Estimation of total and free phenytoin concentrations in patients with hypoalbuminemia admitted in critical care

A DISSERTATION SUBMITTED TO THE TAMIL NADU, DR. M.G.R. MEDICAL UNIVERSITY, IN PARTIAL FULFILMENT OF THE REGULATIONS FOR THE AWARD OF M.D. DEGREE EXAMINATION IN PHARMACOLOGY (BRANCH VI) TO BE HELD IN MAY 2019

Department of Pharmacology and Clinical Pharmacology

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CERTIFICATE

This is to certify that this dissertation entitled “Estimation of total and free phenytoin concentrations in patients with hypoalbuminemia admitted in critical care.” submitted by Dr Premila Magdalene Wilfred, in partial fulfilment of university regulations for the award of M.D. Pharmacology (Branch VI) degree examination of The Tamil Nadu Dr. M.G.R. Medical University, Chennai to be held in May, 2019 is a bonafide original work done under my direct guidance and supervision and completed to my utmost satisfaction.

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This is to certify that this dissertation entitled “Estimation of total and free phenytoin concentrations in patients with hypoalbuminemia admitted in critical care.” is a bonafide original work of Dr Premila Magdalene Wilfred under the guidance of Dr Binu Susan Mathew, Professor and Head, Department of Pharmacology and Clinical Pharmacology, Christian Medical College, Vellore and Dr J V Peter, Professor, Department of Medical Intensive Care Unit, Christian Medical College, Vellore towards partial fulfilment of university regulations for the award of M.D. Pharmacology (Branch VI) degree examination of The Tamil Nadu Dr. M.G.R. Medical University, Chennai to be held in May, 2019.

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DECLARATION

I, Dr Premila Magdalene Wilfred, do hereby declare that this dissertation entitled "Estimation of total and free phenytoin concentrations in patients with hypoalbuminemia admitted in critical care" has been done by me under the direct guidance of Dr Binu Susan Mathew, Professor and Head, Department of Pharmacology and Clinical Pharmacology, Christian Medical College, Vellore and Dr J V Peter, Professor, Medical Intensive Care Unit, Christian Medical College, Vellore in partial fulfilment of university regulations for the award of M.D. degree in Pharmacology (Branch VI). I have not submitted this dissertation in any part or full to any other university or towards any other degree.

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ABSTRACT

Title

Estimation of total and free phenytoin concentration in low albumin patients admitted in critical care

Background

Phenytoin has been widely used in the prophylaxis and treatment of epilepsy. However, dosing phenytoin is challenging because it exhibits non linear pharmacokinetics, zero order elimination and a multitude of drug interactions. Phenytoin is also highly protein bound (90%). It is the unbound or free form which can cross the blood brain barrier and causes both the therapeutic and toxic effects. A fall in serum albumin concentration can alter phenytoin binding capacity. In this situation, estimating total phenytoin concentration which includes the bound and unbound portions will provide discrepant results compared to the actual free phenytoin concentration.

Aim

- To compare free phenytoin concentration measured by three methods (Direct serum measurement, Routine method and by the Sheiner Tozer calculation method).
- To estimate total phenytoin concentration by two method (Direct serum measurement and Sheiner Tozer calculated total method) and if feasible, to

develop a model to predict the free measured phenytoin concentration from total phenytoin concentration.

Methods:

This was an observational study. Patients in the Medical Intensive Care Unit who were on phenytoin, were observed from the day of admission. When serum albumin level was <3.5 g/dL, blood samples were collected prior to phenytoin administration in patients. Direct measurement of total and free phenytoin concentration was done by High Performance Liquid Chromatography. Free phenytoin was also estimated by the Sheiner Tozer equation and routine method (Direct measured total/10).

Results:

The total and free phenytoin concentration was measured in 57 patients with low albumin. The median and interquartile range for direct measured total and Sheiner Tozer calculated total phenytoin concentration was 9.82(6.02-13.85) and 17.14(10.63-24.53) respectively. There was a mean relative difference of 42% in the total phenytoin direct measured concentration compared to the Sheiner Tozer calculated total phenytoin. Seventy five % of patients if reported only using the direct measured concentration would have total phenytoin concentration within the therapeutic range, whereas these patients would be supratherapeutic by the Sheiner

Tozer calculation. These patients would require a reduction in the phenytoin dose, which would be unlikely if the clinician's judgement is based ONLY on the direct measured total phenytoin.

The median (IQR) for routine, direct measured and Sheiner Tozer free phenytoin was 0.98 (0.60 - 1.39), 1.92 (1.06 -2.76) and 1.71 (1.06 - 2.45) respectively. The correlation coefficient (r^2) for direct measured with routine free phenytoin and with Sheiner Tozer calculated free concentrations was found to be 0.63 and 0.64 respectively. And if the phenytoin concentration was reported using the Sheiner Tozer calculated total, 33% of patients would be falsely reported as therapeutic whilst being supratherapeutic in the direct measured free method.

