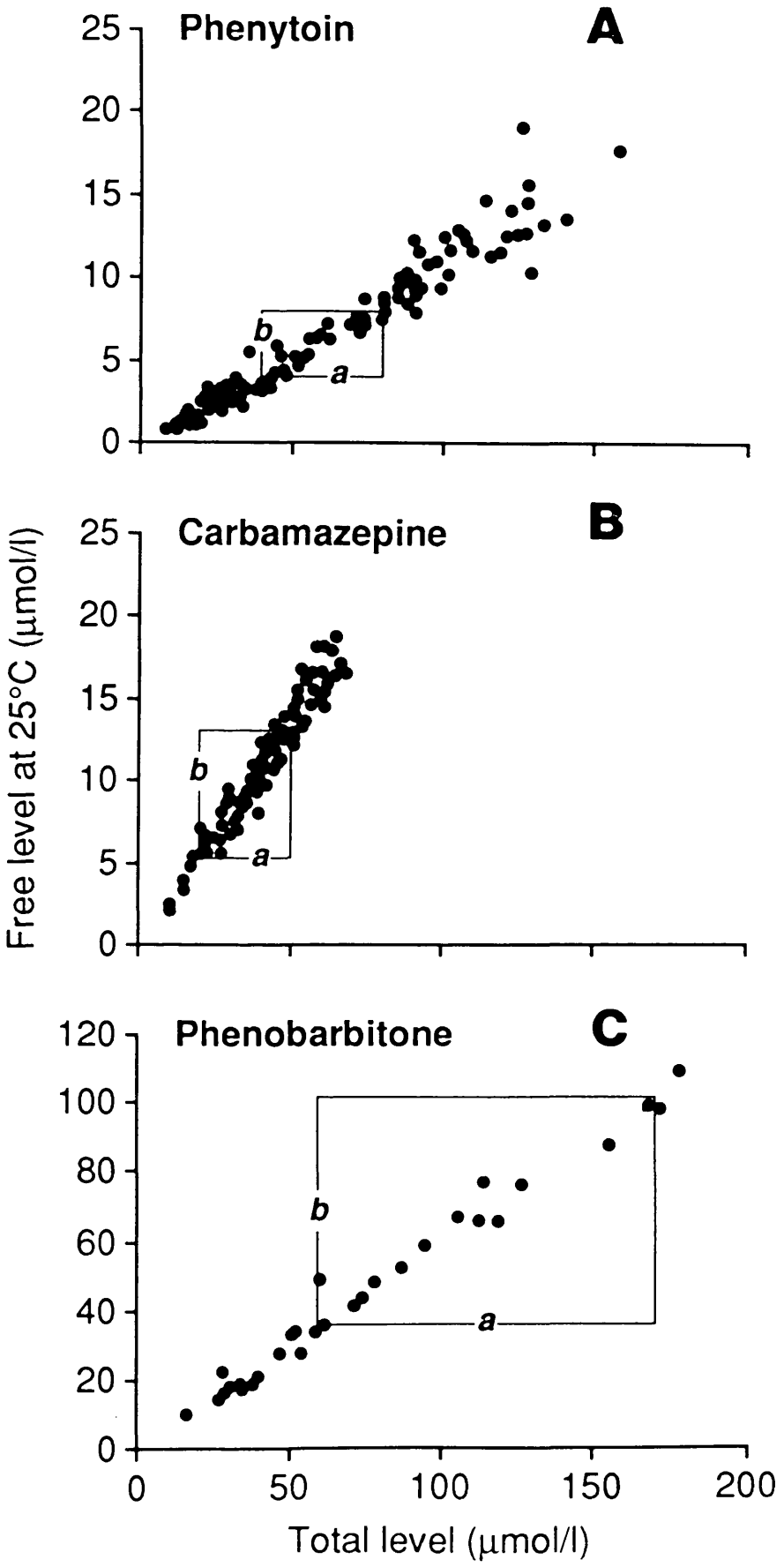

Phenytoin
A
25
20
15
10
5
0
b
a
Carbamazepine
B
Free level at 25°C (µmol/l)
Phenobarbitone
C
120
100
80
60
40
20
0
50
150
200
Total level (µmol/l)

Figure 5.2A F-PHT levels (y) at 37 °C versus the total PHT levels (x).

F-PHT = 0.229 + 0.146 PHT, r = 0.975; p < 0.001; n = 131.

Figure 5.2B F-CBZ levels (y) at 37 °C versus the total CBZ levels (x).

F-CBZ = - 0.281 + 0.311 CBZ, r = 0.960; p < 0.001; n = 121.

Figure 5.2C F-PB levels (y) at 37 °C versus the total PB levels (x).

F-PB = - 0.572 + 0.643 PB, r = 0.970; p < 0.001; n = 36.

The squares in the figure indicate the locally used therapeutic ranges for total levels **(a)** and theoretical target ranges for free levels **(b)**.

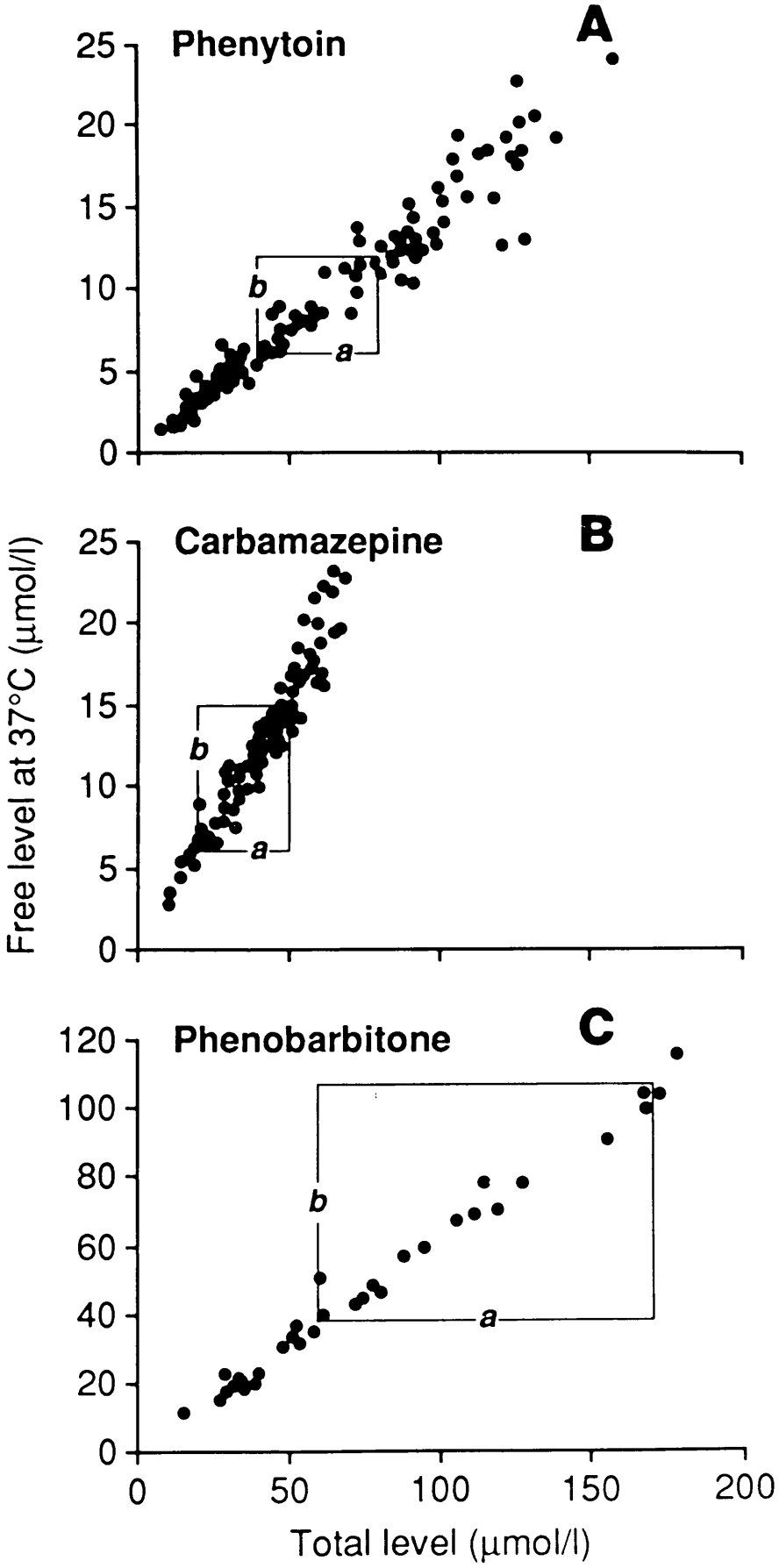

A
Phenytoin
25
20
15
10
5
0
b
a
B
Carbamazepine
Free level at 37°C (µmol/l)
C
Phenobarbitone
120
100
80
60
40
20
0
50
150
200
Total level (µmol/l)

TABLE 5.3 TARGET RANGES FOR F-PHT, F-CBZ AND F-PB AT 25 °C AND 37 °C

	Target range (free level) (derived from regression equations) (μmol/L)		Target range (free level) (calculated using mean values for α) (μmol/L)		Therapeutic range (total level)* (μmol/L)
	25 °C	37 °C	25 °C	37 °C	
PHT	4.0-8.0	6.0-12.0	4.0-8.0	6.0-12.0	40-80
CBZ	5.2-13.0	6.0-15.0	5.2-13.0	6.0-15.0	20-50
PB	36.0-104.0	38.0-108.0	36.0-102.0	38.0-107.0	60-170

* Therapeutic ranges adapted from the literature and used at the National Hospital

5.5 Discussion

The concentration ratios between 25 °C and 37 °C for free levels, shown in Table 5.1, suggest that the separation of free AED's at 25 °C will underestimate F-PHT by 34 %, F-CBZ by 14 % and F-PB by 6 % compared with free levels determined at 37 °C, which more accurately reflect the levels in vivo.

The free fraction (α) of an AED, representing that proportion of total drug not bound to protein, is related to the free level through Equation (3.5) (Chapter 3, page 115). The mean values of α for all three drugs are increased at 37 °C as against the results obtained when carrying out the filtration process at 25 °C, and these changes are statistically significant ($p<0.001$) (Table 5.2). From this study it can be seen that the temperature has a significant effect on the binding of AED's to albumin.

To study the implications of these results in relation to TDM, theoretical target values for free levels at 25 °C and 37 °C for the three AED's were compared with local therapeutic ranges for total levels of PHT, CBZ and PB. Overall there is good correlation between the total levels and free levels for all three AED's (Figures 5.1A to 5.2C). The target ranges calculated using regression equations derived from Figures 5.1A to 5.2 C correspond very closely to the target ranges calculated using mean values for α for PHT, CBZ and PB

(Table 5.3). However the target ranges calculated at 25 °C are lower compared with the target ranges calculated at 37 °C for all three AED's (Table 5.3).

The increase in F-PHT levels determined at 37 °C rather than 25 °C is in relative terms small (amounting to a difference of 5% in the free fraction (α) in the results shown in Table 5.2). However, this change represents an increase in the absolute concentration of F-PHT of more than 50%. These effects are less pronounced for F-CBZ and F-PB, as the proportion of drug bound to protein is less (Table 5.2). Thus it is essential, whenever the measurement of free levels of AED's is carried out, that the temperature used for the separation of free AED from the protein-bound component be specified and the appropriate target range quoted. In order to correlate the concentrations of free AED's with other biochemical parameters, the separation step should preferably be carried out at the physiological temperature.

From a clinical point of view, the measurement of free levels of AED's during monotherapy may not be essential in healthy subjects, because the total level would be expected to reflect the free level. Measurement of free levels of AED's however is important for certain groups of patients such as pregnant women or patients with kidney or liver disease (Rowland, 1980; Perucca, 1984; Levy and Moreland 1984; Rimmer et al, 1984; Levy and Schmidt, 1985; Theodore, 1987; Johno et al, 1988), or in the situation of co-administration of a highly bound

drug such as VPA, which can displace PHT from binding sites. (Patsalos and Lascelles, 1977).

5.6 Summary

Serum samples from patients on PHT, CBZ or PB monotherapy were filtered at 25 °C and 37 °C, and the free levels of the AED's measured by HPLC. The relative difference in free levels were largest for F-PHT, intermediate for F-CBZ and minimal for F-PB at the physiological temperature by comparison with the results obtained at 25 °C. The mean values of α for all three drugs were increased at 37 °C as against the results obtained when carrying out the filtration process at 25 °C, and these changes were statistically significant. Regression analysis of the relationship between free levels and total levels was used to derive temperature dependent target ranges (therapeutic ranges) for free levels of the three AED's.

CHAPTER 6

COMPARISON OF PREDICTED AND OBSERVED F-PHT AND F-CBZ AT 25 °C AND 37 °C IN MONOTHERAPY

6.1 Aim

To assess the accuracy and the precision of a proposed predictive method for F-PHT and F-CBZ levels in patients on monotherapy.

6.2 Introduction

Four methods for predicting F-PHT levels have been published recently, all based on statistical analysis of experimental data (Gugler et al, 1975; Winter and Tozer, 1986; Haidukewych et al, 1989; Dasgupta et al, 1991). Only the Gugler method is known to be based on the results of experiments carried out at the physiological temperature of 37 °C (Gugler et al, 1975). In other published methods the temperature for the separation of F-PHT from its protein-bound component was either stated to be 25 °C or not specified. In this chapter observed (i.e., measured) and predicted F-PHT levels after ultrafiltration both at 25 °C and 37 °C are compared.

The formula which was derived in Chapter 3 for predicting the free level of a drug states that;

$$[D_F] = [D_T]/(1+K_D*[P]) \qquad \text{Equation (3.7)}$$

where $[D_F]$ is the free drug level (unit: μmol/L) and $[D_T]$ is the total drug level (unit: μmol/L), K_D is the apparent dissociation constant (unit: L/μmol) (in the monotherapy situation in this instance) and [P] is the albumin level in serum (unit: μmol/L). If the total drug level, the albumin level and K_D are known, the free level can be calculated. In practice, total drug and albumin levels can be measured in individual samples. However K_D is a derived entity; average values for K_D in the monotherapy situation, which were derived at 25 °C and 37 °C from experimental data using Equation (3.8) in the preceding chapters were used (see Chapters 3 and 4, Table 4.3). These estimates demonstrated a CV below 14 % in all instances, and thus could be acceptable for use in predicting free drug levels for patients on AED monotherapy.

In this chapter this assumption is tested by comparing observed and predicted F-PHT and F-CBZ levels at 25 °C and 37 °C in a group of monotherapy patients. Thus this study also tests the transferability of the constants at two different temperatures at steady-state.

6.3 Materials and Methods

6.3.1 Subjects

Epileptic patients on PHT and CBZ monotherapy (n = 45; female = 21, male = 24) between the ages of 18 and 58 years with normal biochemical profiles, renal function and liver function determined by routine assays comprised the study groups.

The criteria for inclusion in the study were that the patients had been established on their prescribed drug regimens for at least six months and had achieved steady-state serum levels of the prescribed drugs.

6.3.2 Blood Samples

Non-heparinised blood samples were collected, centrifuged and stored as described in Chapter 2.

6.3.3 Analytical Procedures

Separation of F-PHT and F-CBZ from the protein-bound components was carried out at 25 °C and 37 °C using the Amicon Centrifree micropartition system, and the free and total levels of PHT and CBZ were analysed by HPLC as described in Chapter 2. Serum albumin was analysed by RA-XT analyser as described in Chapter 4.

6.3.4 Calculations

Individual values for predicting F-PHT and F-CBZ were calculated using Equation (3.7). Values for K_D(PHT) and K_D(CBZ), substituted in Equation (3.7) for the calculation of F-PHT and F-CBZ levels at 25 °C and 37 °C, were 12.72×10^{-3} $K_{D25°C}$(PHT) and 9.81×10^{-3} $K_{D37°C}$(PHT) and 3.96×10^{-3} $K_{D25°C}$(CBZ) and 3.34×10^{-3} $K_{D37°C}$(CBZ) respectively (Chapter 4; Table 4.3).

Inaccuracy was measured by prediction-error analysis (Sheiner and Beal, 1981). Prediction-error analysis involves calculation of the mean prediction error (MPE). Mean prediction error describes the inaccuracy that may be present, for example a positive value indicates that the prediction method has over-predicted the actual concentration. The MPE is determined by calculating for each patient the prediction error (PE), which is the predicted level minus the observed level. These values are applied to the following equation:-

$$MPE = (1/n) \Sigma (PE)$$

where n is the number of concentration pairs.
The imprecision of the predicted method can then be determined by calculating the Root Mean Squared Error (RMSE), where;

$$RMSE = \surd (1/n) \Sigma (PE)^2$$

The smaller the RMSE, the smaller the imprecision of the prediction method.

The percentage difference between the predicted and observed levels is calculated as follows:-

[(pred - obs)/obs] * 100

6.4 Results

In Figures 6.1A and 6.1B values for observed F-PHT after ultrafiltration of serum samples at 25 °C and 37 °C are correlated with predicted F-PHT values in monotherapy patients. Similarly in Figures 6.2A and 6.2B observed values for F-CBZ at 25 °C and 37 °C are correlated with predicted values for F-CBZ in monotherapy patients. There is very good correlation between the observed and predicted values, with values for $r > 0.9961$. Group means, MPE and RMSE for observed and predicted free levels together with the outcome of the prediction-error analysis are presented in Tables 6.1 and 6.2. The mean difference i.e., mean prediction error (MPE) ± (SD), between predicted and observed F-PHT levels at 25 °C is 0.12 ± (0.22) μmol/L (Table 6.1). Similarly MPE ± (SD) between predicted and observed F-PHT levels at 37 °C is 0.02 ± (0.16) μmol/L (Table 6.2). The corresponding values for the MPE ± (SD) for predicted and observed values for F-CBZ at 25 °C is 0.06 ± (0.31) μmol/L (Table 6.1). Similarly MPE ± (SD) between predicted and observed F-CBZ levels at 37 °C is 0.08 ± (0.29) μmol/L (Table 6.2). These results

indicate that the precision of the prediction method is very satisfactory.

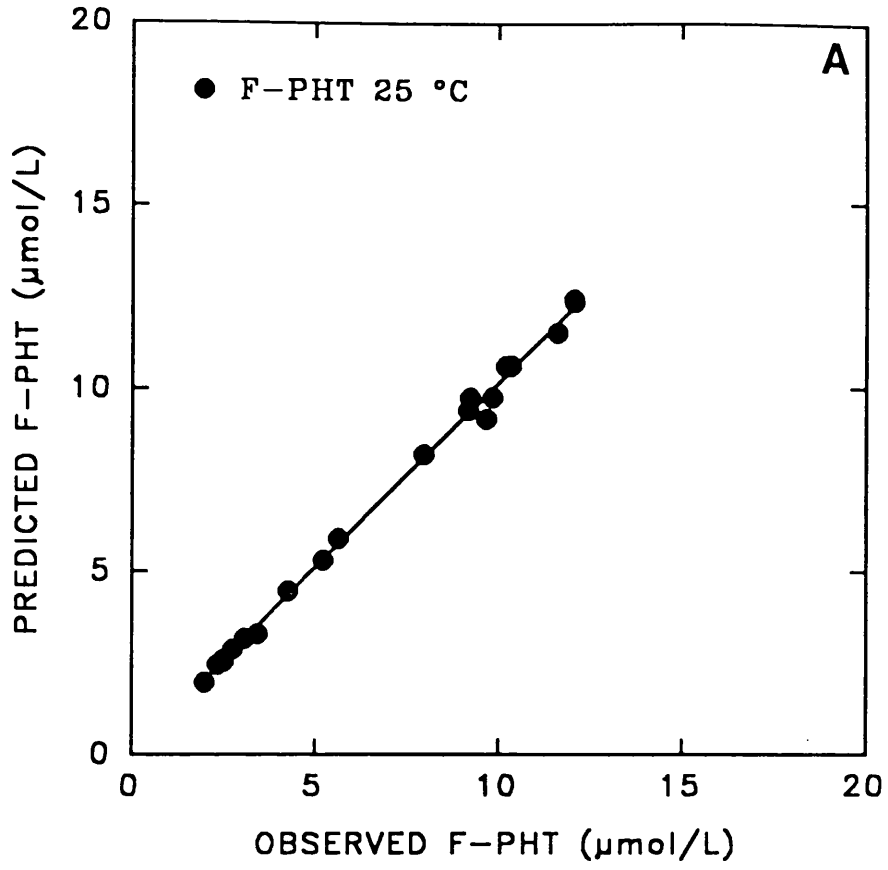
Figures 6.3A and 6.3B show scatter plots for the percentage difference between predicted and observed free levels versus the observed levels for F-PHT at 25 °C and 37 °C. Similarly Figures 6.4A and 6.4B show scatter plots for the percentage difference between predicted and observed free levels versus the observed levels for F-CBZ at 25 °C and 37 °C. It can be seen that for F-PHT 95% of the predicted values fall within ± 4% of the observed measurement and for F-CBZ 95% of predicted values fall within ± 5% of the observed measurement.

Figure 6.1A Observed versus predicted F-PHT levels at 25 °C.

$F\text{-}PHT_{(obs.)} = 0.0359 + 1.071$ $F\text{-}PHT_{(pred.)}$; $r = 0.9984$; $p < 0.001$; $n = 20$.

Figure 6.1B Observed versus predicted F-PHT levels at 37 °C.

$F\text{-}PHT_{(obs.)} = - 0.073 + 1.010$ $F\text{-}PHT_{(pred.)}$; $r = 0.9995$; $p < 0.001$; $n = 20$.


A
F-PHT 25 °C
PREDICTED F-PHT (µmol/L)
OBSERVED F-PHT (µmol/L)
0
5
10
15
20

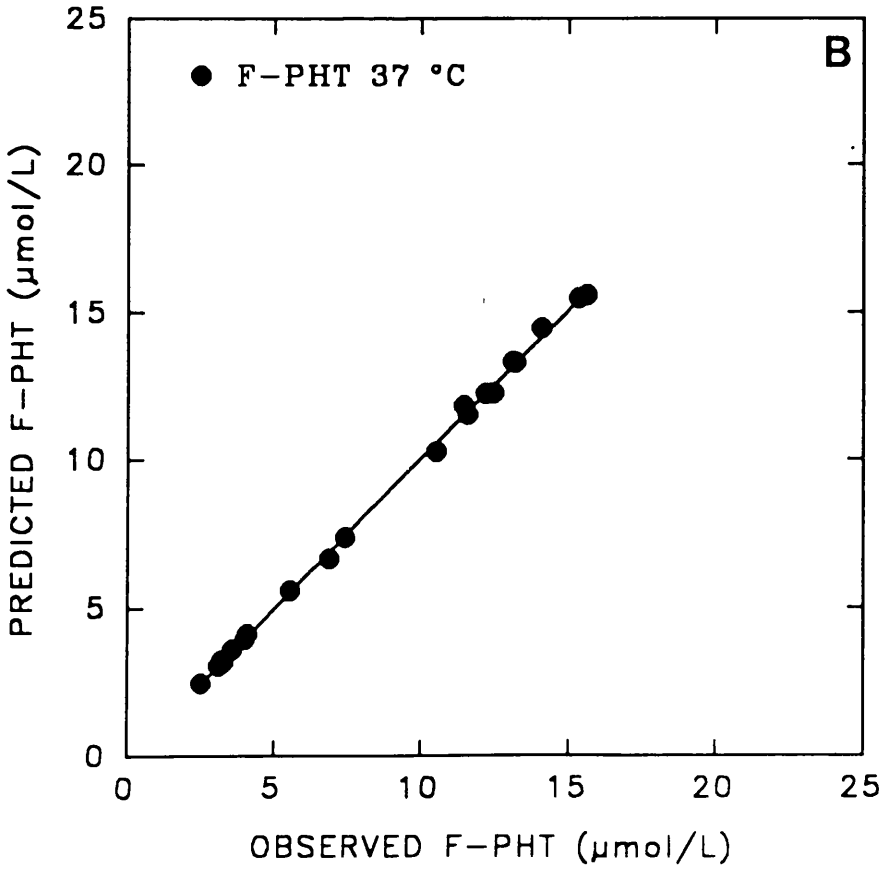
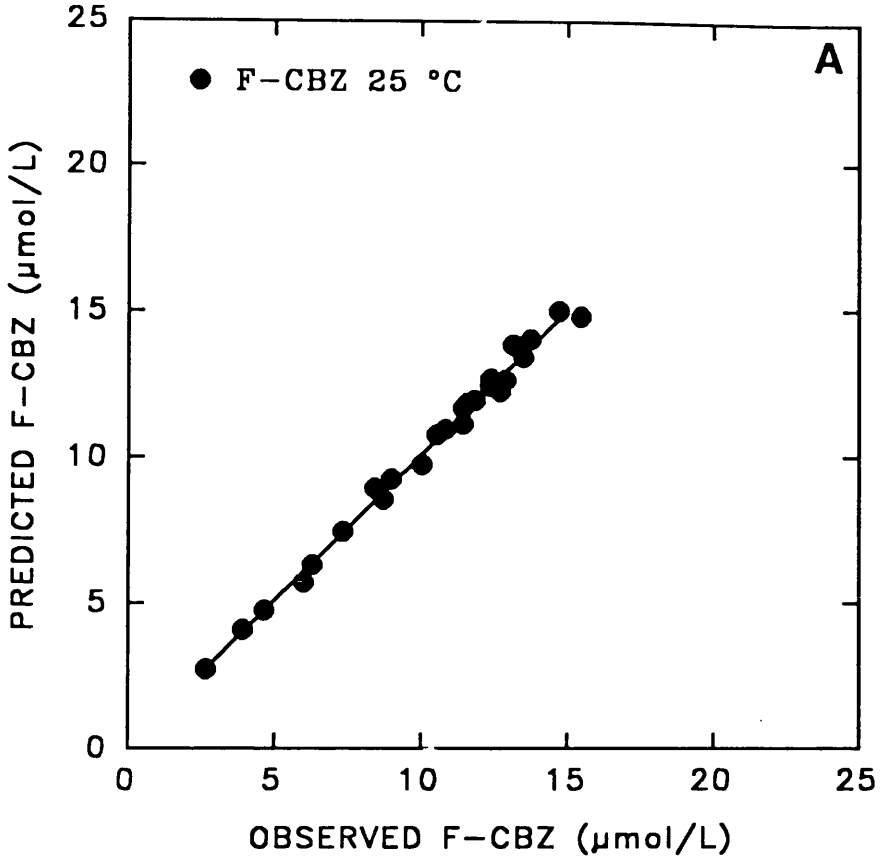

B
F-PHT 37 °C
PREDICTED F-PHT (µmol/L)
OBSERVED F-PHT (µmol/L)
0
5
10
15
20
25

Figure 6.2A Observed versus predicted F-CBZ levels at 25 °C.

$F\text{-}CBZ_{(obs.)} = 0.099 + 0.996$ $F\text{-}CBZ_{(pred.)}$; $r = 0.9961$; $p < 0.001$; $n = 25$.

Figure 6.2B Observed versus predicted F-CBZ levels at 37 °C.

$F\text{-}CBZ_{(obs.)} = 0.246 + 0.972$ $F\text{-}CBZ_{(pred.)}$; $r = 0.9977$; $p < 0.001$; $n = 25$.


