CHAPTER II

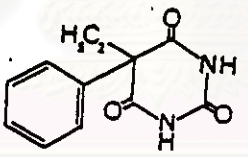
REVIEW OF LITERATURE

I. REVIEW OF PHENOBARBITAL

Structure-Activity

The chemical name for Phenobarbital is 5-phenyl-5-ethylbarbituric acid. The Structure-activity relationship of the barbiturates has been studied extensively. Maximal antiseizure acitvity is obtained when one substituent at position 5 is a phenyl group. The 5, 5-diphenyl derivative has less antiseizure potency the does phenobarbital, but it is virtually devoid of hyphotic activity. By contrast 5, 5-dibenzyl barbituric acid causes convulsions(2).

Structure formula :



Phenobarbital : consists of colourless crystals or a white odorless crystalline powder. It may exhibit polymorphism.

Soluble : 1 in 1000 of water and 1 in 10 of alcohol ; sparingly saluble in chloroform ; soluble in ether ; soluble in aqueous solutions of alkali carbonates and hydroxides an in ammonia. A saturated solution in water has a pH of about 5.

Phenobarbital Sodium : consist of white odourless or almost odourless hygroscopic powder, granule, or flakes. It loses not more than 7% of its weight when dried.

Soluble (Phenobarbital sodium) : 1 in 3 of water and 1 in 25 of alcohol practically insoluble in chloroform and ether. A 10% Solution in water has a pH of 9.2 - 10.2.

Incompatibility : with many other drugs and phenobarbital may precipitated from mixtures containing phenobarbital sodium.

Store : in airtight containers. (18)

Phenobarbital is a weakly acidic compound with a pka of 7.3. These acidic properties have an importance in the distribution and excretion of the drug. At a pH of 7.4 approximately 40% of the drug is un-ionized. Only the un - ionized fraction can cross cellular membranes. Variations of pH modify both renal excretion of Phenobarbital and its distribution between the intra and extracellular compartments.

A variety of methods of determination of phenobarbital levels in biological fluids and tissue are available : initially spectrophotometry, then thin - layer chromatography, gas - liquid chromatography, high - pressure liquid chromatography, gas chromatography - mass spectrometry, and immunoassays. Less precise but easier than chromatographic methods, homogeneous enzyme immunoassay (EMIT) is now widely used(1).

Mechanism of Action

The exact mechanism of action of phenobarbital is unknown, but enhancement of inhibitory processes and diminution of excitatory transmission probably contribute importantly. Phenobarbital inhibits seizures likely involves potentiation of synaptic inhibition through an action on the γ-aminobutyric acid (GABA) receptor.

Recent data indicates that phenobarbital may selectively suppress abnormal neurons, inhibiting the spread and suppressing firing from

the foci. At high concentrations, Phenobarbital decreases sodium and potassium transcellular transport, reducing membrane sodium ion and potassium ion conductance. It also reduces the calcium ion influx in the presynaptic endings. The two actions would result in a decreased release of such neurotransmitters as GABA, Glutamate, aspartate, and acetylcholine, as well as a reduction in the generation of those excitatory and inhibitory post - synaptic potentials that depend on increased sodium conductance. Phenobarbital depresses the cytochrome oxidase system responsible for the synthesis of high - energy phosphate compounds necessary for the maintenance of membrane potentials. It also binds to dihydropicrotoxin receptors and directly activates GABA - related chloride channels, thereby enhancing postsynaptic chloride conductance. However, these effects on ion transport appear more related to its sedative and/or anesthetic properties than to its anti-convulsant action.