Conclusion:

Our study concluded that the total phenytoin concentration based on the Sheiner Tozer corrected equation is different from the direct measured concentration by High Performance Liquid Chromatography, in patients with low albumin. In addition, free phenytoin concentration can only be reported from a direct measured value and not any prediction/equation.

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ABBREVIATIONS

HPLC-High Performance Liquid Chromatography
MICU-Medical Intensive Care Unit
QC-Quality Control
GABA- Gama Aminobutyric acid
RT-retention time
IS-Internal Standard

INTRODUCTION

Epilepsy is a clinical condition that is characterised by recurrent unprovoked seizures. Phenytoin has been widely used in the prophylaxis and treatment of epilepsy because of its good efficacy and low cost.

A challenge in medical practice is administering phenytoin to control seizures. This is because the drug phenytoin exhibits complex pharmacokinetic and pharmacodynamic properties. Phenytoin demonstrates non linear kinetics and hosts multitude of drug drug interactions. Furthermore, phenytoin is 90% bound to albumin. It is the remaining 10% free fraction that is biologically active and responsible for both the therapeutic and toxic effects of the drug. Hence, low drug levels fail in controlling seizures and too high levels can result in toxic symptoms. However, among the antiepileptic drugs, phenytoin has the best documented relationship between blood level and clinical effect

Therapeutic drug monitoring is a valuable tool used to optimise therapy in patients on antiepileptic drugs. This is especially important in the critically ill patient admitted in Medical Intensive Care Unit (MICU). Patients admitted in MICU are prone to develop hypoalbuminemia due to altered protein binding, old age, uraemia and sepsis. A fall in serum albumin concentration can alter phenytoin binding capacity. In this situation, estimating total phenytoin concentration which includes the bound and free portions will provide discrepant results compared to the actual free phenytoin concentration.

In many institutions the total phenytoin concentrations is routinely measured. However, in the critically ill to estimate the free phenytoin concentration two calculation methods are widely used. A more common method is dividing the total phenytoin concentration by 10 (assuming 10% of total phenytoin is free). Secondly, a standard formula that predicts the effect of serum albumin on phenytoin concentration is the Sheiner Tozer equation.

The accuracy of the above two calculation methods has been extensively argued by many authors. Studies done over the last few decades on critically ill patients have shown that measurement of free phenytoin concentration would improve efficacy whilst minimising toxic effects.

This study was done among the critically ill to determine if the concentration of free phenytoin in serum can be accurately predicted from a total phenytoin concentration.

AIMS AND OBJECTIVES

AIM:

To estimate the total and free phenytoin concentration in patients admitted to the Medical Intensive Care Unit (MICU) with low albumin and to develop a model to predict the free measured phenytoin concentration from total phenytoin concentration.

OBJECTIVES:

1. To measure the total and free serum phenytoin concentration using High Performance Liquid Chromatography (HPLC) in patients with low albumin admitted in Medical Intensive Care Unit- **Direct measured total and free phenytoin concentration**

2. To estimate the calculated total and calculated free phenytoin concentration using the standard formula -Sheiner Tozer Equation in these patients – **Sheiner Tozer calculated total and free phenytoin concentration.**

3. To estimate the routine free phenytoin concentration by dividing direct measured total phenytoin concentration by 10 [assuming 10% of the drug is the free form]- **Routine free phenytoin concentration.**

4. To compare the total phenytoin concentration obtained by two methods: Direct measured total phenytoin and Sheiner Tozer calculated total phenytoin concentration.

5. To compare the free phenytoin concentration obtained using Direct measurement with that obtained using the Sheiner Tozer calculated Method and Routine method.

6. To develop and validate a regression model to predict free phenytoin concentration using the total phenytoin concentration.

7. To develop and validate a HPLC method for direct measurement of free phenytoin concentration.

REVIEW OF LITERATURE

Seizure – A seizure is defined as a behavioural change that occurs due to excessive synchronization of neurons located in the cortex of the cerebrum(1). Seizures are classified into four main groups generalized, focal, unknown (eg: epileptic spasms) and unclassified.

Epilepsy- is defined by the International League against Epilepsy -2017 as a brain *disease* characterized by one of the following:

1. One unprovoked (or reflex) seizure and a probability of further seizures similar to the general recurrence risk after two unprovoked seizures (eg, ≥60 percent) occurring over the next 10 years;

2. Two unprovoked seizures more than 24 hours apart;

3. Diagnosis of an epilepsy syndrome occurring due to continually lowered threshold(2).

Burden of Epilepsy-

According to the World Health Organization statement released in February 2018, approximately 50 million people in the world suffer from epilepsy(3). 8-10% of the world population can experience an episode of seizure in their lifetime(4). This makes epilepsy one of the most common neurological disorders worldwide.

