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"PHARMACOKINETICS OF PHENYTOIN, FOSPHENYTOIN AND CHLORAMPHENICOL IN THE RABBIT AND RAT"

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A thesis submitted in partial fulfillment for the degree of Master of Science in Pharmacology and Toxicology in the University of Nairobi.

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2000

DECLARATION

This thesis is my original work and has not been presented for a degree in any other University.

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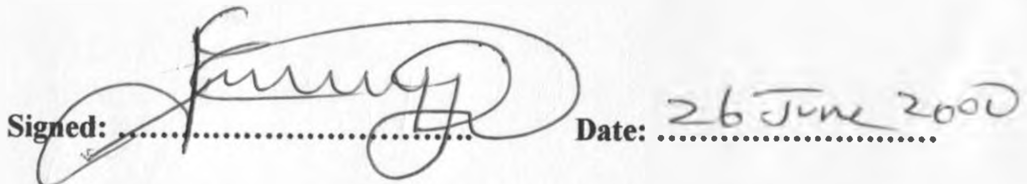
This thesis has been submitted for examination with our approval as University supervisors.



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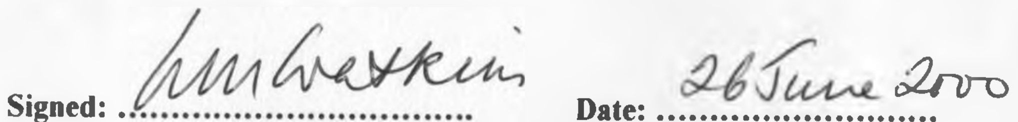

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DEDICATION

To my dear wife, Dorcas Wanjiru, and our daughters, Catherine Muthoni and Diana Wambui, for their patience, understanding and unwavering support.

And to all men and women of goodwill all over the world, who make sacrifices to ensure that the lives of the disadvantaged and less fortunate are more meaningful.

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ABBREVIATIONS

AUC	area under the curve
AUFS	absorbance units full scale
CAP	chloramphenicol
CAPS	chloramphenicol succinate
BCG	bromocresol green
BCP	bromocresol purple
cm	centimetre
°C	degree Celsius
C_{max}	maximum concentration
CV	coefficient of variation
EDTA	ethylene diamino tetraacetate
FOS	fosphenytoin
FPHT	fosphenytoin (unhydrolyzed form)
g	gram
μg	microgram
g	gravity
GLC	gas-liquid chromatography
HPLC	high-performance liquid chromatography
p-HPPH	5-(4-hydroxyphenyl)-5-phenylhydantoin
h	hour
i.d	internal diameter
i.m.	intramuscular
IS	internal standard

i.v.	intravenous
kg	kilogram
l	litre
µl	microlitre
M	molar
mM	millimolar
mg	milligram
min	minute
ml	millilitre
mm	millimetre
MPPH	5-(*p*-methylphenyl)-5-phenylhydantoin
MS	mass spectrometer
nm	nanometre
µm	micrometre
o.d	external diameter
ODS	octadodecyl silica
PEs	phenytoin equivalents
pH	-log (base 10) of hydrogen ion concentration
p*Ka*	log (base 10) of dissociation constant for an acid
PH	peak height ratio
PHT	phenytoin
psi	pascal per square inch
SD	standard deviation
sec	second
TBA	tetrabutylammonium hydrogen sulfate
TLC	thin layer chromatography
TMAH	tetramethylammonium hydroxide

T_{max}	time to maximum concentration
TMPAH	tetramethylphenylammonium hydroxide
uv	ultra-violet
vis	visible
v/v	volume per volume
WHO	World Health Organization
w/w	weight per weight

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ABSTRACT

Seizures commonly complicate cerebral malaria and are associated with increased risk of death and neurological sequelae. Phenytoin is used for treatment of seizures that are refractory to other treatment, but it has limitations due to its poor aqueous solubility. Its metabolism may also be inhibited by chloramphenicol. Fosphenytoin, a water-soluble phenytoin prodrug, has been introduced for clinical use. There is a need to investigate the pharmacokinetics of fosphenytoin in African children with severe malaria. A preliminary study on the pharmacokinetics of phenytoin, fosphenytoin and chloramphenicol was carried out in the rabbit and rat.

Phenytoin pharmacokinetics following i.v. and i.m. administration of fosphenytoin sodium (10 mg/kg phenytoin equivalents) were compared with those obtained following administration of standard phenytoin sodium injection (10 mg/kg) in adult New Zealand White rabbits (N=24; 2.1±0.41 kg), anaesthetized with pentobarbitone sodium (30 mg/kg). In a separate series of experiments, the effect of coadministration of chloramphenicol (25 or 50 mg/kg of chloramphenicol sodium succinate) on the pharmacokinetics of phenytoin following i.v. administration of fosphenytoin (30 mg/kg phenytoin equivalents)

was investigated in female Wistar rats (N=60, 253.1±31.6 g), anaesthetized with ether.

In the rabbit, similar plasma phenytoin concentrations were obtained following i.v. administration of fosphenytoin, and an equivalent dose of phenytoin sodium. Median maximum plasma phenytoin concentrations *(C_{max})* was 158% higher (*P*=0.0277) following i.m. administration of fosphenytoin sodium compared to i.m. administration of phenytoin sodium. The median area under the plasma total and free phenytoin concentration-time curve from time zero to 120 min *(AUC_{0-120})* following i.m. administration was also significantly higher (*P*=0.0277) in fosphenytoin treated rabbits (723.3 µg/ml.min) compared to the phenytoin (261.2 µg/ml.min) group. However, there was no significant difference (*P*=0.0464) in AUC_{0-180} between fosphenytoin (1023.1 µg/ml.min) and phenytoin (1183.4 µg/ml.min) treated rabbits following i.v. administration. There was also no significant difference in the median times to achieve maximum plasma phenytoin concentrations *(T_{max})* between fosphenytoin (30.0 min) and phenytoin (24.8 min) treated rabbits following i.m. administration (*P=0.675*). Mean plasma albumin concentrations were comparable in both groups of animals (*P*=0.9304). Fosphenytoin was rapidly converted to phenytoin both after i.v. and i.m. administration, with plasma fosphenytoin concentrations declining rapidly to undetectable concentrations within 10 min

following administration via either route. These results confirm the rapid and complete hydrolysis of fosphenytoin to phenytoin *in vivo*, and the potential of the i.m. route for administration of fosphenytoin delivering phenytoin in clinical settings where i.v. administration is not feasible.

Following i.v. administration of fosphenytoin in rats, plasma phenytoin concentrations were similar to those obtained after coadministration of fosphenytoin and 25 mg/kg of chloramphenicol succinate (*P=0.281*). The $AUC_{0\text{-}7h}$ was approximately 9% and 60% higher following coadministration of fosphenytoin and 25 and 50 mg/kg of chloramphenicol succinate, respectively. Chloramphenicol concentrations were approximately twofold higher after administration of 50 mg/kg compared to 25 mg/kg, but were below the reported therapeutic range (10-20 μg/ml). The $AUC_{0\text{-}7h}$ was 11.30 and 20.54 μg•h/ml following administration of 25 and 50 mg/kg of chloramphenicol succinate, respectively. The results confirm that both fosphenytoin and chloramphenicol succinate are quantitatively hydrolyzed *in vivo* in the rat, and that the interaction between phenytoin and chloramphenicol *in vivo* in the rat is dose-dependent. The results of this study emphasize the importance of monitoring the plasma concentrations of phenytoin when it is concurrently administered with chloramphenicol in clinical practice.

CHAPTER ONE

INTRODUCTION

1.0 MALARIA

1.1 Malaria as a global problem

Malaria remains one of the world's most common and major public health problems. About 40 per cent of the world's population (approximately 2.4 billion people) live in malaria-endemic areas (Sturchler, 1990; WHO, 1997). An estimated 300 to 500 million clinical cases and about 1.5 to 2.7 million malaria deaths are reported in the world annually (WHO, 1997; WHO, 1998). The greatest disease burden falls on sub-Saharan Africa, where *Plasmodium falciparum* is estimated to cause at least a million deaths among children every year (Greenwood *et al.*, 1991; WHO, 1997). In sub-Saharan Africa, malaria is the leading health problem as well as a leading cause of childhood mortality and morbidity, causing the death in one out of 20 children (5 per cent) under the age of five years (WHO, 1998).

In Kenya, malaria is an important health problem. The disease remains the major cause of morbidity and mortality, with over four million cases estimated to occur annually in the country (WHO, 1997). The disease accounts for about 30% of all illnesses nationally, although the distribution is not uniform throughout the country (Ministry of Health, 1993; 1992). Mortality among children in Africa is high but the magnitude of the problem is unknown as most children die outside health facilities (Greenwood *et al.*, 1987; D'Alessandro,

1997). It is estimated that approximately 26,000 children die every year (72-childhood malaria death each day) from the direct consequences of infection (Snow *et al.*, 1998).

The problem of malaria in Kenya, and the rest of the East African countries, has worsened in recent years, as evidenced by increased cases of severe malaria and malaria mortality among children and adults (WHO, 1998). The most prevalent malaria species, *Plasmodium falciparum*, has developed resistance to commonly used antimalarial drugs, including chloroquine, the cheapest and most widely available antimalarial drug (Zucker *et al.*, 1996). This has led to therapeutic failure, necessitating the use of more expensive drugs, and in some cases longer treatment courses (Olliaro *et al.*, 1996). These alternative drugs cannot be afforded by the majority of patients who need them, resulting in more cases of severe illness and death. To contain the situation, urgent attention needs to be focused on problems related to treatment and management of both severe and uncomplicated malaria. Early diagnosis, prompt and effective treatment and appropriate management of severe and uncomplicated malaria remains the most important strategy for the control of malaria-related mortality in Kenya (Ministry of Health, 1998).

1.2 Severe malaria and associated complications

Infection with *Plasmodium falciparum* causes severe malaria including cerebral malaria (Warrel *et al.*, 1990; Greenwood *et al.*, 1990; Greenwood *et al.*, 1987), with a mortality rate of between10 and 40% (Molyneux *et al.*, 1989). Cerebral malaria is a common complication in childhood malaria in sub-Saharan Africa (Asindi *et al.*, 1993; Molyneux *et al.*, 1989). Complications associated with severe malaria and which are thought to contribute to high mortality include severe metabolic acidosis, hypoglycaemia, respiratory distress and seizures (English *et al.*, 1996; Marsh *et al.*, 1995; Molyneux *et al.*, 1989; Warrel *et al.*, 1987). Seizures associated with severe malaria are common in sub-Saharan Africa (Brewster *et al.*, 1990; Marsh *et al.*, 1995), with a case-fatality rate of 10-30% among hospital admissions (Greenwood *et al.*, 1987; Warrel *et al.*, 1987). These seizures are often prolonged, frequently progress to status epilepticus, and have been associated with poor prognosis (Molyneux *et al.*, 1989; Brewster *et al.*, 1990).

Multiple seizures are often refractory to treatment and children with multiple seizures are twice as likely to die compared to children with fewer seizures (Shorvon, 1994). Among the survivors, protracted or repeated seizures are associated with an increased risk of development of permanent neurological sequelae, characterized by speech impairment, cortical blindness, hemiplegia, and epilepsy (Molyneux *et al.*, 1989; Bondi, 1990; Brewster *et al.*, 1990; Bondi,

(Langslet *et al.*, 1978), and this may lead to fatal respiratory depression. Lastly, although diazepam enters the brain readily and stops status epilepticus quickly, due to its high lipid solubility, it quickly redistributes to other fatty tissues, causing brain and plasma concentrations to fall rapidly (Bone, 1993). The rapid fall in plasma diazepam concentrations following administration of a single i.v. dose may lead to recurrence of further uncontrolled seizures (Langslet *et al.*, 1978), if diazepam alone is used to control the seizures. Moreover, peripheral venous access is sometimes technically difficult in young children who are vigorously convulsing. Thus, there is a need to investigate alternative routes of administration of diazepam. Currently, the suitability of the rectal route for diazepam administration in young children with seizures associated with severe malaria is being investigated in Kilifi, located in the Coast Province.

Phenobarbitone has been used as an anticonvulsant for many years. It is widely available in Africa since it is cheap and highly effective in the treatment of both generalized and partial status epilepticus (Shorvon, 1994). A single i.m injection of phenobarbitone (3.5 mg/kg) is effective in preventing seizures in cerebral malaria (White *et al.*, 1988; Murphy and Waruiru, 1996), and 10 mg/kg, repeated once to a maximum total dose of 20 mg/kg/24 h has been recommended in children with cerebral malaria in Kenya (Ministry of Health, 1998). However, therapeutic concentrations are not achieved following i.m. administration of a 10 mg/kg dose to African children with severe malaria

(Winstanley *et al.*, 1992). Intramuscular administration of a 20 mg/kg prophylactic dose, while achieving therapeutic concentration in plasma and halving seizure frequency, is also associated with a doubling in mortality in childhood cerebral malaria, especially when administered concomitantly with diazepam (Crawley *et al.*, 2000). The main disadvantage of phenobarbitone in the treatment of cerebral malaria is related to the fact that the drug can cause respiratory depression and hypotension. Therefore, there is an urgent need for a pharmacokinetic and clinical evaluation of alternative anticonvulsants for seizure control in childhood malaria. Priority research should be focused on those anticonvulsants less likely to potentiate the respiratory depressant effect of diazepam.

Paraldehyde is an old drug rarely used in the West, but is effective in controlling status epilepticus in children (Lombroso, 1974) and is routinely used in Kenya (Ministry of Health, 1998) and other parts of Africa (Molyneux *et al.*, 1989) for controlling seizures refractory to diazepam. However, the pharmacokinetics of paraldehyde in African children is unknown, and is currently being investigated in our laboratory.

An important subgroup of children with severe malaria has multiple seizures which are difficult to control with the commonly available anticonvulsants described above. Children with seizures refractory to other treatments are

usually treated with phenytoin, which is very effective in these situations (Shorvon, 1994). It rapidly penetrates the blood-brain barrier (Ramsey *et al.*, 1979) with the added advantage of being long acting (ideal for prophylaxis) and, except at very high concentrations, devoid of respiratory or cardiac depression (Wilder *et al.*, 1995). Thus, it can be administered after or together with a benzodiazepine, without exacerbating the CNS depressant effects of the latter.

Phenytoin is used routinely in the paediatric clinic at Kilifi on the Kenyan coast to control seizures associated with severe malaria, and which are refractory to other anticonvulsants.. However, the dosage regimens of phenytoin used are empirical since its pharmacokinetic parameters have not been studied in the African children. The pharmacokinetics of phenytoin in children with severe malaria is currently being investigated in Kilifi.

Parenteral phenytoin is the formulation used for acute control of seizures. There are two main disadvantages of parenteral administration of phenytoin. Firstly, it cannot be administered i.m. due to precipitation of phenytoin at the site of injection, leading to delayed absorption and tissue necrosis (Serrano and Wilder, 1974). Secondly, phenytoin sodium injection contains propylene glycol and alcohol, and is buffered at high pH of between 10-12. These hydroalcoholic solutions may cause irritation at the site of injection, and cardiovascular effects

(Cranford *et al.*, 1978). Therefore, i.v. administration of the injection is done under controlled conditions.

Fosphenytoin (5,5- diphenyl-3-[(phosphonooxy)methyl]- 2,4-imidazolidinedione, disodium salt), the disodium phosphate ester of phenytoin (3-hydroxymethyl-5,5-diphenylhydantoin), is a newly developed water-soluble pro-drug for the parenteral administration (Leppik *et al.*,1990). The greater water solubility of fosphenytoin overcomes most of the previous problems and limitations associated with parenteral phenytoin sodium administration. The advantages of fosphenytoin according to various studies (Varia and Stella, 1984d; Boucher *et al.*, 1989; Leppik *et al.* 1990; Jamerson *et al.*, 1994; Fischer *et al.*, 1995; Boucher *et al.*, 1996; Fierro *et al.*, 1996; Uthman *et al.*, 1996) are: (a) It can be administered either intramuscularly or intravenously and, since the injection contains no propylene glycol and is not buffered at high pH, causes fewer side effects compared with phenytoin, (b) It offers improved compatibility with commonly used intravenous fluids, and causes less irritation and phlebitis at the injection site, (c) It can be administered at infusion rates up to three times the maximum rate for phenytoin (150 mg phenytoin equivalents/min for fosphenytoin versus 50 mg phenytoin /min) (d) It is rapidly and completely absorbed from the intramuscular site and (e) It is rapidly and completely hydrolyzed *in vivo* by blood and tissue phosphatases to generate phenytoin after i.v. or i.m. administration, with approximately 100 per cent bioavailability.

Thus, fosphenytoin may offer both practical and clinical advantages over intravenous phenytoin sodium. It would be very useful in rural parts of Africa since it can be administered intramuscularly at peripheral health centres not only to stop seizures but also to provide prophylactic cover against subsequent seizures.

Fosphenytoin has no significant pharmacological activity of its own, and the clinical effects following its administration are due to the generated phenytoin (Boucher *et al.*, 1979; Leppik *et al.*, 1990; Jamerson *et al.*, 1994; Fischer *et al.*, 1995; Parke-Davis, 1996). Since phenytoin is cleared slowly from the body (Arnold and Gerber, 1970), the sustained levels of phenytoin can provide prophylaxis against further seizures. Thus, a combination of fosphenytoin with diazepam has been suggested as ideal for management of seizures. The diazepam would rapidly terminate the seizures, hence its usefulness in status epilepticus, while the phenytoin derived from fosphenytoin would provide prophylaxis cover for the patient over several hours. Although the pharmacokinetics and safety of fosphenytoin have been evaluated in Caucasian paediatric patients (Boucher, 1996; Fierro *et al.*, 1996; Morton *et al.*, 1996), no such studies have been carried out in African children.

There is a need for studies to (a) evaluate how rapidly phenytoin is generated from fosphenytoin, following both i.m.and i.v. administration of fosphenytoin,

and (b) compare plasma total and free phenytoin concentrations following administration of phenytoin and fosphenytoin via both routes..

Phenytoin is extensively bound to plasma (Lightfoot and Christian, 1966) and may be displaced by other drugs. There is a correlation between the extent of binding to albumin and the concentration of albumin in plasma (Hooper *et al.*, 1973). The concentration of unbound phenytoin in plasma correlate with the anticonvulsant effect (Shoeman and Azarnoff, 1975), toxic effects and clearance from the body (Rowland and Tozer, 1989). In children with severe malaria, unbound phenytoin concentrations may be altered due to hypoalbuminemia as a result of malnutrition or disease, by displacement of phenytoin from binding sites or by inhibition of the metabolism of phenytoin. Almost all children admitted to the Kenya Medical Research Institute (KEMRI) Ward in Kilifi are routinely treated with chloramphenicol for suspected or confirmed bacterial meningitis. A proportion of these children receives concurrent phenytoin for convulsions which are refractory to treatment with other anticonvulsants. However, chloramphenicol has been reported to cause modest to marked elevation in plasma phenytoin concentrations (Koup, 1978; Nation *et al.*, 1990), as a result of inhibition of phenytoin metabolism. It is likely that the pharmacokinetic properties of both drugs determine the degree and clinical significance of this interaction. Therefore, there is a need to investigate the effect of concomitant administration of chloramphenicol on the

pharmacokinetics of phenytoin in children with severe malaria and bacterial meningitis.

1.3 Null hypothesis

1. There is no difference in area under the curve (AUC) for plasma phenytoin concentration between rabbits treated with fosphenytoin and those treated with an equivalent dose of phenytoin sodium.
2. Prior administration of chloramphenicol has no effect on plasma total phenytoin concentrations, following administration of fosphenytoin in the rat.

1.4 Objectives of the study

The specific objectives of the preliminary pharmacokinetic studies were to:

(1) Define the optimum sampling protocol necessary to describe the pharmacokinetics of fosphenytoin and generate pharmacokinetic data.

(2) Compare the rate of conversion of fosphenytoin, the inactive prodrug, to phenytoin following i.m. and i.v. administration in the rabbit.

(3) Compare the absolute bioavailability of phenytoin by assessing the AUC of total and free phenytoin following i.v. administration of fosphenytoin and an equimolar dose of phenytoin in the rabbit.

(4) Investigate the effect of concomitant i.v. administration of chloramphenicol sodium succinate on plasma concentrations of phenytoin after i.v. administration of fosphenytoin in the rat.

1.5 Justification of the study

Uncontrolled seizures associated with severe malaria contribute to mortality, and can lead to the development of neurological disorders among survivors. Early, effective and appropriate management of severe and uncomplicated malaria remains the most important strategy for the control of malaria-related mortality in sub-Saharan Africa. If treatment and prophylaxis with anticonvulsant drugs can reduce the incidence of seizures complicating cerebral malaria, it is possible that this may reduce the morbidity and mortality and the incidence of neurological sequelae among survivors. The goal of therapy is to stop the seizures as quickly as possible and to minimize adverse physiological consequences of status epilepticus. Priority areas of research include understanding the pharmacokinetics of currently available and alternative anticonvulsant drugs, and defining appropriate combinations that can be used at both peripheral health and higher health care centres for treatment of acute seizures and also for providing antiseizure prophylaxis.

Fosphenytoin is one such promising drug whose pharmacokinetics needs to be evaluated. These preliminary studies focus on the pharmacokinetics of

fosphenytoin and phenytoin in the rabbit and rat. The data obtained in the preliminary studies will be used to plan future studies on the pharmacokinetics of fosphenytoin in children with seizures associated with severe malaria.

