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Liquid chromatographic determination of free drug concentration of anticonvulsants in serum

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Liquid Chromatographic Determination of Free Drug Concentration of Anticonvulsants in Serum

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THESIS

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ABSTRACT

A method has been developed for determining the free drug concentration of carbamazepine, ethosuximide, phenobarbital, phenytoin and primidone by liquid chromatography. The procedure employs five different extraction methods. The first two methods are used for total drug determination. In Method A serum proteins are precipitated with acetonitrile containing the internal standard (hexobarbital). The supernate is then injected into the HPLC. Method B, using the Bond-Elut reverse-phase column, extracts the anticonvulsant drugs from the serum into methanol. Method C, using Worthington "Ultrafree" membrane, prepares the protein-free ultrafiltrate for the analysis of the free drug concentrations. The ultrafiltrate is obtained by excerting a vacuum through a tuberculin syringe. Method D, using Amicon membrane, is used to obtain the protein free ultrafiltrate by centrifugal force for the analysis of free drugs. Method E, equilibrium dialysis, separates the free drug from bound by osmosis. The reverse-phase C-18 column is eluted with a mobile phase consisting of acetonitrile/methanol/phosphate buffer (20/5/75) at the rate of 3.0 mL/min at 50° C and the column effluent is monitored at 195 nm.

The sensitivity of the method is to 0.05 mg/L for ethosuximide and primidone and to 0.1 mg/L for phenobarbital, phenytoin, and carbamazepine. Drug recovery ranged from 95.4% to 121.8% for Method D. Within-day precision at 10 mg/L ranged from 1.9% to 4.6% and at 1 mg/L from 4.6% to 9.5%. Day-to-day precision at 10 mg/L ranged from 2.6% to 5.4% and at 1 mg/L ranged from 11.0% to 14.2%. Comparison of Method D with EMIT

method gave correlation coefficient of 0.940 for phenobarbital, 0.842 for phenytoin, and 0.783 for carbamazepine. Comparison of Method C with Method D gave a correlation coefficient of 1.00 for primidone, 0.957 for phenobarbital, 0.925 for phenytoin, and 0.887 for carbamazepine.

INTRODUCTION

1. Definition and Classification of Epilepsy

Epilepsy is a collective designation for a group of chronic central nervous system disorders having in common the occurrence of sudden and transitory episodes (seizures) of abnormal phenomena of motor (convulsion), sensory, autonomic or psychic origin (1). Pronounced brain wave alterations in the electroencephalogram (EEG) are nearly always detected during these episodes. Epilepsy is a syndrome rather than a specific disease entity. Epilepsies can be classified by anatomic site of onset or the cause. The etiologic classification of the epilepsies in use at the National Institute of Neurological Disease and Blindness is comprised of 10 categories: 1) genetic and birth defects, 2) infectious disorders, 3) toxic factors, 4) trauma or physical agents, 5) circulatory disturbances, 6) metabolic and nutritional disturbances, 7) neoplasms, 8) heredofamilial and degenerative diseases, 9) psychogenic causes, and 10) unknown causes (2). The first nine classifications are secondary or symptomatic epilepsy. The last is primary or idiopathic epilepsy. In idiopathic epilepsy the associated disease must be treated as well.

It is more useful to classify epilepsies according to the anatomic site or type of seizure for the drug treatment. The International Classification of Epileptic Seizures based on the clinical manifestations and the EEG pattern is listed in Table 1 (2).

Anticonvulsant drugs have become increasingly effective for the treatment of epilepsy due to early and precise diagnosis, improved understanding and usage of specific drugs, and the widespread use of

dependable therapeutic drug level monitoring. The choice of drug depends primarily on the seizure type, which must be identified before administration of the antiepileptic drug. Once differential diagnosis has been made, treatment is initiated with only one drug. If there is no urgency to protect the patient from seizures, the gradual increase of dosage to achieve the therapeutic blood concentration will minimize side effects. If this approach does not achieve proper seizure control, or if toxic symptoms appear, another drug is then tried. Penry (3) suggests replacing the first drug with another while Johannessen (4) suggests adding a second drug. If this second drug achieves proper seizure control, the first drug is reduced or withdrawn. Anticonvulsant drugs should not be administered at intervals greater than one serum half life. Administration at greater intervals would lead to fluctuations greater than 50% of the serum drug concentration. The lowest concentration should be high enough to avoid seizures and the highest concentration should be low enough to avoid toxic symptoms. Single drug therapy is preferable whenever possible. However, more than half of the people with epilepsy have more than one type of seizure with wide intraindividual and interindividual variability in magnitude, duration, and frequency of occurrence. Due to the complexity of dosage regimens, the narrow therapeutic index, and the individual variability in drug utilization, serum level monitoring is of great importance for individualized drug regimen.

2. The Uses for the Five Major Anticonvulsants

The uses for the five major anticonvulsants discussed here are as follows (1,3):

Phenytoin (diphenylhydantoin; Dilantin) is used for the treatment of many seizure types including generalized convulsive seizures (grand mal), elementary partial seizures and complex partial seizures (psychomotor and temporal lobe). It is not effective against absence seizures or febrile convulsions.

Phenobarbital (Luminal^{R}) is also useful for the treatment of generalized convulsion seizures and most forms of partial seizures as well as febrile convulsions. It is rarely effective.

Primidone (Mysoline^{R}) is effective against all types of epilepsy except absence seizures. It is used primarily for complex partial (psychomotor) and generalized convulsive seizures.

Ethosuximide (Zarontin^{R}) is used for generalized non-convulsive seizures (absence or petit mal).

Carbamazepine (Tegretol^{R}) is used for partial seizures with complex symptoms (psychomotor), elementary partial seizures, generalized convulsive seizures, and is the drug of choice for trigeminal neuralgia (tic douloureux). Carbamazepine has also been used for tabetic lightening pains, paraesthesia associated with Lhermitte's sign, tonic seizures in multiple sclerosis, and post sympathectomy neuralgia (5). It is not useful in petite mal absence seizures (6).

3. Pharmacokinetic Data for the Anticonvulsants

The general pharmacokinetic data for these antiepileptic drugs is shown in Table 2 (1,3,4,5,7).

The anticonvulsants have dose-related and nondose-related toxicities.

4. Variables That Will Effect the Dosage

The usefulness of determining blood levels of anticonvulsant drugs in the management of epileptic patients has been well established (3,4,8-11). The therapeutic monitoring of serum levels are usually indicated in patients with poor seizure control of toxic symptoms.

The blood levels of antiepileptic drugs combined with good clinical judgment, facilitates the formulation of optimum dosage that will yield the best therapeutic results. However, there are great variations among patients due to individual and day-to-day variables that effect the relationship between dose, blood level, and therapeutic effect. Among the important variables to be considered are age, pregnancy, drug compliance, malabsorption, drug interactions, genetic or drug induced metabolic abnormalities, hepatic and/or renal function, and protein binding.

A. Age

Age is a very important factor that modifies drug kinetics and drug responses. In the newborn oral drug absorption is slow and incomplete, so drugs should be given intramuscularly. However, infants and children absorb antiepileptic drugs faster than adults (4), with the exception of carbamazepine. Carbamazepine induces its own metabolism. If a fetus had transplacental exposure to carbamazepine, the newborn will have plasma half lives similar to adults who have received multiple doses. The steady state plasma concentration in children is comparable to that of adults on corresponding doses (5). Serum protein binding is also age dependent. The unbound (free) fraction is increased in newborn from 52% to 64% for phenobarbital and from 10% to 20% for phenytoin. Hyperbilirubinemia will

increase the free fraction further. However, the free fraction stabilizes from infancy to adulthood. The drug metabolism is very slow in neonates and urinary excretion is reduced due to organ immaturity. Consequently, the serum half life is considerably longer in the newborn than in the adult. However, drug clearance increases progressively and after about 30 days the half life decreases in infants and becomes about three times shorter than in adults. The metabolic rates increase steadily through the first six years of life, reduce again at around six years of age, and reach adult levels at around 10 years of age. A marked decrease in antiepileptic drug metabolism is observed during puberty. At this time, serum level monitoring is important to avoid subtle chronic toxicity and increased seizures due to toxic drug levels. The elderly have a gradual decrease in normal physiologic functions. Hypoalbuminemia is frequently associated with increase in free drug concentrations. Decreased urinary output requires reduced drug doses. The elderly frequently suffer from concomitant disease, and therefore receive other drugs complicating the pharmacokinetics due to drug interaction. Therefore, careful monitoring of the very young, pubescent, and elderly is necessary (4).

B. Pregnancy

During pregnancy serum levels drop due to decreased absorption and metabolism. An increased dose is necessary to reduce the risk of seizure. Despite the rare teratogenic defects associated with anticonvulsant drugs, the risk of damage to both the mother and the fetus from a seizure outweighs the risk of continued medication to the normal development of the fetus (4).