Seizures can be effectively managed with appropriate antiseizure drug therapy. But the cost of treatment is high. With more than 85% of people with epilepsy residing in developing countries, treatment should be effective and economical to the patient(5).

About 12 million Indians are affected by epilepsy(6). This accounts for one sixth of the world's epilepsy burden. A recent meta-analysis in India reported a prevalence of 3.0-11.9 per1,000 population and incidence of 0.2-0.6 per 1,000 population per year(6).

Causes of seizures

In 2017, International League against Epilepsy Commission for Classification of Epilepsy classified epilepsy based on the aetiology into 6 types- genetic, structural, metabolic, immune, infectious, and unknown causes(7,8). Genetic epilepsy results from an inherited abnormality in the brain. Structural causes of seizures are further classified into congenital (eg: tuberous sclerosis) and acquired (eg: meningeal infection, stroke, head injury) causes. Metabolic causes include conditions such as creatine deficiency syndromes and glucose transporter deficiency etc. Immune mediated causes are due to inflammation in the brain, as occurs in Rasmussen encephalitis. Infections causing epilepsy include tuberculosis, malaria and Human immunodeficiency syndrome. Epilepsy of unknown causes includes cases where the etiology of the seizure is not known. Approximately one third of patients fall in this category.

Evaluation:

The primary aim in evaluating a patient with the first episode of a seizure is to determine whether the cause of the seizure is of structural origin or a treatable systemic cause. A complete physical and neurological examination will be able to direct the treating physician to the various types of laboratory (eg: blood glucose, electrolytes, renal and liver function tests and toxicology screening) and

neuroimaging tests (magnetic resonance imaging, computed tomography) to be ordered(9). Additional tests include an electroencephalogram (10)and a lumbar puncture if required.

MANAGEMENT OF EPILEPSY

Majority of seizures resolve spontaneously within two minutes. But, it is well known that epilepsy is associated with disability, poor psychosocial outcomes, higher rates of psychiatric comorbidity, and most importantly a threefold increase in mortality (11). Therefore, patients presenting with an acute episode of seizure must undergo a complete evaluation for metabolic causes and if found be treated accordingly. The remaining patients require administration of antiseizure medication.

The management of epilepsy consists of three main goals:

1. Controlling the seizure
2. Avoiding or minimizing side effects
3. Restoring or maintaining quality of life

Drug selection

Initially seizures are managed with a single antiseizure drug. Almost 50% of these patients remain seizure free with a single antiseizure drug(12,13). Currently, there are almost 27 drugs that have been approved for controlling seizures(14). Choice of medicine is based on the seizure type, characteristics of the drug and patient's specific variables such as age and associated co morbidity(15). An evidence-based guideline of the American Academy of Neurology and the American Epilepsy

Society also suggests individualizing drug therapy based on the risk of seizure recurrence against the risk of developing adverse effects while on the drug(16).

For treatment to be a success, the treating physician should counsel both the patient and the family members(17). Non-adherence to treatment has been shown to increase the risk of injury, hospitalization and even mortality(18).

Antiseizure drugs

Antiseizure drugs can be classified based on their mechanism of action (19). (Table: 1)

PHARMACOLGY OF PHENYTOIN

Houston Merritt and Tracy Putnamwere were the first to discover the anticonvulsant and sedative effect of the drug phenytoin in the year 1938 (20,21). The molecular formula of phenytoin is $C_{15}H_{12}N_2O_2$and its molecular weight is 252.273 g/mol(22).

Though phenytoin is an old antiepileptic drug it continues to be used in the treatment of epilepsy. This is because the drug has become well known for its therapeutic efficacy(23–26). In addition, phenytoin is extensively used in developing countries because it is highly cost effective.

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Table: 1 Antiseizure drug :classification and mechanism of actions(27)*:*

Drugs that affect voltage-dependent sodium channel
• Carbamazepine • Eslicarbazepine • Lamotrigine • Lacosamide • Phenytoin • Zonisamide • Oxcarbazepine
Drugs that affect calcium currents
• Ethosuximide
Drugs that affect GABA Activity
• Benzodiazepines • Phenobarbital • Tiagabine • Vigabatrin
Drugs that affect glutamate receptors
• Perampanel
Drugs with multiple mechanisms of action
• Felbamate • Topiramate • Valproate
Drugs with other mechanisms of action
• Brivaracetam • Gabapentin • Levetiracetam • Pregabalin

PHARAMACOLOGY OF PHENYTOIN

MECHANISM OF ACTION

Phenytoin blocks voltage dependent neuronal sodium channels(23–25). This results in the block of action potential, stabilisation of neuronal membranes and decrease in neurotransmitter release(19). Phenytoin is currently used in the management of generalized, focal seizures and in status epilepticus (31–33). And can be used as a second-line agent for patients with mixed seizures (tonic-clonic and myoclonic).