A
F-CBZ 25 °C
PREDICTED F-CBZ (µmol/L)
OBSERVED F-CBZ (µmol/L)
0
5
10
15
20
25

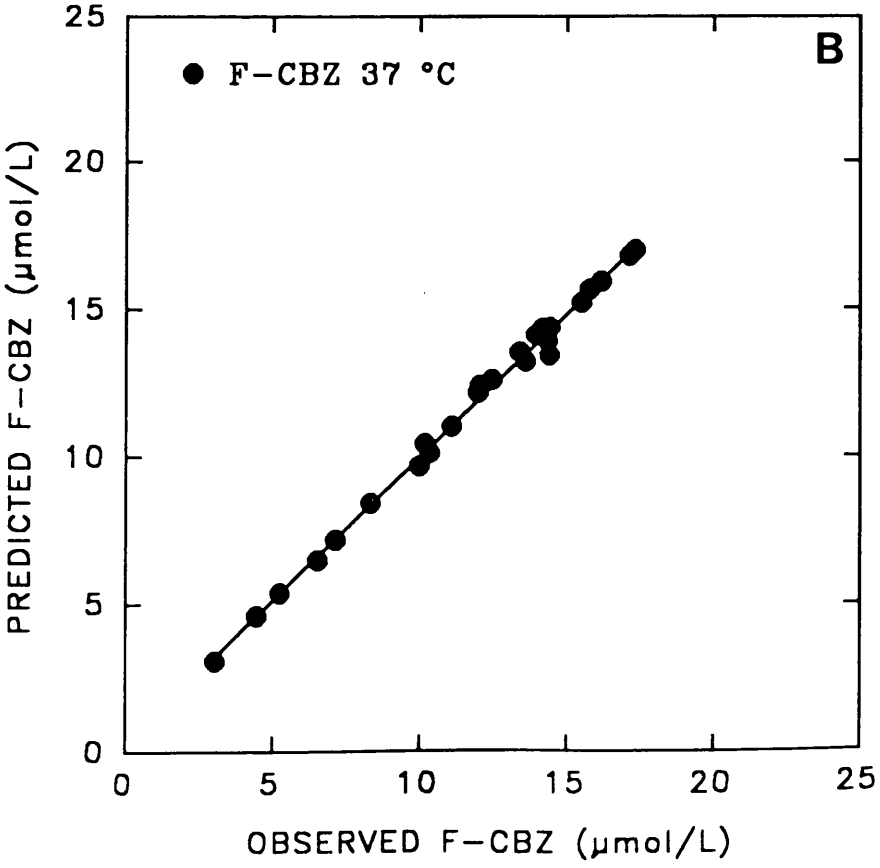

B
F-CBZ 37 °C
PREDICTED F-CBZ (µmol/L)
OBSERVED F-CBZ (µmol/L)
0
5
10
15
20
25

TABLE 6.1 PREDICTION ERROR ANALYSIS FOR F-PHT AND F-CBZ LEVELS AT 25 °C

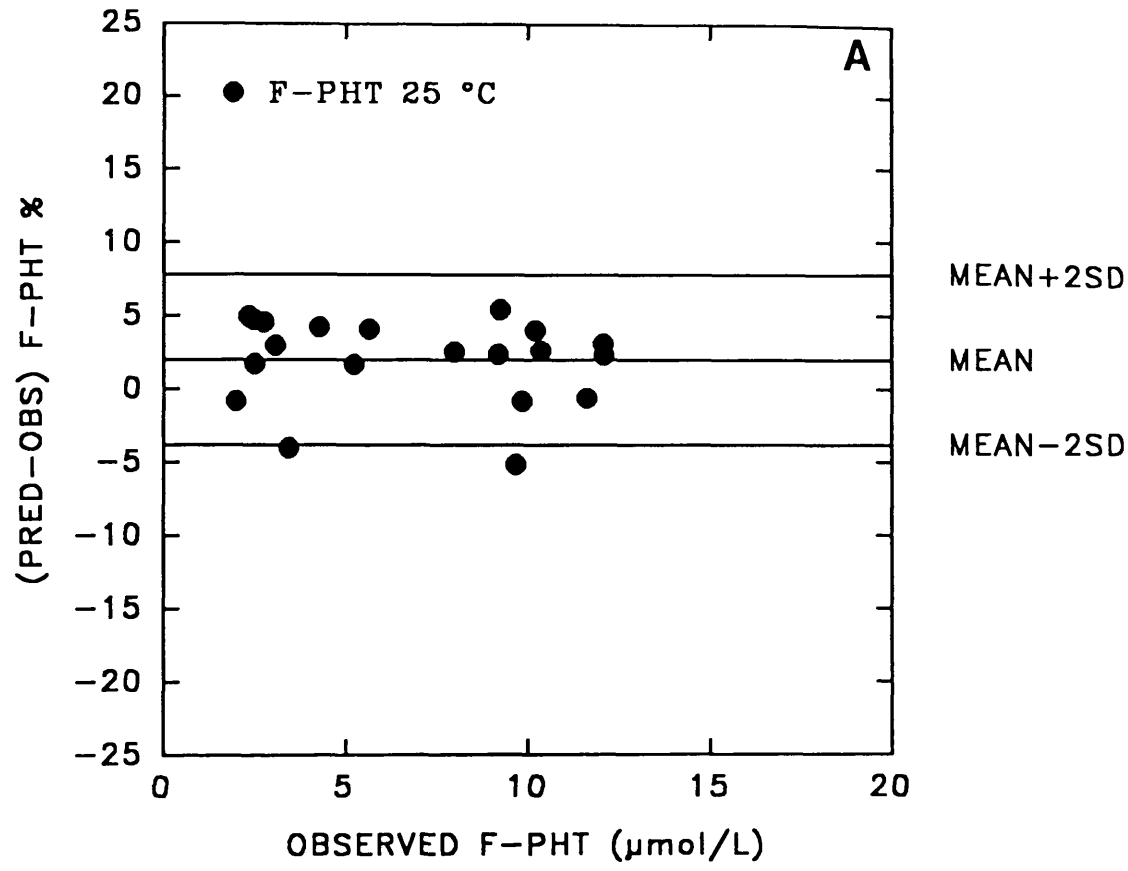
	N	Mean observed free level (μmol/L)	Mean predicted free level (μmol/L)	MPE (μmol/L)	RMSE
F-PHT	20	6.79 ± 3.73	6.91 ± 3.79	0.12 ± 0.22	0.346
F-CBZ	25	10.22 ± 3.45	10.28 ± 3.45	0.06 ± 0.31	0.244

TABLE 6.2 PREDICTION ERROR ANALYSIS FOR F-PHT AND F-CBZ LEVELS AT 37 °C

	N	Mean observed free level (μmol/L)	Mean predicted free level (μmol/L)	MPE (μmol/L)	RMSE
F-PHT	20	8.66 ± 4.70	8.68 ± 4.75	0.02 ± 0.16	0.141
F-CBZ	25	11.72 ± 4.01	11.64 ± 3.91	- 0.08 ± 0.29	0.282

Figure 6.3A Difference (%) between predicted and observed F-PHT versus observed F-PHT at 25 °C.

Figure 6.3B Difference (%) between predicted and observed F-PHT versus observed F-PHT at 37 °C.


A
F-PHT 25 °C
(PRED-OBS) F-PHT %
OBSERVED F-PHT (µmol/L)
MEAN+2SD
MEAN
MEAN-2SD
25
20
15
10
5
0
-5
-10
-15
-20
-25
0
5
10
15
20

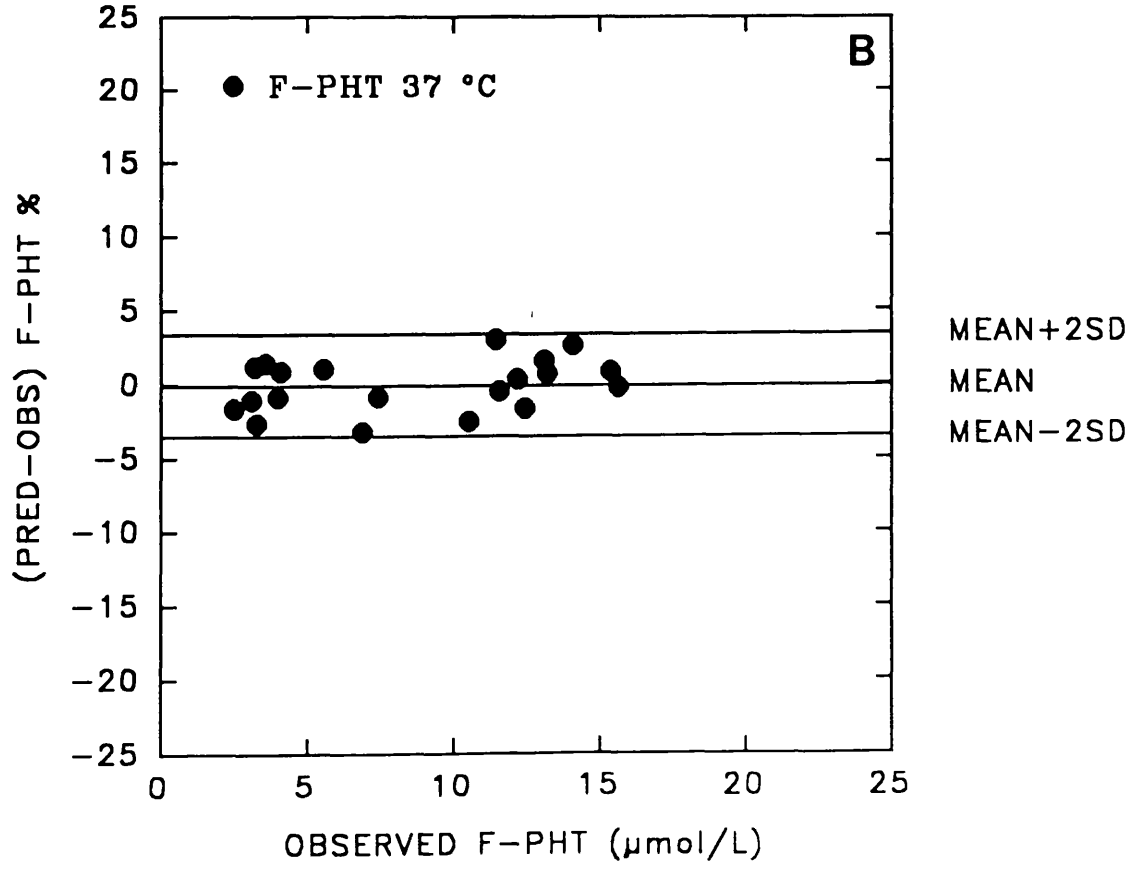
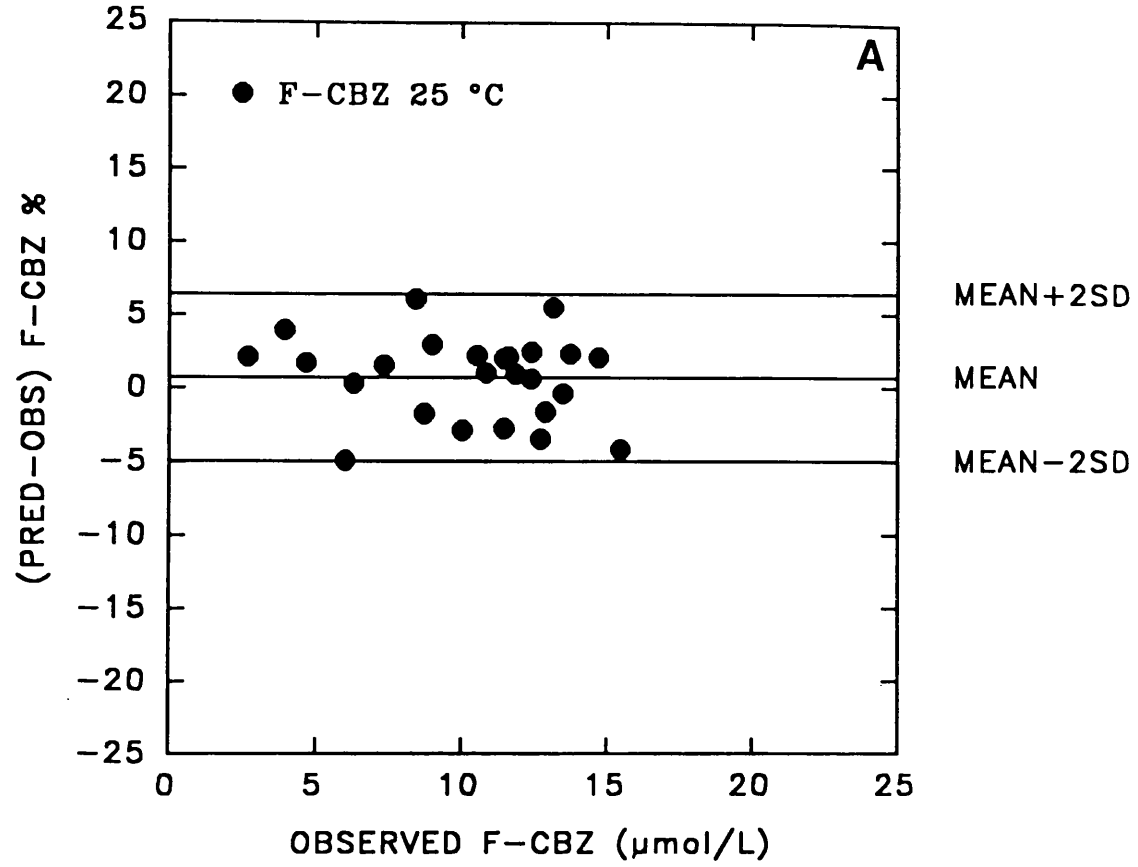
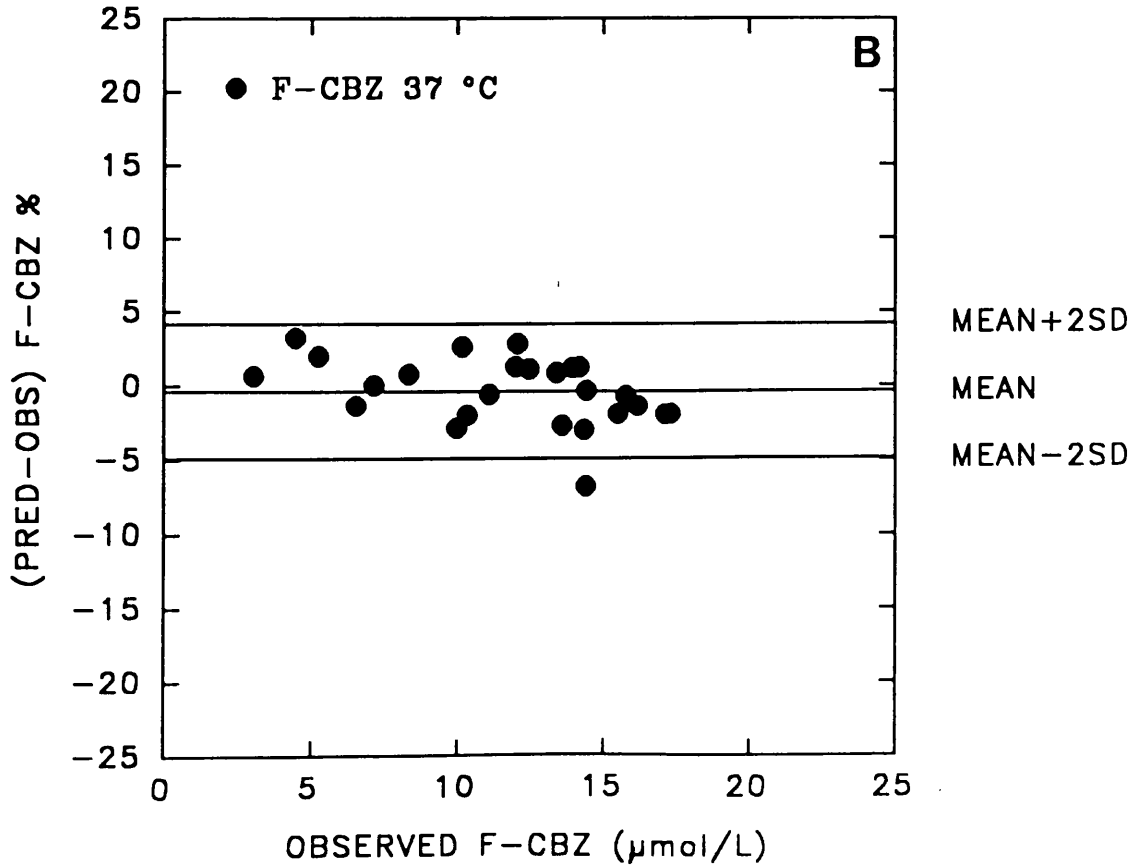

B
F-PHT 37 °C
(PRED-OBS) F-PHT %
OBSERVED F-PHT (µmol/L)
MEAN+2SD
MEAN
MEAN-2SD
25
20
15
10
5
0
-5
-10
-15
-20
-25
0
5
10
15
20
25

Figure 6.4A Difference (%) between predicted and observed F-CBZ versus observed F-CBZ at 25 °C.

Figure 6.4B Difference (%) between predicted and observed F-CBZ versus observed F-CBZ at 37 °C.


A
F-CBZ 25 °C
(PRED-OBS) F-CBZ %
OBSERVED F-CBZ (µmol/L)
MEAN+2SD
MEAN
MEAN-2SD
25
20
15
10
5
0
-5
-10
-15
-20
-25
0
5
10
15
20
25


B
F-CBZ 37 °C
(PRED-OBS) F-CBZ %
OBSERVED F-CBZ (µmol/L)
MEAN+2SD
MEAN
MEAN-2SD
25
20
15
10
5
0
-5
-10
-15
-20
-25
0
5
10
15
20
25

6.5 Discussion

In this chapter evidence is presented that the predictive method described can be used for calculating F-PHT and F-CBZ levels accurately and with good precision from measurements of total drug levels, albumin levels and average values of K_D (Figures 6.1A to 6.2B and Tables 6.2 and 6.3). The MPE between predicted and observed F-PHT level of 0.12 ± 0.22 μmol/L for 25 °C and 0.02 ± 0.16 μmol/L for 37 °C should be compared with the target ranges for F-PHT of 4 to 8 μmol/L and 6 to 12 μmol/L at 25 °C and 37 °C respectively (see Chapter 5, Table 5.3). Similarly, the MPE between predicted and observed F-CBZ level of 0.06 ± 0.31 μmol/L for 25 °C and 0.08 ± 0.29 μmol/L for 37 °C should be compared with the respective target ranges for F-CBZ of 5.2 to 13 μmol/L and 6 to 15 μmol/L (see Chapter 5, Table 5.3). It can be seen that in all cases the MPE is very small compared with the target ranges.

For F-PHT 95% of the predicted values fall within ± 4% of the observed measurement (Figures 6.3A and 6.3B). Similarly, for F-CBZ 95% of the predicted values fall within ± 5% of the observed measurement (Figures 6.4A and 6.4B). These results indicate that the prediction of F-PHT and F-CBZ levels by the proposed method here can be applied in the clinical situation.

The correlations between predicted and observed F-PHT and F-CBZ levels are very good (Figures 6.1A to 6.2B). It is evident from these results that values for K_D are

transferable within a population under steady state conditions. These results also indicate that the value for K_D is transferable between groups of patients with albumin values in serum within the reference range for normal subjects. It is reasonable to assume that values for K_D will not vary significantly between groups of patients when an identical methodology and conditions are used. Nevertheless each laboratory should establish its own values for K_D for its own population.

Long-term performance of quantitative methods including HPLC methods can be monitored by (i) using certified calibration materials and control materials and (ii) by local quality control programes and external quality assessment schemes. However, the K_D values used in this study would not necessarily be applicable to epileptic patients with concomitant hepatic and renal disorders. This is not because the Law of Mass Action is, as such, not applicable to a situation with e.g., hypoalbuminaemia, but because of increased concentrations of endogenous substances that directly or indirectly (allosterically) may change the affinity between AED's and albumin (Muller and Wollert, 1979; Fehske et al, 1981).

6.6 Summary

A prediction method for the estimation of F-PHT and F-CBZ, based on the measurement of total levels and albumin levels and using a value for K_D that had been experimentally derived, has been evaluated. The study has

demonstrated that the method predicts F-PHT or F-CBZ levels with a degree of accuracy and precision that should be clinically acceptable.

CHAPTER 7

THE EFFECTS OF DRUG DISPLACEMENT BY VPA ON APPARENT DISSOCIATION CONSTANTS (K_D), FREE FRACTIONS (α) AND TARGET RANGES FOR PHT, CBZ AND PB AT 25 °C AND 37 °C

7.1 Aim

To obtain estimates of K_D, α and target ranges for PHT, CBZ and PB in patients co-medicated with VPA and compare these estimates with K_D, α and target ranges for PHT, CBZ and PB in the situation of monotherapy.

7.2 Introduction

The affinity between albumin and PHT, CBZ or PB can be expected to change if other drugs bind to albumin either by competing for the same binding sites or by allosteric effects. The interaction would be expected to change the apparent values for K_D, α and target ranges for these drugs in the polytherapy situation, and make predictions of free levels of AED's invalid when they are based on the K_D values for single AED's.

It has not been judged realistic to derive a formal mathematical description of this situation, because of the complex nature of interactions. Instead selected

examples, including clinically relevant drug combinations, have been used to investigate the effect of an "interacting drug" on K_D and α for the "mother drug" and to investigate the possibility of establishing K_D values for polytherapy at least for these combinations. For example, co-administration of a highly bound drug such as VPA which can displace PHT from binding sites. (Patsalos and Lascelles, 1977 Bruni et al, 1980; Perucca, 1984; Baird-Lambert et al, 1987; Pisani et al, 1990).

Other clinically relevant drug combinations include CBZ and VPA or PB and VPA. For example Mattson and co-workers (Mattson et al, 1982) investigated the interaction between CBZ and VPA in vitro using ultrafiltration. They found that F-CBZ levels increased at all concentrations of VPA. This interaction suggests that when CBZ and VPA are taken together there is a greater risk of CBZ toxicity than with CBZ monotherapy (Mattson et al, 1982). VPA therapy has been reported to increase plasma PB levels significantly (Schobben et al, 1975; Vakil et al, 1975; Adams et al, 1978; Wilder et al, 1978; Patel et al, 1979; 1980; Bruni et al, 1980a; Kapetanovic et al, 1981; Fernandez de Gatta et al, 1986), to prolong half-life of PB and to decrease PB clearance (Kapetanovic et al, 1981), specifically its non-renal clearance (Patel et al, 1979; 1980), as reflected in decreased urinary excretion of the major metabolite p-hydroxy phenobarbitone (Bruni et al, 1980). This interaction is clinically important, with increasing somnolence, sometimes resulting in coma

within days or weeks after the initiation of VPA administration (Eadie and Tyrer, 1989). It was therefore decided to compare estimations of K_D, α and target ranges for PHT, CBZ and PB in patients co-medicated with VPA with estimates obtained in the situation of PHT, CBZ or PB monotherapy.

7.3 Material and Methods

7.3.1 Subjects

Samples were obtained from epileptic patients between the ages of 22 and 58 years with normal biochemical profiles, renal function and liver function, receiving PHT, CBZ or PB in combination with VPA as is indicated in Table 7.1 (Groups B, D and F). Data from Chapter 4 for patients on AED monotherapy (Groups A, C and E) have been included for the purpose of comparison (Table 7.1). The criteria for inclusion in the study were that the patients had been established on their prescribed drug regimens for at least six months and had achieved steady-state serum levels of prescribed drugs.

7.3.2 Blood Samples

Non-heparinised samples were collected, separated and stored as described in Chapter 2.

7.3.3 Analytical Procedures

Separation of F-PHT and F-CBZ from the protein-bound components was carried out at 25 °C and 37 °C using the Amicon Centrifree micropartition system, and the free and total levels of PHT and CBZ were analysed by HPLC as described in Chapter 2. Serum albumin was analysed by RA-XT analyser as described in Chapter 4.

7.3.4 Calculations

Values for K_D (unit: L/μmol) were calculated using Equation (3.8), (Chapter 3, page 116);

$$K_D = ([D_T] - [D_F])/([D_F]*[P]) \qquad \text{Equation (3.8)}$$

where $[D_T]$ is the total drug level (unit: μmol/L), $[D_F]$ is the free drug level (unit: μmol/L) and [P] is the albumin level (unit: μmol/L) in serum.

Values for the free fraction (α) were calculated using Equation (3.5), (Chapter 3);

$$\alpha = [D_F]/[D_T] \qquad \text{Equation (3.5)}$$

TABLE 7.1 DRUG REGIMENS OF PATIENTS IN MONOTHERAPY AND POLYTHERAPY STUDY GROUPS

Group	Number of patients		Medication range (mg/day)			
	(male)	(female)	PHT	CBZ	PB	VPA
A	20	8	(200 - 400)	--	--	--
B	16	12	(200 - 400)	--	--	(400 - 2600)
C	14	16	--	(600 - 2000)	--	--
D	15	13	--	(600 - 2000)	--	(600 - 2000)
E	16	9	--	--	(60 - 180)	--
F	5	8	--		(60 - 180)	(600 - 2000)

7.4 Results

7.4.1 K_D, α and Target Ranges at 25 °C and 37 °C for PHT in Patients Co-medicated with VPA

Values for K_D and α at 25 °C and 37 °C for patients on PHT combined with VPA (Group B) are presented in Tables 7.2 and 7.3 respectively; CV is less than 17% for both parameters. The estimate of K_D(PHT+VPA) at 25 °C is 29% lower compared to K_D(PHT), and the estimate of α is 40% higher compared with the situation of PHT monotherapy. Similarly the estimate of K_D(PHT+VPA) at 37 °C is 39% lower compared to K_D(PHT), and the estimate of α is 43% higher compared with PHT monotherapy.

The relationships between F-PHT and total PHT at 25 °C and 37 °C in patients taking PHT with VPA (Group B) are presented in Figures 7.1A and 7.1B respectively. In both instances close but separate correlations (with different slopes) are found.

The equations for the regression lines were then used in calculating target values for F-PHT in patients receiving PHT and VPA, using established therapeutic ranges for total PHT (see Chapter 5). A comparison between the target values for F-PHT monotherapy and F-PHT polytherapy (patients receiving PHT with VPA) at 25 °C and 37 °C is presented in Table 7.4.

7.4.2 K_D, α and Target Ranges at 25 °C and 37 °C for CBZ in Patients Co-medicated with VPA

Values for K_D and α at 25 °C and 37 °C for patients on CBZ combined with VPA (Group D) are presented in Tables 7.2 and 7.3 respectively; CV for the estimate is less than 14% for both parameters. The estimate of K_D(CBZ+VPA) at 25 °C is 15% lower compared to K_D(CBZ), and the estimate of α is 15% higher compared with the situation of CBZ monotherapy. Similarly the estimate of K_D(CBZ+VPA) at 37 °C is 22% lower compared to K_D(CBZ), and the estimate of α is 17% higher compared with the situation of CBZ monotherapy.

The relationships between F-CBZ and total CBZ at 25 °C and 37 °C in patients taking CBZ with VPA (Group D) are presented in Figures 7.2A and 7.2B respectively. In both instances close but separate correlations (with different slopes) are found.

The equations for the regression lines were then used in calculating target values for F-CBZ in patients receiving CBZ and VPA, using established therapeutic ranges for total CBZ (see Chapter 5). A comparison between the target values for F-CBZ monotherapy and F-CBZ polytherapy (patients receiving CBZ with VPA) at 25 °C and 37 °C are presented in Table 7.4.

7.4.3 K_D, α and Target ranges at 25 °C and 37 °C for PB in Patients Co-medicated with VPA

Values for K_D and α at 25 °C and 37 °C for patients on PB combined with VPA (Group F) are presented in Tables 7.2 and 7.3 respectively. CV for the estimate is less than 6% for both parameters. The estimate of K_D(PB+VPA) at 25 °C is 11% lower compared with K_D(PB), and the estimate of α is 11% higher compared with the situation of PB monotherapy. Similarly the estimate of K_D(PB+VPA) at 37 °C is 20% lower compared to K_D(PB), and the estimate of α is 12% higher compared with the situation of PB monotherapy.

The relationships between F-PB and total PB at 25 °C and 37 °C in patients taking PB with VPA (Group E) are presented in Figures 7.3A and 7.3B respectively. In both instances close but separate correlations (with different slopes) are found.

The equations for the regression lines were then used in calculating target values for F-PB in patients receiving PB and VPA, using established therapeutic ranges for total PB.

A comparison between the target values, for F-PB monotherapy and F-PB polytherapy (patients receiving PB with VPA) at 25 °C and 37 °C are presented in Table 7.4.

