THE PHARMACOKINETIC INTERACTIONS BETWEEN VALPROIC ACID AND ACYCLOVIR ASSESSED *IN VITRO* AND IN A RABBIT MODEL

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ABSTRACT

Valproic acid is an antiepileptic drug that is widely used for treatment of epilepsy, while acyclovir is an antiviral drug indicated for treatment of infections caused by herpes simplex type I & II and varicella-zoster viruses. Given the high prevalence of people with conditions for which chronic use of valproic acid is indicated, and the notion that valproic acid increases the antiviral activity of acyclovir, it is not uncommon for the two drugs to be used concomitantly. As such, recent reports on the interaction between valproic acid and acyclovir with break through convulsions were a cause for concern. Since understanding the mechanism of this interaction is vital to the establishment of concrete guidelines on the use of the two drugs in patients, the aim of this study was to investigate the possible pharmacokinetic interaction between acyclovir and valproic acid.

First, a high performance liquid chromatography (HPLC) method for analysis of acyclovir in plasma was developed. It involved simple protein precipitation of 200 µl of plasma with perchloric acid, followed by centrifugation after which 20 µl of the supernatant was injected in the HPLC. The sample was eluted with acetonitrile: octanesulfonic acid: ammonium acetate-citrate (vol./vol.; 5%:11.88%:83.12%) at 1.5 ml/min over a Luna C_{18} (4.60 x 150 mm) 5µ analytical column. Gancyclovir was used as the internal standard. Under these conditions, gancyclovir eluted at 3.4 min and acyclovir at 4.5 min. Over the calibration range of 10 - 100 µg/mL, linearity was demonstrated by a linear regression equation of $y = 0.03196 – 3.207x$ with a regression coefficient $r^2 = 0.995$, and accuracy by a percentage coefficient of variation (CV%) of less than 15%. The method was successfully used to analyze acyclovir in a rabbit treated with acyclovir single dose.

Thereafter, the possibility of a direct interaction between acyclovir and valproic acid *in vitro* was investigated by monitoring the concentrations of valproic acid

and acyclovir at different pH (pH 7.4 or pH 3 or pH 10) and temperatures (25^{o}C and 37^{o}C) when mixed in a 1:1 molar ratio or prepared separately in phosphate buffer. The samples were incubated at 25^{o}C for 2 hours and a further 1 hour at 37^{o}C, and aliquots were drawn at 10 min., 2 and 3 hours to measure the concentration of valproic acid and acyclovir (n=3). The average concentrations of valproic acid and acyclovir from the samples containing the single drug were not different ($P > 0.05$) from those in the mixture of both drugs at the different temperatures and pH. However, when the temperature and pH were evaluated separately, there was a trend whereby, at high temperature (37^{o}C), the concentrations of acyclovir (percentage detected) tended to be higher in the mixture (87%) than when it was alone (84%), while those of valproic acid tended to be lower in the mixture (89%) than when it was alone (92%). This same trend was observed at acid or alkaline pH. In conclusion, although temperature and pH did not induce significant effects on the concentrations of both acyclovir and valproic acid, increased concentrations of acyclovir were associated with reduced concentration of valproic acid when the two drugs were mixed under constrained conditions. These observations suggested a possible direct interaction between the two drugs.

This final part of the study was undertaken to investigate the effect of co-administration of valproic acid and acyclovir on the pharmacokinetic parameters of each other in a rabbit model. Fifteen white New Zealand rabbits were divided into 3 groups A, B and C whereby group A received acyclovir only, group B received valproic acid only, and group C received a combination of acyclovir and valproic acid. In a cross-over design, the intravenous route was studied first, followed by the oral route after a two-week wash out period. Blood samples were drawn over a 10 hr period and the pharmacokinetic parameters were derived from the concentrations. After intravenous administration, the area under the plasma concentration time curve (AUC) and plasma concentrations of acyclovir in group C were higher than in group A, while the volume of distribution (Vd) and

plasma clearance (CLp) of acyclovir in group C were only 12.8% and 10.36% of those of group A, respectively. A similar trend was observed after oral administration. However, the bioavailability (F) of acyclovir was 8.4% in group A versus 1.5% in group C. Of note, the concentrations and kinetic parameters of valproic acid between the two groups after oral and intravenous administration were not different. In conclusion, co-administration of single doses of acyclovir and valproic acid led to reduced oral bioavailability of acyclovir, but increased concentrations of acyclovir due to reduced volume of distribution and clearance and this was most probably due to inhibition of the membrane transport proteins for acyclovir by valproic acid.

Overall, a simple and accurate HPLC method for analysis of acyclovir in plasma was successfully developed, and a possibility of direct interaction between the two drugs was observed both *in vitro* and *in vivo*. These observations call for a cautious approach to the concomitant use of the two drugs until human studies are done.

DECLARATION OF INDEPENDENT WORK

I, Magdalena F.P.C van Jaarsveld hereby declare that the dissertation hereby submitted by me for the M.Med.Sc degree in Pharmacology at the University of the Free State is my own independent work and has not previously been submitted by me at another university or faculty for admission to a degree or diploma of any other qualification.

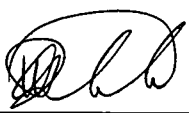
M.J. Jaarsveld
Signature

13 Maart 2008
Date

SUPERVISOR'S DECLARATION

I, Professor A. Walubo, the supervisor of this dissertation entitled: The pharmacokinetic interaction between valproic acid and acyclovir assessed *in vitro* and in a rabbit model, hereby certify that the work in this project was done by Magdalena van Jaarsveld at the department of Pharmacology, University of the Free State.

I hereby approve submission of this dissertation and also affirm that it has not been submitted previously to this or any other institution for admission to a degree or any other qualification.



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13th March '08

Date

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ABBREVIATIONS

ACV	acyclovir
ACEIs	angiotensin converting enzyme inhibitors
ADRs	adverse drug reactions
AUC	area under plasma concentrations versus time
CLp	clearance
Cmax	maximum concentration
CV	coefficient of variation
CYP450	cytochrome P450
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assays
FIA	fluorescence immunoassays
F	bioavailability
GABA	γ-aminobutryric acid
GABA-T	γ-aminobutryric acid transferase
GC	gas chromatographic
GHB	γ-hydroxybutyrate
GIT	gastro-intestinal tract
HDAC	histone deacetylase
HPLC	high performance liquid chromatography
$5HT_{1D}$	serotonin
IS	internal standard
Ke	elimination rate constant
MCT	monocarboxylate transporters
MECA	5'-*N*-methylcarboxyamidoadenosien
MRT	mean residence time
NMDA	*N*-methyl-d-aspartate
NMR	
OATP1B1	organic anion transporting polypeptide 1B1
hOAT1	organic anion transporter type 1

hOCT1	organic cationic transporter type 1
P-gp	P-glycoprotein
RIA	radioimmunoassay
$T_{½}$	half-life
Tmax	time to reach Cmax
UV	ultraviolet
VA	valproic acid
Vd	volume of distribution
WHO	World Health Organization

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CHAPTER 1

GENERAL INTRODUCTION

Technological advance over the past 60 years has led to improved health care that is partly due to invention of new drugs. Currently, there are thousands of drugs on the market compared to the few hundreds by the turn of the 19th century. Unfortunately, this has been associated with, among other things, increase in number of adverse drug reactions (ADRs). Of concern here are adverse drug reactions due to drug-drug interactions. Whereas drug-drug interactions are difficult to quantify, they form a significant portion of ADRs and they can be life-threatening. However, with constant development of new information on drugs, it is clear that most drug-interactions can be predicted and therefore be prevented.

Knowledge about drug interactions is often gained through reports from physicians observing abnormal effects after co-administration of two or more drugs to a patient (s). In many of these cases, it is after such a report that further investigation would lead to explanations not previously realized or known. In this case, a report by Parmeggiani and co-workers (1995) on a pharmacokinetic interaction between the acyclovir and valproic acid whereby the concentrations of valproic acid were reduced following the start of acyclovir therapy and was associated with break through convulsions, was a cause for concern. It was also supported by other workers (Moattari *et al.*, 2002) who, while investigating the potentiation of the antiherpetic effect of acyclovir by valproic acid, observed a possibility of a direct interaction between acyclovir and valproic acid. This is an observation for which the mechanism is still unknown and has not been investigated.

Valproic acid is a well known antiepileptic drug that is widely used as a first-line agent or adjunctive therapy for most types of seizure. The drug is also used as a prophylactic agent for migraine and for control of acute manic phase of bipolar disorders. It is has a therapeutic concentration range of 40 – 100 mg/L with dose related and well recognized central nervous system side effects when serum levels are above 100 mg/L (Cloyd *et al.*, 1986). Therefore, therapeutic drug monitoring plays an important role in the treatment of patients with valproic

acid, and any drug interaction that could influence the serum concentration of valproic acid is of clinical importance. Interactions that increase the serum concentration of valproic acid can lead to clinical signs of toxicity and interactions that decrease the serum concentration of valproic acid could lead to therapeutic failure, including increased frequency of convulsions in epileptic patients.

Acyclovir is an antiviral drug indicated for treatment of infections caused by herpes simplex, both type I and II, and varicella-zoster viruses. Acyclovir provides reduction in the acute pain experienced by patients with herpes zoster and it also provides protection from the cytomegalovirus in immunosuppressed patients. The drug is usually well tolerated with low toxicity when prescribed in therapeutic dosages, and serum concentration monitoring of this drug is not common practice. The oral preparation is well tolerated and more than 90% of the drug is eliminated unchanged by glomerular filtration and tubular secretion (O'Brien *et al.*, 1989).

Acyclovir is a drug with increasing potential for use given the high prevalence of viral conditions for which it is indicated, particularly in patients with immunosuppression, while, as cited earlier, valproic acid is one of the most widely used anti-epileptic drugs. This, together with the notion that valproic acid increases the antiviral activity of acyclovir, increases the potential for co-administration of the two drugs.

The reported interaction between acyclovir and valproic acid could have led to a change in the plasma concentration of either drugs. Unfortunately, there was no information on the concentrations of acyclovir during the clinical incident reported by Parmeggiani and co-workers (1995). This is important because the convulsions could have been due to reduced plasma levels of valproic acid or high concentrations of acyclovir (Hayden, 1996; O'Brien and Compoli-Richards, 1989; Richards *et al.*, 1983; Wagstaff *et al.*, 1994) or both. Of note, convulsions expose patients to risk of death or neurological complications owing to cerebral hypoxia and edema that may develop. Furthermore, it was noted that poorly controlled epilepsy was a major risk factor for sudden unexpected deaths in patients treated with more than one drug (Nilsson *et al.*, 1999). As such, the interaction between valproic acid and acyclovir with break through convulsions is a cause for concern. It was envisaged that understanding the mechanism of this interaction is vital to the establishment of concrete guidelines on the use of the two drugs in

patients. Therefore, this study was undertaken to investigate the effect of co-administration of the two drugs on the pharmacokinetic parameters of each other.

CHAPTER 2

LITERATURE REVIEW: PART I

AN OVERVIEW OF THE PHARMACOLOGY OF VALPROIC ACID AND ACYCLOVIR

2.1 INTRODUCTION

In order to understand the rationale for the possible interaction between valproic acid and acyclovir, one needs to understand the basic pharmacology of both drugs. Therefore, in this section, the pharmacology of valproic acid and acyclovir is outlined.

2.2 VALPROIC ACID

Valproic acid is an antiepileptic drug with a wide spectrum of action and is one of the four first-line drugs for long-term treatment of epilepsy. It is a branched-chain carboxylic acid that is not structurally related to any other anticonvulsant (Hayden, 1996).

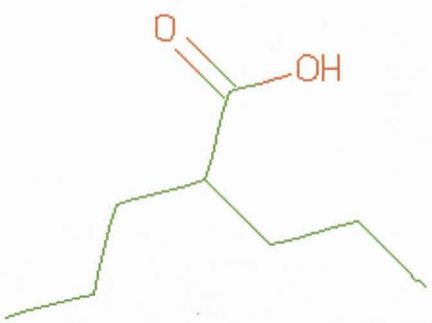


Figure 2.1. Structure of valproic acid.

The drug was first synthesized in 1881, but had been used as an organic solvent for other compounds that were being screened for anticonvulsive activity until the early 1960's when it was accidentally found to show efficacy against epilepsy (Hayden, 1996).

2.2.1 Mechanism of action

The mechanism of action of valproic acid is complex and not completely understood. The drug acts through more than one mechanism to provide its broad pharmacological activity.

a) γ-Aminobutryric acid mechanism: Reports showed that this agent increases brain concentrations of γ-aminobutryric acid (GABA), a neurotransmitter well known for its inhibitory effects in the central nervous system, through at least three different mechanisms (Hayden, 1996; Owens and Nemeroff, 2003). First, by blocking GABA degradation by inhibiting γ-aminobutryric acid transferase (GABA-T). Secondly by increasing GABA synthesis, probably through its action on glutamic acid decarbocylase and thirdly by decreasing GABA turnover (fig. 2.2).

b) Sodium channels: Similar to phenytoin, valproic acid also appears to inhibit voltage-activated sodium channels by prolonging the recovery phase of the activated channels, but reports are still inconsistent (Owens and Nemeroff, 2003).

c) γ-Hydroxybutyrate mechanism: A study done by Vayer and co-workers (1988) also showed that valproate inhibits the formation of the amino acid γ-hydroxybutyrate (GHB) by inhibiting nicotinamide adenine dinucleotide phosphate-dependent aldehyde reductase, a biochemical action that is possibly responsible for valproic acid's efficacy in treating absence seizures.

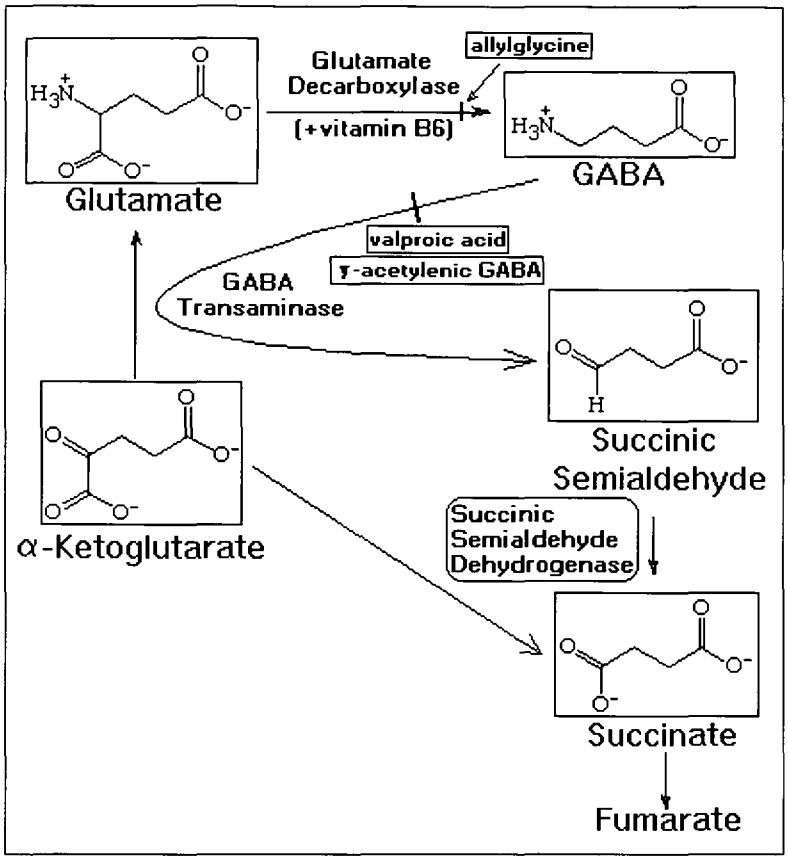


Figure 2.2: The GABA mechanism of action of Valproic acid (Owens and Nemeroff, 2003)

d) *N*-methyl-d-aspartate mechanism: Also, the inhibiting effect of valproic acid on the *N*-methyl-d-aspartate (NMDA) receptor has been studied (Kenta *et al.,* 2006) and may play an important role.

2.2.2 Formulation

Valproic acid is produced in two forms, namely, sodium valproate and valproic acid. Sodium valproate is the sodium salt of valproic acid and it is converted to valproic acid in the gastrointestinal tract such that 150 mg of valproic acid is therapeutically equivalent to 200 mg of sodium valproate (Gibbon, 2003). The drug is also available as a mixture of equal portions of valproic acid and sodium valproate (Divalproex sodium), which slowly dissociates and is absorbed in the intestine (McNamara, 1996). This controlled-release tablet was developed to avoid the gastrointestinal symptoms associated with the immediate release products (DeVane, 2003). Valproic acid is completely ionized at physiological pH in blood and therefore, the

valproate ion is regarded to be the active form, regardless of whether the salt or acid form were administered (Porter and Meldrum, 2004).

2.2.3 Absorption

The oral bioavailability of valproic acid ranges from 90 to 100%. In the gastrointestinal tract, valproic acid dissociates into valproate ions which are absorbed rapidly and completely mainly via the proton-coupled monocarboxylate transporters (MCT) (McNamara, 1996) and/ or the anion-exchange transporter MAE2 (Tsuji, 2002).

Peak concentration following oral administration is observed in 1 to 4 hours although this can be delayed by enteric-coated tablets and delayed release tablet formulations. Although food delays the absorption of valproic acid, the extent of absorption is usually not affected (Davis *et al.,* 1994). Diurnal variation in valproic acid absorption has been noted in healthy volunteers in a study done by Ohdo and co-workers (1990). This study compared the steady-state peak plasma concentration of 400 mg valproic acid administered in the morning and evening and they observed higher serum peak concentrations in the morning compared to the evening. Nevertheless, the significance of this observation has not been explained.

2.2.4 Distribution

Valproic acid is highly bound to proteins, primarily albumin, with a total protein binding of approximately 90%. The high protein binding together, with the fact that valproate is highly ionized, limit its distribution mainly to the extracellular water compartment leading to the volume of distribution of approximately 0.15 L/kg. Previous studies demonstrated the concentration of valproic acid in the brain cortex and cerebrospinal fluid to be approximately 10 % of the plasma concentration (Loscher *et al.,* 1988; Shen *et al.,* 1992). Interestingly, this is more or less similar to the concentration of the free fraction in the serum yet, besides passive diffusion, the drug is supposedly transported into the central nervous system mainly by a carrier-mediated process (Scism *et al.*, 2000).

Of note, the binding of valproic acid to plasma proteins is saturable within the therapeutic range. Therefore, when the plasma concentration of valproic acid is in the upper therapeutic range (> 80 mg/ L), the molar concentration of valproic acid may exceed the molar concentration of albumin leading to an increased free fraction of valproate. This may lead to increased

distribution of valproic acid to the central nervous system which may cause toxicity. Therefore, conditions associated with decreased plasma albumin such as extreme age, chronic liver disease and renal impairment may predispose patients to increased free fraction of valproic acid and associated toxicity.

2.2.5 Elimination

Valproic acid is primarily eliminated by hepatic metabolism whereby 30-50% undergoes glucuronidation, up to 40% mitochondrial beta and omega oxidation, and 15-20% microsomal oxidation to numerous metabolites. Less than 3-5% of the administered dose is excreted unchanged in the urine. The plasma clearance ranges from 0.4 to 0.6 L/h (Cloyd *et al.*, 1986). At least 11 metabolites have been identified, and the most common metabolites in the urine are valproate glucuronide and 3-oxo-valproate. Of note, the 3-oxo-valproate metabolite may possess anticonvulsant properties, but the plasma concentration is too low to be considered significant (Davis *et al.,* 1994).

The pharmacokinetics of valproic acid in children did not show any significant difference form adults, although in children 2 to 10 years of age, increased plasma clearance with shorter half-lives were observed, while infants showed a prolonged average elimination half-life (Buck, 1997).

The elimination of valproic acid is to some extent nonlinear due to saturable glucuronidation within the therapeutic plasma concentration range, leading to reduced clearance and a rise in steady-state plasma concentrations as well as the half life of the drug, hence, therapeutic drug monitoring. The therapeutic range of valproic acid is 40 to 100 mg/L. Although several non-linear pharmacokinetic factors, as described before, confound the interpretation of the relationship between plasma drug concentration and clinical efficacy, monitoring of serum concentration is particularly important to asses possible dose related adverse effects.

2.2.6 Adverse Effects

Valproic acid is associated with fewer neurological adverse effects than other antiepileptic drugs. The most commonly reported adverse effects of valproate acid include gastro-intestinal disturbances and bodyweight gain. Gastrointestinal adverse effects include dyspepsia, heartburn and nausea and were observed in 6 to 45% of patients (Davis *et al.,* 1994 and

Perucca, 2002). With the introduction of enteric-coated formulations, these adverse effects were reduced to 3 to 6% of patients (Davis *et al.,* 1994 and Perucca, 2002). Weight gain of 8 to 14 kg has been reported in 8 to 59 % of patients and was associated with an increased appetite, increased availability of long-chain fatty acids due to competitive displacement from their albumin binding sites and reduction in thermogenesis (Davis *et al.*, 1994).

Also, the use of valproic acid has been associated with a dose-related reduction in platelets, as well as inhibition of the second stage of platelet aggregation in 1% up to 30% of patients (Barr *et al.,* 1982; Allarakhia, 1996). The incidence is related to the serum concentration of valproic acid and is also increased in children. This effect may be significant in patients using other drugs that affect platelet function or during surgical procedures.

Neurological side effects include fine tremors of the hands which were observed in 1 to 5% of patients while drowsiness and ataxia are the other more common dose related adverse effects (Davis *et al.,* 1994).

Whereas up to 10% of patients on valproic acid may exhibit dose-related transient rise in liver enzymes, valproic acid is associated with a rare but severe hepatotoxicity, possibly due to accumulation of the 4-en-valproic acid metabolite. Children under the age of two years appear to be at greatest risk for this fatal adverse effect, particularly those receiving multiple anticonvulsants, those with congenital metabolic disorders and those with mental retardation or organic brain disease (Buck, 1997). Another severe but rare adverse effect of valproic acid is the hyperammonaemia which is associated with inborn errors of the urea metabolism (Coulter *et al,* 1980 and Feil *et al*, 2002).

2.2.7 Anti-cancer effect of valproic acid

Most recently, Phiel and co-workers (2001) as well as Göttlicher and co-workers (2001) described valproic acid as a potent inhibitor of histone deacetylase (HDAC), a negative regulator of gene expression, which made valproic acid a novel candidate for cancer therapy. Both concluded that valproic acid suppresses tumor growth and metastasis as well as induces tumor differentiation *in vitro* and *in vivo.* Subsequently, several reports have appeared on this mechanism which also, although not conclusive, has been linked to its pro-viral activity (Göttlicher *et al,* 2001, Phiel *et al,* 2001and Ginger, 2005).

2.2.8 Drug interactions

Valproic acid is involved in a number of well documented drug-drug interactions mainly due to its high protein binding, enzyme inhibition and possible effect on membrane transporters.

For instance, because both valproic acid and aspirin are highly bound to plasma proteins, concomitant use of the two drugs was associated with increased free fraction of valproic acid (Orr *et al.*, 1982). It is important to note is that aspirin also inhibits the beta-oxidation of valproic acid, leading to significant increase in the concentration of the free fraction of valproic acid with possible toxicity. On the other hand, valproic acid significantly increased the free fraction of phenytoin by displacing phenytoin from its binding site on the plasma protein as well as inhibiting the cytochrome P450 2C9 enzyme, the enzyme mainly responsible for the metabolism of phenytoin (Levy and Koch, 1978; Monks *et al,* 1978). Valproic acid differs from other older generation anti-epileptic drugs (carbamazepine, phenytoin, phenobarbital and primidone) by being a liver enzyme inhibitor rather than inducer. In addition to inhibition phenytoin metabolism, valproic acid can also inhibit the enzymes involved in the oxidation of phenobarbital, carbamazepine and the glucuronidation of lamotrogine (French and Gidal, 2000 and DeVane, 2003).

Another serious drug interaction concerning valproic acid is with carbapenem antibiotics. Co-administration of valproic acid with merepenem leads to a drastic decrease in the plasma valproic acid levels and recurrence of epileptic seizures (Nacarkucuk *et al.*, 2004). Although several mechanisms underlying this pharmacokinetic interaction has been proposed, the exact mechanism is still not completely understood. Yokogawa and co-workers (2001) observed increase in the total clearance of valproic acid glucoronidated metabolites while Torii *et al.* (2001) and Ogawa *et al.* (2006) suggested that carbapenem antibiotics may affect absorption and tissue distribution of valproic acid via membrane transporters.

2.3 ACYCLOVIR

The discovery of acyclovir nearly 30 years ago was regarded as a breakthrough in the management of herpes virus infections because, compared to existent antiviral drugs, acyclovir was more effective and safer. Acyclovir has since become the standard therapy for herpes simplex and varicella zoster virus infections.

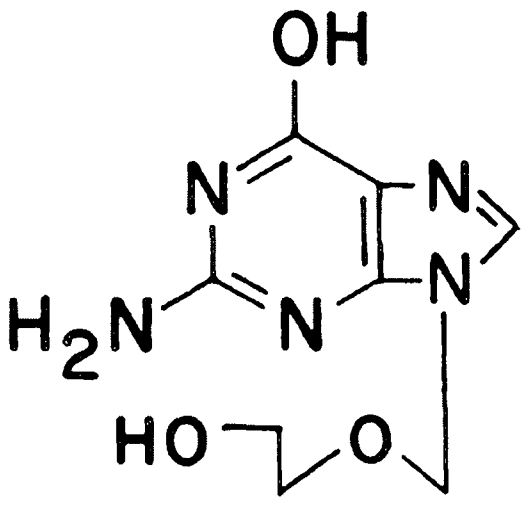


Figure 2.3: Structure of acyclovir

Acyclovir is a synthetic purine nucleoside analogue (9-[(2-hydroxy-ethoxy)methyl]-9H-guanine) with a highly selective antiviral activity.

2.3.1 Mechanism of action

Acyclovir is activated by herpes virus-encoded thymidine kinases to acyclovir monophosphate. The monophosphate is further converted into diphosphate by cellular guanylate kinase and then into triphosphate by other cellular enzymes. Acyclovir triphosphate stops replication of herpes viral DNA in three ways: firstly by competitive inhibition for viral DNA polymerase; secondly by incorporation into and termination of the growing viral DNA chain; and lastly, by inactivation of the viral DNA polymerase (Elion, 1982; Naesens and De Clercq, 2001). Of note, the reaction of thymidine kinase is specific for the viral enzyme because the host cell thymidine kinase is less effective at catalyzing this reaction. Thereafter, the host enzymes are used for the diphosphorylation and triphosphorylation reactions. This results in acyclovir triphosphate

concentrations 40 to 100 times higher in herpes simplex virus-infected than in uninfected cells (Elion, 1982).