C. Patient Compliance

Early studies of the relationship between blood levels and therapeutic ineffectiveness uncovered that many patients with epilepsy do not take the prescribed dosage (13,14). On the other end of the spectrum, elevated levels were found in patients who feared having seizures and consequently overdosed themselves. Another source of unreliable intake was among small babies who received liquid medication. Parents accidentally overdosed their babies by using a tablespoon for a teaspoon, double dosing when the baby spit out part of the medicine, and concentrating the drug at the bottom of the bottle by forgetting to shake it before pouring (10). Blood level determination and urine metabolite level determination can differentiate noncompliance, rapid metabolism, and malabsorption.

D. Malabsorption

Although malabsorption is seen on patients with specific absorptive defects (14), it is more frequently seen in patients as the result of co-medication (15). Low phenytoin levels were observed in patients in conjunction with antacids. However, when antacids were administered two or three hours after ingestion of phenytoin, the plasma levels in these same patients increased two- to three-fold (15).

E. Drug Interaction

Drug interactions can change the serum levels of the anticonvulsant drugs, thereby altering the therapeutic outcome. Drug interactions may alter absorption, protein binding, receptor action metabolism, and excretion of any therapeutic agent. Most drug interactions are caused by changes in

biotransformation by the microsomal enzyme induction or inhibition. Special consideration should be given to antiepileptic combinations. Phenytoin blood level concentrations are decreased when given with carbamazepine. Conversely, phenytoin may decrease the blood level of carbamazepine. Phenytoin phenobarbital interactions are variable and complex. Phenobarbital levels can increase and lead to intoxication when sodium valproate is added. The conversion of primidone to phenobarbital may be increased in the presence of phenytoin. The other drug interactions for primidone are the same as would be anticipated for phenobarbital. Ethosuximide levels also increase when combined with sodium valproate. Carbamazepine induces its own metabolism (autoinduction) and may decrease when phenytoin and/or phenobarbital are administered. However, the serum level shows considerable individual variations. Therefore, monitoring the blood levels of the anticonvulsant drugs provides the best means of regulating the dose. Some examples involving antiepileptics and other drugs are given in Tables 3-6 (4).

F. Altered Biotransformation

Most antiepileptic drugs undergo biotransformation in the liver resulting in inactivation and/or increased excretion. The unbound fraction of the drug is metabolized by the microsomal enzyme system. If the system is oversaturated, the blood level and the percent free drug increases (4,16). If the capacity is reduced by concomitant drugs or other causes, the blood level may be further increased. Familial parahydroxylation deficiency (12) and folic acid deficiency may result in elevated antiepileptic drug levels in the plasma. Interindividual differences in metabolism complicate dosage and the prediction of blood levels. Because carbamazepine induces its own metabolism, the

plasma concentration of this drug cannot readily be predicted from the dose prescribed (5). As stated previously, the antiepileptic drugs as well as other drugs given in co-medication can enhance or inhibit the liver metabolizing enzymes, altering the blood levels of the prescribed drug.

G. Renal and Liver Dysfunction

Renal or liver dysfunction can dramatically alter the pharmacokinetics of a drug. Absorption, distribution, biotransformation, and elimination can be altered by kidney or liver diseases. Such dysfunction will necessarily alter the dose: blood level:therapeutic effect relationship. To prevent excessive accumulation of the drugs, dosage adjustments must be made and closely monitored. Unfortunately, present methods of analysis reflect only total blood level concentration. In liver and renal disease the plasma protein binding may be altered due to the decrease in albumin concentration, resulting in the alteration in the volume of distribution. These changes result in the increase of the active free drug component even though the total drug level remains steady or decreases (9,18). Metabolic acidosis and alkalosis associated with renal disease also effect the free drug level. Acidic pH increases the unbound fraction of phenytoin. Acidosis also increases the brain and muscle concentrations of phenytoin (17). Paradoxically, lower plasma levels of phenytoin are observed in uremic patients. The metabolic product HPPH accumulates rapidly and excessively, competes with the phenytoin, which in turn is more rapidly removed from the plasma of uremic patients (18). However, if the total blood level is determined by enzyme multiplied immunoassay (EMIT), false high results occur. These patients may receive lowered doses resulting in inadequate seizure control (19). Free drug levels

would be a better indicator of therapeutic drug level for patients with renal and/or liver disease.

H. Protein Binding

After a drug enters the blood stream, drug distribution is determined by certain physiological factors and the pharmacokinetic properties of the drug. Drugs bound to plasma proteins are essentially limited to the plasma. Protein binding produces a buffer that increases the duration of action and decreases the maximum intensity of a drug. Only the unbound drug is in equilibrium across the membranes. Drugs exert their action on the dynamics of the organism by binding to a tissue receptor site that alters the function of the cells by either returning the system to normal activity or preventing abnormal activity (1). The distribution of drugs to the central nervous system is particularly restricted to small molecules or highly lipid soluble drugs. Protein does not cross a healthy intact blood brain barrier. Antiepileptics are believed to control seizures by preventing or reducing excessive neuronal discharges, reducing the spread of excitation from seizure foci, and preventing disruption of normal neuronal function when binding to the neurons. Significant correlation between the brain and plasma concentration of antiepileptics has been established (20-24). Equilibrium exists between the bound drug, plasma free drug, and the drug present at the receptor site. The ratio of free to bound drug reaches steady state when all the binding sites have been occupied by the drug.

Drugs may bind to many different sites including various blood constituents. However, plasma proteins are the most clinically significant sites. Plasma proteins capable of binding drugs include prealbumin, albumin,

alpha, beta, and gamma globulins. Albumin constitutes approximately 50% of the total plasma proteins and binds the widest range of drugs. Basic drugs are frequently bound to glycoproteins (25). Phenytoin has been shown to bind to thyroid binding globulin (3). The amount of drug bound to plasma proteins is dependent on the number of protein binding sites available. If the sites are saturated, an increased dose may cause a marked elevation in the free, pharmacologically active drug, even though total concentration is only slightly increased (6,16,26).

Many factors can alter the affinity of a drug for protein binding sites which in turn alters the free to bound drug ratio. Hypoalbuminemia, which decreases the number of available binding sites, will increase the free to bound ratio at a given dose. Other drugs may compete with the antiepileptic drugs for binding sites. Valproic acid displaces phenytoin (7,15,16). Salicylic acid, phenylbutazone, sulfafurazole, and isoniazid also displace phenytoin. Drug displacement is usually followed by an immediate increase in free drug, an enhanced pharmacological effect, and a more rapid elimination from the body. The consequences of drug displacement are presented in Table 7 (27).

Protein binding is also affected by renal disease. Chronic uremics have increased levels of free drug due to a decreased level of albumin, an accumulation of metabolites and fatty acids that compete for the binding sites, and a qualitative change in the albumin of uremics that decreases its affinity for drugs (18,19). The free drugs also increase with acidosis (17). Hepatic disease, such as alcoholic liver disease and acute viral hepatitis increase free drug. The free drug correlates with the albumin, globulin, and bilirubin levels. The binding affinity of albumin is also altered with age. It decreases in old age (4,28). The protein configuration of fetal albumin is

different. Phenytoin binding is 80-95% in newborns and 90-95% in adults. Binding affinity changes with chronic anticonvulsant drug treatment (29) and is altered by the presence of bilirubin and fatty acids (27).

5. Relationship Between Drug Concentration and Clinical Effectiveness

Free drug levels can provide clinically significant information not revealed by total drug concentrations. Small changes in the free drug level of highly bound drugs can have a significant therapeutic effect without marked differences in the total drug level. Phenytoin, phenobarbital, and carbamazepine are antiepileptics that are protein bound to a significant degree. An assay of the percent free drug of these antiepileptics would be useful, particularly considering the large interindividual differences which occur in percent bound (5,26).

Phenytoin is about 90% protein bound, mainly to albumin. Phenobarbital is about 50% protein bound to albumin and different globulins. Primidone is only bound to a minor extent, however, it is converted to phenobarbital which is protein bound. Carbamazepine is bound to protein approximately 75%, however, its binding affinity for albumin is low and other proteins are involved. Ethosuximide does not appear to be bound to serum protein at all (15,30).