At "therapeutic" concentrations phenobarbital produces few changes in membrane conductances. It probably exerts its anticonvulsant action by specifically increasing postsynaptic GABA ergic inhibition and simultaneously reducing glutamic excitation. (1, 2, 19, 20)

Phamacological Properties

Most barbiturates have antiseizure properties. However, the capacity of some of these agents, such as Phenobarbital, to exert maximal antiseizure action at doses below those required for hypnosis determines their clinical utility as antiepileptic drug. Phenobarbital is effective in the treatment of partial seizures and generalized tonic - clonic seizures, though the drug is often tried for virtually every seizure type, especially when

attacks are difficult to control. There is little evidence for its effectiveness in generalized seizure such as absence, atonic attacks (2, 20, 21).

Route of Administration

Phenobarbital can be given by the oral, intramuscular or intravenous route, as the base or the sodium salt. But in first 3 weeks of life, the absorption of the drug will fluctuate. After third week, rate of absorption from gastrointestinal tract will increase more than in adults (22).

Therapeutic Efficacy

The goal of antiepileptic drug therapy is the best possible seizure control with minimal side effect. Phenobarbital is a relatively safe and generally effective drug for the treatment of many types of seizures. As for other antiepileptic drugs, there is considerable interindividual variation in the serum concentration of phenobarbital required for seizure control.

Relation of serum level to seizure control for Phenobarbital was elegantly determined . Buchtal et al observed untreated patients and Phenobarbital was administered in small doses that were gradually increased. The average level at which the EEG and clinical response occured was 10μg/ml(23). From a large sample of patients, Booker (3) concluded that the subjects who respond to treatment with phenobarbital will do so with plasma levels between 10 and 40 μg/ml. In other words, an initial dosage sufficient to produce steady-state levels in the 10 - 15 μg/ml range is recommended. When seizures persist, the dose should be progresssively increased. When a rapid efficacy is needed, a loading dose of twice the

usual daily maintenance dose, given for 4 days, brings the serum concentration to the steady-state value within 3 days. (1)

The optimal phenobarbital plasma concentration might vary with the type of seizure. Schmidt et al (24) found that higher concentrations were necessary for control in epilepsy with partial seizure (37 μg/ml) than in epilepsy with tonic - clonic seizures alone (18 μg/ml).

The therapeutic levels of phenobarbital in most patients range from 10 to 40 μg/ml. Documentation of effectiveness is best in febrile seizures, and levels below 15 μg/ml appear ineffective for prevention of febrile seizure recurrence. The upper end of the therapeutic range is more difficult to define, as many patients appear to tolerate levels above 40 μg/ml (20).

The suggested therapeutic range for neonates is 15 - 25 μg/ml , for infants and children is 10 - 20 μg/ml (12). The recommended maintenance dose for adult is 3 - 5 mg/kg/day , for infants is 5 to 10 mg /kg/day , and for children is 2 to 4 mg/kg/day (12). As show in table 1, Morselli concludes from many variation to recommend loading dose, maintenance dose and therapeutic range

Table A Kinetic Variables, Therapeutic Levels, Suggested Loading and Maintenance Doses of Phenobarbital in Three Age Group (12)

Phenobarbital	Neonates*	Infants	Children
Route of administration	IV, IM	oral, IM, IV	oral, IM, IV
Loading dose	5-10 mg/kg IV	15-20 mg/kg	10 mg/kg
Maintenance dose	5 mg/kg/day	5-10 mg/kg/day	2-4 mg/kg/day
Volume of distribution (L/kg)	0.6 - 1.5	0.5 - 1.3	0.4 - 0.6
Protein binding (% bound)	30-43	46-48	48-50
Plasma half-life (h)	130-500	30-60	25-70
Suggested therapeutic range (mg/L)	15-25	10-20	10-20

* In newborns the plasma half-life may be considerable shorter in case of induction in utero.

Adverse effect and toxicity

Neurotoxicity

Sedation

The hallmark of barbiturate toxicity in adults is surely sedation. Complaints of fatigue and tiredness are difficult to quantify are often variable and subtle. Two sorts of sedation are produced by Phenobarbital. Some degree of drowsiness is common in the first days of

therapy and usually passes without any reduction of Phenobarbital dosage. A different sedation exists in many chronically treated patients. It is usually but not always dose - related. It consists in intellectual blunting, sluggishness, decreased libido, impotence, apathy, and depression more than in drowsiness (2, 26).