TABLE 7.2 THE EFFECT OF VPA ON K_D AT 25 °C AND 37 °C FOR PHT, CBZ AND PB

Group	N	Drug regimen	K_D x 10^{-3} ± SD (L/μmol) 25 °C	CV%	K_D x 10^{-3} ± SD (L/μmol) 37 °C	CV%
A	28	PHT	12.72 ± 1.43¥	11	9.81 ± 1.26¥	13
B	28	PHT + VPA	9.01 ± 1.51***	17	5.97 ± 0.85***	14
C	30	CBZ	3.96 ± 0.46¥	12	3.34 ± 0.47¥	14
D	30	CBZ + VPA	3.36 ± 0.48***	14	2.61 ± 0.22***	8
E	25	PB	1.12 ± 0.15¥	13	0.98 ± 0.14¥	14
F	13	PB + VPA	0.91 ± 0.03***	3	0.78 ± 0.05***	6

¥ Data from Chapter 4, Table 4.3

*** t-test (un-paired) $p < 0.001$

TABLE 7.3 THE EFFECT OF VPA ON FREE FRACTION (α) AT 25 °C AND 37 °C FOR PHT, CBZ AND PB

Group	N	Drug regimen	(α ± SD) 25 °C	CV%	(α ± SD) 37 °C	CV%
A	28	PHT	0.10 ± 0.01¥	13	0.14 ± 0.02¥	14
B	28	PHT + VPA	0.14 ± 0.02***	15	0.20 ± 0.02	11***
C	30	CBZ	0.26 ± 0.02¥	9	0.30 ± 0.03¥	10
D	30	CBZ + VPA	0.30 ± 0.03***	10	0.35 ± 0.02	6***
E	25	PB	0.57 ± 0.04¥	7	0.60 ± 0.04¥	7
F	13	PB + VPA	0.63 ± 0.08***	3	0.67 ± 0.01	3***

¥ Data from Chapter 4, Table 4.4

*** t-test (un-paired) $p < 0.001$

Figure 7.1A F-PHT at 25 °C versus total PHT for patients on **PHT and VPA.**

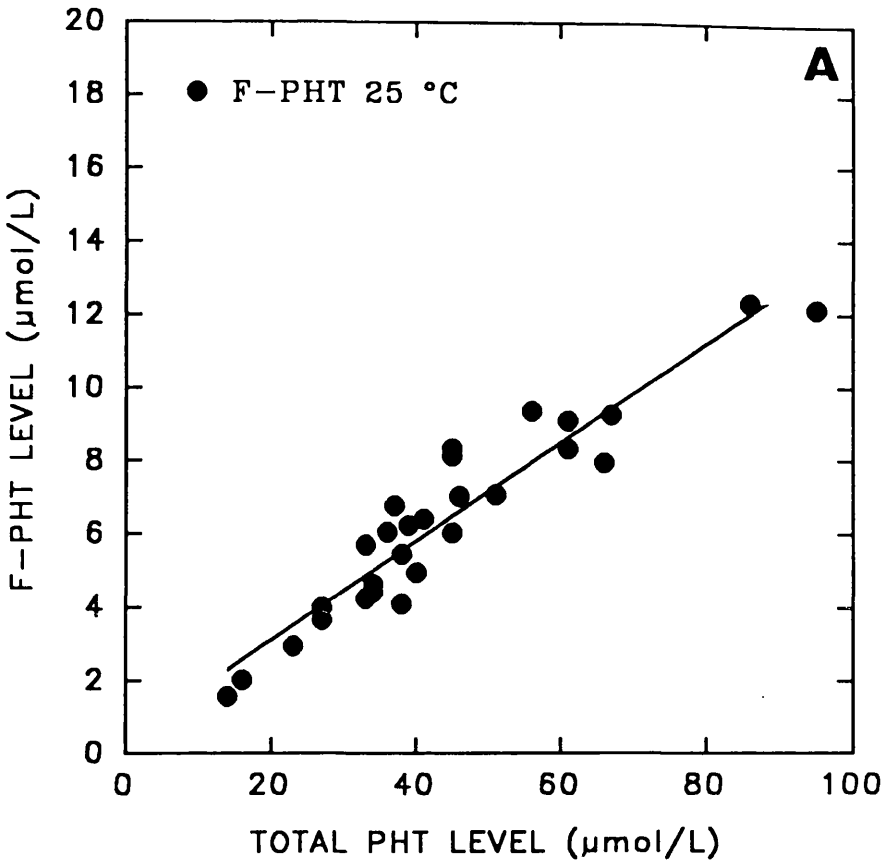
F-PHT = 0.407 + 0.135PHT;

r = 0.9455; p<0.001; n = 28.

Figure 7.1B F-PHT at 37 °C versus total PHT for patients on **PHT and VPA.**

F-PHT = 0.706 + 0.184PHT;

r = 0.9697; p<0.001; n = 28.


A
F-PHT 25 °C
F-PHT LEVEL (μmol/L)
TOTAL PHT LEVEL (μmol/L)
0
2
4
6
8
10
12
14
16
18
20
0
20
40
60
80
100

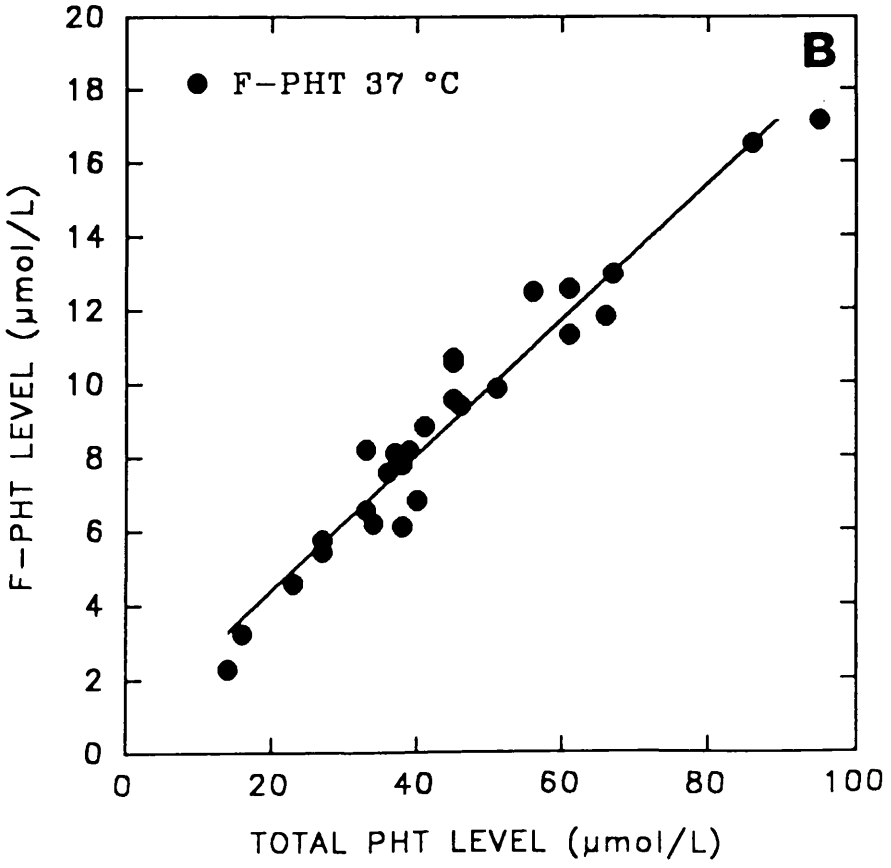

B
F-PHT 37 °C
F-PHT LEVEL (μmol/L)
TOTAL PHT LEVEL (μmol/L)
0
2
4
6
8
10
12
14
16
18
20
0
20
40
60
80
100

Figure 7.2A F-CBZ at 25 °C versus total CBZ for patients on **CBZ and VPA.**

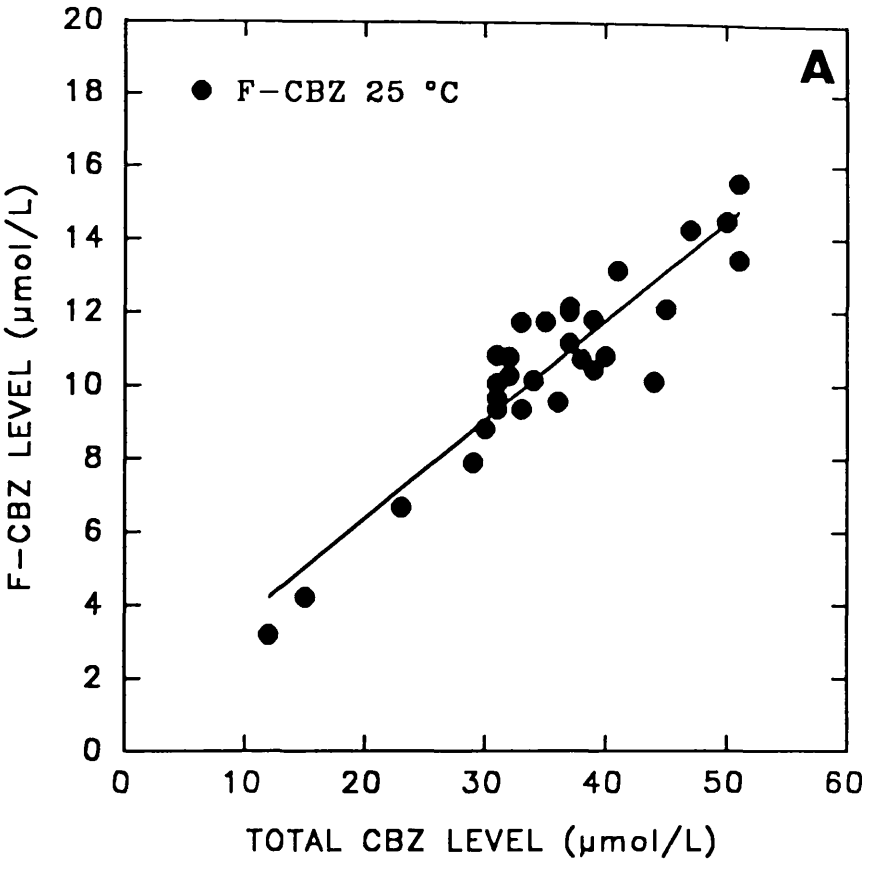
F-CBZ = 0.976 + 0.270CBZ;

r = 0.9200; p<0.001; n = 28.

Figure 7.2B F-CBZ at 37 °C versus total CBZ for patients on **CBZ and VPA.**

F-CBZ = 0.562 + 0.336CBZ;

r = 0.9770; p<0.001; n = 30.


A
F-CBZ 25 °C
F-CBZ LEVEL (µmol/L)
TOTAL CBZ LEVEL (µmol/L)
0
2
4
6
8
10
12
14
16
18
20
0
10
20
30
40
50
60

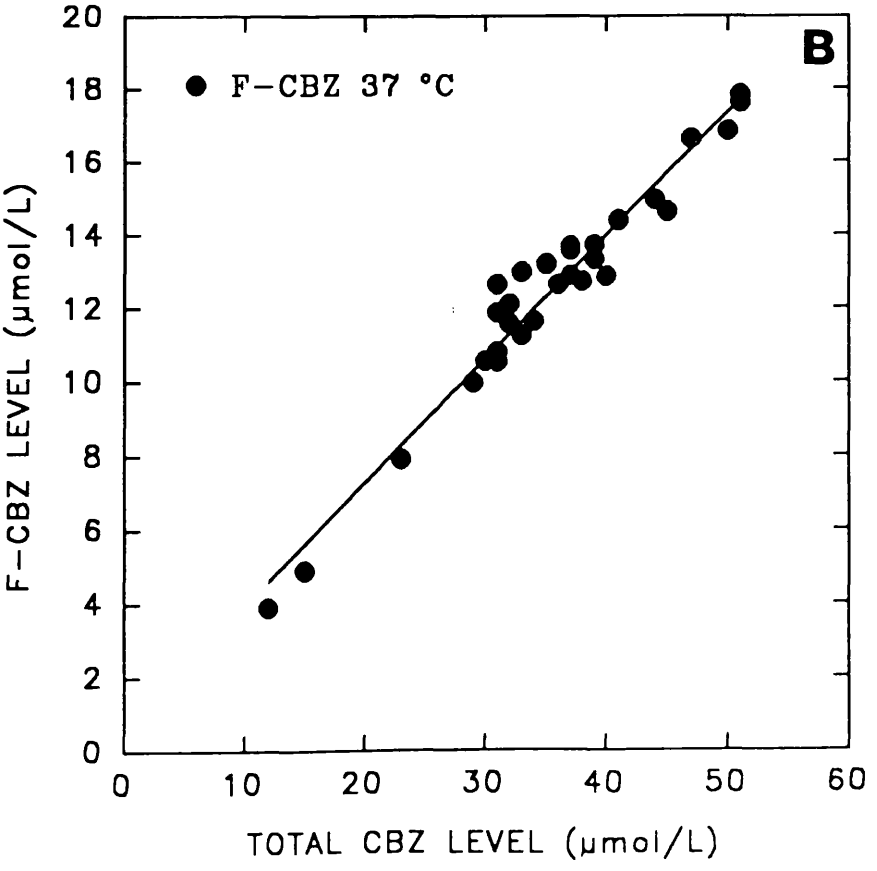

B
F-CBZ 37 °C
F-CBZ LEVEL (µmol/L)
TOTAL CBZ LEVEL (µmol/L)
0
2
4
6
8
10
12
14
16
18
20
0
10
20
30
40
50
60

Figure 7.3A F-PB at 25 °C versus total PB for patients on **PB and VPA.**

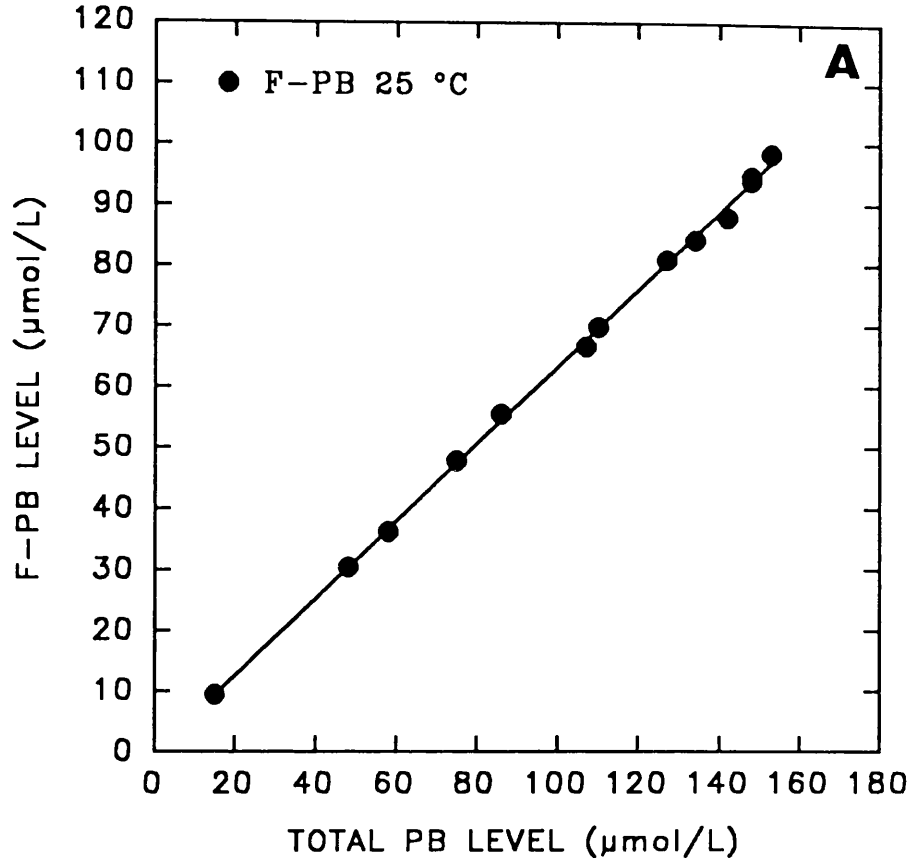
F-PB = - 0.209 + 0.636PB;

r = 0.9995; p<0.001; n = 13.

Figure 7.3B F-PB at 37 °C versus total PB for patients on **PB and VPA.**

F-PB = 0.051 + 0.666PB;

r = 0.9988; p<0.001; n = 13.


A
F-PB 25 °C
F-PB LEVEL (µmol/L)
TOTAL PB LEVEL (µmol/L)
0
10
20
30
40
50
60
70
80
90
100
110
120
0
20
40
60
80
100
120
140
160
180

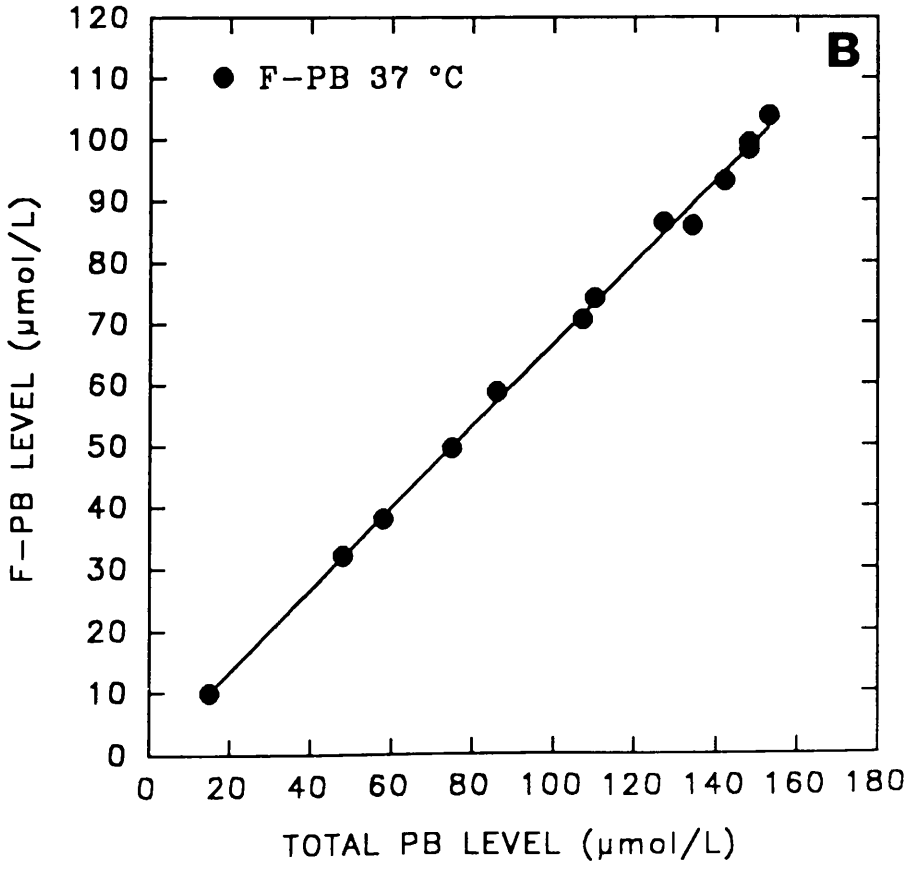

B
F-PB 37 °C
F-PB LEVEL (µmol/L)
TOTAL PB LEVEL (µmol/L)
0
10
20
30
40
50
60
70
80
90
100
110
120
0
20
40
60
80
100
120
140
160
180

TABLE 7.4 TARGET RANGES FOR F-PHT, F-CBZ AND F-PB AT 25 °C AND 37 °C FOR PATIENTS CO-MEDICATED WITH VPA

	Target range (free level) (derived from regression equations) (μmol/L)		Therapeutic range (total level)* (μmol/L)
	25 °C	37 °C	
PHT	4.0 - 8.0*	6.0 - 12.0*	40 - 80
PHT+VPA	5.8 - 11.2	8.1 - 15.4	
CBZ	5.2 - 13.0*	6.0 - 15.0*	20 - 50
CBZ+VPA	6.4 - 14.5	7.3 - 17.4	
PB	36.0 - 104.0*	38.0 - 108.0*	60 - 170
PB+VPA	38.0-108.0	40.0-113.0	

* Data from Chapter 5, Table 5.3.

7.5 Discussion

The present study shows that in the presence of VPA, the value of K_D decreases in the order K_D(PHT) > K_D(CBZ) > K_D(PB) and the value of α increases in the order PHT > CBZ > PB at 25 °C and 37 °C, compared with the situation of monotherapy (Tables 7.2 and 7.3). These decreases in the value of K_D and increases in the value of α are statistically significant (Tables 7.2 and 7.3). The largest effects are seen in the case of PHT, compared with CBZ and PB. These results indicate that PHT is the most easily displaced from albumin by VPA and PB the least, with CBZ falling between PHT and PB.

Target ranges derived using regression equations at 25 °C and 37 °C show that these ranges shifted upwards for F-PHT, F-CBZ and F-PB at both temperatures in patients co-medicated with VPA, compared to the situation for the patients on monotherapy (Table 7.4). This shift in target ranges for polytherapy patients indicates that the free drug levels for PHT, CBZ and PB have increased in the presence of VPA.

7.6 Summary

In this chapter significant changes in K_D and free fraction (α) for PHT, CBZ or PB were observed in groups of patients co-medicated with VPA compared to those

patients on monotherapy. The decrease in the value of K_D and increase in the value of α in polytherapy patients compared with monotherapy patients are statistically significant. The target ranges for both 25 °C and 37 °C were raised for F-PHT, F-CBZ and F-PB in patients co-medicated with VPA compared with the patients on monotherapy.

CHAPTER 8

COMPARISON OF PREDICTED AND OBSERVED F-PHT AND F-CBZ AT 37 °C IN PATIENTS CO-MEDICATED WITH VPA

8.1 Aim

To assess the accuracy and the precision of the predictive method for F-PHT and F-CBZ in patients co-medicated with VPA.

8.2 Introduction

It has been shown in the preceding chapter (Chapter 7), that VPA displaces PHT from albumin and increases the free fraction and F-PHT level in serum (Bruni et al, 1980; Perucca, 1984; Baird-Lambert et al, 1987). Similarly, VPA displaces CBZ from albumin and increases the free fraction and F-CBZ level in serum (Mattson et al, 1982). Prediction of F-PHT in the polytherapy situation is considered to be complex and thus not feasible (Dasgupta et al, 1991), and to date only one method has been published for predicting F-PHT levels in patients co-medicated with VPA (Haidukewych et al, 1989). In this chapter information is presented to assess the applicability of the formula derived in Chap-

ter 3, for the purpose of predicting F-PHT and F-CBZ levels in patients co-medicated with VPA.

The formula derived in Chapter 3 for predicting the free level of a drug, repeated here for convenience, is as follows:-

$$[D_F] = [D_T]/(1+K_D*[P]) \qquad \text{Equation (3.7)}$$

where $[D_F]$ is the free drug level (unit: μmol/L) and $[D_T]$ is the total drug level (unit: μmol/L), K_D is the apparent dissociation constant (unit: L/μmol) in the polytherapy situation and [P] is the albumin level (unit: μmol/L) in serum. If the total drug level, the albumin level and K_D are known, the free level can be calculated.

In practice total drug and albumin levels can be measured in individual samples. However K_D, is a derived entity. Therefore average values for K_D in the monotherapy and polytherapy situation were derived from experimental data in the preceding chapter (Chapter 7). These estimates demonstrated a low CV (< 14 %) in all instances and thus could be acceptable for use in predicting free levels. In this chapter this assumption has been tested by predicting F-PHT and F-CBZ at 37 °C in patients co-medicated with VPA. This study also

tests the transferability of the constants in polytherapy patients.

8.3 Materials and Methods

8.3.1 Subjects

Epileptic patients on PHT and VPA and CBZ and VPA (n = 42; female = 16, male = 26) between the ages of 26 and 58 years with normal biochemical profiles, renal function and liver function comprised the study groups.

The criteria for inclusion in this study were that the patients had been established on their prescribed drug regimens for at least six months and had achieved steady-state serum levels of prescribed drugs.

8.3.2 Blood Samples

Non-heparinised samples were collected, separated and stored as described in Chapter 2. The samples were divided into groups G and H according to the combination of drugs prescribed for the patients as shown in Table 8.1.

8.3.3 Analytical Procedures

Separation of F-PHT and F-CBZ from the protein-bound components was carried out at 37 °C using the Amicon Centrifree micropartition system, and the free and total levels of PHT and CBZ were analysed by HPLC as

described in Chapter 2. Total serum levels of VPA were measured by the FPIA procedure as described in Chapter 2, using valproic acid kits. Serum albumin was analysed by RA-XT analyser as described in Chapter 4.

8.3.4 Calculations

Individual values for predicting F-PHT and F-CBZ were calculated using Equation (3.7). The values for K_D(PHT+VPA) and K_D(CBZ+VPA) used in the Equation (3.7) for the calculation of F-PHT and F-CBZ were 5.9731 x 10^{-3} and 2.6059 x 10^{-3} respectively (Table 7.2, Chapter 7).

Inaccuracy was measured by prediction-error analysis (Sheiner and Beal, 1981), (see Chapter 6 for further details on the calculation of prediction-error analysis).

The percentage difference between the predicted and observed levels is calculated as follows:-

[(pred - obs)/obs] * 100

TABLE 8.1 DRUG REGIMENS OF PATIENTS IN PHT AND CBZ STUDY GROUPS

Group	Number of patients		Medication range (mg/day)		
	(male)	(female)	PHT	CBZ	VPA
G	16	6	(200 - 400)	--	(400 - 2600)
H	10	10	--	(600 - 1800)	(600 - 3000)

8.4 Results

In Figures 8.1A and 8.1B observed values for F-PHT and F-CBZ are correlated with predicted F-PHT and F-CBZ in patients co-medicated with VPA. There is very good correlation between the observed and predicted values for free levels of both drugs with r-values > 0.968. Group means (SD) for observed and predicted free levels together with the outcome of the prediction-error analysis are presented in Table 8.2. The mean prediction error (MPE) ± (SD) between predicted and observed F-PHT levels is - 0.06 ± (0.32) μmol/L. The corresponding value for F-CBZ is - 0.10 ± (0.68) μmol/L (Table 8.2). These results indicate that the performance of the prediction method is very satisfactory and that values for K_D are transferable under steady state conditions.

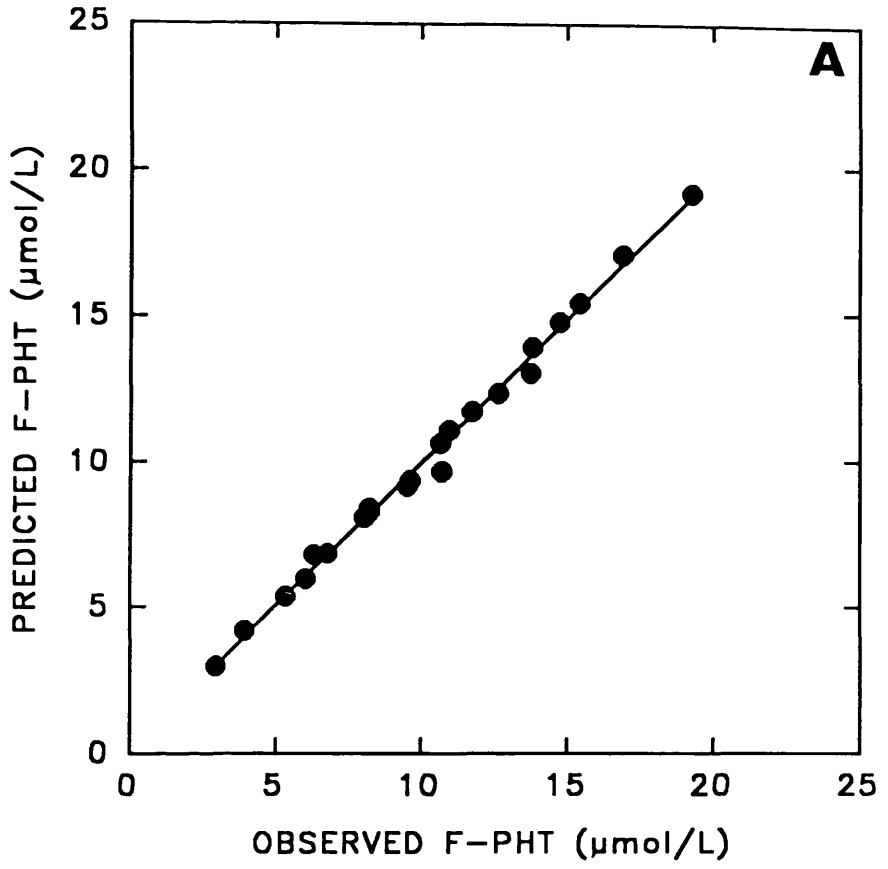
Figures 8.2A and 8.2B show scatter plots for the percentage difference between predicted and observed free levels versus the observed levels for F-PHT and F-CBZ levels respectively. For F-PHT, 95% of the predicted values fall within ± 7% of the observed measurement and for F-CBZ, 95% of predicted values fall within ± 8% of the observed measurement.