CHAPTER TWO

LITERATURE REVIEW

2.0 PHENYTOIN, FOSPHENYTOIN AND CHLORAMPHENICOL

2.1 Phenytoin and Fosphenytoin

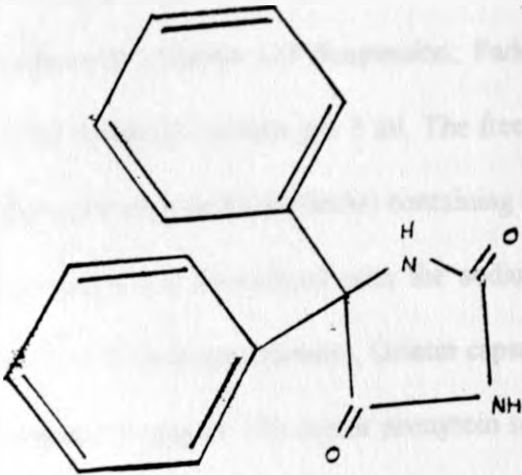
2.1.1 Physico-chemical properties

Phenytoin is the generic name for 5,5-diphenylhydantoin (acid form). Its chemical name is 5,5-diphenyl-2,4-imidazolidine. The free acid has a molecular formula of $C_{15}H_{12}N_2O_2$ and a molecular weight of 252.26. The sodium salt has a molecular weight of 274.25 (91.98% acid). It is an odourless, white powder with a melting point of 294-297°C. Phenytoin is a weak organic acid (Newton and Kluza, 1980; Schwarz *et al.*, 1977) exhibiting poor aqueous solubility (Varia *et al.*, 1984b; Newton and Kluza, 1980). The apparent dissociation constant pK_a (pH at which 50% of the drug is ionized) is between 8.1-9.2. The acid is essentially non-ionized at pH 5.4, with a solubility of about 19.4 µg/g at 25.4°C. At pH 7.4, where the acid is about 80% non-ionized, it has a water solubility of 20.5 µg/g at 25.2 °C. Parenteral sodium phenytoin is formulated with 40% propylene glycol and 10% ethanol in water for injection adjusted to pH 12 with sodium hydroxide (Browne, 1997). This formulation contains 50 mg phenytoin sodium per ml, equivalent to 46 mg phenytoin acid per ml. The relatively high pH of the phenytoin injection contributes to local adverse effects at the injection site. The propylene glycol component is partly responsible for

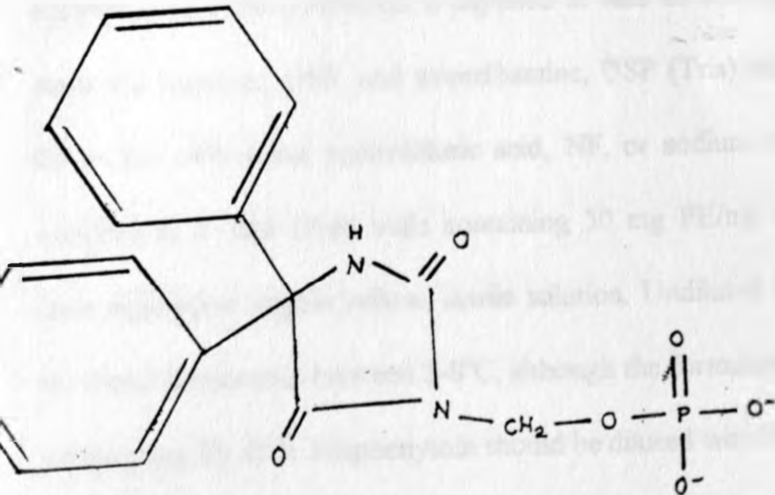
the reported hypertension and cardiac arrhythymias associated with parenteral sodium phenytoin (Louis *et al.*, 1967).

Fosphenytoin sodium (ACC-9653, Cerebyx®; Parke-Davis, Ann Arbor, MI) is the disodium phosphate ester of 3-dihydroxymethyl-5,5-diphenylhydantoin. Fosphenytoin was first synthesized by Stella and Higuchi in 1973 (Stella and Higuchi, 1973). The chemical name for fosphenytoin is 5,5-diphenyl-3-[(phosphonooxy)methyl]-2,4-imidazolidinedione, disodium salt. It is an off-white agglomerated powder with a molecular formula of $C_{15}H_{13}N_2O_6P.2Na$. Its molecular weight is 406.24, compared with 275.25 for sodium phenytoin. Thus, 1.5 mg of fosphenytoin liberates 1.0 mg of phenytoin. The compound is stable under normal conditions of use. Fosphenytoin doses are expressed as phenytoin equivalents (PEs) and 75 mg of fosphenytoin is equivalent to 50 mg PEs. The water solubility of fosphenytoin at 37°C is 7.5×10^4 µg/ml, compared with 20.5 µg/ml for phenytoin and hence it is over 4000 times more soluble (Browne *et al.*, 1993; Varia *et al.*, 1984).

Fig. 1: Structural formulae of phenytoin and fosphenytoin



Phenytoin



Fosphenytoin

2.1.2 Formulations

Phenytoin acid is used as an aqueous oral suspension (Paediatric *Dilantin-30* Suspension and *Dilantin-125* Suspension; Parke-Davis), containing 30 mg or 125 mg of phenytoin sodium per 5 ml. The free acid is also used in formulating chewable tablets (*Dilantin Infatabs*) containing 50 mg phenytoin acid per tablet. Other products are formulated with the sodium salt of phenytoin (phenytoin sodium, 91.98% acid equivalents). Gelatin capsules (*Dilantin Sodium Kapseals*) contain either 30 mg or 100 mg of phenytoin sodium (equivalent to 27.6 mg or 92.0 mg of phenytoin acid equivalents) per capsule. The injectable phenytoin sodium salt is also used as a parenteral formulation (*Parenteral Dilantin*).

Fosphenytoin sodium injection is supplied in vials as a ready-mixed solution in water for injection, USP, and tromethamine, USP (Tris) buffer adjusted to pH 8.6 to 9.0 with either hydrochloric acid, NF, or sodium hydroxide, NF. It is available in 2- and 10-ml vials containing 50 mg PE/ml, which appears as a clear, colourless to pale yellow, sterile solution. Undiluted fosphenytoin should be stored refrigerated between 2-8°C, although the formulation is stable at room temperature for 48 h. Fosphenytoin should be diluted with 5% dextrose or 0.9% normal saline prior to intravenous injection to give a concentration of 1.5-2.5 mg PE/ml. The diluted fosphenytoin solution is stable for 8 h at room temperature and 24 h under refrigerated conditions (Parke-Davis, 1996).

2.1.3 Pharmacokinetics

For it to exert its pharmacologic effects, phenytoin must reach its receptors in the brain and other tissues. This process involves movement of phenytoin from the site of administration, distribution in blood and other extracellular fluids to the cells, synapses and other receptors sites, passage across the cell membranes into cells and across subcellular organelles. The amount of phenytoin that reaches a receptor and its duration of action depend on its rate of biotransformation and excretion from the body.

2.1.3.1 Absorption

The oral and parenteral routes of administration present problems that are mainly related to the low aqueous solubility of phenytoin acid (14 μg/ml at room temperature).

2.1.3.1.1 Oral administration

The absorption of phenytoin from its site of administration depends on its pKa and lipid solubility, the pH of the medium in which phenytoin is dissolved, its solubility in the medium, and its concentration. Due to its weakly acidic nature (p*Ka* 8.31) and poor aqueous solubility (100 μg/ml), phenytoin often shows erratic absorption following oral administration (Tyrer *et al.*, 1960). Following oral administration, phenytoin is rapidly and passively absorbed across the intestinal mucosa in the unionized form but absorption is limited by its extremely low solubility in gastrointestinal fluids (Dill *et. al.*, 1956). Absorption is erratic

and dissolution-rate-limited, and occurs as the drug goes into solution in the intestinal fluids. In humans after oral administration of a single dose, maximum blood concentrations are generally reached 4-8 h after drug administration, but the peak may be reached as early as 3 h or as late as 12 h after ingestion of the drug (Dill *et al*., 1956). The times to reach maximum phenytoin concentrations were reported to increase progressively from 8.4 to 13.2 to 31.5 h after 400, 800 and 1600 mg doses of phenytoin, respectively (Jung *et al*., 1980). The suspension formulation possesses particular problems with oral absorption. The administration of phenytoin suspension in conjunction with enteral nutrition supplements through nasogastric feeding tubes may be associated with reduced phenytoin absorption, subtherapeutic concentrations and breakthrough seizures (Bauer, 1982; Saklad *et al.* 1986).

2.1.3.1.2 Parenteral administration

Phenytoin sodium is given both intravenously and intramuscularly to patients who cannot take the drug orally or those who require a rapid onset of action. However, both of these routes of administration have limitations. Phenytoin is a weak acidic drug (Schwarz *et al.*, 1977; Newton and Kluza, 1980) exhibiting poor aqueous solubility (Newton and Kluza, 1980; Varia *et al.*, 1984b). These properties lead to erratic and incomplete absorption of the drug after intramuscular injection due to precipitation of the free acid. Thus, it has to be administered as a slow intravenous infusion at a rate of less than 50 mg

phenytoin per min over 2 h to prevent precipitation and toxicity from the propylene glycol which is used to make it go into solution. The parenteral sodium phenytoin is formulated in an aqueous alkaline pH (≈12) and contains 40% propylene glycol and 10% ethanol. The parenteral formulation is very toxic after rapid i.v injection (Zoneraich *et al.*, 1976), due to the fact that propylene glycol is cardiotoxic. The parenteral dosage form also presents other problems, especially if admixing or dilution is desired (Newton and Kluza 1980). Phenytoin is never administered intramuscularly due to the slow and incomplete absorption (Serrano *et al.*, 1973; Wilder and Ramsey, 1976).

When fosphenytoin sodium is administered by intravenous infusion, maximum plasma concentrations are achieved at the end of the infusion (Gerber *et al.*, 1988). Fosphenytoin has a half-life of 15 min (Boucher, 1996; Eldon *et al.*, 1993).Fosphenytoin is completely bioavailable following i.m. administration of Cerebyx® (Parke-Davis, 1996). Maximum plasma concentrations occur at approximately 30 min after drug administration. Plasma fosphenytoin concentrations following intramuscular administration are lower but more sustained than those following intravenous administration due to the time required for absorption of fosphenytoin from the injection site.

2.1.3.2 Distribution

Phenytoin is extensively (>90%) and reversibly bound to plasma proteins after entering the blood, but the free form enters tissues and is bound to proteins and phospholipids. The total concentration in these tissues is higher than in the extracellular fluid, but the free levels are similar (Dill *et al.*, 1956). The drug is also stored in fat. Concentrations of phenytoin in transcellular fluids such as cerebral spinal fluid, gastrointestinal fluids, bile, milk, saliva and plasma are the same as the free levels in the blood (Dill *et al.*, 1956). Phenytoin is highly bound to plasma proteins, primarily albumin, although to a lesser extent than fosphenytoin. In the absence of fosphenytoin, which displaces phenytoin from the plasma protein binding site (Eldon *et al.*, 1993), approximately 12% of total plasma phenytoin is unbound over the clinically relevant concentration range. In the presence of fosphenytoin, the free fraction of phenytoin increases (Hussey *et al.*, 1990; Eldon *et al.*, 1993). Free phenytoin fraction also increases with increasing fosphenytoin plasma concentrations and with increasing fosphenytoin infusion rates (> 50 mg PE/min). Prior administration of diazepam has no effect on protein binding of fosphenytoin or phenytoin (Hussey *et al.*, 1990).

Binding of phenytoin to plasma proteins can be inhibited by drugs such as salicylates, thyroxine, phenylbutazone, and others that compete for the binding sites of the proteins and displace phenytoin from the sites (Lunde *et al.*, 1970; Monks *et al.*, 1978; Fraser *et al.*, 1980). Endogenous compounds such as fatty

acids and bilirubin in the neonate also displace phenytoin from plasma proteins and are a potential source of drug interactions. The unbound fraction of phenytoin has been reported to increase by as much as 200% in the presence of salicylates (Lunde *et al.*, 1974) which occurs only with high doses of salicylate and is not clinically important (Leonard *et al.*, 1981). However, 80% of children admitted to the Kilifi District Hospital with severe malaria have detectable salicylate in plasma of which 20% have potentially toxic concentrations (English *et al.*, 1996). The increased free level increases the anticonvulsant effect which depends on the unbound fraction and not the total plasma concentration (Shoeman and Azarnoff, 1975). It also allows more of the phenytoin to reach the liver per unit time, which results in increased biotransformation and thus decreased plasma total phenytoin concentrations. Phenytoin binding is also decreased in ureamia and hepatic disease (Hooper *et al.*, 1973; Shoeman and Azarnoff, 1975).

Following absorption, phenytoin distributes freely in the body because at the pH of plasma (7.4), it exists predominantly in the non-ionized form, which allows rapid movement across cell membranes by diffusion. Phenytoin reaches its maximum volume of distribution (V_d) within 15 minutes after absorption. The average value for V_d based on the total plasma concentrations is about 0.78 L/kg in humans (Trieman and Woodbury, 1995).

Fosphenytoin is extensively bound (95-99%) to human plasma proteins, primarily albumin (Eldon *et al.*, 1993). Binding to plasma proteins is saturable with the result that the percent bound decreases as total phenytoin concentrations increase (Hussey *et al.*, 1990). Fosphenytoin displaces phenytoin from protein binding sites (Eldon *et al.*, 1993). The volume of distribution of fosphenytoin ranges from 4.3 to 10.8 L/kg and increases with dose and rate and in a saturable manner to the same proteins (albumin) as phenytoin.

2.1.3.3 Metabolism and excretion

Phenytoin is eliminated almost entirely by metabolic transformation before excretion in the form of metabolites. Less than 5% of the total drug administered is excreted unchanged in the urine (Browne *et al.*, 1993). The principal metabolic pathway of phenytoin in humans is the 5-(4-hydroxyphenyl)-5-phenylhydantoin (*p*-HPPH), and the dihydrodiol pathway, accounting for 70-90% of administered phenytoin. *Para*-HPPH accounts for 67-88%, and dihydrodiol accounts for 7-11% of human metabolites of phenytoin (Browne *et al.*, 1989). The first step in this pathway is the formation of an arene oxide via the cytochrome oxidase enzyme system arene oxidase. The arene oxide is converted spontaneously to *p*-HPPH and is converted by the enzyme epoxide hydrolase to dihydrodiol. Most *p*-HPPH is excreted as a glucuronide and only small amounts of free *p*-HPPH are found in human urine.

The metabolism of phenytoin is saturable at the normal therapeutic concentrations. Thus, the plasma half-life of the phenytoin in humans is variable, dose-dependent and obeys saturation (Michaelis-Menten) kinetics. The half-life after oral administration of doses that result in therapeutic levels has been reported to average about 22 h, with a range of 7-42 h (Dill and Glazko, 1956; Arnorld and Gerber, 1970). The half-life after i.v. administration of phenytoin has been reported to be shorter, ranging from 10-15 h (Glazko *et al.*, 1969). The reported difference results from the slow rate of absorption of phenytoin from the gut, which maintains the plasma concentrations at a high level for a longer period of time. The half-life determined after intravenous administration of phenytoin would be a more accurate reflection of the true elimination half-life. However, because phenytoin half-life increases with the plasma drug concentration and thus dose (because of saturation elimination kinetics) and exhibits large individual variability, an average value is of limited usefulness. The marked variation in plasma phenytoin half-life emphasizes the importance of tailoring the dose of the drug to each patient and of monitoring the patient by measurement of plasma phenytoin levels.

A number of drug interactions result in alteration of the disposition of phenytoin. Alcohol, barbiturates, and carbamazepine induce oxidative enzymes; this induction results in increased metabolism of phenytoin, reduced serum concentrations of both total and free phenytoin, and reduced pharmacological

effect. Drugs such as chloramphenicol and isoniazid compete with phenytoin metabolism, resulting in increase of both total and free phenytoin concentrations and enhancement of pharmacological effect. Salicylate, valproic acid and phenylbutazone compete with phenytoin for plasma binding sites (Nation *et al.*, 1990).

Following parenteral administration of Cerebyx®, fosphenytoin is rapidly and completely converted to the anticonvulsant phenytoin by phosphatases present in the liver, red blood cells and many other cells. The conversion half-life has been reported to be about 3 min in the dog (Lai *et al*, 1987) and less than 1 min in the rat (Varia and Stella, 1984a). In humans, the conversion half-life of fosphenytoin to phenytoin has been reported to be about 8-15 min, with modest interindividual variability (Gerber *et al.*, 1988; Boucher *et al.*, 1989; Leppik *et al.*, 1990; Browne *et al.*, 1993; Eldon *et al.*, 1993;). The conversion half-life appears to be independent of plasma phenytoin and fosphenytoin concentrations (Browne *et al.*, 1993; Eldon *et al.*, 1993; Leppik *et al.*, 1990). The clearance of fosphenytoin is about 200 ml/min at lower dosing and infusion rates, and increases to about 400 ml/min at higher dosing and rates, probably due to changes in distribution (Browne *et al*, 1990; Eldon *et al.*, 1993). The conversion half-life of fosphenytoin to phenytoin has been reported to be less in patients with hepatic renal disease, possibly due to differences in protein binding (Aweeka *et al.*, 1989). Intravenous and intramuscular administration of

equimolar doses of fosphenytoin and phenytoin resulted in similar plasma phenytoin concentrations in the dog (Varia and Stella, 1984b; Chan *et al.*, 1988). The pharmacological and toxicological effects of fosphenytoin include those of phenytoin.

The hydrolysis of fosphenytoin to phenytoin yields two other metabolites, phosphate and formaldehyde. Formaldehyde is subsequently converted to formate, which is in turn metabolized via folate dependent mechanism. Direct renal excretion of fosphenytoin is small and clinically insignificant (Parke-Davis, 1996). Phenytoin derived from fosphenytoin concentration is eliminated in the same way as phenytoin derived from other formulations (Leppik *et al.*, 1990; Browne *et al.*, 1989). No drugs have been reported to interfere with the conversion of fosphenytoin to phenytoin (Parke-Davis, 1996).

2.1.4 Antiseizure spectrum and pharmacodynamics

Fosphenytoin has no known intrinsic pharmacologic activity before its conversion to phenytoin. Furthermore, its very low lipid solubility suggests that it would be impermeable to the blood-brain barrier, and therefore lack anticonvulsant activity before its conversion to phenytoin. However, since it is rapidly and completely converted *in vivo* to its active metabolite phenytoin after parenteral administration, its toxicological effects are essentially the same as those of phenytoin.

Phenytoin is known to prevent seizures in a variety of animal models of epilepsy (Löscher and Schmidt, 1988). These animal models include partial seizures in kindled rats (McNamara *et al*., 1989; Lothman *et al*., 1991) and generalized tonic-clonic seizures in mice or rats (Krall *et al*., 1978) or clonic seizures in audiogenic mice. Results from these models indicate that phenytoin inhibits the spread of seizure activity within the brain and raises the threshold for localized seizures from electrical stimulation (Lothman *et al*., 1991). This spectrum of anticonvulsant activity in animal models suggests therapeutic activity against partial seizures and tonic-clonic seizures in humans but not against absence or myoclonic seizures. Activity in these models has been useful to predict clinical effectiveness in humans (Löscher and Schmidt, 1988).

The cellular mechanisms that account for anticonvulsant action of phenytoin are not fully known. However, phenytoin is active at a number of pharmacological sites in brain tissues. The most notable of these sites are voltage-sensitive sodium channels of nerve cell membranes where phenytoin acts as a voltage-dependent and use-dependent blocker of sodium channels, having pronounced effects only when cell membranes are depolarized and sodium channels opened repeatedly (Macdonald, 1989). In addition, phenytoin interacts with voltage-sensitive calcium channels (Twombly *et al*., 1988) and enhances the activity of sodium-potassium ATPase of neurons and glial cells (Guillaume *et al*, 1989). The action of phenytoin at voltage-sensitive sodium and calcium channels of

cardiac muscle probably accounts for the antiarrhythmic action of phenytoin.

2.1.5 Toxicity of phenytoin and fosphenytoin

The toxic effects of phenytoin depend upon the route of administration, the duration of exposure, and the dosage. When it is administered intravenously at an excessive rate in the emergency treatment of status epilepticus, the most notable toxic signs are cardiac arrhythmias, with or without hypotension and/or CNS depression (Louis *et al.*, 1967). These complications may be minimized by administering the drug at a rate of less than 50 mg/min. Acute oral overdosage results in cerebellar atrophy. Toxic effects associated with chronic dosage are also dose-related cerebellar vestibular effects including other CNS effects, behavioural changes, increased frequency of seizures, gastrointestinal symptoms, gingival hyperplasia, osteomalacia and megaloblastic anaemia (Winter and Tozer, 1986).

Fosphenytoin has fewer local adverse effects. The more important adverse events caused by the i.v. use of fosphenytoin or phenytoin are cardiovascular collapse and/or central nervous system depression (Parke-Davis, 1996). Hypotension can occur when either drug is administered rapidly by the i.v. route. The rate of administration is very important, and it should not exceed 150 PE/min. The adverse clinical events most commonly observed with the use of fosphenytoin in clinical trials were nystagmus, diziness, pruritus, parethesia,

headache and ataxia. These are commonly associated with conventional phenytoin sodium and are likely to represent effects of the parent drug after conversion (Kutt *et al.*, 1964).