In attempts to establish the relationship between tissue drug concentration, plasma drug concentration, and the clinical effectiveness of a drug, correlation studies have been made of the concentrations of the drugs in the epileptogenic brain and plasma. Sherwin (21) found a phenobarbital brain:plasma ratio of 0.91 ± 0.08 and a phenytoin ratio of 1.5 in patients undergoing neurosurgery for focal epilepsy. All previous studies had been done

on autopsy specimens. Vajda compared brain, cerebrospinal fluid (CSF), and plasma from 12 epileptic patients undergoing temporal lobectomy (20). Brain:plasma ratio for phenobarbital was 0.59 ± 0.21 with a CSF:plasma ratio of 0.46 ± 0.12. Phenytoin gave brain:plasma ratio of 0.75 ± 0.19 and a CSF:plasma ratio of 0.12 ± 0.04. Houghton (22) included primidone in his study and found linear correlation between plasma and brain concentrations, assumed that CSF is an ultrafiltrate of plasma, and derived the percentage of unbound drug in plasma for phenytoin, phenobarbital, and primidone as 10-14%, 43%, and 81% respectively. A linear relationship between brain and plasma concentrations of both carbamazepine and its epoxide (carbamazepine-10,11-epoxide) gave additional support to the assumption that plasma level monitoring is therapeutically valuable (23). The relationship between plasma drug levels and specific receptor sites in the epileptic brain continues to be investigated (24).

It is generally assumed that the unbound fraction of the antiepileptic drug in the serum is in equilibrium with and equal to the total concentration of the drug in the cerebrospinal fluid. It is not common practice to do spinal taps because the risk is not justified for routine monitoring. However, CSF concentrations are still being used as a research technique to calculate unbound fraction. Carbamazepine, a relatively new antiepileptic drug on the market, has been investigated using this method for protein binding investigation (31,32). Marked individual differences between dosage and total concentration of carbamazepine in serum and CSF indicates that dosage must be adjusted for each patient (32). Important interindividual differences in plasma protein binding in patients undergoing chronic treatment lead to a study that compared and confirmed the relationship between CSF levels of

carbamazepine and its epoxide and the free drug values determined by ultrafiltration (29).

6. Use of Saliva and Tears to Assay Free Anticonvulsant Drugs

Saliva was found to be a convenient fluid to assay the pharmacologically active portion of anticonvulsant drugs. Bochner found significant correlation between salivary phenytoin values and plasma ultrafiltrates (33). There was less scatter between salivary and plasma ultrafiltrates than between either of these measurements and the total drug concentration in plasma. Troupin compared phenytoin, phenobarbital, primidone and carbamazepine in saliva, CSF, and dialyzed plasma. Generally, good correlation was found for phenytoin and carbamazepine. However, primidone was inexplicably higher in saliva than dialyzed plasma and lowest in CSF. Phenobarbital in CSF fluid and dialyzed plasma agreed but the saliva levels were unpredictably lower. Phenobarbital is ionized at the pH of serum and does not readily diffuse into saliva. Schmidt measured phenytoin, phenobarbital, and primidone in saliva, plasma, and CSF (35). Saliva was collected over a 20-minute period without stimulation. Saliva:plasma ratios and CSF:plasma ratios were compared for individual drugs and in patients receiving co-medication. The ratios were similar for phenytoin and primidone but the saliva:plasma ratio versus the CSF:plasma ratio was lower for phenobarbital due to the difference in pH between saliva and CSF. A corrected formula was proposed for phenobarbital to account for the pH of the saliva. Many assays have been proposed to measure saliva anticonvulsants and correct the phenobarbital pH problem. Cook measured saliva and plasma anticonvulsants by RLA and GLC-mass spectrometry (36). Again, phenytoin saliva:plasma ratios correlated whereas

phenobarbital saliva:plasma ratios did not. A correction factor was proposed assuming an average saliva pH of 6.5. This worked for a small concentration but was not linear. A calibration curve was proposed to correct the concentration with respect to the pH of saliva. McAulliffe (37) compared EMIT and GLC assays of phenytoin, phenobarbital, primidone, carbamazepine, and ethosuximide and compared the saliva, dialyzed plasma, and total plasma levels. Good correlation was found for all except phenobarbital. This time the saliva pH was measured, a calibration curve and correction factor was applied to give better correlation between the saliva and plasma concentrations (38). Horning compared EMIT and GC/MS assays for phenytoin, phenobarbital, primidone, and ethosuximide and compared the saliva to the plasma concentration. The free fraction comparison was based on previously established studies. The saliva:plasma ratio for carbamazepine, phenytoin, and primidone correlated with the accepted range of free:total ratios. A correction factor was applied to correlate the phenobarbital ratios. EMIT (enzyme immunoassay) results were generally higher than GC/MS values for phenytoin and phenobarbital and were attributed to the presence of metabolites that were measured along with the parent drug. Estimations of free phenobarbital levels using saliva and elaborate correction equations continue to be proposed (39).

Saliva concentration offers a non-invasive technique which involves little discomfort, risk, or skill in collection. However, the fluid secreted by the three major salivary glands differs considerably in composition and varies with age, sex, type of stimulation, length of stimulation, and time of day. Rylance studied saliva anticonvulsant levels in children and found few variables in children with respect to concurrent drug use, saliva flow rate, and pH except

for phenobarbital (40). Goldsmith's study of salivary anticonvulsant levels in children were unusually high in some children who chewed their tablets (41).

There are several problems in obtaining reliable results from saliva. The major salivary glands differ in composition and concentration of the drug. The different methods of collection effect the results; differences in stimulation and time of salivary flow effect the pH; finally, there are the problems with drooling often seen in epileptics with underlying cerebral disease (40). With these problems in mind, Monaco proposed the use of tears as a practical indicator of free anticonvulsant drug levels (42). Monaco compared phenobarbital and carbamazepine levels in saliva, tears, CSF, and plasma using EMIT. The tear:plasma ratio and tear:CSF ratio correlated better than the saliva:plasma ratio and the saliva:CSF ratio. Tears are more stable, have minimum pH changes, and have a relatively constant protein content comparable to CSF (0.6-0.8 g/dL). Tear assays for phenobarbital do not need a pH correlation correction. Monaco assayed phenytoin and primidone by EMIT for plasma, tears, saliva, and CSF (43). Tear to plasma and CSF ratios correlated better than saliva and tears to CSF.

7. Use of Equilibrium Dialysis and Ultrafiltration to Assay Free Anticonvulsant Drugs

Ultrafiltration and equilibrium dialysis have long been standard methods to study protein binding (6,16,26,28,44). Equilibrium dialysis is long, involved, and impractical for routine drug monitoring. Early ultrafiltration methods required difficult manipulations of tubing (16) or altered dilutions due to moistening of the membrane, low recovery due to membrane binding requiring a correction curve, and protein leakage. Membranes were developed that did

not bind the anticonvulsants but still required premoistening and subsequent correction for dilution, but compared well with equilibrium dialysis (44).

In the study of free drugs, the introduction of permselective membranes has reduced the complexity and time involved in obtaining free drug samples. The question arises about the accuracy of ultrafiltration. Concentrations errors could be introduced into ultrafiltration as the changes in concentration of proteins, ions, and water occur during centrifugation. However, studies comparing ultrafiltration to equilibrium dialysis have ruled this out (45,46). Ultrafiltration has several advantages over equilibrium dialysis. First, it is rapid and requires no sample storage which might lead to increased accumulation of fatty acids, which in turn affect protein binding. Next, it requires no addition of competitive buffer components and electrolytes which might contaminate the specimen and alter the results. However, it is important to keep in mind the possibility of drug adsorption by the membrane, the possible polarization of protein on the membrane, and the difference in binding at different temperatures (6,16,44). Another major advantage of ultrafiltration of specimens is that the specimen is ready for direct injection into HPLC with no concern about protein causing an increase in back pressure or deterioration of column performance (48).

Ultrafiltration assays are being developed for other drugs besides anticonvulsants (47,49,50). Garlock and Pippenger reported ultrafiltration method for anticonvulsants. They compared equilibrium dialysis to ultrafiltration, on samples from patients on multiple versus single drug therapy, and found a good correlation.

Joern presented a gas-chromatographic assay of free phenytoin using the ultra-filtration process (50). The storage of plasma before filtration was

studied under a wide variety of times and temperature and found to have an insignificant effect upon filtration. However, serum results were unreproducible. The ultrafiltrates of serum contained protein.

High pressure or high performance liquid chromatography lends itself quite well to the analysis of free anticonvulsants. Due to its efficiency the analysis of small quantities presents no problem. The ability to analyze many drugs simultaneously is particularly advantageous considering the frequency of multiple drug therapy. HPLC is also rapid, specific, precise, and non-destructive to the analyte being analyzed. HPLC requires minimal sample manipulation, it requires the deproteinization of the specimen. Deproteinization is accomplished by the ultrafiltration method.

8. Characteristics of Membranes used in Equilibrium Dialysis and Ultrafiltration

Membranes serve as a selective barrier. Synthetic membranes are classified structurally (what they are), or functionally (how and under what conditions they perform). Three important parameters are thickness, water content, and permeability. The structure may be molecular, colloidal, or macrostructure which will determine the relative pore size, pore-size distribution, resistance to pressure, temperature, hydrolysis, microbial decomposition, and diffusion coefficient. The most difficult and consequently most costly to produce are the highly structured colloidal polymeric membranes used in ultrafiltration.