Figure 8.1A Observed versus predicted F-PHT levels at 37 °C.

$F\text{-}PHT_{(obs.)} = 0.122 + 0.983$ $F\text{-}PHT_{(pred.)}$; $r = 0.9972$; $p < 0.001$; $n = 22$.

Figure 8.1B Observed versus predicted F-CBZ levels at 37 °C.

$F\text{-}CBZ_{(obs.)} = 0.969 + 0.928$ $F\text{-}CBZ_{(pred.)}$; $r = 0.9853$; $p < 0.001$; $n = 20$.


A
PREDICTED F-PHT (μmol/L)
OBSERVED F-PHT (μmol/L)
0
5
10
15
20
25

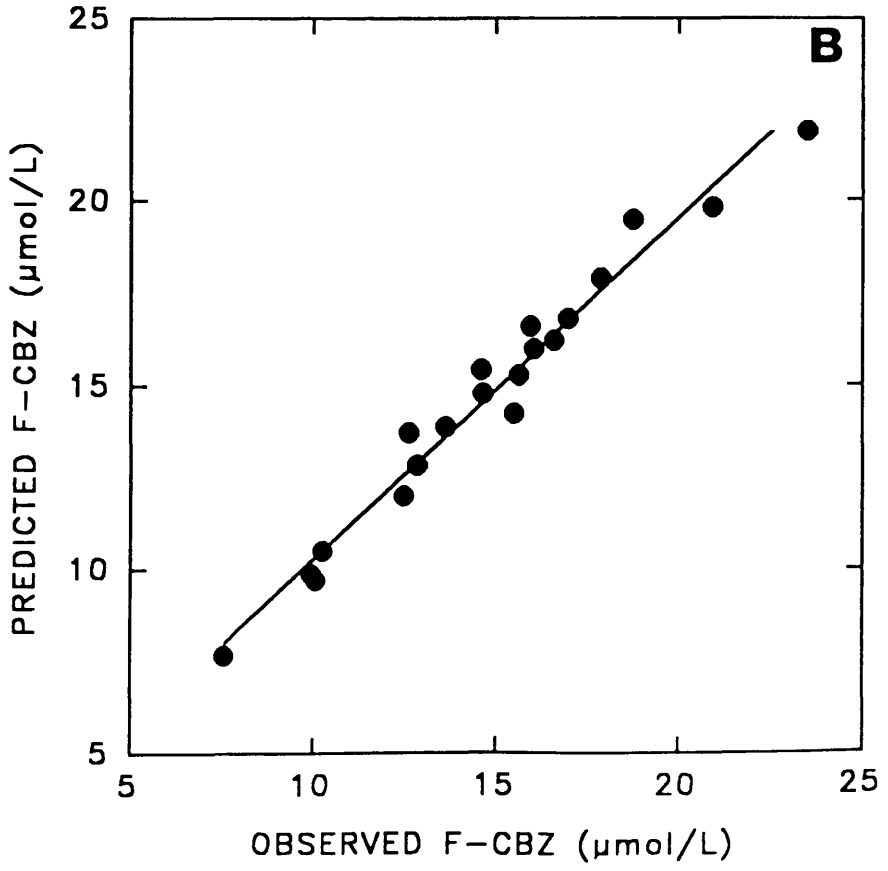
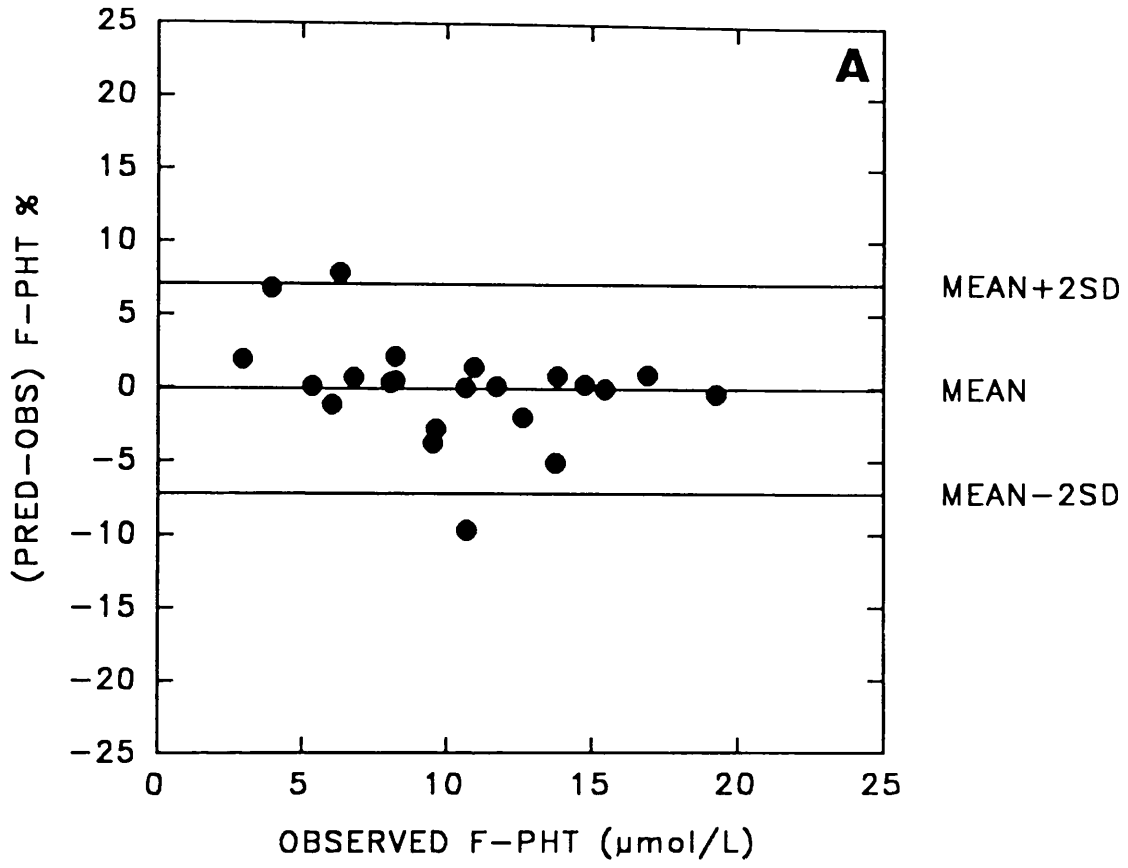

B
PREDICTED F-CBZ (μmol/L)
OBSERVED F-CBZ (μmol/L)
5
10
15
20
25

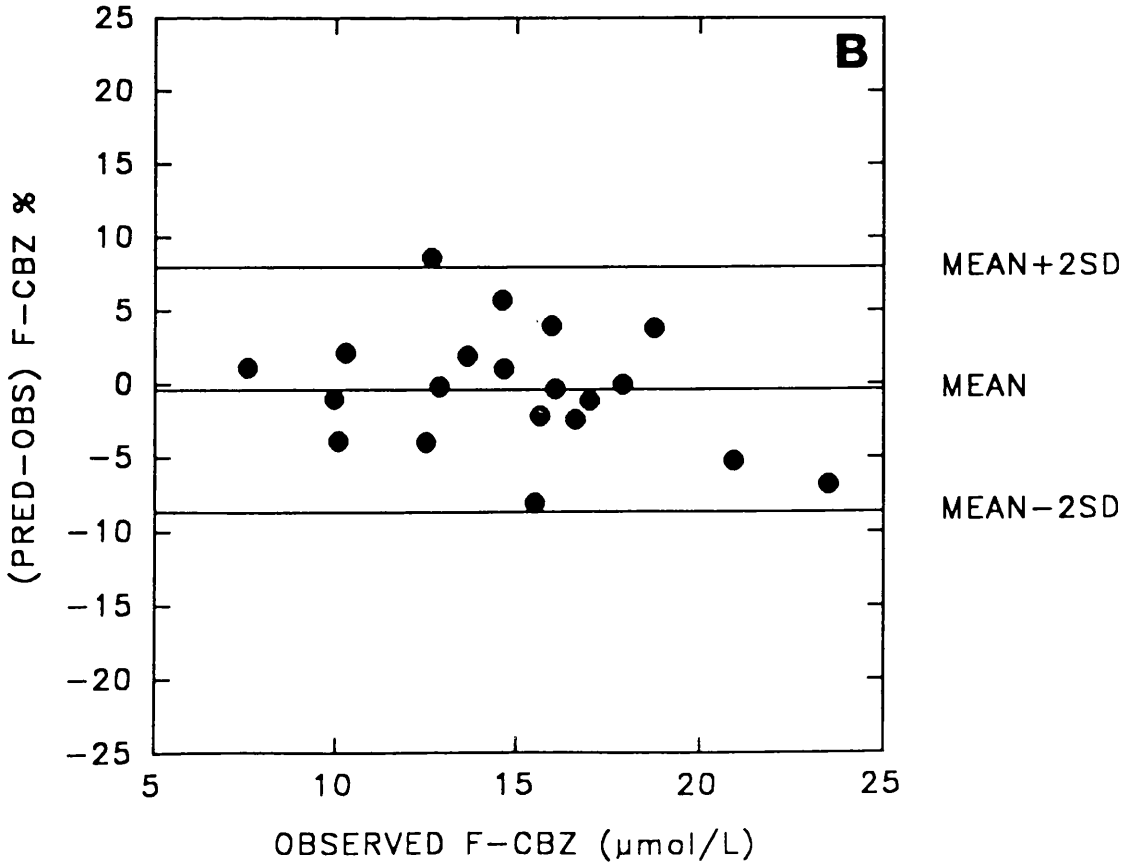
TABLE 8.2 PREDICTION ERROR ANALYSIS FOR F-PHT AND F-CBZ LEVELS

	N	Mean observed free level (μmol/L)	Mean predicted free level (μmol/L)	MPE (μmol/L)	RMSE
F-PHT	22	10.25 ± 4.27	10.19 ± 4.21	- 0.06 ± 0.32	0.130
F-CBZ	20	14.83 ± 3.86	14.73 ± 3.64	- 0.10 ± 0.68	0.800

Figure 8.2A Difference (%) between predicted and observed F-PHT versus observed F-PHT at 37 °C.

Figure 8.2B Difference (%) between predicted and observed F-CBZ versus observed F-CBZ at 37 °C.


A
25
20
15
10
5
0
−5
−10
−15
−20
−25
0
5
10
15
20
25
(PRED−OBS) F−PHT %
OBSERVED F−PHT (μmol/L)
MEAN+2SD
MEAN
MEAN−2SD


B
25
20
15
10
5
0
−5
−10
−15
−20
−25
5
10
15
20
25
(PRED−OBS) F−CBZ %
OBSERVED F−CBZ (μmol/L)
MEAN+2SD
MEAN
MEAN−2SD

8.5 Discussion

In this chapter evidence is presented that the predictive method described in Chapter 3 can be used for calculating F-PHT and F-CBZ levels accurately from measurements of total drug level, albumin levels and an average value of K_D, in the situation of drug displacement (Figures 8.1A and 8.1B). The MPE between predicted and observed F-PHT value of 0.06 ± 0.32 μmol/L for 37 °C should be compared with the target range for F-PHT of 6 to 12 μmol/L (see Chapter 5). Similarly the MPE between predicted and observed F-CBZ value of 0.10 ± 0.68 μmol/L for 37 °C should be compared with the target range for F-CBZ of 6 to 15 μmol/L (see Chapter 5). It can be seen that the MPE is very small compared with the target ranges for both drugs. For F-PHT 95% of the predicted values fall within ± 7% of the observed measurement (Figure 8.2A). Similarly for F-CBZ 95% of the predicted values fall within ± 8% of the observed measurement (Figure 8.2B). These results indicate that the prediction of F-PHT and F-CBZ levels by the method proposed here can be applied routinely in the clinical situation.

The correlations between predicted and observed F-PHT and F-CBZ levels are very good (Figures 8.1A to 8.2B). These results indicate that the value for K_D is transferable between groups of patients with albumin values in serum within the reference range for normal subjects. Thus, it is reasonable to assume that values for

K_D will not vary significantly between groups of patients when a identical methodology and condition are used. However the K_D values used in this study, though appropriate for healthy epileptic subjects, would not necessarily be applicable to epileptic patients with concomitant hepatic and renal disorders for the reasons discussed in Chapter 6.

To date one method for the prediction of F-PHT levels in patients co-medicated with VPA has been described (Haidukewych et al, 1989). In this case the experimental procedure for separating F-PHT from its protein-bound component was carried out at 25 °C (Haidukewych et al, 1989). The present method predicted F-PHT and F-CBZ levels at 37 °C, which would reflect the free levels at physiological temperature. There are no data published for the performance of the Haidukewych method at physiological temperature.

8.6 Summary

A prediction method for the estimation of F-PHT and F-CBZ in the situation of drug displacement, based on the measurement of total levels and albumin levels and a value for K_D that had been experimentally derived, has been evaluated. The study has demonstrated that a value of K_D derived from epileptic patients without complicating disease can be used to estimate F-PHT or F-CBZ levels in patients co-medicated with VPA with a degree of accuracy and precision that should be clinically acceptable.

CHAPTER 9

COMPARISON OF METHODS FOR PREDICTING F-PHT LEVELS AT 25 ·C IN MONOTHERAPY AND POLYTHERAPY PATIENTS

9.1 Aim

To compare the predictive method developed in this thesis with the methods of Sheiner-Tozer, Haidukewych and Dasgupta.

9.2 Introduction

Four methods for predicting F-PHT levels in serum have recently emerged in the literature. All four methods are based on statistical analysis of experimental data (Gugler et al, 1975; Winter and Tozer, 1986; Haidukewych et al, 1989; Dasgupta et al, 1991) (See Chapter 1 for a review of these methods).

The Gugler method does not provide an equation for calculating predictive values, and thus has not been included in this comparison. The Sheiner-Tozer method is intended for predicting F-PHT in hypoalbuminaemic subjects but it has been assumed that the method is also applicable in subjects with normal albumin levels. However, there is no indication in the Sheiner-Tozer

method at what temperature separation of F-PHT was carried out in order to obtain the experimental data for the derivation of the statistical equation. The Haidukewych and Dasgupta methods, however were both developed for prediction of F-PHT levels at 25 °C. The Haidukewych method is intended for predicting F-PHT in patients co-medicated with VPA, without measuring albumin. This is the only published method devised for predicting F-PHT levels in the situation of polytherapy.

Since little in the way of a comparison between these various methods has so far been published, a comparison has been made in this chapter between the prediction method developed in this thesis and the previously published methods for predicting F-PHT levels in serum. The average values of K_D for F-PHT used in the predictive formula derived here (see Chapter 3) were those calculated at 25 °C, because the experimental procedures for deriving statistical equations for the Haidukewych method and Dasgupta method were carried out at 25 °C. An assumption was made that separation of F-PHT from the protein-bound component was also carried out at 25 °C to obtain data for the Sheiner-Tozer equation, since this was the standard temperature used in many procedures at that time.

9.3 Materials and Methods

9.3.1 Subjects

Epileptic patients on PHT monotherapy and PHT with VPA (n = 40; female = 19, male = 21) between the ages of 22 and 54 years with normal biochemical profiles, renal function and liver function comprised the study groups.

The criteria for inclusion in this study were that the patients had been established on their prescribed drug regimens for at least six months and had achieved steady-state serum levels of prescribed drugs. The investigation was carried out up to nine months after the data were collected for estimating values for K_D (from a separate group of patients on AED monotherapy and polytherapy; see Chapter 7).

9.3.2 Blood Samples

Non-heparinised samples were collected separated and stored as described in Chapter 2. The samples were divided into groups I and J according to the combination of drugs prescribed for the patients as shown in Table 9.1.

9.3.3 Analytical Procedures

Total levels of PHT were analysed by HPLC as described in Chapter 2. Total serum levels of VPA were measured

by the FPIA procedure as described in Chapter 2, using valproic acid kits. Serum albumin was analysed by RA-XT analyser as described in Chapter 4.

9.3.4 Calculations

The following equations were used for calculating F-PHT:-

1. The present method (Method A):-

$$[D_F] = [D_T]/(1+K_D*[P]) \qquad \text{Equation (3.7)}$$

Individual values for predicted F-PHT for monotherapy and polytherapy were calculated using Equation (3.7), where $[D_F]$ is the free drug level (unit: μmol/L) and $[D_T]$ is the total drug level (unit: μmol/L), K_D is the apparent dissociation constant (unit: L/μmol) in the monotherapy and polytherapy situations, and [P] is the albumin concentration (unit: μmol/L) in serum. The average value for K_D(PHT) at 25 °C in the monotherapy situation is 12.72×10^{-3} and for K_D(PHT) in the polytherapy situation 9.01×10^{-3} (Chapter 7, Table 7.2).

2. Sheiner-Tozer method (Method B):-

$$PHT_{adjusted} = PHT_{observed}/(0.205 * albumin) + 0.1$$

Assuming a normal free fraction of 0.1 for PHT, one can predict F-PHT level from the adjusted PHT as follows:-

$$F\text{-}PHT = PHT_{adjusted} * 0.1$$

The conventional units for PHT and F-PHT in the above equation was μg/ml, the conversion factor used to calculate the SI unit of μmol/L for PHT and F-PHT is 3.964. Similarly the conventional unit for serum albumin is g/L, and the conversion factor used for the SI unit is 14.7059.

3. Haidukewych method (Method C):-

$$F\text{-}PHT = (0.095 + 0.001\ [VPA])\ [PHT];$$

where VPA and PHT represent total levels of drugs in plasma. The conventional unit for PHT, F-PHT and VPA is μg/ml. The conversion factor used to obtain the SI unit for PHT and F-PHT is 3.964 and for VPA 6.9348.

4. Dasgupta method (Method D):-

$$F\text{-}PHT = (83.1 * [PHT]/[ALBUMIN]) - 0.6);$$

The conventional unit for F-PHT, PHT and albumin is μmol/L.

The methods A to D are summarised in Table 9.2. When comparing values for F-PHT for the different methods it is assumed that the estimate obtained by the present method **(Method A)** represents the reference value. Thus F-PHT (Method A) appears as the independent variable in the graphs (Figures 9.1A, 9.2A and 9.3A) and not as is often the case the average of the two methods being compared (Bland and Altman, 1986).

TABLE 9.1 DRUG REGIMENS OF PATIENTS IN PHT STUDY GROUPS

Group	Number of patients		Medication range (mg/day)	
	(male)	(female)	PHT	VPA
I	14	6	(250 - 400)	--
J	9	11	(200 - 400)	(600 - 2000)

TABLE 9.2 PREDICTION METHODS FOR F-PHT

	METHOD A (Present method)	**METHOD B** (Sheiner-Tozer method)	**METHOD C** (Haidukewych method)	**METHOD D** (Dasgupta method)
Temperature	25 C°/37 C°	not stated	25 C°	25 C°
Drug	PHT and PHT/VPA CBZ and CBZ/VPA	PHT	PHT/VPA	PHT
Albumin	Normal	Hypoalbuminaemic	Normal	Normal

9.4 Results

9.4.1 Comparison of Method A with the Sheiner-Tozer Method (Method B)

Figure 9.1A shows the regression analysis of predicted F-PHT by methods A and B in patients receiving PHT monotherapy (Group I). There is good correlation between the values for F-PHT predicted by the two methods. Figure 9.1B shows scatter plots for the percentage difference between predicted F-PHT levels by Method B and predicted F-PHT levels by Method A versus the predicted F-PHT levels by Method A. For Method B, 95% of the predicted F-PHT values fall within ± 3% of the predicted values by Method A.

9.4.2 Comparison of Method A with the Dasgupta Method (Method D)

Figure 9.2A shows the regression analysis of predicted F-PHT by methods A and D in patients receiving PHT monotherapy (Group I). There is good correlation between the values for F-PHT predicted by the two methods. Figure 9.2B shows scatter plots for the percentage difference between predicted F-PHT levels by Method D and predicted F-PHT levels by Method A versus the predicted F-PHT levels by Method A. For Method D, 95% of the predicted F-PHT values fall within ± 20% of the predicted values by Method A.

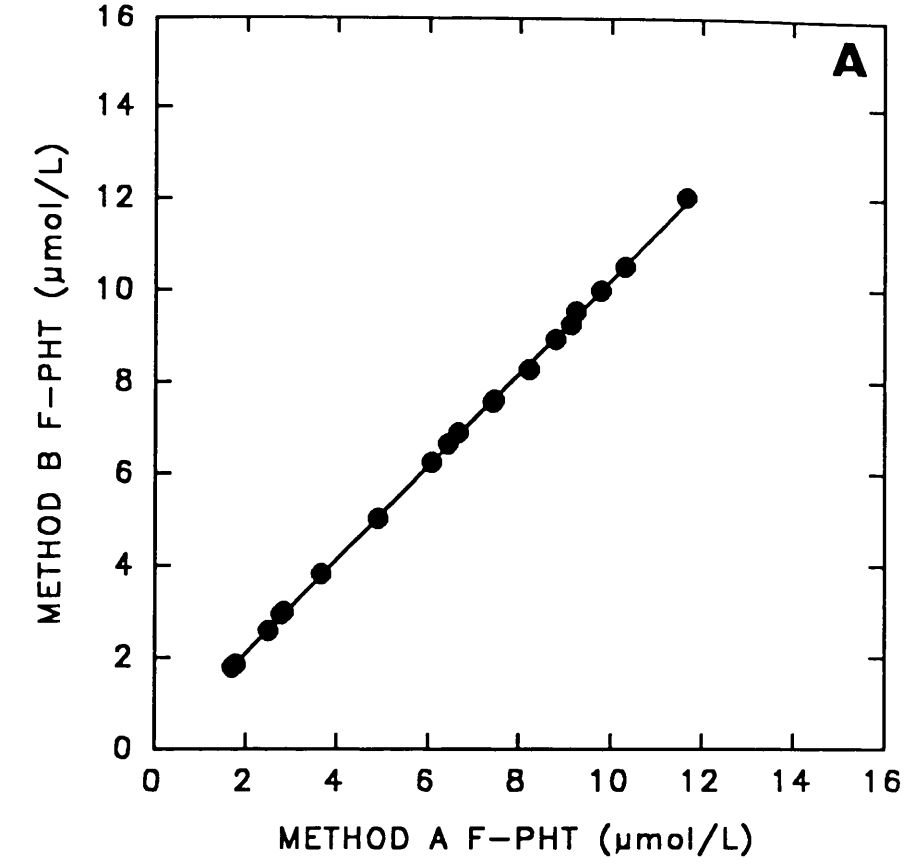
9.4.3 Comparison of Method A with the Haidukewych Method (Method C)

Figure 9.3A shows the regression analysis of predicted F-PHT by methods A and C in patients receiving PHT and VPA (Group J). There is reasonably good correlation between the values for F-PHT predicted by the two methods. Figure 9.3B shows scatter plots for the percentage difference between predicted F-PHT levels by Method C and predicted F-PHT levels by Method A versus the predicted F-PHT levels by Method A. For Method C, 95% of the predicted F-PHT values fall within ± 30% of the predicted values by Method A.

Figure 9.1A Predicted F-PHT levels by Method A **(Author's Method)** versus predicted F-PHT levels by Method B **(Sheiner-Tozer Method)** in patients taking **PHT.**

$F\text{-}PHT_{(method\ B)} = 0.056 + 1.020\ F\text{-}PHT_{(method\ A)}$; $r = 0.9998$; $p < 0.001$; $n = 20$.

Figure 9.1B Difference (%) between predicted F-PHT levels by Method B **(Sheiner-Tozer Method)** and Method A **(Author's Method)** versus predicted F-PHT levels by Method A.


A
METHOD B F-PHT (µmol/L)
METHOD A F-PHT (µmol/L)
0
2
4
6
8
10
12
14
16

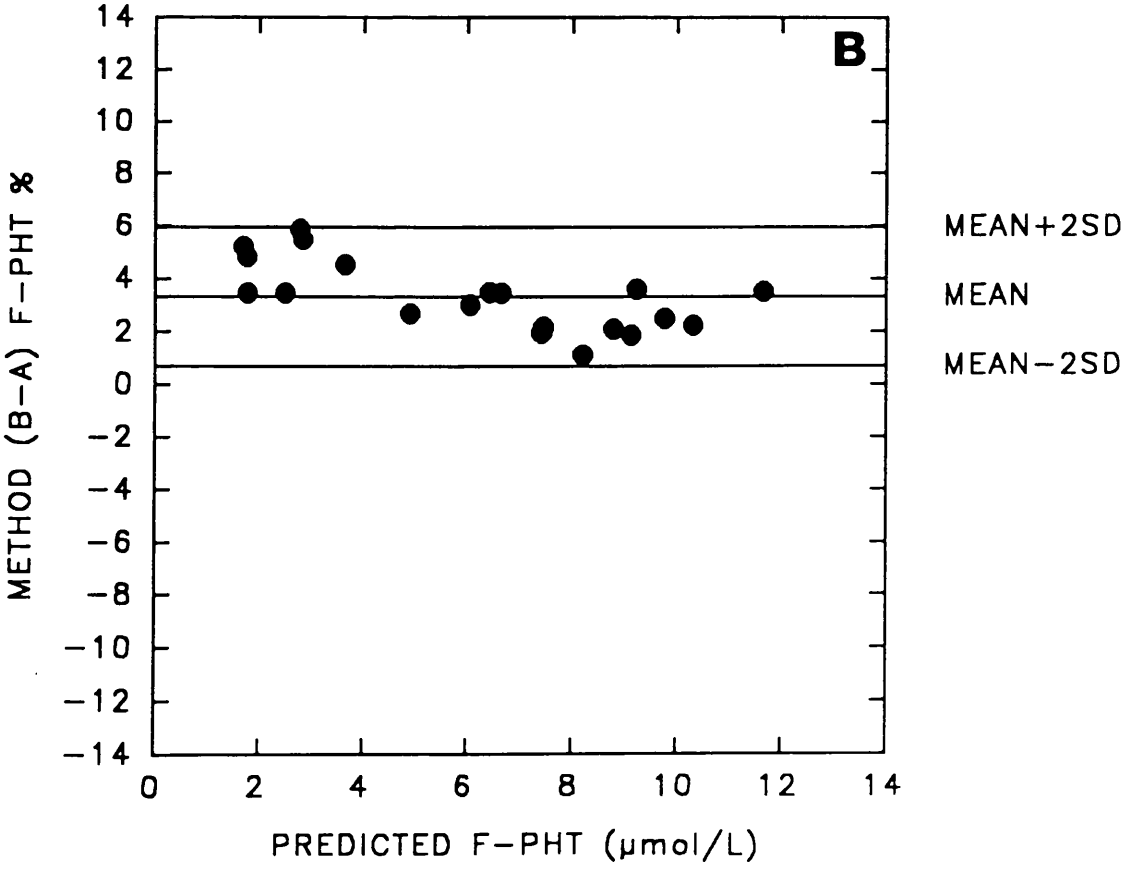
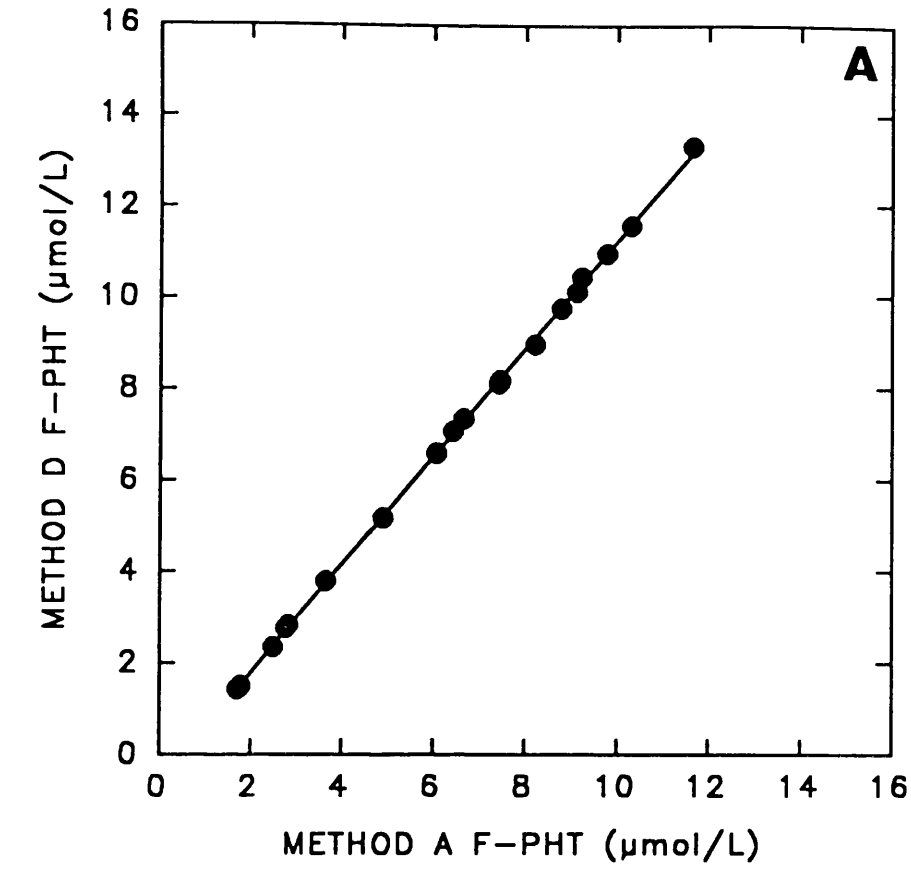

B
METHOD (B-A) F-PHT %
PREDICTED F-PHT (µmol/L)
MEAN+2SD
MEAN
MEAN-2SD
14
12
10
8
6
4
2
0
-2
-4
-6
-8
-10
-12
-14

Figure 9.2A Predicted F-PHT levels by Method A **(Author's Method)** versus predicted F-PHT levels by Method D **(Dasgupta Method)** in patients taking **PHT.**

$F\text{-}PHT_{(method\ D)} = -0.581 + 1.181\ F\text{-}PHT_{(method\ A)}$; $r = 0.9998$; $p < 0.001$; $n = 20$.