2.1.6 Methods of analysis in biological fluids

2.1.6.1 Spectrophotometric methods

Originally, phenytoin concentrations were measured by ultraviolet spectrophotometry, with or without preliminary derivatization of the drug. These methods included a colorimetric method (Dill *et al.* 1956), a spectrophotometric method (Svensmark and Kristensen, (1963), oxidative procedures (Wallace *et al.* 1966), and a simple fluorometric assay procedure (Dill and Glazko 1972; Dill *et al.* 1976). The methods were relatively cumbersome, sometimes of marginal sensitivity, were nonspecific, and were replaced with chromatographic techniques.

2.1.6.2 Thin-Layer Chromatography (TLC)

Several TLC methods have been described for the quantitation of phenytoin (Olesen, 1967), including high-performance TLC (Davis and Fenimore, 1981). The extracted drug was separated by TLC. Quantitation was achieved by either ultraviolet scanning of the plate or by elution of the drug from the plate and recording of its ultraviolet absorption. Many of the TLC methods are specific and reproducible, but they are rather complicated and time-consuming with

have low output, are now replaced by other procedures.

2.1.6.3 Gas-Liquid Chromatography (GLC)

Gas chromatographic techniques were the primary techniques used to analyze anticonvulsant drugs in biological fluids for many years. GLC permits simultaneous analysis of both the parent drug and major metabolites. The chromatographic properties of phenytoin are greatly improved by derivatization, which offers more symmetrical peaks and better separation, better thermal stability, and shorter retention times. MacGee (1970) developed an on-column methylation technique with tetramethylammonium hydroxide (TMAH) and trimetylphenylammonium hydroxide (TMPAH) has been widely used. 5-(4-methylphenyl)-5-phenylhydantoin (MPPH) is also widely used as an internal standard (Chang and Glazko, 1968; 1970) due to its close structural similarity to phenytoin..

Detection is commonly by flame ionization detector, which has adequate sensitivity for the assay of phenytoin. The nitrogen-phosphorus detector and the electron capture detector have particular advantages, since they offer increased specificity and sensitivity. Smaller sample volumes can be analyzed by using a nitrogen-sensitive detector, which also allows a reduction in clean-up procedures due to its insensitivity to carbon. Derivatization of phenytoin to a corresponding halogenated analogue is required when using an electron capture

detector.

2.1.6.4 High-Performance Liquid Chromatography (HPLC)

Determination of phenytoin and other antiepileptic drugs by liquid chromatography is a suitable alternative to gas chromatography for compounds that lack volatility or thermal stability. Liquid chromatographic separations are based on solubility and not on vapour pressure as in gas chromatography. The mobile and stationary phases usually affect separation, whereas in gas chromatography only the stationary phase contributes to the separation. Columns frequently used for phenytoin include modified silica gel C-8 or C-18, for reversed phase chromatography. Anders and Latorre (1970) reported the separation of phenytoin and p-HPPH by HPLC on an ion-exchange column. Evans (1979) used a straight phase HPLC on a polar silica gel column to measure phenytoin and phenobarbital. Adams and Vandemark (1976) demonstrated the higher versatility of reversed-phase by separating phenytoin and a number of other antiepileptic drugs. Kabra *et al.* (1976, 1977) used MPPH as a more appropriate internal standard for phenytoin assay using silica acid columns and reversed-phase columns. Other investigators have used various reversed-phase columns for the determination of phenytoin and other antiepileptic drugs by HPLC (Soldin and Hill, 1976; Slonek *et al.*, 1978). Chrisofides and Fry (1980) described reversed ion pair HPLC, using tetrabutylammonium phosphate.

HPLC methods for determination of plasma concentration of fosphenytoin and phenytoin have been reported (Gerber *et al*., 1988; Leppik *et al*., 1990; Herbranson *et al*., 1993; Cwik *et al*., 1997). Liquid chromatography offers several advantages over gas chromatographic methods, such as lack of derivatization, faster separation, better sample stability, and smaller sample size. HPLC methods remain in use in laboratories which have a relatively low throughput of antiepileptic drug assays and a need to measure a variety of drugs. In the present study, both fosphenytoin and phenytoin were assayed by HPLC.

2.1.6.5 Mass Spectrometer (MS)

Methods involving mass spectrometers are the ultimate methods and serve as reference sources, but are hardly applicable in routine laboratories because of the cost involved. Gas chromatographic-mass spectrometric and HPLC-mass spectrometric assay permit enhanced selectivity and specificity, but the cost of the instrumentation required has largely restricted such methods to research purposes.

2.1.6.6 Immunoassay methods

Several immunoassay methods have been used for the determination of serum phenytoin levels. These assays utilize a variety of methods of quantitating the *in vitro* product of a reaction between the drug under study and an antibody raised against it. They include radiation measurement (Cook *et al.*, 1973), linked enzyme-catalyzed reactions (Scharpe *et al.*, 1976), liposome lysis (Kubotsu *et al.*, 1992), fluorescence polarization (Lu-Steffes *et al.*, 1982), substrate labeled fluorescence (Wong *et al.*, 1979) and electron spin resonance (Montgomery *et al.*, 1975). The immunoassay methods offer better economics than HPLC, are convenient and can give results quite quickly. They are very sensitive, but some of the antibodies used in these methods may cross-react with metabolites of the drug in question, including biologically active metabolites. Hence, immunoassay methods may sometimes yield unreliable results which do not coincide with those produced by intrinsically more specific methods. This may mislead the subscriber if an inactive metabolite is measured as well as the drug in question, particularly if the relative proportions of the two substances are unusual, as may occur if the metabolite accumulates in renal failure e.g. p-hydroxy phenytoin (Robberts and Rainey, 1993), or as a result of pharmacokinetic interaction.

Therapeutic drug monitoring has been a hallmark of phenytoin use for many years. The target concentrations for total phenytoin range from 10-20 μg/ml

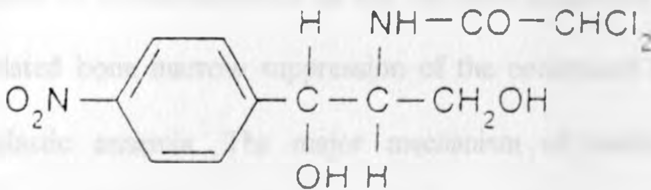
(40-80 µmol) and for unbound phenytoin from 1.0-2.0 µg/ml. The goal of therapy are the same for fosphenytoin, but it is important to note the potential for falsely elevated phenytoin concentrations using immunoanalytic methods for up to 2 h or more after fosphenytoin dosing. Prior to complete conversion, immunoanalytical techniques such as TDx/TDxFL® (fluorescence polarization; Abbott Laboratories, North Chicago, IL) and EMIT 2000 (enzyme multiplied; Syva, St. Louis, MO) may significantly overestimate plasma phenytoin concentrations in the presence of fosphenytoin because of cross-reactivity with fosphenytoin (Kugler *et al.*, 1994). The degree of error is dependent on the plasma phenytoin and fosphenytoin concentrations, which are influenced by fosphenytoin dose, route and rate of administration, and time of sampling relative to dosing, as well as the analytical method. Chromatographic methods quantify phenytoin concentrations accurately in biological fluids in the presence of fosphenytoin. Prior to complete conversion, blood samples for phenytoin monitoring should be collected in tubes containing ethylene diamine tetraacetate (EDTA) as an anticoagulant to minimize ex vivo conversion of fosphenytoin to phenytoin (Kugler *et al.*, 1994).

Chloramphenicol

.1 Introduction

loramphenicol {(*d*-theo-(–)-2,2-Dichloro-N-[β-hydroxy-α-(hydroxymethyl)--nitrophenylethyl]acetamide} was the first broad-spectrum antimicrobial, lated in 1947 from *Streptomyces venezuelae*, a soil actinomycete (Ehrlich *et* 1947). It was initially called *chloromycetin*, because of its two chlorine ms. It has a molecular formula of $C_{11}H_{12}Cl_2N_2O_5$ and molecular weight of .1. It is a fine, white to grayish-white or yellowish-white, and odourless npound with a bitter taste. It is readily soluble in organic solvents but tively insoluble in water (Wade and Reynolds, 1977).

. 2: Structural formula of chloramphenicol



2.2.2 Mechanism of action

Chloramphenicol is a broad-spectrum antibiotic and inhibits protein synthesis in the bacteria. It acts primarily by binding reversibly to the larger 50S subunit of the 70S ribosomes at a locus that prevents the attachment of the amino acid-containing end of the aminoacyl-*t*RNA to its binding region (Pestka, 1971). Without this attachment, the association of the amino acid substrate with peptidyltransferase does not occur, and peptide bond formation is prevented. This block in protein synthesis produces a static effect against most sensitive microorganisms. It is bacteriostatic to some organisms like *Staphylococcus aureus*, *Staphylococcus epidermidis*, Gram-negative enterics, *Salmonella*, *Shigella*, and streptococci. However, chloramphenicol is bactericidal against some meningeal pathogens such as *Haemophilus influenzae*, *Streptococcus pneumoniae* and *Neisseria meningitidis* (Rahal and Simberkoff, 1979). Although the mammalian cells contain primarily 80S ribosomes that are unaffected by chloramphenicol, the mitochondria contain 70S particles. The effect of chloramphenicol on this has been suggested as a cause for the dose-related bone marrow suppression of the compound but not the idiosyncratic aplastic anaemia. The major mechanism of resistance of bacteria is by inactivation of chloramphenicol by acetyltransferase.

2.2.3 Pharmacokinetics

2.2.3.1 Absorption

Three preparations of chloramphenicol are most commonly used in clinical practice: a crystalline powder for oral administration, a palmitate ester for oral administration as a suspension, and a succinate ester for parenteral administration. Both esters are inactive, requiring hydrolysis to chloramphenicol for antibacterial activity. The palmitate ester is hydrolyzed in the small intestine to active chloramphenicol prior to absorption (Kauffman *et al.*, 1981). Chloramphenicol is then absorbed from the gastrointestinal tract, and peak plasma concentrations (10-13 μg/ml) occur within 2-3 h after the administration of a one gram dose. In patients with gastrointestinal disease or in newborns, the bioavailability is greater for chloramphenicol, probably because of the incomplete hydrolysis of the palmitate (Smith and Weber, 1983).

The preparation of chloramphenicol for parenteral use is the water soluble-soluble, inactive sodium succinate preparation (chloramphenicol succcinate). Similar concentrations are achieved after intravenous and intramuscular administration (Shann *et al.*, 1985). Chloramphenicol succinate is rapidly cleared from plasma by the kidneys. The renal clearance of the prodrug may affect the overall bioavailability of chloramphenicol, because about 20-30% of the dose may be excreted before hydrolysis. Poor renal function in the neonate and other states of renal insufficiency result in increased plasma concentrations

of chloramphenicol succcinate and of chloramphenicol. (Slaughter *et al.*, 1980). Decreased esterase activity has been reported in the plasma of neonates and infants. This results in prolonged period to reach peak concentrations of active chloramphenicol (4 h) and a longer period over which renal clearance of chloramphenicol succcinate can occur (Kauffman *et al.*, 1981).

2.2.3.2 Distribution

Physical-chemical characteristics of chloramphenicol enable the drug to diffuse rapidly throughout the body. Chloramphenicol is widely distributed in the body fluids and readily reaches therapeutic concentrations in CSF, where values are about 60% of those in plasma (range, 45-99%) in the presence or absence of meningitis (Friedman *et al.*, 1979). The drug may actually accumulate in brain tissue (Kramer *et al.*, 1969). The drug is present in saliva and bile, and is secreted into the milk. It readily traverses the placental barrier, resulting in fetal concentrations of between 30-80% of the maternal serum value. In bile, the majority of the drug is conjugated, and therefore inactive, implying that the drug is unsuitable for treatment of urinary tract infections.

2.2.3.3 Metabolism and excretion

Chloramphenicol succinate and palmitate esters undergo hydrolysis to yield active chloramphenicol. Chloramphenicol is metabolized primarily in the liver, where it is conjugated with glucuronic acid, forming a monoglucuronide. The

major glucuronide metabolite of chloramphenicol is water-soluble and has no antimicrobial activity. It is excreted via the bile into the small intestine where it is hydrolyzed by bacterial β-glucuronidase. The free chloramphenicol is reabsorbed and conjugated with glucuronic acid again. Eventually this enterohepatic recirculation results in 80-90% of the monoglucuronide being excreted into the urine. Only about 5-10% of the administered dose is recovered in the urine as biologically active chloramphenicol. Chloramphenicol can also undergo a reduction at the nitro position to an amine, which occurs in the gastrointestinal tract after excretion of glucuronide and chloramphenicol into the bile.

Chloramphenicol has been shown to interact with a number of drugs, because of competition for hepatic microsomal enzymes. Chloramphenicol inhibits the metabolism of tolbutamide, chlorproamide, phenytoin and warfarin (Rose *et al.*, 1969; Petitpierre and Fabre, 1970; Young and Lietman, 1978; Christensen and Skousted, 1969). Chronic administration of phenobarbital or acute administration of rifampicin shortens the half-life of chloramphenicol, presumably because of enzyme induction, and may result in subtherapeutic concentrations of the drug (Powell *et al.*, 1981; Prober, 1985).

2.2.4 Analytical methods for determination of chloramphenicol in plasma

Various methods have been developed for the determination of the concentration of chloramphenicol in biological fluids, including bioassay, gas chromatography and high performance liquid chromatography. Methods for chloramphenicol determination should differentiate between the prodrug forms, chloramphenicol palmitate or succinate, and their active metabolite, chloramphenicol.

2.2.4.1 Colorimetric methods

These methods measure the amine formed after the reduction of aromatic nitro groups of chloramphenicol. Mason *et al.* (1979) reported that the inactive prodrug and the glucuronide-metabolite-containing nitro groups interfere with chloramphenicol measurement. The method is inexpensive, but it has poor specificity, and is therefore not suitable for the measurement of chloramphenicol concentrations in biological fluids.

2.2.4.2 Microbiological methods

The most frequently used techniques for monitoring chloramphenicol serum concentrations are microbiological assays. These are relatively inexpensive and easy to use, but are limited by poor sensitivity and specificity (Bannatyne and Cheung, 1979), as well as lack of precision and accuracy in interpreting the inhibition zones. The radioenzymatic assay is rapid, precise, sensitive and

specific but disadvantages include high cost, handling of radioactive materials, and its inability to measure the prodrug and chloramphenicol metabolites (Smith and Smith, 1978).

2.2.4.3 Gas-liquid chromatography (GLC)

GLC methods are accurate, precise, sensitive, and specific, but require lengthy extraction procedures, and thus are cumbersome (Least *et al.*, 1977).

2.2.4.4 High-performance liquid chromatography (HPLC)

HPLC methods are accurate, precise, specific and have excellent sensitivities for measurement of chloramphenicol and its prodrug and major metabolites (Aravind *et al.*, 1981; Aravid et al., 1982). They are rapid and have made therapeutic drug monitoring practical for chloramphenicol . In the present study, an HPLC method is used to assay for both chloramphenicol and chloramphenicol succinate.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Chemicals and reagents

Phenytoin (PHT; 5,5-diphenylhydantoin), 5-(*p*-methylphenyl)-5-phenylhydantoin (MPPH), the interna standard (IS), chloramphenicol base (CAP) and chloramphenicol sodium succinate (CAPS) were purchased from the Sigma-Aldrich Co. Ltd. (Gillingham, Dorset, UK). Fosphenytoin sodium (FOS) powder and fosphenytoin sodium injection (*Cerebyx*®, 50 mg Phenytoin Equivalents/ml) were generously donated by Parke-Davis Pharmaceutical Research (Ann Arbor, MI, USA). Phenytoin sodium injection (*Dilantin*®, 250 mg/5ml; Parke-Davis) and chloramphenicol sodium succinate (CAPS) (*Chlorocide-1gm*; Regal Pharmaceuticals Ltd., Nairobi) were purchased locally. Acetonitrile, ethyl acetate, diethyl ether, glacial acetic acid, and orthophosphoric acid (all HPLC grade) were purchased from BDH (Poole, Dorset, UK). Tetrabutylammonium hydrogen sulfate (TBA, AnalaR® grade) was obtained from Janssen Chimica, Belgium. Sodium hydroxide and sodium acetate (both AnalaR® grade) were purchased from BDH (Poole, Dorset, UK). Centrifree® micropartition devices with YMT membranes (Amicon Inc., Beverly, MA, USA) were purchased from Millipore Limited (Harrow, Middlesex, UK). All other chemicals and reagents were of analytical or reagent grade. Deionized water was prepared from an Elgacan® C114 Ultra Pure Water System (The Elga Group, Buckinghamshire, England).

3.2 Experimental Animal Procedures

3.2.1 Rabbits

3.2.1.1 Housing and feeding

A total of 24 (9 males, 15 females) New Zealand White rabbits (2.09 ± 0.41 kg; mean ± SD) from the University of Nairobi Animal Unit were used for the study. The animals were fed on standard commercial rabbit pellets (Unga Feeds Limited, Nairobi) and were allowed access to fresh drinking water *ad libitum*.

3.2.1.2 Drug administration and blood sampling

Each rabbit was weighed and anaesthetized with sodium pentobarbitone (30 mg/kg body weight) via the marginal ear vein. The femoral artery was exposed and cannulated (polyethylene tubing, 0.4 mm i.d., 1.8 mm o.d.; Portex, Hyde, UK) followed by administration of 10 mg/kg of either phenytoin sodium or fosphenytoin sodium equivalents either via the marginal ear vein, or i.m. through the gluteral muscle. Blood samples (1.5 ml) were withdrawn via the femoral artery cannula into lithium heparinised tubes (Vacutainer®, Becton Dickinson, Lutherford, New Jersey, USA) at the following times: predose and at 1, 3, 5, 10, 15, 20, 30, 45, 60, 90, 120, 180, 240 and 300 min after drug administration. The cannula was flushed with an equal volume of heparinised sterile normal saline after each blood sample to prevent blood clotting in the

cannula. Anaesthesia was maintained throughout the experiment by administering maintenance doses of sodium pentobarbitone (10 mg/kg body weight) as appropriate. Blood was immediately centrifuged (1000 x *g*, 10 min) at room temperature and plasma separated and stored at -20^0C until analysis for fosphenytoin and phenytoin was done by HPLC. The animals were sacrificed at the end of the study with an overdose (60 mg/kg) of sodium pentobarbitone.

3.2.2 Rats

3.2.2.1 Housing and feeding

Female Wistar (albino) rats (N=60; 253.13 ± 31.60 g; mean ± SD) were used. The animals were acquired from the Department of Pharmacology and Pharmacognosy, Faculty of Pharmacy, University of Nairobi. They were housed in plastic cages with mesh-wire lids at ambient temperature. Wood-shavings and sawdust were used as bedding materials. They were fed on standard commercial mice pellets obtained from a reputable manufacturer (Unga Feeds Ltd., Nairobi) and fresh clean water *ad libitum*.

3.2.2.2 Drug administration and blood sampling

The rats were anaesthetized using diethyl ether soaked on piece of cotton wool. The tail was immersed into warm water (40°C) in a beaker to dilate the tail vein. The normal body temperature of the rat was maintained at 37°C using an incandescent lamp. A 26 gauge x 0.5-inch needle was inserted into the vein.

Fosphenytoin sodium (30 mg phenytoin equivalents/kg) alone, or concomitantly with chloramphenicol sodium succinate (*Chlorocide® 1gm*, 100 mg/ml in sterile normal saline) was administered as a slow intravenous infusion over 2 min via the tail vein.

Four rats were sacrificed by cervical dislocation at 0.5, 1, 2, 3, 4, 6 and 7 h after drug administration. Blood (3 ml) was withdrawn by cardiac puncture and transferred into tubes (Vacutainer®, Becton Dickinson, Lutherford, New Jersey, USA) containing lithium heparin. The blood was immediately centrifuged (1000 g for 10 min) at room temperature and plasma was harvested. The plasma was immediately frozen at −20°C until assayed for total phenytoin, chloramphenicol and chloramphenicol sodium succinate by HPLC.

3.3 Analytical Procedures

3.3.1 Fosphenytoin and phenytoin assay

The HPLC assay for phenytoin (PHT) and fosphenytoin (FOS) was based on the procedure previously described by Cwik *et al.* (1997), with some modifications.

3.3.1.1 Standard solutions

A stock solution of FOS (4.0 mg/ml) was prepared in deionized water and stored at –20°C until required. 8-ml aliquots of blank plasma were collected into tubes (Vacutainer®) containing lithium heparin and spiked with various quantities of the FOS stock solution. The plasma standard samples were vortex-mixed and stored at –20°C until use.

PHT stock solution (400 µg/ml) was prepared in acetonitrile. A 1-ml aliquot of the solution was evaporated to dryness under a gentle stream of nitrogen gas at 40°C and reconstituted with 10 ml of pooled drug-free plasma by tumbling (35 inversions per min for 1 h) on a blood tube rotator (Stuart SB1, Jencons, Beldfordshire, UK). The solution was sonicated for 30 min to ensure complete dissolution. The standard solutions were stored at –20°C until use.