In vitro, acyclovir also showed some activity against Epstein-Barr virus but activity against cytomegalovirus is limited mainly because the cytomegalovirus lacks a gene for thymidine kinases and the viral DNA polymerase is poorly inhibited by acyclovir triphosphate (Crooks *et al.*, 1991; Harding *et al.,* 1991).

Also, by yet unknown mechanisms, acyclovir has also been shown to increase healing of skin rashes, reduce severity of acute and chronic pain as well as the intraocular complications associated with herpes zoster infections (Crooks *et al.,* 1991; Harding *et al.,* 1991).

2.3.2 Formulation

Acyclovir is available as capsules, ointment, syrup and as powder which is reconstituted for intravenous use.

2.3.3 Absorption

Generally, the absorption of acyclovir is incomplete with an oral bioavailability of 10 to 30% and peak plasma concentration is reached within 1.5 to 2.5 hours. Also, it was shown that the bioavailability of acyclovir reduces with increasing dose (O'Brien and Compoli-Richards, 1989), most probably due to saturable absorption mechanisms.

Absorption occurs in the small intestine mainly by passive diffusion (Lewis *et al.,* 1986), though membrane protein transporters have been implicated. For instance, the organic cation transporter family as well as the organic anion transporter family show some affinity for acyclovir (Takede *et al.,* 2002; Ho and Kim, 2005). Since transporters exhibit saturable characteristics, this could partly explain the reduced bioavailability with increasing dose. The role of transporter mechanism is enhanced by the absorption characteristics of valacyclovir. Valacyclovir is the L-valyl ester of acyclovir that was developed to improve the bioavailability of acyclovir. It was observed that after uptake by the oligopeptide transporter, valacyclovir is rapidly converted to acyclovir inside the cell, leading to a 5-fold increase in the bioavailability of acyclovir (Weller *et al.,* 1993).

2.3.4 Distribution

Acyclovir is widely distributed to body fluids and tissues, mucosa and herpetic vesicular fluid. The cerebro-spinal fluid concentrations are about 50% of the serum levels (Safrin, 2004). Plasma protein binding is between 9 to 30 % irrespective of the plasma concentration (De Mirande *et al.,* 1982). Although limited information is available on the actual volume of distribution of acyclovir, most probably due to variable diffusion characteristics of acyclovir, a volume of distribution of 0.69 ± 0.19 L/kg has been reported (Hayden, 1996).

2.3.5 Elimination

The kidneys are the main route of elimination of acyclovir mainly by glomerular filtration and tubular secretion. The clearance of acyclovir was reported to be approximately 3.37 times creatinine clearance + 0.41 ml/min./kg. About 80% of the dose is recovered in urine as unchanged drug after intravenous administration. Tubular secretion is thought to be via the organic anion transporters but this requires further investigations. Most of the remaining dose (less than 15%) is excreted as inactive metabolites namely, 9-carboxymethoxymethylguanine or other minor metabolites (Hayden, 1996; O'Brien and Compoli-Richards, 1989; Richards *et al.,* 1983; Wagstaff *et al.,* 1994; etc.). Unfortunately, it was not indicated how these metabolites are formed. The half-life is approximately 3 hours in patients with normal renal function and 20 hours in patients with anuria.

2.3.6 Adverse effects

Because acyclovir is not activated by uninfected cells, the safety profile of this drug is favorable hence the drug is in general well tolerated. However, acyclovir has been associated with nausea, diarrhea, rash, headache, renal insufficiency and neurotoxicity (Hayden, 1996). A reversible renal dysfunction occurs in approximately 5% of patients and this is probably related to the high urinary levels leading to crystalline nephropathy. Rapid intravenous administration, existing dehydration and inadequate urine flow increase this risk, which usually resolve with drug cessation and volume expansion (Hayden, 1996; O'Brien and Compoli-Richards, 1989; Richards *et al.,* 1983; Wagstaff *et al.*, 1994; etc.). Neurotoxicity is seen in 1 to 4 % of patients and may manifest as alteration in sensation, tremors, myoclonus, delirium, seizures, and possibly, extrapyramidal symptoms. These symptoms are more common in patients receiving high dosages of acyclovir and in the presence of severe renal failure (Hayden, 1996; O'Brien and Compoli-Richards, 1989; Richards *et al.,* 1983; Wagstaff *et al.*, 1994; etc.).

2.3.7 Drug interactions

Drug interactions with acyclovir have been mainly observed at tubular level. Probenecid decreased the renal clearance of acyclovir, suggesting that the renal organic anion transporters are responsible for the tubular secretion of this drug (Laskin *et al.*, 1982; Izzedine *et al.,* 2005). Acyclovir by itself, may decrease the renal clearance of other drugs such as methotrexate, by competitive inhibition of active renal secretion of the drug (Laskin *et al.*, 1982). Secondly, concomitant use of cyclosporine and other nephrotoxic agents enhance the risk of acyclovir induced nephrotoxicity (Bradley *et al.*, 1997).

CHAPTER 3

LITERATURE REVIEW PART II

AN OVERVIEW ON MECHANISMS OF DRUG-DRUG INTERACTIONS

3.1 INTRODUCTION

Drug interactions are part of adverse drug reactions responsible for considerable morbidity and mortality in patients on multiple drug therapy. Whereas an adverse drug reaction is defined by the World Health Organization (WHO) as 'any response to a drug which is noxious and unintended and occurs at normal doses' a drug-drug interaction is 'the modification of the effect of one drug by prior or concomitant administration of another drug' (Edwards and Aronson, 2000). Unfortunately, despite the clear distinction by definition, in practice, there is a general failure to separate the two and therefore accurate determination of the incidence of drug-drug interactions. This is mainly due to, among other things,: the different study methods used by different investigators, e.g. retrospective versus epidemiological studies (Jankel *et al.,* 1993); sample size where studies with large samples were associated with a lower incidence of drug-drug interactions than those with smaller samples (Becker *et al.,* 2007); and age where the elderly population were associated with higher incidence of drug interactions than the young (Becker *et al.,* 2007).

Nevertheless, it is known that adverse drug reactions affect millions of patients annually and are responsible for up to 6% of hospital admissions at any stage (Lazarou *et al.*, 1998; Einarson, 1993; Stockley, 2002; and Pirmohamed *et al., 2004*). In the United States alone, adverse drug reactions were responsible for more than 100 000 deaths per year (Lazarou *et al.*, 1998; Pirmohamed *et al., 2004*). On the other hand, not withstanding the afore-mentioned problems, reports on hospital admissions due to drug-drug interactions indicate an incidence of up to 2.8% (Jankel and Gitterman, 1993; Hamilton *et al.*, 1998).

3.2 IMPORTANCE OF DRUG-DRUG INTERACTIONS

Therapeutic value: Although concern for drug-drug interactions is often directed at undesirable effects, some drug-drug interactions have been utilized in therapeutics. For instance, in ritonavir – boosted anti-retroviral regimens, where ritonavir, a potent cytochrome P450 enzyme inhibitor, is combined with lopinavir (ritonavir 100mg/lopinavir 200mg; Kelatra®). This leads to increase in the concentrations of lopinavir, thereby enhancing lopinavir's therapeutic effectiveness and reducing risk of adverse effects of the lopinavir due to a lower dose requirement (Product Information: Kaletra, 2005).

Adverse effects: However, more often, drug-drug interactions lead to diminution of the therapeutic effectiveness and/or toxicity. For example, carbamazepine induces the metabolism of warfarin and therefore can lead to reduced effectiveness of warfarin (Massey, 1983), while drugs such as terfenadine, astemizole and cisapride were withdrawn from the American market due to serious drug-drug interactions associated with QT prolongation when combined with enzyme inhibitors such as erythromycin (Product Information: Propulsid®, 2000, Product Information: Seldane®, 1998). Even then, clinically significant drug-drug interactions are commonly associated with drugs that have a narrow therapeutic index, are highly bound to plasma proteins and exhibit non-linear kinetics (saturable metabolism), for example phenytoin. Also, drug interactions are common in patients on chronic medication, particularly in the severely ill or the elderly patients, and this is compounded by a high incidence of multiple drug therapy and impaired organ (liver or kidney) function in these patients.

3.3 MECHANISM OF DRUG-DRUG INTERACTIONS

Drug-drug interactions may be due to pharmaceutical, pharmacokinetic and pharmacodynamic interactions. A pharmaceutical drug interaction is due to chemical incompatibility of two or more drugs when mixed together before administration. For instance penicillin and aminoglycoside, such as gentamycin, solutions will form a precipitate if mixed together (Farchione, 1981). Pharmacokinetic drug-drug interactions occur where one drug alters the rate or extent of any of the pharmacokinetic processes, namely drug absorption, distribution, metabolism or excretion of another drug. Pharmacodynamic interactions are where one drug affects the pharmacological activity of another drug without affecting its pharmacokinetics.

3.3.1 Pharmacokinetic Drug-drug interactions

3.3.1.1 Absorption

There are several mechanisms by which one drug may alter the absorption of another drug. This could be due to physiochemical interaction where one drug changes the gastrointestinal pH, leading to a change in ionization of the other drug, thereby affecting its ability to cross membranes. For example drugs such as ranitidine and omeprazole, that increase gastric pH, can reduce the absorption of ketoconazole (Blum *et al.,* 1991; Lelawongs *et al.*, 1988; Product Information: Prilosec(R), omeprazole, 1997). Also, intercalation with di- and trivalent cations in antacids or preparations containing calcium, magnesium, zinc and iron, leads to chelation of tetracyclines and quinolone antibiotics and consequently decrease their absorption (D'Arcy and McElnay, 1987).

Secondly, changes in gastrointestinal motility could affect both the rate and/or the extent of drug absorption. Drugs that increase gastric emptying may result in earlier and higher peak concentrations for drugs that are rapidly absorbed from the small intestine whereas drugs that decrease gastric emptying may result in decreased or delayed drug absorption. In general, opioids and drugs with anticholinergic effects decrease absorption of paracetamol due to reduced gastric emptying and intestinal motility, while metoclopramide increases the absorption of paracetamol because it increases gastric emptying (Anderson *et al.,* 1999).

Recently, drug-drug interactions involving membrane transport proteins in the gastro-intestinal tract (GIT) have been reported. For instance verapamil increases the bioavailability of digoxin due to inhibition of P-glycoprotein (P-gp) in the GIT (Verschraagen *et al.*, 1999). This is owing to the fact that P-gp is normally responsible for excretion of digoxin in the lumen.

3.3.1.2 Interactions during distribution

Drug-drug interactions during distribution are mainly due to competitive displacement from plasma proteins, mainly albumin and alpha(α)$_1$-acid glycoproteins. This commonly involves drugs that are highly bound to albumin and most likely to the same binding site. Although the determinant of binding affinity are not known, some drugs such as warfarin are easily displaced by other drugs, for example aspirin. However, this type of interaction is seldom of clinical importance because, usually, the increase in free fraction of the displaced drug will also lead to increase in drug distribution, metabolism and/or excretion (Hansten, 1994).

Another mode of distribution based drug-drug interaction is competitive drug displacement from tissue binding sites. Here, one drug displaces another drug from tissue binding sites, thereby increasing the free fraction and/or leading to reduced therapeutic effect of the other drug. An example of the former is quinidine which displaces digoxin from non-specific tissue binding sites leading to increase in the free fraction of digoxin (Hansten, 1994), while for the latter, colchicine displaces paclitaxel from specific active tissue binding sites (presumably microtubules) leading to reduced tissue concentrations of paclitaxel (Sadeque *et al.*, 2000). The significance of this interaction is not yet clear but is believed to be associated with reduced therapeutic effect of paclitaxel.

As observed earlier under absorption, membrane transporters also affect the tissue distribution of some drugs, thereby leading to limitation and probably selective distribution of drugs to some tissues. For example, pravastatin, a 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor, is mainly distributed to the hepatocytes owing to uptake by the organic anion transporting polypeptide 1B1 (OATP1B1; Cordon-Cardo *et al.,* 1990; Thiebaut *et al.*, 1987). Cyclosporine and gemfibrozil inhibit this transporter, therefore, increase the plasma concentrations of pravastatin, placing the patient at an increased risk of myotoxicity, when co-administered (Pertti *et al.,* 2006). However, the impact on therapeutic effect of pravastatin has not been reported.

Another important membrane transporter in drug distribution is P-glycoprotein (P-gp), an efflux pump responsible for extrusion of drugs from the cells. P-gp is expressed on the luminal surface of intestinal epithelia, the renal proximal tubule, the bile canalicular membrane of hepatocytes, the placenta and on the apical side of the blood-brain barrier. Although not proven clinically for most drugs, P-gp has been used to describe clinical situations relating to penetration of drugs into the central nervous system. For example, in 8 healthy volunteers, quinidine, an inhibitor of P-gp, led to increased CNS effects of loperamide, a known substrate of P-gp, (Sadeque *et al.,* 2000). Also, increased CNS toxicity was observed in some clinical studies where P-gp inhibitors were given concomitantly with anticancer agents (Fisher *et al.*, 1996; Miller *et al.*, 1998; Fracasso *et al.*, 2000).

Enterohepatic circulation is important for distribution of some drugs such as oral contraceptives and rifampicin. Such drugs are usually excreted in the bile as unmetabolized drugs which are then reabsorbed (e.g. rifampicin), or as conjugated metabolites which are hydrolyzed by

intestinal bacteria thereby releasing the parent drug for re-absorption (e.g. oral contraceptives; Weaver and Glasier, 1999). In the case of oral contraceptives, co-administration with broad spectrum antibiotics can lead to contraceptive failure because the antibiotics kill the bacteria, leading to reduced enterohepatic recirculation and therefore, plasma concentrations (Bolt *et al.*, 1975).

3.3.1.3 Interactions during metabolism

Drug-drug interactions during drug metabolism are commonly associated with drugs that are metabolized by cytochrome P450 (CYP450) enzymes, a super family of enzymes responsible for most of the drug metabolism in the body (Correia, 2004). Of note, CYP450 enzymes exhibit selectivity towards different drug substrates. Drug interactions at CYP450 enzyme level occur mainly due to induction or inhibition of CYP450 enzyme activity by some drugs. Enzyme induction leads to increased activity of CYP450 enzyme, while enzyme inhibition leads to reduced activity of the enzymes. Therefore, induction of the CYP450 drug metabolizing enzymes would lead to increased metabolism and decrease in the substrate drug's plasma concentration with probable therapeutic failure (Correia, 2004). On the other hand, enzyme inhibition would lead to increase in the plasma concentration of the substrate drug with exaggerated and prolonged pharmacologic or toxic effects. Table 3.1 is an illustration of some of the important drug substrates with inhibitors and inducers of their major CYP450 metabolizing enzymes (Correia, 2004).

3.3.1.4 Interactions during excretion

The kidney is the primary organ of drug excretion, therefore, drug-drug interactions at this level are more prominent than in other excretory organs such as the lungs and biliary system. Renal based drug-drug interactions can occur during glomerular filtration, active tubular secretion and re-absorption. Drugs such as indomethacin, a non-steroidal anti-inflammatory agent, that change the renal blood flow may potentially affect the glomerular filtration rate with consequent reduction in excretion of drugs such as amikacin that are eliminated mainly by glomerular filtration (Zarfin *et. al.*, 1985). Also, factors such as change in urinary pH, volume of urine as well as urinary flow rate can increase or decrease re-absorption of a drug depending on the physico-chemical properties of the drug (De Vrueh *et al.,* 1998). Lastly, competition for tubular secretory transporters can lead to increased or reduced drug excretion depending on the individual drug's affinity for the transporter. For example, probenecid inhibits excretion of

penicillin by competitive inhibition for the organic anion transporter at the proximal renal tubular epithelial cells (Munnich *et al.,* 1974, De Vrueh *et al.,* 1998).

3.3.2 Pharmacodynamic drug-drug interactions

This type of drug interaction relates to alteration in the pharmacological activity of the interacting drugs without change in the pharmacokinetics of the drugs. It often manifests as either increased or decreased clinical effect or side effect of one or both drugs.

Competitive or non-competitive antagonism: This drug interaction may be due to competitive or non-competitive antagonism at the site of action. For example, the interaction between propranolol, a non-selective beta blocker, and salbutamol, a $beta_2$-receptors stimulant, where propranolol inhibits the stimulation of $beta_2$-receptors by salbutamol leading to therapeutic failure in asthma.

Physiological antagonism: Another mode of interaction is by direct or indirect physiological antagonism. Here, two agonists interact with two receptor systems in two different effector systems, producing effects that oppose each other. For example, the physiological antagonism between adrenaline and pilocarpine on the iris. Whereas pilocarpine stimulates the muscarinic receptors of the pupillary constrictor muscle leading to miosis, adrenaline stimulates the $alpha_1$ adrenergic receptors of the radial muscle leading to mydriasis (Katzung, 2004). Therefore, if for any reason, e.g., cardiac arrest, a patient on treatment with pilocarpine for acute angle glaucoma receives adrenaline, it may lead to therapeutic failure due to the opposing effect of adrenaline to pilocarpine.

Synergism: Pharmacodynamic drug interactions may also occur as a result of synergism leading to exaggerated therapeutic or side effects. For example, in case of the former, severe hypotension due to inadvertent use of high doses of hypotensive agents such as atenolol, a beta blocker, and furosemide, a diuretic, in combination. With regard to side effects, increased sedation may be observed when patients on diazepam, a benzodiazepine, for anxiety, use codeine, an opioid, for pain.

Table 3.1: Selected Substrates, Inhibitors and Inducers of Specific CYP450 (Correia, 2004)

CYP Isoform	Substrate	Inhibitor	Inducer
1A2	Clozapine Imipramine	Cimetidine Fluoroquinolones Fluvoxamine	Tobacco
2C19	Diazepam Phenytoin Amitriptyline Clomipramine Cyclophosphamide	Fluvoxamine Ketoconazole Lansoprazole Omeprazole	
2C9	Tolbutamide Glyburide Irbesartan Phenytoin Tamoxifen Warfarin	Amiodarone Fluconazole Isoniazid Tolbutamide	Rifampicin
2D6	Propafenone Timolol Amitriptylline Clomipramine Imipramine Haloperidol Risperidone Codeine Dextromethorphan Flecainide Mexiletine Ondansetron Tamoxifen Tramadol Venlafaxine	Amiodarone Chlorpheniramine Cimetidine Clomipramine Fluoxetine Haloperidol Methadone Paroxetine Quinidine Ritonavir	
2E1	Paracetamol Chlorzoxazone Ethanol	Disulfiram	Ethanol
3A4	Clarithromycin Erythromycin Quinidine Alprozolam Diazepam Midazolam Cylosporin Tacrolimus Indinavir Ritonavir Saquinavir Amlodipine Diltiazem Felodipine Nifedipine Verapamil Atorvastatin Simvastatin Methadone Tamoxifen Trazodone Vincristine	Indinavir Nelfinavir Ritonavir Saquinavir Amiodarone Cimetidine Clarithromycin Diltiazem Erytromycin Fluvoxamine Grapefruit juice Itraconazole Ketoconazole Verapamil	Carbamazepine Phenobarbital Troglitazone Rifabutin Rifampin St.John,s wort Pheytoin

3.4 A POSSIBLE INTERACTION BETWEEN VALPROIC ACID AND ACYCLOVIR

Despite the wealth of knowledge on drug-drug interactions, as illustrated in the previous reviews, there is an information gap on the mechanism of interaction between valproic acid and acyclovir. Specifically, although the interaction was suggested to be pharmacokinetic, the elimination characteristics of the two drugs are not related; valproic acid is extensively metabolized in the liver while acyclovir is mainly eliminated by renal excretion. Also, it is unlikely to be due to displacement from the protein binding sites, because, although valproic acid is highly protein bound (90%), acyclovir is not (15-30%). This leaves the processes of absorption and/or tissue distribution as the centre of focus. In fact, the mechanism of absorption for the two drugs is still not well known; both drugs are absorbed by passive diffusion of variable magnitude and by membrane transporters that have not been characterized fully (Lewis *et al.*, 1986; Takede *et al.,* 2002; Ho and Kim, 2005; McNamara, 1996 and Tsuji, 2002).

However, this is complicated by the possibility of a direct interaction between the two drugs (Moattari *et al.,* 2002). Of concern in that report were the disproportionate concentrations of the two drugs such that they do not mimic a clinical situation.

There are two instances where direct interaction may occur; in the GIT before absorption or in the body after absorption. In case of the latter, high concentrations of valproic acid were used, versus the trace concentrations for acyclovir. Specifically, the concentrations of valproic acid of 4 mM (664.8 µg/ml) are far higher than the therapeutic range of 40 – 100 µg/ml, while that of acyclovir was 5 µM (0.005 µg/ml) which would be too low in patients where trough concentrations range from 0.5 – 1.5 µg/ml (Product Information: ZOVIRAX ®, 2005).

In case of the GIT, the concentration ratio should be approximate to the dosage used in the clinic. Usually 200 mg (1.2 mmol) of valproic acid and 200 mg (1 mmol) of acyclovir are recommended per dosing interval. This implies that the ratio of valproic acid to acyclovir in the reaction mixture should be at least 1.2:1. Therefore, there is a need to re-investigate the possibility of a direct interaction between valproic acid and acyclovir under more realistic conditions and this requires comprehensive *in vivo* and *in vitro* pharmacokinetic studies. However, because the pharmacokinetic studies will require analyzing the valproic acid and acyclovir, a review of the analytical methods shall be undertaken in the next section.

CHAPTER 4

LITERATURE REVIEW: PART III

REVIEW OF ANALYTICAL METHODS FOR ACYCLOVIR AND VALPROIC ACID

4.1 ACYCLOVIR

A variety of analytical methods have been used to measure the concentrations of acyclovir in plasma, serum and urine. They are mainly immunoassays (Lycke *et al.*, 1989; Wood *et al.*, 1994; Sarva *et al.*, 1996 and Tadepalli *et al.*, 1986), spectrophotometry (Ahmed *et al.*, 2004), and high performance liquid chromatography (HPLC) (Boulieu *et al.*, 1997; Ramakirshna *et al.*, 2000; Kok-Khiang *et al.*, 1996; Swart *et al.*, 1994 and Brown *et al.*, 2002), techniques.

The immunoassay methods include both radioimmunoassay (RIA) and enzyme-linked immunosorbent assays (ELISA). Although radioimmunoassay methods are sensitive and proved to be technically simple, these methods are overall costly, as they still entail time consuming experimental procedures. For instance, they need antibodies and make use of radioactive material with short shelf-life and inconvenient disposal properties (Lycke *et al.*, 1989 and Wood *et al.*, 1994). The enzyme-linked immunosorbent assay method described by Tadepalli and co-workers (1986) is specific and sensitive, but it requires expensive antibodies as well as a lengthy separation procedures.

Regarding chromatographic techniques, the method described by Bahrami and co-workers (2005), although very sensitive, where complicated by a liquid-liquid extraction pre-treatment procedure and the use of vanillin as internal standard. Furthermore, their method required a large serum sample volumes of 1 ml, which is difficult to obtain from small animals. The method described by Fernandez and co-workers (2003), although sensitive, was complex. They used 5'-*N*-methylcarboxyamidoadenosien (MECA) as the internal standard, a solid-phase extraction and they also needed a large sample volume (1 ml) as well as the analyte volume (100 µl) injected in the chromatograph. The other methods suffer from long retention times of over 11 minutes (Boulieu *et al.*, 1997 and Ramakirshna *et al.*, 2000), extremely large sample volumes of

5 ml (Boulieu *et al.*, 1997) and some required more specialized equipment like fluorescence detection (Kok-Khiang *et al.,* 1996) and an automated sample preparation systems (Swart *et al.*, 1994).

However, the method described by Brown and co-workers (2002), appeared to be more favorable as it involved a small sample volume (0.2 ml) that was extracted by precipitation and filtration and detection was by UV. Also, a small injection volume of 20 µl was used. Unfortunately, some of the conditions and materials used in this method are not available in our setting. Therefore, a method for analysis of acyclovir was developed by modifying this procedure.

4.2 VALPROIC ACID

Because clinical use of valproic acid requires drug concentration monitoring, there has been a concerted effort to develop analytical procedures that can be easily used in the clinic. Unfortunately, this has been hampered by the unfavorable physical-chemical characteristics of the drug. For gas chromatographic (GC) methods, valproic acid is a small and highly volatile molecule that is difficult to process, (i.e., extraction, evaporation and derivatization), with consequent loss in sensitivity (Hershey *et al.*, 1979; Bialer *et al.,* 1984; Nursen *et al.*, 1988; Darius and Meyer, 1994 and Anari *et al.*, 2000). On the other hand, the high performance liquid chromatography (HPLC) methods have been frustrated by the absence of a suitable chromophore in valproic acid's chemical structure in that it cannot be detected by conventional spectrophotometry such as ultra violet (UV). Therefore, most HPLC methods include prior derivatization of valproic acid to enable detection by UV-absorption or laser induced fluorescence methods (Ming-Chun *et al.,* 2004; Amini *et al.*, 2006 and Hao Cheng *et al.*, 2007). Unfortunately, although the HPLC methods are robust, these procedures are too complicated and expensive.

Currently, the fluorescence immunoassays (FIA) is the most widely used method for routine monitoring of valproic acid in clinics. This is because of rapid turnaround times, good sensitivity and ease, as well as availability of drug assay kits for most therapeutically monitored drugs including valproic acid. Also, excellent correlation was demonstrated between fluorescence polarization immunoassay and some of the HPLC methods for valproic acid, implying that they

are reliable (Steijns *et.al.*, 2002). The fluorescence immunoassays method has been used in our laboratory for patient therapeutic drug monitoring of valproic acid for over fifteen years and it has been proven as accurate and reliable. Therefore, for the purposes of this study, it was felt appropriate to analyse valproic acid by this method.

CHAPTER 5

OBSERVATIONS FROM THE REVIEW, AIM, SPECIFIC OBJECTIVES AND EXPECTED OUTCOME

5.1 OBSERVATIONS FROM THE REVIEW

In summary, it was observed that:

5.1.1 There is evidence of a drug interaction between acyclovir and Valproic acid.

5.1.2 The mechanism of this interaction is not known.

5.1.3 The drug interaction is clinically significant as it was possibly the cause of convulsion in the patient.

5.2 AIM

To investigate the possible pharmacokinetic interaction between acyclovir and valproic acid.

5.3 SPECIFIC OBJECTIVES

5.3.1 To adopt a high performance liquid chromatography method for analysis acyclovir in plasma.

5.3.2 To investigate the possibility of a direct interaction between acyclovir and valproic acid *in vitro*.

5.3.3 To investigate the effect of co-administration of acyclovir and valproic acid on each other's pharmacokinetics after oral and intravenous administration in a rabbit model.

