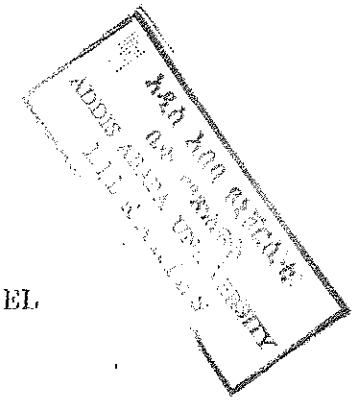
ADDIS ABABA UNIVERSITY

SCHOOL OF GRADUATE STUDIES

BLOOD LEVEL DETERMINATIONS AND PHARMACOKINETIC STUDIES OF ANTICONVULSANTS IN ETHIOPIAN EPILEPTICS



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Blood Level Determinations And Pharmacokinetic Studies of Anticonvulsants in Ethiopian Epileptics

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Abstract

A gas chromatographic method was employed to monitor plasma levels of phenytoin and phenobarbital in 101 Ethiopian epileptic patients who were on chronic drug treatment and kinetics and plasma level-response relationship was studied. 40% of the patients were on single drug treatment while the remaining were taking both phenytoin and phenobarbital. A very low correlation between dose and plasma level was observed and there was high variation among individuals. Although 86% of the patients were receiving 300 mg of phenytoin per day, some patients showed plasma levels as low as 5 μg/ml and others as high as 60 μg/ml.

About 70% of patients were seizure-free at the range of 10-20 μg/ml phenytoin levels. However the best range for maximum seizure control in this study was 20-30 μg/ml. At lower levels, that is below 10 μg/ml, and at higher levels above 30 μg/ml, the seizures were poorly controlled. The therapeutic range of phenobarbital for optimum control of seizure was found to be comparable with that of phenytoin (20-30 μg/ml).

The kinetics of phenytoin is best described by Michaelis-Menten kinetics. The most important parameters K_m and V_{max} were estimated and found to be 6.5 mg/lt and 6.9 mg/kg/d respectively. The maximum metabolic capacity, V_{max}, for

1. INTRODUCTION

During the past 20 years, the value of monitoring plasma antiepileptic drug levels as an adjunct to the provision of better patient care has been repeatedly demonstrated. Clinical studies have shown a strong correlation between plasma antiepileptic drug concentrations and seizure control, and it has been suggested that nearly 80% of all epileptic patients could become seizure-free with the currently available anti-epileptic drugs if their plasma drug concentrations accurately monitor.[1] Because of variations in individual pharmacokinetic and pharmacodynamic characteristics and other external factors such as product formulation etc, there is gross individual variation in the relationship between plasma level and seizure control. Thus a wide range of plasma levels may be found in patients receiving the same dose of anticonvulsants. Similarly, the degree of seizure control or the intensity of intoxication may vary widely among different patients with the same blood levels. There is therefore, no universal therapeutic level for any antiepileptic drug applicable to all patients. As there is no study so far on the therapeutic levels as well as other pharmacokinetic parameters of these drugs in plasma of inhibitants of this country, we set out to monitor blood levels of some chronic patients with the aim of generating data that may be of use in the treatment of other epileptics in the country. This study was a collaborative work conducted with physicians at Neurology unit of Tikur Anbessa hospital, Medical Faculty.

2. QUANTITATIVE ANALYSIS OF ANTICONVULSANTS

2.1. General consideration

Samples of biological origin are often of complex nature and drugs are present in low concentrations. When there is a need to determine their quantities in such samples, good selectivity and high sensitivity of analytical method is therefore of utmost importance. Various methods have been developed for quantitative determination of anti-convulsants in biological fluids. The summary is given below (Table 1.)

Table 1. Comparison of the various phenytoin assay methods[2]

Analytical method	Specificity for phenytoin	Minimum sample volume (ml)	Analysis cost	Specialized operator training	Analysis time	Comments
HPLC	1	0.2	3	3	2	Overall, probably the method of choice
GC						
on column methylation	1	0.5	2	4	2	Method used in most of the recent literature
Precolumn methylation	1	0.5	2	4	3	
RIA	2	0.2	3	2	2	Requires a scintillation counter
SP	4	1	1	2	3	Now less frequently used

Arbitrary ranking scale of 1 (excellent) to 4 (poor)

HPLC = High performance liquid chromatography

GC = Gas chromatography

RIA = radioimmunoassay SP = Spectrophotmetry

2.2. Gas chromatography in Assay of Anticonvulsants

The first accurate antiepileptic drug level determination was realised about 20 years ago by using gas chromatographic method. GC can be used to separate compounds which exhibit a measurable vapour pressure at temperatures below 300°c. It can also be used for substances from which a stable, volatile derivative can be prepared. Most GC separations are carried out on columns packed with inert support, coated with a film of liquid stationary phase. The compounds are eluted by an inert mobile gas such as nitrogen. The separation of the compounds depends upon their varying tendency to be dissolved in the stationary phase.

Literally thousands of partition liquid ranging from non-polar to very polar have been described in the literature. The general rule "like dissolves like" is a good one to follow when selecting liquid phases, thus polar liquid phases are best for nonpolar samples. Unless the sample dissolves well in the liquid phase, little or no separation occurs. The gas phase is inert and separation occurs only in the liquid phase. Of the 250 liquid phases that can be found in most catalogues only a few meet the high temperature, low viscosity and stability requirements of the antiepileptic drug level field. Only phenyl/ methyl silicone operates well at temperature up to 300°C, is neutral and offers the right range

of polarity for good separation. One such example is OV-17 (Ohio Valley Speciality Co; Merietta, Ohio).

Apart from packed column, a long capillary column in which the walls act as support for stationary liquid phase can be used. Such columns have greater separation capabilities and lower limits of detection.

The successful use of any column lies on the efficiency and sensitivity of the detector. It is usually located at the exit of the separation column and senses the arrival of the separated components as they leave the column and provides a corresponding electrical signal. There are more than 20 different detector types for use in GC. The most common ones are flame ionization detector (FID), thermal conductivity detector (TCD) and electron capture detector (ECD) of which FID is currently the most popular detector in use in anticonvulsant analysis because of its high sensitivity, wide range and great reliability.

For any compound to be analysed by GC, the pre-requisite is its volatility. Unfortunately most anticonvulsants are not easily volatile and, therefore gas chromatographic analysis of such drugs is very difficult. To increase the volatility and hence to reduce absorptive property or tailing and to make the analysis of the polar molecule easier it is essential to modify the functional groups of anticonvulsants chemically with the aid of derivatizing agent.

An internal standard is a reference compound usually having similar chemical and physical properties with the component analysed, used to compare or test a measurement. In GC, there are two different possible ways of analysis: the internal standard method and absolute parameter method. In the absolute parameter method we use variable factors to calculate the amount or concentration of a substance in an unknown sample. This method has a number of disadvantages. It requires precise management over all instrumental conditions otherwise:

- slight fluctuation in gas flow and column temperature can alter peak shape.
- slight fluctuation in instrumental condition can significantly alter peak height
- slight fluctuation in weighing and transferring of sample in the instrument causes false reading
- any loss of sample during syringe injection introduces errors in the results.

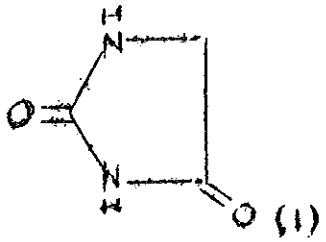
Because of all these undesirable properties, absolute parameter is hardly used in anticonvulsant analysis. Instead analysis with internal standard, which is devoid of all the above undesirable properties is employed. In this method a known amount of internal standard is added to the component sample and a ratio is calculated from the gas chromatographic responses. The relationship between peak height or area and concentration is based on a calibration curve determined experimentally beforehand. In internal standard method slight variation in instrumental conditions, column temperature, gas flow, personal variation in handling the

sample during extraction etc may affect the individual peaks or area but not their ratio. Since the internal standard is expected to have similar chemical and physical properties to that of the component analysed whatever factors affect the peak height of internal standard would have comparable effect on the peak height of the component, but the ratio remains constant.