The IS stock solution was prepared by dissolving MPPH in acetonitrile to give a concentration of 1.0 mg/ml. The working IS solution was prepared by

appropriate dilution of the stock solution with deionized water to give a concentration of 100 µg/ml, and kept at –20°C until use.

3.3.1.2 Calibration curves for fosphenytoin and phenytoin in plasma

The calibration standards of PHT in plasma were prepared over the concentration range of 0.1 to 40 µg/ml by serial dilution of the stock solution of the PHT (40 µg/ml) with drug-free plasma. FOS calibration standards were prepared in a similar manner by appropriate serial dilution of the FOS stock solution (400 µg/ml) to give concentration range of 0.5 to 400 µg/ml.

3.3.1.3 Calibration curves for free fosphenytoin and phenytoin in Krebs buffer

Standards for ultrafiltrate samples used to determine the protein binding of FOS and PHT were prepared in Krebs buffer, which have equivalent contents of plasma ultrafiltrate, because of the difficulty in obtaining large volumes of protein-free rabbit or rat plasma water. Krebs buffer consisted of 5.19 g NaCl (88.8 mM), 0.35 g KCl (4.73 mM), 0.185 g $CaCl_2 \cdot 2H_2O$ (1.27 mM), 0.34 g KH_2PO_4 (2.50 mM), 0.286 g $MgSO_4 \cdot 7H_2O$ (1.18 mM) and 2.09 $NaHCO_3$ (24.9 mM), dissolved in deionized water, adjusted to pH 7.4 with 0.1 M HCl and brought to 1000 ml with deionized water. The standard curves of FOS in Krebs buffer were prepared within the concentration range of 0.5 to 80 µg/ml, and PHT calibration curves had concentrations ranging from 0.1 to 5.0 µg/ml.

3.3.1.4 Extraction recovery of fosphenytoin and phenytoin

The efficiency of the extraction procedure in drug recovery from plasma and Krebs buffer was evaluated by analyzing spiked plasma and Krebs buffer samples containing PHT and FOS. Known amounts of PHT and FOS were added to drug-free plasma or Krebs buffer. Aliquots of 0.1 ml of plasma or Krebs buffer were put in separate centrifuge tubes, and 0.5 μg of the IS (5 μl of 100 μg/ml) added to each tube before extraction as described in section 3.3.1.5. The recovery was defined as the ratio of the peak heights of PHT, FOS or IS from the extracted spiked plasma or buffer to the peak heights obtained by direct injection of equivalent amounts of the drugs and IS on column. The recovery values were estimated as a percentage.

3.3.1.5 Extraction Procedure

Plasma samples were thawed quickly just before extraction and mixed by vortexing for 10 sec. To 100 μl of plasma (blank, standard, control or animal samples) in a 15-ml glass centrifuge tube, was added 5 μl of IS solution (MPPH; 100 μg/ml in deionized water) and 100 μl of 85% orthophosphoric acid, followed by vortexing for 10 sec. Diethyl ether (2 ml) was then added and the tubes tightly capped. Extraction was effected by repeated inversion on a Stuart SB1 tube rotator (Jencons, Beldfordshire, UK) of the mixture (35 inversions per min, 20 min). The samples were then centrifuged (1000 *g* for 10

min) and the upper organic phase was transferred into a clean centrifuge tube and evaporated to dryness in a water-bath (37°C) under a gentle stream of white-spot nitrogen gas (BOC Kenya Limited, Nairobi). The residue was reconstituted in mobile phase (200 μl) and 50-μl aliquots injected onto the HPLC column.

3.3.1.6 Free drug concentration

Centrifree® micropartition system ultrafiltration membrane units were used according to the manufacturer's instructions to separate free drug from protein-bound drug. Plasma (1 ml) was added into the sample reservoir of the Centrifree® and centrifuged (1000 x *g*, 20 min) in a fixed angle centrifuge at room temperature. The filtration cup containing the filtrate was then carefully removed from the centrifuge rotor and capped and stored at –20°C if not assayed immediately. During analysis, the volume of each ultrafiltrate was recorded, followed by the addition of the IS (5 μl of 100 μg/ml MPPH), then vortexing (10 sec). A 50-μl aliquot of the ultrafiltrate was injected onto the HPLC column.

3.3.1.7 Preparation of calibration curves and determination of intra- and inter-assay precision of phenytoin and fosphenytoin

Calibration curves were prepared daily by spiking duplicate drug-free rabbit plasma with PHT (0.01–4.0μg), FOS (0.05–40 μg) and IS (0.5 μg), followed by

extraction as described above. Quality control samples were prepared independently from the calibration curves by spiking drug-free plasma or Krebs buffer (pH 7.4) with known amounts of PHT and FOS. Three different levels, corresponding to low, medium and high concentrations of the quality control samples were prepared. The intra-assay (within-day) precision and accuracy were assessed by analyzing a minimum of five (5) quality control samples for each level of concentration. The inter-assay reproducibility was assessed by analyzing two quality control samples for each concentration level every week for two months.

3.3.1.8 Chromatography

Chromatography was performed using an Isochrom delivery system (SpectraSystem P1000, Spectra-Physics, San Jose, CA, USA) fitted with a Rheodyne (model 7125; Cotati, CA, USA) sample injection valve with a 50-µl loop. Separation was achieved on a reversed-phase (C18) stainless steel column (Ultrasphere ODS, 15 cm × 4.6 mm i.d, 5 µm particle size, Beckman, Instruments, Inc., Fullerton, CA, USA) preceded by a guard column (C18 precolumn; 100 RP18 endcapped, 10 mm × 4.6 mm i.d., 5 µm; Merck, Darmstadt, Germany). The column effluent was monitored using a variable wavelength UV/VIS absorbance detector (SpectraSystem™ UV1000, Spectra Physics) set at 210 nm. The mobile phase comprised 21% v/v acetonitrile in deionized water containing 5 *mM* tetrabutylammonium hydrogen sulfate (TBA)

as an ion-pair reagent. The pH was adjusted to 2.5 with orthophosphoric acid. Flow rate was set at 3.5 ml/min, generating an operating backpressure of about 2300 psi. The mobile phase was ultrasonically degassed (PUK 125, Kerry Ultrasonics Ltd., England) prior to use. Column temperature was maintained at 40°C using a Model 7990 SPACE Column Heater (Jones Chromatography Ltd., Mid Glamorgan, UK). Detector output was monitored using a flatbed chart recorder (Servogor 124, Belmont Instruments, Glasgow, UK) set at a chart speed of 12 cm/min.

3.3.1.9 Plasma albumin concentration determination

The plasma albumin concentrations were determined in the pretreatment samples using the bromocresol green method. Determination of albumin in plasma or serum is based on the binding behaviour of the protein with the anionic dyes bromocresol green (BCG) or bromocresol purple (BCP) in a manual or automated procedure. Bromocresol green binds quantitatively with albumin forming an intense blue/green complex with an absorbance maximum at 630 nm. The intensity of the colour produced is directly proportional to the albumin concentration in the sample. The specimen is plasma and heparin is used as an anticoagulant. The plasma is stored in a frozen state if the sample is not assayed immediately. The common sources of errors for the method arise from the contamination of reagents, inaccurate pipetting or due to malfunctioning spectrophotometer.

The calibration curve for albumin was prepared from the 45 g/l standard solution of albumin supplied with the kit, by serial dilution to obtain albumin concentrations of 5, 10, 15, 20, 25, 30, 35, 40 and 45 g/l with deionized water. The working standards solutions were ran using the albumin method described above to obtain the corresponding absorbances. The value of the absorbance for each standard solution (vertical axis) was plotted against the corresponding concentration in g/l (horizontal axis). The linearity of the bromocresol green method for albumin is about 60 g/l. The calibration graph was checked using the Randox quality control albumin samples.

3.3.2 Chloramphenicol Assay

3.3.2.1 Standard solutions

Stock solutions of CAP and CAPS (2 mg/ml) were prepared by weighing accurately 20 mg of each compound and dissolving the amount in 10 ml of methanol. Working solutions of the compounds were prepared by serial dilution of the solutions in distilled water. The stock solution (0.2 mg/ml) of the IS (mephenesin) was prepared by dissolving 2 mg of mephenesin in 10 ml of methanol. The working solution of the IS solution (100 μg/ml) was prepared by dilution of this solution in the same solvent. The stock and working solutions of these compounds were stored at 4°C until required.

3.3.2.2 Extraction procedure

To 100 µl sample in a 15 ml centrifuge tube was added IS solution (0.5 µg, 50 µl of 100 µg/ml solution) and 200 µl of cold sodium acetate buffer (pH 4.6). Ethyl acetate (1 ml) was added to each tube, followed by vortexing at high speed for 2 min. After centrifugation (1500 x *g*, 5 min), the organic layer was transferred to a clean tube and evaporated to dryness under a gentle stream of nitrogen (40°C). Samples were reconstituted in mobile phase (100 µl) and 50-µl aliquots injected into the chromatograph.

3.3.2.3 Calibration, recovery and reproducibility of chloramphenicol and chloramphenicol succinate

The calibration curves of CAP or CAPS were prepared by serial dilution of the stock solutions (1 mg/ml) of these compounds in deionized water to give concentrations of 2.5, 5.0, 10, 25, 50, 100, 250 and 500 µg/ml. Calibration curves were prepared within the concentrations of 0.25–50 µg/ml. 100 µl of each calibration point was analyzed as described in section 3.3.2.2.

The efficiency of the extraction procedure in drug recovery from plasma was assessed by analyzing spiked plasma samples containing CAP and CAPS. Known amounts of CAP and CAPS were added separately to drug free plasma. In one set of tubes, aliquots of 0.1ml spiked plasma were put into centrifuge

tubes, while in another set of tubes, equivalent amounts of the drugs were spiked directly into the organic phase (1 ml ethyl acetate). IS (mephenesin) [5 μg (50 μl of 100 μg/ml] was added to each tube before extraction as described in section 3.3.2.2. The recovery was defined as the ratio of the peak height ratio (drug/IS) obtained from the extracted spiked plasma samples to the peak height ratios obtained by direct injection of equivalent amounts of the drugs into the organic phase followed by processing as in section 3.3.2.2. The recovery was expressed as a percentage.

Quality control samples were prepared independently from the calibration curves by spiking drug-free plasma with known amounts of CAP and CAPS corresponding to 1.0 μg/ml and 10 1.0 μg/ml. The intra-assay precision was assessed by analyzing a minimum of five (5) quality control samples for each concentration level. The inter-assay reproducibility was assessed by analyzing a minimum of two quality control samples for each concentration level every week for one month.

3.3.2.4 Chromatography

Chromatography was performed using an Isochrom delivery system (SpectraSystem™ P100, Spectra Physics, San Jose, CA, US) connected to a Rheodyne (model 7125, Cotati, CA, USA) valve injector (50 μl loop). A reversed-phase (C18) stainless steel column (Hypersil 5ODS, 25 cm × 4.6 mm

i.d, 5 µm particle size; Capital HPLC, Wellington House, Macclesfield, UK) preceded by a guard column (C18 precolumn; Lichrospher 4.4 100 RP18 endcapped, 10 × 4.6 mm i.d., 5 µm; Merck, Darmstadt, Germany) was used. The column effluent was monitored using a variable wavelength uv–vis absorbance detector (Model UV100, Spectra Physics) set at 278 nm. The mobile phase consisted of methanol–0.05*M* trichloracetic acid (40:60, v/v); pH adjusted to 4.5 with 5*M* sodium hydroxide solution. Flow rate was set at 1.0 ml/min, which generated an operating backpressure of about 2000psi. The mobile phase was ultrasonically degassed (PUK 125, Kerry Ultrasonics Ltd., England) prior to use. Detector output was monitored by a flatbed chart recorder (Servogor 120, Belmont Instruments, Glasgow, UK) set at a chart speed of 12 cm/min.

3.4 Pharmacokinetic and statistical analysis

Areas under the plasma total and free phenytoin concentration–time curves (AUC) were calculated between time zero and 120 min after drug administration, using the linear trapezoid method (Gibaldi and Perrier, 1982) with the pharmacokinetic software (TopFit® Version 2.0, Schering, Germany). Plasma free phenytoin concentrations were calculated using the free fraction estimated from the 30-min plasma sample. Maximum concentration (C_{max}) and time to maximum concentration (T_{max}) were experimentally observed values from concentration-time curves following intramuscular administration of fosphenytoin or phenytoin sodium. Phenytoin pharmacokinetic parameters following intravenous or intramuscular administration of fosphenytoin or phenytoin were compared using the non-parametric Wilcoxon Sign Rank with level of significance assigned at $P<0.05$. Calculations were performed with the microcomputer software, Unistat® Statistical Package (Unistat Ltd, England). Concentration versus time data in figures were presented as mean value (standard deviation). Pharmacokinetic parameters were presented as median values (interquartile range).

CHAPTER FOUR

RESULTS

4.1 Chromatography

4.1.1 Phenytoin and fosphenytoin

4.1.1.1 Chromatograms

Chromatograms of extracts of drug-free plasma spiked with the IS (MPPH) (A), plasma spiked with PHT, FOS and and IS (B) and a plasma sample obtained 1 min following administration of a single intravenous 10 mg phenytoin equivalents/kg dose of fosphenytoin and spiked with the IS (C) are shown in Fig 3. The corresponding chromatograms of plasma ultrafiltrate are shown in Fig 4. The chromatograms are free of interference from endogenous compounds and PHT, FOS and the IS were resolved to baseline over the concentration ranges represented by the calibration curves of these compounds in both plasma and Krebs buffer. The retention times were 7.5, 11 and 15 min for PHT, FOS and IS, respectively. Commonly used antimalarial drugs (quinine, quinidine, chloroquine, pyrimethamine, sulfadoxine, proguanil, chlorcycloguanil) and other anticonvulsant drugs (phenobarbitone and diazepam) used in children with seizures associated with malaria did not interfere with the assay.

Fig. 3: HPLC chromatograms of:

A. Extracted pre-dose plasma containing 5 μg/ml internal standard (MPPH);

B. Extracted spiked plasma sample containing 4 μg/ml PHT (2), 20 μg/ml FOS (3) and 5 μg/ml of internal standard (MPPH) (4);

C. Extracted plasma sample obtained 1 min after intravenous administration of 10 mg PEs/kg fosphenytoin sodium to a rabbit (No. 7). PHT and FOS concentrations were 22.56 and 26.73 μg/ml, respectively.

Peaks: 1 = injection event; 2 = PHT; 3 = FOS; 4 = internal standard (MPPH).

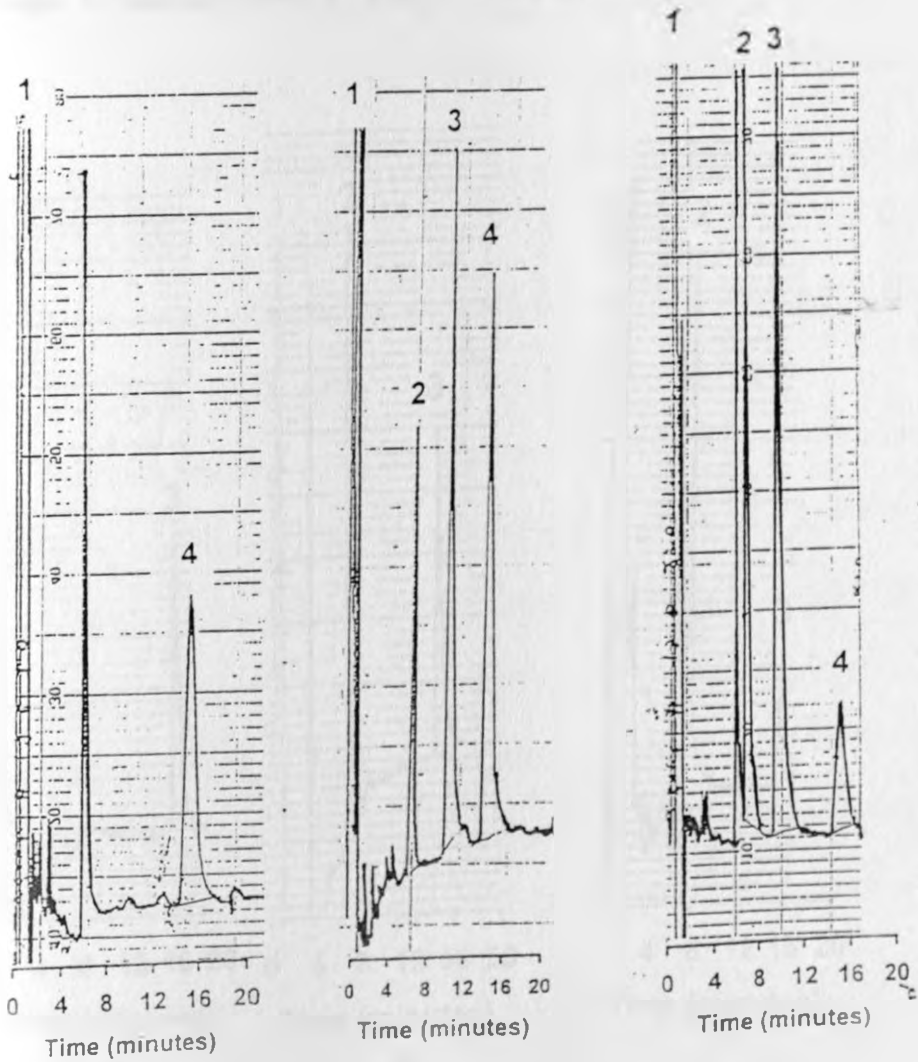


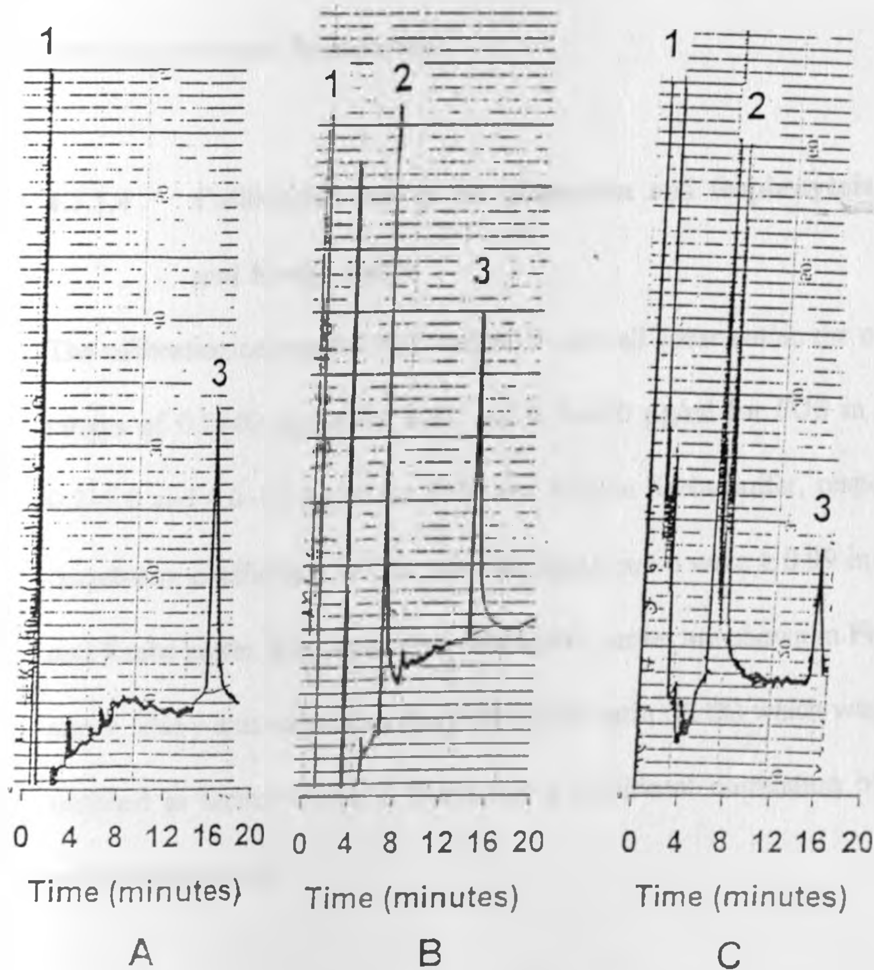
Fig. 4: HPLC chromatograms of:

A. Extracted blank Krebs buffer spiked with 5 μg/ml internal standard (MPPH);

B. Spiked Krebs buffer sample containing 2 μg/ml PHT (2) , and internal standard 5 μg/ml MPPH (3);

C. Plasma ultrafiltrate sample obtained 30 min after a single intravenous dose of fosphenytoin to a rabbit (no. 9). PHT concentration was 1.87 μg/ml. FOS was not detected.

Peaks: 1 = injection event; 2 = PHT; 3 = internal standard (MPPH).



4.1.1.2 Detection limits

The limits of quantification of the assay for PHT and FOS from a 100-µl plasma sample were 0.2 and 0.5 µg/ml, respectively (peak > ×4 the baseline noise, at 0.5 AUFS). In Krebs buffer, the limits of detection were 0.1 and 0.5 µg/ml for PHT and FOS, respectively.

4.1.1.3 Extraction efficiency

The analytical recoveries of phenytoin and fosphenytoin in both plasma and Krebs buffer are shown in Tables 1 and 2, respectively. The extraction technique had an efficiency of over 76% in both plasma and Krebs buffer for both phenytoin and fosphenytoin.