5.4 EXPECTED OUTCOME

5.4.1 A high performance liquid chromatography method for measuring the concentration of acyclovir in plasma.

5.4.2 Knowledge of whether there is a direct chemical interaction between acyclovir and valproic acid.

5.4.3 Knowledge on whether co-administration of acyclovir and valproic acid affects the pharmacokinetics of each other.

5.4.4 Recommendations to enable proper use of these drugs concomitantly in the clinic.

CHAPTER 6

Determination of Acyclovir Concentration in Plasma Samples by High performance Liquid Chromatography

6.0 SUMMARY

A high performance liquid chromatography (HPLC) method for analysis of acyclovir in plasma was developed. It involved simple protein precipitation of 200 µl of plasma with perchloric acid, followed by centrifugation after which 20 µl of the supernatant was injected in the HPLC. The sample was eluted with acetonitrile: octanesulfonic acid: ammonium acetate-citrate (vol./vol.; 5%:11.88%:83.12%) at 1.5 ml/min over a Luna C_{18} (4.60 x 150 mm) 5µ analytical column. Gancyclovir was used as the internal standard. Under these conditions, gancyclovir eluted at 3.4 min and acyclovir at 4.5 min. Over the calibration range of 10 - 100 µg/mL, linearity was demonstrated by a linear regression equation of $y = 0.03196 - 3.207x$ with a regression coefficient $r^2 = 0.995$, and accuracy by a percentage coefficient of variation (CV%) of less than 15% . The method was successfully used to analyze acyclovir in a rabbit treated with acyclovir single dose.

6.1 INTRODUCTION

In this chapter, a high performance liquid chromatography method for determination of the concentration of acyclovir in plasma is described. As indicated earlier, it is based on the method reported by Brown and co-workers (2002).

6.2 METHODS

6.2.1 Apparatus

A precision balance from Scaltec Instruments (Hamburg, Germany) was used for weighing the milligram amounts of drug standards and other reagents, while Finnpipettes® from Thermo Labsystems (Midland, Canada) and Hamilton® syringes (Bonaduz, Switzerland) were used for pipetting and spiking small plasma sample volumes, respectively. A Thermolyne Maxi-Mix Vortex Mixer (Daigger & Co. Inc. United States of America) and a bench-top Minispin centrifuge (Hamburg, Germany) were used for mixing and quick spinning of the samples, respectively.

6.2.2 Reagents and chemicals

The analytical standards of acyclovir and gancyclovir (internal standard) were obtained from Sigma Chemical Co. (Steinheim, Germany), while general grade citric acid [$C(OH)(COOH)(CH_2.COOH)_2.H_2O$], ammonium acetate [$CH_3.COONH_4$], ortho-phosphoric acid (H_3PO_4) and perchloric acid ($HClO_4$) were from Merck Laboratories (Darmstadt, Germany). Sodium 1-octanesulfonate [$CH_3(CH_2)_7SO_3Na$] was from Tokyo Kasei (Tokyo, Japan) and HPLC-grade acetonitrile was from Burdick & Jackson (Muskegon, USA).

6.2.3 Preparation of mobile phase

The mobile phase was prepared from three solvents A, B and C. Solvent A consisted of a 10 mM ammonium acetate-citrate buffer that was prepared by dissolving 385 mg ammonium acetate and 1050 mg citric acid in 500 ml deionised water. Solvent B consisted of 3.7 mM octanesulfonic acid that was prepared by dissolving 400 mg of octanesulfonic acid in 500 ml deionised water, while solvent C was HPLC-grade acetonitrile.

The working mobile phase was prepared by, first, mixing 875 ml of solvent A with 125 ml of solvent B and adjusting pH to 3.08 with phosphoric acid. Thereafter, 50 ml of solvent C was added to 950 ml of the mixture of solvents A and B leading to final ratios of acetonitrile: octanesulfonic acid: ammonium acetate-citrate (vol./vol.; 5%:11.88%:83.12%).

6.2.4 Sample preparation

First, standard solutions containing 1.0 mg/ml acyclovir and internal standard were made separately in deionized water. Since these solutions were reported to be stable for only 2 weeks (Brown et al 2002) at 2-8 °C, they were replaced every two weeks. Thereafter, standard plasma samples used for calibration were prepared by spiking 1 ml of plasma with appropriate volumes of acyclovir standard solution to obtain final concentrations of 100, 80, 60 40, 20 and 10 μg/ml.

6.2.5 Sample extraction

To 200 μl of the standard plasma sample was added 20 μl of the internal standard solution and vortexed for 15 seconds. Thereafter, 60 μl of 2 M perchloric acid solution was added to precipitate the proteins, then vortexed for 15 seconds, after which it was centrifuged at 1000 *g* for 10 minutes and 20 μl of the supernatant was injected into the HPLC system.

6.2.6 Chromatographic system and conditions

Chromatographic system: The HPLC system consisted of Hewlett-Packard 1100 Series system that comprised of a quaternary pump, degasser, auto-sampler and variable-wavelength UV detector ((Palo Alto, USA), and data was collected using Chem Stations Software.

Chromatographic conditions: Chromatographic separation of the drugs was achieved by running the mobile phase at a flow rate of 1.5 ml/ min over a Luna C_{18} (4.60 x 150 mm) 5µ analytical column coupled to Security Guard Cartridge C_{18} (4 x 3 mm) pre-column. The eluent was detected by UV at wavelength 254 nm.

6.3 STANDARDIZATION

Calibration was done by analyzing plasma samples spiked with acyclovir at concentrations of 100, 80, 60, 40, 20, and 10 µg/mL on different days for seven days. The calibration curves were constructed by plotting peak area ratio of acyclovir to the internal standard versus the spiked concentrations, after which it was analyzed by linear regression using the GraphPad® Instat statistical program.

Accuracy was determined by evaluating for the percentage coefficient of variation (CV%) at each concentration using the following formula: (standard deviation/mean) x 100.

Possibility of interference from other drugs was not assessed, because the study was done in rabbits where use of other drugs was not expected during this experiment. Stability testing was not reported here, because it is part of the next chapter.

6.4 APPLICATION

The method was tested by analyzing plasma samples from animals (rabbits) administered with acyclovir intravenously. The details on the animal study and procedures including ethics approval are described in chapter 8.

6.5 RESULTS

6.5.1 Chromatographic performance

Figures 6.1a to 6.1e are the representative chromatograms for the standard solutions (fig. 6.1a and b), blank plasma (fig 6.1c) and spiked plasma (fig 6.1d and e). From the standard solution, it is clear that the peaks were well resolved, sharp and symmetrical, reflecting a good column performance, while blank plasma shows that there was no interference from plasma peaks. This observation was also confirmed in the spiked samples. Under these conditions, the retention time for acyclovir was 4.5 min and for gancyclovir was 3.4 min. The total run time was 10 minutes, while sample extraction was 15 minutes (including 10 minutes for centrifugation), giving an overall turnaround time of 25 minutes.

6.5.2 Calibration Curve

Table 6.1 shows data of the seven days' calibration curves, while figure 6.2 shows the plot of the average 7-day calibration curve. The curves were linear as indicated by the linear regression equation of $y = 0.03196 - 3.207x$ and a regression coefficient $r^2 = 0.995$. Also, the accuracy was sufficient with CV% of less than 7% for concentrations from 40 µg/ml and above, and less than 15% for concentrations from 20 µg/ml and below.

6.5.3 Application

Figure 6.3 shows the chromatogram of acyclovir in plasma from a rabbit after single dose intravenous administration with acyclovir. A separate calibration curve was used to derive concentrations of acyclovir (Fig. 6.4). Details on the pharmacokinetics are reported in chapter 8.

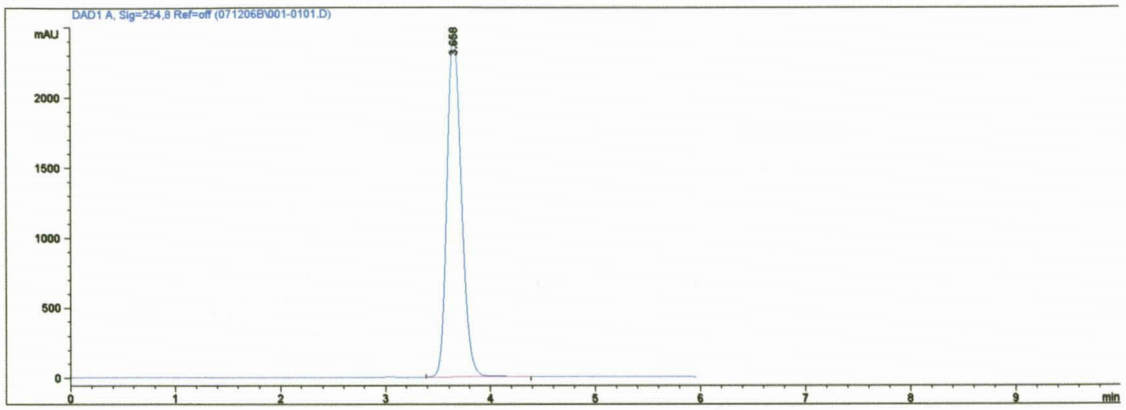


Figure 6.1a: Mobile phase spiked with acyclovir

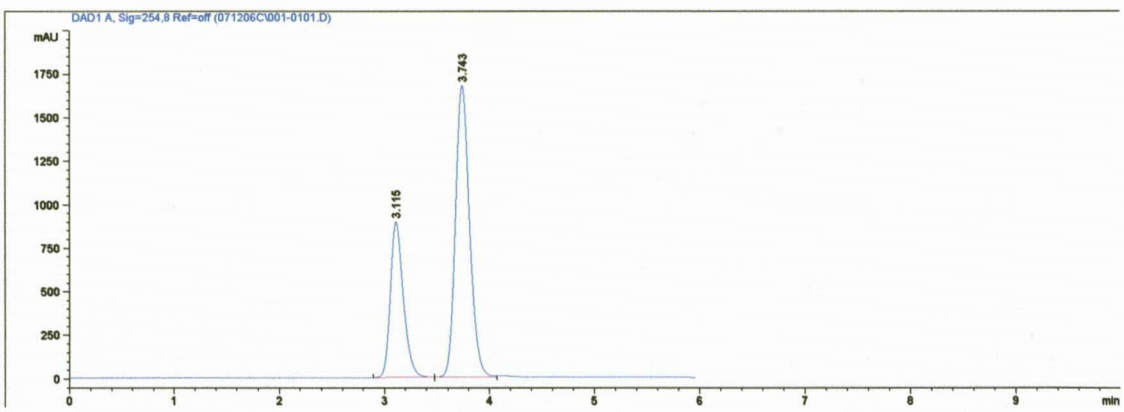


Figure 6.1b: Mobile phase spiked with internal standard (ganciclovir) and acyclovir

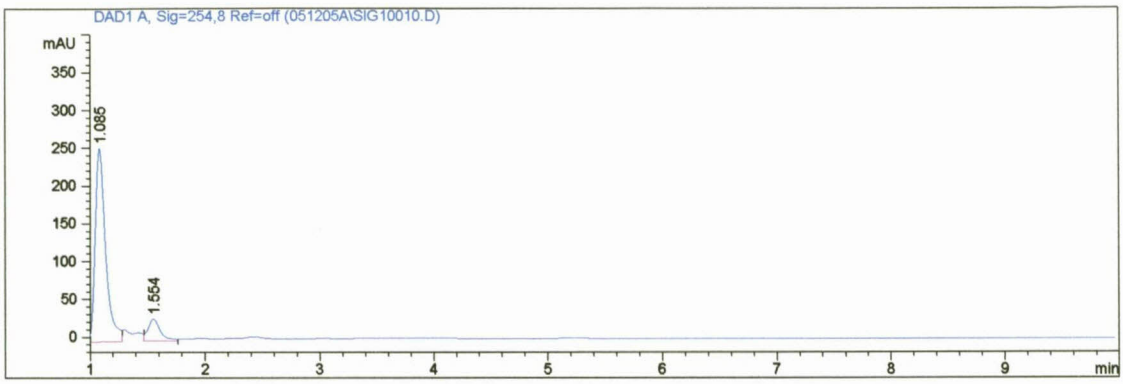


Figure 6.1c: Blank plasma sample

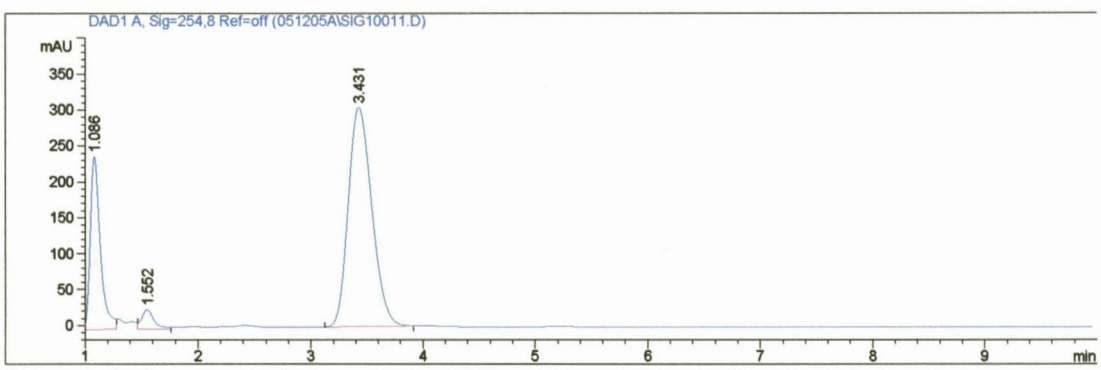


Figure 6.1d: Plasma sample spiked with internal standard (IS)

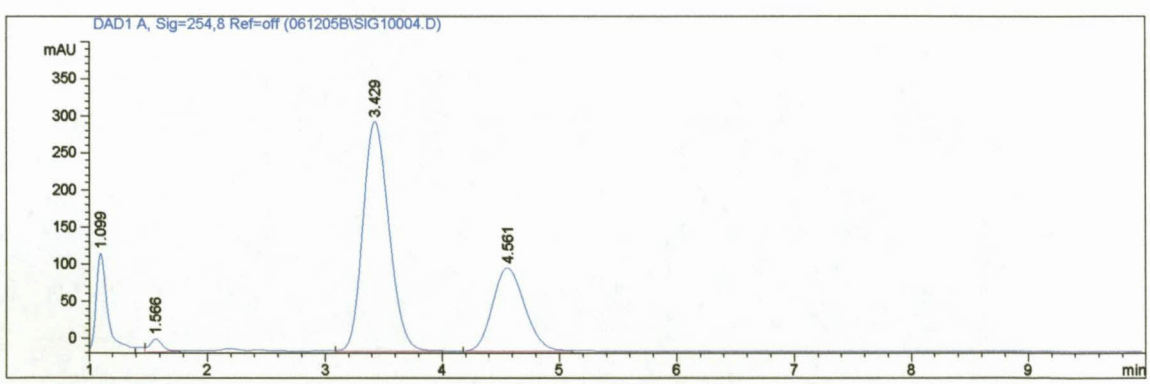


Figure 6.1e: Plasma sample spiked with internal standard (IS) and acyclovir 60 µg/ml

ble 6.1: HPLC calibrations for acyclovir using ratios of area ACV/area IS

onc.	calibr CV%	calibr	calibr	calibr	calibr	calibr	calibr	Mean	SD	CV %
g/ml)	day 1	day 2	day 3	day 4	day 5	day 6	day 7			
	0.094	0.09	0.12	*0.16	0.11	0.12	0.1	0.1134	0.024	12.3
	0.18	0.18	0.2	0.25	0.2	0.23	0.19	0.204	0.026	13.0
	0.43	0.43	0.46	0.43	0.43	0.45	0.4	0.433	0.019	4.4
	0.6	0.61	0.6	0.66	0.6	0.63	0.64	0.62	0.02	3.9
	0.72	0.72	0.73	0.77	0.69	0.83	0.8	0.751	0.050	6.7
0	0.96	0.96	0.95	0.93	0.8	0.93	0.96	0.927	0.058	6.3

) = standard deviation

/ = coefficient of variation

utlier

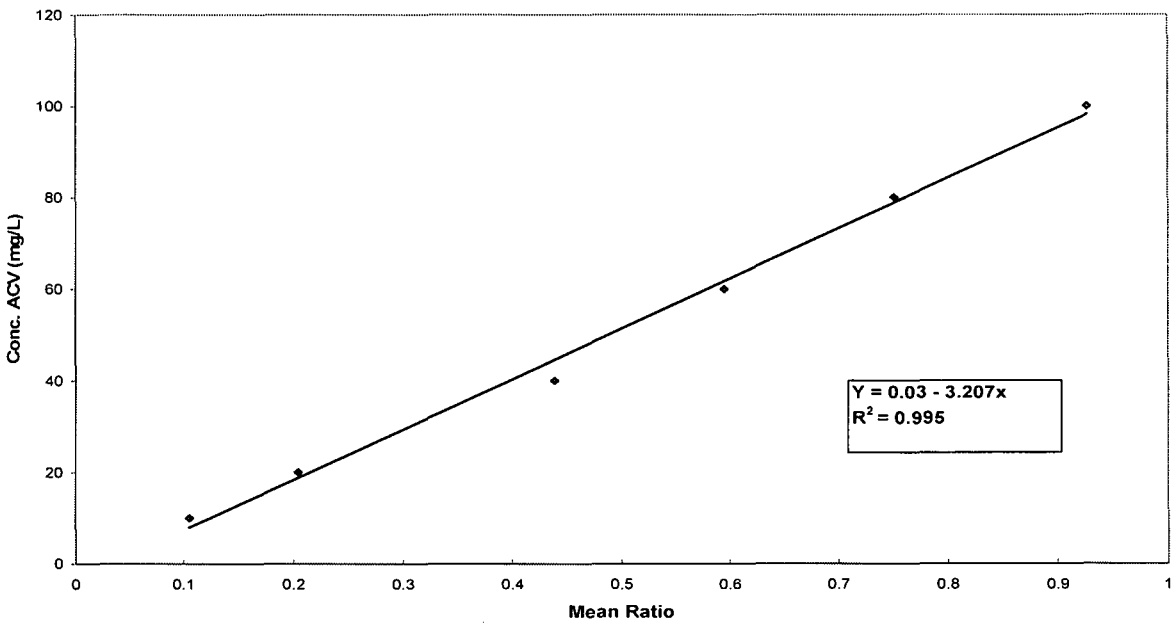


Figure 6.2: Calibration curve of acyclovir

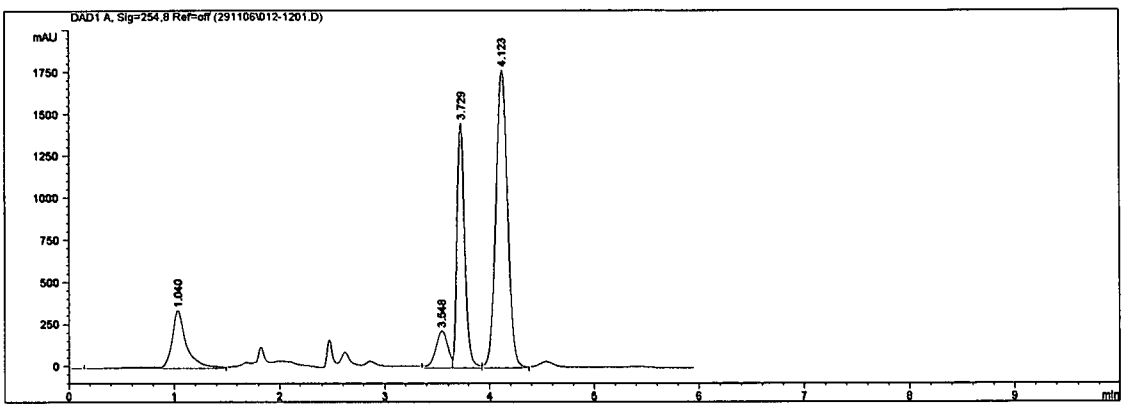


Figure 6.3: Plasma sample of rabbit 15; 60 minutes after receiving 60 mg/kg acyclovir intravenously and spiked with IS; the concentration was 33.55 µg/ml

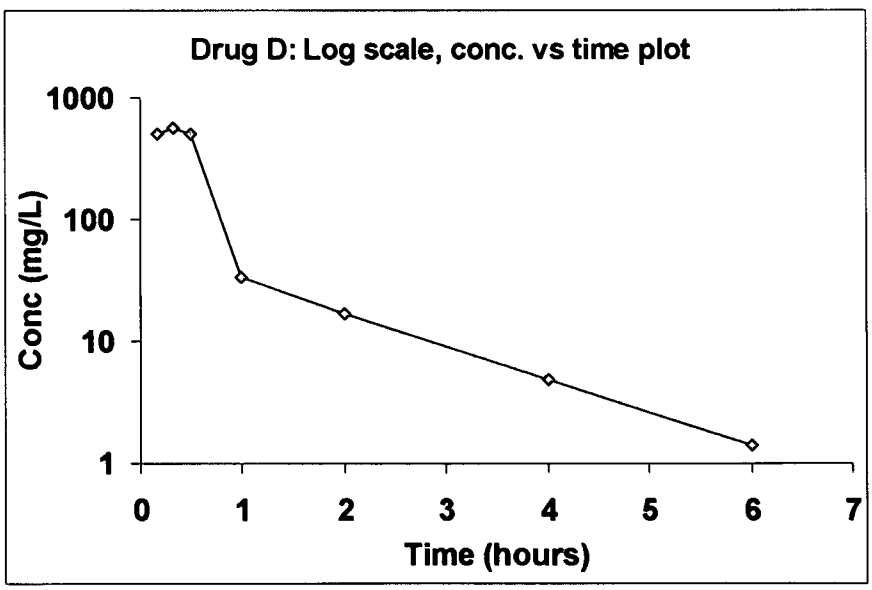


Figure 6.4: Log plot of animal 15 after single dose acyclovir IV over 6h period

6.5 Discussion

A simple, fast, accurate and inexpensive high performance liquid chromatography method for determination of acyclovir in plasma was successfully developed. Variation of this method from the original methods of Brown and co-workers (2002) was mainly; in the mobile phase composition where they did not use acetonitrile and the analytical column where they used a C8 analytical column instead, as well as filtration of samples which was not done in this method. However, this method exhibited shorter retention times and, although filtration of samples can prolong the analytical column life, this is not essential if the protein precipitation and centrifugation are done well. The new method proved to be accurate over a wide spectrum of acyclovir plasma concentrations (10 – 100 µg/ml) and was used successfully to determine the concentrations of acyclovir in rabbits.

CHAPTER 7

THE EFFECT OF TEMPERATURE AND pH ON VALPROIC ACID AND ACYCLOVIR *IN VITRO*.

7.0 SUMMARY

The possibility of a direct interaction between acyclovir and valproic acid *in vitro* was investigated by monitoring the concentrations of valproic acid and acyclovir at different pH (pH 7.4 or pH 3 or pH 10) and temperatures (25^{o}C and 37^{o}C) when mixed in a 1:1 molar ratio or when prepared separately in phosphate buffer. The samples were incubated at 25^{o}C for 2 hours and a further 1 hour at 37^{o}C, and aliquots were drawn at 10 min., 2 and 3 hours to measure the concentration of valproic acid and acyclovir (n=3). The concentrations of valproic acid and acyclovir when prepared separately were not different ($P > 0.05$) from those in the mixture of both drugs at the different temperatures and pH.

However, when the temperature and pH were evaluated separately, there was a trend whereby, at high temperature (37^{o}C), the concentrations of acyclovir (percentage detected) tended to be higher in the mixture (87%) than when it was alone (84%), while those of valproic acid tended to be lower in the mixture (89%) than when it was alone (92%). This same trend was observed at acid or alkaline pH. In conclusion, although temperature and pH did not induce significant effects on the concentrations of both acyclovir and valproic acid, increased concentrations of acyclovir were associated with reduced concentration of valproic acid when the two drugs were mixed under constrained conditions. These observations suggested a possible direct interaction between the two drugs.

7.1 INTRODUCTION

This part of the study is based on the report by Moattari and co-workers (2002) who, while investigating the potentiation of the antiherpetic effect of acyclovir by valproic acid, observed the possibility of a direct interaction between acyclovir and valproic acid. However, the

experimental conditions were not described in full, yet, as cited earlier, they appeared not clinically tenable. Specifically, the high concentrations of valproic acid in the reaction medium versus trace concentrations for acyclovir. Therefore, this part of the study was undertaken to investigate the possibility of a direct interaction between valproic acid and acyclovir in conditions (temperature and pH) that may be clinically attainable.

7.2 METHODS

7.2.1 Materials and regents

Acyclovir and valproic acid sodium salts were purchased from Sigma Chemical Co. (Steinheim, Germany) while di-sodium hydrogen orthophosphate dihydrate ($Na_2HPO_4.2H_2O$) was purchase from Merck Laboratories (Darmstadt, Germany).

7.2.2 Preparation of standard solutions:

Stock solutions containing 5 M of valproic acid and acyclovir were prepared separately in 20 ml of sodium phosphate buffer, pH 7.4. For valproic acid, 16.62 mg was used while, for acyclovir, 22.52 mg was used. The stock solutions were always kept at 4°C in the refrigerator. The working buffer was 1 M sodium phosphate at different pH, i.e., pH 3, 7.4, and 10.

7.2.3 Procedure

The test samples consisted of 1 ml of buffer containing either 112.6 μg/ml (5 M) acyclovir (**ACV only**) or 72.13 μg/ml (5 M) valproic acid (**VA only**) or both (**VA +ACV**) in a 1:1 molar ratio. For each experiment, the samples were prepared in the appropriate buffer, i.e., either pH 3, 7.4 or 10. They were briefly vortexed and then incubated at 25°C for 2 hours and a further 1 hour at 37°C. Aliquots of 200 μl were drawn immediately and then at 2 and 3 hours, and were used for measuring the concentration of valproic acid and /or acyclovir. The experiment was repeated three times (n=3) on different days. All the samples were immediately stored at -20 degrees until determination of the concentrations. The concentrations of valproic acid were determined using an enzyme-linked immunoabsorbent assays from Abbott Diagnostics while the concentrations of acyclovir were determined using the HPLC method developed earlier.

Analysis of valproic acid: The concentrations of valproic acid were measured by Fluorescence Polarization Immunoassay using an automated immunoassay analyzer (Axsym System, Abbott Laboratories, Illinois, USA). This involved use of kits in which drug standards for

calibrations were supplied. The kits were used according to the manufacturer's instructions (Abbott, Diagnostics, USA). Samples of 100 µl each were run in duplicates and the average was the ultimate concentration measured.