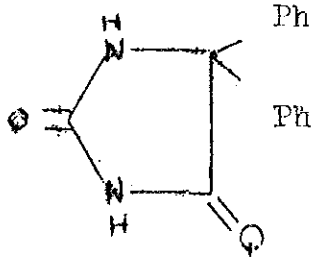
3. CLINICAL PHARMACOLOGY OF PHENYTOIN AND PHENOBARBITAL

3.1. Diphenylhydantoin

Diphenylhydantoin commonly called phenytoin, is the derivative of hydantoin ring shown below (1)



(1)



(2)

Chemically it is 2,4-imidazolidinedione. The first hydantoin to be used widely in medicine was 5-ethyl-5-phenylhydantoin which was known as nirvanol. This hydantoin was introduced as a hypnotic in 1916 and was reported to have about the same intensity of action as phenobarbital, but less toxic (latter found to be dangerous in the continued use.)

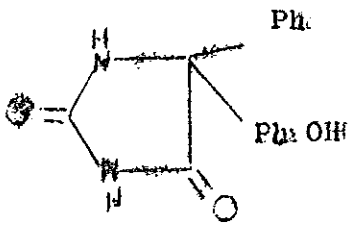
The hydantoin derivative which has acheived the greatest popularity in medicine is 5,5-diphenylhydantoin or phenytoin (2). The use of phenytoin in the treatment of epilepsy was first recommended in 1938 by Merritt and Putnam.[3] Since then phenytoin has become the most widely used agent in the management of seizure disorders. It is effective in the treatment of primary or secondary generalized tonic-clonic epilepsy and elementary and complex partial seizures as well as status epileptious.

Absorption :- phenytoin is a weak acid hence is expected to be absorbed from stomach. However because of its low solu-

small intestine. Even there absorption is slow and may be incomplete and fraction of dose absorbed varies. Furthermore, the rate of absorption varies considerably among dosage forms. Peak serum concentrations occur between 4 and 12 hr. after a dose of Dilantin[R] withsa mean of approximately 6 hr. However, for some preparations and in some individuals the time to at ain peak level may be even more than 12 hours. This slow absorption and relatively slow elimination of the drug have led to the recommendation of a once daily administration regimen. The intramuscular route of administration is generally avoided because of precipitation of phenytoin at the site of injection.

Distribution :- phenytoin is reported to distribute through volumes of 0.5 lt/kg body weight in adults.[4] It is also highly bound to serum albumin with reported values 87% to 90% at 37°C.[5,6] The drug binds to brain tissue to about the same extent as it binds to serum proteins, and therefore the brain and serum concentrations are almost identical.[7] Whole blood concentrations are less than those found in plasma, whereas cerbrospinal fluid and saliva concentrations are nearly identical to the free (non protein bound) fraction of plasma phenytoin.[8]

Metabolism :- Elimination of phenytoin occurs primarily by biotransformation to several inactive hydroxylated metabolites. The 5-(p-hydroxyphenyl)-5-phenylhydantoin (p-HPPH) (3), is the major metabolite which accounts for 60 to 90% of an oral dose of phenytoin.[4]



p-PHHP

Phenytoin is extensively metabolised in the liver, less than 5% appearing unchanged in the urine. The rate at which it is metabolised is under genetic control which is thought to be polygenic in nature. There is a continuous unimodal distribution of rates of metabolism with very slow and very fast metabolisers at the extreme ends of the distribution. Race may also be important; such that negroes metabolise phenytoin more slowly than caucasians.[9]

The conversion of phenytoin to its major metabolite by microsomal enzymes in the liver follows zero-order kinetics. i.e. the liver is unable to increase rate of metabolism as serum phenytoin increases. Above the saturation limit of the liver the dose have non-linear relationship with serum concentration. At therapeutic concentrations the rate of metabolism is close to this limit. For this reason, the metabolism of phenytoin is said to be capacity-limited or to show saturability. Slow metabolisers are more to receive toxic doses of the drug, just as fast metabolisers are likely to undertreated because these patients dose requirements fall outside the usual prescribing range.

The rate limiting enzymatic reaction is assumed to follow typical Michaelis-Menten kinetics in which the rate of the reaction, depends upon the substrate concentration, here the concentration in plasma, C, as follows

$$v = \frac{V_m \cdot C}{k_m + C} \quad \text{---------- (1)}$$

where V_m is maximum rate of metabolism (metabolic capacity) and k_m is a constant at one-half of the maximum. The value of these constants usually vary from 100 - 1000 mg/day for V_m and 1 to 15 µg/ml or more for km. The consequences of the capacity limited metabolism of the drug are most readily observed under steady-state conditions. For an intravenous infusion, the rate of administration (R) is equal to rate of elimination.

$$R = \frac{V_m \cdot C_{ss}}{k_m + C_{ss}} \quad \text{---------- (2)}$$

where C_{ss} is the steady or plateau concentration; on rearranging we get

$$V_m = R + k_m \frac{R}{C_{ss}} \quad \text{---------- (3)}$$

on plotting V_m versus K_m, the y-intercept is R and the x-intercept is $-C_{ss}$. This relationship is useful for estimating the values of pharmacokinetic parameters, V_m and k_m. The value of R/C_{ss} is conventionally called clearance[2]. As the rate of administration approaches the maximum rate of metabolism, the steady-state concentration increases disproportionately and approaches infinity. Values of R greater than V_m are not applicable because steady state can't occur under this condition. For phenytoin C_{ss} almost always exceeds the value of km, thus capacity-limited metabolism is evident.

Phenytoin has non linear elimination, because of which the time to reach steady-state concentrations varies with the rate of administration and depends upon the value of V_m and k_m.[10] This dependence, assuming a constant rate of infusion of phenytoin is shown as

$$V \cdot dc/dt = R - \frac{V_m \cdot C}{K_m + C} \quad \text{- - - - - - - - - -} \quad (4)$$

where $V \cdot dc/dt$ is not rate of change of phenytoin in the body, e.g. it is the difference between the intake and output rates.

From this equation the time required to acheive 90% of the steady-state value is acheived and is equal to

$$t_{90\%} = \frac{k_m \cdot V_m \cdot C}{(V_m - R)2} \cdot (2.203\ V_m - 0.9R) \text{ - - - } (5)$$

From this equation one can understand that as rate of administration (R) appraches V_m, the value of $t_{90\%}$ increases dramatically. If they are equal, steady state is never achieved. The higher the rate of administration the longer it takes to approach steady state concentration. The time to accumulate to a given plateau concentration varies with values of k_m and V_m. The smaller the value of k_m the longer it takes to reach plateau. Furthermore because of capacity limited metabolism of phenytoin, a small change in the bio-availability can produce a dramatic change in the steady-state concentration.

Renal excretion :- Less than 5% of daily dose is excreted unchanged in the urine.[11] 70% - 80% being eliminated as p-HPPH. The ratio i.e. i = DPH/P- HPPH in urine dependes on two factors where DPH stands for phenytoin and p-HPPH is the

the majour metabolite. The first factor is genetic difference and the second one is the steady state plasma concentration of phenytoin. Since its metabolism follows zero-order kinetics, as phenytoin concenteration increases in serum, the hydroxylation mechanism becomes saturated and p-HPPH production fails to rise in proportion and therefore more phenytoin appears in the urine.