Neurological side effects

Increasing the dosage of phenobarbital eventually leads to neurological signs dysarthria, incoordination, ataxia, and nystagmus appear as serum levels exceed 40 mg/ml(2,26).

Behavior

Instead of the sedative effect of phenobarbital common in adults, a paradoxical effect of the drug in children and in elderly may produce insomnia, irritability, aggressiveness, and hyperactivity or even hyperkinetic syndrome. The pattern of behavior included signs of distractability, shortened attention span, fluctuation of mood, and aggressive outbursts(2, 26). Unfavorable behavioral changes have been estimated to occur in 20% to 75% of children receiving phenobarbital, hyperactivity being the most common symptom(27, 28).

In a study of 109 children treated daily with phenobarbital following the first febrile convulsion, 42% developed a behavior disorder, usually hyperactivity. The behavior disturbance usually appeared within several months, was not correlated with high blood barbiturate levels, disappearal in 73%, and improved in all children when Phenobarbital was discontinued (27).

Affect

Phenobarbital therapy can produce alteration of affect (26).

Cognition

A side effect of Phenobarbital of considerable potential importance, especially in children, is a possible disturbance in cognitive function. Problem with memory or compromised work and school performance may develop independent of sedation and hyperkinetic activity, although these factors may play a contributory role.

Farwell et al (28) concluded that phenobarbital depresses cognitive performance in children treated for febrile seizures and that this disadvantage, which may outlast the administration of the drug by several months, is not effect by the benefit of seizure prevention. In the study of 217 children between 8 and 36 months of age, they compared the intelligence quotients (IQs) of a group assigned to Phenobarbital with the IQs of a group assigned to placebo, assigned to phenobarbital than in the placebo group. Six months later, after the medication had been tapered and discontinued, the mean IQ was 5.2 points lower in the group assigned to phenobarbital(29).

A placebo - controlled study of side effects of phenobarbital in toddlers. There were no significant differences in IQ between placebo and phenobarbital groups after 8 to 12 months of therapy. However, detrimental effects of phenobarbital were found in memory, for which serum level influenced scores, and in comprehension, in that length of treatment time affected performance(30). There was a negative result study performed on 50 children with a history of febrile convulsions. Psychometric tests were no significant differences between 2 groups (31).

Phenobarbital overdose

Phenobarbital overdose causing serum levels in excess of 50 to 60 μg/ml leads to progressive neurologic dysfunction and depression in consciousness(26). Sedation and ataxia occur in chronically treated patients at phenobarbital serum concentrations of 35 to 80 μg/ml. Coma with intact reflexes of 65 to 117 μg/ml, and coma without reflexes is associated with levels greater than 100 μg/ml (32).

Hematological toxicity

Megaloblastic Anemia

Megaloblastic Anemia is a disorder with characteristic abnormalities of the blood and bone marrow induced by impaired DNA synthesis. Phenobarbital can induce megaloblastic anemia by impaired folate absorption or utilization (33)

Folate Deficiency

Serum and red cell folate defficiency is relatively common. The mechanism of action of phenobarbital in reducing folate status may inhibit absorption or induce hepatic enzymes that require folate. (33)

Vitamin K

Hemorrhagic diathesis in neonates born from epileptic mothers taking Phenobarbital has been reported (34). The coagulation defect is similar to that observed in vitamin K deficiency : a decrease in the activity of clotting factors II (prothrombin), VII, IX, and X, with normal levels of clotting factors V, VII, and fibrinogen. Supplementation of vitamin K is recommended for epileptic pregnant women who are near term (2).

Red Cell aplasia

Phenobarbital also reported to induce red blood cell aplasia.