Drug recovery: Percentage recovery of the respective drugs was calculated as a fraction of the "total amount of drug measured in the mixture" *(concentration x sample vol.)* divided by the "total amount spiked" multiplied by 100.

- Fraction recovered (%) = [(concentration x sample vol.)/ (amount spiked)] x 100

The rationale for the experimental conditions: The pH and temperature used were based on the conditions of administering drugs orally to patients, where the pH 3 depicted the pH of the stomach, pH 7.4 the neutral medium in which the drugs are normally mixed, and the alkaline pH 10 for the lower GIT (normal pH is above 8). Regarding temperature, the 25°C was for the standardized room temperature at which drugs are normally mixed, while the 37°C was for normal body temperature.

7.2.4 Statistical analysis

Results are reported as mean ± SD and comparison of data was done with the ANOVA Tukey-Kramer Multiple Comparisons Test with level of significance set at p value < 0.05.

7.3 RESULTS

7.3.1 Effect of temperature and pH on drug concentrations:

In table 7.1, it is clear that at pH 7.4, the concentrations of both valproic acid and acyclovir were not significantly effected by changes in temperature whether the two drugs were in combination or not ($p<0.05$). Also, table 7.2 shows that at pH 3, the concentrations of both valproic acid and acyclovir were not significantly effected by changes in temperature whether in single drug solution or when in a mixture ($p<0.05$). Again, table 7.3 shows that at pH 10, the concentrations of both valproic acid and acyclovir were not significantly effected by changes in temperature ($p<0.05$).

7.3.2 Effect of temperature and pH on drug recovery:

However, when the total amount of drug spiked in the original sample was related to the amount of drug detected after incubation at different temperatures and pH, there were some differences of interest.

a) Effect of temperature: Although there was no significant difference, at the higher temperature of 37°C, more acyclovir was detected versus less valproic acid from the samples containing the mixture of both drugs. As shown in figure 7.1, the fraction of acyclovir detected in samples containing both drugs was substantially higher (87%) than the fraction detected in samples containing acyclovir only (84%), while in figure 7.2, the fraction of valproic acid detected in the same samples of the mixture of the two drug was less (89%) than the fraction detected in sample containing valproic acid only (92%).

b) Effect of pH: In figure 7.3, the fraction of acyclovir detected in samples containing the mixture of both drugs, particularly at acidic pH, was higher (87%) than the fraction detected in samples containing acyclovir only (84%). However, in figure 7.4, the opposite happened to valproic acid; at all the pH tested, lower fraction of valproic acid was detected in samples from the mixture compared to the fraction detected in samples containing valproic acid only.

Table 7.1: The mean (± standard deviation) concentrations (μg/ml) of valproic acid and acyclovir in single drug solution (VA only; ACV only) and in a mixture (VA + ACV) at different temperatures (25°C and 37°C) at pH 7.4 (n=3)

Time:		**10 min (25° C)**	**2 hr (25° C)**	**3 hr (37° C)**
VA only:	VA	64.85 ±7.970	59.21±9.830	66.55 ±5.990
ACV only:	ACV	83.91± 2.877	84.86±8.629	98.20±5.073
VA + ACV:	VA	62.30±5.580	64.49±2.280	62.07±7.720
	ACV	89.32±10.226	91.49±12.590	97.00±7.251

Table 7.2: The mean (± standard deviation) concentrations (μg/ml) of valproic acid and acyclovir in single drug solution (VA only; ACV only) and in a mixture (VA + ACV) at different temperatures (25°C and 37°C) at pH 3 (n=3)

Time:		**10 min (25° C)**	**2 hr (25° C)**	**3 hr (37° C)**
VA only:	VA	67.24± 8.360	66.95±1.900	65.14±3.120
ACV only:	ACV	87.73±11.624	106.52±15.220	88.66±8.192
VA + ACV:	VA	61.61± 6.070	65.33±6.850	64.61±7.560
	ACV	97.35±12.905	101.02±1.291	96.60±5.260

Table 7.3: The mean (± standard deviation) concentrations (μg/ml) of valproic acid and acyclovir in single drug solution (VA only; ACV only) and in mixture (VA + ACV) at different temperatures (25°C and 37°C) at pH 10 (n=3)

Time:		**10 min (25° C)**	**2 hr (25° C)**	**3 hr (37° C)**
VA only:	VA	68.97±4.370	67.90±4.720	68.02±4.090
ACV only:	ACV	90.69±2.876	91.80±9.246	96.25±10.889
VA + ACV:	VA	64.60±5.270	64.39±5.060	65.58±2.500
	ACV	96.66±11.291	88.19±3.640	99.83±12.248

Figure 7.1: The fraction of acyclovir detected in single drug solution (ACV only) and in the mixture with valproic acid (VA +ACV) at different temperatures (25°C and 37°C)

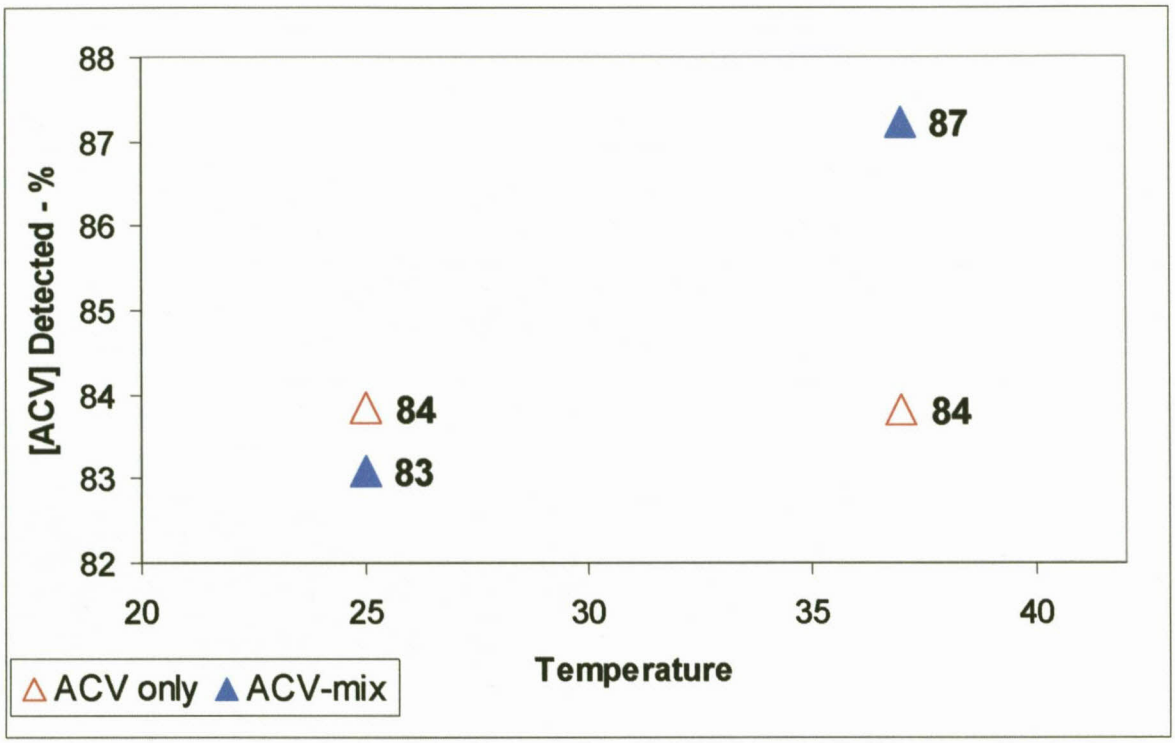


Figure 7.2: The fraction of valproic acid detected in single drug solution (VA only) and in the mixture with acyclovir (VA + ACV) at different temperatures (25°C and 37°C)

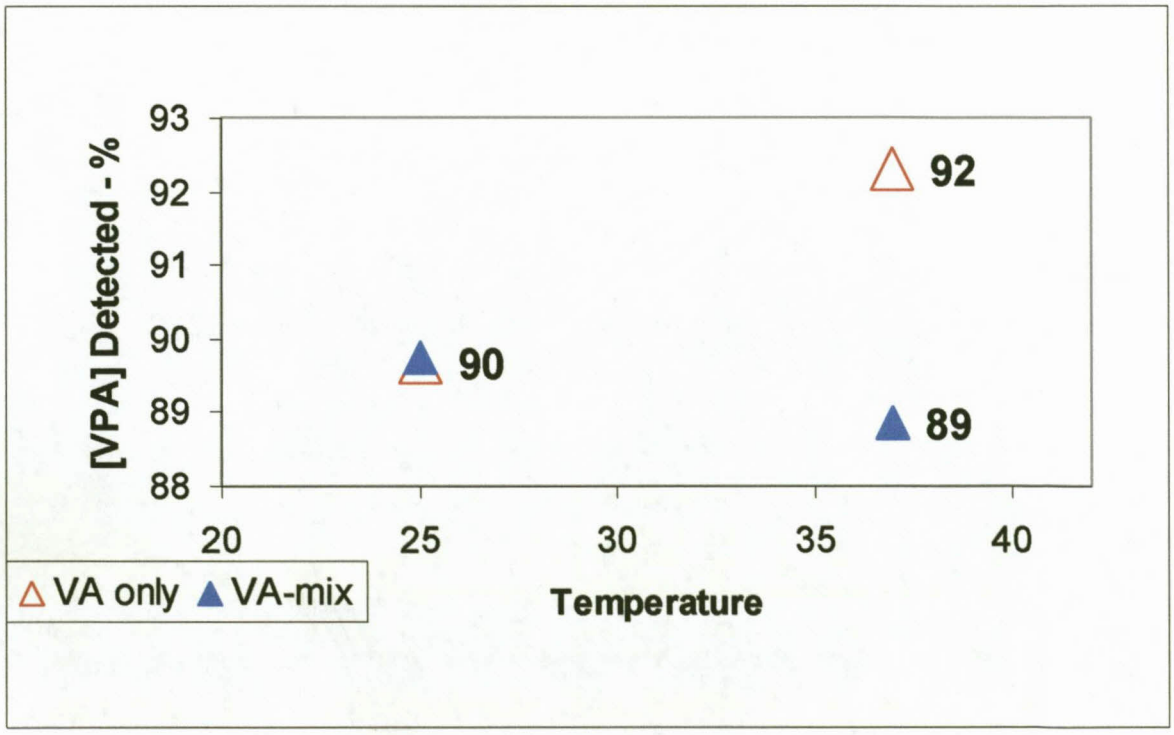


Figure 7.3: The fraction of acyclovir detected as single drug solution (ACV only) and in the mixture with valproic acid (VA + ACV) at different pH values.

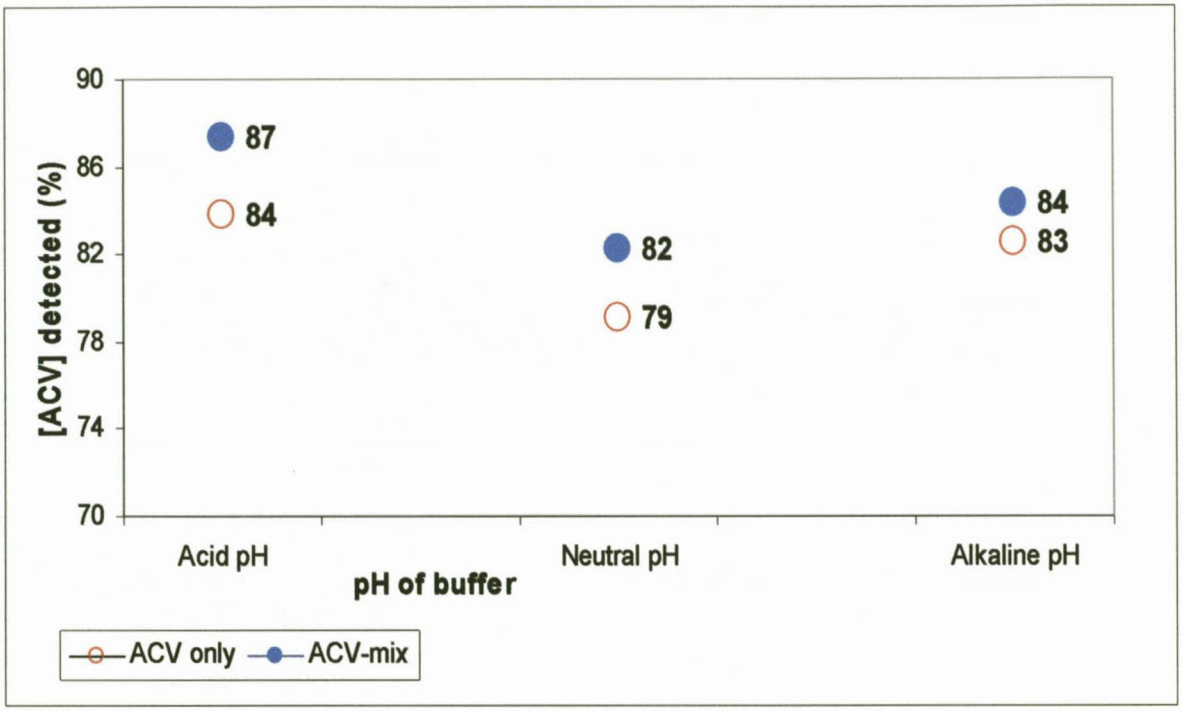
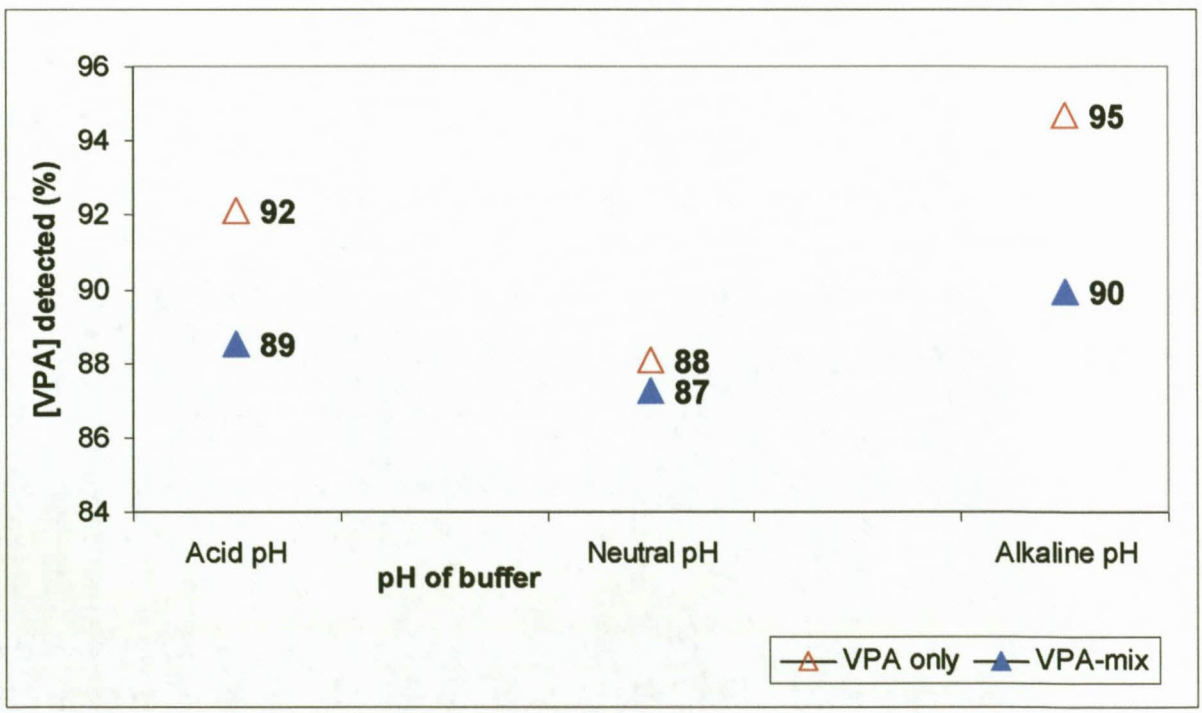


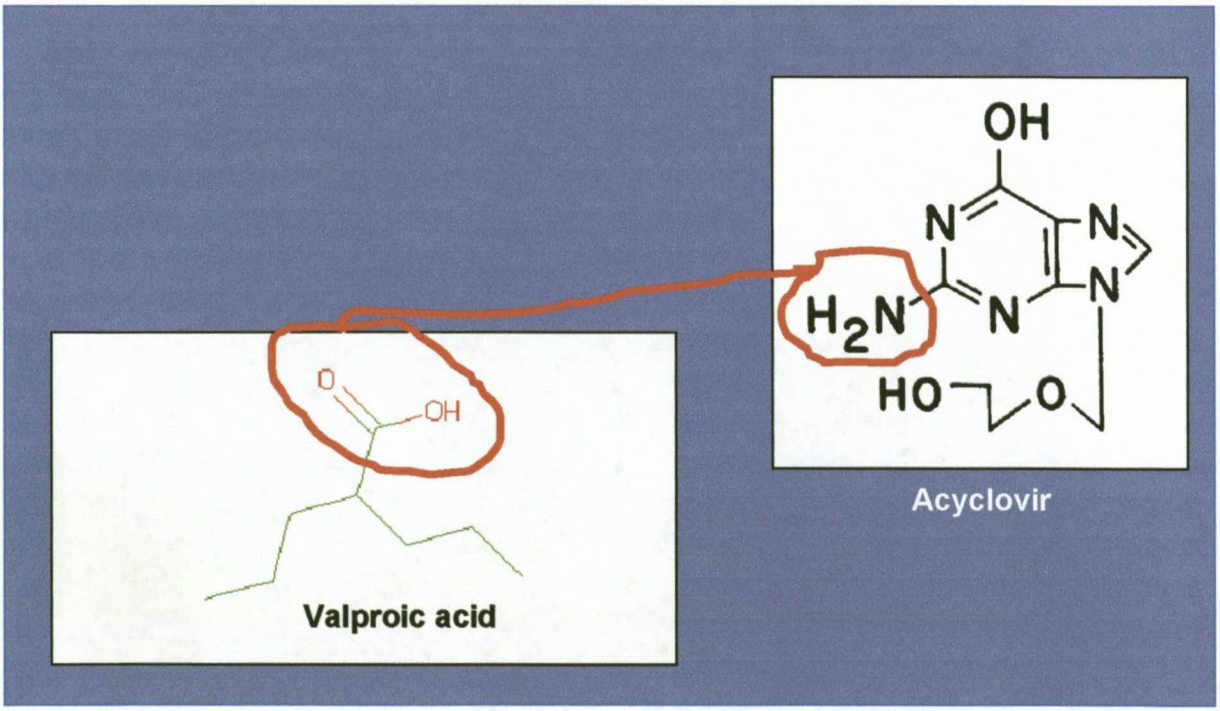
Figure 7.4: The fraction of valproic acid detected as single drug solution (VA only) and in the mixture with acyclovir (VA +ACV) at different pH values.



7.4 DISCUSSION

In this study it has been shown that when the two drugs were mixed, recovery of acyclovir was increased while that of valproic acid was reduced. This implies that there is a direct interaction between the two drugs whereby some valproic acid is lost. This observation augurs well with the findings of the case report where co-administration of the two drugs led to reduced valproic acid concentrations (Parmeggiani *et al.*, 1994). However, the fact that this was not observed at room temperature (25°C; Fig. 7.1 and 7.2) implies that the interaction requires a catalyst, such as higher temperature, pH or other conditions. Of note, the pH exhibited a consistent trend of higher recoveries for acyclovir and lower recoveries for valproic acid in samples containing both drugs (Fig. 7.3 and 7.4). In fact, Moattari and co-workers (2002) found a reproducible direct interaction between valproic acid and acyclovir, and they postulated that, under lower pH in their experimental conditions, valproic acid interacted with the amino group of acyclovir giving rise to a new compound (Fig. 7.5). In this study, the concentrations of valproic acid were measure by fluorescent enzyme-linked immunoabsorbent assay while those of acyclovir by HPLC, which would not exclude the formation of a new compound if it were structurally related to the parent compounds.

Figure 7.5: Postulated sites of interaction between valproic acid and acyclovir



These observations imply that acyclovir may reduce the bioavailability of valproic acid irrespective of pH though less so at neutral pH, and this may be augmented by normal body temperature (37°C). With regard to extreme pH (i.e., pH 3 and 10), it implies the interaction may occur in the stomach where pH is acidic as well as in the lower GIT where the pH is more alkaline.

In conclusion, the present results imply that mixing acyclovir and valproic acid may lead to reduced recovery of valproic acid. However, the implications of these observations require further studies *in vivo* to confirm whether co-administration of the two drugs affects the bioavailability of valproic acid, and this is part of the objective for the next chapter.

CHAPTER 8

PHARMACOKINETIC INTERACTION BETWEEN VALPROIC ACID AND ACYCLOVIR AFTER INTRAVENOUS AND ORAL ADMINISTRATION IN RABBITS

8.0 SUMMARY

This part of the study was undertaken to investigate the effect of co-administration of valproic acid and acyclovir on the pharmacokinetic parameters of each other. Fifteen white New Zealand rabbits were divided into 3 groups A, B and C whereby Group A received acyclovir only, group B received valproic acid only, and group C received a combination of acyclovir and valproic acid. In a cross over design, the intravenous route was studied first, followed by the oral route after a two-week wash out period. Blood samples were drawn over 10 hr period and the pharmacokinetic parameters were derived from the concentrations. After intravenous administration, the area under the plasma concentration time curve (AUC) and plasma concentrations of acyclovir in group C were higher than in group A, while the volume of distribution (Vd) and plasma clearance (CLp) of acyclovir in group C were only 12.8% and 10.36% of those of group A, respectively. A similar trend was observed after oral administration. However, the bioavailability (F) of acyclovir was 8.4% in group A versus 1.5% in group C. Also, the concentrations and kinetic parameters of valproic acid between the two groups after oral and intravenous administration were not different. In conclusion, co-administration of single doses of acyclovir and valproic acid led to reduced oral bioavailability of acyclovir, but increased concentrations of acyclovir due to reduced volume of distribution and clearance and this was most probably due to inhibition of the membrane transport proteins for acyclovir by valproic acid.

8.1 INTRODUCTION

This part of the study was undertaken to investigate the effect of co-administration of the two drugs on the pharmacokinetic parameters of each other. Because the concentrations of both drugs were measured, this enabled us to account for any changes in the pharmacokinetics of either drug.

8.2 MATERIALS AND METHODS

8.2.1 Materials

Intravenous formulations for acyclovir and valproic acid were purchased from the local in-hospital Medi-Clinic pharmacy. For acyclovir, the formulation was Zovirax® 250 mg/vail manufactured by Glaxo Welcome, while for valproic acid, it was Epilim® 400mg/vial by Sanofi-Synthlabo. However, because the acyclovir formulations were too dilute and expensive, the oral formulations for acyclovir were prepared from laboratory standards using normal saline. Acyclovir powder was a donation from the local representative of Glaxo Welcome (Brentford, United Kingdom). The oral formulation for valproic acid was a syrup (Epilim® 200mg/5 ml; Sanofi-Synthlabo) and was purchased from a local pharmacy. The rabbits were obtained from the University Animal House where they were cared for before and during the study period.

8.2.2 Animal experiment

The study was approved by the Animals Ethics committee of the University of the Free State. Fifteen white New Zealand rabbits of either sex were used. The animals were divided into 3 groups A, B and C whereby Group A received acyclovir only, group B received valproic acid only, and group C received a combination of acyclovir and valproic acid. The intravenous dose of acyclovir was 60 mg/kg (similar to the dosages used by Brown *et. al,* 2002), while the oral dose was 300 mg/kg. For valproic acid, the intravenous and oral doses were the same, 20 mg/kg (similar to dosages used in pediatric patients). In a crossover design, the intravenous route was studied first, followed by the oral route after a two-week washout period. The high dose of oral acyclovir (300 mg/kg) was to compensate for the low oral bioavailability of 10- 20% (Safrin *et al.*, 2004). This was not necessary for valproic acid because it has a bioavailability of more than 80% (Porter *et al.*, 2004). Intravenous administration was via the lateral ear vein while a plastic catheter was used for oral administration of the drugs in solution. Blood samples (0.5-1 ml) were drawn from the central ear artery over a ten-hour period. Each sample was

centrifuged immediately after collection and the plasma was stored at -20° until ready for drug concentration assay (figure 8.1).

8.2.3 Analysis of Drugs

Acyclovir was analyzed by a validated high performance liquid chromatography (HPLC) method described earlier (chapter 6), while the concentrations of valproic acid were measured by Fluorescence Polarization Immunoassay as indicated and described in chapter 4 (4.4).

8.2.4 Pharmacokinetic data analysis

Pharmacokinetic parameters of acyclovir and valproic acid for each animal were obtained from plasma concentration versus time plots by non-compartmental methods, whereby the area under plasma concentrations versus time (AUC) were calculated using the trapezoidal rule with extrapolation to infinity (Ct/Ke) (Gibaldi *et al.*, 1984). The elimination rate constant (Ke) for the decline of plasma concentrations was obtained by linear regression on log plasma concentration against time plots. Thereafter, the following equations were used to derive the relevant pharmacokinetic parameters:

- Half-life ($T_{½}$) = $\frac{0.693}{Ke}$ Eq.1
- Volume of distribution (Vd) = $\frac{Dose}{AUCxKe}$ Eq. 2
- Clearance (CLp) = $\frac{Dose}{AUC}$ Eq. 3
- Mean residence time (MRT) = $\frac{1}{Ke}$ Eq. 4

The bioavailability (F) was determined using the equation:

- F = $\frac{AUCo}{AUCiv}$ Eq. 5

Of note, because the oral dose of acyclovir was different and higher than the intravenous dose, the F was obtained using a calculated AUCiv if the oral dose were given intravenously as follows:

- AUCiv = $\frac{Dose(oral)}{(VdxKe)}$ Eq. 6

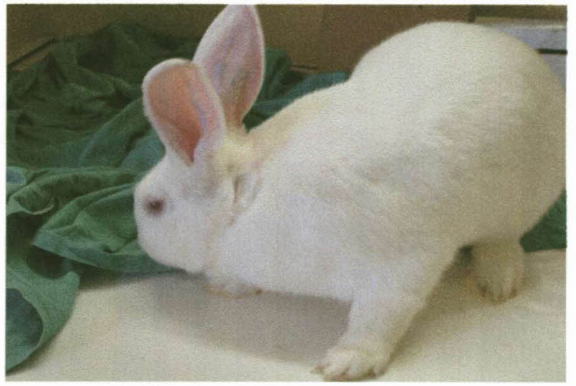
where Vd and Ke were those obtained after the intravenous dose of 60 mg/kg.