4.1.1.4 Calibration curves for phenytoin and fosphenytoin in plasma and Krebs buffer

The calibration curves for PHT and FOS were all linear within the concentration ranges of 0.2–40 µg/ml for PHT and 0.5–400 µg/ml for FOS in plasma, and 0.2–5.0 and 1.0–80 µg/ml for PHT and FOS in Krebs buffer, respectively. The correlation coefficients (r^2) for the calibration curve were ≥ 0.99 in both plasma and Krebs buffer. Representative calibration curves are shown in Figures 5, 6, 7 and 8. The y-axis represents the peak height ratio (PHR) which was obtained as outlined in section 3.3.2.3. There was a significant correlation between PHR and concentrations.

Table 1: Recoveries of phenytoin and fosphenytoin in samples of spiked plasma

Compounds	Concentration (µg/ml)	Replicates (n)	% Recovery (Mean ±SD)
PHT	0.8	10	98.29 ± 3.55
	5.0	11	81.28 ± 7.90
	35.0	11	76.88 ± 3.56
FOS	2.0	5	90.17 ± 15.62
	400	5	92.92 ± 10.54

Table 2: Recoveries of phenytoin and fosphenytoin in samples of spiked Krebs buffer.

Compounds	Concentration (µg/ml)	Replicates (n)	% Recovery (Mean ± SD)
PHT	0.3	7	91.64 ± 11.26
	5.0	11	81.28 ± 7.90
FOS	2.0	7	97.61 ± 9.81
	80.0	7	94.23 ± 9.87

Fig. 5: Calibration curve of phenytoin in spiked plasma.

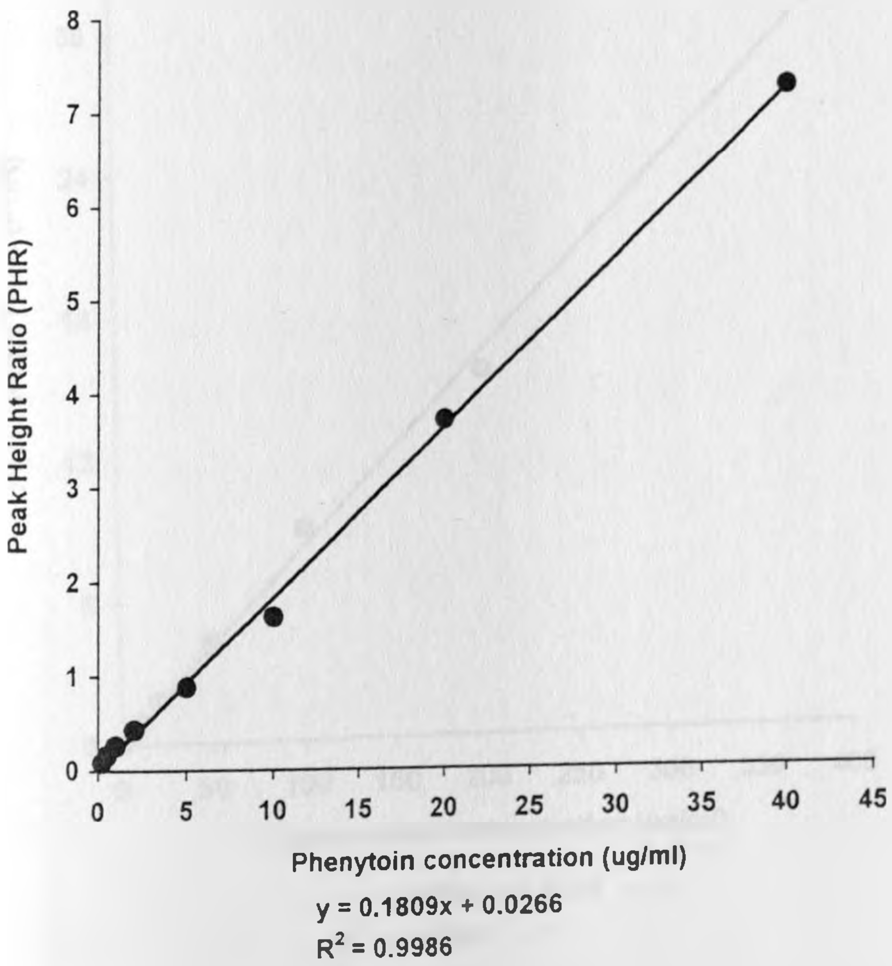
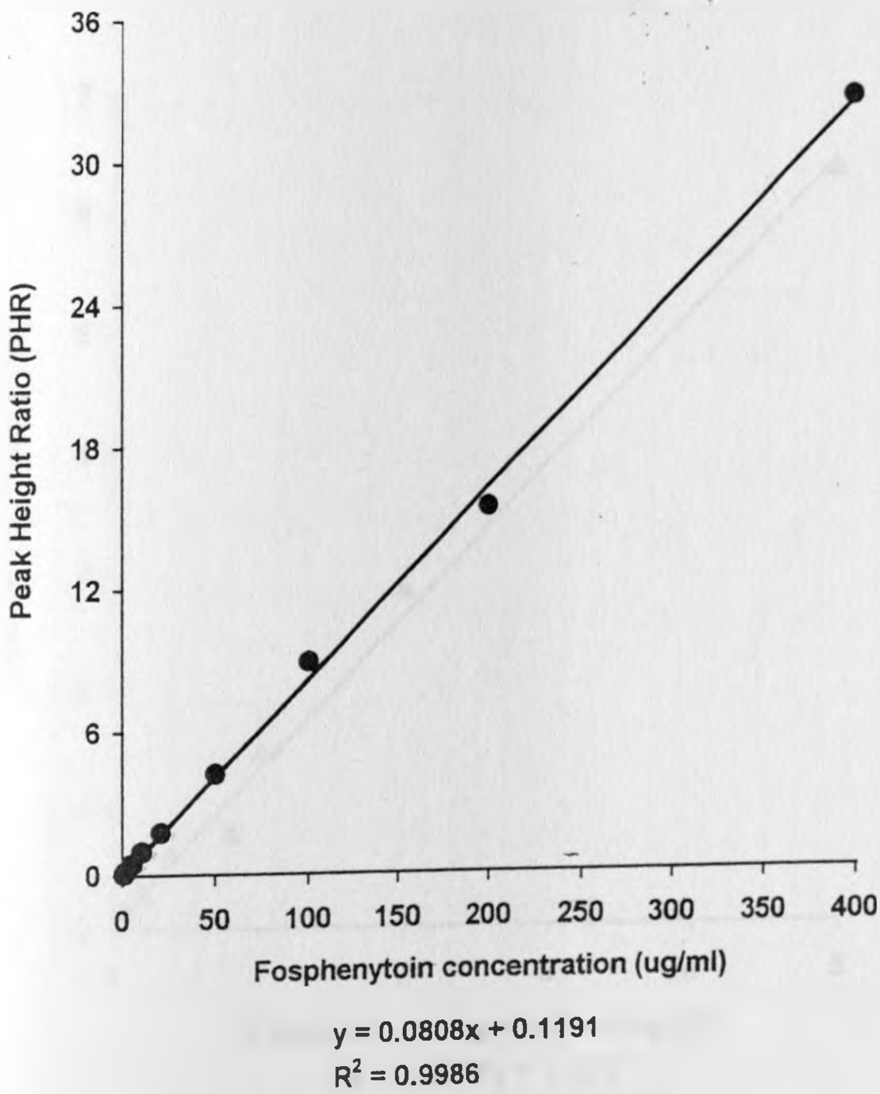


Fig. 6: Calibration curve of fosphenytoin in spiked plasma.



$y = 0.0808x + 0.1191$

$R^2 = 0.9986$

Fig. 7: Calibration curve for determination of free phenytoin concentrations in plasma ultrafiltrate

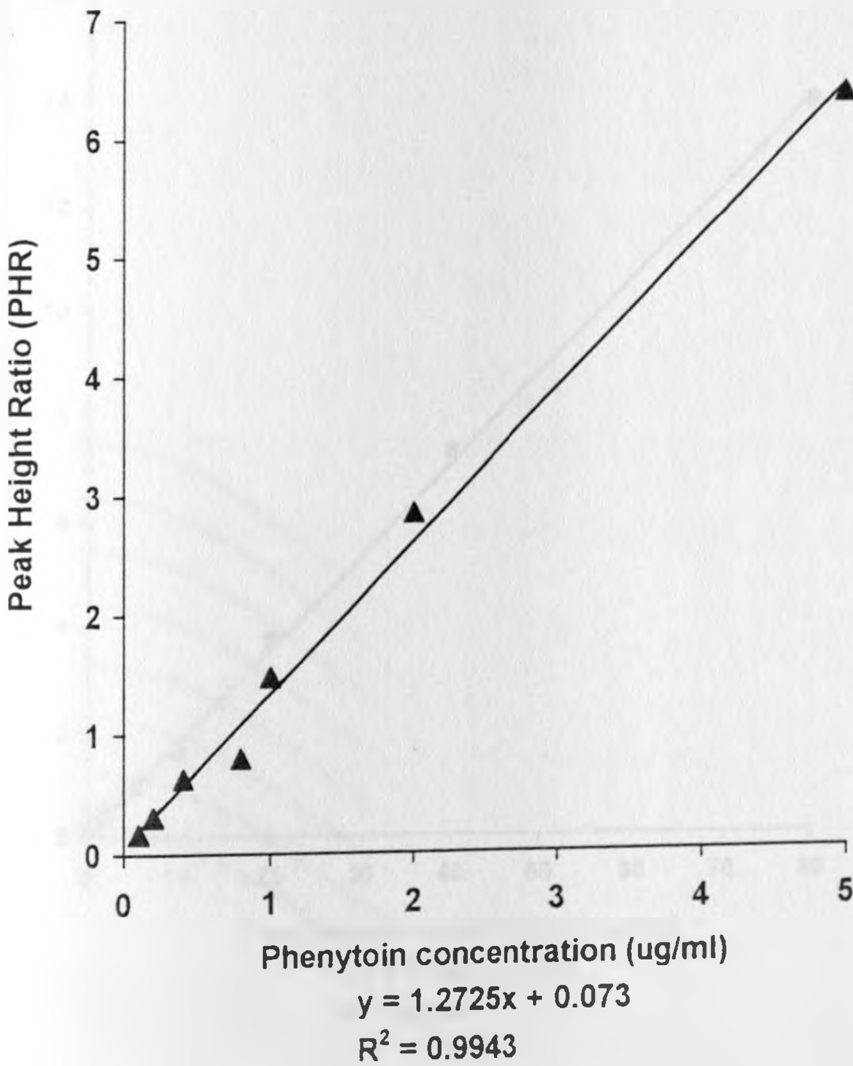
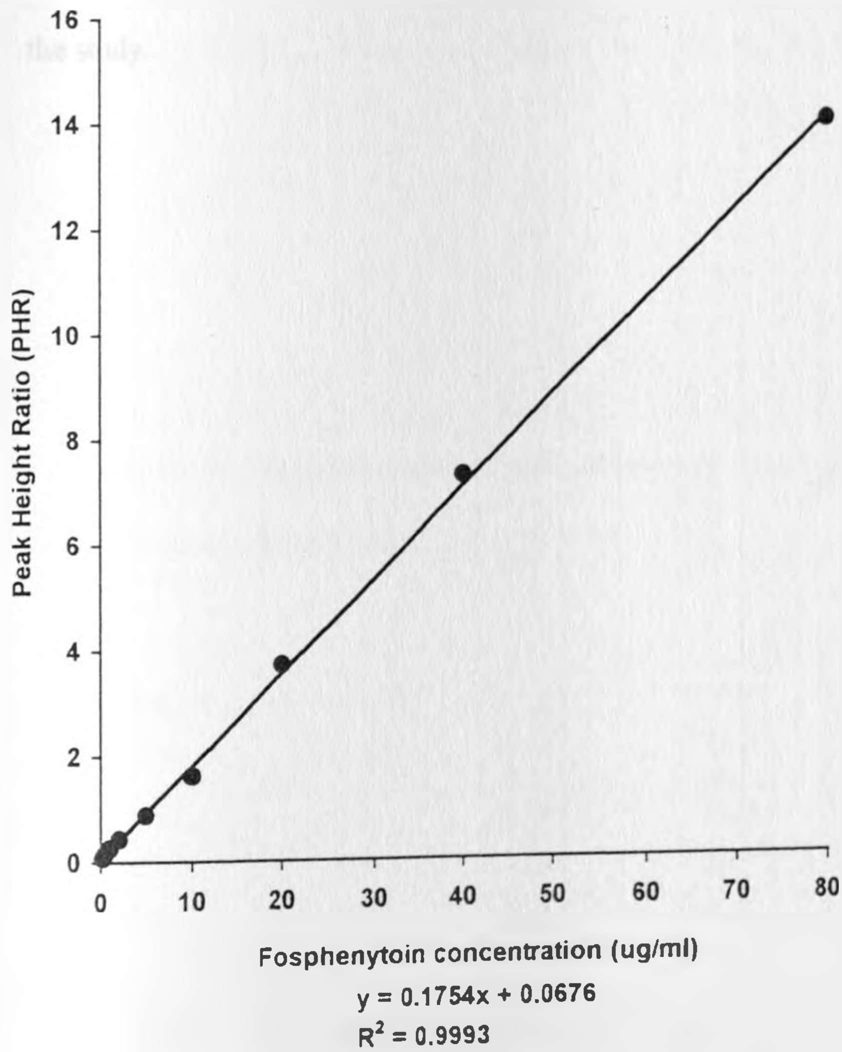


Fig. 8: Calibration curve for determination of free fosphenytoin concentrations in plasma ultrafiltrate



4.1.1.5 Intra- and inter-assay precision of phenytoin and fosphenytoin in plasma and Krebs buffer

The intra- and inter-assay coefficients of variation for PHT and FOS are shown in Table 3. The values were < 20% at the three concentrations of the quality control samples. This indicates that the assay method was consistent throughout the study.

Table 3: Intra-and inter-assay precision of phenytoin and fosphenytoin in plasma.

Compound	Concentration (μg/ml)	Intra-assay (CV%)	Inter-assay (CV%)	Replicates (n)
PHT	1.8	15.19	8.32	7
	18.0	6.52	12.98	7
	34.0	8.56	12.74	7
FOS	1.5	6.95	8.76	5
	18	11.46	9.80	7
	320	12.04	6.53	8

Table 4: Intra- and inter-assay precision and accuracy of phenytoin and fosphenytoin in Krebs buffer.

Compound	Concentration (μg/ml)	Intra-assay (CV%)	Inter-assay (CV%)	Replicates (n)
PHT	0.3	14.62	7.4	7
	2.5	11.80	4.98	7
	4.0	8.90	5.34	7
FOS	1.5	17.9	20.91	5
	45.0	4.05	14.81	7
	70.0	8.56	9.85	8

4.1.2 Chloramphenicol and chloramphenicol succinate

4.1.2.1 Chromatograms

Chromatograms of extracts of drug-free plasma spiked with the IS (mephenesin) (A); plasma spiked with CAP and CAPS and IS (B); and a plasma sample obtained 30 min after coadministration of a single i.v 30 mg phenytoin equivalents/kg dose of fosphenytoin sodium and 50 mg/kg dose of CAPS and spiked with the IS are shown in Fig 9. The chromatograms are free of interference from endogenous compounds. CAP, CAPS and the IS were resolved to baseline over the concentration ranges represented by the calibration curves of these compounds in plasma. The retention times were 8.5, 12.0 and 14.5 min for CAP, CAPS and IS, respectively. Commonly used antimalarial drugs e.g. quinine, quinidine, chloroquine, pyrimethamine, sulfadoxine, proguanil, chlorcycloguanil and other anticonvulsant drugs, including phenytoin, fosphenytoin, phenobarbitone and diazepam did not interfere with the assay.

Fig. 9: HPLC chromatograms of:

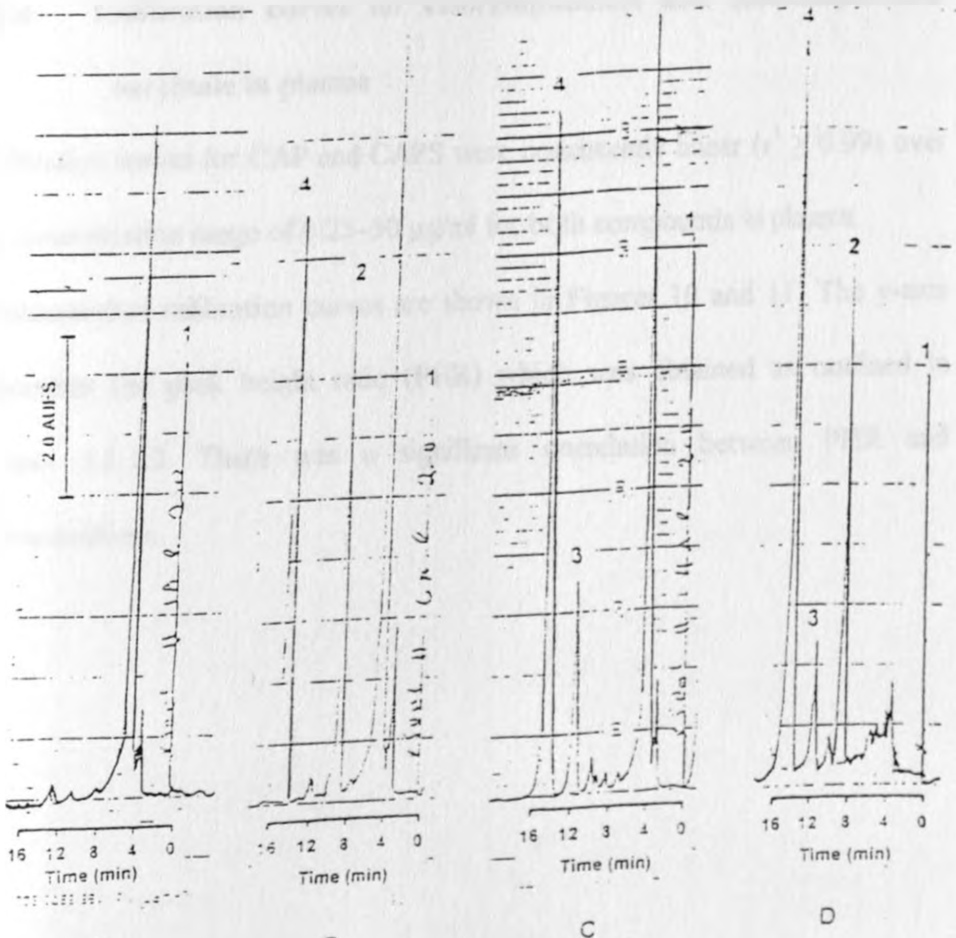
A: Extracted blank plasma;

B: Extracted spiked plasma sample containing 5.0 µg/ml CAP (2) and 5.0 µg of the internal standard (mephenesin) (4);

C: Extracted spiked plasma sample containing 1.0 µg/ml CAPS (3) and 5 µg of the internal standard (mephenesin) (4); and

D: Extracted plasma sample obtained 30 min after intravenous administration of 30 mg PEs/kg fosphenytoin sodium and 50 mg/kg chloramphenicol succinate to a rat. CAP and CAPS concentrations were 4.04 and 1.97 µg/ml, respectively.

Peaks: 1 = injection event; 2 = CAP; 3 = CAPS; 4 = internal standard (mephenesin).



4.1.2.2 Detection limits and retention times

The limits of quantification of the assay for CAP and CAPS from a 100-µl plasma sample were 0.10µg/ml (peak > ×4 the baseline noise) at 0.5 absorbance units full scale.

4.1.2.3 Extraction efficiency

The analytical recoveries of chloramphenicol and chloramphenicol succinate in plasma are shown in Table 5. The extraction technique using ethyl acetate as the extraction solvent had an efficiency of over 76% for both CAP and CAPS.

4.1.2.4 Calibration curves for chloramphenicol and chloramphenicol succinate in plasma

Calibration curves for CAP and CAPS were consistently linear ($r^2 \geq 0.99$) over the concentration range of 0.25–50 µg/ml for both compounds in plasma. Representative calibration curves are shown in Figures 10 and 11. The y-axis represents the peak height ratio (PHR) which was obtained as outlined in section 3.3.1.2. There was a significant correlation between PHR and concentrations.

Table 5: Recoveries of chloramphenicol and chloramphenicol succinate in samples of spiked plasma.