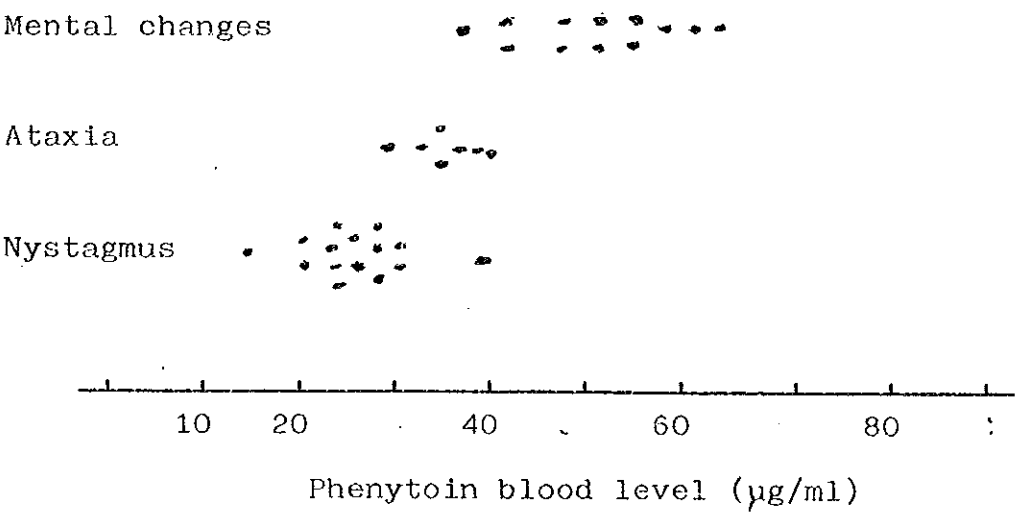
Plasma levels in relation to concentration and response

A positive correlation has been shown between the severity of the seizure process and the amount of antiepileptic drug needed to control the seizure experimentally. However there is high variation among individuals. Several retrospective studies described patients whose seizures were well controlled by low levels of phenytoin, while others continued having seizures with high plasma levels.[12] In general there is no universal therapeutic level for any antiepileptic drug applicable to all patients. Analysis of large groups of patients continue to have seizures and above which seizures in many patients are controlled or improved. The upper limits of the clinically useful plasma level range are those above which disturbing signs and symptoms of intoxication appear in most patients.

Clinical sign and toxicities of phenytoin were frequently encountered with levels above 20 µg/ml. Most researchers suggested a therapeutic range of 10-20 µg/ml with optimum control of fits but minimum toxicity. Lund[13] found that higher concentration were associated with better control of epilepsy. In two other studies, the relationship was reversed,[14]

whilst others have found no relationship. However there is general agreement that clinical sign or phenytoin intoxication become frequent when plasma level rises to somewhere between 20-30 µg/ml. The incidence of toxic effects at a particular concentrations varies considerably from one report to another, no doubt depending on the clinicians criteria for intoxication. Some report that there are patients with levels greater than 40 µg/ml without any clinical sign; conversely there are some patients who show nystagmus at serum levels below 15 µg/ml.[15] Nystagmus is usually accepted as the first sign of elevated phenytoin concentration, as it's probably the most frequent objective symptom that can be documented. Fig. 1 shows the onset of central nervous system side effects in relation to phenytoin concentration.[2]

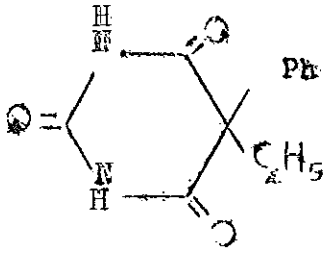
Fig. 1. The onset of CNS side effects in relation to phenytoin concentration.



In conclusion a knowledage of the serum level makes it possible to achieve optimal treatment without risk of over-dosage. The inflexible use of a range 10-20 µg/ml imposes unnecessary and harmful limits on clinical judgement.

3.2. Phenobarbital

Phenobarbital (4) was synthesized by Horlein of the Bayer dye works and submitted for clinical trials. The following year (in 1912) it was found to be effective against epilepsy. It was the first effective organically synthesized anticonvulsant introduced into medicine and is effective in tne management of generalize tonic-clonic, complex paritial status epilepticus and febrile seizures.



(4)

In animal tests, phenobarbital has been found to have much greater anticonvulant activity relative to hypnotic activity than barbituric acids having only alkenyl substituents in the 5-position.

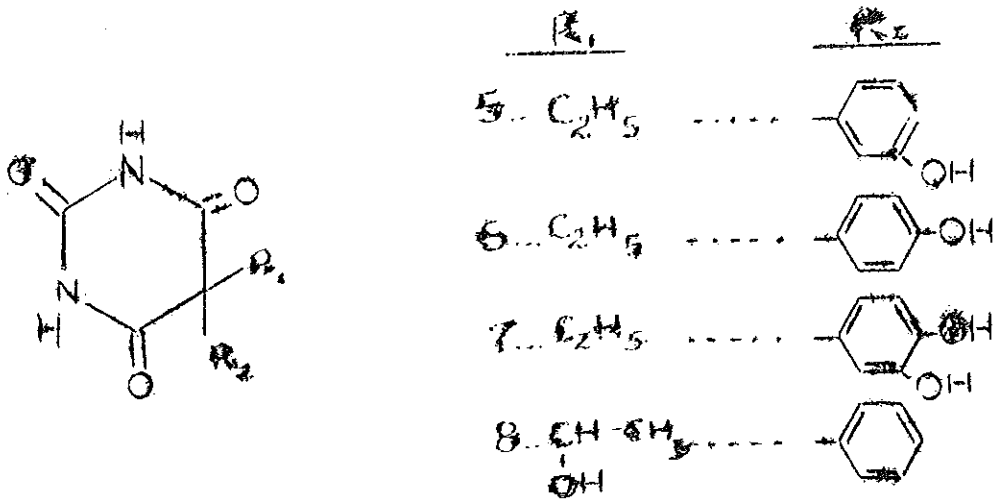
Absorption: - Phenobarbital is administered both orally and intramuscularly. Comparative studies of both routes demonstrated that the bioavalability is similar. It is generally concluded that the absorption of orally administered phenobarbital is 80% to 90% complete[16] peak serum concentrations are similar after oral and itramuscular phenobarbital administration and are achieved within a few hours.

Distribution: - The reported volumes of distribution for phenobarbital range from approximately 0.6 - 1.0 lt/kg.[17] Approximately 50% to 60% of plasma phenobarbital is bound to protein. Phenobarbital's concentration in body fluids is dependent on the pH and protein concentration of the specific

fluid. Saliva, with a lower pH than plasma, has somewhat lower phenobarbital concentration.[18] This concentration is similar to the free plasma concentration.

Metabolism: - Phenobarbital is metabolised in the liver and six significant metabolites have been identified of which compound 6 is by far the most significant.

Fig 2. Phenobarbital and its metabolites.[1]



Phenobarbital is known to stimulate the metabolism of a number of drugs, but there is no evidence that it induces its own metabolism.

Renal excretion:- Phenobarbital is by far the most persistant (long acting) of any barbiturates in medicinal use. Its elimination kinetics are generally reported in terms of half-life which in adults averages about 4 days (range 49-160 hr). [17] Furthermore about 25% of a dose of phenobarbital is eliminated unchanged in the urine. pH influences tubular reabsorption with excretion increases with increase pH.

Dose veruses concentration and response:-

The clinical value of phenobarbital estimation is less applicable because the relationship between the serum concentration and its action on central nervous system may not be as close as has been assumed. In some reports it is indicated that a minimum effective level is probably between 10 and 15 µg/ml which will produce approximately a 90% reduction in electro-encephalographically detected proxysmal activity in epileptics.[19] In many other studies the normal recommended therapeutic level suggested is 20-30 µg/ml. Some reported higher serum concentrations are required for more severe epilepsy and suggested upper limits range from 30 to 40 µg/ml.[20] In general at present time, the linearity of the dose-serum concentration relationship is somewhat contraversial. Traditional dogma states that a linear relationship exists, but a curvilinear relationship was recently reported in a retrospective study of 2 patients.[21] Clarification of this issue must await further study.

It is well recognised that tolerance occurs to the sedative effects but is uncertain for antiepileptic activity. Reports of drowsiness at plasma concentrations as low as 25 µg/ml and toxic patients with serum concentrations above 60 µg/ml have produced a considerable dilemma in setting a useful limit to the therapeutic range. In conclusion it is likely that the relationship between plasma level and tissue effect is not a constant one but depends on the degree of tolerance that has developed.[22]

4. AIM OF THE PROJECT

This study is concerned with the most widely used anti-convulsants namely phenytoin and phenobarbital. Both drugs show interesting pharmacokinetic properties. Buchthal[23] was the first to define a therapeutic range of plasma concentration for phenytoin. As clinical signs and sign of toxicity were frequently encountered with levels above 20 µg/ml they considered that a therapeutic range of 10-20 µg/ml would give optimum control of fits without toxicity. This view has been reiterated by Kutt and Mc Dowell[24] and this range has been widely used in the management of phenytoin therapy. However, Bucthal and his co-workers studied only a small number of patients with severe epilepsy and their conclusions may not be valid for ambulant patients with mild or moderate epilepsy. Although a number of retrospective studies have been performed in patients of this type, the results have been conflicting. Lund[15] found that higher concentrations were associated with better control of epilepsy, but Triemdman, Fishman and Yahr and Haerer[25] and Grace[12] had previously found no correlation. In two studies, the relationship was reversed, i.e. a lower plasma concentration was found in patients with adequately controlled fits.