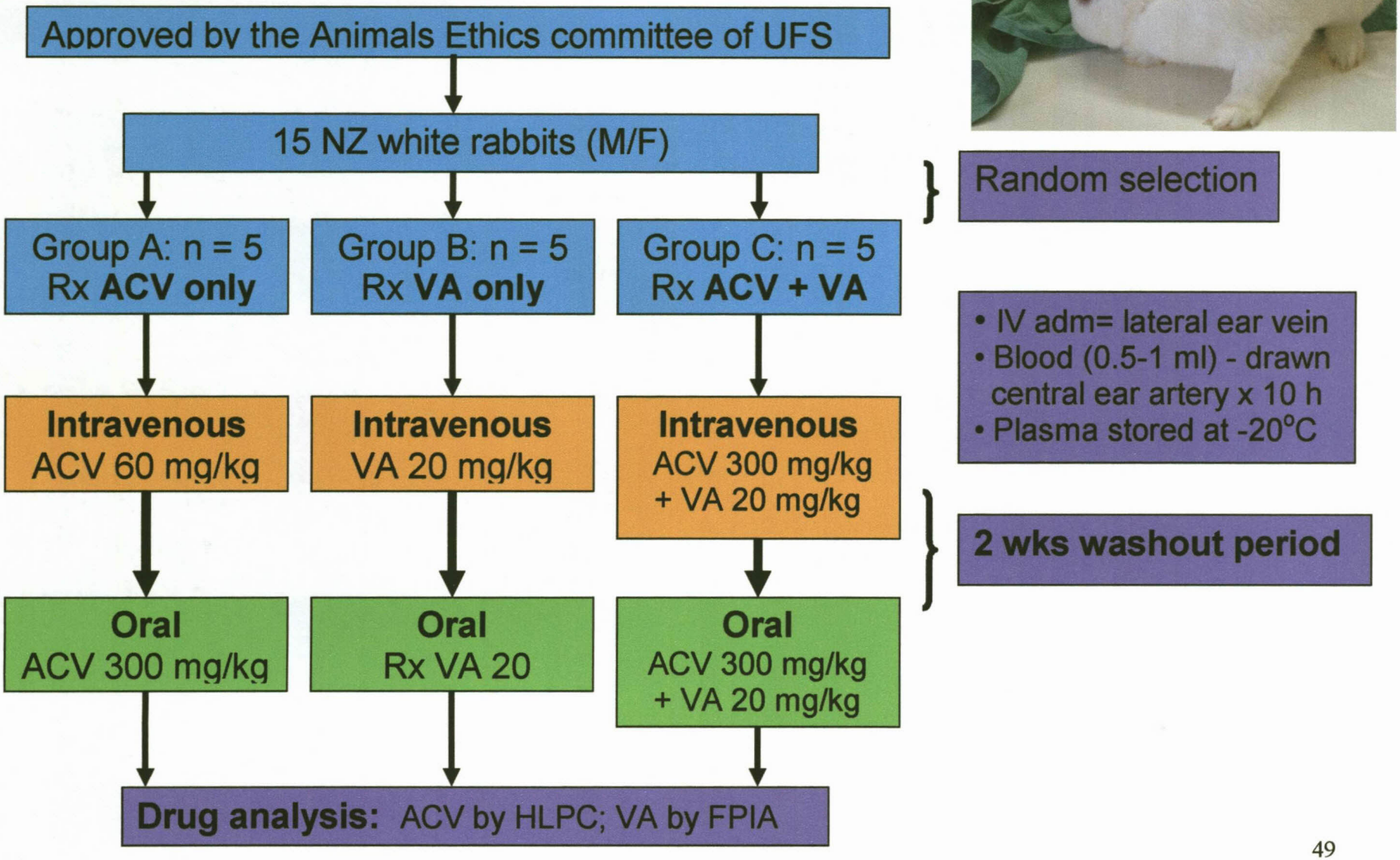
The maximum concentration (Cmax) and the time to reach Cmax (Tmax) were read from the concentration-time curves.

8.2.5 Statistical data analysis

Data was analyzed by non-parametric methods using the Graph Pad statistical program. Accordingly, parameters were reported as median and range, and the Mann Whitney Test was used for data comparison with the level of significance set at $p < 0.05$.

Figure 8.1: An illustration of the experimental design





8.3 RESULTS

Figure 8.2 and Table 8.1 show that, after intravenous administration, the corresponding plasma concentrations of acyclovir in animals that received a combination of acyclovir and valproic acid (group C) were more than 10 times higher than in those that received acyclovir only (group A). Also, acyclovir was almost undetectable at 4 hours in group A, while in group C, it was still measurable 8 hours after administration. This was confirmed by a higher AUC for group C than group A, ($p < 0.0001$); AUC for group A was 10.30% of that for group C (Table 8.2). Furthermore, the volume of distribution (Vd) and plasma clearance (CLp) of acyclovir were significantly lower in group C than group A, i.e., 12.8% and 10.36% of group A values, respectively. The p-values were less than 0.0001 for both Vd and Clp. These observations imply that valproic acid inhibited the distribution and clearance of acyclovir. A similar trend was observed after oral administration (Figure 8.3 and Table 8.1). The corresponding plasma concentrations of acyclovir for group C were 3-4 times higher than for group A, and acyclovir was measurable at 10 hours in group C versus 8 hours in group A. Again, the Vd and CLp of acyclovir were lower in group C than group A, i.e., 12.35% and 12.90% of group A values, respectively (Table 8.2). The p-values were less than 0.0079 for both the Vd and CLp.

Surprisingly, the AUCs were not significantly different between the two groups A and C, but values for group C were higher than in group A (Table 8.2). It was also observed that absorption of acyclovir was slower in group A than in group C as evidenced by the Tmax of 3 hr in group A versus 0.5 hr in group C. The corresponding Cmax were 4.6 mg/L for group A and 18.4 mg/L for group C. The latter could not make sense until it was found that the bioavailability (F) of acyclovir in group C (median, 1.5%) was only 17.9% of that for group A (median, 8.4%) (Table 8.2). This implies that valproic acid interfered with the absorption of acyclovir from the gastrointestinal tract, but high plasma concentrations of acyclovir were observed from the small amount (1.5%) that was absorbed because valproic acid also interfered with the distribution and clearance of acyclovir.

Figure 8.4 and 8.5 as well as Table 8.3 show the concentration of valproic acid after intravenous and oral administration in groups B and C. There was no significant difference in the concentrations of valproic acid between the two groups after oral and intravenous

administration. Similarly, the kinetic parameters of valproic acid after administration with and without acyclovir were not significantly different after either route of administration (Table 8.4). However, there was a trend of decreased bioavailability when the two drugs were combined. The bioavailability decreased from 92.1 % (67-109), in the animals that received valproic acid alone, to 81.7% (54.7 – 108) in the group that received both drugs.

The inhibition of acyclovir by valproic acid appeared to be concentration dependent with a threshold concentration of valproic acid between 60 and 30 mg/L. This was indicated by the rapid fall in the plasma concentrations of acyclovir when the concentrations of valproic acid were within this range after intravenous administration in Group C. When valproic acid concentrations were between 77.95 mg/L at 1 hr and 53.3 mg/L at 2 hr, the corresponding concentrations of acyclovir fell rapidly from 362 mg/L to 16.5 mg/L, respectively. In effect, there was a significant rapid drop of over 50 % in the concentration of acyclovir, implying that after the threshold concentration, there was rapid elimination and/or distribution of acyclovir. For the oral route, this was not obvious, but it helped in setting the lower limit of 30 mg/L. When valproic acid concentrations were between 38.95 mg/L at 2 hr and 27.56 mg/L at 3 hr, the corresponding concentrations of acyclovir fell rapidly from 8.66 mg/L and 4.78 mg/L, respectively, a drop of 55%. The concentration for valproic acid of 27.56 mg/L at 3 hr after oral administration implies that the threshold concentration was higher than this hence the range of 60 to 30 mg/L was suggested.

Figure 8.2: The median plasma concentrations of acyclovir in rabbits after intravenous administration of acyclovir alone (solid line) and acyclovir with valproic acid (dotted line), (n = 5).

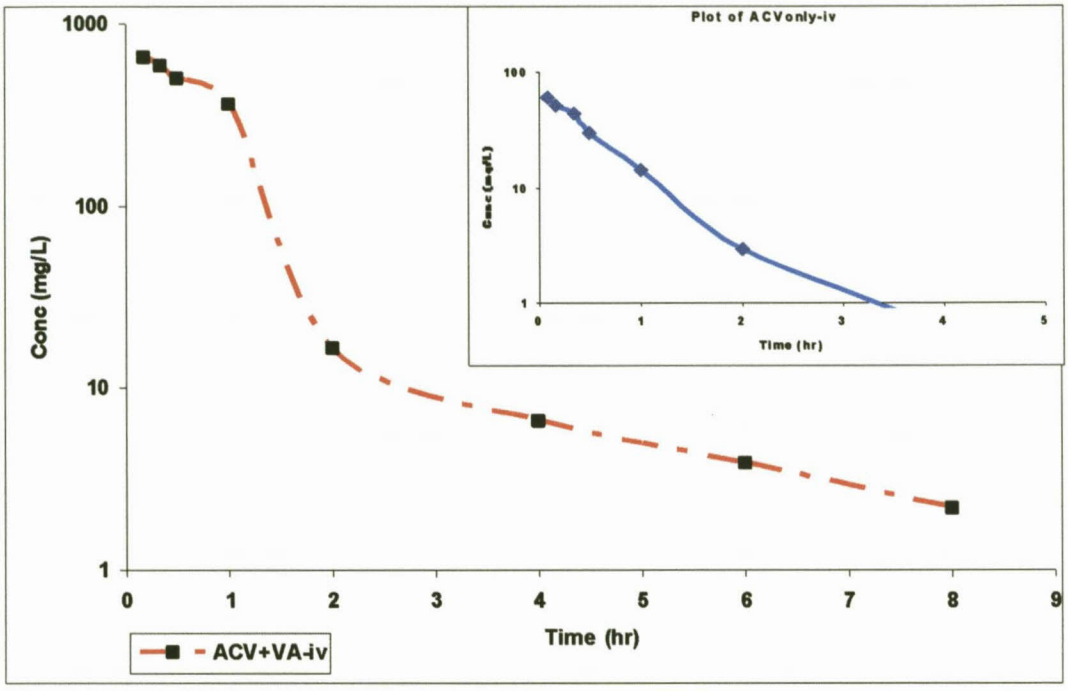


Figure 8.3: The median plasma concentrations of acyclovir in rabbits after oral administration of acyclovir alone (solid line) and acyclovir with valproic acid (dotted line), (n = 5).

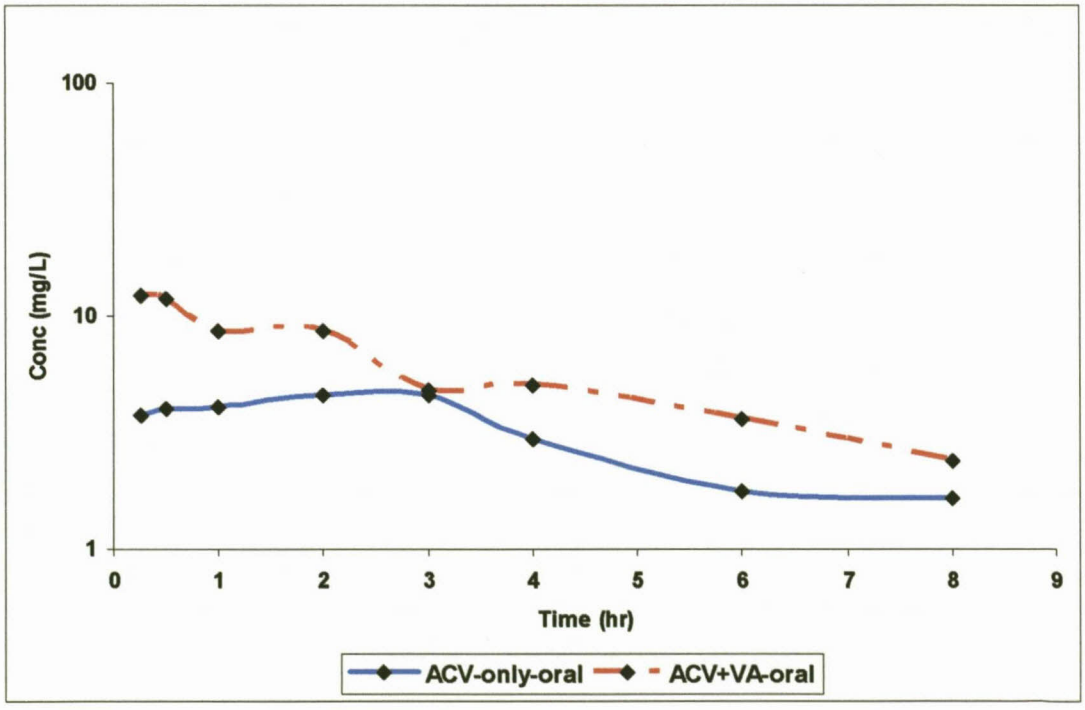


Figure 8.4: The median plasma concentrations of valproic acid in rabbits after intravenous administration of valproic acid alone (solid line) and valproic acid with acyclovir (dotted line), (n = 5).

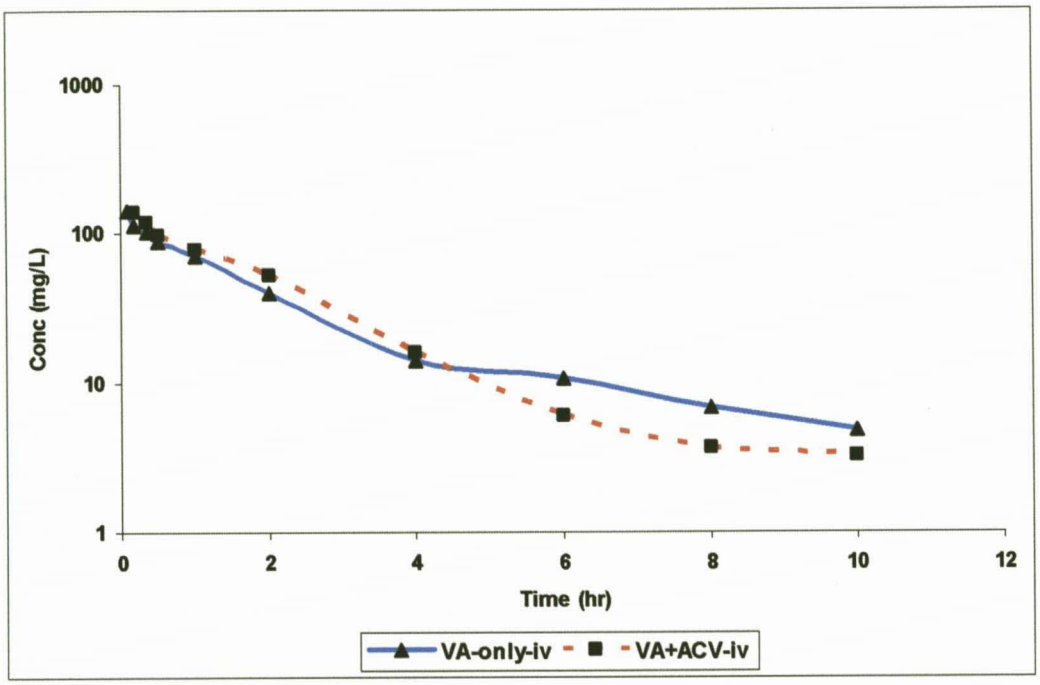


Figure 8.5: The median plasma concentrations of valproic acid in rabbits after oral administration of valproic acid alone (solid line) and valproic acid with acyclovir (dotted line), (n = 5).

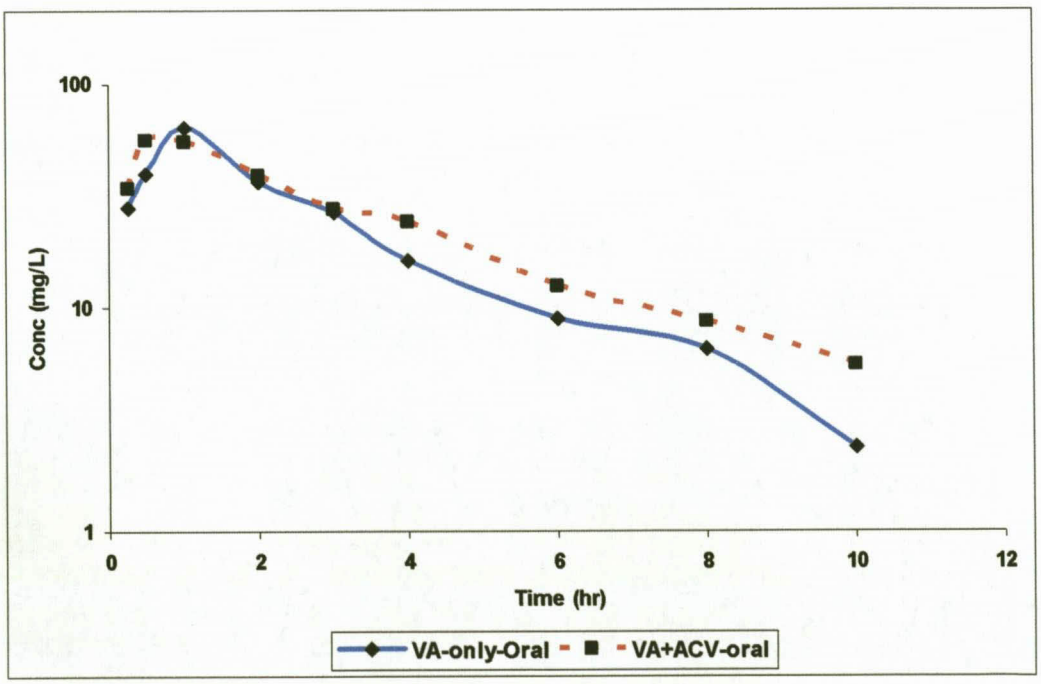


Table 8.1: Plasma concentrations (mg/L) of acyclovir, median (range), in rabbits after intravenous and oral administration of acyclovir alone (group A) and acyclovir with valproic acid (group C), (n = 5).

Intravenous:

Time (hr):	**0.17**	**0.34**	**0.5**	**1**	**2**	**4**	**6**	**8**	**10**
Group A:	**51.4** (42-58)	**43.1** (33-47)	**29.8** (14-40)	**14** (2-19)	**2.9** (3-7)	**0.6** (0.3-0.9)	-	-	-
Group C:	**647** (504-911)	**591** (561-820)	**499** (55-742)	**362** (34-568)	**16.5** (15-24)	**6.6** (3-15)	**3.9** (1.4-4.4)	**2.18** (1-5)	-

Oral:

Time (hr):	**0.25**	**0.5**	**1**	**2**	**3**	**4**	**6**	**8**	**10**
Group A:	**3.7** (3.1-5.2)	**3.97** (1.6-4.2)	**4.06** (1.4-10.7)	**4.58** (2-7)	**4.6** (2-5)	**2.95** (2.87-6.47)	**1.7** (1.5-2.5)	**1.6** (1-2)	- -
Group C:	**12.2** (0.8-30)	**11.86** (0.7-25)	**8.6** (4.1-18.3)	**8.66** (4-14)	**4.78** (1.6-9)	**5.0** (1.7-8.5)	**3.6** (1.06-8.4)	**2.4** (0.5-4)	**0.59** (0.36-2.5)

Table 8.2: Plasma pharmacokinetic parameters of acyclovir, median (range), in rabbits after intravenous and oral administration of acyclovir alone (group A) and acyclovir with valproic acid (group C).

Route	Acyclovir after intravenous administration		Acyclovir after oral administration	
	Group A	**Group C**	**Group A**	**Group C**
No. rabbits	**5**	**5**	**5**	**5**
Sex	2M 3F	2M 3F	2M 3F	2M 3F
Wt (kg)	**3.6** (3.5-3.8)	**4** (3.5-4.2)	**3.8** (3.5-4.2)	**4** (3.5-4.3)
Dose (mg)	**216** (210-288)	**240** (210-250)	**1140** (1050-1260)	**1200** (1050-1290)
Co (mg/L)	**69.1** (64-83)	**529** (126-1005)*	**11.5** (6.9-18.5)	**12.2** (6.7-28)
Ke (/hr)	**0.86** (0.67-1.8)	**0.84** (0.5-1.5)	**0.32** (0.2-0.4)	**0.28** (0.2-0.5)
Half-life (hr)	**0.80** (0.4-1.0)	**0.83** (0.5-1.5)	**2.14** (1.6-3.5)	**2.49** (1.4-3.5)
AUC (mg.hr/L)	**81.65** (46 -101)	**751** (509-1094)*	**29.9** (21.3-38.7)	**44.5** (18.1-76.3)
Vd (L)	**3.05** (2.6-3.8)	**0.34** (0.2-0.9)*	**8.03** (6.3-23.4)	**1.44** (0.8-2.0)*
CLp (L/hr)	**2.76** (2.1-4.7)	**0.30** (0.2-0.4)*	**2.76** (2.1-4.7)	**0.401** (0.4 - 0.4)*
MRT(hr)	**1.16** (0.6-1.5)	**1.19** (0.6-2.2)	**3.1** (2.3-5.0)	**3.59** (2.0-5.1)
F (%)	---	---	**8.4** (5.0-11.0)	**1.5** (0.6-2.3)*
Tmax (hr)	---	---	**3.0** (1.0 – 4.0)	**0.5** (0.5 – 2.0)*
Cmax (mg/L)	---	---	**4.6** (2.9-10.7)	**18.4** (5.5-30.6)*

*** = significant difference, $p < 0.05$**

Key: AUC = Area under the plasma concentration-time curve; **CLp** = plasma clearance; **Cmax** = maximum concentration; **Co** = concentration after instantaneous distribution at time zero; **F** = bioavailability; **Ke** = elimination rate constant; **MRT** = mean residence time; **Tmax** = the time to reach maximum concentration; **Vd** = volume of distribution; and **Wt** = weight

Table 8.3: Plasma concentrations (mg/L) of valproic acid, median (range), in rabbits after intravenous and oral administration of valproic a alone (group B) and valproic acid with acyclovir (group C), (n = 5).

Intravenous:

Time (hr):	**0.17**	**0.34**	**0.5**	**1**	**2**	**4**	**6**	**8**	**10**
Group B:	**114.48** (98.6-131)	**104.5** (90.7-114)	**89.82** (84.2-101.5)	**71.25** (58.5-72.3)	**40.36** (33.5-58)	**14.16** (11.7-25)	**10.77** (3.4-23.5)	**6.89** (1.9-18)	**4.81** (1.2-14)
Group C:	**138.5** (117-149)	**119.19** (90-135)	**99.56** (88-100)	**77.95** (62-86.5)	**53.3** (32-60)	**15.96** (6.9-21)	**5.98** (3.7-12.4)	**3.66** (2.8-12)	**3.25** (1.5-8.2)

Oral:

Time (hr):	**0.25**	**0.5**	**1**	**2**	**3**	**4**	**6**	**8**	**10**
Group B:	**27.74** (12.6-55.6)	**39.68** (14.7-78)	**63.75** (16.2-71)	**36.69** (18-55.7)	**26.69** (14.9-33.9)	**16.28** (12.8-27)	**8.94** (7.6-23.7)	**6.5** (3.1-15)	**2.38** (2.2-8.5)
Group C:	**34.32** (24.3-95.5)	**56.01** (25-105)	**55.17** (26.6-92.8)	**38.95** (22.7-68.3)	**27.56** (15.4-61.2)	**23.92** (10.5-46.3)	**12.36** (4.5-22.9)	**8.61** (1.8-17.6)	**5.55** (0.9-17)

Table 8.4: Plasma pharmacokinetic parameters of valproic acid, median (range), in rabbits after intravenous and oral administration of valproic acid alone (group B) and valproic acid with acyclovir (group C).

Route	Valproic acid after intravenous administration		Valproic acid after oral administration	
Group	Group B	Group C	Group B	Group C
No. rabbits	**5**	**5**	**5**	**5**
Sex	2M 3F	2M 3F	2M 3F	2M 3F
Wt (kg)	**3.5** (3.1-3.8)	**4** (3.5-4.2)	**3.8** (3.4-4.5)	**4** (3.5-4.3)
Dose (mg)	**70** (62-76)	**80** (70-84)	**76** (64-90)	**80** (70-86)
Co (mg/L)	**106.6** (78-123)	**113** (92.4-124)	**67.99** (22.5-113)	**40.12** (26.7-167)
Ke (/hr)	**0.33** (0.19-0.5)	**0.39** (0.3-0.5)	**0.29** (0.13-0.42)	**0.28** (0.1-0.6)
Half-life (hr)	**2.07** (1.4-3.6)	**1.79** (1.4-2.3)	**2.37** (1.67-5.2)	**2.49** (1.3-13.4)
AUC (mg.hr/L)	**318.0** (224 -406)	**281.1** (227-371)	**227.0** (127-306)	**254.7** (154-580)
Vd (L)	**0.67** (0.7-0.95)	**0.65** (0.62-0.92)	**1.36** (0.76-4.6)	**1.17** (0.63-2.67)
CLp (L/hr)	**0.22** (0.18-0.34)	**0.28** (0.19-0.37)	**0.36** (0.21-0.63)	**0.29** (0.14-0.56)
MRT(hr)	**2.98** (2.0-5.2)	**2.58** (2.1-3.4)	**3.42** (2.4-7.5)	**3.59** (1.8-19)
F (%)	---	---	**70.1** (40-101.5)	**87.7** (67.9-106.2)
Tmax (hr)	---	---	**1.0** (0.5-2)	**1.0** (0.25-2)
Cmax (mg/L)	---	---	**64.2** (18-78)	**68.28** (37.8-105)

Key: AUC = Area under the plasma concentration-time curve; **CLp** = plasma clearance; **Cmax** = maximum concentration; **Co** = concentration after instantaneous distribution at time zero; **F** = bioavailability; **Ke** = elimination rate constant; **MRT** = mean residence time; **Tmax** = the time to reach maximum concentration; **Vd** = volume of distribution; and **Wt** = weight

8.4 DISCUSSION

This part of the study has achieved its aim of determining the pharmacokinetics of acyclovir and valproic acid when administered together in a rabbit model, thereby revealing an interaction that was difficult to predict from our current knowledge on the pharmacokinetics of the two drugs (Hayden *et al.*, 1996; McNamara *et al.*, 1996). The results of this study are relevant because the rabbit model is commonly used for evaluating the pharmacokinetics of acyclovir (Good *et al.*, 1982; Stagni *et al.,* 2004 and Chetoni *et al.*, 2004). Indeed, some of the kinetic parameters or trends as such, in the control group A were similar to those reported in humans. For instance, as shown in Table 8.2, the volume of distribution after intravenous administration of acyclovir alone to group A was 0.85 L/kg versus 0.69 ± 0.19 L/kg in humans. The total clearance of 46 ml/min was almost three times the glomerular filtration rate of the rabbit (17 ml/min), and this is similar to humans where clearance of acyclovir is 3.37 times the creatinine clearance (Cl_{cr}) and both depict tubular secretion (Laskin *et al.*, 1982; De Miranda *et al.*, 1982; Korner *et al.,* 1963; Vadstrup *et al.*, 1983). Also, after oral administration of acyclovir alone to group A, the bioavailability was within the range of 10-20% as observed in humans, while the maximum concentration (Cmax) was in the therapeutic range of 5 to 15 mg/L. As such, although human studies will still have to be done for concrete answers, these results can be used to elucidate the possible mechanism of the interaction between the two drugs.