Functional terms commonly associated with membrane processes are diffusion, osmosis, reverse osmosis, ultrafiltration, and dialysis. Diffusion refers to the migration of a substance across a concentration gradient.

Osmosis is the phenomenon whereby solvent permeates a semipermeable membrane separating two solutions of different concentrations. The flow of solvent occurs from the less concentrated to the more concentrated solution. When pressure in excess of the osmotic pressure is applied to the more concentrated solution, the net flow of solvent is into the more dilute solution and is known as reverse osmosis. The term ultrafiltration is virtually interchangeable with reverse osmosis. However, there is a tendency to confine the term ultrafiltration to situations involving separation of larger particles of comparatively low concentrations by viscous flow mechanism rather than by diffusion. Dialysis is a diffusion phenomenon in which there is a permeation of some solute species, but not others. Because the driving force of equilibrium dialysis is a concentration gradient, the process takes time to reach equilibrium (55).

The membranes used in equilibrium dialysis are molecular in structure, easy to manufacture, and have a well controlled pore size, but has been made possible due to the development of anesotropic or asynometric semipermeable polymeric membranes covering a broad range of pore sizes (from 10-200 Angstroms) which have extraordinarily high water permeabilities at low operating pressures. Ultrafiltration membranes are composed of two layers. A fine film of the microporous polymers overlays a coarsely porous foam. The film serves as the molecular separation barrier and the foam provides the mechanical support. The thin skin accounts for the high water permeability and additionally the pores are not blocked or plugged by the solute molecules.

Equilibrium dialysis is the standard technique for analysis in protein drug bonding studies. However, it is too time consuming to be used in a routine clinical laboratory. Empirical (26) and theoretical (45) studies have proven

that ultrafiltration is equivalent to equilibrium dialysis, but much simpler to carry out. The concern that sample volume and pressure would effect the results of ultrafiltration has not proven to be a problem (57). However, protein leakage is a consistent problem.

Ultra-free anticonvulsant drug filters from Worthington, a division of Millipore Corporation, are described in their brochure as having a 40,000 molecular weight cut-off which retains more than 99% of the plasma albumin. The driving force is obtained by aspiration of a syringe plunger to provide a vacuum source.

Amicon's type YMB filters are described in their pamphlet as anisotropic, hydrophobic, with low-non-specific binding properties. They retain serum protein greater than 99.6%, PVP K15 less than 50% and raffinose less than 5.0%.

The following discussion presents an assay for free anticonvulsant drugs using ultrafiltration membranes and equilibrium dialysis by the method developed by Kabra et al. The method is sensitive to 0.05 mg/L for ethosuximide and primidone and to 0.1 mg/L for phenobarbital, phenytoin, and carbamazepine, linear from 0.2 mg/L to 50 mg/L, and gives a recovery from 95.4% to 121.8%.

MATERIALS AND METHODS

Instrumentation: A series 3 liquid chromatograph, equipped with a Model LC-100 column oven, a Model LC-75 variable wavelength detector, and a Sigma 10 chromatograph data station, all from Perkin Elmer Corporation, Norfolk, CT,

was used. A Rheodyne Model 7125 injector with a 100 µL loop was used. A 250 4.6 mm (i.d.) column (Altex, Berkeley, CA) packed with 10 µm particle size C-18 reversed-phase bonded-phase packing (Sherisorb ODS) was mounted in the oven. The column was eluted with methanol/acetonitrile/phosphate buffer (5/20/75 by volume) at the rate of 3.0 mL/min at 50° C and the column effluent was monitored at 195 nm.

Automatic Clinical Analyzer (ACA) III was used for the protein analysis of ultrafiltrates, Dupont, Wilmington, Delaware.

Materials: Equilibrium dialysis, 1 mL cells with cellulose membrane in 0.067 M phosphate buffer at 7.4 pH were from Fisher Products.

Ultrafiltration membranes, MPS-1 units, centrifuge cups and YMB membranes for MPS1 were from Amicon, Danvers, MA.

Ultrafree, ultrafiltration membranes and support stand were from Worthington Diagnostics, a division of Millipore Corporation, Freehold, NJ.

Polypropylene tubes, 1.5 mL capacity and an Eppendorf Model 5412 centrifuge were from Brinkman Instruments Inc., Westbury, NY.

Tuberculin syringes 1 cc disposable were from Sherwood Med. Industries, Delano, FL.

Sure-Sep II plasma separators were used from General Diagnostics, Morris Plains, NJ.

Hamilton glass syringes, 25 µL and 100 µL were from Hamilton Co., Reno, NV.

Bond-Elut, disposable extraction columns and Vac-Elut, a vacuum manifold were from Analytichem International, Harbor City, CA.

International Centrifuge, Model UV was from International Equipment Co., Needham, MA.

1 mL dialysis cells were from Technilabs Instruments, Inc., Pequannock, NJ.

Dialysis membranes with an average pore of 24 A from VWR Scientific, Inc., San Francisco, CA, were pretreated by soaking in water for 10 minutes, ethanol for 15 minutes, and biocarbonate buffer, pH 7.4, for 10 minutes.

Shaker bath for equilibrium dialysis cells at 37° C for 6 hours was from Braun-Melsungen A.G., Germany.

Reagents: Drug Standards: Phenytoin was obtained from the Eastman Kodak Co., Rochester, NY; phenobarbital from University of California at San Francisco, hospital pharmacy; hexobarbital from Sigma Chemical Corp., St. Luis, MO; primidone from Ayerst Laboratories Inc., New York, NY; ethosuximide from Parke, Davis, and Co., Detroit, MI; and carbamazepine from Ciba-Geigy Corp., Summit, NJ. A chromatographic standard was prepared by dissolving 100 mg of hexobarbital in 100 mL of acetonitrile. The working internal standard was prepared by adding 1 mL of stock internal standard to 99 mL of 0.9% saline.

All chemicals used were of reagent grade. Methanol and acetonitrile, HPLC quality, were obtained from J.T. Baker Chemical Co., Phillipsburg, NJ.

Phosphate buffer, 6.7 mM/L, pH 4.4 was prepared by dissolving 1.36 g of KH_2PO_4 in 1 L of water to get a 1 M/L stock buffer. 10 mL of the stock buffer was added to 1490 mL of distilled water.

Mobile phase: 200 mL of acetonitrile and 50 mL of methanol in 750 mL of 6.7 mM/L phosphate buffer.

PROCEDURES

Method A: Acetonitrile Precipitation Method

Pipet 0.5 mL of serum or plasma into 1.5 µL polypropylene microtest tube. Add 0.5 mL of working internal standard (made by diluting stock internal standard 1 to 10 with acetonitrile). Vortex the tube for 10 seconds. Allow it to sit for 10 minutes. Centrifuge the tubes for two minutes in an Eppendorf centrifuge, 10,000 g. Inject 20 µL of drug reference standard into the HPLC. Calculate the relative retention times and response factors for the drugs. Inject 20 µL of the unknown supernatant into the HPLC.

Method B: Bond-Elut Extraction Method

Place Bond-Elut C-18 columns on the top of Vac-Elut chamber. Plug remaining holes with stoppers. Pass two column volumes of methanol and water through each column. For total levels place 200 µL of the control or patient serum onto the appropriate column. For ultrafiltrates add 100 µL of 0.1 M phosphate buffer pH 3.5:100 µL ultrafiltrate, and 100 µL internal standard (in saline) onto the appropriate column. Connect the vacuum. Pass two columns of water through each column. Disconnect vacuum. Place a metal rack of previously numbered tubes inside the vacuum chamber under appropriate column. Pipette 300 µL of methanol onto each column. Connect the vacuum. Disconnect the vacuum and remove the tubes. Shake eluates before injecting 20 µL on HPLC.

Method C

Firmly twist a 1 cc tuberculin syringe into the bottom of the Worthington unit. Place it in support stand. Pipette 2 mL of serum onto reservoir cup (1 mL minimum). Sit for 2 minutes. Withdraw plunger and secure in support stand. After 60 minutes remove the filter unit. Transfer ultrafiltrate to microtest tube. Mix 50 µL ultrafiltrate and 50 µL of internal standard and inject 50 µL into the HPLC.

Method D

Assemble MPS-1 membrane by Amicon support unit with the membrane's shiny side up. Label and attach centrifuge cups to the base of the MPS unit. Pipette 200 to 1,000 µL of serum onto the membrane. Centrifuge at 1,600 x g for 15 minutes. Transfer ultrafiltrate to microtest tube. Mix 50 µL of ultrafiltrate and 50 µL of internal standard and inject into the HPLC.