PHENYTOIN DOSE-

Phenytoin is administered either orally or intravenously. As the plasma half-life of phenytoin ranges between 7-42 hours and serum therapeutic range is attained after 5-7 half-lives (7-10 days), therapeutic drug monitoring of phenytoin is done 5-7 days later. This will accurately determine the steady state serum phenytoin concentration.

Loading dose-

However, with an intravenous loading dose of phenytoin therapeutic concentration may be achieved within 10-30 minutes depending on the rate of infusion(34). The Food and Drug Administration (FDA) recommends intravenous phenytoin to be administered as a loading dose of 10-15 mg/kg at a rate of infusion not exceeding 50mg per minute in adults. This is to be administered in saline This dosing regimen is known to minimise cardiovascular adverse events(26,52).

Maintenance dose

Following the administration of a loading dose of phenytoin, patients should be initiated on a maintenance dose of 100mg (4 to 6 mg/kg/day) thrice daily delivered by the intravenous or oral route(35).

SIDE EFFECTS OF PHENYTOIN

Phenytoin has a narrow therapeutic window which warrants careful therapeutic drug monitoring to prevent toxic effects. When total phenytoin concentration exceeds 20 mcg/mL patients may present with nystagmus. At a higher concentration of 30 mcg/mL, patients may experience ataxia, slurred speech, and incoordination. When total phenytoin blood concentration exceeds 40 mcg/mL, patients may experience mental status changes and coma. But above a phenytoin concentration of 50 mcg/mL, drug-induced seizure may occur (34,36,37).

PHARMACOKINETICS OF PHENYTOIN

In normal individuals, the zero order pharmacokinetics of phenytoin is the major factor which determines decisions regarding the dosing of phenytoin. When the dose of phenytoin needs to be altered, either because of questionable efficacy or a definite side effect, almost all clinicians prefer to make the dose change after measuring the serum phenytoin concentration.

When the serum phenytoin concentration is outside of the therapeutic range, a change of dose is required. But in view of the complex pharmacokinetic properties of phenytoin, the physician has to be very careful whilst changing the dose. It is suggested that only small dose increments should be made during every dosage change and the serum phenytoin concentration be checked at every

point before making any further modifications of the dose. The pharmacokinetic characteristics of phenytoin include(38):

1. Extensive binding to serum proteins
2. Nonlinearity of its elimination kinetics
3. Potent and broad-spectrum inducer of hepatic CYP and UGT-glucuronidation
4. Narrow therapeutic range
5. Other properties

1. Extensive binding to serum proteins-90% bound to serum albumin

Phenytoin, in the blood is extensively (>90%) bound to serum albumin(Figure1). It is the remaining 10% unbound or free fraction that is pharmacologically active and is found to be responsible for both the therapeutic and toxic effects of the drug(39). This is because only the free or unbound fraction crosses the blood brain barrier(40). However certain drugs such as valproate, tolbutamide, some sulphonamides and heparin can also alter the protein binding of phenytoin resulting in an increase in the free fraction (41). This must be kept in mind when comedications are added to a patient already on phenytoin. Also, in conditions such as burns, protein energy malnutrition, hepatic cirrhosis, critically ill patients, old age, nephrotic syndrome and pregnancy, serum albumin can be low. Low serum albumin can increase the biologically active free fraction of phenytoin.

2. Nonlinearity of its elimination kinetics

When serum phenytoin concentration in blood is low, the drug exhibits linear kinetics (Figure 2). However as the serum concentration of phenytoin increases, the drug elimination becomes non linear. Any increase in dose thereafter, leads to

non linear pharmacokinetics and produces unpredictable serum concentrations of phenytoin. At this point, the liver metabolism becomes saturated which leads to the disproportionate increase in serum phenytoin concentration (27). This often leads to the toxic manifestations observed in patients on phenytoin.

3. Potent and broad-spectrum inducer of hepatic CYP (Cytochrome P 450) and UGT (Uridine-diphosphate glucuronosyl transferase)-glucuronidation.

Approximately 95% of phenytoin is metabolised by the CYP2C9. The main metabolite 5-(4'-hydroxyphenyl)-5-phenylhydantoin derivative has been found to be inactive. The other drugs metabolised by CYP enzymes can inhibit phenytoin metabolism and this can result in high serum concentration of phenytoin (42).