Bone disorder

Osteomalacia and rickets induced by long term treatment with anticonvulsant drugs. The disorders are due to a drug - related 2, 5 dihydroxyvitamin D decrease. By increasing hepatic conversion of VitaminD and 25 - hydroxy - vitamin D to more polar biologically inactive metabolites. (2, 33) But there is a study indicated that in ambulatory patients with adequate diet and outdoor activities in Italy clinically irrelevant impairment of bone metabolism. (35)

Disorders of Connective tissue

Phenobarbital produces an increased tendency to fibrosis. Coarsening of facial features is observed in most patients after many years on Phenobarbital. Dupuytren's contracture and frozen - shoulder syndrome are found in Phenobarbital treated patients. (7)

Hepatic toxicity

Liver disease such as acute hepatitis induced by phenobarbital appears not to be dose dependent and has a low incidence.

Phenobarbital are potent inducers of hepatic microsomal enzymes, which can lead to enhanced metabolism of other drugs or endogenous substance such as 6 - hydroxylated steroids. It has been shown to enhance the synthesis of aminolevulinic acid (ALA) synthetase which can cause chemical porphyria.

Phenobarbital also used in the management of thyrotoxicosis, because it stimulated the rate of thyroxine and triiodothyronine clearance.

Bilirubin conjugation has been induced by phenobarbital usefully to treat neonatal hyperbilirubinemia (36), because of induced action of hepatic microsomal enzymes. (7)

Hypersensitivity Reactions

Phenobarbital caused various types of skin reactions (less than 3% of all patients receiving phenobarbital). These usually are mild maculopapular, morbiliform, or scarlatiniform. And there are also reports of exfoliative dermatitis, erythema multiforme, Stevens - Johnson syndrome, or toxic epidermal necrolysis are impressively rare. Hypersensitivity reactions usually occur within 1 to 5 weeks of therapy.

Other adverse reactions

Acute interstitial nephritis ; interference with biochemical test (increase urinary excretion of 6 - hydroxylated steroids etc.) ; increase serum Sex - hormone - binding globulin (SHBG) level, plasma caeruloplasmin, activity of Lactate dehydrogenase, elevate serum ALP, GGT

Pharmacokinetic Properties

1. Absorption

A. Oral - Oral absorption of phenobarbital (acid, tablets) is complete and rapid. The time required to reach peak serum concentration ranges from 0.5 to 4 hours. The rate of absorption from gastrointestinal tract depends on the preparation administered as well as on physiological factors

such as gastric emptying. The rate of disintegration of tablets, the chemical form of the drug, the crystal size, the tendency of the crystals to become wet, and the solubility of the drug in water all influence the speed of entry into the circulation. These variables have been only partially examined in connection with phenobarbital. Despite the clinical impression that barbiturates are absorbed rather rapidly from the gastrointestinal tract, numerous investigations have shown that several hours may elapse before blood concentrations of the drug reach a peak. For example, in three young men given 750 mg of phenobarbital powder with 500 ml of water, Lous observed the highest plasma concentrations 6 to 8 hours after intake. He suggested that the slow rate of absorption in these experiments might be partly attributable to the large dose and the insolubility of the drug.

Although barbiturates can be absorbed from the stomach, under normal circumstances most of the drug enters the circulation from the small intestine. Drugs that affect the circulation or the motility of the gastrointestinal tract may influence the rate of absorption of barbiturates.

In newborns, phenobarbital is absorbed in the small intestine following oral administration. But in first 3 weeks, the absorption is not certainly stable, after that the rate of absorption will be higher than adults. Although bioavailability studies have not been performed in newborns, absorption from the gastrointestinal tract is considered to be complete. The presence of food delays the rate but not the extent of absorption. (1, 32, 37)

The available data indicate that at least 80% and probably more than 90% of phenobarbital administered orally is absorbed. Complete bioavailability (F = 1.0) is supported by the observation that similar plasma concentrations are observed when the same dose of Phenobarbital is given orally and parenterally. (11)

B. Intramuscular and Intravenous

The rate of absorption of phenobarbital after oral administration is not appreciably different from its rate of absorption after intramuscular and intravenous administration and found to be close to unity(32).