Compounds	Concentration (µg/ml)	Replicates (n)	% Recovery (Mean ± SD)
CAP	2.5	7	98.04 ±.2.80
	50.0	7	90.97 ± 4.13
CAPS	2.5	7	87.52 ± 4.50
	50.0	7	73.99 ± 3.78

Fig. 10: Calibration curve of chloramphenicol in spiked plasma

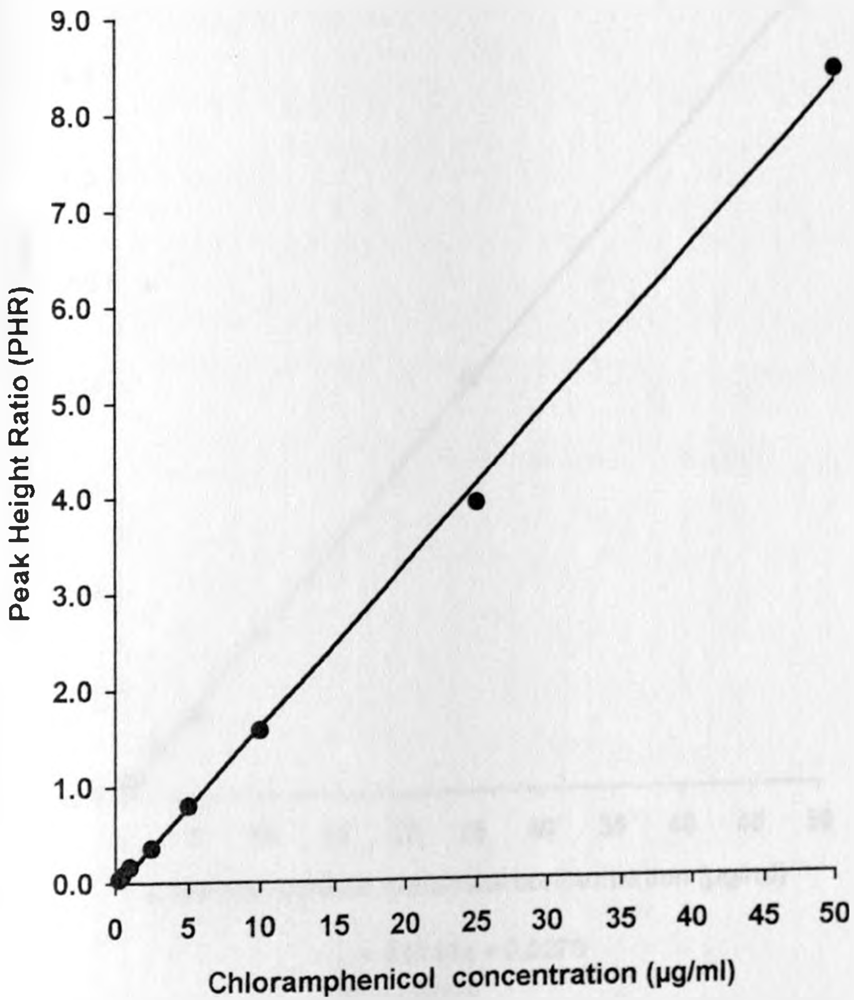
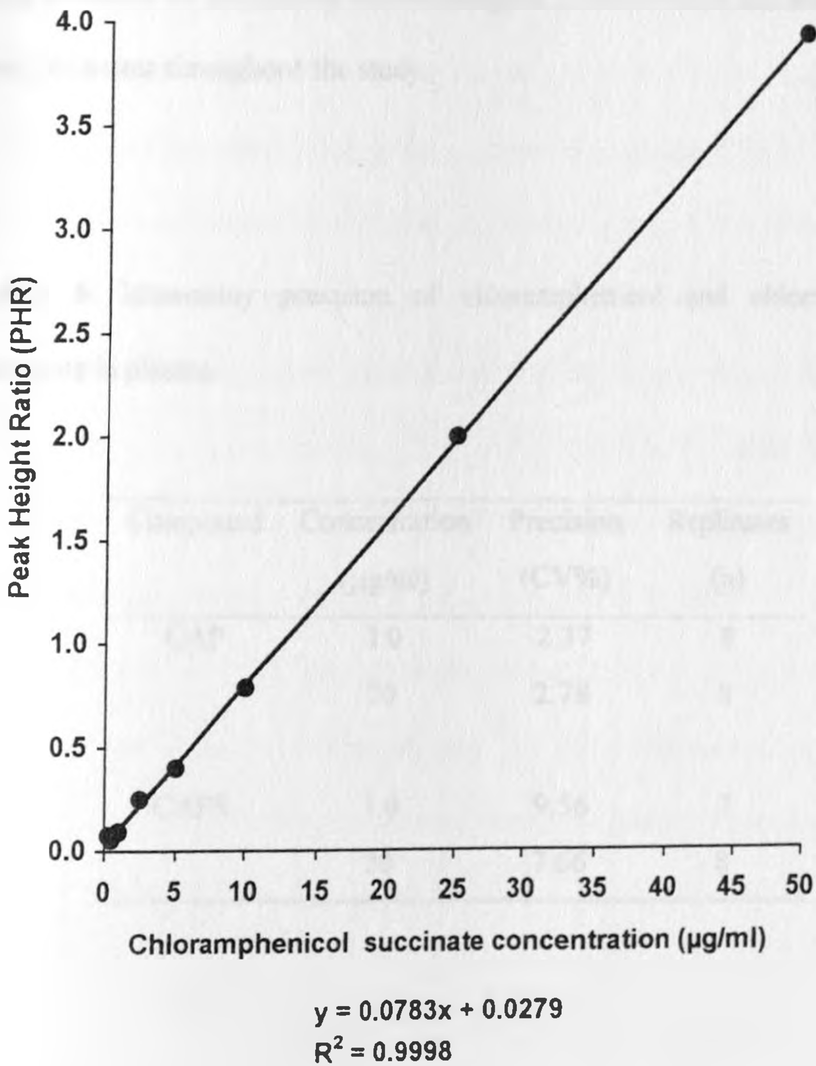


Fig. 11: Calibration curve of chloramphenicol succinate in spiked plasma



4.1.2.5 Intra-assay precision of chloramphenicol and chloramphenicol succinate

The intra-assay coefficients of variation for CAP and CAPS are shown in Table 6. The precision values were measured by the calculated relative standard deviations divided by the mean. These values were <15 % at the three concentrations of the quality control samples. This indicates the assay method was consistent throughout the study.

Table 6: Intra-assay precision of chloramphenicol and chloramphenicol succinate in plasma.

Compound	Concentration (μg/ml)	Precision (CV%)	Replicates (n)
CAP	1.0	2.37	8
	20	2.78	8
CAPS	1.0	9.56	7
	20	7.66	8

4.2 Pharmacokinetic parameters of phenytoin in the rabbit

There was no significant difference in weights of male and female rabbits. Mean plasma albumin concentrations were also not significantly different between males (3.266 ± 1.02; n=9) compared with females (2.971 ± 0.759; n=15) (P=0.423). Mean plasma albumin concentrations were not significantly different in fosphenytoin-treated compared with phenytoin-treated rabbits (Table 7). The total plasma phenytoin concentrations for each rabbit are shown in apendices 3-6, while the free plasma phenytoin are shown in appendices 7-10. The plasma phenytoin concentration-time profiles following i.v. and i.m. administration of fosphenytoin and phenytoin are shown in Figures 12 and 13, respectively. The pharmacokinetic parameters obtained in the study are shown in Table 8. Following i.m. administration, the mean maximum plasma phenytoin concentration (C_{max}) was 158% (P=0.0277) higher in fosphenytoin versus phenytoin treated rabbits. The AUC from time zero to 120 min (AUC_{0-120}) was also significantly higher (P=0.0277) in fosphenytoin treated rabbits compared to the phenytoin group. However, there were no significant differences between the time to achieve maximum plasma drug concentrations (T_{max}) (P=0.675).

Table 7: Mean (SD) weight, plasma albumin concentrations and free phenytoin fractions following intravenous and intramuscular administration of 10 mg/kg phenytoin sodium or fosphenytoin sodium equivalents in six rabbits

Treatment group	**Fosphenytoin**		**Phenytoin**		***P*-value**
	i.v.	**i.m.**	**i.v.**	**i.m.**	
Weight (kg)	2.016 ± 0.276	2.062 ± 0.664	2.177 ± 0.300	2.099 ± 0.394	0.9304
Plasma albumin (g/dl)	2.998 ± 0.958	2.693 ± 0.335†	3.465 ± 1.030	3.168 ± 0.948†	0.4903
Free PHT fraction	0.1941 ± 0.033	0.220 ± 0.137	0.1328 ± 0.036	0.156 ± 0.058	0.3547

† n = 5

Table 8: Median (interquartile range) phenytoin pharmacokinetic parameter values following intravenous and intramuscular administration of fosphenytoin sodium and standard phenytoin sodium in the rabbit (n=6 in all cases). Doses are 10 mg/kg phenytoin equivalents.

	Parameter	**Phenytoin**	**Fosphenytoin**	**P-value**
i.v.	AUC_{0-180} (µg/ml.min)	1183.4 (1108.3–1517.5)	1023.1 (846.8–1375.2)	0.0464
i.m.	AUC_{0-120} (µg/ml.min)	261.2 (152.1–355.3)	723.3 (422.5–987.7)	0.0277
	C_{max} (µg/ml)	2.58 (2.28-2.78)	6.65 (6.01–6.94)	0.0277
	T_{max} (min)	24.8 (3.0–60.0)	30.0 (15.0–45.0)	0.6750

Fig. 12: Mean (SD) plasma phenytoin concentrations versus time following intravenous administration of 10 mg/kg of phenytoin sodium and fosphenytoin sodium equivalents in the rabbit (n=6).

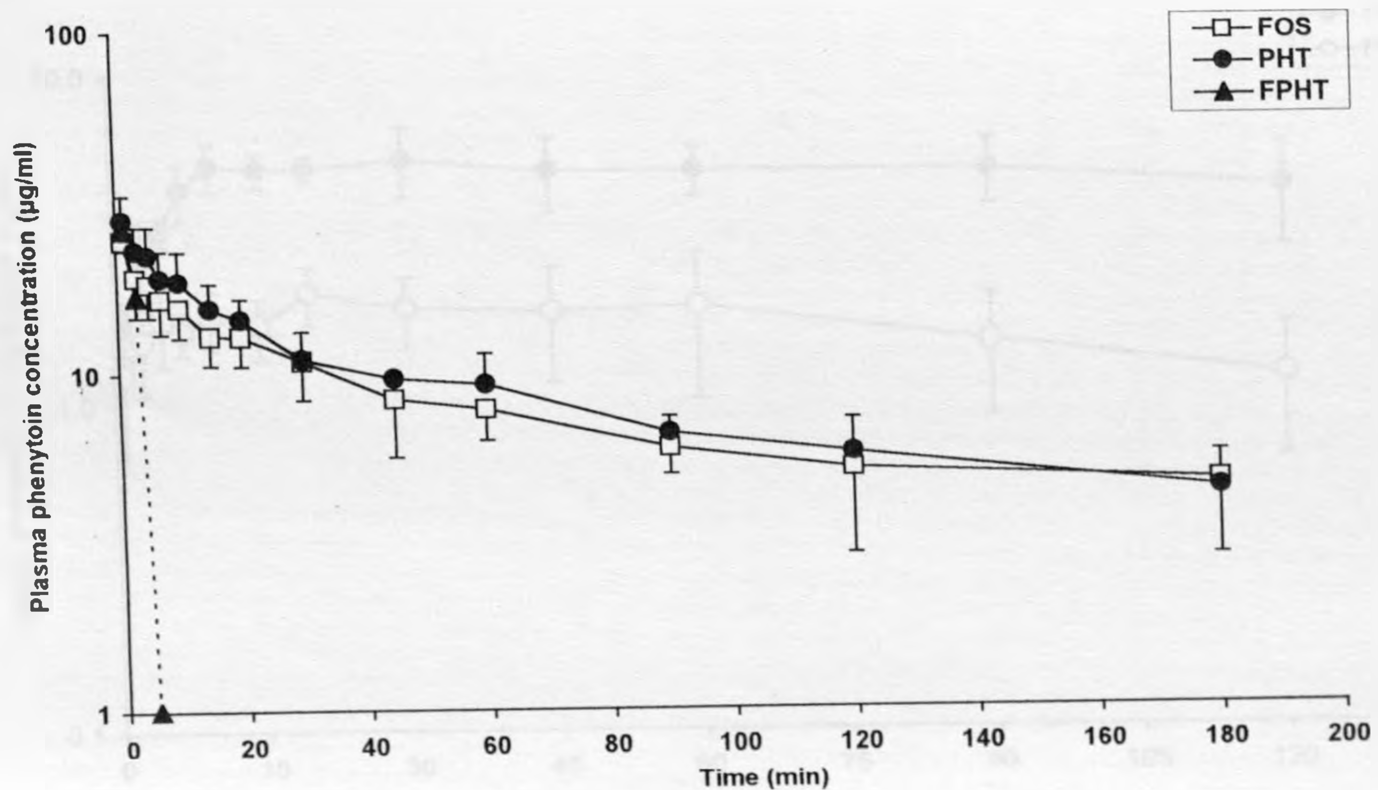


Fig. 13: Mean (SD) plasma phenytoin concentrations versus time following intramuscular administratio of 10 mg/kg of phenytoin sodium and fosphenytoin sodium equivalents in the rabbit (n=6).

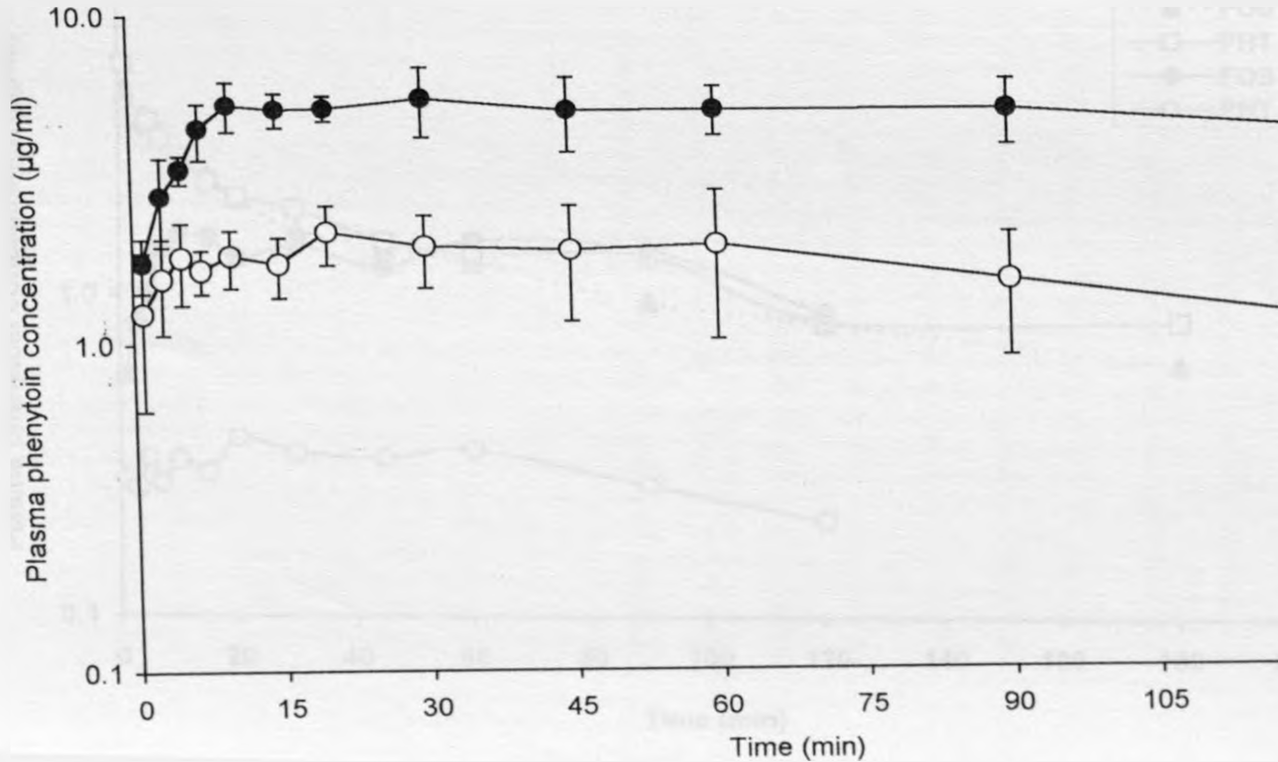
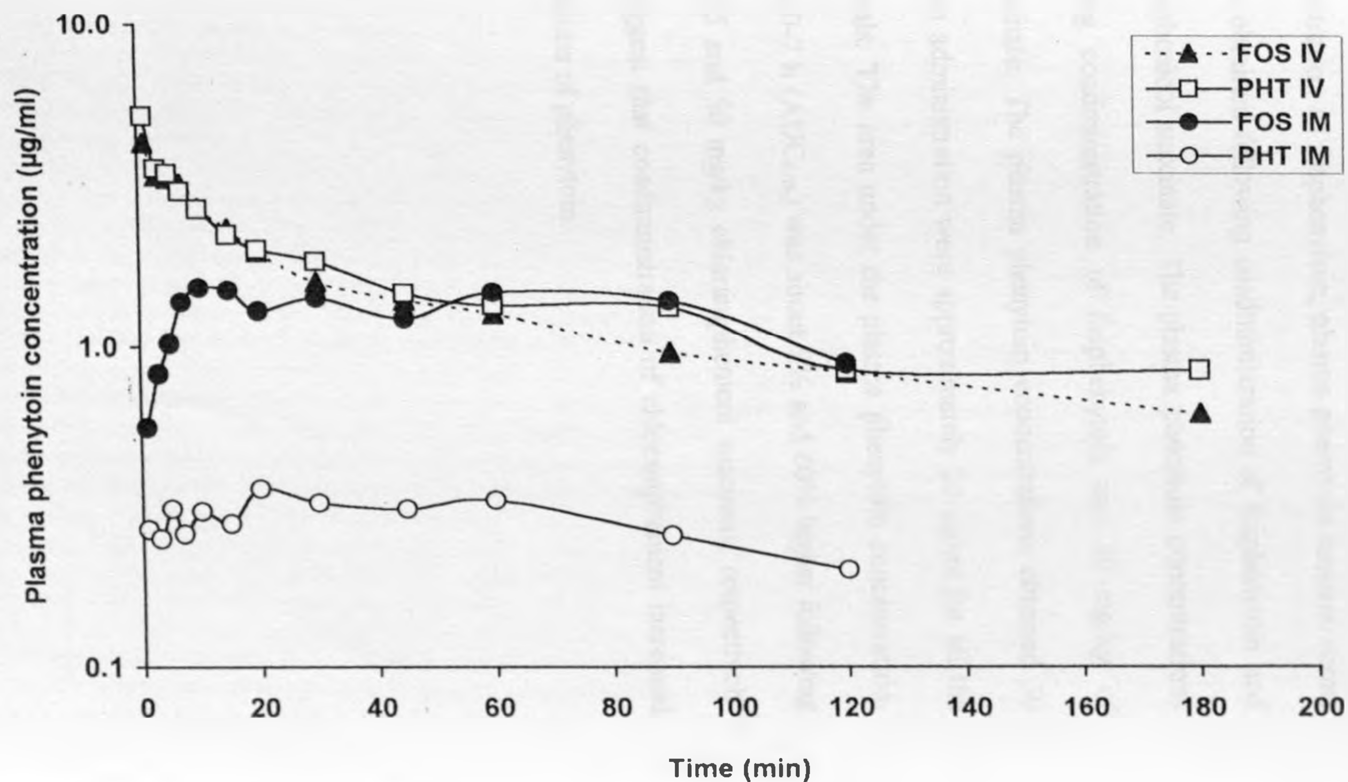


Fig. 14: Mean free plasma phenytoin concentrations following intravenous and intramuscular administration of 10 mg/kg of phenytoin sodium or fosphenytoin sodium equivalents in the rabbit (n=6).



4.3 Plasma phenytoin concentrations in the rat following coadministration of fosphenytoin and chloramphenicol succinate

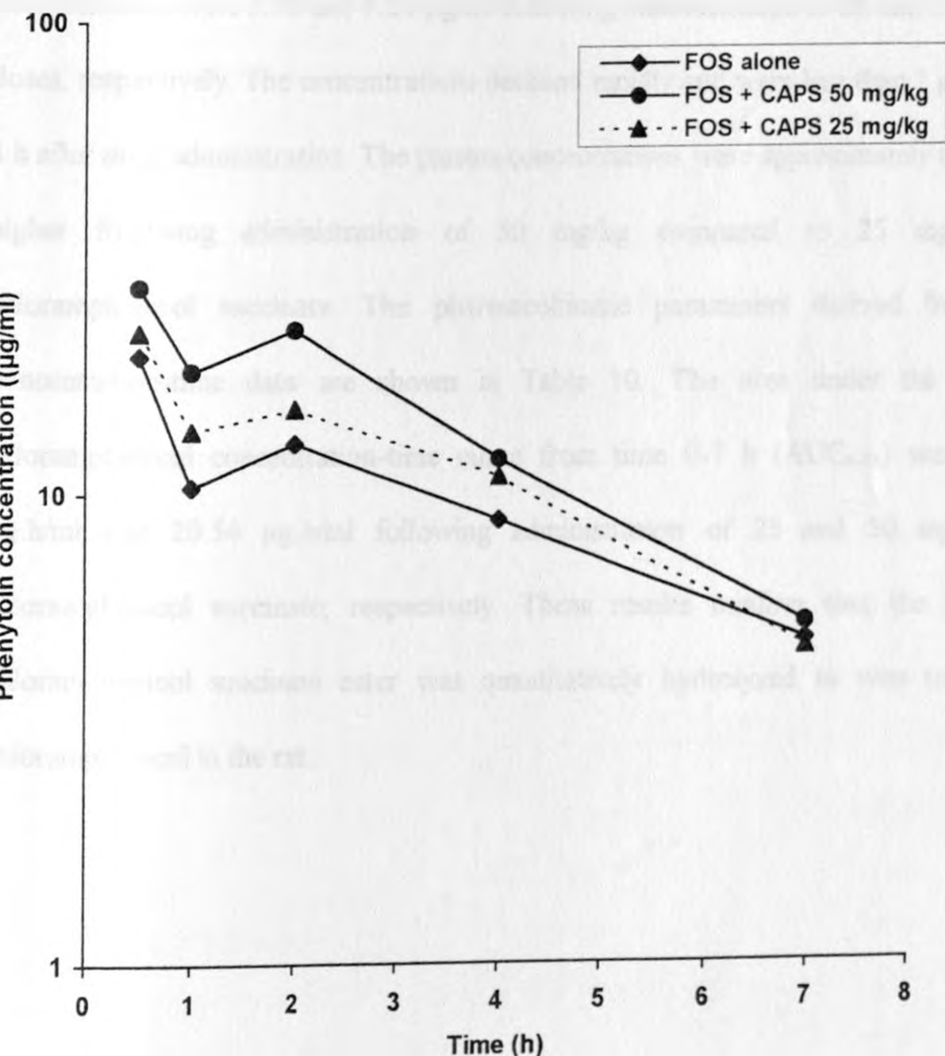
Following i.v. administration of fosphenytoin, plasma phenytoin concentrations were similar to those obtained following coadministration of fosphenytoin and 25 mg/kg of chloramphenicol succinate. The plasma phenytoin concentrations were higher following coadministration of fosphenytoin and 50 mg/kg of chloramphenicol succinate. The plasma phenytoin concentrations obtained 30 min after fosphenytoin administration were approximately 20 µg/ml for all the three groups of animals. The area under the plasma phenytoin concentration-time curve from time 0-7 h ($AUC_{0\text{-}7h}$) was about 9% and 60% higher following coadministration of 25 and 50 mg/kg chloramphenicol succinate, respectively. The $AUC_{0\text{-}7h}$ data suggest that coadministration of chloramphenicol increased the plasma concentrations of phenytoin.