The incidence of toxic effects at a particular concentration varies considerably from one report to another. Although Kutt *et al*[24] found a good relationship between the plasma level and the clinical signs of toxicity a number of authors report patients with levels greater than 40 µg/ml in the absence of clinical signs.[25] Conversely some patients may show toxic symptoms at plasma levels below 15 µg/ml.

Although a number of indications discussed for phenytoin apply also to phenobarbital the clinical value of phenobarbital estimation is by far less important. It is likely that relationship between plasma level and tissue effect of phenobarbital is not a constant one but depends on the degree of tolerance that has developed. Buchthal and Svensmark[28] had found that drowsiness occured at levels of 25-50 µg/ml. Plaa and Hine[29] reported mental slowness and ataxia with plasma levels of 36-77 µg/ml, although several other patients had levels above 30 µg/ml without evidence of intoxication. Sunshine[30] reported levels of up to 60 µg/ml in patients who were free of toxic symptoms.

In general seizure control, plasma level and sign and symptoms relationships show striking variations among patients because of variations in individual pharmacokinetic and pharmacodynamic characteristics and external factors such as product formulation etc or both. Thus, a wide range of plasma levels may be found in patients receiving the same dose of antivonvulsants. Similarly, the degree of seizure control or the intensity of intoxication may vary widely among different patients with the same plasma levels. There is, therefore, no universal therapeutic level for any antiepileptic drug applicable to all patients. Furthermore no study has been made to monitor plasma levels and other pharmacokinetic parameters of these drugs in human plasma of inhibitants of this country.

The main aim of the present study was to determine serum level of phenytoin and phenobarbital in Ethiopian epileptcs inorder to develop plasma concentration versus response relationship and to study the pharmacokinetic parameters which are responsible for the wide variation in response to drugs. A further goal has been to get some data on possible existence of population based genetic variation.

. To acheive this goal it was necessary

= to determine serum levels of anticonvlsants in plasma samples taken from representative epileptics by gas chromatography.

= to develop dose-serum level and plasma level-response relationships.

= to assess whether there is population based variation on the disposition of the drugs.

= to estimate the most important pharmacokinetic parameters and apply them for use in clinical practice.

= to propose the most likely dosage regimen which is sufficient to suppress seizure without affecting normal brain function based on the estimation of pharmacokinetic parameters.

5. MATERIALS AND METHODS

5.1 Subjects

Plasma samples were obtained from 101 epileptic patients ranging from 13-53 years of age and attending the neurology unit at Tikur Anbessa hospital. Forty one of these were on single drug treatment (20 taking phenytoin alone; 21 phenobarbital alone). The rest of the subjects were taking both drugs simultaneously. Almost all of them were on anticonvulsant treatment for months.

5.2 Plasma sample collection:

5 ml venous blood was drawn from one of the arms of each subject; the cells were then separated from plasma by centrifugation and the plasma was stored at - 20°C until analysis.

5.3 Solvent extraction procedure

The extraction procedure was adapted from the one developed by Kupferberg.[31] To 1 ml plasma was added 1 ml (0.25M) phosphate buffer (pH 7.2) and 6 ml ethylene dichloride in a 30 ml ground glass-stoppered centrifuge tube. The mixture was shaken for 15 min, using magnetic stirrer, centrifuged and as much of the organic phase as possible was removed using pasteur pipettes. A second 6 ml of ethylene dichloride was added; the mixture was shaken and centrifuged. Again as much as possible of the organic phase was removed and combined with the original organic extract. A double extraction with ethylene dichloride gave more consistent results than a single

extraction. 10 ml of the ethylene dichloride extract was combined with 5 ml hexan and 4.5 ml 0.2 M Na_3PO_4 in a 30 ml ground glass-stoppered centrifuge tube. The mixture was shaken for 10 min, centrifuged and 3.5 ml of the aqueous phase removed. The phosphate buffer extract was acidified with 0.3 ml of 5 N HCl and 10 ml of ethylene dichloride was added. Again the mixture was shaken for 10 min and centrifuged. Nine ml of the above organic phase was placed in a 12-ml ground-stoppered centrifuge tube along with 1 ml of cholestane (1 µg/ml of methanol) as an internal standard. The mixture was dried on Rota vapour under water vaccum. 40 µl of trimethylanilinium hydroxide solution was added to the dry residue. 1-4 µl of the extract was injected into the chromatograph.

5.4. Apparatus: Gas chromatography.

The gas chromatograph used in this study was a Varian Model 3700 with a coiled glass tubing packed with 3% OV-17 on chromosorb Q. The different experimental conditions and temperature settings are as follows.

Model _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ Varian 3700

Detector _ _ _ _ _ _ _ _ _ _ _ _ _ _ Flame Ionization

Column _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ U-shaped glass tubing

Packing_ _ _ _ _ _ _ _ _ _ _ _ _ _ _ 3% OV-17 on chromosorb Q

Experimental condition :

Inj. Temp._ _ _ _ _ _ _ _300°C

Column Length _ _ _ _ _ 2 m , $\frac{1}{8}$" ID.

Column Temp.

Init. _ _ _ _ _ _ _ _160°C

Final _ _ _ _ _ _ _ _240°C

Rate _ _ _ _ _ _ _ 10°C/ min

Detector Temp_ _ _ _ _300°C

Gas flow rate

H_2 _ _ _ _ _ _ _ _ 30 ml/min

N_2 _ _ _ _ _ _ _ _ 30 ml/min

Air _ _ _ _ _ _ _200 ml/min

Attenuater elec_ 8 x 10^{-10}A

The trimethylanilinium hydroxide solution, a derivatizing reagent, was prepared as described by Brockmann-Hannsen and Oki.[32]

6. RESULT AND DISCUSSION

6.1 Analysis and discussion of the methodology employed

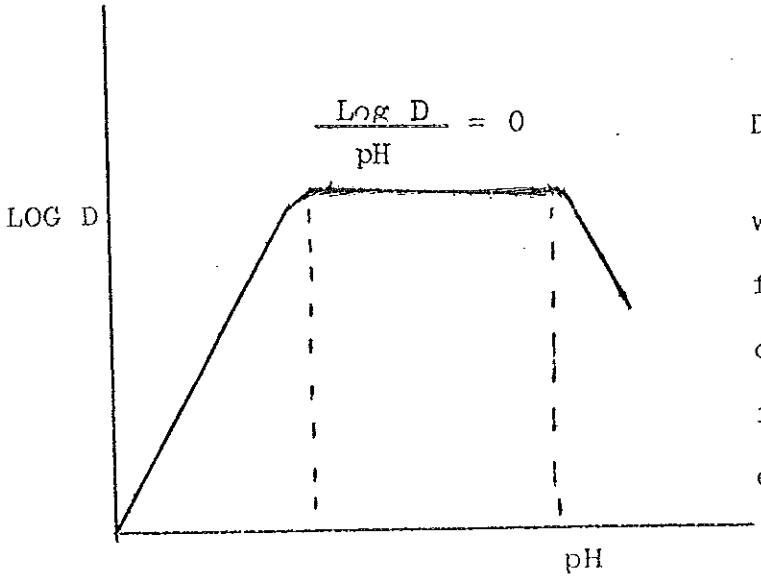
The GC technique employed in this experiment was the one that was developed by Kupferberg[31] with some modifications and it has 3 major steps.

a. Solvent extraction:-

There are two major factors that affect solvent extraction. The solvent itself and the pH under which the extraction is conducted. The solvent used in this experiment was 1,2-dichloroethane, other than ethylene dichloride which had been used earlier, some attempts have been made to determine the partition ratio of some other halogenated solvents but the one recommended in the original paper was found to be best.