2. Distribution

The distribution characteristics of phenobarbital can be separated into an early distribution phase and late phase

2.1 .Early distribution - Shortly after absorption, phenobarbital is present in high concentrations in the more vascular organs. The outstanding exception is the brain. Phenobarbital appears to penetrate the brain slowly, and when it is used to treat status epilepticus, an immediate effect is not expected. The ability of a drug to enter the brain and accumulate in the cerebrospinal fluid is related to its oil/water partition coefficient. The coefficient of phenobarbital is low.

2.2 Late distribution - A few hours after administration drug becomes approximately uniformly distributed throughout the brain and is found in nearly equal concentrations in all tissues of the body. The cerebrospinal fluid and saliva, which only small amounts of protein, exhibit lower concentration of phenobarbital than are observed in plasma. The cerebrospinal fluid to plasma ratio (about 0.47) is close to the free fraction of phenobarbital in plasma (0.40) , and the saliva to plasma ratio is about 0.35. The lower level in saliva can be explained by nonionic diffusion serum concentration ratios are approximately 0.4, which can result in both therapeutic serum concentrations and clinical signs of toxicity in the nursed infant. (32 , 37)

Phenobarbital is bound to both plasma and tissue proteins, but mainly to plasma albumin, and its free fraction increases in hypoalbuminemia. (32) Phenobarbital is 40% to 60% bound to plasma albumin and bound to a similar extent in tissues, including brain. In neonates protein binding of phenobarbital is low (about 10 - 30%) and will be lower when there is a hyperbilirubinemia (free 80 to 90 %). As a result, the new born brain is exposed to a higher free fraction of phenobarbital than an adult brain (22).

The steady - state volume of distribution (Vd) is 0.4 - 0.6 L/kg in adults and children (22, 38, 39) because neonates have low protein binding capacity so they have high Vd (0.6 - 1.5 L/kg) (40) and 0.5 - 1.3 L/kg in infant(22).

3. Metabolism

Phenobarbital is partly metabolized and partly excreted unchanged in the urine. About 25% is excreted unchanged in urine, its lipid solubility being sufficiently low for it to be incompletely reabsorbed from the renal tubules. The remaining 75% is metabolized, Its metabolism follows straight forward linear pharmacokinetics. The steady state concentration is proportional to dosage. It is primarily metabolized in the liver by the hepatic microsomal enzyme system. There are two major metabolic pathways. Hydroxylation of the phenyl ring gives rise to p - hydroxy phenobarbital with, as a probable precursor, an arene oxide not measurable in body fluids. Parahydroxyphenobarbital is the main primary metabolite. A significant fraction of the parahydroxy metabolite is glucuronidated. Other minor metabolites such as N-glucoside, dihydroxy, or cathecol compounds

have also been identified. The metabolites are excreted directly or are subsequently conjugated and excreted as glucuronides or sulfates. Together these pathways account for 30% to 50% of a dose. About 25% of the drug is excreted unchanged in the urine(2, 32).

Boreus et al. (1978) compared urinary excretion patterns of phenobarbitone in newborns and adults and found striking similarities. Both groups excreted 16 to 17% of a dose as unchanged drug and 9 to 10% as the p-hydroxymetabolite. However, there was a significant difference in urinary excretion of the metabolite conjugated to glucuronide, with adults excreting 15% of a dose in this form compared with 5% in neonates, suggestion a reduced capacity to conjugate p-hydroxyphenobarbitone in the neonate but not to hydroxylate phenobarbitone.