Figure 9.2B Difference (%) between predicted F-PHT levels by Method D **(Dasgupta Method)** and Method A **(Author's Method)** versus predicted F-PHT levels by Method A.


A
METHOD D F-PHT (µmol/L)
METHOD A F-PHT (µmol/L)
0
2
4
6
8
10
12
14
16

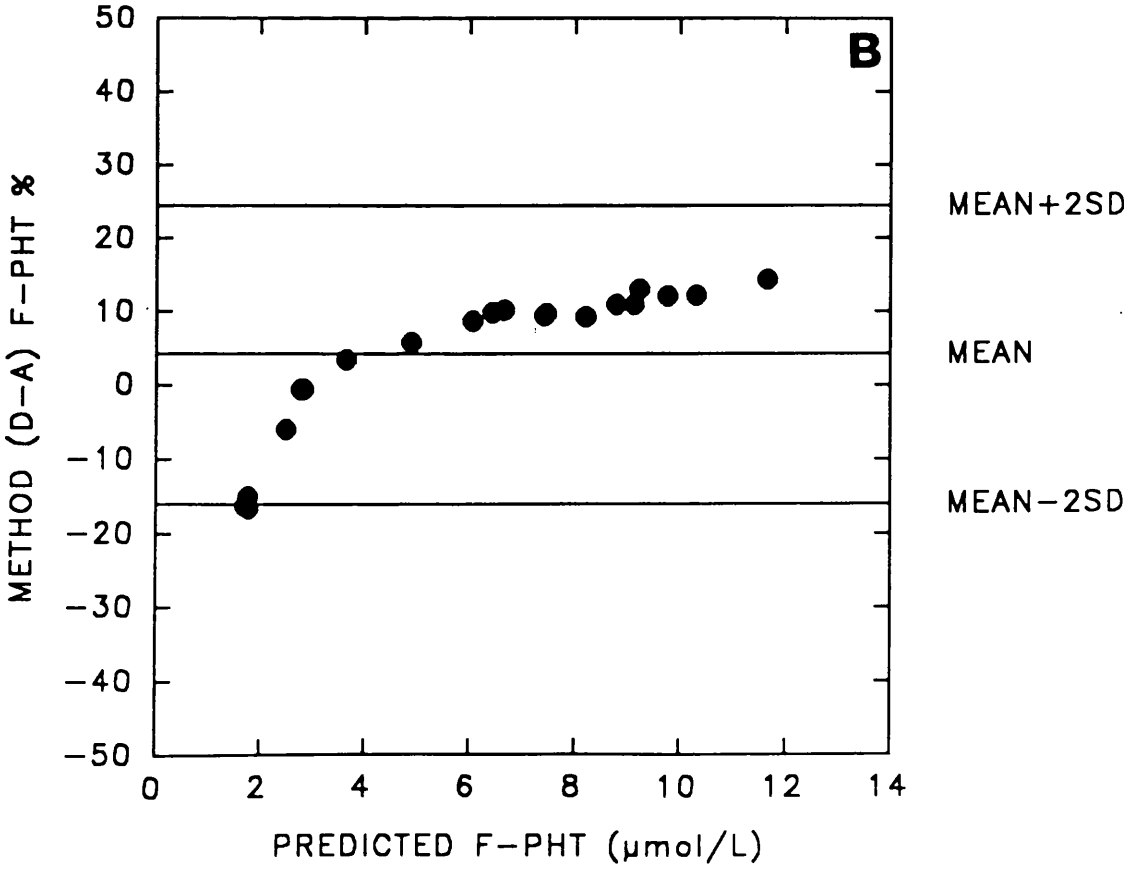
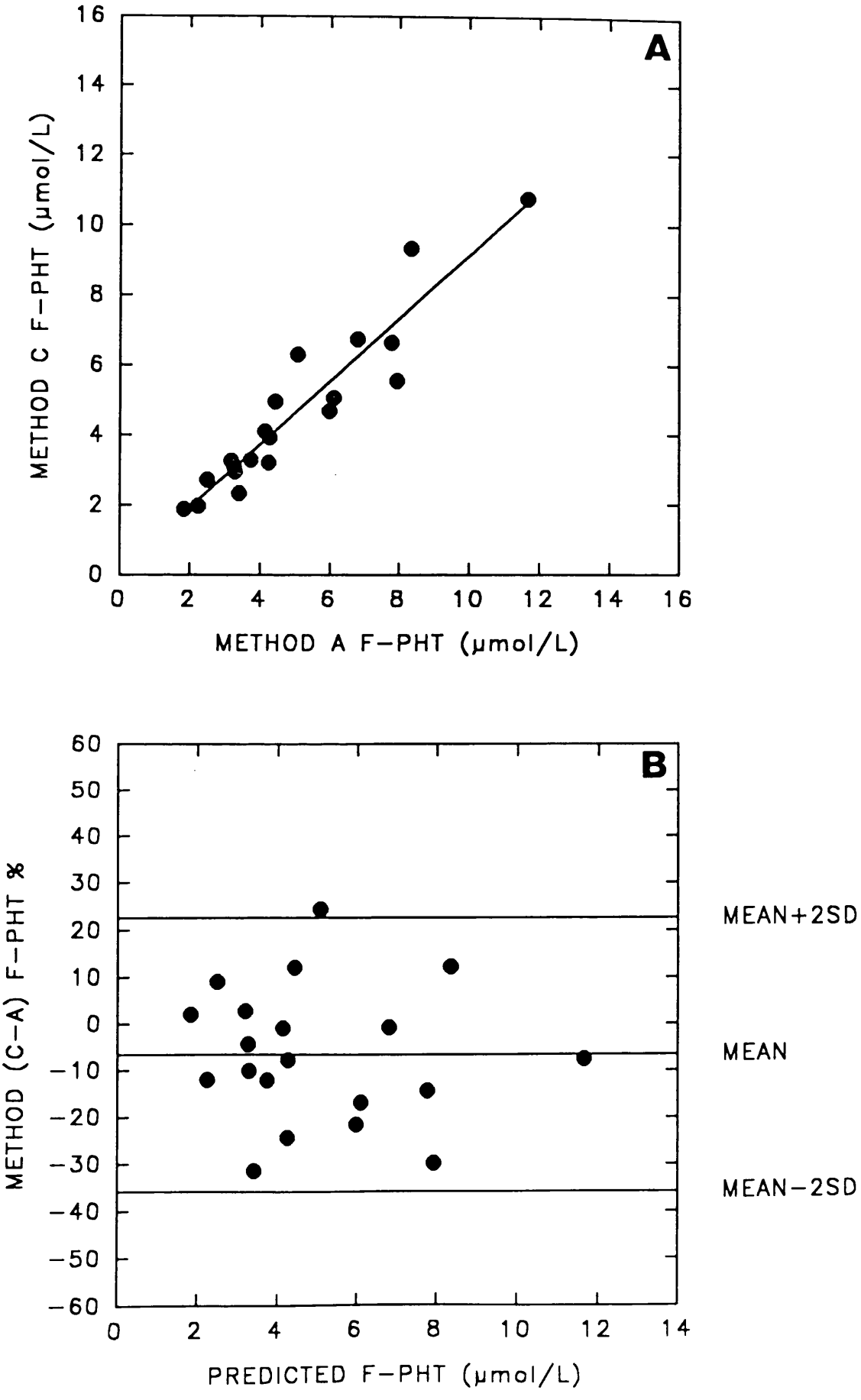

B
METHOD (D-A) F-PHT %
PREDICTED F-PHT (µmol/L)
MEAN+2SD
MEAN
MEAN-2SD
50
40
30
20
10
0
-10
-20
-30
-40
-50
0
2
4
6
8
10
12
14

Figure 9.3A Predicted F-PHT levels by Method A **(Author's Method)** versus predicted F-PHT levels by Method C **(Haidukewych Method)** in patients taking **PHT** and **VPA.**

$F\text{-}PHT_{(method\ C)} = 0.112 + 0.905\ F\text{-}PHT_{(method\ A)}$; $r = 0.9414$; $p < 0.001$; $n = 20$.

Figure 9.3B Difference (%) between predicted F-PHT levels by Method C **(Haidukewych Method)** and Method A **(Author's Method)** versus predicted F-PHT levels by Method A.


A
METHOD C F-PHT (μmol/L)
METHOD A F-PHT (μmol/L)
0
2
4
6
8
10
12
14
16
B
METHOD (C-A) F-PHT %
PREDICTED F-PHT (μmol/L)
MEAN+2SD
MEAN
MEAN-2SD
-60
-50
-40
-30
-20
-10
0
10
20
30
40
50
60

9.5 Discussion

In this chapter the method derived from the Law of Mass Action for predicting F-PHT levels in monotherapy and polytherapy patients (Method A) has been compared with three published predictive methods. In general, all the methods give good correlation with Method A. But Method B gives the best results by comparison with methods C and D. In Method B, 95% of the predicted F-PHT values fall within ± 3% of the predicted values by Method A (Figure 9.1B). However in Method B the predicted F-PHT is slightly over-estimated by comparison with Method A (Figure 9.1B). This bias may be due to the different analytical methods used in methods A and B.

There is good correlation between predicted F-PHT levels by methods A and D ($r = 0.9998$) but there is a trend in the prediction by Method D to underestimate F-PHT at lower levels and conversely to over-estimate F-PHT at higher levels (Figure 9.2B). For Method D, 95% of the predicted F-PHT values fall within ± 20% of the predicted values by Method A, which is methodologically unacceptable.

Method C compared with Method A gives slightly lower r-value ($r = 0.9414$). The reason for this comparatively weak correlation may be that albumin is a important factor in all the other methods for predicting F-PHT, while Method C does not include a value for albumin in the estimation of F-PHT. For Method C, 95% of the pre-

dicted F-PHT values fall within ± 30% of the predicted values by Method A. Which seems methodologically unacceptable.

The proposed method, Method A, can be considered a reliable method for predicting F-PHT levels in serum because correlation with measured values of F-PHT is very good (Chapters 6 and 8). Of the published methods, the Sheiner-Tozer method (Method B), which gives the best results in a comparison with Method A, would be acceptable in clinical practice. However it is important to realise that methods C and D were developed to predict F-PHT levels at 25 °C, and for Method B the temperature was not specified. The predicted values obtained by these methods will thus not necessarily reflect the F-PHT levels at the physiological temperature, i.e., it is not known how methods C and D would perform when applied to obtain results at 37 °C.

9.6 Summary

A comparison of the present method of predicting F-PHT levels with three published methods has demonstrated that the Sheiner-Tozer method is the only published method acceptable for predicting F-PHT levels. Even though the Haidukewych method and Dasgupta method give good correlation with the present method, by comparison 95% of the predicted F-PHT values fall within ± 20% and ± 30% respectively of the predicted values from the present method, which seems methodologically unacceptable.

CHAPTER 10

GENERAL DISCUSSION

In this thesis an HPLC micromethod has been developed for routine measurement of total and free AED levels. This method, combined with ultrafiltration, has been used to carry out a systematic study of the effect of temperature on the separation of free AED's from protein-bound components.

Facilities for the measurement of F-PHT or F-CBZ levels are not generally routinely available, but this study has shown that free levels of these AED's can be predicted in patients on monotherapy and polytherapy from measurements of total drug and albumin levels in serum.

10.1 Methodology for the Determination of Free Levels of AED's.

10.1.1 Equilibrium Dialysis

The advantages and disadvantages of equilibrium dialysis have been discussed in Chapter 1. Although equilibrium dialysis may be regarded as the "reference" method for the determination of free levels, it seems to be less suited for routinely monitoring the free drug levels in serum, primarily because the procedure takes between four and ten hours to complete.

10.1.2 Ultrafiltration

Ultrafiltration methods have the advantage of being fast and simple. Therefore, such methods would seem to be particularly suitable for routine monitoring of free drug levels. However, there are several potential problems associated with ultrafiltration procedures. In particular, interference caused by drug absorption to the ultrafiltration device or protein leakage into the filtrate must be ruled out. The results of the recovery experiments using pure drug standards carried out in the present study have demonstrated that there is no significant absorption of AED's onto the ultrafiltration device used (Chapter 2), and experiments carried out using pooled sera quality control materials have demonstrated good precision.

Monitoring free drug levels requires both an appropriate ultrafiltration device and a sensitive analytical method. Measurement of free drug levels poses a special problem for drugs that are highly bound, since very low concentrations of free drug are present in the ultrafiltrate. Most HPLC instruments are sensitive enough to quantitate such drug levels, and the good correlation over a wide range of drug concentrations between the HPLC micromethod described in this study and the standard FPIA method shows that this HPLC method is accurate and reliable for the routine monitoring of total and free levels of AED's.

10.1.3 Choice of Methodology for the Measurement of Free Drug Levels.

Monitoring of total drug levels is still the standard approach for TDM in clinical laboratories. Free drug level monitoring can assume a more important role now that there are refinements in the technology to make such measurement easier and faster. Commercial availability of ultrafiltration devices may also make routine free level measurements more convenient. Both HPLC and FPIA are appropriate methods for the routine analysis of free levels of AED's when combined with ultrafiltration. The major disadvantage of the FPIA method for analysis of samples from patients on polypharmacy is that each drug must be analysed separately using expensive kits. This means that FPIA is less cost effective than the HPLC method. For routine use, a combination of ultrafiltration and an HPLC method would be a cost effective way of providing free level measurement as discussed on Chapter 2, but the conditions need to be standardised and target ranges for free levels accurately defined for those conditions.

10.1.4 Temperature Effects on the Separation of Unbound AED's.

The present study has not only shown clearly that drug binding tends to decrease with increasing temperature (Chapter 4) but has established the theoretical basis for these observation. In particular there is a marked

decrease in binding of PHT to albumin compared to other AED's. The separation of free AED's at 25 °C will underestimate F-PHT by 34%, F-CBZ by 14% and F-PB by 6% (Table 5.1, Chapter 5). The difference in the free fraction (α) at 37 °C compared to 25 °C is statistically significant for all the drugs investigated, namely PHT, CBZ and PB (Chapters 4 and 5). Also, it is evident from this study that target ranges for free levels of AED's at 37 °C are at a higher level compared with target ranges calculated for 25 °C (Chapter 5).

The observed increase in F-PHT levels determined at 37 °C rather than 25 °C is small in relative terms, amounting to a difference of 5% in the free fraction (α). However, in the case of PHT this change represents an increase in the absolute concentration of F-PHT of more than 50%, since the drug is approximately 90% bound to albumin. Ultrafiltration at ambient temperature or a set temperature of 25 °C does not accurately reflect the free levels of AED's in patients sera in vivo. Therefore it is recommended that the separation step using ultrafiltration should be carried out at the physiological temperature wherever possible. Alternatively, the appropriate target ranges for free levels determined as described in Chapter 5 should be used. Recently it has been shown that temperature has a significant effect not only on free drug levels but also in the measurement of free-thyroxine hormone. The conclusion from that study was that measurement of free hormone should be carried out at 37 °C and reference

values should be defined at 37 °C (Van der Sluijs Veer et al, 1992).

10.1.5 Quality Control of Drug Determinations

Scientists have long accepted the need for quality control to check the accuracy of their laboratory results. The HEATHCONTROL (originally BARTSCONTROL) AED quality control scheme assesses the accuracy and precision of commonly used analytical methods for the measurement of total levels of AED's (Wilson et al, 1983). To date, there are no quality control schemes available for free drug level measurement and there is no recommended standard temperature for the separation of free levels of AED's from the protein-bound component using ultrafiltration. Drug estimations at low concentrations are generally difficult to carry out and the need for quality control is therefore much greater. Also the separation of unbound drug is an additional stage in the analytical procedure and is likely to increase the analytical variability. This cannot be independently monitored in the absence of a quality control scheme. Hence, the introduction of a quality control scheme for the measurement of free levels of AED's is necessary. If such a scheme is developed, it will eventually lead to a standardisation of techniques for the separation and measurement of free levels of AED's.

10.2 Drug Displacement

There are few clinically significant AED interactions resulting from alterations in protein binding. One of the most important displacement interactions is that between PHT and VPA (Dahlqvist et al, 1979; Friel et al, 1979; Monks and Richens, 1980; Kober et al, 1980; Perucca et al, 1980; Johno et al, 1988).

Altered protein binding can result in clinically significant changes in free drug level for patients on some drug regimes. In these instances, total drug levels may be misleading and free drug levels may be required for optimal patient management. Addition of VPA to the drug regimen of patients maintained on PHT has been reported to precipitate toxicity in at least 25% of the study patients, as well as decreased seizure control in up to 13% (Windorfer et al, 1975; Bardy et al, 1976; Windorfer and Sauer, 1977; Wilder et al, 1978). In these cases of drug interaction, free drug levels may correlate better with clinical status than total drug levels. Thus, for patients treated with PHT and VPA, monitoring of F-PHT levels is recommended (Baird-Lambert et al, 1987).

This present study shows that VPA displaces PHT, CBZ and PB from their binding site(s) on plasma proteins. Also it is evident from this study that in the presence of VPA, the value of K_D decreases in the order K_D(PHT) > K_D(CBZ) > K_D(PB) and the value of α increases in the order PHT > CBZ > PB both at 25 °C and 37 °C compared

with the situation of monotherapy. This decrease in the value of K_D and increase in the value of α is statistically significant (Chapter 7). The largest effect is seen in the case of PHT compared with CBZ and PB. These results indicate that PHT is the most easily displaced from albumin by VPA and PB the least with CBZ falling in between PHT and PB.

Most of the previously published work has concentrated on the displacement of PHT by VPA. Very limited information is available on the clinical effect of interactions between CBZ and VPA or PB and VPA. Additional controlled studies may be necessary to confirm the clinical significance of these interactions, and aid in better management of patients with epilepsy.

10.3 Empirical Methods for Predicting Free Drug Levels

10.3.1 An Equation Derived From the Law of Mass Action to Predict F-PHT or F-CBZ Levels in Monotherapy and Polytherapy Patients

In this thesis evidence is presented that the predictive method developed for estimating F-PHT and F-CBZ levels can be used in monotherapy and polytherapy patients with methodologically acceptable accuracy and precision from measurements of total drug levels, albumin levels and average value for K_D. (Chapters 6 and 8). A comparison of the present method for predicting F-PHT levels with three other published methods has demonstrated that the Sheiner-Tozer method is the only

one acceptable for predicting F-PHT levels. Even though the Haidukewych and Dasgupta methods give good correlation with the present method, by comparison 95% of the predicted F-PHT values fall within ± 20% and ± 30% of the predicted values obtained by the method developed in this study. This would probably not be acceptable for clinical purposes.

10.4 Clinical Significance of Free Drug Level Monitoring

The conflicting opinions regarding the necessity for routinely monitoring free drug levels reflect our still limited knowledge in this area. Several reviews have appeared on the relevance of altered protein binding to drug dosage and on the interpretation of serum drug level (Koch-Weser and Sellars, 1976; Rowland, 1980; Greenblatt et al, 1982; Levy and Moreland, 1984; Kwong, 1985; Theodore, 1987). It is generally assumed that only the free drug can reach sites of action and exert a pharmacologic effect. Although this hypothesis may be overly simplistic, it appears that, at least for certain drugs, the pharmacologic effect is indeed more closely related to the free drug level than to the total drug level. The present technology for free drug measurement is more complex and costly compared to the routine assays for total drug. Hence, to-date clinical correlation of drug effects with serum levels has been based on total drug levels, and therapeutic ranges are defined for total drug. As long as protein binding is normal and constant (i.e., normal liver and kidney function and no drug interactions) within patient

populations, free drug is a fairly constant fraction of total drug. In this situation, measurement of total drug level provides an adequate reflection of free drug level, making routine measurement of free drug unnecessary. This has been demonstrated to be the case in a detailed report on epileptic patients who were on PHT (Rimmer et al, 1984). However, it had been previously suggested that for PHT, clinical response and toxicity correlate better with F-PHT level rather than total PHT level (Booker and Darcey, 1973; DeMonaco and Lawless, 1983). This has been recently confirmed by Peterson and his co-workers (Peterson et al, 1991). The formulae developed in this thesis can be used to estimate F-PHT levels in the clinical situation, thus quantifying these effects

The free drug level is determined both by drug dosage and the clearance of the unbound drug. In terms of drug distribution, the free level of most drugs depends only on tissue binding (Levy and Moreland, 1984). On the other hand, the free fraction (α) is dependent on the apparent dissociation constant (K_D) for the binding of drug to albumin as well as albumin concentration (Chapters 4 and 5). The total drug level, therefore, depends on both free level and free fraction. This has important implications for the interpretation of drug results in clinical practice. If the free fraction varies considerably within or between individuals, the concentration of free level in serum is not always proportional to the total drug level. This may lead to misinterpretation of total drug levels and to error in

drug dosage. Therefore, measurement of both free and total drug levels should be considered for highly protein-bound drugs that have a variable free fraction in situation, where more then two AED's are used.

Free drug level monitoring has been proposed for drugs like PHT (Booker and Darcey, 1973; Kilpatrick et al, 1984; Oellerich and Muller-Vahl, 1984; Perucca, 1984; Barre et al, 1988), carbamazepine, (Mackichan et al, 1981; Perucca, 1984; Barre et al, 1988), and valproic acid (Levy, 1980; Perucca, 1984; Barre et al, 1988). However it should be emphasized that the target ranges for F-PHT, F-CBZ and F-PB as determined in this study are based on the therapeutic ranges for total AED levels (Chapter 5). Much research remains to be carried out to improve our knowledge of the clinical relevance of free drug level measurement, and particular attention needs to be given to controlled prospective studies, which are necessary to establish target ranges for free levels. So far, the literature has little to offer on this important issue.

To summarise, measurement of free AED levels is a most valuable research tool, but introduction of this technique into routine clinical practice requires the establishment of clinically relevant target ranges, as well as the further development of simple and reliable methodology such as has been described in this study. The establishing of a quality control scheme which will encourage the standardisation of procedures for TDM of free drug levels would also be advantageous. Until such

time, free level AED monitoring will remain primarily as a research tool in specialised centres.

10.5 Conclusion

The indications for free drug monitoring are still understood only for a limited number of drugs and certain clinical situations. Until new and easier techniques for free drug measurement are devised, TDM will continue to be based on measurement of total drug levels, which will suffice for the management of many but not all patients with epilepsy. The need for free drug monitoring, however, will increase as we advance our understanding of protein binding as well as of the effects of altered binding on drug metabolism and clearance. As clinical experience is gained on the relationship between free drug level and drug effects, therapeutic ranges based on free drug rather than total drug levels will be established, and informed clinicians will find free drug measurement valuable, especially in the management of clinical situations where altered binding can lead to unexpected and unpredictable free drug levels. This clinical demand in its turn will become the impetus for the technological innovations that are needed to make free drug monitoring the routine TDM activity of clinical laboratories.