The other factor that influences extraction is the pH and of course pKa. The effect of pH on solvent extraction is shown in Fig 3.

Fig. 3. Effect of pH on solvent extraction.



$$D = \frac{C_{OR}}{C_{AQ}} \quad \text{- - - - - - - (6)}$$

where D is partition coefficient C_{OR} and C_{AQ} are concentration of the drug in organic phase and aqueous phase respectively.

Since only the undissociated form of the drug can be transferred into the organic layer, at low pH extraction is poor but with increase in pH, amount of the drug in the organic phase increases and eventually reaches a maximum level. This pH-range, where log D/pH = 0, is suitable to effect maximum extraction of drug from the aqueous to an organic phase.

b. Derivatization:- The first requirement for any compound to be analysed by GC is its volatility at the temperature of the injector system. However both drugs are not easily volatile. Thus direct GC analysis of these compounds is very difficult. Therefore the compounds must be chemically modified in order to convert them to volatile derivatives. This is acheived by a methylation reaction shown below.

$$C_6H_5\,N(CH_3)_3^+\ OH^- + R_2\text{-}NH \xrightarrow{\Delta} C_6\,H_5N(CH_3)_2 + R_2\,NCH_3 + H_2O$$

Trimethyl anilinium Hydroxide

6. Chromatographic analysis and interpretation

Four chromatograms are shown on Fig. 4. The first three are from plasma of 3 epileptic patients who were on chronic anticonvulsant treatment. The last chromatogram is the control.

In all of the chromatograms there are 3 peaks - The first peak appearing after the elution of solvent corrosponds to phenobarbital, the second to phenytoin and the last to the internal marker or the internal standard.

Interpretation was performed by measuring the peak height ratio obtained by dividing the peak height of the drug by that of the internal standard. This is converted into concentration by using a calibration curve which was prepared beforehand. The standard curve used in this study is shown in Fig. 5.

Figure 4. Gas chromatograms of plasma samples taken from 3 patients who where taking 300 mg of phenytoin and 100 mg of phenobarbital for long time. Chromatogram(d) is from ethanolic solution to which 20 µg of phenytoin and 30 µg/ml phenobarbital is added.

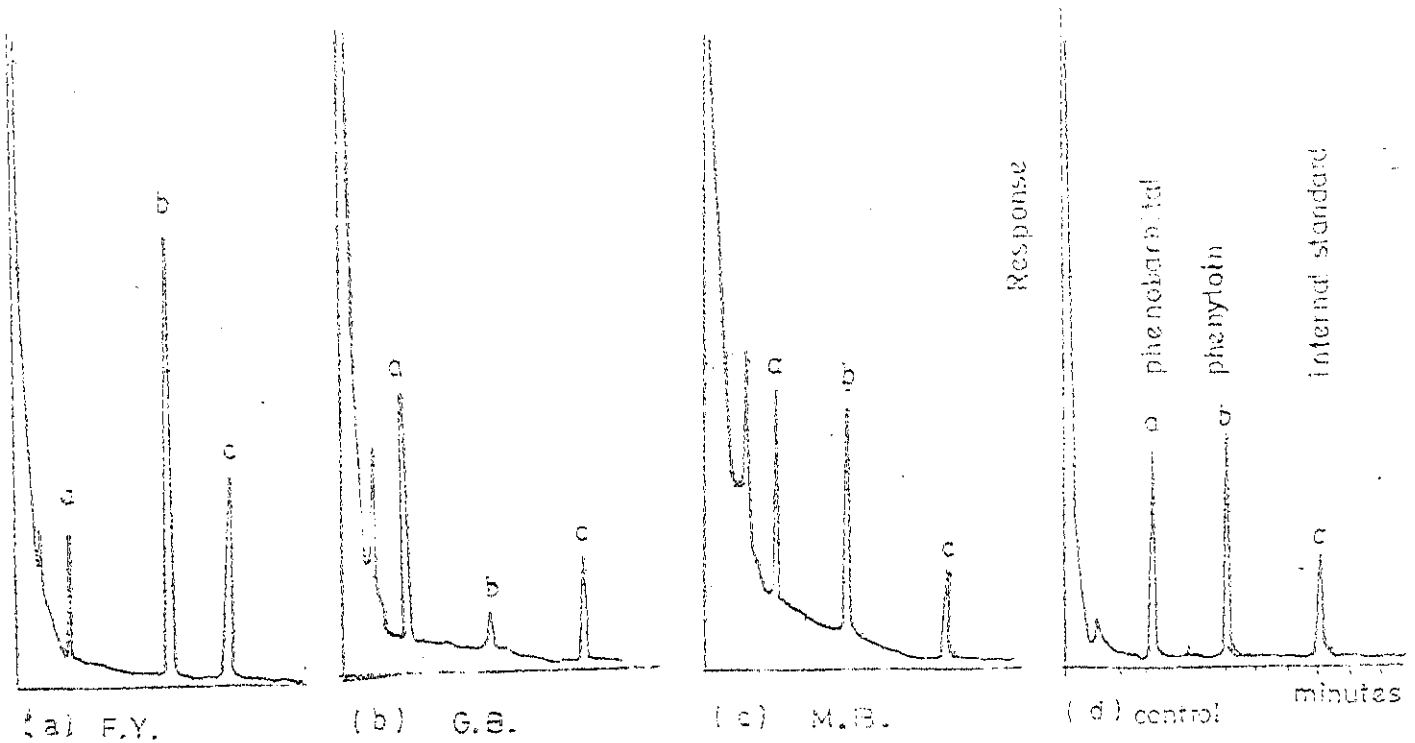
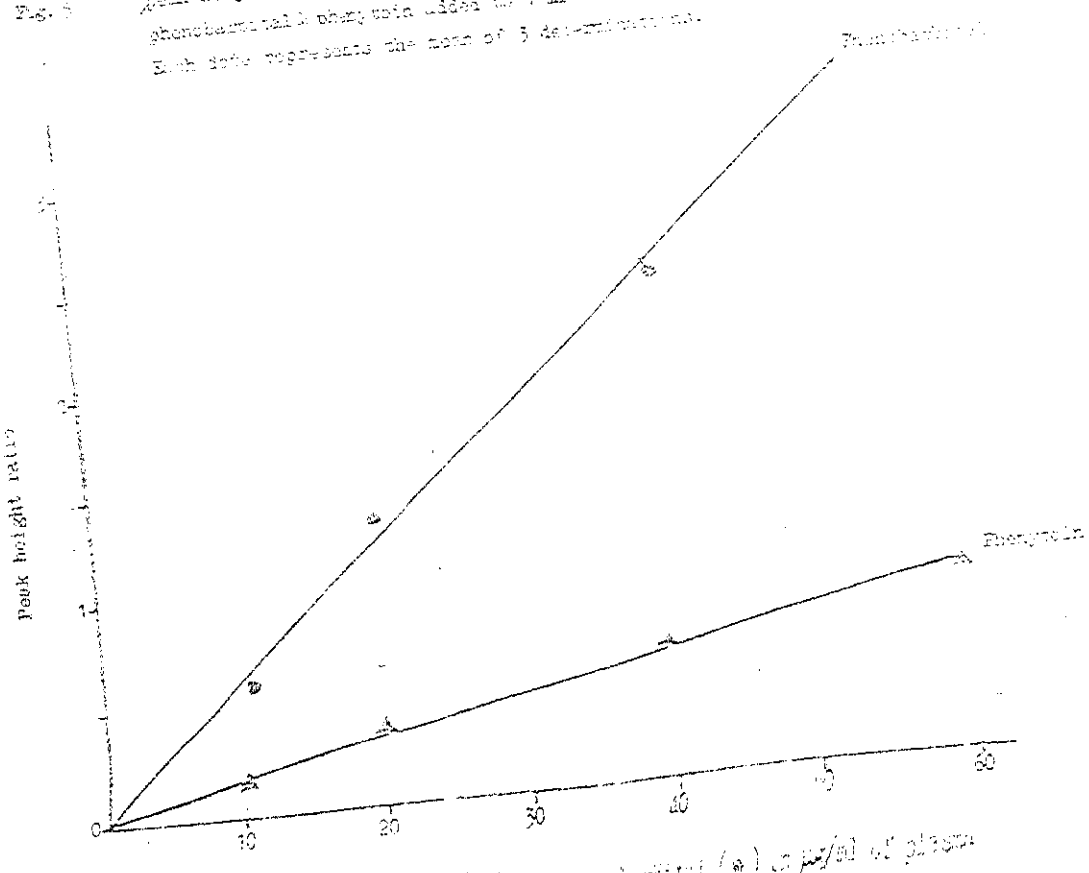


Fig. 5 Peak height ratio of cholesterol (as internal standard) to a fixed amount of phenobarbitone (▲) and phenytoin added to 1 ml of normal plasma and then extracted. Each data represents the mean of 3 determinations.



when patients are given the conventional dose, their blood level is expected to fall in the recommended therapeutic range and the chromatogram should exactly looks like the one as shown in the control, but the picture is different when you come to the real situation.