Although phenobarbital is a potent inducer of cytochrome P 450 enzymes, and in animals induces its own metabolism (41), autoinduction does not seem to be significant in man (42)

4. Excretion

The half - life of a drug is the combined result of metabolism and urinary excretion of the unchanged compound. Both hepatic metabolism and renal clearance of phenobarbital are very slow. They vary considerably among patients. The total body clearance of phenobarbital is 3.7 +/- 0.7 ml/hr/kg. The renal clearance (1.5 ml/hr/kg) is urine-flow as well as pH-dependent. Phenobarbital is excreted from the kidney into acidic urine largely in the un-ionized form. It is readily reabsorbed from the kidney tubule. Shifts in urinary pH modify phenobarbital clearance. In alkaline urine the excreted phenobarbital becomes ionized and cannot be reabsorbed.

Of all antiepileptic drugs, phenobarbital exhibits the longest elimination half-life. The half-life in adults (75 to 126 hours). It is shorter in neonates (130-500 hr.) and longer than infant (30-60 hr.) and children (25-70 hr.) (2, 22, 32).

Drug Interaction

Probable Mechanisms involved in pharmacokinetic interactions.

Absorption

Phenobarbital is usually almost completely absorbed, and there have been no reports of its absorption being directly altered by other drugs through chelation. It is conceivable that drugs that greatly facilitate intestinal motility and emptying time may reduce the amount of drug absorbed. Absorption of griseofulvin, however, has been suspected to be reduced by phenobarbital.

Protein Binding

Phenobarbital is approximately 50% bound to plasma protein. It is unlikely that it would displace other drugs or be displaced by other drugs to any significant extent. This mechanism has not yet been implicated as a major factor in any of the reported interactions.

Altered Biotransformation

Induction and inhibition of biotransformation have been key elements in the majority of interactions between phenobarbital and other drugs.Phenobarbital is the prototype among inducers of the hepatic mixed-function oxidase system which effects the biotransformation of numerous drugs and endogenous substances.

The mode of action by which phenobarbital produces induction is complex and only partially understood. It appears that an increase of production and a decrease of degradation of enzyme both occur(43).

Clinical interactions manifesting in changes of Phenobarbital kinetics by other drug

Moderate Clinical significance

- Valproic acid

The interaction between phenobarbital and valproic acid is probably one of the clinically most important interaction in this group , because it always be used together. When Phenobarbital and valproic acid are used together, plasma phenobarbital will increase (44). The clinical manifestation was increasing somnolence, sometime resulting in coma, within days or weeks after the initiation of valproic acid administration.

The mechasnism by which valproic acid caused phenobarbital accumulation is thought to involve inhibition of phenobarbital metabolism. Valproic acid inhibit phenobarbital hydroxylation, a decrease of urinary p-hydroxyphenobarbital was also noted following addition of valproic acid. Another theoretically possible mechanism that may ontribute to phenobarbital accumulation is a reduction of its renal excretion by valproic acid lowering the pH., the role of pH in the mechanism of this interaction remains uncertain. Most likely, the valproic acid - induced phenobarbital accumulation is caused by a combination of factors , some of which are still undetermined.

Elevate plasma phenobarbital concentration

- chloramphenicol, Primidone, Propoxyphene

MAOI - Prolong effect of phenobarbital

Minor Clinical Significance

Large dose of Vitamin B6 and triacetyloleandomycin reduce plasma phenobarbital concentration.

Clinical interactions manifested in changes of kinetics of other agents by Phenobarbital.

Major Clinical Significance

A declines in anticoagulant effect of bishydroxycoumarin and warfarin after the start of phenobarbital administration has been reported. Although the mechanism of reduction of anticoagulant activity by phenobarbital is generally thought to be caused by induction of the anticoagulant metabolism, interference with absorption may also be partly involved.

Moderate Cilinical Significance

Phenobarbital reduces plasma level of tricyclic antidepressant, beta blocker , theophylline, chloramphenicol, chlorpromazine, Quinidine, Doxycycline disopyramide.