Though monotherapy is preferred in seizure control, other antiseizure drugs may be added to control a seizure. These antiseizure drugs that are added may also alter serum phenytoin concentration.

Drugs that decrease the metabolism of phenytoin are valproic acid, isoniazid, cimetidine, phenylbutazone, chloramphenicol and some sulphonamides(43–45). Drugs that increase the metabolism of phenytoin include rifampicin.

The efficacy of levetiracetam, carbamazepine, valproate and topiramate may decrease when combined with phenytoin(46,47). Phenytoin induces the metabolism of the drug warfarin resulting in a subtherapeutic international normalized ratio. In this situation, a clinician has to increase the dose of warfarin.

Figure: 1 The Extraordinary Ligand Binding Properties of Human Serum Albumin Mauro Fasano1IUBMB Life, 57(12): 787 – 796, December 2005

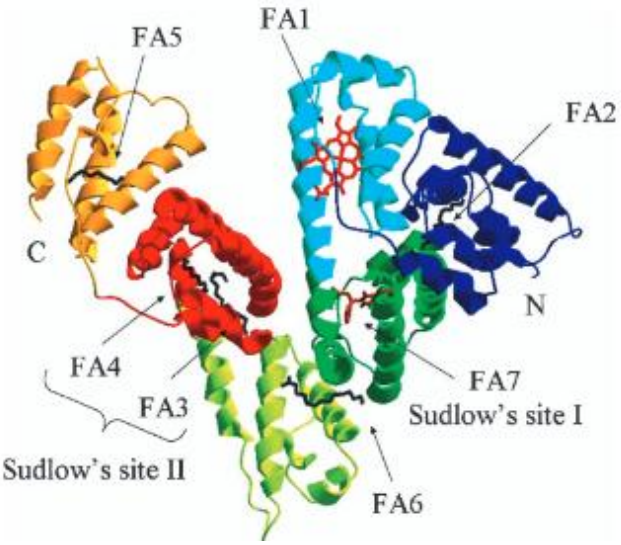
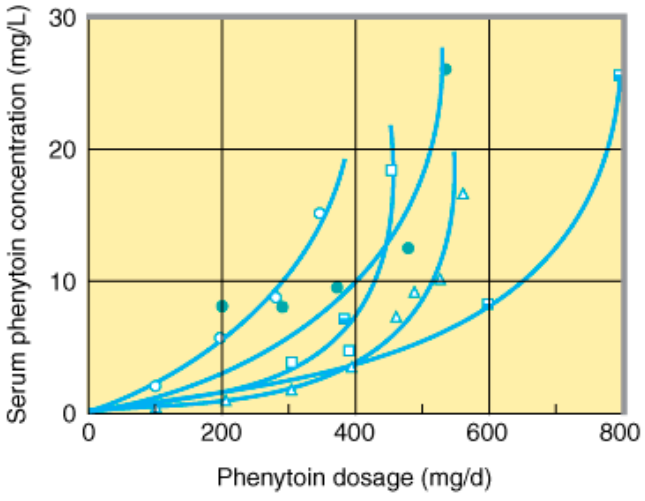


Figure 2: Non linear kinetics



Source: Katzung BG, Masters SB, Trevor AJ: *Basic & Clinical Pharmacology, 11th Edition:* http://www.accessmedicine.com

Table 2: Drugs affecting the metabolism of phenytoin (Adapted from uptodate 2018)

A. Drugs that increase the metabolism of phenytoin and thereby decrease phenytoin concentrations (Total and Free):

Folic acid
Dexamethasone
Phenobarbitone
Diazepam
Rifampicin
Nitrafurantoin
Methadone
Oestrogens

B. Drugs that decrease the metabolism of phenytoin and thereby increase phenytoin concentrations (Total and Free):

Valproic acid
Carbamazapine
Warfarin
Isoniazid
Cimetidine, Ranitidine
Omeprazole
Ibuprofen
Metronidazole
Chloramphenicol
Fluoconazole
Amiodarone

4. Narrow therapeutic range

The normal therapeutic range for total phenytoin (bound and unbound) concentration is 10-20µg/ml(48). As 90% of phenytoin is bound to serum albumin, the remaining 10% is free phenytoin.

So, Free phenytoin concentration= Total phenytoin concentration divided by 10.

The corresponding therapeutic range of free phenytoin concentration is 1-2µg/ml(49).

Therapeutic range for phenytoin is as follows:

- Total phenytoin concentration: 10 to 20 µg/ml
- Free phenytoin concentration: 1 to 2 µg/ml

When drug concentrations lie below the reference range (*subtherapeutic*), the patient can have a seizure and when values exceed the therapeutic range (*supratherapeutic*) the patient can manifest with toxic features.