The chromatogram of the first patient shows peak height for phenytoin which is equivalent to 60 µg/ml, that the second patient on the other hand displays a peak height which is equivalent to only 5 µg/ml and in the chromatogram of the third patient an intermidiate plasma level that falls within the recommended therapeutic range is observed. Thus, although patients are given nearly equal doses; it is very difficult to anticipate what the blood levels would be as there is poor correlation between dose taken and the resultant blood level. A number of studies clearly showed that there is a good correlation between the concentration of the drug in plasma and the clinical response. Therefore problems regarding clinical manifestation and responses to these drugs could be better understood by referring to plasma levels rather than to the dose administered.

6.2 Statistical Analysis

Humanbeings are too variable to allow a strictly controlled study since each patient is unique. However, broadly speaking there is some relationship in response to some given treatment. Hence based on these general relationship some primary statistical parameters have been determined. The summary is given in Table 2.

The second column shows the average plasma levels. The average plasma levels of both drugs fall in the recommended therapeutic range. In fact plasma phenytoin level was at the upper boundary range or slightly higher. The third column indicates the standard deviation. Its value tells us how far the data are scattered around the central or the mean. Usually the values are below 1. In this study the values of standard deviation are as high as 10 times. That means the plasma levels are highly scattered away from the central value. This deviation is best shown by the values of relative standard deviation (RSD) in the next column. This clearly shows that there is high variation among individuals.

Other statistical parameters such as student t-test and confidence limit were also estimated. In general, from the statistical analysis one could observe that there is good agreement shown among the means indicating the reliability of the method used.

Table 2. Statistical parameters summary

Medication		$\bar{X}$	σ	RSD
Phenytoin (300 mg)		19.3	7.8	40%
Phenobarbital (100 mg)		22.2	9.8	45%
Combined	Phenytoin	21.0	10.6	48%
Treatment	Phenobarbital	20.4	9.3	42%

Observation :- Good agreement shown among the pairs of means indicates reliability of method used.

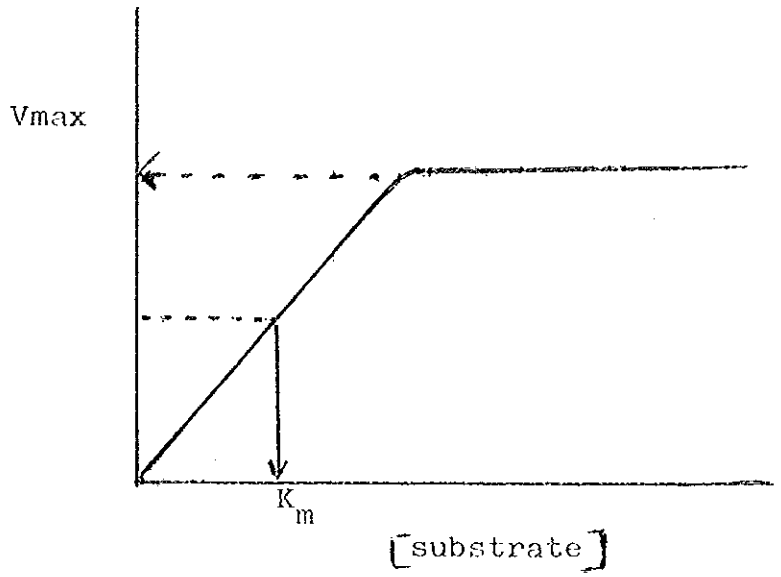
- High values of $\hat{\sigma}$ and RSD indicates high variation among individuals.

6.3 PHARMACOKINETIC DATA AND CLINICAL APPLICATION

6.3.1. Michaelis - Menten kinetics and their application

The conversion of phenytoin to its metabolite by microsomal enzymes in the liver follows zero-order kinetics. In this type of kinetics only a constant quantity of the substrate drug is transformed per unit time and the rate is independent of the amount of the drug present in the blood. Initially when the concentration of the drug is low, the metabolising enzyme is in excess hence it can't show its effect fully. As the concentration of the drug increases, the enzyme effect also increases and comes to a point where its activity reaches maximum. At this point the enzyme becomes a limiting factor; such type of kinetics are best described by Michaelis-Menten kinetics. (Fig. 6)

Fig. 6. Effect of substrate concentration on the enzyme activity



$$v = \frac{V_{max}\, C_{ss}}{K_m + C_{ss}} \quad \text{---(8)}$$

v = Metablic rate

V_{max} = Maximum metabolic capacity

K_m = Michaelis-Menten constant

C_{ss} = Substrate concentration at steady state

In the Michaelis-Menten kinetics, there are two useful parameters, K_m, drug concentration in plasma at which the rate of elimination is one-half of its maximum value and V_{max}, the maximum rate of metabolism. It's now generally accepted that the kinetics of phenytoin metabolism are best explained by Michaelis-Menten equation.[3]

In this study K_m and V_{max} values were calculated for 62 adult epileptics (13-53 yr age range). The studies were performed in individuals with steady-state plasma phenytoin concentrations. The summary of pharmacokinetic values obtained is given in Table 3. Results of other reports (for comparative purpose) are also included in the second and third line.

The mean value for V_{max} reported in the population studied in this study was 6.9 mg/kg/d. This means that 6.9 mg of the drug is converted into its metabolites per kg body weight in one day. This value is found to be slightly lower than the values reported in previous studies. This difference is because of several reasons. One such reason may be related to racial factors. The patients under the study may metabolise the drug more slowly than the populations studied

Table 3. Phenytoin Pharmacokinetic Summary

Stydy Population	Age Range (yr)	K_m (mg/kg d)	V_m (t/kg/d)	Cl (lt/kg/hr)	References
62	13-53	6.5 0.1-26.9	6.9 5.5-8.6	0.0127 0.0059-0.031	Results obtained in this study
21	Adults	5.8	8.1	-	Eadie et al [33] (1976)
-	Adults	7.5	9	-	Matike et al [34] (1981)

in other countries. If this holds true it is in close agreement with the results obtained by Arnold and Gerber[9] in 1970. The other possible reason may be due to age factor which will be discussed later.

K_m has no physical meaning. Its numerical value is that of the drug concentration at half maximum velocity. At this concentration, half of the enzyme molecules are bound to the drug. In this study the value of K_m is 6.5 mg/lt.

6.3.2 Influence of age on pharmacokinetic parameters

Metabolism of phenytoin to its metabolites is age dependent. Children metabolise at a faster rate than adults. This phenomenon is also further confirmed in this study. A total of 56 epileptics with different age groupings were studied for their metabolic capacity. The results are shown in Table 4.However a detailed analysis indicates that there are statistically valid differences in V_{max} between age classes, Strongest between young (16-19) and patients over 30. There appears to be a trend of reduction in V_{max} untill age about 30 and then little further reduction. The correlatien coefficient estimated and its value (r=0.18) does not give a straight line relationship. The theoretical curve showing general relationship of V_{max} with age is given in Fig. 7

Fig. 7. Theoretical curve showing general relationship of V_{max} with age.