Method E: Equilibrium Dialysis

Place 800 µL of serum and bicarbonate buffer, pH 7.4, into the two half cells of a 1 mL dialysis cell. Separate by a dialysis membrane pretreated by soaking in water (10 min), ethanol (15 min), and buffer (10 min). Equilibrate the cells at 37° C for 6 hours. Remove the plasma and buffer, measure the volume, and freeze until assayed. For assay, mix 100 µL of plasma or buffer with 100 µL of internal standard and inject into the HPLC.

RESULTS AND DISCUSSION

Analytical Variables

The chromatographic conditions were selected: wavelength at 195 nm, oven temperature at 50° C, flow rate at 3.0 mL/min, phosphate buffer at pH 4.4, and mobile phase containing methanol/acetonitrile/phosphate buffer (5/20/70 by volume), and a reverse-phase C-18 column. These conditions were previously established in this laboratory as optimum conditions to assay phenobarbital, phenytoin, primidone, ethosuximide, and carbamazepine (53,54,55).

Method A was used for deproteinizing serum. Method B used extraction method by adsorbing the drugs onto a C-18 Bond-Elut column, allowing the proteins to be washed off the column with water, and the drugs were then eluted with methanol. This method of sample preparation was rapid, simple, and effective.

Method C and Method D were followed according to the manufacturer's instructions. Chromatographs are presented in Graphs 1 and 2.

Initially a high speed centrifuge (Damon/IEC PR-6000) was used to spin the specimens for Method D, but a standard floor centrifuge was equally convenient and efficient, without the added disadvantage of exceeding the manufacturer's recommended centrifugal force.

Method C, Worthington, requires more specimens, takes longer to process each specimen, and produces less ultrafiltrate than Method D. However, it is easier to assemble and can be used without a centrifuge.

Quantitation

The chromatographic conditions employed in this assay gave homogenous and symmetrical peaks; therefore, peak height ratio could be used to quantitate the drugs. The calculations used are as follows:

$$\text{response factor (RF)} = \frac{\text{peak height of I.S. (internal standard)}}{\text{peak height of drug}}$$

$$\text{drug. conc. in mg/L} = \frac{\text{peak height of drug}}{\text{peak height of I.S.}} \times \text{RF} \times \text{conc. of I.S.}$$

Precision

Day to day and within day precision studies were carried out in two pools at different concentrations. The results are illustrated in Table 8. The within day precision at 10 mg/L ranged from 1.9% to 4.6% and at 1 mg/L ranged from 4.6% to 9.5%. Day to day precision at 10 mg/L ranged from 2.6% to 5.4% and at 1 mg/L ranged from 11.0% to 14.2%.

Sensitivity

The method is sensitive to 0.05 mg/L for ethosuximide and primidone and to 0.1 mg/L for phenobarbital, phenytoin, and carbamazepine based on a signal to noise ratio of three.

Recovery

A standard of 50 mg/L of drugs and internal standard was made in 0.9% saline and processed by Method D, and recovery was calculated against the standard injected directly onto the HPLC. This was not done for Method C as

it consistently gave higher values than Method D. Recovery ranged from 95.4% to 121.8%. The results are listed in Table 9.

Linearity

Linearity was assessed by running drug standards from 0.2 mg/L to 50 mg/L in saline with 25 mg/L of internal standard. The samples were run in triplicate. The peak areas were measured by the Sigma 10 data system. The ratios of the drugs to the internal standard were calculated and found to be linear from 0.2 mg/L to 50 mg/L. The results are presented in Table 10 and Graph 3.

Specificity

Previous studies in this lab indicated that only ethotoin interferes with the analysis of phenobarbital (53,54). Lipemic, icteric, and hemolyzed specimens do not interfere with the analysis.

Patient Samples

More than 200 patient samples were analyzed for the five drugs. The samples were collected in a red or green top tube, spun down with a Sure-Sep II, a serum-plasma separator, and refrigerated at 4° C. The total levels were determined within two days. Unfortunately, no record was made of which tubes were serum and which were plasma. The serum or plasma was then poured off and frozen until they were processed by ultra filtration. Fifty-five of the specimens were run by both Method C and Method D. The patient results are presented in Tables 11-15.

The percent free drug was calculated. The mean, standard deviation and two standard deviation range were calculated and presented in Table 16. The previously reported percent of free drugs is also listed in this table. The ethosuximide and primidone gave poor comparison. However, the more highly bound drugs gave better correlation. The reported percent free for phenobarbital is 50%. We obtained a mean of 53.1% but a large standard deviation of 24.58%. Carbamazepine has a reported percent free of 25%. We obtained a mean of 35.3% with a standard deviation of 17.48%. Phenytoin is reported as 10% free. We obtained a mean of 12.38% free with a standard deviation of 8.84, a rather good comparison.

Five samples analyzed in duplicate by both Method C and Method D were selected for protein determination. The serum total proteins and the ultrafiltrate proteins were measured on the ACA. The total proteins are reported by Dupont to be linear from 0-12 g/L and the ultrafiltrates were analyzed by the CSF protein method which is linear from 0-200 mg/L. The results are presented in Table 17.

In these samples the membranes retained the serum proteins by more than 99%. The ultrafiltrates from the samples with higher results by Method C were not tested for protein leakage. That problem has been previously reported by other investigators using Worthington ultrafilters (49).

The analysis of ethosuximide and primidone was questionable. There were some extraneous peaks at the retention time for ethosuximide in some samples. It is probable that the small amount of protein present in the ultrafiltrate was still interfering with the analysis of ethosuximide and primidone. The samples were cleaned up using Method B. This improved the

shape of the peaks and gave a chromatograph with a baseline separation of primidone and ethosuximide.

Accuracy

Of the many patient samples processed, few gave more than 150 µL of ultrafiltrate. Of the 150 µL of ultrafiltrate, 50 µL was used in the LC assay. 100 µL was needed for the EMIT assay for a comparison study. Only 13 phenytoin, 7 carbamazepines and 8 phenobarbitals were available for comparison with EMIT. The results are listed in Table 18.

Despite the small numbers, phenobarbital and phenytoin gave a good correlation by EMIT. Carbamazepine, however, did not have a satisfactory comparison.

Samples were also run by equilibrium dialysis and analyzed on the HPLC. The results are listed in Table 19 and compared in Table 20. The percentage of free drug was calculated by dividing the concentration for the drug in the buffer solution by the concentration of the total drug in the serum before dialysis (30).

The overall comparison of the percent free drugs by the two methods was poor. The samples were frozen and thawed repeatedly before they were run by equilibrium dialysis and this might have altered the protein binding which would account for the poor results.

The results of Method C and Method D were compared for each drug and presented in Table 21. Primidone, phenobarbital, and phenytoin showed a good comparison. Carbamazepine showed a fair comparison.

DISCUSSION

Method A was used to deproteinize the serum protein. Method B was used to absorb the drugs onto a C-18 column, wash the proteins off the column with water, and elute the drugs with methanol. Method A was adequate but Method B was rapid, simple, and effective.

Method C and Method D used a membrane to retain the serum protein and allow the collection of protein free ultrafiltrates. Method C required more specimens, took longer to process each specimen, produced less ultrafiltrate, had protein leakage problems, and was difficult to obtain from the manufacturer. However, it was easier to assemble, disposable, and could be used without a centrifuge. Method D produced more than adequate ultrafiltrate volume, good recovery and dependable protein free ultrafiltrate which in turn gave cleaner chromatographs. However, Method D required a centrifuge, was extremely difficult to assemble, and the membrane assembly unit needed to be washed between each use. In a clinical laboratory where ability to accommodate large sample volume, turn around time, and ease of use are critical, Method C would be the most compatible if the manufacturer could be depended upon to provide the membranes when ordered. This has not been the case in our experience.

Method E is a standard technique for analysis in protein binding studies. However, it is too time consuming to be used in a routine clinical laboratory. Empirical (26) and theoretical (45) studies have proven that ultrafiltration is equivalent to equilibrium dialysis but much simpler to carry out. In this paper, comparison of percent free drug by equilibrium versus percent free drug by ultrafiltration was poor.

The HPLC method described in this paper is very good for analyzing free drug levels. The method has good within-day and day-to-day precision. The method is sufficiently sensitive, linear from 0.2 mg/L to 50 mg/L, and specific. Only ethotoin interferes with the analysis of phenobarbital.

Serum or plasma samples were collected on more than 200 patients. They were refrigerated at 4° C and the total levels were determined within two days. The samples were then frozen until they were processed by ultrafiltration or equilibrium dialysis. Serum and plasma behave differently when stored for long periods of time or at low temperature. Storage of plasma before filtration under a wide variety of times and temperature has been found to have an insignificant effect. However, serum results were unreproducible (50). This may be the source of the poor correlation between the ultrafiltrate free drug concentration and the equilibrium dialysis free drug concentration. We recommend the use of plasma and avoid freezing or prolonged storage time.