5. Miscellaneous: Apart from the pharmacokinetic factors mentioned above multiple factors can alter the free fraction of phenytoin. A few of them include:

i) Patients with hypercholesteremia have exhibited high free phenytoin concentration. This is because free fatty acids displace phenytoin from the binding site(50–52).

ii) Protein binding of phenytoin in diabetics is also found to be lower than for the non diabetics (53).

iii) Circulating high levels of bilirubin competitively bind to albumin raising free phenytoin concentration (44).

The above mentioned factors emphasize the need for measuring free phenytoin concentration in serum.

MEASUREMENT OF FREE PHENYTOIN- EXISTING OPTIONS INCLUDE:

Mathematical equations to obtain free phenytoin concentrations from the total concentration have been developed(50,54,55).

1. Routine method -Measured total phenytoin concentration divided by 10 [assuming 10% of the drug is the free form] which is the method routinely adopted in many hospitals.

2. Sheiner Tozer calculated method- A mathematical equation developed to help clinicians predict free or unbound phenytoin concentrations based on the total phenytoin concentration and the serum albumin.

Sheiner Tozer equation-

i. *In patients with normal renal function*

- Total corrected phenytoin concentration = Measured phenytoin concentration (mg/L)/ {(0.2*serum albumin [g/dl]) +0.1}

Unit of Phenytoin concentration is mg/L and albumin is in g/dL

ii. *End stage renal disease* is defined as a Glomerular Filtration Rate (GFR) <15 ml/min/1.73 m^2 by the Kidney Disease Outcome Quality Initiative of the National Kidney Foundation. In patients with end stage renal disease there is reduced protein binding secondary to uraemia and the equation is as follows:

- Total corrected phenytoin concentration =Measured phenytoin concentration (mg/L)/ {(0.1*serum albumin [g/dl]) +0.1}

To obtain the free phenytoin concentration the predicted total phenytoin concentration obtained from Sheiner Tozer mathematical formula is divided by 10 (or multiplied by 0.1)

3. <u>Direct measurement of phenytoin</u>- Third option is direct measurement of free phenytoin concentration by HPLC.

MEASUREMENT OF PHENYTOIN IN THE CRITICALLY ILL PATIENTS

However, in critically ill patients there are many factors that might influence the pharmacokinetics of phenytoin, so this equation may not be accurate enough to guide dosing in this population (30, 36).

Albumin is synthesised by the liver hepatocytes and is the most abundant protein in plasma. In a healthy adult approximately, half the total protein is albumin(42). The normal albumin level is 3.5g/dl-5.0gm/dl(56). Albumin is a negative acute phase reactant(57). In critical illness, the concentration of acute phase reactants (albumin) declines (58). This results in an increase in the serum free phenytoin concentration (59,60). In this population (MICU patients), apart from a low albumin state, there are other factors such as uraemia and drug-drug interactions that bring about further changes in the biologically active free fraction of phenytoin. Surprisingly, even the use of Sheiner Tozer equation may not give accurate estimate of the concentration of free phenytoin in serum.(37, 39, 49). For this reason, free phenytoin concentrations are measured in serum in patients

admitted in ICU (39,50,61–66). Some common methods in use are mentioned below.

DRUG ANALYSIS TECHNIQUES

Drug analysis techniques include(54,59,67,68)

1. Immunochemical techniques- A rapid and easy method.

2. Chromatographic methods- a specific method. Chromatography methods include High performance liquid chromatography (HPLC) and Liquid chromatography -Mass Spectrometry (LCMS).

THERAPEUTIC DRUG MONITORING

Therapeutic drug monitoring is an effective method used to measure plasma drug concentrations(59,67,69,70). The indications for therapeutic drug monitoring include individualizing therapy, monitoring compliance and diagnosing toxicity(69,71,72). Interpreting plasma drug concentration is not simple and requires assessment of other factors such as clinical response, dosing schedule and timing of sampling(71).

Measurement of Free Phenytoin

Total phenytoin concentration is routinely performed in many hospitals in India. But, the measurement of free phenytoin concentration is relevant in patients with low albumin(50,73,74).

The problems in the measurement of free phenytoin concentration include:

- High cost-The estimated cost of measurement of free phenytoin concentration can almost be double that of total phenytoin concentration(38).

- Labor intensive- Measurement of free phenytoin concentration involves an extra step of ultrafiltration(75).

- Delay in results-In small hospitals, samples must be batched and free phenytoin concentration results may be delayed(38).