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APPENDIX I

(Raw data used in comparison between the FPIA method and the HPLC method; Chapter 2)

	PHT FPIA	PHT HPLC	CBZ FPIA	CBZ HPLC	PB FPIA	PB HPLC
			(μmol/L)			
1	28	28	19	17	64	67
2	75	79	35	33	164	170
3	35	36	41	38	54	52
4	14	14	29	27	111	104
5	66	68	33	29	32	28
6	21	24	36	33	142	144
7	18	19	33	29	60	66
8	27	29	25	22	67	66
9	79	79	37	32	169	173
10	33	37	39	37	54	54
11	5	7	20	18	52	49
12	38	40	35	33	63	65
13	28	30	42	39	169	174
14	28	28	18	16	56	54
15	76	80	42	40	69	67
16	37	37	31	29	65	68
17	88	90	37	36	69	66
18	66	70	55	53	171	171
19	65	70	35	34	67	67
20	68	70	40	38	56	55
21	29	29	19	17	170	171
22	28	29	34	33	52	50
23	34	37	42	40	67	64
24	29	27	26	24	62	59
25	86	89	34	32	169	170
26	26	28	42	40	49	47
27	78	80	20	18	72	73
28	35	37	36	34	73	80
29	18	20	42	41	55	53
30	28	30	21	19	188	193
31	44	47	34	33	58	56
32	22	24	42	41	122	119
33	34	32	19	17	56	55
34	9	10	34	34	168	173
35	7	8	40	41	67	67
36	26	29	35	36	70	74
37	46	50	40	39	57	54
38	19	22	30	28	115	117
39	48	47	32	29	-	-
40	92	90	39	36	-	-
41	29	32	39	42	-	-
42	14	15	26	24	-	-
43	38	40	24	23	-	-
44	14	16	24	22	-	-
45	29	26	26	24	-	-
46	85	83	18	15	-	-
47	17	16	27	30	-	-
48	38	40	14	13	-	-
49	46	44	39	40	-	-
50	18	20	20	18	-	-

APPENDIX I (Continued)

	PHT FPIA	PHT HPLC	CBZ FPIA	CBZ HPLC	PB FPIA	PB HPLC
			(μmol/L)			
51	30	32	6	4	-	-
52	76	74	33	32	-	-
53	-	-	39	40	-	-
54	-	-	41	39	-	-
55	-	-	27	26	-	-
56	-	-	19	18	-	-
57	-	-	32	33	-	-
58	-	-	26	24	-	-
59	-	-	41	41	-	-
60	-	-	26	25	-	-

APPENDIX I (Continued)

	F-PHT HPLC	F-PHT FPIA	F-CBZ HPLC	F-CBZ FPIA	F-PB HPLC	F-PB FPIA
			(μmol/L)			
1	5.72	5.91	3.34	4.57	30.97	32.24
2	10.43	10.01	7.12	8.29	18.09	18.21
3	12.49	13.82	20.80	0.58	29.89	31.88
4	3.72	4.27	8.11	10.24	104.37	108.94
5	14.74	17.65	11.19	12.95	95.35	97.57
6	1.19	2.16	9.83	10.58	18.29	8.08
7	13.24	13.88	7.08	7.83	14.38	14.65
8	9.55	10.22	5.98	6.39	65.32	66.00
9	0.97	1.07	5.62	6.31	16.05	16.12
10	9.00	11.01	7.26	8.25	63.35	67.59
11	2.28	2.48	5.41	5.63	141.84	146.17
12	1.58	1.68	9.41	10.62	31.99	33.59
13	3.61	3.98	5.98	5.63	32.11	32.30
14	2.73	4.11	8.57	8.51	74.65	77.76
15	13.16	13.80	9.56	10.45	55.66	57.57
16	4.03	5.24	9.45	9.90	45.94	47.84
17	2.24	2.66	12.04	11.38	18.76	17.75
18	2.80	2.65	8.31	9.14	110.91	113.99
19	2.61	2.27	8.69	9.69	34.71	36.12
20	2.01	2.61	9.14	8.29	28.79	30.52
21	0.91	0.80	10.82	10.45	26.83	27.29
22	1.17	1.54	14.26	13.08	214.76	219.47
23	3.48	4.37	8.42	9.14	51.14	53.03
24	8.99	10.82	4.55	5.42	31.58	32.56
25	8.89	11.50	11.12	10.37	38.16	41.46
26	4.64	6.29	10.12	9.44	41.12	43.31
27	4.99	5.85	7.12	6.98	47.75	49.22
28	8.48	9.88	5.18	4.86	21.15	20.78
29	12.05	13.22	15.49	14.64	44.10	43.41
30	3.57	4.32	6.92	6.18	27.74	27.77
31	3.71	4.20	8.55	10.28	99.53	98.77
32	6.95	7.97	5.55	4.32	30.98	31.56
33	2.73	2.65	8.61	8.17	31.24	32.12
34	3.99	5.14	19.12	20.82	21.14	20.90
35	2.58	2.95	11.42	10.58	17.81	16.56
36	3.59	3.55	6.43	5.61	31.32	32.48
37	2.53	2.31	8.95	7.85	31.18	31.92
38	6.98	8.87	11.99	11.11	31.94	32.14
39	3.08	2.57	12.89	12.30	31.08	30.38
40	3.14	4.13	12.10	11.24	96.99	98.13
41	2.11	2.07	7.58	7.01	–	–
42	3.63	4.39	5.72	5.32	–	–
43	2.98	1.87	10.83	9.46	-	-
44	4.23	5.37	10.89	10.18	-	-
45	3.58	4.02	6.62	6.83	-	-
46	3.53	5.07	7.40	6.86	-	-
47	4.44	3.60	7.07	6.68	-	-
48	3.56	4.22	8.04	7.83	-	-
49	4.75	4.12	8.61	9.35	-	-
50	2.85	1.29	10.72	9.96	-	-
51	2.18	2.23	9.59	9.28	-	-
52	3.16	4.01	4.05	4.88	-	-
53	4.06	5.90	8.53	9.68	-	-
54	4.52	4.29	21.9	22.51	-	-
55	2.15	2.26	9.64	10.26	-	-
56	11.36	12.51	10.49	11.11	-	-

	F-PHT HPLC	F-PHT FPIA	F-CBZ HPLC	F-CBZ FPIA	F-PB HPLC	F-PB FPIA
			(μmol/L)			
57	6.90	6.27	12.59	12.50	-	-
58	3.26	3.26	8.82	8.61	-	-
59	3.68	3.17	9.86	10.44	-	-
60	2.40	2.17	7.68	7.39	-	-
61	7.36	8.92	12.91	13.82	-	-
62	0.79	1.16	4.79	5.50	-	-
63	2.26	2.61	7.44	8.01	-	-
64	3.35	3.90	12.30	13.34	-	-
65	1.89	1.84	4.96	5.35	-	-
66	2.79	2.42	8.49	9.79	-	-
67	6.86	8.29	5.78	6.17	-	-
68	4.19	3.77	5.85	6.58	-	-
69	9.85	11.02	2.27	2.41	-	-
70	1.18	1.09	11.58	11.79	-	-
71	1.62	1.96	13.59	13.78	-	-
72	2.75	2.28	9.93	9.55	-	-
73	3.80	2.75	3.50	3.90		
74	1.13	1.25	8.66	9.42		
75	5.62	6.01	7.63	8.20		
76	7.90	9.94	10.30	10.96		
77	12.24	13.67	9.55	10.53		
78	3.71	4.21	11.55	11.39		
79	2.25	1.15	7.93	8.67		
80	1.63	0.73	7.76	8.97		
81	6.02	7.21	11.48	12.02		
82	3.23	3.49	10.74	10.76		
83	2.09	2.13	12.19	13.71		
84	6.69	7.42	10.85	11.03		
85	3.86	3.73	6.01	6.27		
86	8.67	9.12	10.76	11.07		
87	7.39	7.36	8.97	8.98		
88	4.83	4.18	8.99	9.96		
89	3.56	4.19	7.11	7.32		
90	7.69	7.25	3.36	4.67		
91	4.36	3.57	7.34	7.46		
92	7.31	8.48	6.95	7.41		
93	5.83	5.07	11.77	12.52		
94	11.15	12.56	8.93	9.36		
95	-	-	4.42	4.74		
96	-	-	9.18	9.21		
97	-	-	11.96	12.10		

APPENDIX I (Continued)

(Raw data used in the stability of free levels of AED's in serum during sample storage; Chapter 2)

	F-CBZ (DAY1)	F-CBZ (DAY84)	F-PHT (DAY1)	F-PHT (DAY84)	F-PB (DAY1)	F-PB (DAY84)
			(μmol/L)			
1	9.68	9.83	10.20	10.34	34.24	32.61
2	11.11	10.87	4.29	4.77	18.21	18.09
3	12.50	12.70	11.59	11.45	31.88	33.87
4	8.61	8.81	6.17	6.42	108.94	109.69
5	10.44	9.54	6.27	6.46	95.57	100.69
6	7.39	7.42	7.79	8.27	18.08	18.75
7	13.82	14.01	4.35	4.85	14.65	14.91
8	8.01	8.58	2.58	2.82	66.00	69.36
9	13.34	13.97	8.54	9.13	16.12	16.75
10	8.01	8.58	6.62	7.01	67.59	69.40
11	13.34	13.97	1.39	1.46	33.59	34.10
12	5.35	6.36	3.18	3.08	32.30	33.94
13	6.58	6.48	3.16	3.27	47.84	47.17
14	11.79	11.99	3.87	4.08	17.75	17.76
15	13.78	14.11	15.57	15.98	36.12	36.30
16	9.55	9.33	1.20	1.44	27.29	27.70
17	3.90	4.27	10.23	10.24	53.03	53.86
18	9.42	9.58	6.29	6.88	32.56	31.08
19	8.20	8.58	8.42	8.36	41.46	41.71
20	10.96	10.73	11.55	11.54	43.31	45.24
21	8.67	8.62	8.72	9.08	49.22	47.75
22	11.07	10.36	9.66	9.91	20.78	21.38
23	4.67	4.69	10.08	10.36	43.41	41.46
24	7.46	7.35	9.38	10.16	27.77	28.44
25	7.41	7.38	10.35	10.68	98.13	98.98
26	12.52	11.62	4.19	4.44	98.77	98.81
27	4.74	4.64	10.20	10.34	31.56	32.78
28	13.96	14.40	1.64	1.56	20.90	21.07

APPENDIX II

(Raw data used in Chapter 4)

	F-PHT µmol/L 15 °C	F-PHT µmol/l 25 °C	F-PHT µmol/L 37 °C	PHT µmol/L	Albumin g/L	Albumin µmol/L
1	8.45	11.45	14.08	102	42	618
2	4.68	6.42	7.83	56	44	647
3	5.16	6.46	7.84	57	42	618
4	6.14	8.27	10.26	91	48	706
5	3.92	4.85	6.19	47	46	677
6	2.17	2.82	3.59	22	37	544
7	7.10	9.13	11.52	74	48	706
8	5.19	7.01	8.82	73	47	691
9	0.93	1.46	1.87	15	42	618
10	0.97	1.56	1.92	18	49	721
11	2.15	3.08	4.15	23	38	559
12	2.37	3.27	4.23	37	49	721
13	2.87	4.08	5.30	31	40	588
14	13.51	15.98	20.28	128	40	588
15	0.90	1.41	1.67	14	40	588
16	8.51	10.24	12.87	88	51	750
17	5.44	6.88	8.35	59	42	618
18	6.47	8.36	10.47	88	52	765
19	9.57	11.54	14.33	92	41	603
20	7.33	9.08	11.05	81	42	618
21	8.16	9.91	12.28	88	48	706
22	8.01	10.36	13.03	86	47	691
23	8.35	10.16	12.77	99	46	677
24	8.91	10.68	12.88	129	50	735
25	7.41	8.75	11.30	80	41	603
26	8.15	9.37	11.79	84	44	647
27	11.23	12.77	17.66	127	45	662
28	8.45	9.30	11.93	92	45	662

	F-CBZ µmol/L 15 °C	F-CBZ µmol/L 25 °C	F-CBZ µmol/L 37 °C	CBZ µmol/L	Albumin g/L	Albumin µmol/L
1	11.16	11.11	12.94	46	52	765
2	12.25	12.50	14.45	47	46	677
3	8.66	8.61	9.90	36	44	647
4	9.64	10.44	11.70	40	52	765
5	6.87	7.39	8.64	31	46	677
6	12.57	13.82	14.94	51	48	706
7	7.81	8.01	9.97	40	49	721
8	12.46	13.34	13.79	45	49	721
9	5.09	5.35	6.17	18	49	721
10	6.07	6.17	7.33	23	48	706
11	6.46	6.58	7.37	23	48	706
12	2.43	2.41	3.36	10	52	765
13	11.95	11.79	13.86	42	44	647
14	13.42	13.78	16.09	48	45	662
15	4.11	3.90	5.37	15	52	765
16	9.14	9.42	11.21	30	40	588
17	8.67	8.20	9.58	28	43	632

	F-CBZ	F-CBZ	F-CBZ	CBZ	Albumin	Albumin
	µmol/L	µmol/L	µmol/L	µmol/L	g/L	µmol/L
	15 °C	25 °C	37 °C			
18	10.93	10.96	12.40	38	44	647
19	11.10	11.39	12.58	42	51	750
20	8.91	8.67	9.81	34	51	750
21	8.82	8.97	10.30	30	40	588
22	11.50	12.02	12.17	45	46	677
23	10.11	10.76	11.53	42	49	721
24	13.62	13.71	14.14	54	46	675
25	10.42	11.03	11.26	39	44	647
26	5.84	6.27	6.64	22	45	662
27	10.33	11.07	10.98	40	47	691
28	7.05	7.32	7.83	28	50	735

	F-PB	F-PB	F-PB	PB	Albumin	Albumin
	µmol/L	µmol/L	µmol/L	µmol/L	g/L	µmol/L
	15 °C	25 °C	37 °C			
1	32.78	32.61	34.34	54	44	647
2	17.53	19.46	19.55	38	46	677
3	32.37	33.87	36.36	52	36	529
4	109.38	109.69	116.55	178	45	662
5	100.67	100.69	103.68	172	48	706
6	18.25	18.75	20.50	34	44	647
7	13.71	14.91	15.16	27	47	691
8	66.87	69.36	69.94	119	48	706
9	15.90	16.75	17.82	30	46	676
10	45.40	47.17	48.09	78	42	618
11	17.17	17.76	17.73	31	53	779
12	35.75	36.30	39.47	62	50	735
13	27.80	27.70	30.59	48	44	647
14	52.69	53.86	57.70	88	44	647
15	32.55	31.08	34.74	54	44	647
16	41.62	41.71	43.68	72	47	691
17	43.24	45.24	46.84	81	46	677
18	19.64	21.38	22.93	40	46	677
19	41.77	41.46	44.41	74	49	721
20	26.94	28.44	31.45	54	49	721
21	98.52	98.98	105.01	167	40	588
22	97.94	98.81	100.25	168	48	706
23	33.26	33.27	34.88	54	44	647
24	21.44	21.07	22.03	40	50	735
25	15.57	16.40	18.40	35	51	750

APPENDIX III

(Raw data used in chapter 5)

	F-PHT 25 °C µmol/L	F-PHT 37 °C µmol/L	PHT µmol/L
1	17.65	23.99	158
2	8.42	12.46	89
3	2.16	4.15	25
4	0.86	1.57	11
5	13.24	20.48	132
6	10.22	15.49	102
7	1.07	2.18	15
8	8.01	12.61	81
9	2.48	4.34	31
10	5.24	8.01	55
11	2.66	4.96	32
12	2.65	4.44	28
13	2.27	3.89	24
14	2.61	4.44	28
15	0.80	1.50	8
16	4.37	6.28	44
17	10.82	18.31	95
18	11.50	15.60	118
19	6.29	8.81	58
20	4.20	6.28	44
21	2.65	3.19	21
22	5.14	7.72	53
23	2.95	4.60	29
24	3.55	5.97	41
25	2.31	4.01	27
26	8.87	13.38	90
27	2.51	3.39	19
28	4.13	7.45	48
29	2.07	3.85	27
30	4.39	6.85	47
31	1.87	3.79	26
32	5.37	6.42	36
33	4.02	5.97	31
34	5.07	7.88	53
35	3.60	5.97	41
36	4.12	6.47	48
37	1.29	2.71	19
38	2.26	3.95	26
39	12.51	18.77	121
40	6.27	10.96	63
41	3.26	5.49	40
42	3.17	4.80	35
43	2.17	3.50	25
44	8.92	11.90	85
45	1.16	2.33	17
46	2.61	4.66	32
47	1.84	2.88	21
48	2.42	4.04	30
49	9.29	13.13	92
50	3.77	6.53	43
51	11.02	13.45	98
52	1.09	2.16	16
53	1.96	3.85	27
54	2.28	5.77	34

	F-PHT 25 °C µmol/L	F-PHT 37 °C µmol/L	PHT µmol/L
55	1.25	1.78	13
56	13.48	19.22	140
57	1.15	1.96	11
58	0.73	1.28	9
59	7.21	8.38	62
60	3.49	5.39	40
61	2.13	3.57	24
62	7.42	8.45	71
63	3.73	5.35	33
64	9.12	13.11	90
65	7.36	11.51	79
66	7.25	13.70	73
67	3.37	6.26	43
68	8.48	11.20	80
69	5.07	7.43	51
70	12.56	18.06	124
71	7.50	10.85	73
72	1.72	3.02	22
73	1.68	2.79	18
74	0.87	1.54	11
75	7.10	11.10	70
76	3.18	4.64	26
77	11.59	14.08	102
78	6.17	7.83	56
79	6.27	7.84	57
80	7.79	10.26	91
81	4.35	6.19	47
82	2.58	3.59	22
83	8.54	11.52	74
84	6.62	9.82	73
85	1.39	1.87	15
86	1.64	1.92	18
87	3.18	4.15	23
88	3.16	4.23	37
89	3.87	5.30	31
90	15.57	20.28	128
91	1.20	1.67	14
92	10.23	12.87	88
93	6.29	8.35	59
94	8.42	10.47	88
95	11.55	14.33	92
96	8.72	11.05	81
97	9.66	12.28	88
98	10.08	13.03	86
99	9.38	12.77	99
100	10.35	12.88	129
101	8.75	11.30	80
102	8.79	11.46	85
103	12.82	17.94	105
104	9.37	11.79	84
105	12.77	17.66	127
106	9.30	11.93	92
107	1.27	2.67	17
108	1.97	4.04	23
109	3.44	6.45	28
110	4.64	8.33	52

	F-PHT 25 °C µmol/L	F-PHT 37 °C µmol/L	PHT µmol/L
111	2.18	3.40	23
112	7.10	12.84	74
113	5.22	8.88	47
114	1.99	3.51	16
115	2.65	5.04	26
116	2.34	4.65	19
117	3.29	5.07	28
118	1.63	2.65	16
119	5.81	8.42	45
120	2.83	4.01	24
121	12.46	19.45	107
122	11.24	18.45	116
123	14.60	18.33	114
124	12.20	15.06	90
125	18.88	22.54	127
126	11.64	15.62	110
127	14.06	19.19	123
128	12.58	16.05	100
129	14.46	18.47	128
130	9.83	12.45	90
131	12.77	16.95	106

	F-CBZ 25 °C µmol/L	F-CBZ 37 °C µmol/L	CBZ µmol/L
1	12.95	15.94	51
2	10.58	12.75	44
3	7.83	10.45	34
4	6.39	7.45	22
5	6.31	7.88	26
6	8.25	10.97	34
7	5.63	6.39	27
8	10.62	12.57	44
9	5.63	6.39	23
10	8.51	10.51	33
11	9.14	11.07	36
12	9.69	12.28	43
13	10.45	11.27	40
14	9.14	10.60	35
15	5.42	6.00	18
16	10.37	11.55	41
17	9.44	11.01	38
18	4.83	5.93	17
19	14.64	18.77	61
20	6.18	7.88	26
21	5.61	6.67	21
22	11.11	12.94	40
23	12.30	13.66	41
24	7.01	8.84	21
25	5.32	6.72	20
26	6.83	8.39	31
27	6.68	8.83	30
28	7.83	9.70	33
29	11.11	12.94	46
30	12.50	14.45	47

	F-CBZ 25 °C µmol/L	F-CBZ 37 °C µmol/L	CBZ µmol/L
31	8.61	9.90	36
32	10.44	11.70	40
33	7.39	8.64	31
34	13.82	14.94	51
35	8.01	9.97	40
36	13.34	13.79	45
37	5.35	6.17	18
38	6.58	7.37	23
39	2.41	3.36	10
40	11.79	13.86	42
41	13.78	16.09	48
42	3.90	5.37	15
43	9.42	11.21	30
44	8.20	9.58	28
45	10.96	12.40	38
46	11.39	12.58	42
47	8.67	9.81	34
48	8.97	10.30	30
49	12.02	12.17	45
50	10.76	11.53	42
51	13.71	14.14	54
52	11.03	11.26	39
53	6.27	6.64	22
54	10.07	11.98	40
55	7.12	7.83	28
56	3.36	4.51	15
57	7.34	7.89	27
58	6.95	7.54	32
59	11.77	12.36	45
60	9.18	9.87	36
61	15.84	16.30	62
62	13.73	16.75	55
63	16.45	19.39	65
64	11.19	13.84	45
65	6.30	6.92	24
66	12.42	15.90	51
67	13.02	16.73	54
68	16.04	20.19	56
69	15.46	17.32	52
70	12.86	14.41	51
71	16.52	18.14	57
72	14.29	16.76	51
73	15.51	17.28	52
74	16.67	18.49	54
75	18.17	21.59	59
76	16.49	20.05	60
77	18.15	22.22	62
78	8.60	10.80	29
79	18.83	23.26	65
80	17.82	22.03	64
81	16.49	22.78	69
82	11.60	13.39	45
83	8.39	9.59	29
84	9.33	11.13	36
85	8.54	9.20	33
86	15.46	17.73	58
87	15.33	16.97	61

	F-CBZ 25 °C µmol/L	F-CBZ 37 °C µmol/L	CBZ µmol/L
88	8.43	9.64	35
89	4.66	5.26	18
90	10.53	12.00	39
91	9.67	11.05	38
92	13.13	14.67	51
93	12.37	13.93	48
94	12.39	14.16	46
95	9.81	10.93	39
96	11.51	12.72	46
97	13.00	14.91	47
98	13.27	15.15	48
99	11.02	12.57	41
100	12.72	13.98	49
101	14.70	17.32	57
102	10.99	12.19	45
103	12.24	13.52	46
104	2.10	2.86	10
105	9.31	10.77	39
106	17.01	19.59	67
107	12.03	13.61	51
108	10.85	12.06	41
109	11.33	12.50	47
110	6.00	6.99	22
111	10.03	11.09	37
112	15.04	16.63	53
113	12.70	14.33	48
114	6.29	6.60	26
115	12.55	14.44	45
116	12.45	13.57	43
117	8.71	9.32	33
118	10.84	12.29	44
119	11.45	12.45	41
120	13.36	14.31	54
121	14.97	16.41	62

	F-PB 25 °C µmol/L	F-PB 37 °C µmol/L	PB µmol/L
1	18.21	19.55	38
2	33.87	36.36	52
3	108.94	116.55	178
4	97.57	103.68	172
5	18.08	20.50	34
6	14.65	15.16	27
7	66.00	69.94	119
8	16.12	17.82	30
9	47.84	48.09	78
10	17.75	18.73	31
11	36.12	39.47	62
12	27.29	30.59	48
13	53.03	57.70	88
14	41.46	43.68	72
15	43.31	46.84	81
16	20.78	22.93	40
17	43.41	44.41	74
18	27.77	31.45	54

	F-PB 25 °C µmol/L	F-PB 37 °C µmol/L	PB µmol/L
19	98.13	105.01	167
20	98.77	100.25	168
21	20.90	22.03	40
22	16.56	18.40	35
23	117.26	119.78	128
24	32.60	33.44	51
25	17.86	19.69	35
26	10.10	11.23	16
27	47.99	51.21	61
28	33.50	34.92	59
29	21.99	22.90	29
30	58.70	59.71	95
31	75.77	78.48	128
32	66.63	67.24	106
33	77.48	78.53	115
34	36.16	38.53	60
35	65.49	68.44	112
36	87.84	90.58	156

APPENDIX IV

(Raw data used in chapter 6)

	F-PHT µmol/L 25 °C	F-PHT µmol/L 37 °C	PHT µmol/L	Albumin g/L	albumin µmol/L
1	4.28	5.55	42	45	662
2	2.50	3.28	23	43	632
3	10.20	13.20	90	40	588
4	9.23	12.18	88	43	632
5	3.44	4.10	28	40	588
6	5.64	7.43	52	42	618
7	2.48	3.22	23	42	618
8	10.34	13.10	92	41	603
9	9.66	11.58	88	46	676
10	7.98	10.54	74	43	632
11	5.22	6.88	47	42	618
12	1.99	2.51	16	38	559
13	12.05	15.62	110	42	618
14	12.06	15.35	107	41	603
15	2.75	3.56	26	43	632
16	2.34	3.10	19	36	529
17	11.59	14.08	102	42	618
18	9.18	11.46	85	43	632
19	9.83	12.45	90	44	647
20	3.07	4.00	28	42	618

	F-CBZ µmol/L 25 °C	F-CBZ µmol/L 37 °C	CBZ µmol/L	Albumin g/L	Albumin µmol/L
1	13.73	16.15	55	50	735
2	6.30	7.15	24	48	706
3	13.48	15.50	51	48	706
4	12.86	14.41	51	52	765
5	11.60	14.39	45	48	706
6	8.99	10.18	33	44	647
7	15.46	17.13	58	50	735
8	8.43	10.34	35	50	735
9	4.66	5.26	18	48	706
10	10.53	12.00	39	45	662
11	13.13	15.77	51	46	676
12	12.37	13.93	48	49	721
13	14.70	17.32	57	48	706
14	2.66	3.05	10	46	676
15	10.85	12.06	41	47	691
16	3.92	4.46	15	46	676
17	12.39	14.16	46	45	662
18	6.00	6.55	22	49	721
19	10.03	11.09	37	48	706
20	12.70	14.33	48	50	735
21	11.45	13.57	43	46	676
22	8.71	9.98	33	49	721
23	11.84	13.39	44	46	676
24	11.45	12.45	41	46	676
25	7.34	8.35	27	45	662

APPENDIX V

(Raw data used in chapter 7)

	F-PHT µmol/L 25 °C	F-PHT µmol/L 37 °C	PHT µmol/L	VPA µmol/l	Albumin g/L	Albumin µmol/L
1	4.25	6.56	33	237	46	676
2	4.63	6.20	34	369	47	691
3	2.01	3.23	16	275	45	662
4	8.34	11.32	61	172	47	691
5	12.31	16.50	86	381	47	691
6	5.70	8.22	33	552	47	691
7	6.76	8.11	37	354	43	632
8	6.40	8.83	41	553	45	662
9	4.10	6.12	38	194	44	647
10	6.04	7.60	36	324	45	662
11	8.34	10.68	45	261	43	632
12	3.99	5.74	27	129	44	647
13	6.03	9.57	45	100	50	735
14	1.56	2.28	14	75	47	691
15	4.94	6.83	40	164	43	632
16	7.07	9.86	51	379	44	647
17	6.23	8.19	39	262	42	618
18	8.13	10.58	45	376	43	632
19	4.41	6.22	34	386	47	691
20	7.02	9.39	46	443	48	706
21	3.66	5.43	27	389	45	662
22	9.10	12.57	61	494	47	691
23	2.95	4.58	23	426	49	721
24	7.96	11.82	66	353	46	676
25	12.14	17.14	95	182	46	676
26	5.44	7.81	38	372	47	691
27	9.36	12.49	56	442	51	750
28	9.26	12.96	67	379	50	735

	F-CBZ µmol/L 25 °C	F-CBZ µmol/L 37 °C	CBZ µmol/L	VPA µmol/L	Albumin g/L	Albumin µmol/L
1	4.21	4.90	15	384	48	706
2	10.12	14.93	44	201	49	721
3	12.11	14.61	45	512	47	691
4	3.18	3.89	12	173	50	735
5	8.84	10.56	30	227	48	706
6	14.27	16.61	47	264	47	691
7	14.51	16.84	50	320	46	676
8	11.80	13.71	39	454	50	735
9	15.54	17.82	51	279	51	750
10	7.90	9.98	29	437	50	735
11	9.36	10.81	31	273	47	691
12	9.66	10.54	31	432	50	735
13	6.68	7.93	23	341	46	676
14	10.45	13.33	39	385	50	735
15	9.57	12.66	36	177	43	632
16	10.82	12.87	40	516	50	735
17	10.07	11.88	31	474	48	706
18	13.16	14.38	41	116	49	721
19	11.16	12.89	37	170	49	721
20	10.29	11.59	32	631	51	750

21	11.75	13.20	35	522	46	676
22	12.15	13.58	37	418	50	735
23	11.74	12.99	33	520	47	691
24	10.79	12.12	32	438	46	676
25	10.83	12.66	31	484	45	662
26	10.15	11.65	34	167	48	706
27	12.04	13.68	37	476	50	735
28	9.37	11.27	33	500	49	721
29	10.73	12.74	38	365	50	735
30	13.44	17.62	51	319	50	735

	F-PB µmol/L 25 °C	F-PB µmol/L 37 °C	PB µmol/L	VPA µmol/L	Albumin g/L	Albumin µmol/L
1	69.87	74.10	110	317	43	632
2	94.68	99.46	148	331	43	632
3	36.18	38.20	58	403	46	676
4	94.02	98.30	148	443	42	618
5	98.47	103.76	153	510	41	603
6	81.01	86.38	127	323	44	647
7	84.28	85.89	134	89	43	632
8	30.43	32.21	48	483	45	662
9	47.78	49.65	75	207	40	588
10	66.72	70.56	107	373	46	676
11	87.98	93.23	142	146	45	662
12	55.49	58.75	86	433	42	618
13	9.38	9.98	15	840	45	662

APPENDIX VI

(Raw data used in chapter 8)

	F-PHT µmol/L 37 °C	PHT µmol/L	VPA µmol/L	Albumin g/L	Albumin µmol/L
1	19.26	95	355	45	662
2	16.91	86	264	46	676
3	9.53	43	131	42	618
4	15.43	75	376	44	647
5	14.75	68	529	41	603
6	13.80	64	528	41	603
7	12.61	59	453	43	632
8	8.22	38	264	41	603
9	11.72	55	460	42	618
10	8.05	40	323	45	662
11	10.65	49	534	41	603
12	10.94	52	476	42	618
13	9.63	48	446	47	691
14	2.92	15	295	46	676
15	6.03	29	384	44	647
16	13.74	68	358	48	706
17	10.70	47	380	44	647
18	5.34	26	408	44	647
19	6.78	32	567	42	618
20	3.92	20	344	43	632
21	6.29	33	448	44	647
22	8.21	43	294	47	691

	F-CBZ µmol/L 37 °C	CBZ µmol/L	VPA µmol/L	Albumin g/L	Albumin µmol/L
1	14.64	42	458	48	706
2	14.60	45	932	50	735
3	23.50	63	258	49	721
4	20.91	57	423	49	721
5	12.88	37	460	49	721
6	10.08	29	361	52	765
7	13.64	40	225	49	721
8	7.56	22	152	49	721
9	15.63	44	307	49	721
10	16.04	46	252	49	721
11	12.65	39	456	48	706
12	18.75	56	180	49	721
13	12.50	35	299	50	735
14	15.95	42	265	40	588
15	16.60	46	205	48	706
16	9.96	28	579	48	706
17	16.97	47	83	47	691
18	15.50	41	538	49	721
19	10.28	29	300	46	676
20	17.88	48	429	44	647

APPENDIX VII

(Raw data used in chapter 9)

	PHT µmol/L	Albumin µmol/L	Albumin µmol/L
1	16	43	632
2	24	46	676
3	101	41	603
4	91	48	706
5	56	44	647
6	47	46	676
7	22	37	544
8	88	52	765
9	86	47	691
10	57	42	618
11	99	46	676
12	74	48	706
13	92	45	662
14	15	42	618
15	18	49	721
16	73	47	691
17	23	38	559
18	31	40	588
19	59	42	618
20	80	41	603

	PHT µmol/L	VPA µmol/L	Albumin g/L	Albumin µmol/L
1	14	269	50	735
2	40	220	42	618
3	52	83	42	618
4	32	416	47	691
5	28	155	49	721
6	44	81	48	701
7	58	459	45	662
8	31	258	49	721
9	38	492	49	721
10	50	276	48	706
11	21	372	41	603
12	52	229	43	632
13	15	252	43	632
14	21	112	39	574
15	29	110	44	647
16	22	272	43	632
17	28	315	42	618
18	23	327	47	691
19	19	333	50	735
20	78	298	43	632

Selected publications related to thesis

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A Micromethod for the Estimation of Free Levels of Anticonvulsant Drugs in Serum

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A micromethod for estimating free levels of phenobarbitone, phenytoin and carbamazepine in patients' sera is described. Serum samples are subjected to a process of ultrafiltration, the filtrates treated with acetonitrile and the drug concentration quantified using high performance liquid chromatography. The stability of free levels in specimens before and after storage is investigated. The method is reproducible and mean recovery exceeds 98.5% showing that there is no significant absorption of drug onto the filters used. There is no interference from other substances normally present in patients' sera and there is a good correlation between results obtained by this method and a fluorescence polarisation immunoassay with correlation coefficient between 0.975 and 0.999. Serum samples can be stored for a lengthy period before ultrafiltration without adverse effects. The relevance of the method to patient care is discussed.