Table 9: Mean plasma phenytoin concentrations (µg/ml) following intravenous administration of 30 mg PEs/kg of fosphenytoin sodium concomitantly with either 25 or 50 mg/kg of chloramphenicol succinate in the rat. Each value is the mean for four rats.

Sampling Time (h)	Plasma phenytoin concentrations (µg/ml)		
	FOS Alone	FOS + 25 mg/kg CAP succinate	FOS + 50 mg/kg CAP succinate
0.5	19.6	21.6	21.1
1	10.4	14.4	18.6
2	12.9	16.0	22.6
3	13.9	ND	10.7
4	8.8	10.5	11.4
6	7.6	ND	22.5
7	4.9	2.95	8.4
Parameters			
AUC_{0-7h} (µg.h/ml)	71.45	77.62	112.85
$AUC_{0-\infty}$ (µg.h/ml)	93.40	85.90	165.7

Key: FOS-fosphenytoin; PEs-phenytoin equivalents; CAP-chloramphenicol;

Fig. 15: Mean plasma phenytoin concentrations (μg/ml) versus time following intravenous administration of 30 mg phenytoin equivalents/kg of fosphenytoin sodium concomitantly with either 25 or 50 mg/kg of chloramphenicol succinate in the rat.



.4 Plasma chloramphenicol concentrations in the rat

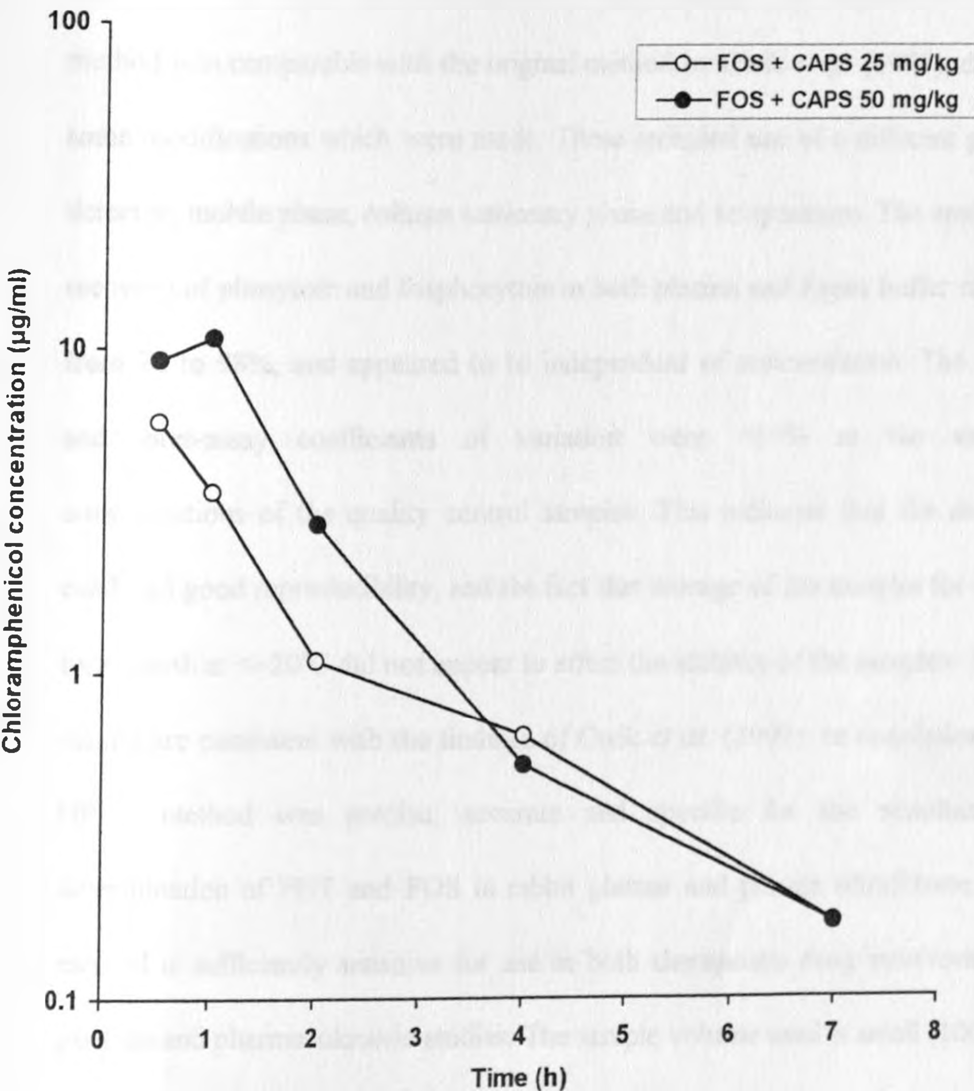
he chloramphenicol concentrations obtained following coadministration of osphenytoin and 25 or 50 mg/kg chloramphenicol succinate are shown in Table 10. he mean plasma chloramphenicol concentrations achieved at 0.5 h after drug dministration were 5.98 and 9.24 μg/ml following administration of 25 and 50 mg/kg oses, respectively. The concentrations declined rapidly and were less than 1 μg/ml by h after drug administration. The plasma concentrations were approximately two-fold gher following administration of 50 mg/kg compared to 25 mg/kg of loramphenicol succinate. The pharmacokinetic parameters derived from the oncentration-time data are shown in Table 10. The area under the plasma loramphenicol concentration-time curve from time 0-7 h ($AUC_{0\text{-}7h}$) was 11.30 g.h/ml and 20.54 μg.h/ml following administration of 25 and 50 mg/kg of loramphenicol succinate, respectively. These results confirm that the inactive loramphenicol succinate ester was quantitatively hydrolyzed *in vivo* to active loramphenicol in the rat.

Table 10: Mean plasma chloramphenicol concentrations (μg/ml) following intravenous coadministration of 30 mg PEs/kg of fosphenytoin sodium and 25 or 50 mg/kg of chloramphenicol succinate in the rat. Each value is the mean for four rats.

Sampling Time (h)	Plasma chloramphenicol concentrations (μg/ml)	
	FOS + 25 mg/kg CAP succinate	FOS + 50 mg/kg CAP succinate
0.5	5.98	9.24
1	3.63	10.81
2	1.10	2.89
4	0.64	0.52
7	0.17	0.17
Parameters		
k_{el} (h^{-1})	0.375	0.551
$t_{1/2}$ (h)	1.83	1.26
AUC_{0-t} (μg.h/ml)	11.30	20.54
$AUC_{0-\infty}$ (μg.h/ml)	11.75	20.84
Cl_T (ml/min)	35.50	40.00
V_d (L/kg)	5.62	4.35

Key: FOS-fosphenytoin; PE-phenytoin equivalents; CAP- chloramphenicol

Fig. 16: Mean plasma chloramphenicol concentrations versus time following intravenous administration of 30 mg phenytoin equivalents/kg of fosphenytoin sodium concomitantly with either 25 or 50 mg/kg of chloramphenicol succinate in the rat. Each point is the mean for four rats



CHAPTER FIVE

DISCUSSION

5.1 Chromatography

The HPLC method exhibited good resolution of PHT, FOS and the IS, with high specificity as no interfering peaks were detected. The sensitivity of this method was comparable with the original method by Cwik *et al.* (1997), despite some modifications which were made. These included use of a different pump, detector, mobile phase, column stationary phase and temperature. The analytical recovery of phenytoin and fosphenytoin in both plasma and Krebs buffer ranged from 76 to 98%, and appeared to be independent of concentration. The intra- and inter-assay coefficients of variation were <10% at the various concentrations of the quality control samples. This indicates that the method exhibited good reproducibility, and the fact that storage of the samples for up to two month at <–20°C did not appear to affect the stability of the samples. These results are consistent with the findings of Cwik *et al.* (1997). In conclusion, the HPLC method was precise, accurate and specific for the simultaneous determination of PHT and FOS in rabbit plasma and plasma ultrafiltrate. The method is sufficiently sensitive for use in both therapeutic drug monitoring in patients and pharmacokinetic studies. The sample volume used is small (100 μl), which is important in young children with severe falciparum malaria, who are

often anaemic, and sample volume must be kept to a minimum. The extraction procedure is short (20 min), and the retention time is about 15 min. This allows rapid screening of phenytoin samples following administration of fosphenytoin when the use of immunoassays is inappropriate.

5.2 Pharmacokinetic parameters of phenytoin in the rabbit

Following i.v. administration of fosphenytoin, plasma phenytoin concentrations were similar to those obtained after i.v. administration of an equivalent dose of phenytoin sodium (Fig 12). The percentage ratio of the mean AUC from 0-180 min after i.v. administration of fosphenytoin was 90.2 of that following administration of phenytoin sodium, suggesting complete hydrolysis of fosphenytoin to phenytoin in the rabbit. These results are consistent with previous findings (Varia *et al.*, 1984a,b) in the beagle dog and rat. It should be pointed out that AUC was calculated by assuming that the pharmacokinetics of phenytoin were not saturable at the dose used in the present study. The use of the linear trapezoid method would be inappropriate if the concentrations produced in the present study resulted in non-linear kinetics (Jusko *et al.*, 1976). The estimation of AUC was restricted to the first 120 min after drug administration since autoinduction of phenytoin metabolism develops rapidly, especially during chronic administration (Cusack *et al.*, 1987).

Fig. 12 shows the plasma phenytoin concentrations following i.v. administration of fosphenytoin sodium and phenytoin sodium. Although the maximum phenytoin concentration (C_{max}) is practically the same following i.v. administration of fosphenytoin sodium and phenytoin sodium, this is not a true reflection of what would be expected in clinical practice. In the latter case, phenytoin sodium is normally infused at a slower rate (20-60 min) to minimize cardiovascular side effects. This would result in lower C_{max} following administration of phenytoin sodium compared to fosphenytoin sodium. However, the rate of entry of phenytoin into brain, which is the more important factor related to activity, has been shown (Walton *et al.*, 1999) to be faster following phenytoin sodium administration at a slower rate compared to administration of fosphenytoin sodium. Thus, it is possible that even in the present study, phenytoin entry into brain was faster following administration of phenytoin sodium. Brain phenytoin concentrations were, however, not investigated in the present study.

Administration of effective anticonvulsants can be useful in preventing seizures associated with severe malaria. Intramuscular (i.m.) administration of fosphenytoin was also evaluated in the present study. Intramuscular

administration of fosphenytoin would be particularly useful in most parts of rural Africa where facilities for i.v. drug administration are scarce. Higher (approximately two-fold) plasma phenytoin concentrations were achieved following administration of fosphenytoin sodium compared to phenytoin sodium (Table 8 and Fig 13). In practice, i.m. administration of fosphenytoin would be useful in seizure prophylaxis, or for maintenance doses following initial i.v. administration of a loading dose of phenytoin . For acute seizure control, however, i.m. administration of fosphenytoin would have to be combined with i.v. administration of a more rapidly acting anticonvulsant such as diazepam to allow time for complete hydrolysis of fosphenytoin.

5.3 Interaction between phenytoin and chloramphenicol in the rat

Chloramphenicol is an inexpensive, readily available, broadspectrum antibiotic, which is used in developing countries. Relatively little is known, however, about the interaction between phenytoin and chloramphenicol. This study provided some evidence of a pharmacokinetic interaction between the two drugs *in vivo* in the rat. Chloramphenicol sodium succinate is the inactive water-soluble prodrug for intravenous administration that is rapidly hydrolyzed within the body to biologically active chloramphenicol. The hydrolysis of chloramphenicol succinate to chloramphenicol is incomplete following intravenous administration

of the prodrug. The hydrolysis of chloramphenicol succinate ranged from 55 to 95% (0.69 ± 0.13, mean ± sd) following i.v. administration of chloramphenicol succinate in 12 patients aged 2.5 months to 20 years. The remaining amount (45 to 5%) was excreted or eliminated from the body before it could be converted to the active product (Nahata and Powell, 1981). In another study in 18 children, Kauffman *et al.* (1981) reported that 36% of chloramphenicol succinate was collected in urine unchanged, and higher relative bioavailability was observed using the oral chloramphenicol palmitate than from intravenous chloramphenicol succinate. These observations emphasis the importance of evaluating the bioavailability of chloramphenicol even when the chloramphenicol succinate is administered intravenously. In the present study, however, the chloramphenicol levels found in plasma were very low. Therefore, it was not possible to estimate the relative bioavailability of chloramphenicol following administration of the succinate. The low levels of chloramphenicol succinate found in the present study could probably be due to elimination of the pro-drug in the urine before hydrolysis, or rapid conversion of the drug to the active form *in vivo.*

Coadministration of fosphenytoin and chloramphenicol resulted in modest elevation of plasma phenytoin, which is in agreement with the report by Nation

et al. (1990) in human subjects. The plasma phenytoin concentrations (about 20 μg/ml) obtained 30 min after fosphenytoin administration in the rat were similar to those obtained by Walton *et al.* (1999) following administration of a similar dose in the rat via the femoral artery. This suggests that the administration of drug via the tail vein is as effective as the femoral vein.

Several cases have been reported in the literature indicating that administration of chloramphenicol to patients receiving phenytoin can result in phenytoin toxicity (Ballek *et al.*, 1973; Rose *et al.*, 1977; Koup *et al.*, 1978). Rose *et al.* (1977) reported a marked elevation of serum phenytoin concentrations in a patient who was on maintenance doses of phenytoin and was administered chloramphenicol. The serum concentrations declined after cessation of chloramphenicol therapy. In another study by Ballek *et al.* (1973), a patient who was on maintenance doses of phenytoin experienced toxicity characterized by nystagmus on lateral gaze when chloramphenicol was added to the drug regimen.

The potential for interaction between the two drugs has been recognized, but only one study investigating the influence of chloramphenicol on phenytoin pharmacokinetics in humans has been conducted (Christensen and Skovsted,

1969). In that study it was reported that chloramphenicol, added to the daily drug regimen of two patients stabilized on phenytoin, caused an increase in serum phenytoin concentrations. In three other patients, the elimination half-life of a radiolabelled phenytoin given i.v. was increased considerably. It was suggested that chloramphenicol inhibited the metabolic biotransformation of phenytoin, and that chloramphenicol should be used with caution in patients receiving phenytoin.

Phenytoin is eliminated from the body largely by hepatic biotransformation. This pathway is readily saturated at moderate doses of the drug and is modified by a number of drug interactions and disease states (Christensen and Skovsted, 1969). Chloramphenicol causes plasma concentrations of phenytoin to be elevated by inhibiting its metabolism (Christensen and Skovsted, 1969). Phenytoin possesses a narrow therapeutic index. Its metabolism is saturable. When the metabolizing enzyme system approaches saturation, small changes in enzyme activity produce disproportionately large increase in plasma phenytoin concentration. The rise of serum plasma concentration occurs as a result of the pronounced non-linearity or dose-dependence in phenytoin disposition. Dosing of the drug in excess of this metabolic rate produces a rapid increase in the phenytoin plasma concentrations. Any factor that inhibits the rate of formation

of *p*-HPPH will accentuate this non-linearity, and disproportionate increases in phenytoin plasma concentration will occur at low doses. In the present study, the concentrations of phenytoin were approximately twofold higher following coadministration of fosphenytoin and 50 mg/kg single dose of chloramphenicol succinate. In clinical practice, fosphenytoin or phenytoin is infused over 20 min, followed by maintenance doses 12 hourly for up to 48 h. In addition, chloramphenicol is usually administered in multiple doses for the treatment of meningitis. It is, therefore, likely that clinically significant interaction between chloramphenicol and phenytoin may occur under most clinical situations. Phenytoin has been reported to decrease the plasma concentrations and increase the total body clearance of chloramphenicol, probably by inducing hepatic microsomal enzymes (Koup *et al.*, 1978). About 90% of chloramphenicol is inactivated in the liver by conjugation with glucuronic acid or by reduction to arylamines before being excreted in the urine, while the remaining 10% is excreted unchanged in the urine. The decrease in chloramphenicol plasma levels is probably explained by the induction of hepatic microsomal enzymes by phenytoin. Plasma concentrations should be monitored when these two agents are administered concomitantly (Powell and Nahata, 1981). It is important for the clinician to be on alert for toxicity from other agents which are metabolized

by the liver while administering chloramphenicol and plasma levels should be monitored when the two drugs are administered concomitantly.

Infants and young children with bacterial meningitis are often treated with phenytoin to prevent convulsions. Chloramphenicol is the drug of choice for the treatment of *H. influenza* meningitis. In Kenya, concomitant administration of phenytoin and chloramphenicol is currently a routine clinical practice especially when treating children with severe malaria. In Kilifi on the Kenyan coast, the majority of children admitted to paediatric ward with seizures refractory to treatment with other anticonvulsants are administered phenytoin. However, due to the difficulty in excluding meningitis in cases of suspected cerebral malaria, many centres, including Kilifi, routinely initiate antimalarial and antibiotic treatments, in addition to anticonvulsants when indicated, as a standard approach. Thus, chloramphenicol is routinely used for treatment of suspected or confirmed bacterial meningitis. The results of the present study have demonstrated the possibility of an interaction between these two drugs *in vivo*. Inter- and intra-subject variability in the metabolism of chloramphenicol and phenytoin necessitates the importance of close monitoring of the plasma concentrations of both drugs and possible dosage adjustments.

CONCLUSIONS

The results of the present study confirm several points regarding fosphenytoin.

1. The drug is rapidly and completely hydrolyzed into phenytoin *in vivo*, following both i.v. and i.m. administration.

2. Maximum plasma phenytoin concentrations would be achieved within 30 min after i.m. administration of fosphenytoin in the rabbits and rats.

3. Intramuscular (i.m.) fosphenytoin could offer a practical alternative to i.v. phenytoin, especially for seizure prophylaxis.

4. Coadministration of fosphenytoin sodium and chloramphenicol succinate causes a modest increase in phenytoin concentrations *in vivo* in the rat. The interaction is dose-dependent, and it is therefore important to monitor the plasma concentrations of both drugs when they are concurrently administered in clinical practice.

5. Based on the results of the present study, further investigations of the pharmacokinetics of phenytoin in African children following i.v. administration of phenytoin sodium and fosphenytoin sodium and i.m. administration of fosphenytoin sodium would be undertaken.

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APPENDICES

Appendix 1: Details of experimental rabbits used in the study.

Rabbit No.	Sex	Weight (kg)	Drug formulation and route		Plasma albumin (g/dl)	Free Phenytoin Fraction
2	M	2.130	FOS	i.v.	2.66	0.1074
3	F	2.185	PHT	i.v.	2.58	0.1671
4	M	2.470	FOS	i.v.	2.09	0.3248
5	M	2.276	PHT	i.m.	2.95	0.0845
6	M	2.048	PHT	i.v.	2.68	0.0786
7	M	1.748	FOS	i.v.	2.72	0.1847
8	M	1.715	FOS	i.m.	2.78	0.1792
9	M	1.872	FOS	i.v.	2.67	0.1679
10	M	1.990	FOS	i.m.	2.16	0.4289*
11	M	2.104	FOS	i.v.	2.99	0.1803
12	F	1.630	FOS	i.m.	3.10	0.2208
13	M	1.765	FOS	i.m.	2.88	0.1053
14	F	1.882	FOS	i.m.	2.45	0.1008
15	M	3.392	FOS	i.m.	2.79	0.0683
16	F	2.328	PHT	i.v.	2.66	0.1354
17	M	2.128	PHT	i.m.	2.79	0.1699
18	M	2.145	PHT	i.m.	2.95	0.1551
19	F	2.680	PHT	i.v.	2.35	0.1494
20	F	2.049	PHT	i.m.	2.54	0.1872
21	F	1.576	PHT	i.v.	4.15	ND
22	F	1.778	PHT	i.m.	4.81	0.1283
23	F	2.688	PHT	i.m.	4.75	0.1379
24	M	1.778	PHT	i.v.	4.59	0.1332
25	M	1.770	FOS	i.v.	4.86	0.2474

Key: M–male; F–female; FOS-fosphenytoin; PHT-phenytoin; ND–not determined; *–value an outlier; excluded during data analysis.