It reduces therapeutic effect of acetaminophen, methadone, oral contraceptive, and reduces plasma concentration of corticosteroids, when used with alcohol and benzodiazepine ; it reduces effect of calcium channel blocker. When phenobarbital is used with CNS depressants, it will increase CNS depression effect. The nephrotic effect of methoxyflurane or methoxyflurane and tetracycline or aminoglycoside will increase when they are used with phenobarbital. It also increase toxic effect of meperidine.

Minor clinical significance

Phenobarbital reduces plasma concentration of carbamazepine, cimetidine, phenylbutazone, digitoxin, griseofulvin, metronidazole, phenytoin, and rifampicin. The excretion of antipyrine, clonazepam, and doxorubicin

will increase when used with phenobarbital. The metabolism of cyclophosphamide and lidocaine are also effected by phenobarbital. (43 - 46)

Relation of dose to plasma level

The relationship of dose of phenobarbital to plasma level at steady state is significantly influenced by age. Because phenobarbital's half life is long, even compared with a 24 - hour dosing interval, and because its kinetics are not dose dependent, the relationship between dose and plasma level at steady state is more stable than for many other antiepileptic drugs.

In adults, each mg/kg per day gives a plasma level of approximately 10 mg/ml (47, 48). In children, plasma clearance is more rapid, the half - life averages around 50 hour, and the ratio of plasma level to dose at steady state is lower. These effect are related to basal metabolic rate. Body weight or age, however, are used to determine and give adequate results in clinical practice. Dose in very young children need to be two or more times those given to adults to achieve the same steady state plasma level. The average of the level dose ratios reported by several workers of patients in age group 1 to 5 years is 4.4, 5 to 10 years is 5.6, 10 to 15 is 7.0, and in adults is 9.6 (3). In one study, the dose ratio appeared to decline with increasing age in infants up to age 1 year, then to increase with age in patients above 1 year old (49). Co - administration of other drugs can affect the kinetics of phenobarbital, generally increasing the ratio of plasma level to dose.

The purpose of this study was to investigate to effect of dose, age and associated therapy on phenobarbital serum levels in Thai paediatric patients,

II. Review of homogeneous enzyme - immunological assay technology (CEDIA®)

The CEDIA homogeneous enzyme immunoassay is for the quantitation of phenobarbital in human serum or plasma using automated clinical chemistry analyzers. Measurements are used in the diagnosis and treatment of phenobarbital overdose and in monitoring levels of phenobarbital to ensure proper therapy.

The assay is based on the bacterial enzyme - galactosidase. The enzyme has been genetically engineered into two inactive fragments, the Enzyme Donor and the Enzyme Acceptor. Enzyme Donor and Enzyme Acceptor spontaneously reassociate to form fully active enzyme. In an assay format, the active enzyme cleaves a substrate generating a color change that can be measured on a spectrophotometric clinical chemistry analyzer. Figure A (Appendix A) showed the mechanism of how CEDIA® works.

In CEDIA assays, analyte is attached to Enzyme Donor in a way that does not interfere with this spontaneous reassociation. Sample is mixed with Enzyme Acceptor and analyte - specific antibody. Enzyme Donor is then added. If analyte is present in the sample, antibody will bind to the analyte ; Enzyme Donor will be free to form active enzyme with Enzyme Acceptor. If there is no analyte present in the sample, the antibody will bind to Enzyme Donor and inhibit the reassociation of Enzyme Donor and Enzyme Acceptor. No active enzyme will be formated. CEDIA assays are linear because the amount of enzyme form is directly proportional to the amount of analyte present. In the CEDIA Phenobarbital Assay system, the displacement format is used to provide improved sensitivity and precision through reduced background noise and concomitant increase of signal - to - noise ratio. In the assay, analyte in the sample displaces a

traction of the antibody Enzyme Donor phenobarbital conjugate complex. Subsequently, Enzyme Acceptor Reagent is added and the reactants are incubated to allow complementation with free Enzyme Donor-phenobarbital conjugate. The amount of active enzyme formed and resultant absorbance change are directly proportional to the amount of analyte present in the sample.