Determination of the percent free drug for ethosuximide and primidone are not clinically useful. Ethosuximide is essentially 100% free drug and can be determined adequately by Method A or B and reported as total concentration. Primidone is only 10% protein bound and can also be determined adequately by Method A or B and reported as total concentration. Both primidone and ethosuximide come off early in the HPLC assay and are frequently in the serum protein peaks. The ultrafiltrates were cleaned up using Method B to reevaluate the primidone and ethosuximide levels. Ethosuximide was rarely ordered as an assay, gave poor results, and should not be done by ultrafiltration. Primidone is ordered a little more frequently and does not co-elute with the serum proteins as severely as ethosuximide by this

assay. Method C and Method D did not agree well. Method C and Method D gave lower free drug levels than would be expected for a drug that is 90% free drug. Method C and D obtained only 74% free drug. Method B, however, when used to clean up the ultrafiltrate obtained 90.6% free drug. This agrees with what would be expected; however, it requires an ultrafiltration and a clean up step. This consumes time and materials without any return in important information. Primidone should not be done by ultrafiltration. However, primidone is converted to phenobarbital which is protein bound.

The reported percent free drug for phenobarbital is 50%. We obtained a mean of 53.1% but a large standard deviation of 24.58%. Carbamazepine has a reported percent free of 25%. We obtained a mean of 35.3% with a standard deviation of 17.48%. Phenytoin is reported as 10% free. We obtained a mean of 12.38% free with a standard deviation of 8.84.

Sixteen patient samples were run by Method D and Method E. The correlation coefficient was 0.870. The ethosuximide results were on patient number 6413. The free results were 109.6% and 101.2%. The ultrafiltrate and equilibrium buffer were cleaned up using Method B and reassayed. The free results were 20.7% and 4.2%. The correlation coefficient was 0.989. However, an N of 2 is insufficient, especially since it is the same sample run twice. The results should have been the same. Primidone had an N of 4 and a correlation coefficient of 0.616. Primidone's ultrafiltrate and equilibrium buffer were also cleaned up using Method B and reassayed. One sample showed an increase in percent free for Method D and a decrease for Method E. The other sample stayed the same. The results are not reproducible. Phenobarbital had a correlation coefficient of -0.093 and carbamazepine had a correlation coefficient of -0.590. Carbamazepine showed a poor correlation

and phenobarbital showed no correlation. Phenytoin had a correlation coefficient of 0.996 on six samples. This is an excellent correlation coefficient. Overall, however, the comparison of the percent free drugs by the two methods was poor.

Determining the percent free drug concentration of phenobarbital, carbamazepine, and phenytoin is clinically significant. Many factors can alter the affinity of a drug for protein binding sites which in turn alters the free drug concentration. Many factors can affect the number of protein binding sites available which in turn also alters the free drug concentration. It is the free drug that crosses the blood-brain barrier, reaches the tissue receptor site which alters the function of the cells, returning the system to normal, preventing abnormal activity or if in excess, inducing toxicity.

This assay may prove to be useful for free drug determination of phenytoin, phenobarbital, and possibly carbamazepine in cases where normal total drug levels are associated with poor patient control or suspected toxicity due to protein binding complications.

CONCLUSION

Free drug levels can provide clinically significant information not revealed by total drug concentrations. Small changes in the free drug level of highly bound drugs can have a significant therapeutic effect without marked differences in the total drug level. Phenytoin, phenobarbital, and carbamazepine are anticonvulsants that are protein bound to a significant degree. An assay of the percent free drug of these anticonvulsants would be

helpful. The procedure described here offers the necessary sensitivity, specificity, precision, and linearity to assay the free drug concentration of the five major anticonvulsants simultaneously. Bond-Elut sample extraction compares well with acetonitrile protein precipitation method for sample preparation with the added advantage of sample purity, speed, and simplicity. Worthington and Amicon filters were comparable. However, Worthington filters appear to leak more protein. On the other hand, Worthington filters are easier to handle, do not require a centrifuge, and deliver a larger ultrafiltrate volume. This assay compares well with EMIT for phenobarbital and phenytoin, but not for carbamazepine. The average percent free drugs also compare well with the established percent free drug concentration for phenobarbital, phenytoin, and carbamazepine. It compares very poorly for the ethosuximide and primidone. The procedure does not compare well with equilibrium dialysis. The possible source of the inconsistency may be due to sample collection and storage. In conclusion, it is recommended that only plasma be used and freezing should be avoided.

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Table 1

International Classification of Epileptic Seizures

I. Partial seizures (seizures beginning locally)

- A. Partial seizures with elementary symptomatology (generally without impairment of consciousness):
  1. With motor symptoms (including jacksonian seizures)
  2. With special sensory or somatosensory symptoms
  3. With autonomic symptoms
  4. Compound forms
- B. Partial seizures with complex symptomatology (generally with impairment of consciousness) (temporal lobe or psychomotor seizures):
  1. With impairment of consciousness only
  2. With cognitive symptomatology
  3. With affective symptomatology
  4. With "psychosensory" symptomatology
  5. With "psychomotor" symptomatology (automatisms)
  6. Compound forms
- C. Partial seizures secondarily generalized

II. Generalized seizures (bilaterally symmetric and without local onset)

1. Absences (petit mal)
2. Bilateral massive epileptic myoclonus
3. Infantile spasma
4. Clonic seizures
5. Tonic seizures

6. Tonic-clonic seizures (grand mal)
7. Atonic seizures
8. Akinetic seizures

III. Unilateral seizures

IV. Unclassified epileptic seizures

Table 2

Pharmacokinetic Data

Drug	Oral Absorption	Time to Peak level (h.)	Half-life (h.)	Approx. % Protein Bound	Therapeutic Range (ug/ml)	Major Metabolic Product(s)	Excretion
Phenytoin	slow variable	2-8	8-60	90	10-20	HPPH 5 (p-hydroxyphenyl)-5-phenylhydantoin	urine 5% unchanged
Phenobarbital	slow complete	2-8	50-120	50	10-40	parahydroxyphenobarbital	urine 25% unchanged
Primidone	rapid complete	1-9	10	10	5-10	phenobarbital + PEMA (phenylethylmalonamide)	urine 40% unchanged
Carbamazepine	slow variable	6 12	15-20 [1.] 18-65 [2.]	75	5-10	carbamazepine 10-11-epoxide	72% urine 1% unchanged 28% feces
Ethosuximide	slow complete	3-7	60	0	40-100	2(1-hydroxyethyl)-2-methyl succinimide	urine 10-20% unchanged

1. single oral dose
2. multiple dosing decreases half life due to autoinduction

Table 3.

Interaction Between Phenytoin and Other Drugs

Influence of Other Drugs on Phenytoin	Influence of Phenytoin on Other Drugs
Increased Effect:	**Increased Effect:**
Alcohol	Phenobarbital
Chloramphenicol	
Chlordiazepoxide	**Decreased Effect:**
Chlorpromazine	Anticonceptives
Cycloserine	Antidiabetics
Diazepam	Carbamazepine
Dicoumarol	Clonazepam
Disulfiram	Dexamathasone
Estrogens	Digitoxin
Ethosuximide	Dicoumarol
Halothane	L-Dopa
Isoniazid	Doxycycline
Naproxene	Furosemide
Para-aminosalicylic acid	Hydrocortisone
Phenobarbital	Methadone
Phenyramidol	Methyl prednisone
Phenylbutazone	Quinidine
Prochlorperazine	Sodium valproate
Salicylic acid	Tricyclic antidepressants
Sodium valproate	
Sulfaphenazole	
Sulthiame	
Tolbutamide	
Decreased Effect:	
Alcohol	
Carbamazepine	
Phenobarbital	
Pyridoxine	

Table 4.

Effects of Phenytoin

Substance	Effect
ACTH	Increased
Cortisol	Decreased
Dexamethasone	Decreased
DNA synthesis	Decreased
Folic acid	Decreased
17-ketosteroids, urine	Decreased
PBJ	Decreased
Thyroid hormones	Decreased
Vitamin K dependent coagulation factors	Decreased
Vitamin B_{12}	Decreased
Vitamin D	Decreased

Table 5.

Interaction Between Barbituates and Other Drugs

Influence of Other Drugs on Barbituates	Influence of Barbituates on Other Drugs
Increased Effect:	Increased Effect:
Alcohol	CNS Depressants
Chloramphenicol	
Dicoumarol	Decreased Effect:
Meprobamate	Amidopyrine
Methyl phenidate	Carbamazepine
Morphine derivatives	Corticosteroids
Para-aminosalicylic acid	Dicoumarol
Phenothiazine	Digitoxin
Phenytoin	Doxycycline
Reserpine	Griseofulvin
Sodium valproate	Methyl dopa
	Phenazone
	Phenobarbital
	Phenylbutazone
	Phenytoin
	Propranolol
	Quinidine
	Steroids
	Tricyclic antidepressants

Table 6.