KEY WORDS: free drug levels; ultrafiltration; anticonvulsants; phenobarbitone; phenytoin; carbamazepine.

Several of the commonly prescribed anticonvulsant drugs are bound to plasma protein to a considerable extent. In the case of phenytoin (DPH), over 90% of the drug is protein bound (1–3). Carbamazepine (CBZ) and sodium valproate are more than 70% bound and phenobarbitone (PHB) is approximately 50% bound (1–3).

It is now recognised that the estimation of non-protein bound levels (free levels) of anticonvulsant drugs in serum is a useful adjunct in the treatment of patients with epilepsy under circumstances likely to be associated with altered binding capacity (1,2,4–7). This occurs in particular during pregnancy and the puerperium (1,8,9), or as a consequence of certain disease states such as uraemia (1–3,5,6,10). It is also recognised that the extent to which a particular anticonvulsant drug is bound to plasma protein may change when the drug régime is altered (1,2,6). The addition or withdrawal of one anticonvulsant drug may have a marked effect on protein binding characteristics when several anticonvulsant drugs are competing for binding sites on protein molecules (3,11–14).

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The separation of protein-bound from unbound anticonvulsant drugs in serum or plasma is usually performed by equilibrium dialysis, ultracentrifugation or ultrafiltration techniques (2,3,6,7,15–25). These procedures may be carried out in the presence of trace quantities of labelled drug so that estimation of the labelled drugs present in the exudate or supernatant gives a measure of the level of unbound drug (5,17,21,22,25). Alternatively the estimation of free drugs is carried out by gas liquid chromatography, high performance liquid chromatography (HPLC) or immunoassay techniques (4,7,10,15,17,19,20,23,24,26–30). These methods may yield comparable results (17,25,26), but all have associated problems which may render it difficult to determine the correct analytical result (6,7,17,18,20,26), and no one method has so far gained acceptance as being entirely suitable for routine use. Nevertheless, it appeared that a procedure which combined ultrafiltration and HPLC techniques might have the necessary qualities of flexibility, speed and accuracy for the provision of a therapeutic monitoring service for free drugs (7,23). We now present a micromethod based on the adaptation of a simple ultrafiltration technique followed by analysis by HPLC for the estimation of free levels in serum of PHB, DPH and CBZ and the major metabolite of CBZ, carbamazepine-10,11 epoxide (EPOX), which itself has anticonvulsant activity (31,32); the internal standard is 5-p-tolyl-5-phenylhydantoin (MPPH). We have also examined the correlation between this method and a fluorescence polarisation immunoassay (FPIA) procedure from the Abbott Company (Maidenhead, UK) (27). In view of the statement in their protocol that serum samples for the analysis of free levels of DPH may be stored frozen for 7 days before filtration (33), we carried out an investigation into the stability of free levels of all three drugs in patients' serum samples in order to determine suitable storage procedures.

Materials and methods

PATIENTS' SAMPLES

Blood samples were collected from patients on PHB, DPH and CBZ for routine measurement and

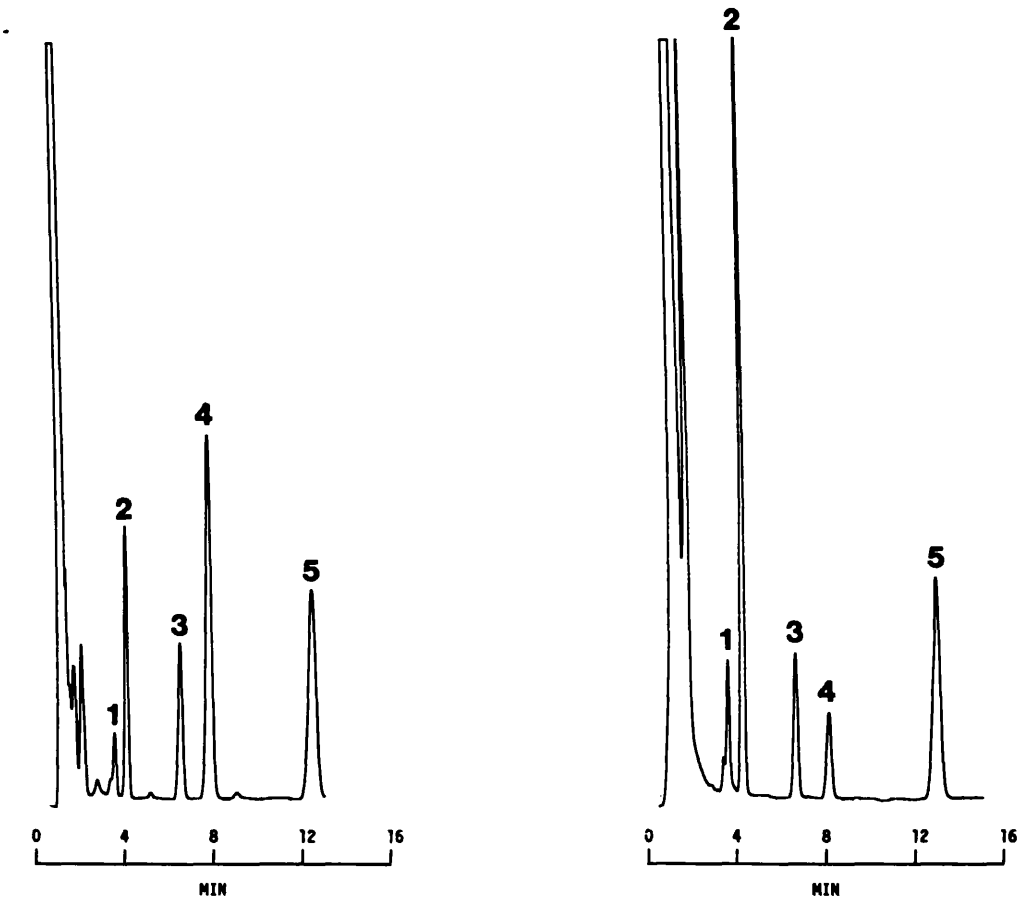

Figure 1—Chromatograms of drugs extracted from (a) spiked physiological saline and (b) an ultrafiltrate from a patient's sample. Peak: 1, carbamazepine-10,11-epoxide; 2, phenobarbitone; 3, carbamazepine; 4, phenytoin; and 5, 5-p-tolyl-5-phenylhydantoin (internal standard).

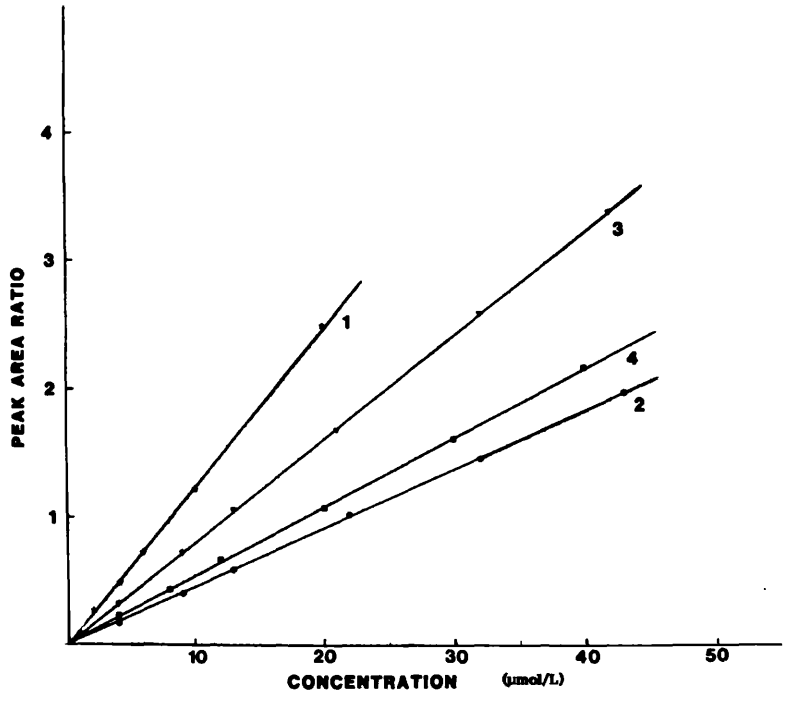

Figure 2—Calibration graphs for anticonvulsant drugs. 1, carbamazepine-10,11-epoxide; 2, phenobarbitone; 3, carbamazepine; and 4, phenytoin.

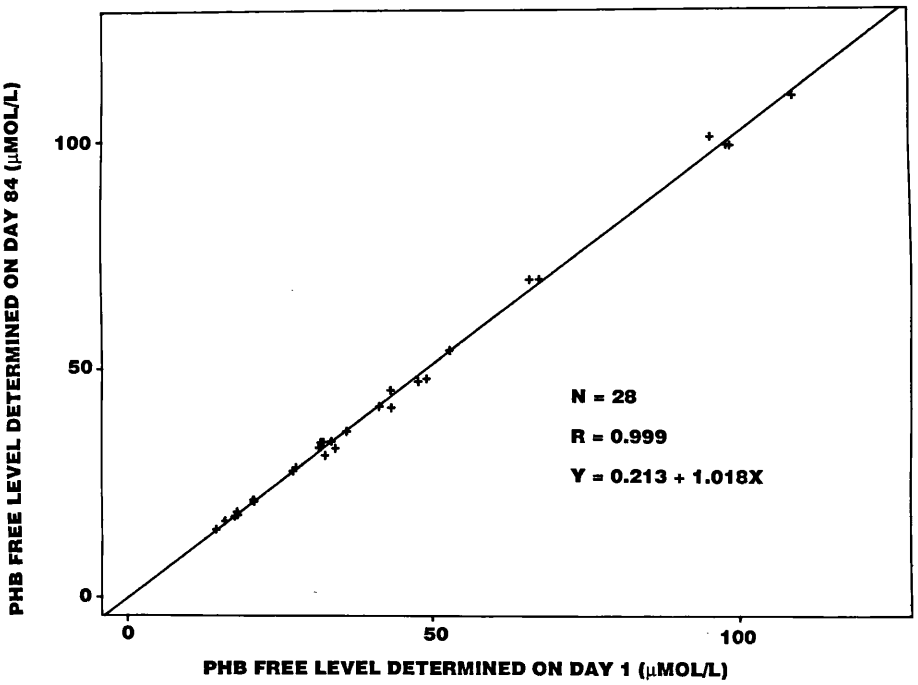

Figure 3—Correlation between analysis of patients' serum samples for free levels of phenobarbitone immediately after collection and after 84 days.

centrifuged within 2 h. The sera thus obtained were stored at −20 °C until required and then allowed to equilibrate at room temperature for 60 min before the filtration process. A number of serum samples chosen at random from patients on monotherapy were filtered and analysed for free levels of PHB, DPH and CBZ immediately after collection and again after storage at −20 °C for 84 days.

Materials

Stock solutions of PHB (BDH, Poole, UK), DPH (Warner-Lambert, Eastleigh, UK), CBZ and EPOX (Ciba-Geigy Pharmaceuticals, Horsham, UK) and the internal standard MPPH (Aldrich, Gillingham, Dorset, UK) were made up to a concentration of 10 mmol/L in methanol. These solutions were stable for three months when stored at 4 °C.

All solvents used were of HPLC grade or equivalent (far-UV range where available). Methanol was obtained from BDH and acetonitrile and HPLC-grade water from FSA (Loughborough, UK). Phosphate buffer (0.05 mol/L, pH 5.6) was prepared using 'Analar' potassium dihydrogen phosphate and dipotassium hydrogen phosphate (BDH) and HPLC-grade water.

Methods

FPIA assay

Determinations of the free levels of DPH and CBZ were carried out using the appropriate kits for the TDX analyser (Abbott) according to the manufacturer's instructions. Determinations of free levels of PHB were carried out using Phenobarbital II assay kits (Abbott).

HPLC assay

Apparatus

The liquid chromatographic system used was a Spectra-Physics SP 8780 equipped with an SP 8780 XR autosampler, Spectroflow 783 UV detector and SP 4270 integrator/printer plotter with LABNET data system (Spectra-Physics, Maidenhead, UK). Chromatograms were run at ambient temperature on a Merck HI-BAR column (250 × 4.0 mm) packed with Lichrosorb RP select B, 5 μm (BDH). A mobile phase of acetonitrile: phosphate buffer (32:68) with a flow rate of 1.4 mL/min at 175 bar was used. The column effluent was monitored at 215 nm with a sensitivity range of 0.02 absorption units full scale and a chart speed of 0.5 cm/min.

Preparation of standard solutions

Working standards of PHB, DPH, CBZ and EPOX were made by diluting the stock 10 mmol/L solutions with physiological saline solution to give a range of concentrations from 2 μmol/L to 42 μmol/L. The anticonvulsant drug concentrations were determined by the ratio of the peak areas of each drug to the peak area of internal standard plotted against concentrations of the drug.

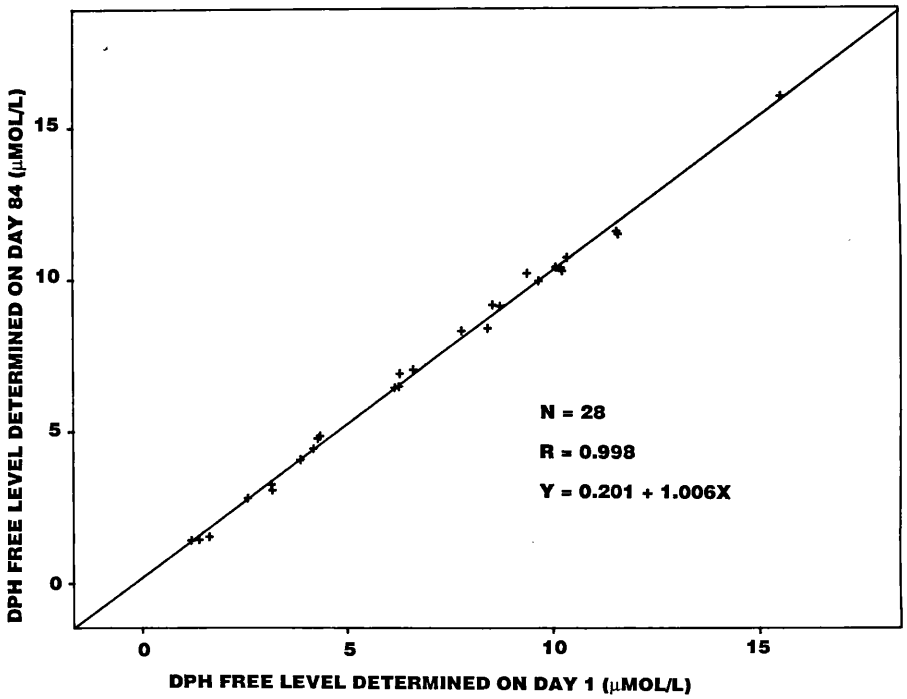

Figure 4—Correlation between analysis of patients' serum samples for free levels of phenytoin immediately after collection and after 84 days.

PROCEDURE

Filtration

Serum (250 μL) was pipetted into the sample reservoir of an Amicon Centrifree micropartition system (Amicon, Stonehouse, UK) in accordance with the manufacturer's instructions and centrifuged at 1000 × g for 20 min at a temperature of 25 °C in a Sorvall RC-5B refrigerated superspeed

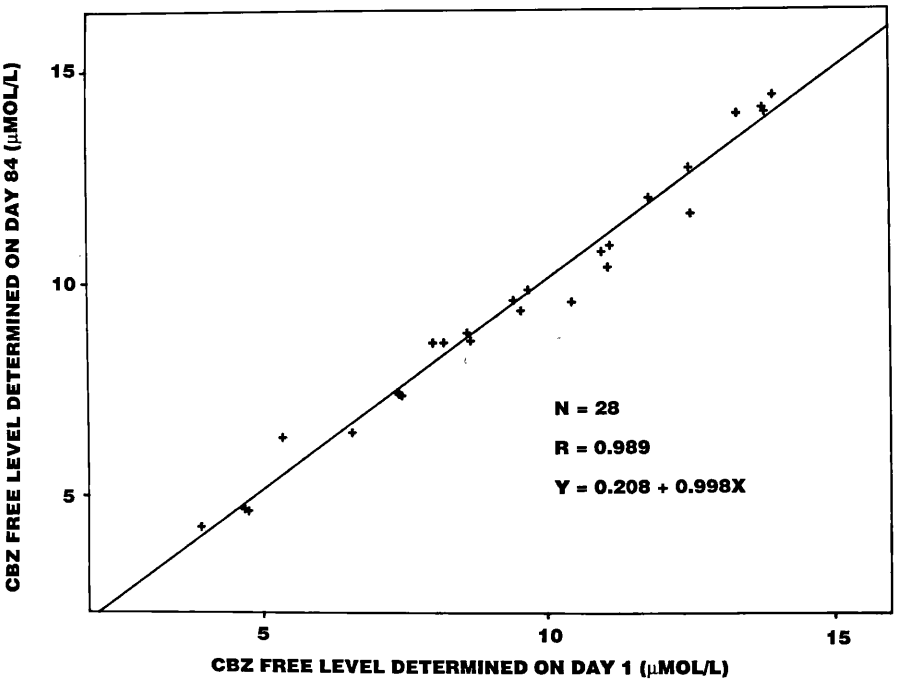

Figure 5—Correlation between analysis of patients' serum samples for free levels of carbamazepine immediately after collection and after 84 days.

TABLE 1
Recovery of Anticonvulsant Drugs Added to Physiological Saline

Drug	Concentration of Prepared Standards[a] (μmol/L)	Measured Concentration (μmol/L)	Recovery %	SD	CV %
Phenobarbitone	21.53	21.43	99.5	0.61	2.83
Phenytoin	3.96	3.90	98.5	0.34	8.62
Carbamazepine	4.23	4.18	98.8	0.37	8.92
Carbamazepine -10,11-epoxide	3.96	4.01	101.3	0.14	3.37

[a]Mean of 30 determinations.

centrifuge fitted with an SM24 rotor (Du Pont, Stevenage, UK).

Sample preparation

A 30 μL volume of the filtrate in the micropartition system filtrate cup was pipetted into a 1.5 mL polypropylene centrifuge tube with cap (Sarstedt, Leicester, UK) and 75 μL of the diluted internal standard solution (10 μmol/L in acetonitrile) added. The tube was shaken for 60 s on a Vibrax-IKA shaker (Sartorius-IKA, Epsom, UK) and 90 μL of the solution was pipetted into a 0.1 mL glass insert in a 1 mL glass vial (Chrompak, London, UK) and 20 μL injected in the chromatograph by the automatic injection procedure. A range of concentrations of the drug standards diluted with physiological saline was treated similarly in order to prepare calibration graphs.

Results

Figure 1 shows chromatograms of (a) a standard extract from physiological saline and (b) a patient's serum filtrate. It can be seen that the resolution of the peaks is very satisfactory using the chromatographic conditions described. Several extraneous peaks are seen close to the solvent front but there is no interference either from materials normally present in serum or from other drugs commonly prescribed in a neurological hospital. The limit of detection for drugs in patients' samples is 0.1 μmol/L with the detector range setting maintained at 0.02 absorption units full scale. The complete chromatographic run takes 15 min.

Figure 2 shows calibration graphs between 2 and 42 μmol/L for PHB, DPH, CBZ and for EPOX between 1 and 20 μmol/L obtained by the analysis of the drug standards prepared in physiological saline. It can be seen that the graphs are linear for all the drugs within these ranges.

Figures 3–5 show the results of analysis of random patients' samples for free levels of PHB, DPH and CBZ immediately after collection and after storage for 84 days at −20 °C. The correlation between the two sets of analytical results is extremely good in each case since the correlation coefficient lies between 0.989 and 0.999. This indicates that serum samples can be stored frozen for a considerable length of time and then batched for filtration without prejudicing the accuracy of the assay.

Recovery was determined by analysing drug standards made up in physiological saline, using the standard procedure. The results presented in Table 1 show recoveries approaching 100% for PHB, DPH, CBZ and EPOX, indicating that there is little or no absorption of the drugs onto the membranes of the micropartition systems. Similar results were obtained when the volume to be filtered was reduced from 250 μL to 100 μL.

The imprecision of the HPLC assay was determined from analyses made on pooled serum. Patients' serum samples known by previous measurement to contain PHB, DPH and CBZ were pooled and filtered with thorough mixing, aliquoted into bottles and stored at −20 °C until required for analysis. Within-batch imprecision was determined from 24 analyses of this material carried out on one day, and between-batch imprecision from three analyses on 11 separate occasions. It can be seen from the results given in Table 2 that the standard

TABLE 2
Imprecision of HPLC Assay for Free Levels of Anticonvulsant Drugs in Pooled Serum Samples

	Within Batch Imprecision				Between Batch Imprecision			
Drug	*n*	Mean (μmol/L)	SD	CV %	*n*	Mean (μmol/L)	SD	CV %
Phenobarbitone	24	47.23	1.32	2.80	33	46.40	0.75	1.62
Phenytoin	24	4.47	0.40	9.06	33	4.13	0.53	12.79
Carbamazepine	24	11.14	0.69	6.19	33	10.02	1.14	11.39
Carbamazepine -10,11-epoxide	24	3.68	0.17	4.56	33	3.91	0.52	13.36

TABLE 3
Imprecision of FPIA Assay for Free Levels of Anticonvulsant Drugs in Pooled Serum Samples

	Within Batch Imprecision				Between Batch Imprecision			
Drug	n	Mean (μmol/L)	SD	CV %	n	Mean (μmol/L)	SD	CV %
Phenobarbitone	24	45.90	0.69	1.51	33	47.13	1.03	2.19
Phenytoin	24	4.23	0.31	2.54	33	4.14	0.31	7.33
Carbamazepine	24	10.42	0.39	3.78	33	9.97	0.48	4.83

deviation is less than 1.32 and the coefficient of variation less than 13.36% in all instances, which is acceptable for routine measurements of anticonvulsant drugs present in low concentration. The same procedure was adopted to determine the imprecision of the FPIA assay for PHB, DPH and CBZ. It can be seen from the results shown in Table 3 that the standard deviation is less than 1.03 and the coefficient of variation less than 7.33% in all instances, which is very satisfactory.

A comparison of our HPLC procedure with the FPIA method for PHB, DPH and CBZ gave a correlation coefficient of 0.999 (n = 40), 0.980 (n = 94), and 0.975 (n = 97) respectively.

Discussion

Much of the controversy over the need for measurement of free levels of anticonvulsant drugs in patient care has centred on doubts about the accuracy of the results obtained using the various separation techniques (3,7,34). Although equilibrium dialysis is usually regarded as a reference method, and has the advantage that it can be carried out at physiological temperature (20,22), it is too time-consuming to be practicable as a routine analytical procedure (3,7,15,18,23). Dialysis time and buffer concentration must be carefully selected and controlled, and highly ionized drugs may be absorbed onto the dialysis membrane (3,7,18,35). In addition, a formula has to be applied in each case to correct for the fall in concentration resulting from the redistribution of the drug between plasma and buffer compartments in the dialysis cell (3,7,18,35). Stringent precautions must be taken to avoid bacterial contamination during the lengthy processes of incubation and dialysis.

The process of ultracentrifugation even using the recently introduced microscale assay techniques takes 4 h or more to complete (18,21,22,24,25). Possible sources of error are back diffusion of drug from the pellet into the supernatant (21) or sedimentation of small amounts of unbound drug from the supernatant (36), and temperature needs to be carefully controlled to avoid alterations in the binding charateristics of the drugs (18,22,24,25).

With its qualities of speed and simplicity, the technique of ultrafiltration seemed to have the greatest potential for use in the routine situation (6,7,23,37), although absorption of drugs onto the filters or leakage of protein into the ultrafiltrate were reported in early experiments (7,26,37–39). Other possible sources of error were thought to be disturbances of the binding equilibrium (3,7,18,21,24) caused by concentration effects analogous to those observed with equilibrium dialysis (2,3,6,7,18,21,24). Strict temperature control was also necessary (2,3,6,7, 18,23,24,26,37).

These major problems are largely overcome by use of the method described here. The excellent results obtained in recovery experiments using spiked samples show that there is no significant absorption of drugs onto the filters used, which is in agreement with previous findings (23,25). The method is simple to operate and we find that filtration followed by preparation of 50 samples for HPLC takes only 90 min, in contrast to the time reported for equilibrium dialysis and ultracentrifugation techniques (7,18,22,25,35,36). Temperature control to within ± 1 °C is easily achieved with the Sorval refrigerated centrifuge at a setting of 25 °C. It has been reported that when the ultrafiltration procedure is carried out at 15 °C rather than 25 °C, the results obtained from the analysis of PHB, DPH and CBZ are not significantly different (22,24,37,40,41). On the other hand, there are significant differences between results obtained from the analyses of free levels of DPH after filtration at 20 °C or 25 °C and 37 °C (40,41) which may indicate that DPH is less strongly bound to protein than PHB or CBZ. In view of these findings, it might be considered desirable for each laboratory carrying out analysis of free levels of anticonvulsants to establish a therapeutic range for DPH at the particular temperature used for the separation process.

The simple HPLC method we use has considerable advantages. Each HLPC run for a sample containing PHB, DPH, CBZ and EPOX takes 15 min, but use of the automatic injection procedure allows samples to be chromatographed overnight in a programmed run. In addition, by using the simple clean-up treatment described, at least 2000 samples can be chromatographed before any indication of peak trailing which would mark the deterioration of the HPLC column; the inclusion of a pre-column, which would cause peak spreading and increased

retention time, is therefore unnecessary. The extremely low levels of drug present after the separation process require that the analytical procedures used should be considerably more sensitive than those used in the analysis of total levels of drug, which comprise both free and protein-bound elements. In the case of DPH and CBZ, free levels higher than 20 μmol/L are very rarely seen except in overdose situations. However, for PHB, free levels may exceed 42 μmol/L, and we have found in practice that the standard curve is linear up to 200 μmol/L. Another indication of the validity of our HPLC procedure is provided by the good correlation between this method and the FPIA (TDX) procedure.