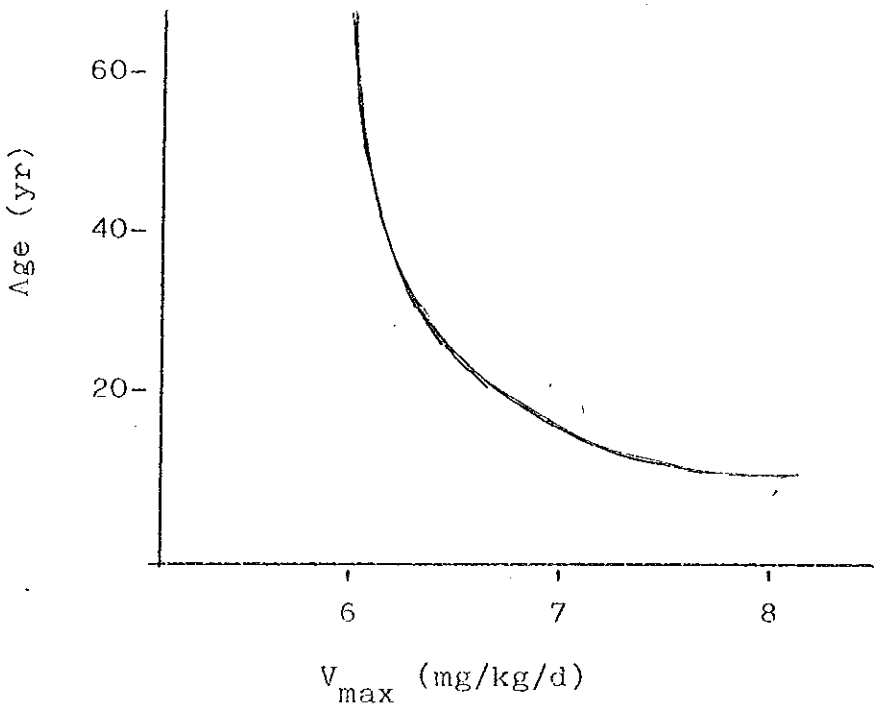


Table 4. Metabolic capacity in relation to age range in a total of 56 chronic epileptics.

Age range (yr)	Mean age (yr)	V_{max} (mg/kg/d)
16-19	17.5	7.4
20-29	24.1	7.1
30-39	35.3	6.8
40-53	46.3	6.3

A similar decrease in metabolic capacity with increasing age were reported in other studies. This is shown below (Table 5). For the ease of comparison the results achieved in this study are also listed.

Table 5. Metabolic capacity in relation to age

	Age range (yr)						References
	0-10	10-19	20-29	30-39	40-49	50	
V_{max} (mg/kg/d)	-	7.4	7.1	6.8	6.1	-	Results obtained in this study
	-	-	7.5 (20-39)	-	6.6 (40-59)	6.0 (60-79)	Bauer & Blouin[35] (1982)
	20.4 (0.6-28)	8.7 (18-66)	- -	- -	- -	- -	Blain et al[36] (1981)
	17.9 (1)	9.6 (10-22)	-	- -	- -	- -	Dodson (1982)[37]

The decrease in rate of elimination capacity with increase in age was also found to be true for phenobarbital. Since phenobarbital does not undergo capacity-limited metabolism Michaelis-Menten kinetics is not applicable; however this effect is shown by estimating the ratio of plasma level to daily dose (E) using the following equation.

$$E = \frac{C_{SS}}{R} \qquad \text{----------(9)}$$

where C_{SS} = steady state concentration and R is daily dose

Table 6. Phenobarbital elimination rate in relation to age range in a total of 61 epileptics

Age range (yr)	E
16 - 19	0.062
20 - 29	0.065
$\geqslant$ 30	0.070

The value of E increases as age range increases. However the correlation observed was not linear.

6.3.3. Dosage adjustment based on steady-state concentrations

The estimated dosage for a given plasma level is calculated using the following equation.

$$\text{Dose (mg/kg/d)} = \frac{V_{max} \cdot C_{ss}}{K_m + C_{ss}} \quad \text{----------} \quad (10)$$

In this study the estimated dosage using the above equation calculated and the results are shown below (Table 7)

Table 7. Estimated dosage in relation to age

age range (yr)	mean age (yr)	estimated dose (mg/kg/d)	% of reduction of estimated dose to maintain C_{ss}
16-29	17.5	6.3	-
20-29	24.1	5.0	21
30-39	35.9	4.5	29
⩾ 40	46.3	4.3	32

The last column in Table 7 gives percentages of reduction of estimated dose to maintain steady state concentrations. It was estimated that the elderly (40-53 yr) require 32% less phenytoin per day than 16-19 yrs group to maintain steady state concentration of 20 µg/ml.

The average estimated dose was 5.2 mg/kg/d, a figure which is less than that of V_{max}. The value of estimated dose greater than the value of V_{max} is not applicable because no steady state concentration can be acheived. For phenytoin the therapeutic concentration almost always exceeds (97% the value of K_m. Thus it's evident that phenytoin undergoes

capacity-limited metabolism.

6.4. Dose versus concentration and response relationship

6.4.1. Dose in relation to blood level

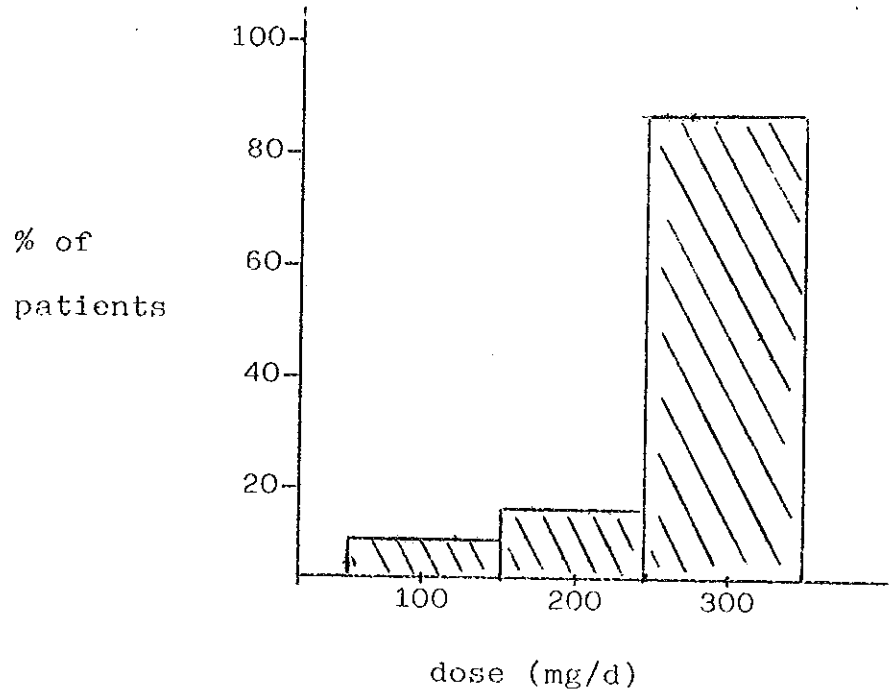
Literature plasma level ranges in relation to dose and the actual values obtained in this study are given in Table 8.