Interactions Between Carbamazepine and Other Drugs

Influence of Other Drugs on Carbamazepine	Influence of Carbamazepine on Other Drugs
Increased Effect:	Decreased Effect:
Dextroproxyphene	Clonazepam
	Dicoumarol
Decreased Effect:	Doxycycline
Phenobarbital	Phenytoin
Phenytoin	Sodium valproate

Table 7.

The Consequences of Drug Displacement from Serum Albumin

	Immediately After Displacement	New Steady State
Free drug fraction in serum	Increased	Increased
Free drug concentration in serum	Increased	Unchanged
Total drug concentration in serum	Unchanged	Decreased
Pharmacologic activity	Increased	Unchanged
Glomerular filtration	Increased	Unchanged
Tubular secretion	Variable	Unchanged
Diffusion into liver cell	Increased	Unchanged
Active hepatic uptake	Variable	Unchanged

Table 8.

Precision

Within Day (n = 10)

Day to day (n = 13)

Drug	mg/l	±SD	%CV	mg/L	±SD	%CV
Ethosuximide	9.415	0.175	1.86	9.744	0.514	5.27
	0.853	0.506	5.93	0.837	0.944	11.272
Primidone	10.785	0.236	2.19	9.771	0.384	3.932
	0.869	0.829	9.536	0.8311	0.109	13.186
Phenobarbital	10.105	0.215	2.13	9.744	0.514	5.272
	0.930	0.492	5.29	0.844	0.925	10.959
Phenytoin	9.87	0.429	4.34	9.903	0.261	2.639
	0.939	0.428	4.55	0.869	0.117	13.431
Carbamazepine	10.146	0.466	4.59	9.927	0.538	5.421
	1.011	0.5243	5.187	0.9510	0.135	14.150

Table 9.

Recovery

Drug	Standard	Method D	% Recovery
Ethosuximide	48.229	46.011	95.4
		46.338	96.08
Primidone	50.1404	48.3284	96.39
		48.5096	96.75
Phenobarbital	50.4946	49.0237	97.09
		48.5096	96.75
Phenytoin	26.8174	32.6759	121.84
		33.4732	124.81
Carbamazepine	49.9331	48.7889	97.71
		48.8972	97.93

Table 10

Correlation and Regression Statistics

	0.2 to 50 mg/L	0.2 to 25 mg/l
Ethosuximide		
	N = 8	N = 7
	r = 0.996	r = 0.999
	slope = 0.142	slope = 0.120
	Y intercept = -0.094	Y intercept = 0.013
Primidone		
	N = 8	N = 7
	r = 0.997	r = 0.999
	slope = 0.164	slope = 0.141
	Y intercept = -0.040	Y intercept = 0.070
Phenobarbital		
	N = 8	N = 7
	r = 0.996	r = 0.999
	slope = 0.208	slope = 0.174
	Y intercept =-0.141	Y intercept = 0.020
Phenytoin		
	N = 8	N = 7
	r = 0.995	r = 1.000
	slope = 0.237	slope = 0.192
	Y intercept = -0.229	Y intercept = -0.014
Carbamazepine		
	N = 8	N = 7
	r = 0.995	r = 0.999
	slope = 0.141	slope = 0.116
	Y intercept = -0.142	Y intercept = -0.024

Table 11

Ethosuximide

Sample number	Total mg/L	Method B Free	Method D Free	%Free B	%Free D
6413	25	5.18	27.4	110	20.7
8161	42	26.2	23.5	62.4	55.6
8507	56	0.46	36.5	0.8	65.2

Table 12

Primidone

Sample number	Total mg/L	Method B Free	Method C Free	Method D Free	%Free B	%Free C	%Free
1402A	8			6.1			76.3
599A	2			1.9			95.0
600A	18			13.4			74.4
2556A	6			3.8			63.3
8037	18		10.90	10.75		60.6	59.7
1162	11			5.35			48.6
7782	2		2.67	1.5		134.0	75.0
562	3		2.87	2.97		95.6	99.0
6388	9			4.14			46.0
6457	9			3.58			39.8
1014	9			3.45			38.3
1	10			6.26			62.6
2	5	4.73		4.28	94.6		85.6
3	3.4	1.69		1.83	49.7		53.8
4	9	7.17		5.67	79.7		63.0
5	12			11.60			96.7
6	17.8			19.57			109.9
7	6.1	4.06		3.48	66.6		57.0
8	7	6.05		6.27	86.4		89.6
9	8.5	8.87		8.29	104.4		97.5
10	12.8	9.02		6.17	70.5		48.2
11	7.4	7.15		6.02	96.6		81.4
12	14	13.89		8.27	99.2		59.1
13	3.85	3.70		3.55	96.1		92.2
14	7.7			6.09			79.1
16	10.2			7.20			70.6
17	13	14.40		10.09	110.8		77.6
18	11.2	10.86		7.17	96.7		64.1
19	13	16.02		11.32	123.2		87.1
20	7.6	7.09		4.86	93.3		63.9

Table 13

Phenobarbital

Sample number	Total mg/L	Method C Free	Method D Free	%Free C	%Free D
2442A	42		19.4		46.2
7402A	27		16.1		59.6
6924A	12		6.2		51.7
370A	9		5.3		58.9
599A	26		14.3		55.0
600A	22		11.7		53.2
2556A	22		9.8		44.5
7338A	11		6.6		60.0
7990	24	10.42	8.57	43.4	35.7
8037	7	2.61	2.35	37.7	33.6
5199	30	16.34	15.71	54.5	52.4
6604	21	10.85	9.637	51.7	45.9
7999	21	8.29	7.25	39.5	34.5
198	18	8.99	7.76	49.9	43.1
1162			16.29		
6415	19		9.12		48.0
7782	22	12.81	10.11	58.2	45.9
562	21	12.37	10.11	58.9	48.1
2157	25	14.77	11.65	59.1	46.6
9373		10.14	8.72		
2970	15	6.77		45.1	
3684	2	0.36		18.0	
5671	23	9.49	10.7	41.3	46.5
5686	7	4.00	5.59	57.1	80.0
7239	8		2.64		33.0
7243	8		12.53		156.6
8165	15		0.28		1.8
6388	26		11.84		45.5
6457	20		7.55		37.7
1014	14		5.82		41.6
2134	3	2.90	2.40	96.6	80.0
8591	28	14.08	12.33	50.3	44.1
1721	7	8.28	6.69	118.3	95.6
7239	8		2.64		33.0
9556	19		11.03		58.1

Table 14

Phenytoin

Sample number	Total mg/l	Method C Free	Method D Free	%Free C	%Free D
460A	1		0.4		40
6924A	35		3.5		10
370A	3		1.6		53.3
2556A	9		0.6		6.6
3792A	7		0.9		12.9
3942A	6		0.5		8.3
4082A	9		0.6		6.6
6921A	7		0.5		7.1
7053A	8		0.6		7.5
7252A	6		0.5		8.3
7990	11	1.85	1.11	16.8	10.1
5199	2	0.38	0.1	19.0	5.0
6435	8	1.38	0.91	17.3	11.4
6604	1	0.5		50.0	
7999		2.81			
9402	21	2.71	2.48	12.9	11.8
9785	28	3.23	2.21	11.5	7.9
1162			1.85		
7782	28	3.83	2.46	13.7	8.8
562	25	4.11	2.26	16.4	9.0
335	14	1.97	1.23	14.1	8.8
9255	3		0.25	0.25	8.3
255	3	0.4		13.3	
9373	13	1.85	2.05	14.2	15.8
80	2		0.50		24.9
332	18	1.52	1.60	8.4	8.9
2970	12	1.51		12.6	
3142	7	0.71	0.65	10.2	9.2
3415	7		0.79		8.8
3443	9	0.97	0.78	10.8	8.7
3684	2	0.17	0.15	8.5	7.5
3920	28	3.03	1.97	10.8	7.0
5113	4	0.51	0.53	12.9	13.3
5403	16	2.07	1.65	12.9	10.3
5671	9	0.60	0.64	6.7	7.1
5743	6	0.80	0.83	13.3	13.8
5979	18		1.16		6.4
7296	6		0.44		7.3
7637	5		0.27		5.4
4030	12		0.85		7.1
6388	11		0.99		9.0
6457	5		0.41		8.2
6462	10		0.81		8.1
7239	2		0.26		13.0
8165	4		0.38		9.5
9556	8		1.03		12.9
9885	6		0.58		9.7