Mechanism of interaction: It has been shown for the first time that after acute co-administration of acyclovir and valproic acid, valproic acid inhibited the tissue distribution and clearance of acyclovir, thereby leading to higher plasma concentrations of acyclovir. In addition, valproic acid inhibited the absorption of acyclovir from the gastrointestinal tract. Acyclovir, on the other hand, did not effect the concentrations of valproic acid. The latter is contradictory to the earlier clinical observation of reduced concentrations of valproic acid and phenytoin 4 days after start of acyclovir, and this was associated with breakthrough convulsions (Parmeggiani *et al.,* 1995). Also, 5 days after discontinuation of the acyclovir, the patient continued to convulse. Unfortunately, as cited earlier, the concentration of acyclovir was not measured and these events occurred on chronic co-administration of the drugs. By 'chronic co-administration' we mean that the reduction in the plasma concentration of valproic

acid could not be attributed to induction by phenytoin because the patient had been stabilized on both drugs for a long period and this was confirmed by concentration monitoring.

Nevertheless, high concentrations of acyclovir have been associated with central nervous system side effects that include convulsions (Safrin *et al.*, 2004). Therefore, the delayed onset of convulsions in the afore mentioned case report was, most probably, because valproic acid interfered with the absorption of acyclovir, leading initially to low plasma concentrations that took time to reach toxic levels as a result of the inhibited elimination. Secondly, and probably the most important, it could be because valproic acid inhibited the distribution of acyclovir to the central nervous system, and this would remain so as long as adequate levels of valproic acid were maintained. This meant that, by day 4, the levels of acyclovir were so high that a reduction in the concentrations of valproic acid, be it due to low trough levels or patient non-compliance, would lead to dis-inhibition of acyclovir tissue distribution, leading to a gash of acyclovir into the tissues, including the central nervous system, thereby causing convulsions. Non-compliance may be due to the illness under therapy or central nervous system side effects of acyclovir that precedes convulsions such as lethargy, tremors, confusion, hallucinations, agitation, disorientation, etc (Micromedex®,2007; Arndt *et al.*, 1988)

Similarly, the occurrence of convulsions 5 days after stopping acyclovir, as well as the continuous seizures that required higher doses of phenytoin to control was, most probably, because acyclovir levels were still high owing to the continual administration of valproic acid. This means that the occurrence of acyclovir induced seizures was determined by the fluctuations in valproic acid levels against a high concentration of acyclovir. In effect, this study appears not to support the claim that acyclovir causes low levels of valproic acid.

On the other hand, it had been held that valproic acid increases the anti-viral activity of acyclovir by stimulating and/or activation of viral replication, which enhances the phosphorylation of acyclovir and therefore its antiviral action (Kuntz-Simon *et al.*, 1995; Kabiri *et al.*, 2001). However, a recent report showed that it was the concentration of valproic acid rather than acyclovir that were the major determinant of the increased antiviral activity (Moattari *et al.*, 2002). They (Moattari *et al.*, 2002) insinuated that the increased antiviral

activity was due to metabolites of valproic acid and/or a product of an interaction of valproic acid with acyclovir. The observations in our study lend support to this notion by suggesting that the high concentrations of acyclovir would provide a raw medium for this interaction. In this perspective, it is possible that the high concentrations of acyclovir lead to continued consumption of valproic acid to form the so called new product/compound (Moattari *et al.*, 2002). This eventually caused the concentrations of valproic acid to drop with chronic administration. This would then explain the delay in occurrence of convulsions as due to time taken to reduce the concentration of valproic acid to sub-therapeutic levels. In this way, the findings of this study would rhyme with the claim that acyclovir may cause low levels of valproic acid.

Alternatively, the interaction may be similar to that of valproic acid with meropenem. Although the exact mechanism is still unresolved, the reduction in valproic acid concentration by meropenem was attributed to increased metabolism (glucuronidation) of valproic acid by meropenem (Nacarkucuk *et al.,* 2004 and Yamamura *et al.,* 1999). Of note, both acyclovir and meropenem are substrates of membrane transporters and are actively secreted at the renal tubule, and co-administration of either drug with valproic acid was associated with a drop in the plasma concentrations of valproic acid. Although breakthrough convulsions have been reported with the meropenem interaction with valproic acid as well (Spriet *et al.,* 2007; Santucci *et al.*, 2005), reducing the concentration of valproic acid per se does not mean occurrence of convulsions because meropenem can cause convulsions in 1-2% of patients (Micromedex®,2007). This means that an insult stimulus plays a role and, in this case, acyclovir would be implicated. In general, this single dose study has not ruled out the possibility of acyclovir reducing the plasma concentrations of valproic acid. As such, further investigations are required to determine the mechanism by which acyclovir may reduce the concentration of valproic acid on chronic co-administration.

Role of membrane transporters: The rapid absorption of acyclovir when it was combined with valproic acid can best be explained after appreciating the cause of delayed absorption in group A in which acyclovir was administered alone. It is known that acyclovir is absorbed from the gastrointestinal tract via the peptide and organic anion transporters and by passive

diffusion (O'Brien *et al.,* 1989). The importance of the transporter mechanism of absorption was further confirmed by the invention of val-acyclovir, a drug with a higher affinity for the transporter and therefore higher bioavailability than acyclovir due to its amino acid ester moiety. Therefore, the slow absorption of acyclovir in group A where it was administered alone was probably due to the associated efflux of the drug by the transporters themselves. It was shown *in vitro* that after absorption by the CaCo2 cells, val-acyclovir was hydrolyzed and the freed acyclovir was transported back into the medium at the mucosal side (De Vrueh *et al.,* 1998). Therefore, in group C where acyclovir was co-administered with valproic acid, the inhibition of transporters by valproic acid improves the efficiency of passive diffusion (i.e., fast, with no efflux), but because absorption of acyclovir by passive diffusion is limited, it wanes off quickly and there is no improvement in the overall bioavailability.

The reduced volume of distribution of acyclovir when it was combined with valproic acid indicates that valproic acid inhibits the entry of acyclovir into cells and/or distribution into tissues. This suggests that, as it were for the absorption of acyclovir from the gastrointestinal tract, acyclovir distribution into cells is via transporters.

The transporter mechanisms could also explain the marked reduction in clearance of acyclovir when the two drugs were combined after either route of administration. For instance, it is known that approximately 90% of acyclovir is excreted unchanged via the kidneys, and that the renal clearance of acyclovir is reduced by probenecid, a drug known to inhibit the organic anion transporters (Laskin *et al.,* 1982). More recently, other workers observed active renal secretion of acyclovir in rats by the organic anion transporter type 1 (hOAT1) as well as organic cationic transporter type 1 (hOCT1), and they did not rule out involvement of other transporters (Takeda *et al.,* 2002). Inhibition of these transporters by valproic acid would therefore explain the reduction in the renal clearance of acyclovir, but this remains to be confirmed.

Alternatively, the reduced volume of distribution of acyclovir when combined with valproic acid could be due to increased protein binding of acyclovir. Unfortunately, protein binding and free drug were not determined in this study. Total plasma concentrations were used because they are the most commonly monitored in the clinic. Nevertheless, only the transporter mechanism, and not protein binding, can explain the cause for the reduced volume of

distribution and clearance as well as the reduced bioavailability after oral administration of acyclovir. On the other hand, it was observed that inhibition of acyclovir by valproic acid was concentration dependent with a threshold concentration of valproic acid between 60 and 30 mg/L. Since the therapeutic range for valproic acid is 40 – 100 mg/L, this means that valproic acid should not be used together with acyclovir because the therapeutic concentrations can potentially inhibit acyclovir distribution and clearance. Nevertheless, this relationship would need clarification by dose-response studies.

Direct interaction: It appears the reduction in valproic acid bioavailability when combined with acyclovir would not be of clinical significance. Nevertheless, this observation is consistent with the *in vitro* findings of possible direct interaction between the two drugs.

In conclusion, co-administration of single doses of acyclovir with valproic acid led to reduced oral bioavailability of acyclovir, but increased concentrations of acyclovir due to reduced volume of distribution and clearance, and this was, most probably, due to inhibition of the membrane transport proteins for acyclovir by valproic acid. These observations call for a cautious approach to the concomitant use of the two drugs until human studies are done.

CHAPTER 9

CONCLUSIONS AND FUTURE STUDIES

9.1 CONCLUSIONS

The objectives of this study were achieved as follows:

9.1.1 A method for measuring the concentration of acyclovir in plasma by high performance liquid chromatography was successfully validated and applied in the study.

9.1.2 A possibility of direct chemical interaction between acyclovir and valproic was observed both *in vitro* and *in vivo*.

9.1.3 When acyclovir and valproic acid were co-administered, valproic acid significantly affected the pharmacokinetics of acyclovir. Valproic acid led to reduced oral bioavailability of acyclovir but increased concentrations of acyclovir due to reduced volume of distribution and clearance.

9.1.4 The role of membrane transport proteins in the absorption and distribution of acyclovir was suggested.

9.1.5 These observations call for a cautious approach to the concomitant use of the two drugs until human studies are done.

9.2 FUTURE STUDIES

9.2.1 There is a need to determine the compound formed in the direct interaction between acyclovir and valproic acid. This would require to undertake NMR studies on the mixture of acyclovir and valproic acid under the conditions set earlier, i.e., acidic, neutral and alkaline pH.

9.2.2 Regarding the animal studies, there is a need to undertake studies in humans to determine whether the same interaction occurs in humans and whether renal excretion of acyclovir is affected, which was not possible in rabbits.

9.2.3 Also, there is a need to identify the transporters involved and the implications to other drugs that are substrates of these transporters. Such study can be done by use of selective markers for membrane transporters.

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APPENDICES

The effect of temperature and pH on the concentration of Valproic acid

The concentration of valproic acid (ug/ml) alone in sample:

Sample	DAY 1	DAY 2	DAY 3	Mean	SD	Temp	pH
A2	57.36	73.22	63.98	**64.85**	7.97	0°C	7.4
A3	47.88	64.34	65.42	**59.21**	9.83	25°C	7.4
A4	59.64	70.22	69.78	**66.55**	5.99	37°C	7.4
A6	57.6	72.42	71.7	**67.24**	8.36	0°C	3
A7	64.98	68.78	67.08	**66.95**	1.90	25°C	3
A8	61.8	67.98	65.64	**65.14**	3.12	37°C	3
A10	64.08	72.48	70.34	**68.97**	4.37	0°C	10
A11	62.76	68.9	72.04	**67.90**	4.72	25°C	10
A12	63.3	70.48	70.28	**68.02**	4.09	37°C	10

The concentration of valproic acid (ug/ml) when in combination with acyclovir in sample:

Sample	DAY 1	DAY 2	DAY 3	Mean	SD	Temp	pH
C2	55.86	65.38	65.66	**62.30**	5.58	0°C	7.4
C3	61.86	65.9	65.72	**64.49**	2.28	25°C	7.4
C4	53.16	66.88	66.16	**62.07**	7.72	37°C	7.4
C6	55.2	62.36	67.26	**61.61**	6.07	0°C	3
C7	58.86	64.62	72.5	**65.33**	6.85	25°C	3
C8	57.12	64.48	72.24	**64.61**	7.56	37°C	3
C10	58.74	68.94	66.12	**64.60**	5.27	0°C	10
C11	60.6	62.42	70.14	**64.39**	5.06	25°C	10
C12	63	65.74	68	**65.58**	2.50	37°C	10

The Effect of Temperature and pH on the concentration of Acyclovir

The concentration of acyclovir (ug/ml) alone in sample:

Sample	DAY 1	DAY 2	DAY 3	Mean	SD	Temp	pH
B2	82.56	82.05	87.11	**83.91**	2.79	0°C	7.4
B3	76.18	89.63	88.76	**84.86**	8.63	25°C	7.4
B4	101.83	99.43	93.34	**98.20**	5.07	37°C	7.4
B6	80.60	83.53	99.08	**87.73**	11.62	0°C	3
B7	97.78	105.60	115.26	**106.52**	15.22	25°C	3
B8	80.85	92.77	92.36	**88.66**	8.19	37°C	3
B10	89.49	88.90	93.69	**90.69**	2.88	0°C	10
B11	85.11	90.16	100.11	**91.80**	9.25	25°C	10
B12	97.84	86.61	104.29	**96.25**	10.89	37°C	10

The concentration of acyclovir (ug/ml) when in combination with valproic acid in sample:

Sample	DAY 1	DAY 2	DAY 3	Mean	SD	Temp	pH
B2	82.74	86.10	99.11	**89.32**	10.23	0°C	7.4
B3	79.36	99.46	95.66	**91.49**	12.59	25°C	7.4
B4	93.46	104.12	93.43	**97.00**	7.25	37°C	7.4
B6	88.66	93.76	109.62	**97.35**	12.91	0°C	3
B7	100.22	101.00	101.82	**101.02**	1.29	25°C	3
B8	94.16	93.92	101.72	**96.60**	5.26	37°C	3
B10	107.69	90.61	91.68	**96.66**	11.29	0°C	10
B11	88.78	78.80	97.57	**88.19**	3.64	25°C	10
B12	109.53	88.85	101.12	**99.83**	12.25	37°C	10

APPENDIX B

Valproic acid concentrations after oral and IV adminstration in rabbits (n = 5)

Valproic acid alone: intravenously

Animal	Gender	Wt (kg)	Dose (mg)	0.17	0.34	0.5	1	2	4	6	8	10
30	M	3.4	70	119.3	104.5	85.85	72.29	40.36	13.32	6.78	2.57	2.09
13	F	3.7	74	98.58	92.16	84.24	58.53	33.61	25.2	23.45	18.31	13.74
19	F	3.1	62		109.8	90.63	71.25	58.04	15	13.36	9.91	6.39
67	F	3.5	70	109.6	90.65	89.82	71.83	45.74		10.77	6.89	4.81
15	M	3.8	76	130.9	114.1	101.5	66.2	33.46	11.68	3.41	1.85	1.2
Median		3.5	70	**114.5**	**104.5**	**89.82**	**71.25**	**40.36**	**14.16**	**10.77**	**6.89**	**4.81**
Minimum		3.1	62	98.58	90.65	84.24	58.53	33.46	11.68	3.41	1.85	1.2
Maximum		3.8	76	130.9	114.1	101.5	72.29	58.04	25.2	23.45	18.31	13.74

Valproic acid with acyclovir: intravenously

Animal	Gender	Wt (kg)	Dose (mg)	0.17	0.34	0.5	1	2	4	6	8	10
23	F	4.2	84	138.5	134.7	99.74	78.96	32.02	6.87	3.68	2.76	1.45
19	F	4	80	148	132.9	100.1	71.62	54.56	15.96	4.37	3.11	2.49
15	M	4.2	84	116.7	90.07	87.46	61.86	39.74	12.49	5.98	3.66	3.25
4	M	3.5	72	149.2	116.3		86.5	59.9	21	12.37	11.9	8.22
67	F	3.5	70	118.4	119.2	99.37	77.95	53.3	20.85	10.88	6.45	3.84
Median		4	80	**138.5**	**119.2**	**99.56**	**77.95**	**53.3**	**15.96**	**5.98**	**3.66**	**3.25**
Minimum		3.5	70	116.7	90.07	87.46	61.86	32.02	6.87	3.68	2.76	1.45
Maximum		4.2	84	149.2	134.7	100.1	86.5	59.9	21	12.37	11.9	8.22

Valproic acid alone: oral

Animal	Gender	Wt (kg)	Dose (mg)	0.25	0.5	1	2	3	4	6	8	10
30	M	4.2	80	12.64	14.73	16.23	18.06	14.88	12.75	8.94	8.19	2.38
13	F	3.8	76	55.59	78.07	63.75	36.69	22.36	14.32	7.56	6.43	2.3
19	F	3.6	72	23.85	39.12	71.29	55.73	33.93	20.96	8.98	3.13	2.21
67	F	3.4	64	27.74	39.68	38.69	33.27	31.72	26.96	23.68	15.04	8.45
15	M	4.5	90	37.91	58	64.21	45.41	26.69	16.28	8.06	6.5	4.98
Median		3.8	76	**27.74**	**39.68**	**63.75**	**36.69**	**26.69**	**16.28**	**8.94**	**6.5**	**2.38**
Minimum		3.4	64	12.64	14.73	16.23	18.06	14.88	12.75	7.56	3.13	2.21
Maximum		4.5	90	55.59	78.07	71.29	55.73	33.93	26.96	23.68	15.04	8.45

Valproic acid with acyclovir: oral

Animal	Gender	Wt (kg)	Dose (mg)	0.25	0.5	1	2	3	4	6	8	10
23	F	4.2	84	34.32	32.66	37.84	22.72	15.4	10.52	7.69	4.87	5.55
19	F	4	80	95.45	105	26.63	25.22	19.81	24.52	16.86	17.62	17
15	M	4.2	84	29.48	57.65	72.07	55.1	27.56	15.78	4.51	1.84	0.93
4	M	3.5	72	41.51	56.01	55.17	38.95	33.81	23.92	12.36	8.61	6.21
67	F	3.5	70	24.26	24.99	92.82	68.28	61.16	46.27	22.86	9.7	4.56
Median		4	80	**34.32**	**56.01**	**55.17**	**38.95**	**27.56**	**23.92**	**12.36**	**8.61**	**5.55**
Minimum		3.5	70	24.26	24.99	26.63	22.72	15.4	10.52	4.51	1.84	0.93
Maximum		4.2	84	95.45	105	92.82	68.28	61.16	46.27	22.86	17.62	17

APPENDIX C

The kinetic parameters of valproic acid after oral and IV administration in rabbits (n=5)

Valproic acid alone: intravenously

Animal number	Gender	Wt (kg)	Dose (mg)	Co (mg/L)	Ke (/hr)	Half-life (hr)	AUCiv (mg.hr/L)	Vd (L)	CLp (L/hr)	MRT (hr)
30	M	3.5	70	106.603	0.335	2.067	317.959	0.657	0.220	2.983
13	F	3.7	74	77.642	0.191	3.620	405.557	0.953	0.182	5.223
19	F	3.1	62	94.541	0.292	2.373	323.778	0.656	0.191	3.425
67	F	3.5	70	106.603	0.335	2.067	317.959	0.657	0.220	2.983
15	M	3.8	76	112.488	0.503	1.379	223.759	0.676	0.340	1.989
Median		**3.5**	**70**	**106.603**	**0.335**	**2.067**	**317.959**	**0.657**	**0.220**	**2.983**
Minimum		3.1	62	77.642	0.191	1.379	223.759	0.656	0.182	1.989
Maximum		3.8	76	112.488	0.503	3.620	405.557	0.953	0.340	5.223

Valproic acid with acylovir : intravenously

Animal number	Gender	Wt (kg)	Dos (mg)	Co (mg/L)	Ke (/hr)	Half-life (hr)	AUCiv (mg.hr/L)	Vd (L)	CLp (L/hr)	MRT (hr)
23	F	4.2	84	111.359	0.485	1.429	226.466	0.765	0.371	2.061
19	F	4.0	80	123.959	0.448	1.548	281.140	0.636	0.285	2.234
15	M	4.2	84	92.417	0.388	1.787	233.308	0.929	0.360	2.579
4	M	3.5	72	112.981	0.297	2.332	371.311	0.653	0.194	3.366
67	F	3.5	70	114.538	0.361	1.921	311.582	0.623	0.225	2.771
Median		**4.0**	**80**	**112.981**	**0.388**	**1.787**	**281.140**	**0.653**	**0.285**	**2.579**
Minimum		3.5	70	92.417	0.297	1.429	226.466	0.623	0.194	2.061
Maximum		4.2	84	123.959	0.485	2.332	371.311	0.929	0.371	3.366

Valproic acid alone: oral

Animal number	Gender	Wt kg	Dose (mg)	Co (mg/L)	Ke (/hr)	Half-life (hr)	AUCiv (mg.hr/L)	Vd (L)	CLp (L/hr)	MRT (hr)	Bioav: AUCor/AUCiv	Tmax	C max
30	M	4.2	80	22.465	0.136	5.096	127.063	4.630	0.630	7.354	0.400	2.000	18.060
13	F	3.8	76	76.650	0.351	1.973	208.346	1.039	0.365	2.848	0.514	0.500	78.070
19	F	3.6	72	113.100	0.415	1.669	226.953	0.764	0.317	2.408	0.701	1.000	71.290
67	F	3.4	64	41.560	0.133	5.211	305.696	1.574	0.209	7.520	0.961	0.500	39.680
15	M	4.5	90	67.990	0.292	2.375	227.071	1.358	0.396	3.427	1.015	1.000	64.210
Median		**3.8**	**76**	**67.990**	**0.292**	**2.375**	**226.953**	**1.358**	**0.365**	**3.427**	**0.701**	**1.000**	**64.210**
Minimum		3.4	64	22.465	0.133	1.669	127.063	0.764	0.209	2.408	0.400	0.500	18.060
Maximum		4.5	90	113.100	0.415	5.211	305.696	4.630	0.630	7.520	1.015	2.000	78.070

Valproic acid with acyclovir: oral

Animal number	Gender	Wt kg	Dose (mg)	Co (mg/L)	Ke (/hr)	Half-life (hr)	AUCiv (mg.hr/L)	Vd (L)	CLp (L/hr)	MRT (hr)	Bioav: AUCor/AUCiv	Tmax	C max
23	F	4.3	86	40.120	0.279	2.488	153.703	2.009	0.560	3.590	0.679	1.000	37.840
19	F	4.0	80	26.650	0.052	13.402	579.746	2.669	0.138	19.339	1.062	0.500	104.990
15	M	4.2	84	140.920	0.551	1.259	204.690	0.745	0.410	1.816	0.877	1.000	72.070
4	M	3.7	74	26.650	0.247	2.802	254.657	1.175	0.291	4.043	0.686	0.500	56.010
67	F	3.5	70	167.440	0.353	1.965	315.636	0.629	0.222	2.836	1.013	2.000	68.280
Median		**4.0**	**80**	**40.120**	**0.279**	**2.488**	**254.657**	**1.175**	**0.291**	**3.590**	**0.877**	**1.000**	**68.280**
Minimum		3.5	70	26.650	0.052	1.259	153.703	0.629	0.138	1.816	0.679	0.500	37.840
Maximum		4.3	86	167.440	0.551	13.402	579.746	2.669	0.560	19.339	1.062	2.000	104.990

APPENDIX D

Acyclovir concentrations after oral and IV adminstration in rabbits (n = 5)

Acyclovir alone: intravenously

Animal	Gender	Wt (kg)	Dose (mg)	0.17	0.34	0.5	1	2	4
10	M	3.5	210	41.55	44.18	39.75	19	6.83	0.88
16	M	3.8	288	47.08	43.11	29.74	17.38	2.65	0.31
24	F	3.6	216	54.41	33.25	14.02	2.26		
12	F	3.5	210	51.37	46.81	33.53	10.56	2.81	
32	F	3.8	225	57.73	40.3	23.43		2.95	0.6
Median		3.6	216	**51.37**	**43.11**	**29.74**	**13.97**	**2.88**	**0.6**
Minimum		3.5	210	41.55	33.25	14.02	2.26	2.65	0.31
Maximum		3.8	288	57.73	46.81	39.75	19	6.83	0.88

Acyclovir with valproic acid: intravenously

Animal	Gender	Wt (kg)	Dose (mg)	0.17	0.34	0.5	1	2	4	6	8
19	F	4	240	712.9	584	582.6	396.8	15.44	3.045		
4	M	3.5	210	646.6		55.34	53.04	23.03	14.58		4.998
23	F	4.2	250	911.8	821	742.1	567.8	15.97	6.584	3.855	2.181
67	F	3.5	210	644.7	597.3	434.6	362	23.84	11.41	4.386	1.059
15	M	4.2	250	504.9	561	498.9	33.55	16.46	4.778	1.407	
Median		4	240	**646.6**	**590.6**	**498.9**	**362**	**16.46**	**6.584**	**3.855**	**2.181**
Minimum		3.5	210	504.9	561	55.34	33.55	15.44	3.045	1.407	1.059
Maximum		4.2	250	911.8	821	742.1	567.8	23.84	14.58	4.386	4.998

Acyclovir alone: oral

Animal	Gender	Wt (kg)	Dose (mg)	0.25	0.5	1	2	3	4	6	8
10	M	3.8	1140	3.259	4.177	4.067	4.575	4.575	2.948	1.693	
16	M	3.8	1140	3.743	1.582	1.379	1.99	2.004	2.878	2.543	1.065
24	F	4	1200	4.858	1.61	3.644	3.873	4.147	2.962	1.815	1.531
12	F	3.5	1050	3.1	4.187	4.541	4.875	5.195	6.467	1.551	1.746
32	F	4.2	1260	5.189	3.973	10.74	6.993	4.737	2.87		2.086
Median		**3.8**	**1140**	**3.743**	**3.973**	**4.067**	**4.575**	**4.575**	**2.948**	**1.754**	**1.639**
Minimum		3.5	1050	3.1	1.582	1.379	1.99	2.004	2.87	1.551	1.065
Maximum		4.2	1260	5.189	4.187	10.74	6.993	5.195	6.467	2.543	2.086

Acyclovir with valproic acid : oral

Animal	Gender	Wt (kg)	Dose (mg)	0.25	0.5	1	2	3	4	6	8	10
19	M	4	1200	2.127	11.86	8.648	4.126	3.846	2.602	8.375	2.886	2.492
4	M	3.7	1110	22.25	21.15	9.099	9.06	5.392	5.263	3.632		0.366
23	F	4.3	1290	30.55	25.3	18.36	14.04	8.844	8.5	5.025	4.315	1.4
67	F	3.5	1050	0.854	0.933	4.11	8.663	4.783	5.025	2.309	1.888	0.587
15	F	4.2	1260		0.684	5.472	4.047	1.63	1.679	1.057	0.54	0.556
Median		**4**	**1200**	**12.19**	**11.86**	**8.648**	**8.663**	**4.783**	**5.025**	**3.632**	**2.387**	**0.587**
Minimum		3.5	1050	0.854	0.684	4.11	4.047	1.63	1.679	1.057	0.54	0.366
Maximum		4.3	1290	30.55	25.3	18.36	14.04	8.844	8.5	8.375	4.315	2.492