Table 8. Literature blood level ranges and the actual values of phenytoin and phenobarbital obtained in this study.[38]

Medication dose	Expected plasma level (µg/ml)		Actual plasma level obtained (µg/ml)	
	range	Av	range	Av
Phenytoin 300 mg/d	5-20	10	5-60	19.3
Phenobarbital 60-100 mg/d	10-30	20	5-45	22

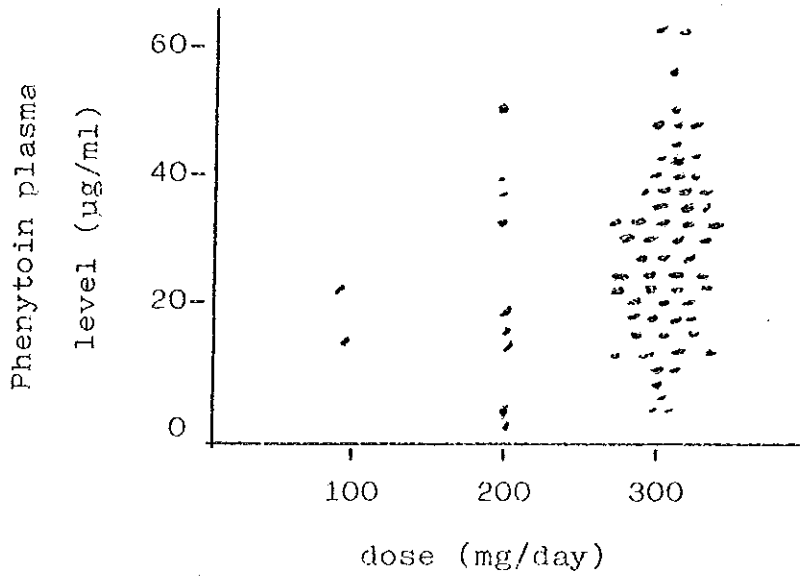
A total of 72 adult patients were given 100 mg, 200 and 300 mg of phenytoin once daily for months and plasma levels were determined. The majority of patients (86%) were receiving 300 mg/d. The data obtained is shown in Fig.8.

Fig. 8. Dose distribution of phenytoin in a total of 72 adult epileptic patients



Since the half-life of the drug is sufficiently long, the plasma concentrations determined represent the steady-state plasma level. It is a well known fact that there is high variation of plasma levels for a given daily dose. This fact also holds true in patients considered in this study. Fig. 9 shows phenytoin plasma level distribution in relation to dose.

Fig. 9. Relationship between prescribed dose of phenytoin and the concentration in plasma of patients with epilepsy.



For a daily dose of 300 mg of phenytoin the plasm levels vary from 5 µg/ml to 60 µg/ml. There could be several reasons why low or high plasma levels in relation to dose is brought about.

In patients who were treated with both drugs simultaneously, the physiological response induced would be the combined effect and the response-dose relationship is very difficult to predict. Such high variation in plasma level in relation to dose is also true in the case of phenobarbital. This non-linear relationship between dose and plasma level makes epileptic treatment difficult at present time.

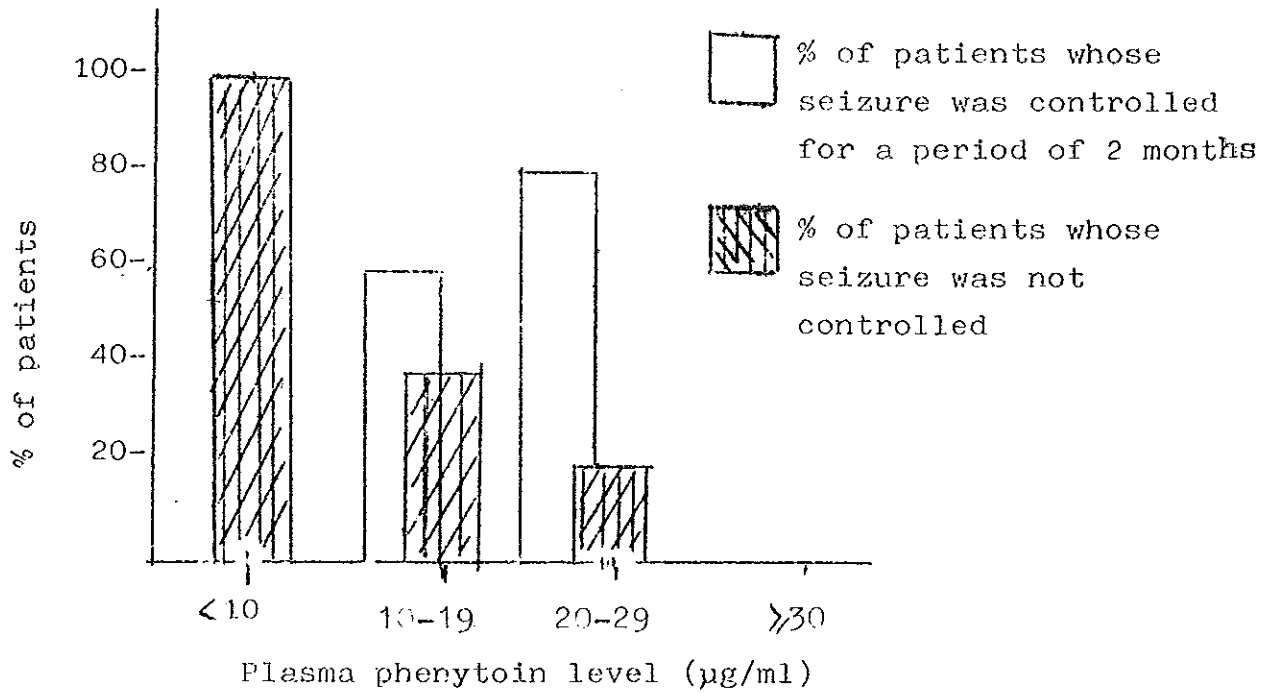
6.4.2 Plasma level in relation to response.

Although the correlation between plasma level and

clinical response is high, the correlation between dose and plasma level is very low not only among subjects but also in the same subject under different situations. For these reasons, problems in the clinical use of phenytoin are better understood by referring to plasma level rather than dose.

Treatment with phenytoin, phenobarbital or with any of the presently available antiepileptic drugs does not cure epilepsy, but only suppresses the recurrence of seizure. Thus seizure-free days is common measuring parameter of response of patients towards antiepileptic treatment. The plasm phenytoin level in relation to percentage of seizure-free patients is given in Figure 10.

Fig. 10. Plasma phenytoin level in relation to response in a total of 56 epileptic patients



Out of the total number of patients, 4 had plasma levels below 10 µg/ml and none-of them had controlled seizure. There were 25 patients with plasma level in the range of 10-19 µg/ml out of which 15 were with controlled seizure and out of the 27 patients who were having plasma level above 29 µg/ml, 21 patients were controlled seizure. Thus the majority of patients on phenytoin had excellent control of their seizures in the range of 20-30 µg/ml and occasionaly above. Out of the 77 patients who were taking phenobarbital either singly or combined with phenytoin, 56.8% of patients had phenobarbital levels in the therapeutic range of 10-30 µg/ml, 18.9% had levels below 10 µg/ml and 24.3% had levels above 30 µg/ml. Seizure free periods also correlated positively with the plasma level of phenobarbital.

The highest percentage of patients show toxic symptoms above 30 µg/ml of plasma phenytoin level and above 40 µg/ml of plasma phenobarbital level. However there are still few patients with plasma level at 15 µg/ml of phenytoin and 20 µg/ml of phenobarbital and show toxic symptoms. Ingeneral seizure free peroids and toxic sign and symptoms correlated positively with plasma level of the anticonvulsants.[39]

Conclusion

1. A poor correlation and wide variation in plasma level to a given dose of both drugs have been observed.
2. Clinical response correlated positively with the plasma level of the anticonvulsants. However it is less significant for phenobarbital.
3. The majority of patients on phenytoin had excellent control of their seizure in the range of 20-30 μg/ml and occasionally above. The same is found true for those patients who were on phenobarbital.
4. Michaelis-Menten parameters have been determined and the figures seem to indicate that the patients considered were slow metabolisers.
5. The most likely phenytoin dosage regimen based on pharmacokinetic parameters was estimated and the mean is found to be 5.2 mg/kg/d.
6. Low V_{max} and high plasma level values probably indicate population based genetic variation.
7. A statistically valid difference in V_{max} between age classes was observed. Strongest between young (16-19 yr) and patients over 30. There appears a trend of reduction in V_{max} until age about 30 and then little further reduction.
8. A wide variation in response to a given dose among individuals is observed and thus routine monitoring of plasma level of these drugs specially in patients with renal and hepatic disorders and are on multiple drug treatment is highly recommended.

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D E C L A R A T I O N

I, the undersigned, declare that this thesis is my work and that all sources of material used for the thesis have been duly acknowledged.

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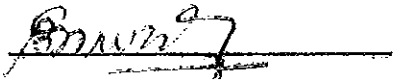
Signature : 

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