Table 15

Carbamazepine

Sample number	Total mg/L	Method C Free	Method D Free	%Free C	%Free D
246A	6		0.7		11.7
7402A	6		1.1		18.3
6921A	1		0.5		50.0
7474	7	1.82	1.46	26.0	20.9
198	2	0.52	0.47	26.0	23.5
1204	9		1.33		14.8
3044	9	1.42	1.80	15.8	20.0
6915	7		1.49		21.3
7212	9		1.36		15.1
2157	6	3.41	3.96	56.8	66.0
2335	3	2.22	2.12	74.0	70.7
4181	4	2.75	2.31	68.8	57.8
6188	8	3.20	4.35	40.0	54.4
6970	6	2.27	3.32	37.8	55.3
8551	9		2.56		28.4
8591	7		3.76		53.7
332	3		0.31		10.3
3920	3	0.83	1.24	27.7	41.3
5064	8	2.17	3.34	27.1	41.8
5743	6	1.66	2.74	27.7	49.0
5896	7		2.22		31.6
8165			2.56		
403	7		1.53		21.9
1945	8		2.11		26.4
4455	10		2.31		23.1
6359	9		2.77		30.8
6388	3		1.82		60.7
6457			0.19		
6462	9		1.34		14.9
7023	8		2.37		29.6
360	9	3.00	2.86	33.3	31.8
1640	6		1.87		31.1
160	8		2.56		32.0

Table 16
Statistics of % Free Drug

	Reported % Free (Table 2
Ethosuximide	
Method B and D	100 %
N = 6	
mean = 52.45	
Std. dev. = 38	
Range (2 S.D.) = -23.75 to 128.65	
Primidone	
Method B	greater than 90%
N = 14	
mean = 90.55	
Std. dev. = 90.55	
Range (2 S.D.) = 52.7 to 128.4	
Method C and D	
N = 33	
mean = 74.1	
Std. dev. = 21.57	
Range (2 S.D.) =30.94 to 117.22	
Phenobarbital	
Method C and D	50 %
N = 47	
mean = 53.1	
Std. dev. =24.58	
Range (2 S.D.) = 3.93 to 102.26	
Phenytoin	
Method C and D	10 %
N = 63	
mean = 12.38	
Std. dev. = 8.84	
Range (2 S.D.) = -5.29 to 30.0	
Carbamazepine	
Method C and D	25 %
N = 43	
mean = 35.3	
Std. dev. = 17.48	
Range (2 S.D.) = 0.36 to 70.30	

Table 17

Sample number	Total Protein g/L	CSF Protein Method C mg/L	CSF Protein Method D mg/L
8591	7.64	2.32	1.19
2335	3.83	3.68	1.12
9373	4.73	0.72	1.19
332	5.64	2.48	1.12
2157	7.81	3.88	1.12

Table 18.

Correlation and Regression Statistics

Comparison of HPLC Versus EMIT

Drug	Paramaters
Phenobarbital	N = 7
	r = 0.940
	slope = 1.181
	Y intercept = -0.417
Phenytoin	N = 13
	r = 0.842
	slope = 1.050
	Y intercept = 0.061
Carbamazepine	N = 7
	r = 0.783
	slope = 1.195
	Y intercept = 0.493

Table 19

Results of ultrafiltrates (Method D) and equilibrium dialysis (Method E)

Drug and Sample #	Total Method B	Free Method D	% Free	Serum Method E	Buffer Method E	Calculated % Free
Etho.						
6413	25	27.4	109.6	15.56	25.3	101.2
6413 1.)	25	5.18	20.7	15.56	1.05	4.2
Primi.						
8520 1.)	9.8	8.27	84.4	9.47	8.82	90.0
	9.8	13.89	141.7	9.47	7.63	77.9
275 1.)	10	6.26	62.6	7.32	4.42	44.2
	10	6.26	62.6	7.32	4.94	49.4
Phenobarb.						
7239	8	2.64	33.0	2.92	3.67	45.9
8591	28	14.08	50.3	11.36	10.27	36.7
9556	19	11.03	58.1	7.52	8.99	47.3
Phenytoin						
7239	2	0.26	13.0	2.08	0.24	12.0
7296	6	0.44	73.3	4.37	0.51	85.0
9885	6	0.58	9.7	5.64	0.97	16.2
9556	8	1.03	12.9	6.78	1.30	16.3
7637	5	0.27	5.4	3.46	0.31	6.2
332	18	1.6	8.9	18.33	1.82	10.1
Carbama.						
5896	7	2.21	31.6	5.78	1.57	22.4
4455	10	2.31	23.1	13.10	3.01	30.1
8501	7	3.76	53.7	6.23	1.92	27.4
332	3	0.31	10.3	2.74	1.03	34.3

1.) Sample ultrafiltrate and equilibrium buffer were cleaned up by Method B and reassayed.

Table 20

Correlation and Regression Statistics
Comparison of Method D and Method E
% Free by equilibrium dialysis versus Amicon ultrafiltration
N= 19
r = 0.870
slope = 0.683
Y intercept = 8.751

Ethosuximide
N = 2
r = 0.989

Primidone
N = 4
r = 0.616

Phenobarbital
N = 3
r = -0.093

Phenytoin
N = 6
r = 0.996

Carbamazepine
N = 4
r = -0.590

Table 21

Correlation and Regression Statistics
Comparison of Method C and Method D

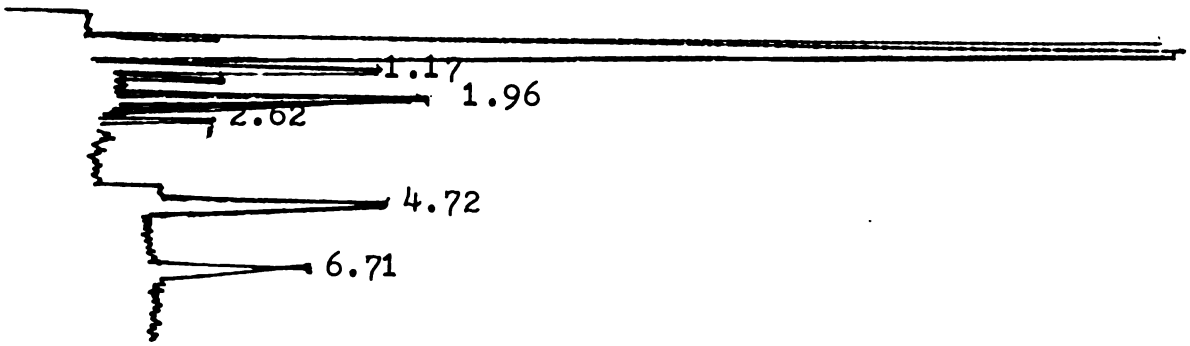
Primidone
N = 3
r = 1.000
Std. dev. = 0.590
slope = 0.890
Y intercept = 1.335

Phenobarbital
N = 15
r = 0.957
Std dev. = 1.186
slope = 1.126
Y intercept = -0.026

Phenytoin
N = 19
r = 0.925
Std. dev. = 0.505
slope = 1.385
Y intercept = -0.034

Carbamazepine
N = 11
r = 0.887
Std. dev. = 0.487
slope = 0.664
Y intercept = 0.443

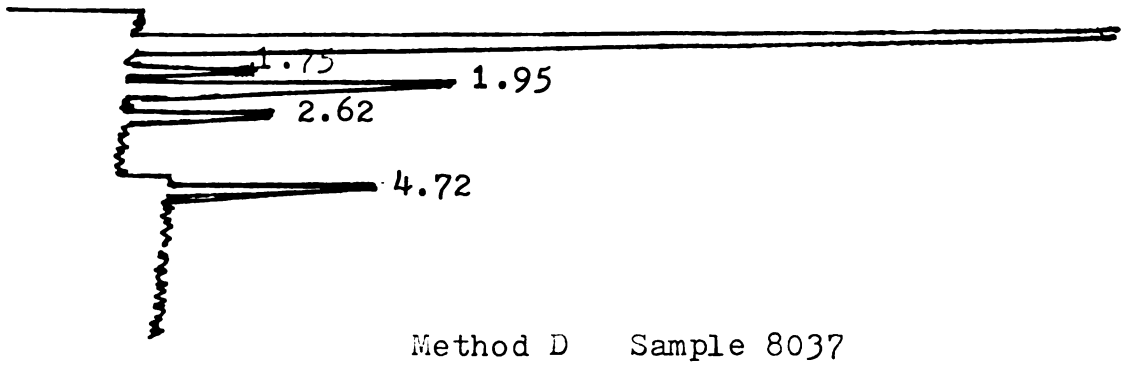
Graph 1



Method C Sample 8037

Time	Drug
1.96	Primidone
2.62	Phenobarbital
4.72	Hexobarbital (I.S.)

Graph 2



Method D Sample 8037

Time	Drug
1.95	Primidone
2.62	Phenobarbital
4.72	Hexobarbital (I.S.)

Graph 3

Linearity

Concentration versus Peak area ratio