Appendix E

The kinetic parameters of acyclovir after oral and IV administration in rabbits (n=5)

Acyclovir alone: intravenously

Animal number	Gender	Wt (kg)	Dose (mg)	Co (mg/L)	Ke (/hr)	Half-life (hr)	AUCiv (mg.hr/L)	Vd (L)	CLp (L/hr)	MRT (hr)
10	M	3.5	210.0	68.877	0.678	1.021	101.526	3.049	2.068	1.474
16	M	3.8	288.0	75.908	0.885	0.783	85.799	3.794	3.357	1.130
24	F	3.6	216.0	83.324	1.795	0.386	46.412	2.592	4.654	0.557
12	F	3.5	210.0	69.073	0.864	0.802	79.903	3.040	2.628	1.157
32	F	3.8	225.0	63.497	0.778	0.891	81.646	3.544	2.756	1.286
Median		**3.6**	**216.0**	**69.073**	**0.864**	**0.802**	**81.646**	**3.049**	**2.756**	**1.157**
Minimum		3.5	210.0	63.497	0.678	0.386	46.412	2.592	2.068	0.557
Maximum		3.8	288.0	83.324	1.795	1.021	101.526	3.794	4.654	1.474

Acylovir with valproic acid: intravenously

Animal number	Gender	Wt (kg)	Dose (mg)	Co (mg/L)	Ke (/hr)	Half-life (hr)	AUCiv (mg.hr/L)	Vd (L)	CLp (L/hr)	MRT (hr)
19	F	4	240.0	1005.934	1.543	0.449	800.948	0.194	0.299	0.648
4	M	3.5	210.0	126.039	0.456	1.521	508.995	0.881	0.401	2.195
23	F	4.2	250.0	640.476	0.838	0.827	1094.113	0.272	0.228	1.193
67	F	3.5	210.0	528.686	0.827	0.838	751.653	0.337	0.279	1.209
15	M	4.2	250.0	370.917	1.031	0.672	675.940	0.359	0.370	0.970
Median		**4**	**240.0**	**528.686**	**0.838**	**0.827**	**751.653**	**0.337**	**0.299**	**1.193**
Minimum		3.5	210.0	126.039	0.456	0.449	508.995	0.194	0.228	0.648
Maximum		4.2	250.0	1005.934	1.543	1.521	1094.113	0.881	0.401	2.195

Acyclovir alone: oral

Animal number	Gender	Wt kg	Dose (mg)	Co (mg/L)	Ke (/hr)	Half-life (hr)	AUCiv (mg.hr/L)	Vd (L)	CLp (L/hr)	MRT (hr)	AUC-F AUCor/AUCiv	Tmax (h)	C max (mg/L)
10	M	3.8	1140.0	11.533	0.324	2.141	25.926	6.493	2.102	3.089	0.048	3.000	4.575
16	M	3.8	1140.0	8.808	0.249	2.788	21.310	13.494	3.354	4.023	0.063	4.000	2.880
24	F	4	1200.0	6.881	0.199	3.477	29.857	23.390	4.662	5.017	0.116	3.000	4.147
12	F	3.5	1050.0	18.511	0.327	2.117	35.702	8.032	2.630	3.054	0.089	4.000	6.467
32	F	4.2	1260.0	16.769	0.435	1.593	38.717	6.347	2.761	2.299	0.085	1.000	10.743
Median		**3.8**	**1140.0**	**11.533**	**0.324**	**2.141**	**29.857**	**8.032**	**2.761**	**3.089**	**0.085**	**3.000**	**4.575**
Minimum		3.5	1050.0	6.881	0.199	1.593	21.310	6.347	2.102	2.299	0.048	1.000	2.880
Maximum		4.2	1260.0	18.511	0.435	3.477	38.717	23.390	4.662	5.017	0.116	4.000	10.743

Acylovir with valproic acid: oral

Animal number	Gender	Wt kg	Dose (mg)	Co (mg/L)	Ke (/hr)	Half-life (hr)	AUCiv (mg.hr/L)	Vd (L)	CLp (L/hr)	MRT (hr)	AUC-F AUCor/AUCiv	Tmax (h)	C max (mg/L)
19	F	4	1200.0	16.751	0.505	1.372	44.464	0.794	0.401	1.979	0.015	0.500	18.364
4	M	3.7	1110.0	11.432	0.196	3.536	56.159	2.048	0.401	5.103	0.020	0.250	22.246
23	F	4.3	1290.0	28.088	0.278	2.491	76.338	1.442	0.401	3.594	0.024	0.250	30.554
67	F	3.5	1050.0	12.220	0.248	2.794	35.977	1.618	0.401	4.031	0.014	2.000	8.663
15	M	4.2	1260.0	6.693	0.334	2.074	18.055	1.201	0.401	2.993	0.006	1.000	5.472
Median		**4**	**1200.0**	**12.220**	**0.278**	**2.491**	**44.464**	**1.442**	**0.401**	**3.594**	**0.015**	**0.500**	**18.364**
Minimum		3.5	1050.0	6.693	0.196	1.372	18.055	0.794	0.401	1.979	0.006	0.250	5.472
Maximum		4.3	1290.0	28.088	0.505	3.536	76.338	2.048	0.401	5.103	0.024	2.000	30.554

APPENDIX F

PUBLICATIONS

A. Conference abstracts

1. **MFPC Van Jaarsveld** and A. Walubo. **The effect of temperature and pH on valproic acid and acyclovir *in vitro*.** 39th Annual Congress of the South African Pharmacology Society. Riverside Hotel, 20 - 23rd September 2006. Also at Faculty Form 2006; Faculty of Health Sciences; UFS.

2. **MFPC van Jaarsveld**, A Walubo and JB du Plessis. **The Effect of Valproic Acid on Acyclovir After Intravenous and Oral Administration in a Rabbit Model**. The First African Conference on Drug metabolism and Development, 21st – 24th May 2007, Kempton-Park, Johannesburg, South Africa. Also, the 36th Annual conference of the American College of Clinical Pharmacology, San Francisico, September, 2007 and Faculty Form 2007; Faculty of Health Sciences; UFS.

THE EFFECT OF TEMPARATURE AND pH ON VALPROIC ACID AND ACYCLOVIR *IN VITRO.*

MFPC van Jaarsveld and A Walubo, Department of Pharmacology, University of the Free State.

Introduction and aim: Valproic acid (VPA) is an antiepileptic drug that is widely used for treatment of epilepsy. Unfortunately, it has a narrow therapeutic concentration range that predisposes it to adverse drug interactions and hence the need for therapeutic drug monitoring. Acyclovir (ACV), on the other hand, is an antiviral drug indicated for treatment of infections caused by herpes simplex type I & II and varicella-zoster viruses, and is often prescribed to patients on treatment with VPA. Parmeggiani and co-workers (1994) reported a pharmacokinetic interaction between ACV and VPA which was heralded by reduced plasma levels of VPA and break through convulsions. This was followed by Moattari and co-workers (2002) who described the possibility of a direct interaction between ACV and VPA. Unfortunately, the mode of interaction between ACV and VPA is still not well understood. Therefore, the aim of this study was to investigate the possibility of a direct interaction between VPA and ACV at different pH and temperatures *in vitro*.

Methods: The concentrations of VPA and ACV, prepared separately or as a mixture of the two, were monitored in buffers at different pH and temperatures. The test samples consisted of 1 ml buffer at pH 7.4 or pH 3 or pH 10, containing either ACV, 112.6 μg/ml (5 mM), or VPA sulphate, 72.13 μg/ml (5 mM), or both in a 1:1 molar ratio. The samples were incubated at 25°C for 2 hours and a further 1 hour at 37°C. Aliquotes of 200 μl were drawn at 10 min. and at 2 and 3 hours to measure the concentration of VPA and /or ACV, and the experiments were repeated three times (n=3) on different days. The concentrations of VPA were determined using an Enzyme-linked Immunoabsorbent Assay from Abbott Diagnostics and the concentrations of ACV were analyzed by a validated HPLC method. Results were compared using ANOVA Tukey-Kramer Multiple Comparisons Test with the level of significance at $p < 0.05$.

Results: The concentrations of VPA and ACV alone were not different ($P > 0.05$) from those in the mixture of both drugs at the different temperatures and pH. However, when the temperature and pH were evaluated separately, there was a trend whereby at high temperature (37°C) the concentrations of ACV (percentage detected) tended to be higher in the mixture (87%) than when it was alone (84%), while those of VPA tended to be lower in the mixture (89%) than when it was alone (92%). This same trend was observed at acid or alkaline pH.

Conclusion: Although temperature and pH did not induce significant effects on the concentrations of both ACV and VPA, increased concentrations of ACV were associated with reduced concentration of VPA when the two drugs were mixed under constrained conditions and these observations require further studies.

THE EFFECT OF VALPROIC ACID ON ACYCLOVIR AFTER INTRAVENOUS AND ORAL ADMINISTRATION IN A RABBIT MODEL

MFPC van Jaarsveld, A Walubo, J du Plessis

Introduction and aim: Valproic acid (VPA), an antiepileptic drug, and acyclovir (ACV), an antiviral drug, is often co-administered to patients. Parmeggiani and co-workers (1994) reported a pharmacokinetic interaction between ACV and VPA which was heralded by reduced plasma levels of VPA and break through convulsions, followed by Moattari and co-workers (2002) who described the possibility of a direct interaction between ACV and VPA. Therefore, the aim of this study was to investigate the effect of co-administration of the two drugs on the pharmacokinetic parameters of each drug.

Methods: New Zealand white rabbits were divided into 3 groups. Group A received ACV, group B VA and group C both drugs. The intravenous dose of ACV was 60 mg/kg and the oral dose 300 mg/kg due to low bioavailability (20%). The dose of VA IV and oral were 20 mg/kg.

Results: The plasma concentrations of ACV (IV) group C were 10 times higher than group A. The AUC group C (median) was significantly higher than for group A, i.e., 751mg.hr/L versus 81.7 mg.hr/L. Furthermore, both the volume of distribution (Vd) and plasma clearance (Clp) of ACV were significantly higher in group A than group C: 3.1 L versus 0.34 L , while CLp was 2.76 L/hr versus 0.29 L/hr. The plasma concentrations of ACV (orally) in group C, were 3-4 times higher than in group A. Similar to IV, the Vd and CLp of ACV were higher in group A than group C. Surprisingly, the absorption of ACV was slower in group A than in group C (Tmax 3hr versus 0.5hr). Also, the bioavailability (F) of ACV was lower in group C (1.6%) than in group A (8%). There was no difference in the concentrations and kinetic parameters of VA between group B and group C.

Conclusion: Co-administration of ACV with VA led to reduced absorption, Vd and CLp of ACV, as well as a modification in the absorption characteristics of ACV, and this was most probably due to inhibition of the membrane transport proteins for ACV by VA acid.

B. Journal article

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SUMMARY

Key terms: valproic acid, acyclovir, drug interaction, high performance liquid chromatography, pharmacokinetic parameters, membrane transport proteins.

Valproic acid is an antiepileptic drug that is widely used for treatment of epilepsy, while acyclovir is an antiviral drug indicated for treatment of infections caused by herpes simplex type I & II and varicella-zoster viruses. Given the high prevalence of people with conditions for which chronic use of valproic acid is indicated, and the notion that valproic acid increases the antiviral activity of acyclovir, it is not uncommon for the two drugs to be used concomitantly. As such, recent reports on the interaction between valproic acid and acyclovir with break through convulsions were a cause for concern. Since understanding the mechanism of this interaction is vital to the establishment of concrete guidelines on the use of the two drugs in patients, the aim of this study was to investigate the possible pharmacokinetic interaction between acyclovir and valproic acid.

First, a high performance liquid chromatography (HPLC) method for analysis of acyclovir in plasma was developed. It involved simple protein precipitation of 200 µl of plasma with perchloric acid, followed by centrifugation after which 20 µl of the supernatant was injected in the HPLC. The sample was eluted with acetonitrile: octanesulfonic acid: ammonium acetate-citrate (vol./vol.; 5%:11.88%:83.12%) at 1.5 ml/min over a Luna C_{18} (4.60 x 150 mm) 5µ analytical column. Gancyclovir was used as the internal standard. Under these conditions, gancyclovir eluted at 3.4 min and acyclovir at 4.5 min. Over the calibration range of 10 - 100 µg/mL, linearity was demonstrated by a linear regression equation of $y = 0.03196 - 3.207x$ with a regression coefficient $r^2 = 0.995$, and accuracy by a percentage coefficient of variation (CV%) of less than 15% . The method was successfully used to analyze acyclovir in a rabbit treated with acyclovir single dose.

Thereafter, the possibility of a direct interaction between acyclovir and valproic acid *in vitro* was investigated by monitoring the concentrations of valproic acid and acyclovir at different pH (pH 7.4 or pH 3 or pH 10) and temperatures (25^{o}C and 37^{o}C) when mixed in a 1:1 molar ratio or prepared separately in phosphate buffer. The samples were incubated at 25^{o}C for 2 hours and a further 1 hour at 37^{o}C, and aliquots were drawn at 10 min., 2 and 3 hours to measure the concentration of valproic acid and acyclovir (n=3). The average concentrations of valproic acid and acyclovir from the samples containing the single drug were not different ($P > 0.05$) from those in the mixture of both drugs at the different temperatures and pH. However, when the temperature and pH were evaluated separately, there was a trend whereby, at high temperature (37^{o}C), the concentrations of acyclovir (percentage detected) tended to be higher in the mixture (87%) than when it was alone (84%), while those of valproic acid tended to be lower in the mixture (89%) than when it was alone (92%). This same trend was observed at acid or alkaline pH. In conclusion, although temperature and pH did not induce significant effects on the concentrations of both acyclovir and valproic acid, increased concentrations of acyclovir were associated with reduced concentration of valproic acid when the two drugs were mixed under constrained conditions. These observations suggested a possible direct interaction between the two drugs.

This final part of the study was undertaken to investigate the effect of co-administration of valproic acid and acyclovir on the pharmacokinetic parameters of each other in a rabbit model. Fifteen white New Zealand rabbits were divided into 3 groups A, B and C whereby group A received acyclovir only, group B received valproic acid only, and group C received a combination of acyclovir and valproic acid. In a cross-over design, the intravenous route was studied first, followed by the oral route after a two-week wash out period. Blood samples were drawn over a 10 hr period and the pharmacokinetic parameters were derived from the concentrations. After intravenous administration, the area under the

plasma concentration time curve (AUC) and plasma concentrations of acyclovir in group C were higher than in group A, while the volume of distribution (Vd) and plasma clearance (CLp) of acyclovir in group C were only 12.8% and 10.36% of those of group A, respectively. A similar trend was observed after oral administration. However, the bioavailability (F) of acyclovir was 8.4% in group A versus 1.5% in group C. Of note, the concentrations and kinetic parameters of valproic acid between the two groups after oral and intravenous administration were not different. In conclusion, co-administration of single doses of acyclovir and valproic acid led to reduced oral bioavailability of acyclovir, but increased concentrations of acyclovir due to reduced volume of distribution and clearance and this was most probably due to inhibition of the membrane transport proteins for acyclovir by valproic acid.

Overall, a simple and accurate HPLC method for analysis of acyclovir in plasma was successfully developed, and a possibility of direct interaction between the two drugs was observed both *in vitro* and *in vivo*. These observations call for a cautious approach to the concomitant use of the two drugs until human studies are done.

OPSOMMING

Sleutelterme: Valproaatsuur, asiklovir, geneesmiddelinteraksie, hoë druk vloeistof chromatografie, farmakokinetiese parameters, membraan transport proteïene.

Valproaatsuur is 'n breë spektrum anti-epileptiese geneesmiddel wat algemeen aangewend word in die behandeling van epilepsie. Hierteenoor is asiklovir 'n antivirale geneesmiddel wat ge-indikeerd is in die behandeling van herpes simpleks tipe I en II asook varisella-zoster virale infeksies. Vanwee die hoë voorkoms van pasiënte met toestande waarvoor chroniese valproaatsuur gebruik aangedui is, tesame met die wete dat valproaatsuur die antivirale aktiwiteit van asiklovir verhoog, is dit nie ongewoon om 'n kombinasie van dié geneesmiddels aan pasiënte voor te skryf nie. Om hierdie rede is die onlangse gevallestudie van 'n interaksie tussen valproaatsuur en asiklovir, wat aanleiding gegee het tot deurbraakkonvulsies, 'n rede tot kommer. Dit is noodsaaklik om die meganisme van dié interaksie te verstaan vir die daarstelling van konkrete riglyne aangaande die gebruik van die middels in pasiënte en daarom was die doel van hierdie studie dus 'n ondersoek na die moontlike interaksie tussen asiklovir en valproaatsuur.

Eerstens is 'n hoë druk vloeistof chromatografie (HPLC) metode ontwikkel waarmee die plasmakonsentrasie van asiklovir bepaal kan word. Die metode het die volgende behels: proteïen presipitering van 200 µl plasma met perchloor suur, gevolg deur sentrifugering en inspuiting van 20 µl van die bodrywende vloeistof in die HPLC. Die monster is oor 'n Luna C_{18} (4.60 x 150 mm) 5µ analitiese kolom gedra deur asetonitriel: oktanesulfoniese suur: ammonium asetaat-sitraat (vol./vol.; 5%:11.88%:83.12%) teen 1.5 ml/min en gansiklovir is as interne standaard gebruik. Onder hierdie toestande het die piek van gansiklovir teen 3.4 min. en die van asiklovir teen 4.5 min. uitgekom. Oor 'n kallibrasie

reikwydte van 10 - 100 µg/mL is lineariteit gedemonstreer deur die liniëre regressie vergelyking y = 0.03196 – 3.207x met 'n regressie koëffisiënt r^2 = 0.995, en akkuraatheid deur 'n persentasie koëffisiënt van variëring (CV%) van minder as 15% . Die metode is suksesvol gebruik om die plasmakonsentrasie van asiklovir te bepaal in die plasma van 'n konyn na toediening van 'n enkeldosering asiklovir.

Hierna is die moontikheid van 'n direkte *in vitro* interaksie tussen asiklovir en valproaatsuur ondersoek deur die konsentrasies van asiklovir en valproaatsuur te monitor by verskillende pH (pH 7.4 of pH 3 of pH 10) en temperature (25^{o}C en 37^{o}C) gemeng in 'n 1:1 molêre verhouding, of afsonderlik, in fosfaatbuffer voorberei. Die monsters is geïnkubeer teen 25^{o}C vir 2 ure en daarna vir nog 1 uur teen 37^{o}C, deelvolumes is geneem teen 10 min., 2 en 3 ure om die konsentrasie van asiklovir en valproaatsuur te bepaal (n=3). Die gemiddelde konsentrasies van asiklovir en valproaatsuur van die monsters wat die afsonderlike geneesmiddels bevat het, het nie verskil (P > 0.05) van die wat die kombinasie geneesmiddel bevat het by die verskillende temperatuur en pH nie. Nietemin, toe die uitwerking van temperatuur en pH op die konsentrasie van die geneesmiddels apart van mekaar geëvalueer is, is 'n neiging geïdentifiseer waar, teen hoër temperatuur (37^{o}C), die konsentrasie van asiklovir (persentasie gemeet) hoër was in die monsters wat beide middels bevat het (87%) vergelyk met die monsters wat 'n enkel geneesmiddel bevat het (84%); in teenstelling hiermee was die konsentrasie van valproaatsuur (persentasie gemeet) laer in die monsters wat beide middels bevat het (89%) vergelyk met die monsters wat 'n enkel geneesmiddel bevat het (92%). Dieselfde neiging is geobserveer met suur en alkaliese pH. Samevattend dus: alhoewel temperatuur en pH nie 'n statisties betekenisvolle effek op die konsentrasies van asiklovir en valproaat suur gehad het nie, is verhoogde konsentrasies van asiklovir geassosiëer met verlaagde konsentrasies van valproaat suur wanneer die twee geneesmiddels gemeng is

onder uiterste fisiologiese toestande. Hierdie observering suggereer die moontlikheid van 'n direkte interaksie tussen die twee geneesmiddels.

Die finale deel van hierdie studie is onderneem om die effek van kombinasietoediening van asiklovir en valproaatsuur op die farmakokinetiese parameters van elke geneesmiddel in 'n konynmodel te ondersoek. Vyftien wit New Zealand konyne is verdeel in 3 groepe A, B en C. Groep A het slegs asiklovir ontvang, groep B het slegs valproaatsuur ontvang en groep C het 'n kombinasie van asiklovir en valproaatsuur ontvang. In 'n oorkruis studie-ontwerp is die intraveneuse roete eerste bestudeer, gevolg deur 'n twee weke uitwasperiode waarna die middels oral toegedien in. Bloedmonsters is getrek oor 'n 10 h periode en die farmakokinetiese parameters is afsonderlik verkry vanaf die konsentrasie versus tydprofiel van elke dier. Na intraveneuse toediening was die area onder die plasma konsentrasie versus tydkurwe (AUC) en die plasma konsentrasies van asiklovir in groep C hoër as dié in groep A, terwyl die volume van distribusie (Vd) en plasma opruiming (CLp) van asiklovir in groep C respektief slegs 12.8% en 10.36% van die waarde van groep A was. 'n Soortgelyke neiging is gevind na orale toediening. Nietemin was die biobeskikbaarheid (F) van asiklovir 8.4% in groep A versus 1.5% in groep C. Interessant is dat gedurende hierdie studie geen statisties betekenisvolle verskil in die plasma konsentrasies sowel as kinetiese parameters van valproaatsuur tussen groep B en groep C gevind na oraal en intraveneuse toediening nie. Samevattend dus, kombinasie toediening van 'n enkel dosering asiklovir en valproaatsuur het gelei tot verminderde biobeskikbaarheid van asiklovir maar verhoogde plasma konsentrasies van asiklovir as gevolg van 'n verlaging in volume van distribusie en opruiming en dit was mees waarskynlik as gevolg van inhibisie van membraan transport proteïene van asiklovir deur valproaatsuur.

Ten slotte dus, 'n eenvoudig dog akkurate HPLC metode is suksesvol ontwikkel waarmee die konsentrasie van asiklovir in plasma bepaal kan word en die moontlikheid van 'n direkte interaksie tussen asiklovir en valproaatsuur is

waargeneem beide *in vitro* en *in vivo*. Hierdie waarneming maan tot versigtige gebruik van hierdie geneesmiddels in kombinasie in pasiënte totdat verdere menslike studies gedoen is.

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Interaction between Valproic Acid and Acyclovir after Intravenous and Oral Administration in a Rabbit Model

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Abstract: This study was undertaken to investigate the effect of co-administration of valproic acid and acyclovir on the pharmacokinetic parameters of each other. Fifteen white New Zealand rabbits were divided into three groups: A, B and C. Group A received acyclovir only, group B received valproic acid only and group C received a combination of acyclovir and valproic acid. In a cross-over design, the intravenous route was studied first, followed by the oral route after a 2-week wash-out period. Blood samples were drawn over 10 hr and the pharmacokinetic parameters were derived from the concentrations. After intravenous administration, the area under the plasma concentration time curve and plasma concentrations of acyclovir in group C were higher than in group A, while the volume of distribution and plasma clearance of acyclovir in group C were only 12.8% and 10.36% of those of group A, respectively. A similar trend was observed after oral administration. However, the bioavailability (F) of acyclovir was 8.4% in group A versus 1.5% in group C. In addition, the concentrations and kinetic parameters of valproic acid between the two groups after oral and intravenous administration were not different. In conclusion, co-administration of single doses of acyclovir and valproic acid led to reduced oral bioavailability of acyclovir, but increased concentrations of acyclovir due to reduced volume of distribution and clearance. These observations call for a cautious approach to the concomitant use of the two drugs until human studies are done.

Valproic acid is an antiepileptic drug that is widely used as a first-line agent or adjunctive therapy for most types of seizures. It has a narrow therapeutic concentration range with well-recognized dose-related side effects of the central nervous system [1]. Therefore, therapeutic drug monitoring is important for optimization of treatment with valproic acid. Acyclovir, on the other hand, is an antiviral drug indicated for treatment of infections caused by herpes simplex, both type I and II, and varicella-zoster viruses [2]. Given the high prevalence of people with conditions for which chronic use of valproic acid is indicated, and the notion that valproic acid increases the antiviral activity of acyclovir, it is not uncommon for the two drugs to be used concomitantly. This is evidenced by reports of possible interaction between the two drugs [3,4]. In the first report, a possible pharmacokinetic interaction between the acyclovir and valproic acid that was associated with break-through convulsions [3], while in the second report it was a coincidental finding whereby, while investigating the potentiation of the antiherpetic effect of acyclovir by valproic acid *in vitro*, a possibility of a direct interaction between acyclovir and valproic acid was suggested [4]. Such an interaction would lead to reduced plasma levels of both drugs because they form a new product.

Unfortunately, there was no information on the concentrations of acyclovir during the clinical incident. This is important because the convulsions may be due to reduced plasma levels of valproic acid or high concentrations of acyclovir, or both. Convulsions expose patients to risk of death or neurological complications owing to cerebral hypoxia and oedema that may develop. Furthermore, it was noted that poorly controlled epilepsy was a major risk factor for sudden unexpected deaths in patients treated with more than one drug [5]. As such, the interaction between valproic acid and acyclovir with breakthrough convulsions is a cause for concern. We envisaged that understanding the mechanism of this interaction is vital to the establishment of concrete guidelines on the use of the two drugs in patients. Therefore, this study was undertaken to investigate the effect of co-administration of the two drugs on the pharmacokinetic parameters of each other.

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Materials and Methods

Materials. Acyclovir and valproic acid intravenous formulations were purchased from a local pharmacy. For acyclovir the formulation was Zovirax®, 250 mg/vial manufactured by GlaxoSmithKline, while for valproic acid, it was Epilim®, 400 mg/vial by Sanofi-Aventis (New York, NY, USA). However, because these formulations were too dilute and expensive, the oral formulations were prepared from laboratory standards using normal saline. Acyclovir powder was a donation from the local representative for GlaxoSmithKline, while valproic acid was purchased from Sigma Chemical Co. (Steinheim, Germany) and both were of analytical grade. The rabbits were obtained from the University Animal House where they were cared for before and during the study period.