THERAPEUTIC DRUG MONITORING OF PHENYTOIN

WHY MEASURE TOTAL PHENYTOIN:

The correlation between phenytoin serum concentration and the biological effects of phenytoin treatment, both in terms of clinical efficacy and toxicity is well established. D J M Reynolds et al have assessed the value of measurement of serum phenytoin concentrations in patients. They concluded that measurement of total serum phenytoin concentration will provide maximum efficacy benefit and prevent toxic side effects(69). Vozeh and colleagues evaluated steady state concentration in 28 patients using nomograms. Their study results suggest that applying nomograms most often underestimates serum concentration and serum phenytoin concentration should be measured especially when considering a change of dose(76).

A. Birnbaum, et al performed an observational study in 32 nursing homes recruiting 56 patients (>65 years) who were on the same dose of phenytoin and not on any interfering comedications for a period of 4 weeks. A total of 258 total phenytoin concentrations were analysed. The results of the study reveal that despite being on the same dose, the total phenytoin concentrations varied as much as two- to threefold. This study highlights the variability of phenytoin concentrations over time and the need for measurement of phenytoin concentrations(63).

These pharmacokinetically guided studies have identified an optimal therapeutic range for phenytoin and have recommended dose adjustment to obtain this target therapeutic range.

However, recent pharmacokinetic studies on patients treated with phenytoin in *intensive care units* show inappropriate dosing and failure to achieve target therapeutic concentrations. This suggests that the free or unbound phenytoin concentration should be measured in this special population.

WHY MEASURE FREE PHENYTOIN

Mathew D. Krasowski and his colleagues studied 756 patient data from electronic medical records from August 1996 to November 2010 where simultaneous measurement of total and free phenytoin of the same blood draw and a plasma albumin within 7 days of measuring phenytoin was analyzed. On applying the Sheiner Tozer equation the calculated phenytoin concentration most often underestimated the measured free serum phenytoin concentration. The Pearson correlation coefficient between total measured phenytoin/10 and measured free measured phenytoin was 0.72 and for free measured phenytoin and Sheiner Tozer calculated free phenytoin concentration was 0.79. The authors also found a poor relationship between free phenytoin concentration and albumin and suggested measurement of free phenytoin concentration whilst dosing patients on phenytoin (38).

Buckley et al performed a retrospective cohort study on 256 patients in the adult Intensive Care Unit in a University Medical Centre between January 1, 2010 and June 21, 2013. After admission into the MICU, blood was simultaneously drawn for measurement of total and free serum phenytoin concentrations with plasma albumin ≤48 hours of phenytoin draws. The study showed the mean Sheiner Tozer

total phenytoin and free levels were 16.1 ± 8.1 and 1.5 ± 0.8 µg/mL, respectively (r = 0.817; P < 0.001). A majority [77% (n=238)] measured free and Sheiner Tozer free concentrations was in agreement of 0.633 (P< 0.001). The level of agreement between measured phenytoin free concentration and Sheiner Tozer calculated free was classified into supratherapeutic, therapeutic and subtherapeutic and found to be 82.3%, 80.8%, and 67.2%, respectively (P= 0.063). The remaining 23% of patients showed disagreement between interpretation of the free and Sheiner Tozer concentrations with the Sheiner Tozer concentration overestimating free measured concentrations in 72% of the patients studied(77). Their results are shown below

Agreement between direct measured free and Sheiner Tozer calculated total phenytoin (categorized into subtherapeutic, therapeutic and supratherapeutic) (n = 238)

	Sheiner Tozer calculated total phenytoin concentration			
Free phenytoin concentration	Subtherapeutic (<10 µg/mL)	Therapeutic (10-20µg/mL)	Supratherapeutic (>20 µg/mL)	Total
Subtherapeutic (<1 µg/mL)	45(19%)	21(8.8%)	1(0.4%)	67(28%)
Therapeutic (1-2 µg/mL)	6(2.5%)	97(41%)	17(7.2%)	120(50%)
Supratherapeutic (>2 µg/mL)	1(0.4%)	8(3.4%)	42(18%)	51(21%)
Total	52(22%)	126(53%)	60(25%)	238

Categorization into Subtherapeutic, Therapeutic and Subtherapeutic in patients where there was disagreement between measured free and Sheiner Tozer total phenytoin serum concentrations (n = 54).

	Sheiner Tozer calculated total phenytoin concentration		
Free phenytoin concentration	Subtherapeutic (<10 μg/mL)	Therapeutic (10 - 20μg/mL)	Supratherapeutic (>20 μg/mL)
Subtherapeutic (<1 μg/mL)	-	21(3%)	1(1.9%)
Therapeutic (1-2 μg/mL)	6(11%)	-	17(31%)
Supratherapeutic (>2 μg/mL	1(1.9%)	8(15%)	-

Their results conclude that free phenytoin measurement would be the ideal method in critical care.