Reagents information and preparation

For detailed reagent information and preparation instructions are on Figure B.1, B.2, and B.3 (Appendix A)

Specimen Collection and Preparation

Serum or plasma (Na or Li heparin ; Na EDTA) samples are suitable for use in the CEDIA phenobarbital assay. Care should be taken to preserve the chemical integrity of the serum or plasma sample from the time it is collected until the time it is assayed. Cap samples, store at 2 - 8 °C and assay within 24 hours, or if the specimen is to be shipped, cap the specimen , and keep it frozen. At - 20 °C and assay within 2 weeks. To protect the integrity of the sample do not induce foaming and avoid repeated freezing and thawing. Clarify specimens containing particulate matter by centrifugation. The timing of specimen collection can influence the relationship between phenobarbital concentration and the clinical response. Other pharmacokinetic factors such as mode of administration, concomitant drug therapy, and biological variations in drug absorption should be taken into consideration.

Calibration

The CEDIA phenobarbital assay produces a linear standard curve using the Low and High Calibrators provided in the kit. Data reduction calculated from point to point interpolation least square linear regression can

be achieved using analyzer software, a personal computer, or calculator. Validate the assay calibration by testing commercial controls with established recovery ranges for the CEDIA phenobarbital assay. The calibration is stable for 5 days with a daily Low Calibrator update.

Assay Range

The CEDIA Phenobarbital Assay is designed to quantitate phenobarbital in patient samples between 0 and the High Calibrator (approximately 80.0 mg/ml or 344.8 mmol/l)

Out of Range Samples

Specimens quantitating greater than the High Calibrator can be reported as greater than the value of the High Calibrator or dilute one part sample with one part Low Calibrator and reassay. The value obtained on reassay should be derived as follows :

Actual Value = (2 x diluted value) - concentration of Low Calibrator

Specimens giving values below the minimum detectable concentration of the assay should be reported as < 1.2 µg/mL (52 Mmol/l)

Quality Control

Each laboratory should establish its own control frequency. Good laboratory practice suggests that at least two levels (low and high medical decision points) of quality control be tested each day patient samples are assayed and each time a calibration is performed. Monitor the control values for any trends or shifts. If any trends or shifts are detected, or if the control does not recover with the specified range, review all operating parameters.

Limitations

The CEDIA Phenobarbital Assay performance has not been established with body fluids other than human serum and plasma (Na or Li heparin ; Na EDTA). The incidence of patients having antibodies to E. coli B-galactosidase is extremely low. However, some samples containing such antibodies can result in artificially high results that do not fit the clinical profile. If this occurs, contact Technical Support for assistance.

Sample containing phenobarbital and the following concentrations of potential interfering substances were quantitated accurately by the CEDIA Phenobarbital Assay : hemoglobin up to 1000 mg/dl, bilirubin up to 60 mg/dl, triglyceride up to 500 mg/dl, total protein up to 13 g/dl, IgA up to 800 mg/dl, IgG up to 4500 mg/dl , IgM up to 770 mg/dl, and Rheumatoid Factor up to 180 IU/ml. For special wash instructions , refer to specific analyzer application protocol.

Specificity

The CEDIA Phenobarbital Assay is very specific , with low cross-reactivity to similar amino acids and drugs. Cross - reactivity is clinically insignificant for the following compounds. Cross-reactivity is expressed as a ratio of the phenobarbital concentration giving 50% of the maximum response to the concentration of the cross-reactant giving the same response.

((Phenobarbital) at ED50/(Cross-reactant) at ED50) x 100

= % cross-reactivity

Figure C (Appendix A) showed % cross-reactivity of the CEDIA phenobarbital Assay.

Sensitivity

The minimum detectable concentration of the CEDIA Phenobarbital Assay is 1.2 μg/ml (5.2 Mmol/l). Figure D (Appendix A) showed method comparison between CEDIA® Phenobarbital Assay and FPIA Phenobarbital Assay