Appendix 2: Absorbances and corresponding albumin concentrations of standards, Randox quality control (QC) and rabbit plasma samples. Samples were analyzed in batches.

Rabbit No.	QC sample	Absorbance		Albumin concentration (g/dl)	
		QC sample	Rabbit sample	QC sample	Rabbit sample
1	Standard	0.384	0.367	4.50	4.29
2	Low Randox	0.207	0.227	2.43	2.66
3	Mid Randox	0.330	0.220	3.87	2.58
4	High Randox	0.415	0.179	4.86	2.09
5			0.252		2.95
6			0.229		2.68
7			0.232		2.72
8			0.238		2.78
9			0.228		2.67
10			0.184		2.16
11	Standard	0.375	0.249	4.50	2.99
12	Low Randox	0.210	0.259	2.52	3.10
13	Mid Randox	0.315	0.240	3.78	2.88
14	High Randox	0.410	0.204	4.92	2.45
15			0.233		2.79
16	Standard	0.378	0.223	4.50	4.50
17	Low Randox	0.207	0.235	2.46	2.48
18	Mid Randox	0.320	0.248	3.81	3.79
19	High Randox	0.413	0.197	4.92	4.95
20			0.214		
21	Standard	0.380	0.217	4.50	4.15
22	Low Randox	0.209	0.253	2.48	4.81
23	Mid Randox	0.320	0.249	3.79	4.75
24	High Randox	0.418	0.241	4.95	4.59
25			0.255		4.86

The albumin concentrations for the Randox quality control (QC) samples were: Low: 2.23–3.01; Middle: 3.23–4.37; and High: 4.07–5.51 g/dl

Appendix 3: Total plasma phenytoin concentrations (μg/ml) following intravenous administration of 10 mg PEs/kg of fosphenytoin sodium in the rabbit (n=6).

Rabbit No	Time after drug administration (min)															
	Predose	1	3	5	7	10	15	20	30	45	60	90	120	180	240	300
2	0.00	ND	ND	21.54	ND	17.15	14.53	14.32	15.81	10.61	9.63	ND	9.59	5.43	3.42	3.24
4	0.00	ND	17.75	ND	21.47	19.39	13.76	14.82	9.56	8.43	7.91	5.37	3.47	3.25	3.61	2.96
7	0.00	22.56	11.97	12.53	12.63	11.32	9.60	9.48	9.37	5.50	7.12	ND	3.38	2.13	*	*
9	0.00	25.51	23.20	17.00	14.10	14.57	11.71	14.66	11.14	12.54	9.61	7.22	4.59	6.42	2.52	3.70
11	0.00	25.52	22.48	21.56	16.43	14.18	11.71	12.19	9.72	6.34	6.63	4.93	4.77	3.82	3.09	3.29
25	0.00	25.56	20.25	19.01	17.88	16.34	15.51	11.07	9.62	7.07	6.07	6.26	5.06	6.94	*	*
N	6	5	5	5	6	6	6	6	6	6	6	4	6	6	4	4
Mean	0.00	24.79	19.13	18.33	16.50	15.49	12.80	12.76	10.87	8.415	7.83	5.95	5.14	4.67	3.16	3.30
SD	0.00	1.495	4.534	3.762	3.440	2.780	2.185	2.201	2.503	2.790	1.514	1.01	2.286	1.896	0.478	0.305

Key: **ND**: not determined; *: no more data points as animal died.

Appendix 4: Total plasma phenytoin concentrations (µg/ml) following intravenous administration of 10 mg/kg of phenytoin sodium in the rabbit (n=6).

Rabbit	Time after drug administration (min)															
No	Predose	1	3	5	7	10	15	20	30	45	60	90	120	180	240	300
3	0.00	ND	ND	30.25	ND	26.43	19.45	16.71	11.49	10.72	11.92	ND	8.67	4.99	6.38	6.45
6	0.00	30.80	26.05	20.08	ND	16.72	14.63	12.36	7.05	9.14	8.59	6.18	5.73	4.13	3.08	*
16	0.00	21.97	19.81	15.95	15.02	13.43	11.33	11.12	10.00	9.57	9.20	7.63	5.50	5.82	4.40	3.48
19	0.00	33.72	27.77	24.31	24.06	17.54	18.01	15.72	11.63	8.46	6.01	6.79	5.11	2.80	3.08	2.51
21	0.00	ND	18.54	21.58	17.10	17.37	13.63	15.51	12.70	9.21	11.09	5.89	5.11	*	*	*
24	0.00	27.19	23.79	22.20	20.21	18.18	15.65	14.53	13.10	10.98	8.96	ND	4.36	*	*	*
N	6	4	5	6	4	6	6	6	6	6	6	4	6	4	4	3
Mean	0.00	28.42	23.19	22.40	19.10	18.62	15.45	14.33	11.00	9.68	9.295	6.623	5.75	4.40	4.24	4.15
SD	0.00	5.062	3.950	4.753	3.936	4.400	2.951	2.155	2.215	0.178	2.075	0.769	1.510	1.290	1.560	2.053

ND: not determined; *: no more data points as animal died.

Appendix 5: Total plasma phenytoin concentrations (μg/ml) following intramuscular administration of 10 mg/kg of phenytoin sodium in the rabbit (n=6).

Rabbit	Time after drug administration (min)															
No.	Predose	1	3	5	7	10	15	20	30	45	60	90	120	180	240	300
5	0.00	ND	0.90	2.47	ND	1.73	1.77	1.55	1.27	0.70	1.16	0.75	0.56	0.46	*	*
17	0.00	2.54	2.18	2.30	2.00	2.10	2.14	2.05	2.06	1.92	1.99	1.70	1.62	1.41	1.15	0.99
18	0.00	1.72	1.67	1.89	1.63	2.35	1.97	2.80	2.28	2.58	3.41	2.16	1.37	1.03	0.98	*
20	0.00	1.07	1.16	1.36	1.68	1.80	1.38	2.51	2.66	2.72	2.78	2.25	1.66	1.29	1.07	0.77
22	0.00	1.38	1.46	1.56	1.67	1.67	1.89	2.28	1.87	1.63	1.05	0.92	0.50	*	*	*
23	0.00	1.77	2.00	1.28	1.31	1.17	1.25	1.83	1.61	1.84	1.49	1.44	1.49	1.32	1.02	0.71
N	6	5	6	6	5	6	6	6	6	6	6	6	6	5	4	3
Mean	0.00	1.239	1.562	1.810	1.658	1.827	1.733	2.170	1.958	1.898	1.980	1.537	1.200	1.102	1.055	0.823
SD	0.00	0.619	0.489	0.496	0.245	0.361	0.348	0.456	0.492	0.728	0.945	0.622	0.529	0.386	0.073	0.147

ND: not determined (sample not collected or lost during extraction procedure) *: No more data points since animal died

Appendix 6: Total plasma phenytoin concentrations (μg/ml) following intramuscular administration of 10 mg PEs/kg of fosphenytoin sodium in the rabbit (n=6).

Rabbit No	Time after drug administration (min)															
	Predose	**1**	**3**	**5**	**7**	**10**	**15**	**20**	**30**	**45**	**60**	**90**	**120**	**180**	**240**	**300**
8	0.00	2.16	3.71	ND	5.32	5.72	5.76	6.01	5.11	4.70	4.98	6.36	5.91	5.16	2.36	*
10	0.00	1.99	3.82	3.89	5.49	5.25	5.48	5.18	5.88	4.06	6.73	5.79	3.48	*	*	*
12	0.00	1.43	1.68	3.54	3.60	5.32	5.15	5.32	8.18	7.28	5.35	4.35	2.89	*	*	*
13	0.00	1.64	3.00	3.10	4.98	6.94	4.90	4.99	4.64	4.55	5.11	*	*	*	*	*
14	0.00	ND	2.27	3.14	3.42	4.52	4.06	4.72	5.60	6.34	5.17	6.61	5.95	4.90	4.09	3.07
15	0.00	1.50	2.34	3.23	4.18	4.34	5.71	ND	4.31	4.00	3.88	3.82	*	*	*	
N	6	5	6	5	6	6	6	5	6	6	6	6	4			
Mean	0.00	1.74	2.80	3.38	4.50	5.35	5.18	5.24	5.62	5.16	5.20	5.21	4.56			
SD	0.00	0.317	0.855	0.333	0.890	0.936	0.639	0.484	1.383	1.344	0.912	1.193	1.603			

ND: not determined *: No more data points since animal died, PEs: phenytoin equivalents

Appendix 7: Free plasma phenytoin concentrations (μg/ml) following intravenous administration of 10 mg PEs/kg of fosphenytoin sodium in the rabbit (n=6).

Rabbit	**Time after drug administration (min)**															
No.	**Predose**	**1**	**3**	**5**	**7**	**10**	**15**	**20**	**30**	**45**	**60**	**90**	**120**	**180**	**240**	**300**
2	0.00	ND	ND	2.32	ND	1.84	1.56	1.54	1.70	1.14	1.04	ND	1.03	0.58	0.37	0.35
4	0.00	ND	1.40	ND	2.31	2.08	1.48	1.59	1.03	0.91	0.85	0.58	0.37	0.35	0.39	0.32
7	0.00	4.17	2.21	2.31	2.33	2.09	1.77	1.75	1.73	1.02	1.32	ND	0.62	0.39	*	*
9	0.00	4.28	4.15	2.85	2.37	2.45	1.97	2.46	1.87	2.11	1.61	1.21	0.77	1.08	0.42	0.62
11	0.00	4.60	4.05	3.89	2.96	2.56	2.11	2.20	1.75	1.14	1.20	0.88	0.86	0.69	0.56	0.59
25	0.00	6.32	5.01	4.70	4.42	4.04	3.84	2.73	2.38	1.75	1.50	1.55	1.25	1.72	*	*
N	6	4	5	5	6	6	6	6	6	6	6	4	6	6	4	4
Mean	0.00	4.84	3.36	3.21	2.88	2.51	2.12	2.05	1.74	1.35	1.25	1.06	0.82	0.80	0.44	0.47
SD	0.00	1.00	1.50	1.05	0.90	0.79	0.87	0.49	0.43	0.48	0.28	0.42	0.31	0.52	0.09	0.16
SEM	0.00	0.25	0.30	0.21	0.18	0.13	0.15	0.08	0.07	0.08	0.05	0.10	0.05	0.09	0.02	0.04

ND: not determined; *: no more data points as animal died; PEs: phenytoin equivalents.

Appendix 8: Free plasma phenytoin concentrations (µg/ml) following intravenous administration of 10 mg/kg phenytoin sodium in the rabbit (n=6).

Rabbit	**Time after drug administration (min)**															
No.	**Predose**	**1**	**3**	**5**	**7**	**10**	**15**	**20**	**30**	**45**	**60**	**90**	**120**	**180**	**240**	**300**
3	0.00	ND	ND	5.15	ND	4.42	3.25	2.79	1.92	1.79	1.99	ND	1.45	0.83	1.07	1.08
6	0.00	2.42	2.05	1.58	ND	1.31	1.15	0.97	0.56	0.72	0.68	0.49	0.45	0.32	0.24	*
16	0.00	2.97	2.68	2.16	2.03	1.82	1.53	1.51	1.35	1.30	1.25	1.03	0.74	0.79	0.60	0.47
19	0.00	5.04	4.15	3.63	3.59	2.62	2.69	2.35	1.74	1.26	0.90	1.01	0.76	0.42	0.46	0.37
24	0.00	3.62	3.17	2.96	2.69	2.42	2.08	1.94	1.74	1.45	1.18	ND	0.58	*	*	*
N	5	4	4	5	3	5	5	5	5	5	5	3	5	4	4	3
Mean	0.00	3.51	3.01	3.10	2.77	2.52	2.14	1.91	1.46	1.30	1.20	0.84	0.80	0.590	0.59	0.64
SD	0.00	1.13	0.89	1.39	0.78	1.18	0.85	0.71	0.55	0.39	0.50	0.31	0.39	0.258	0.35	0.38
SEM	0.00	0.28	0.22	0.28	0.26	0.24	0.17	0.14	0.11	0.08	0.10	0.10	0.08	0.064	0.09	0.28

ND – not determined; * – no more data points as animal died.

Appendix 9: Free plasma phenytoin concentrations (µg/ml) following intramuscular administration of 10 mg/kg phenytoin sodium in the rabbit (n=6).

Rabbit	Time after drug administration (min)															
No	Predose	1	3	5	7	10	15	20	30	45	60	90	120	180	240	300
5	0.00	ND	0.08	0.21	ND	0.25	0.15	0.13	0.11	0.06	0.10	0.06	0.05	0.04	*	*
17	0.00	0.44	0.37	0.39	0.34	0.36	0.37	0.35	0.35	0.33	0.34	0.29	0.28	0.24	0.20	0.17
18	0.00	0.27	0.26	0.29	0.25	0.36	0.31	0.43	0.35	0.40	0.53	0.34	0.21	0.16	0.15	*
20	0.00	0.20	0.22	0.25	0.31	0.34	0.26	0.47	0.50	0.51	0.52	0.42	0.31	0.24	0.20	0.14
22	0.00	0.18	0.19	0.20	0.22	0.22	0.25	0.30	0.24	0.22	0.14	0.12	0.07	*	*	*
23	0.00	0.24	0.28	0.18	0.18	0.16	0.17	0.25	0.22	0.25	0.21	0.20	0.21	0.18	0.14	0.10
N	6	5	6	6	5	6	6	6	6	6	6	6	6	5	4	3
Mean	0.00	0.27	0.23	0.25	0.26	0.27	0.25	0.32	0.30	0.30	0.31	0.24	0.19	0.17	0.13	0.14
SD	0.00	0.10	0.10	0.08	0.07	0.08	0.08	0.12	0.13	0.16	0.19	0.04	0.11	0.08	0.08	0.04
SEM	0.00	0.02	0.02	0.01	0.01	0.01	0.01	0.02	0.02	0.03	0.03	0.01	0.02	0.02	0.02	0.01

ND–Not determined; * – no more data points as animal died.

Appendix 10: Free plasma phenytoin concentrations (µg/ml) following intramuscular administration of 10 mg/kg (PEs) of fosphenytoin sodium in the rabbit (n=6).

Rabbit	**Time after drug administration (min)**															
No	**Predose**	**1**	**3**	**5**	**7**	**10**	**15**	**20**	**30**	**45**	**60**	**90**	**120**	**180**	**240**	**300**
8	0.00	0.39	0.66	ND	0.95	1.07	1.08	1.12	0.96	0.88	0.93	1.19	1.11	0.97	0.44	*
10	0.00	0.85	1.64	1.67	2.35	2.45	2.47	2.58	2.19	2.02	2.14	2.73	2.53	*	*	*
12	0.00	0.32	0.37	0.78	0.82	1.17	1.14	1.17	1.82	1.61	1.18	0.96	0.64	*	*	*
13	0.00	0.17	0.32	0.33	0.52	0.73	0.52	0.53	0.49	0.48	0.53	0.45	*	*	*	*
14	0.00	ND	0.23	0.32	0.34	0.46	0.41	0.48	0.56	0.64	0.52	0.67	0.60	0.49	0.41	0.31
15	0.00	0.10	0.16	0.22	0.29	0.39	0.39	ND	0.29	0.27	0.41	0.39	*	*	*	*
N	6	5	6	5	6	6	6	5	6	6	6	6	4	2	2	2
Mean	0.00	0.36	0.56	0.67	0.88	1.00	1.00	1.18	1.05	0.98	0.95	1.07	1.22			
SD	0.00	0.29	0.55	0.60	0.77	0.78	0.79	0.85	0.78	0.67	0.65	0.87	0.90			
SEM	0.00	0.06	0.09	0.12	0.13	0.13	0.13	0.17	0.13	0.11	0.11	0.15	0.23			

ND – not determined; * – no more data points as animal died; PEs – phenytoin equivalents

Appendix 11: Pharmacokinetic parameter values of each rabbit after intravenous administration of 10 mg/kg phenytoin sodium or fophenytoin sodium equivalents.

Pharmacokinetic parameters					
Fosphenytoin			Phenytoin		
Rabbit No.	AUC_{0-120} µg/ml.min	AUC_{0-180} µg/ml.min	Rabbit No.	AUC_{0-120} µg/ml.min	AUC_{0-180} µg/ml.min
2	1379.2	1829.8	3	1517.5	1927.3
4	1032.0	1233.6	6	1108.3	1404.1
7	846.8	1012.1	16	1122.8	1462.3
9	1209.7	1540.0	19	1180.9	1418.6
11	959.4	1217.1	21	1186.0	ND
25	1014.1	1374.1	24	1238.0	ND

Key: ND- not determined

Appendix 12: Pharmacokinetic parameter values of individual rabbits following intramuscular administration of 10 mg kg of phenytoin sodium.

Rabbit No.	C_{max} µg/ml	T_{max} min	AUC_{0-120} µg/ml.min	AUC_{0-180} µg/ml.min
5	2.47	5	82.95	112.5
17	2.54	1	226.87	317.8
18	3.41	60	283.3	355.3
20	2.51	20	272.8	361.3
22	2.28	20	152.1	ND
23	2.00	3	184.0	268.0

Key: ND- not determined

Appendix 13: Pharmacokinetic parameter values of individual rabbits following intramuscular administration of 10 mg/kg phenytoin equivalents of fosphenytoin sodium.

Rabbit No.	C_{max} µg/ml	T_{max} min	AUC_{0-120} µg/ml.min	AUC_{0-180} µg/ml.min
8	6.01	20	655.6	987.7
10	6.73	45	653.7	ND
12	8.18	45	701.6	918.8
13	6.94	10	422.5	ND
14	6.34	45	663.2	988.8
15	5.71	15	367.6	ND

Key: ND- not determined

Appendix 14: Details of the experimental rats which were given 30 mg/kg phenytoin equivalents of fosphenytoin sodium.

Rat No.	Sex	Weight (g)	Notional sample time	Weight of wet brain (g)
1	F	243	1 h	1.150
2	F	236	2 h	1.258
3	F	213	3 h	1.241
4	F	303	4 h	1.510
5	F	241	1 h	1.879
6	F	206	1 h	1.607
7	F	312	4 h	1.726
8	F	222	2 h	1.823
9	F	290	3 h	1.852
10	F	226	3 h	1.615
11	F	239	1 h	1.467
12	F	246	4 h	1.752
13	F	275	0.5 h	1.799
14	F	245	0.5 h	1.747
15	F	255	7 h	1.756
16	F	205	7 h	1.495
17	F	258	7 h	1.496
18	F	295	7 h	1.763
19	F	264	6 h	1.853
20	F	298	6 h	1.786
21	F	315	6 h	1.900
22	F	273	6 h	1.543
29	F	292	0.5 h	1.646
30	F	233	0.5 h	1.741

Appendix 15: Details of the experimental rats used in the study. The rats were administered 30 mg phenytoin equivalents/kg of fosphenytoin sodium concomitantly with 50 mg/kg of chloramphenicol sodium succinate.

Rat No.	Sex	Weight (g)	Notional sample time	Weight of wet brain (g)
23	F	284	6 h	1.638
24	F	275	6 h	1.716
25	F	257	4 h	1.778
26	F	267	4 h	1.588
27	F	254	2 h	1.828
28	F	268	2 h	1.834
31	F	254	7 h	1.671
32	F	295	7 h	1.760
33	F	250	3 h	1.787
34	F	208	3 h	1.873
35	F	244	1 h	1.744
36	F	272	1 h	1.770
37	F	247	0.5 h	1.639
38	F	219	0.5 h	1.827
39	F	258	3 h	1.772
40	F	249	0.5 h	1.750

Appendix 16: Details of the experimental rats used in the study. The rats were administered 30 mg phenytoin equivalents/kg of fosphenytoin sodium concomitantly with 25 mg/kg of chloramphenicol sodium succinate.

Rat No.	Sex	Weight (g)	Notional sample time	Weight of wet brain (g)
41	F	276	7 h	1.696
42	F	231	7 h	1.881
43	F	215	4 h	1.754
44	F	220	4 h	1.678
45	F	199	2 h	1.658
46	F	292	2 h	1.653
47	F	245	1 h	1.744
48	F	259	1 h	1.613
49	F	352	0.5 h	1.787
50	F	253	0.5 h	1.748
51	F	261	7 h	1.854
52	F	221	7 h	1.749
53	F	234	4 h	1.874
54	F	228	4 h	1.663
55	F	220	2 h	1.686
56	F	252	4 h	1.745
57	F	205	1 h	1.452
58	F	224	1 h	1.616
59	F	238	0.5 h	1.761
60	F	277	0.5 h	1.827

Appendix 17: Preparation of sodium acetate buffer (pH 4.6)

5.4 g of sodium acetate was dissolved in 50 ml of distilled water. The pH was adjusted to 4.6 with glacial acetic acid, and the volume made to 100 ml with distilled water.