Animal experiment. The study was approved by the Animals Ethics Committee of the University of the Free State. Fifteen white New Zealand rabbits of either sex were used. The animals were divided

randomly into three groups: A, B and C. Group A received acyclovir only, group B received valproic acid only and group C received a combination of acyclovir and valproic acid. The intravenous dose of acyclovir was 60 mg/kg while the oral dose was 300 mg/kg. For valproic acid, the intravenous and oral doses were the same, 20 mg/kg. In a cross-over design, the intravenous route was studied first, followed by the oral route after a 2-week wash-out period. The high dose of oral acyclovir (300 mg/kg) was to compensate for the low oral bioavailability of 10–20% [6]. This was not necessary for valproic acid because it has a bioavailability of more than 80% [7]. The dose of valproic acid was based on the dose used for treatment of epilepsy [8], while for acyclovir the dose was within range to that used for treatment of cytomegalovirus [9]. In addition, a similar dose of acyclovir was used in previous experiments involving rabbits by Testereci et al. [10]. Intravenous administration was via the lateral ear vein while a plastic catheter was used for oral administration of the drugs in solution. Blood samples (0.5–1 ml) were drawn from the central ear artery over a 10-hr period. Each sample was centrifuged immediately after collection and the plasma was stored at −20°C until ready for drug concentration assay.

Analysis of valproic acid. Total plasma concentrations of valproic acid were measured by Fluorescence Polarization Immunoassay using an automated immunoassay analyser (Axsym System, Abbott Laboratories, Abbott Park, IL, USA). This involved use of kits with which drug standards for calibrations were supplied. The kits were used according to the manufacturer's instructions (Abbott Diagnostics, Abbott Park, IL, USA) and involved preliminary precipitation of plasma proteins with methanol. Plasma samples of 100 µl each were ran in duplicates and the average was the ultimate concentration measured. This method is robust and accurate, and has been used in our laboratory for patient therapeutic drug monitoring of valproic acid for over 15 years.

Analysis of acyclovir. Total plasma concentrations of acyclovir were analysed by a validated high performance liquid chromatography (HPLC) method based on published methods [11,12]. Briefly, to 200 µl of plasma was added the internal standard, ganciclovir, to a final concentration of 20 µg/ml after which proteins were precipitated by adding 60 µl of 2 M perchloric acid. The sample was vortexed for 15 sec., centrifuged at 1000 ×g for 10 min. and 20 µl of the supernatant was injected into the HPLC. The HPLC system consisted of Hewlett-Packard 1100 series system that included a quaternary pump, degasser, auto sampler and variable-wavelength ultraviolet detector. Separation was achieved using a Phenomenex C_{18} (4.60 × 150 mm) 5 µ analytical column coupled to Security Guard cartridge C_{18} pre-column (4 × 3 mm). The mobile phase consisted of 10 mM acetate/citrate buffer with 3.7 mM aqueous octanesulfonic acid, adjusted with phosphoric acid to pH 3.08. It was pumped at 1.5 ml/min. and the eluate was detected by ultraviolet at 254 nm. Linearity and accuracy were assessed using 7 days' daily calibration curves at acyclovir concentrations of 10, 20, 40, 60, 80 and 100 mg/l. Under these conditions, ganciclovir eluted at 4 min. and acyclovir eluted at 2.77 min. The linear regression equation was $y = 0.03196 - 3.47x$ with a regression coefficient (r) of = 0.995 and coefficient of variation (CV%) of 11.7% at low concentrations (20 mg/l) and 4.68% at higher concentrations (60 mg/l). The method was simple, accurate and fast.

Pharmacokinetic data analysis. Pharmacokinetic parameters of acyclovir and valproic acid for each animal were obtained from plasma concentration versus time plots by non-compartmental methods, whereby the area under plasma concentrations versus time (AUC) were calculated using the trapezoidal rule with extrapolation to infinity (Ct/Ke) [13]. The elimination rate constant (Ke) for the decline of plasma concentrations was obtained by linear regression on log plasma concentration against time plots. Thereafter, the following equations were used to derive the relevant pharmacokinetic parameters: half-life ($t_{1/2}$) = 0.693/Ke; volume of distribution (Vd) = Dose/(AUC × Ke); clearance (CLp) = Dose/AUC and mean residence time (MRT) = 1/Ke. The bioavailability (F) was determined using the equation: F = AUCo/AUCiv, where AUCo is area under curve after oral administration, and AUCiv is area under curve after intravenous administration. Of note, because the oral dose of acyclovir was different and higher than the intravenous dose, the F was obtained using a calculated AUCiv if the oral dose were given intravenously as follows: AUCiv = oral Dose/(Vd × Ke), where Vd and Ke were those obtained after the intravenous dose of 60 mg/kg. The maximum concentration (C_{max}) and the time to reach C_{max} (T_{max}) were read from the concentration-time curves.

Statistical data analysis. Data were analysed by non-parametric methods using the GraphPad statistical programme. Accordingly, parameters were reported as median and range, and the Mann–Whitney test was used for data comparison with the level of significance set at $P < 0.05$.

Results

Figure 1 and table 1 show that after intravenous administration, the corresponding plasma concentrations of acyclovir in animals that received a combination of acyclovir and valproic acid (group C) were more than 10 times higher than in those that received acyclovir only (group A). Furthermore, acyclovir was almost undetectable by 4 hr in group A, while in group C, it was still measurable 8 hr after administration. This was confirmed by a higher AUC for group C than group A ($P < 0.0001$); AUC for group A was 10.30% of that for group C (table 2). Furthermore, the volume of distribution (Vd) and plasma clearance (CLp) of acyclovir were

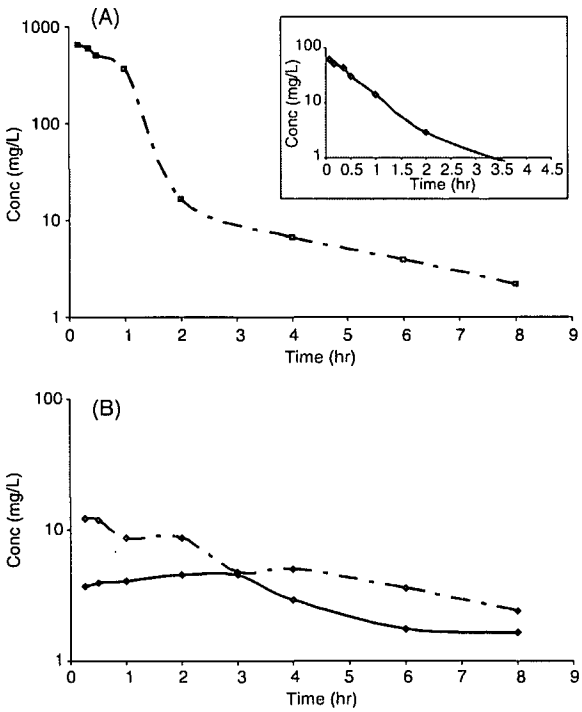

Fig. 1. The median plasma concentrations of acyclovir in rabbits after intravenous (A) and oral (B) administration of acyclovir alone (solid line) and acyclovir with valproic acid (dotted line), (n = 5).

Table 1.

Plasma concentrations (mg/l) of acyclovir, median (range), in rabbits after intravenous and oral administration of acyclovir alone (group A) and acyclovir with valproic acid (group C), (n = 5).

Intravenous

Time (hr)	0.17	0.34	0.5	1	2	4	6	8	10
Group A	51.4 (42–58)	43.1 (33–47)	29.8 (14–40)	14 (2–19)	2.9 (3–7)	0.6 (0.3–0.9)	–	–	–
Group C	647 (504–911)	591 (561–820)	499 (55–742)	362 (34–568)	16.5 (15–24)	6.6 (3–15)	3.9 (1.4–4.4)	2.18 (1–5)	–

Oral

Time (hr)	0.25	0.5	1	2	3	4	6	8	10
Group A	3.7 (3.1–5.2)	3.97 (1.6–4.2)	4.06 (1.4–10.7)	4.58 (2–7)	4.6 (2–5)	2.95 (2.87–6.47)	1.7 (1.5–2.5)	1.6 (1–2)	– –
Group C	12.2 (0.8–30)	11.86 (0.7–25)	8.6 (4.1–18.3)	8.66 (4–14)	4.78 (1.6–9)	5.0 (1.7–8.5)	3.6 (1.06–8.4)	2.4 (0.5–4)	0.59 (0.36–2.5)

significantly lower in group C than group A (i.e. 12.8% and 10.36% of group A values, respectively). The P-values were less than 0.0001 for both Vd and Clp. These observations imply that valproic acid inhibited the distribution and clearance of acyclovir. A similar trend was observed after oral administration (fig. 1 and table 1). The corresponding plasma concentrations of acyclovir for group C were three to four times higher than for group A, and acyclovir was measurable at 10 hr in group C versus 8 hr in group A. Again, the Vd and CLp of acyclovir were lower in the group C than group A (i.e. 12.35% and 12.90% of group A values, respectively) (table 2). The P-values were less than 0.0079 for both the Vd and CLp.

Surprisingly, the AUCs were not significantly different between the two groups A and C but values for group C were higher than in group A (table 2). It was also observed that absorption of acyclovir was slower in group A than in group C as evidenced by the T_{max} of 3 hr in group A versus 0.5 hr in group C. The corresponding C_{max} were 4.6 mg/l for group A and 18.4 mg/l for group C. The latter could not make sense until it was found that the bioavailability (F) of acyclovir in group C (median, 1.5%) was only 17.9% of that for group A (median, 8.4%) (table 2). This implies that valproic acid interfered with the absorption of acyclovir from the gastrointestinal tract, but high plasma concentrations of acyclovir were observed from the small amount (1.5%) that was absorbed because valproic acid also interfered with the distribution and clearance of acyclovir.

Figure 2 and table 3 show the concentration of valproic acid after intravenous and oral administration in groups B

Table 2.

Plasma pharmacokinetic parameters of acyclovir, median (range), in rabbits after intravenous and oral administration of acyclovir alone (group A) and acyclovir with valproic acid (group C).

Route	Acyclovir after intravenous administration		Acyclovir after oral administration	
Group	Group A	Group C	Group A	Group C
No. of rabbits	5	5	5	5
Sex	2M 3F	2M 3F	2M 3F	2M 3F
Wt (kg)	3.6 (3.5–3.8)	4 (3.5–4.2)	3.8 (3.5–4.2)	4 (3.5–4.3)
Dose (mg)	216 (210–288)	240 (210–250)	1140 (1050–1260)	1200 (1050–1290)
Co (mg/l)	69.1 (64–83)	529 (126–1005)*	11.5 (6.9–18.5)	12.2 (6.7–28)
Ke (hr)	0.86 (0.67–1.8)	0.84 (0.5–1.5)	0.32 (0.2–0.4)	0.28 (0.2–0.5)
Half-life (hr)	0.80 (0.4–1.0)	0.83 (0.5–1.5)	2.14 (1.6–3.5)	2.49 (1.4–3.5)
AUC (mg hr/l)	81.65 (46–101)	751 (509–1094)*	29.9 (21.3–38.7)	44.5 (18.1–76.3)
Vd (l)	3.05 (2.6–3.8)	0.34 (0.2–0.9)*	8.03 (6.3–23.4)	1.44 (0.8–2.0)*
CLp (l/hr)	2.76 (2.1–4.7)	0.30 (0.2–0.4)*	2.76 (2.1–4.7)	0.401 (0.4–0.4)*
MRT (hr)	1.16 (0.6–1.5)	1.19 (0.6–2.2)	3.1 (2.3–5.0)	3.59 (2.0–5.1)
F (%)	–	–	8.4 (5.0–11.0)	1.5 (0.6–2.3)*
T_{max} (hr)	–	–	3.0 (1.0–4.0)	0.5 (0.5–2.0)*
C_{max} (mg/l)	–	–	4.6 (2.9–10.7)	18.4 (5.5–30.6)*

*Significant difference, $P < 0.05$.

AUC, Area under the plasma concentration-time curve; CLp, plasma clearance; C_{max}, maximum concentration; Co, concentration after instantaneous distribution at time zero; F, bioavailability; Ke, elimination rate constant; MRT, mean residence time; T_{max}, the time to reach maximum concentration; Vd, volume of distribution; Wt, weight.

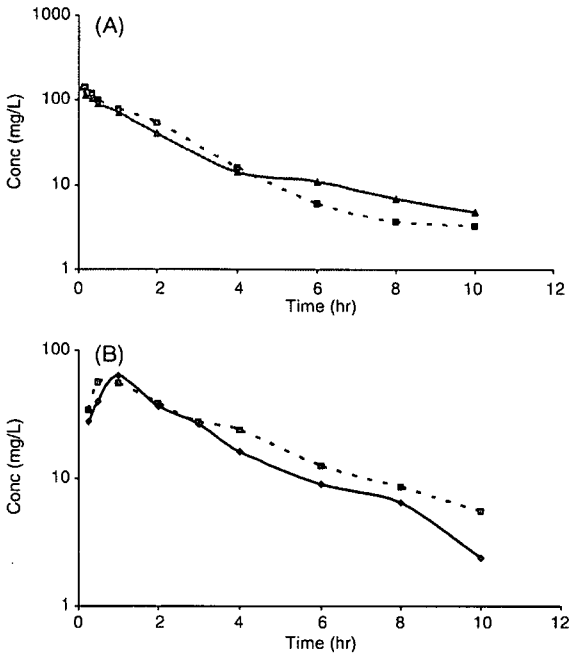

Fig. 2. The median plasma concentrations of valproic acid in rabbits after intravenous (A) and oral (B) administration of valproic acid alone (solid line) and valproic acid with acyclovir (dotted line), (n = 5).

and C. There was no significant difference in the concentrations of valproic acid between the two groups after oral and intravenous administration. Similarly, the kinetic parameters of valproic acid after administration with and without acyclovir were not different after either route of delivery (table 4). This implies that acyclovir had no effect on valproic acid.

The inhibition of acyclovir by valproic acid appeared to be concentration-dependent with a threshold concentration of valproic acid between 60 and 30 mg/l. This was indicated by the rapid fall in the plasma concentrations of acyclovir when the concentrations of valproic acid were within in this range after intravenous administration in group C. When valproic acid concentrations were between 77.95 mg/l at 1 hr and 53.3 mg/l at 2 hr, the corresponding concentrations of acyclovir fell rapidly from 362 mg/l to 16.5 mg/l, respectively. In effect, there was a significant rapid drop of over 50% in the concentration of acyclovir, implying that after the threshold concentration, there was rapid elimination and/or distribution of acyclovir. For the oral route, this was not obvious but it helped in setting the lower limit of 30 mg/l. When valproic acid concentrations were between 38.95 mg/l at 2 hr and 27.56 mg/l at 3 hr, the corresponding concentrations of acyclovir fell rapidly from 8.66 mg/l and 4.78 mg/l, respectively, a drop of 55%. The concentration for valproic acid of 27.56 mg/l at 3 hr after oral administration implies that the threshold concentration was higher than this hence the range of 60 to 30 mg/l was suggested.

Discussion

This study has achieved its aim of determining the pharmacokinetics of acyclovir and valproic acid when administered together in a rabbit model, thereby revealing an interaction that was difficult to predict from our current knowledge on the pharmacokinetics of the two drugs [14,15]. The results of this study are relevant because the rabbit model is commonly used for evaluating the pharmacokinetics of acyclovir [16–18]. Indeed, some of the kinetic parameters or trends as such, in the control group A were similar to those reported in human beings. For instance, in table 2, the volume of distribution after intravenous administration of acyclovir alone to group A was 0.85 l/kg and the total clearance of 46 ml/min. was almost three times the glomerular filtration rate of the rabbit (17 ml/min.), depicting tubular secretion [19–22]. In addition, after oral administration of acyclovir alone to group A, the bioavailability was within the range of 10–20% with the maximum concentration (C_{max}) in the therapeutic range of 5–15 mg/l. As such, although human studies will still have to be done for concrete answers, these results can be used to elucidate on the possible mechanism of the interaction between the two drugs.

It has been shown for the first time that after acute co-administration of acyclovir and valproic acid, valproic acid

Table 3.

Plasma concentrations (mg/l) of valproic acid, median (range), in rabbits after intravenous and oral administration of valproic acid alone (group B) and valproic acid with acyclovir (group C), (n = 5).

Intravenous

Time (hr)	0.17	0.34	0.5	1	2	4	6	8	10
Group B	144.5 (98.6–131)	104.5 (90.7–114)	89.82 (84.2–101.5)	71.25 (58.5–72.3)	40.36 (33.5–58)	14.16 (11.7–25)	10.77 (3.4–23.5)	6.89 (1.9–18)	4.81 (1.2–14)
Group C	138.5 (117–149)	119.19 (90–135)	99.56 (88–100)	77.95 (62–86.5)	53.3 (32–60)	15.96 (6.9–21)	5.98 (3.7–12.4)	3.66 (2.8–12)	3.25 (1.5–8.2)

Oral

Time (hr)	0.25	0.5	1	2	3	4	6	8	10
Group B	27.74 (12.6–55.6)	39.68 (14.7–78)	63.75 (16.2–71)	36.69 (18–55.7)	26.69 (14.9–33.9)	16.28 (12.8–27)	8.94 (7.6–23.7)	6.5 (3.1–15)	2.38 (2.2–8.5)
Group C	34.32 (24.3–95.5)	56.01 (25–105)	55.17 (26.6–92.8)	38.95 (22.7–68.3)	27.56 (15.4–61.2)	23.92 (10.5–46.3)	12.36 (4.5–22.9)	8.61 (1.8–17.6)	5.55 (0.9–17)

Table 4.

Plasma pharmacokinetic parameters of valproic acid, median (range), in rabbits after intravenous and oral administration of valproic acid alone (group B) and valproic acid with acyclovir (group C).

Route	Valproic acid after intravenous administration		Valproic acid after oral administration	
Group	Group B	Group C	Group B	Group C
No. rabbits	5	5	5	5
Sex	2M 3F	2M 3F	2M 3F	2M 3F
Wt (kg)	3.5 (3.1–3.8)	4 (3.5–4.2)	3.8 (3.4–4.5)	4 (3.5–4.3)
Dose (mg)	70 (62–76)	80 (70–84)	76 (64–90)	80 (70–86)
Co (mg/l)	106.6 (78–123)	113 (92.4–124)	67.99 (22.5–113)	40.12 (26.7–167)
Ke (hr)	0.33 (0.19–0.5)	0.39 (0.3–0.5)	0.29 (0.13–0.42)	0.28 (0.1–0.6)
Half-life (hr)	2.07 (1.4–3.6)	1.79 (1.4–2.3)	2.37 (1.67–5.2)	2.49 (1.3–13.4)
AUC (mg hr/l)	318.0 (224–406)	281.1 (227–371)	227.0 (127–306)	254.7 (154–580)
Vd (l)	0.67 (0.7–0.95)	0.65 (0.62–0.92)	1.36 (0.76–4.6)	1.17 (0.63–2.67)
CLp (l/hr)	0.22 (0.18–0.34)	0.28 (0.19–0.37)	0.36 (0.21–0.63)	0.29 (0.14–0.56)
MRT (hr)	2.98 (2.0–5.2)	2.58 (2.1–3.4)	3.42 (2.4–7.5)	3.59 (1.8–19)
F (%)	–	–	92.1 (67–109)	81.7 (54.7–108)
T_{max} (hr)	–	–	1.0 (0.5–2)	1.0 (0.25–2)
C_{max} (mg/l)	–	–	64.2 (18–78)	68.28 (37.8–105)

AUC, Area under the plasma concentration-time curve; CLp, plasma clearance; C_{max}, maximum concentration; Co, concentration after instantaneous distribution at time zero; F, bioavailability; Ke, elimination rate constant; MRT, mean residence time; T_{max}, the time to reach maximum concentration; Vd, volume of distribution; Wt, weight.

inhibited the tissue distribution and clearance of acyclovir, thereby leading to higher plasma concentrations of acyclovir. In addition, valproic acid inhibited the absorption of acyclovir from the gastrointestinal tract. Acyclovir, on the other hand, did not affect the concentrations of valproic acid. The latter is contradictory to the earlier clinical observation of reduced concentrations of valproic acid and phenytoin 4 days after start of acyclovir, and this was associated with breakthrough convulsions [3]. Furthermore, 5 days after discontinuation of the acyclovir, the patient continued to convulse. Unfortunately, as cited earlier, the concentration of acyclovir was not measured and these events occurred on chronic co-administration of the drugs. By 'chronic co-administration', we mean that the reduction in the plasma concentration of valproic acid could not be attributed to induction by phenytoin, because the patient had been stabilized on both drugs for a long period and this was confirmed by concentration monitoring.

Nevertheless, high concentrations of acyclovir have been associated with central nervous system side effects that include convulsions [6]. Therefore, the delayed onset of convulsions in the aforementioned case report was most probably, because valproic acid interfered with the absorption of acyclovir, leading initially to low plasma concentrations that took time to reach to toxic levels as a result of the inhibited elimination. Second, and probably the most important, it could be because valproic acid inhibited the distribution of acyclovir to the central nervous system, and this would remain so, as long as adequate levels of valproic acid were maintained. This meant that by day 4, the levels of acyclovir were so high that a fall in the concentrations of valproic acid, be it due to low trough levels or patient non-compliance, would lead to disinhibition of acyclovir tissue distribution, leading to a gash of acyclovir into the tissues, including the central nervous system, and thereby causing convulsions. Non-compliance may be due to the illness under therapy or central nervous system side effects of acyclovir that precedes convulsions such as lethargy, tremors, confusion, hallucinations, agitation, disorientation, etc. [9,23]. Similarly, the occurrence of convulsions 5 days after stopping acyclovir, as well as the continuous seizures that required higher doses of phenytoin to control was, most probably, because acyclovir levels were still high owing to the continual administration of valproic acid. This means that the occurrence of acyclovir-induced seizures was determined by the fluctuations in valproic acid levels against a high concentration of acyclovir. In effect, this study appears not to support the claim that acyclovir causes low levels of valproic acid.

On the other hand, it had been held that valproic acid increases the antiviral activity of acyclovir by stimulating and/or activation of viral replication, which enhances the phosphorylation of acyclovir and therefore its antiviral action [24,25]. However, a recent report has shown that it was the concentration of valproic acid rather than acyclovir that were the major determinant of the increased antiviral activity [4]. They insinuated that the increased antiviral activity was due to metabolites of valproic acid and/or a product of an interaction of valproic acid with acyclovir. The observations in this study lend support to this notion by suggesting that the high concentrations of acyclovir would provide a raw media for this interaction. In this perspective, it is possible that the high concentrations of acyclovir lead to continued consumption of valproic acid to form the so-called new product/compound [4]. This eventually caused the concentrations of valproic acid to drop on chronic administration. This would then explain the delay in

occurrence of convulsions as due to time taken to reduce the concentration of valproic acid to subtherapeutic levels. In this way, the findings of this study would rhyme with the claim that acyclovir may cause low levels of valproic acid. Of note, elucidation on the structure or mechanism of formation of the so-called new product is beyond the scope of this article.

Alternatively, the interaction may be similar to that of valproic acid with meropenem. Although the exact mechanism is still unresolved, the reduction in valproic acid concentration by meropenem was attributed to increased metabolism (glucuronidation) of valproic acid by meropenem [26,27]. Of note, both acyclovir and meropenem are substrates of membrane transporters and are actively secreted at the renal tubule, and co-administration of either drug with valproic acid was associated with a drop in the plasma concentrations of valproic acid. Although break-through convulsions have been reported with the meropenem interaction with valproic acid as well, reducing the concentration of valproic acid *per se* does not mean occurrence of convulsions. It means that an insult stimulus plays a role and, in this case, acyclovir would be implicated. In general, this single-dose study has not ruled out the possibility of acyclovir reducing the plasma concentrations of valproic acid. As such, further investigations are required to determine the mechanism by which acyclovir may reduce the concentration of valproic acid on chronic co-administration.

The rapid absorption of acyclovir when it was combined with valproic acid can best be explained after appreciating the cause of delayed absorption in group A in which acyclovir was administered alone. It is known that acyclovir is absorbed from the gastrointestinal tract via the peptide and organic anion transporters and by passive diffusion [2]. The importance of the transporter mechanism of absorption was further confirmed by the invention of valacyclovir, a drug with a higher affinity for the transporter and therefore bioavailability than acyclovir due to its amino acid ester moiety. Therefore, the slow absorption of acyclovir in group A where it was administered alone was probably due to the associated efflux of the drug by the transporters themselves. It was shown *in vitro* that after absorption by the CaCo-2 cells, valacyclovir was hydrolysed and the freed acyclovir was transported back into the medium at the mucosal side [28]. Therefore, in group C where acyclovir was co-administered with valproic acid, the inhibition of transporters by valproic acid improves the efficiency of passive diffusion (i.e. fast, with no efflux), but because absorption of acyclovir by passive diffusion is limited, it wanes off quickly and there is no improvement in the overall bioavailability.

The reduced volume of distribution of acyclovir when combined with valproic acid indicates that valproic acid inhibits the entry of acyclovir into cells and/or distribution into tissues. This suggests that, as it were for the absorption of acyclovir from the gastrointestinal tract, acyclovir distribution into cells is via transporters.

The transporter mechanisms could also explain the marked reduction in clearance of acyclovir when the two drugs were combined after either route of administration. For instance, it is known that approximately 90% of acyclovir is excreted unchanged via the kidneys, and that the renal clearance of acyclovir is reduced by probenecid, a drug known to inhibit the organic anion transporters [29]. More recently, other workers observed active renal secretion of acyclovir in rats by the organic anion transporter type 1 as well as organic cationic transporter type 1, and they did not rule out involvement of other transporters [30]. Inhibition of these transporters by valproic acid would therefore explain the reduction in the renal clearance of acyclovir, but this remains to be confirmed.

Alternatively, the reduced volume of distribution of acyclovir when combined with valproic acid could be due to increased protein binding of acyclovir. Unfortunately, protein binding and free drug were not determined in this study. Total plasma concentrations were used because they are the most commonly monitored in the clinic. Nevertheless, only the transporter mechanism and not protein binding, can explain the cause for the reduced volume of distribution and clearance as well as the reduced bioavailability after oral administration of acyclovir. On the other hand, it was observed that inhibition of acyclovir by valproic acid was concentration-dependent with a threshold concentration of valproic acid between 60 and 30 mg/l. Because the therapeutic range for valproic acid is 40–100 mg/l, this means that valproic acid should not be used together with acyclovir because the therapeutic concentrations can potentially inhibit acyclovir distribution and clearance. Nevertheless, this relationship would need clarification by dose-response studies.

In conclusion, co-administration of single doses of acyclovir with valproic acid led to reduced oral bioavailability of acyclovir, but increased concentrations of acyclovir due to reduced volume of distribution and clearance, and this was, most probably, due to inhibition of the membrane transport proteins for acyclovir by valproic acid. These observations call for a cautious approach to the concomitant use of the two drugs until human studies are done.

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