A major advantage of our ultrafiltration/HPLC procedure is that the small size of the serum samples required [250 μL as against the 400–500 μL previously reported as the minimum quantity necessary (2,23,36)] allows application in routine pediatric practice. Moreover, in cases where the amount of serum available is less than 250 μL the method can be scaled down to 100 μL, without the need to modify the filtration apparatus as described in a previous report (42). In this context, the analysis of the free levels of PHB, DPH and CBZ in a single sample is very important since patients with intractable epilepsy, children or adults, are frequently on polypharmacy. The fact that the method can be used for the analysis of the free level in serum of EPOX (the major metabolite of CBZ), is an additional advantage, particularly for pharmacokinetic studies and in cases where CBZ toxicity is suspected even though the levels of CBZ determined are well within the therapeutic range for CBZ (29,30). Both HPLC and FPIA are convenient methods for the routine analysis of free levels of anticonvulsant drugs when combined with ultrafiltration. The major disadvantage of the FPIA method for analysis of samples from patients on polypharmacy is that each drug must be analysed separately. This means that FPIA is less cost effective than the HPLC method. In addition, no kit is available for the analysis of EPOX at present.

The monitoring of free levels of anticonvulsant drugs may not come into use entirely as a replacement for the measurement of total levels but will continue to be required for selected patients (3,18, 24,43–45). We measure free levels of anticonvulsants in addition to the total levels in situations where there may be altered protein binding capacity, including pregnancy or hepatic and renal failure. In these cases, results obtained by analysing both total and free levels provide additional clinical information necessary for patient care.

The method presented here has been found to be easy to operate and convenient to use for routine monitoring. We have analysed over 3000 patients' serum samples for free levels of PHB, DPH or CBZ alone or in combination without encountering any problems of interference from co-prescribed drugs or other substances usually present in patients' sera. We consider that the use of ultrafiltration combined with this simple HPLC procedure represents a useful addition to the techniques available for drug monitoring to the benefit of patients with epilepsy, and in view of the small size of the samples required will have a particular place in pediatric practice. There are no generally accepted reference ranges for free levels of anticonvulsant drugs. To this end extensive work is in progress to establish reference ranges for free levels for our own hospital.

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Temperature Effects on the Estimation of Free Levels of Phenytoin Carbamazepine and Phenobarbitone

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Summary: Serum samples from patients on phenytoin (PHT), carbamazepine (CBZ), or phenobarbitone (PB) monotherapy were filtered at 15, 25, and 37°C and the free concentrations measured by high-performance liquid chromatography. The mean apparent dissociation constants at each temperature were calculated, and were used to predict free drug levels from a further series of patients' samples in which total drug and albumin concentrations only were known. The correlation coefficients (r) between these predicted free levels and experimental results obtained by analysis of the same samples for PHT, CBZ, or PB were 0.977, 0.968, and 0.998, respectively, at 25°C; at 37°C, the corresponding values of r were 0.975, 0.961, and 0.997, respectively. We then determined free fractions (α) of PHT, CBZ, and PB at 25 and 37°C and used these values to derive theoretical target ranges for free levels for each of the three drugs. We discuss the implication of these results for patient care, with special reference to the need to specify temperature and quote the appropriate target range when analyses of free levels of AEDs are carried out. **Key Words:** Protein binding—Anticonvulsants—Temperature effects—Theoretical target ranges.

Several of the commonly prescribed antiepileptic drugs (AEDs) are bound to plasma protein to a varying extent, with albumin quantitatively by far the most important fraction (1–3). The estimation in serum or plasma of the non-protein-bound component of these drugs, which is pharmacologically active, may thus provide useful information for the treatment of patients with epilepsy (1,2). Phenytoin (PHT) is more than 90% bound, carbamazepine (CBZ) is approximately 70% bound, and phenobarbitone (PB) is approximately 50% bound (1,3,4). In view of the high proportion of these drugs bound to protein, the estimation of non-protein-bound concentrations (free levels) of PHT and CBZ is particularly important under circumstances likely to be associated with altered binding capacity (1,2,5,6). This may occur during pregnancy (1,7) or as a consequence of certain disease states such as uraemia (1,3,8) or when a particular drug regimen is altered (1,3,9).

The separation of protein bound from unbound AEDs in serum or plasma is usually carried out by equilibrium dialysis, ultracentrifugation, or ultrafiltration techniques (2,3,5,10–20). Although equilibrium dialysis is widely regarded as the reference method, ultrafiltration is increasingly being used since the introduction of new technology has greatly improved performance (16,19,20). This technique can conveniently be combined with high-performance liquid chromatography (HPLC) for the measurement of free levels of AEDs in the routine situation (6,16,20).

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One advantage of equilibrium dialysis is that it can be carried out either at ambient temperature or at 37°C, whereas the technique of ultrafiltration may be restricted to the lower temperatures by the limitations of the equipment available (16). It is known that drug binding tends to decrease with increasing temperature (21–23) and there might be implications for patient care if a result obtained under the circumstances of ultrafiltration at ambient temperature or a set temperature of 25°C does not accurately reflect the free concentration of anticonvulsant drug in patients' sera in vivo (14). In order to understand changes in free levels of AEDs, which occur in disease states due to altered protein binding, it is necessary to establish theoretical target ranges (reference ranges) for free levels in a large population of patients with epilepsy. Therefore, we now present an extensive study of the process of ultrafiltration for the separation of free levels of PHT, CBZ, and PB (PHT-F, CBZ-F, and PB-F) from the sera of patients on monotherapy, carried out at three different temperatures: 15, 25, and 37°C; we then determine the apparent dissociation constant (K_D), which defines the relationship between free and protein-bound AED concentrations, and the free fraction (α), which represents that fraction of the total concentration of AEDs that is unbound. From these values, we subsequently derive reference ranges for PHT-F, CBZ-F, and PB-F under different experimental conditions.

MATERIALS AND METHODS

Patients' Samples

Nonheparinised blood samples for routine measurement of AEDs were collected from patients on PHT, CBZ, or PB monotherapy only and centrifuged within 2 h. The sera thus obtained were stored at −20°C until required for analysis.

Albumin Assay

Estimation of concentrations of albumin in patients' sera were carried out on a Technicon RA-XT analyser using Technicon albumin reagent kits in accordance with the manufacturer's instructions (Technicon, Basingstoke, U.K.). The results were converted into molar units using the value of 68,000 Da for the molecular weight of albumin (24).

Assay of Free Levels of AEDs

Serum (250 μl) was pipetted into the sample reservoir of an Amicon Centrifree micropartition system (Amicon, Stonehouse, U.K.) in accordance with the manufacturer's instructions, and centrifuged for 20 min at 1,000 g at a temperature setting of 15, 25, or 37°C in a Sorvall RC-5B refrigerated superspeed centrifuge fitted with an SM24 rotor (Du Pont, Stevenage, U.K.). Temperature control to within ±1°C was easily achieved with the Sorvall refrigerated centrifuge at the required settings.

The concentrations of AEDs present in the ultrafiltrates were determined by HPLC essentially as described in Ratnaraj et al. (20). An HPLC system comprising a Spectra-Physics SP 8750 equipped with an SP 8780 XR autosampler, Spectroflow 783 UV detector, and SP 4270 integrator/printer plotter with LABNET data system (Spectra-Physics, Maidenhead, U.K.) was used. Chromatograms were run at ambient temperature on a Merck HI-BAR column (250 × 4.0 mm) packed with Lichrosorb RP select B, 5 μm (BDH, Poole, U.K.). A mobile phase of acetonitrile: 0.05 *M* phosphate buffer, pH 5.6 (32:68) with a flow rate of 1.4 ml/min at 175 bar was used. The column effluent was monitored at 215 nm with a sensitivity range of 0.02 absorption units full scale and a chart speed of 0.5 cm/min.

Assay of Total Concentrations of AEDs

Concentrations of PHT, CBZ, and PB in patients' sera were determined by a modification of the method of Ratnaraj et al. (20). A 30 μl volume of serum was pipetted into a 1.5 ml polypropylene centrifuge tube with cap (Sarstedt, Leicester, U.K.) and 75 μl of internal standard solution (50 μmol/L of 5-*p*-tolyl-5-phenylhydantoin) added. The tube was shaken for 60 s on a Vibrax-IKA shaker (Sartorius-IKA, Epsom, U.K.), centrifuged for 3 min at 9,500 *g* in an Abbott centrifuge (Abbott, Maidenhead, U.K.), 90 μl of the solution pipetted into a 0.1 ml glass insert in a 1 ml glass vial (Chrompak, London, U.K.), and 20 μl injected into the chromatograph by the automatic injection procedure. The chromatographic conditions were as described above, but the

range of the detector was adjusted to 0.04 absorption units full scale.

Theoretical Considerations

The apparent dissociation constants for each of the AEDs (PHT, CBZ, or PB) present in patients' sera were calculated using the conventions of the Law of Mass Action. By this means, the binding of a drug D to protein P in serum could be represented as follows: [D] + [P] $\rightleftharpoons$ [DP]. The apparent dissociation constant K_D is therefore given by

$$K_D = [DP]/[D]\,[P] \quad (1)$$

Levels of unbound AEDs were calculated by the application of related formulae as follows: If the fraction of the total concentration of drugs that is unbound (i.e., the free fraction) is expressed by α, then the unbound concentration (i.e., the free level) can be expressed by αC, where C represents the concentration of drug, and the bound concentration by $(1 - \alpha)C$.

By substitution into Eq. (1),

$$K_D[P] = (1 - \alpha)C/\alpha C$$

Therefore,

$$\alpha = 1/(1 + K_D[P]) \quad (2)$$

but

$$\alpha = [D_F]/[D_T] \quad (3)$$

where D_F is the free (unbound) level and D_T is the total (free + bound) drug level. Thus, by substitution into Eq. (2),

$$[D_F]/[D_T] = 1/(1 + K_D[P]) \quad (4)$$

from which the value of $[D_F]$ can be calculated.

In this study, all such calculations were performed on a Hewlett-Packard Vectra ES/12 microcomputer using a STATPAK program, and results were plotted on a Hewlett-Packard ColorPro 7440A graphics plotter (Hewlett-Packard, Bracknall, U.K.).

RESULTS

The results of the analyses of albumin concentration in the patients' sera used in this study were between 550 and 645 μmol/L. (The reference range for healthy adults is 500–765 μmol/L.)

Figure 1 shows the concentration ratios for PHT-F, CBZ-F, and PB-F in patients' sera at 15, 25, and 37°C with the values for 25°C as reference concentrations, whilst Table 1 gives the arithmetic mean of the concentration ratios for the three drugs under the same analytical conditions.

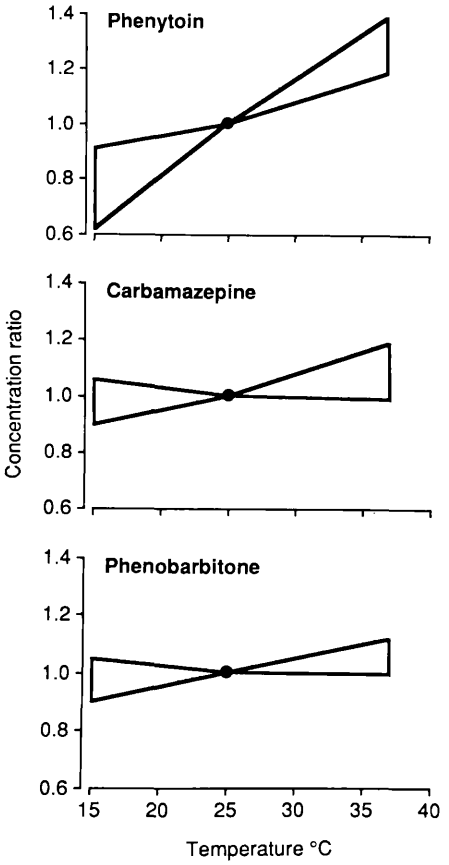

FIG. 1. Concentration ratios for PHT-F (n = 28), CBZ-F (n = 28), and PB-F (n = 25) at 15, 25, and 37°C.

Further information can be obtained by calculating the apparent dissociation constants (K_D) for the AED present in each patient's serum sample at 15, 25, and 37°C using Eq. (1); Fig. 2 shows inverse correlations of K_D vs. temperature for each patient's sample. The application of regression analysis to find the best-fit line, from which the mean of K_D at each temperature can be estimated, is pre-

TABLE 1. *Arithmetic mean of concentration ratios at different temperatures*

	15/25°C	25/25°C	37/25°C
Phenytoin	0.78	1.00	1.26
Carbamazepine	0.98	1.00	1.06
Phenobarbitone	0.98	1.00	1.14

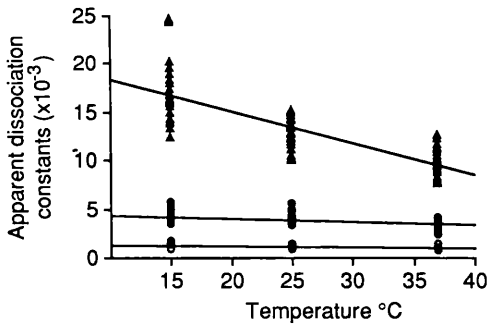

FIG. 2. Regression analysis of apparent dissociation constants (K_D) for PHT (n = 28), CBZ (n = 28), and PB (n = 25) at 15, 25, and 37°C.

sented in Table 2; Table 3 shows the results of the calculations of the arithmetic mean and the coefficient of variation (CV).

Figure 3 shows the results obtained by plotting observed levels (D_F) of PHT-F, CBZ-F, or PB-F, estimated after filtering patients' serum samples at 25 and 37°C, against the predicted values for D_F, which were calculated for the same serum samples from the mean values of K_D by using Eq. (4). Table 4 shows observed mean values of α for all three drugs at 25 and 37°C calculated using Eq. (3), and also predicted mean values of α derived by using Eq. (2).

In Fig. 4, results obtained by analysing a larger series of patients' serum samples for PHT-F, CBZ-F, or PB-F at 25 and 37°C are plotted against the total drug levels from the same samples. In addition, values for therapeutic ranges for total levels of PHT, CBZ, and PB are indicated here. Table 5 shows these quoted therapeutic ranges for PHT, CBZ, and PB together with theoretical target values for therapeutic ranges for free levels of all three drugs at 25 and 37°C. These target values were derived by multiplying the values for therapeutic ranges for total levels by the observed mean values of α from Table 4.

TABLE 2. *Mean values of the apparent dissociation constant (K_D) at different temperatures derived by regression analysis from Fig. 2*

	$K_D \times 10^{-3}$		
	15°C	25°C	37°C
Phenytoin	16.7	13.4	9.5
Carbamazepine	4.2	3.8	3.4
Phenobarbitone	1.2	1.1	1.0

DISCUSSION

The binding of AEDs to albumin in vivo is a complex process; therefore, we restricted this study to the case of patients on AED monotherapy. The binding of PHT, CBZ, and PB to protein was found to be inversely related to temperature with the largest effect seen in the case of PHT (Table 1), and these findings are in accordance with previously published data (3,14,21–23). The depiction of concentration ratios of the free levels of the drugs at 15, 25, and 37°C in Fig. 1, with values at 25°C used for reference, shows the marked decrease in binding of PHT compared with the very much smaller effects for CBZ and PB. As would be expected from the results shown in Table 1, the values of K_D for PHT decreased sharply, concomitantly with the decreased binding at the higher temperature, and the effect was very small for CBZ and PB (Fig. 2). It can be seen that the mean values of K_D for PHT, CBZ, and PB at 15, 25, and 37°C, derived from Fig. 2 by regression analysis, correspond very closely to the calculated arithmetic means for K_D (Tables 2 and 3).

The apparent dissociation constant K_D, which is a theoretical concept, has an important practical application. Results given in Fig. 3 demonstrate that the use of mean values of K_D to calculate free levels from unknown patients' samples using Eq. 4 is a valid procedure; the correlation coefficient (r) between the values obtained by calculation and experimentally determined results for all three drugs lies between 0.961 and 0.998 and all results are within the 95% confidence limits. It follows that provided there is no alteration in protein binding, the values of K_D for a particular AED in monotherapy will remain constant. Therefore, it is possible, having established mean values for K_D at a given temperature for a particular population on AED monotherapy, to use these values to predict free levels accurately for other patients in similar circumstances knowing total levels of drug and albumin only (Fig. 3). In our study, this population comprised patients on PHT, CBZ, or PB monotherapy with normal levels of serum albumin to provide a simple, general case to establish the stability of K_D, as a basis for the investigation of more complex situations.

The free fraction (α) of an AED, representing that proportion of total drug not bound to protein, is related to the free level through both Eqs. (2) and (3). The mean values of the K_D shown in Table 2

TABLE 3. *Arithmetic mean of the apparent dissociation constant (K_D) at different temperatures*

	N	$K_D \times 10^{-3}$ at 15°C	CV %	$K_D \times 10^{-3}$ at 25°C	CV %	$K_D \times 10^{-3}$ at 37°C	CV %
Phenytoin	28	17.0	18.8	12.2	11.3	9.8	12.8
Carbamazepine	28	4.1	12.2	3.9	11.7	3.3	14.2
Phenobarbitone	25	1.2	18.3	1.1	13.2	1.0	14.4

CV, coefficient of variation.

were used to predict the mean of α [Eq. (2)], and the calculated results for this group of patients corresponded very closely to the observed mean of α obtained by application of Eq. 3 (Table 4). The values for α for all three drugs were also increased at 37°C compared to the results obtained when carrying out the filtration process at 25°C, and these changes were statistically significant ($p < 0.001$).

To examine the implications of these results in relation to therapeutic drug monitoring for patients, we calculated theoretical target values for free levels and compared them with local therapeutic ranges for total levels of PHT, CBZ, and PB. It is clear from the data shown in Fig. 4 that estimations of target ranges for free levels carried out at 37°C incline towards toxic values compared with target ranges for 25°C. The increase in levels of PHT-F determined at 37°C rather than 25°C is small in relative terms [amounting to a difference of 5% in the free fraction (α), as shown in Table 5]. However, this change represents an increase in the absolute concentration of PHT-F of more than 50%. These effects are less pronounced for CBZ-F and PB-F since the proportion of drug bound to protein is less (Table 4). The apparently small fractional increase in PHT-F determined after filtration at the physiological temperature of 37°C compared to the more usual 25°C could be of clinical significance and ex-

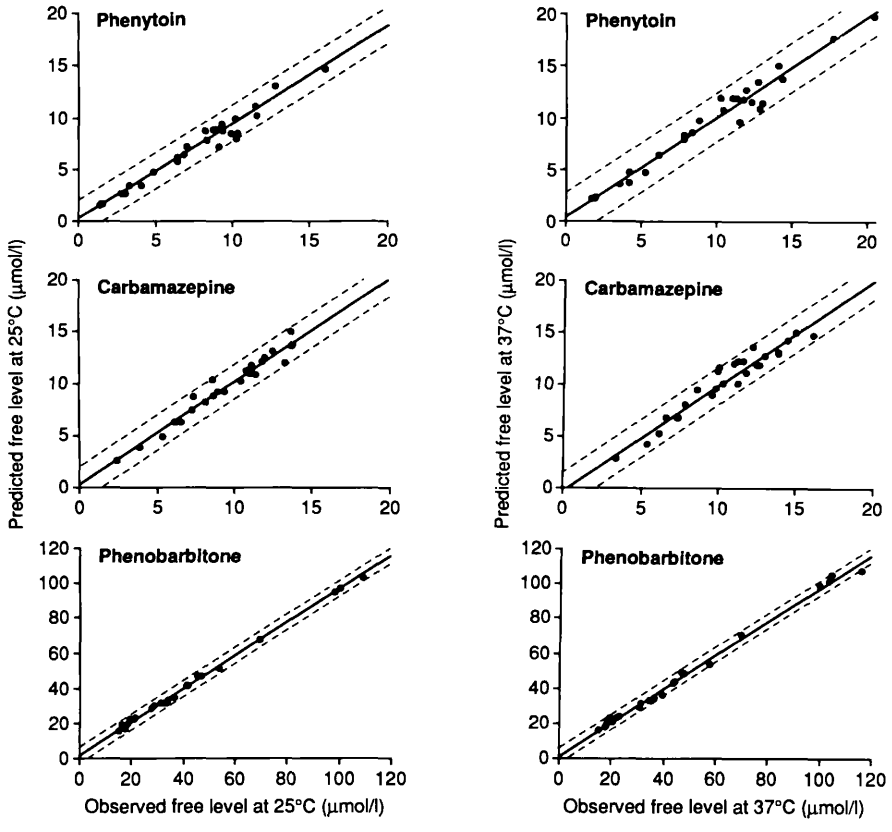

FIG. 3. Predicted free levels (y) of PHT-F, CBZ-F, and PB-F at 25 and 37°C vs. observed free levels (x) PHT-F, CBZ-F, and PB-F. —, Regression line; - - -, 95% confidence interval. Equations for regression line at 25°C: PHT-F (pred.) = 0.204 + 0.911 PHT-F (obs.), $r = 0.977$, $n = 28$; equations for regression line at 37°C: PHT-F (pred.) = 0.401 + 0.967 PHT-F (obs.), $r = 0.975$, $n = 28$. Equations for regression line at 25°C: CBZ-F (pred.) = 0.296 + 0.993 CBZ-F (obs.), $r = 0.968$, $n = 28$; equations for regression line at 37°C: CBZ-F (pred.) = −0.439 + 1.038 CBZ-F (obs.), $r = 0.961$, $n = 28$. Equations for regression line at 25°C: PB-F (pred.) = 1.691 + 0.949 PB-F (obs.), $r = 0.998$, $n = 25$; equations for regression line at 37°C: PB-F (pred.) = 1.139 + 0.959 PB-F (obs.), $r = 0.997$, $n = 25$.

TABLE 4. *Observed free fraction (α) and predicted free fraction (α) at 25 and 37°C*

	N	Observed free fraction (α)	CV %	Predicted free fraction (α)	CV %
Phenytoin					
25°C	131	0.10	16.9	0.10	8.2
37°C	131	0.15	14.3	0.15	7.8
Δ[a]		0.05		0.05	
Carbamazepine					
25°C	121	0.26	8.9	0.27	5.6
37°C	121	0.30	9.8	0.30	5.4
Δ[a]		0.04		0.03	
Phenobarbitone					
25°C	36	0.60	14.3	0.59	3.4
37°C	36	0.63	13.1	0.62	3.2
Δ[a]		0.03		0.03	

[a] $p < 0.001$ for difference between mean free fraction at 25 and 37°C.

plain why drug toxicity of PHT has been observed when total levels of the drug lie within the therapeutic range, as previously suggested (11). In animal experiments, Shoeman and Azarnoff (25) have shown that the anticonvulsant action of PHT depends on the concentration of PHT-F rather than on the total serum concentration or dose of PHT. In general, pharmacological effect and biotransformation are known to be closely related to free drug concentration rather than to the total levels usually determined, which comprise both free and bound elements. Thus, it is essential, whenever the estimation of free levels of AEDs is carried out, that the temperature used for the separation of free AED from the protein-bound component be specified and the appropriate target range be quoted. However, the general question of the choice of temperature at which the ultrafiltration of AEDs is carried out also

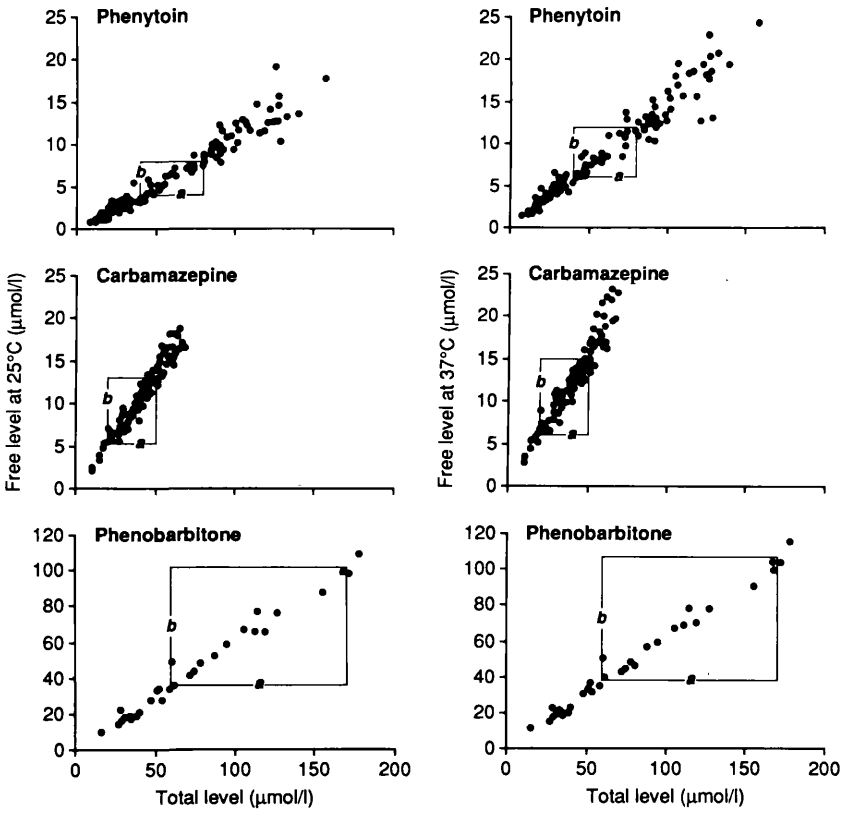

FIG. 4. Free levels (y) of PHT-F, CBZ-F, and PB-F assayed at 25 and 37°C vs. the total levels (x) of PHT, CBZ, and PB. Equations for regression line at 25°C: PHT-F = −0.442 + 0.112 DPH, r = 0.975, $p < 0.001$, n = 130; equations for regression line at 37°C: PHT-F = 0.229 + 0.146 PHT, r = 0.975, $p < 0.001$, n = 130. Equations for regression line at 25°C: CBZ-F = −0.188 + 0.267 CBZ, r = 0.969, $p < 0.001$, n = 121; equations for regression line at 37°C: CBZ-F = 4.028 + 2.966 CBZ, r = 0.960, $p < 0.001$, n = 121. Equations for regression line at 25°C: PB-F = −1.323 + 0.621 PB, r = 0.967, $p < 0.001$, n = 36; equations for regression line at 37°C: PB-F = 0.572 + 0.643 PB, r = 0.970, $p < 0.001$, n = 36. The squares in the figure indicate the therapeutic ranges for total levels (a) and theoretical target ranges for free levels (b).

TABLE 5. *Target ranges for free levels of phenytoin, carbamazepine, and phenobarbitone at 25 and 37°C*

	Theoretical target range (free level) (μmol/L)		Therapeutic range (total level)[a] (μmol/L)
	25°C	37°C	
Phenytoin	4.0–8.0	6.0–12.0	40–80
Carbamazepine	5.2–13.0	6.0–15.0	20–50
Phenobarbitone	36.0–102.0	38.0–107.0	60–170

[a] Therapeutic ranges were adapted from the literature and used at the Natoinal Hospital.

needs to be considered. In order to correlate the concentrations of free AEDs with other biochemical parameters, the separation step should be carried out at the physiological temperature wherever possible.

From a strictly clinical point of view, the measurement of free levels of AEDs may not be essential except for certain groups of patients such as pregnant women or patients with kidney or liver disease (1–3,10,13,26–28). Nevertheless, calculations based on the Law of Mass Action, using data derived from patients on AED monotherapy who have albumin levels within the normal range, will be essential for laboratories needing to establish their own target ranges. In addition, data obtained in this way can be used to predict the free concentration of a particular drug for patients on AED monotherapy. Calculations based on the Law of Mass Action, such as those we use here, may thus increase our understanding of factors involved in the protein binding of AEDs. This may assist both in the control of toxic symptoms and in the treatment of intractable epilepsy, where AED polytherapy is frequently necessary.

The investigation of the binding of AEDs to protein in the group of patients reported here forms a basis for the study of more complex situations. The fact that values for K_D remain stable for a particular population of patients on PHT, CBZ, or PB monotherapy, provided that the binding of the drugs to albumin does not alter, will allow the investigation of certain changes in binding in a very precise way by altering one factor at a time. For example, changes in the value of K_D can be used as an additional parameter when investigating drug interactions in patients on polytherapy. It is known that the addition of a drug to the regimen when a patient is on AED monotherapy will have a profound effect on the binding characteristics of that AED (29). To this end, we are investigating changes in the values of K_D and α in patients on AED polytherapy, and these aspects will be covered in a separate study.

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