Hong et al-performed a study on 49 hypoalbuminemic patients on phenytoin and found a moderate linear correlation between measured and calculated free pheytoin concentrations($r=0.822$, $p<0.001$). The mean difference between the measured and Shenier Tozer free concentrations was (0.65 ± 0.88μg/mL; 95% confidence interval (1.11 to 2.41). Dividing the patients based on albumin concentrations (as seen in the table below) into normal albumin and low albumin, they observed 20% difference in phenytoin concentration.

They suggest the measurement of free phenytoin concentration in low albumin patients(50).

Albumin level and difference in phenytoin concentrations

	Percent difference (≥20%)	Percent difference (<20%)	Total
Albumin (>3.5)	29	5	34
Albumin(≤3.5)	8	7	15
Total	37	12	49

A study done by Sadeghi et al, recruiting 40 head trauma patients admitted in critical care on phenytoin, further emphasized the need for measuring free phenytoin concentration. Their study showed a weak to moderate correlation (r=0.528) between measured free and total phenytoin concentration in severe head injury patients(41).

All the above factors highlight the importance of therapeutic drug monitoring of total and free phenytoin concentration.

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

High performance liquid chromatography is a technique used to measure drug concentrations in plasma. A Russian- Italian botanist, Sir Mikhail S Tswett invented adsorption chromatography, a highly improved form of column chromatography(78). Currently, HPLC is the most widely used chromatographic technique used for measurement of plasma drug concentrations(79,80).

The advantages of HPLC include:

1. Results are Sensitive and accurate(81)

2.Ability to simultaneously measure concentrations of several drugs(79)

3. Biologically active metabolites can be measured

HPLC Principle: works on the principle of column chromatography. In this technique analytes of a biological matrix are identified, separated and quantified.

HPLC works on the principle of either normal phase or reverse phase chromatography (more common).The stationary phase is a nonpolar packed material held in a steel column. The mobile phase is polar and consists of water with organic solvents such as methanol or acetonitrile combined with buffers such as phosphate- K_2HPO (82,83).

Components of HPLC:

HPLC consists of a column, pump (2-4 in number), an injector, a detector, oven, a data processing unit and a display system(computer).

Column

The column consists of a stainless steel (most common) or glass tube 50-300mm long and 2-5mm in diameter. The longer the column the better the separation. The tube contains silica-based matrix. Silica is polar or hydrophilic in nature making it suitable for normal phase chromatography. By binding silica to hydrophobic bonded phases such as -C18, -C8 , -C4 and phenyl, silica is made non polar(used in reverse phase chromatography)(82). This converts the reactive silanols (-Si-OH) on the surface of silica to nonreactive silanols. But, even after this a few residual silanol groups may persist. Silonols are acidic in nature and can react with analytes that are basic and produce tailing of peaks. To prevent this, they are further bonded using "end capping". The commonly used end capping agent is CL(CH3)2SiCH3. Carbon load is a measure of the number of bonded ligands attached to the surface of the packing material. The particle size in the column ranges between 2-25um. The commonly used size is 5um.

Pump

The pump generates pressure to deliver an isocratic or gradient flow of solvent (analyte) through the column. The flow rate is controlled by the pump. A normal flow rate is 1-2 ml/min. Pumps are designed to generate a pressure of upto 6000psi. Depending on the analyte the pump can be adjusted to deliver an isocratic or gradient flow of solvent.

- ***Isocratic elution***-Here the composition of the mobile phase remains constant throughout the analysis. .Eg: A 30% acetonitrile +70% water that remains constant through the procedure. This method is used to separate

components of a mixture that have a similar affinity for the stationary phase. In this type of analysis as the retention time of the analyte increases, the peak becomes broader making it difficult to identify them as a definite peak.

- ***Gradient Elution***-The composition varies during the procedure usually from low eluting strength (eg 10% acetonitrile) to a higher eluting strength (eg: 70% acetonitrile). This allows late eluting compounds to elute faster and decreasing the retention time. Peaks are thus well defined.

Injector

An injector can be automated or manual. Automated injector is used when there are there are many samples. The needle of the injector is flushed prior to each sampling.

Guard column

Situated between the injector and the column, the guard column functions as a filter retaining particulate matter present in the sample. This increases the life of the column.

Detector

The most common detector used is the ultraviolet detector employed in this study. It works on the principle of detecting UV rays by a sensor(83). An analyte eluting a column absorbs UV light and changes the amount detected by the sensor. The amount of UV light absorbed by the analyte is proportional to the analytes concentration.

Software and Data system

The *computer* is used to set parameters such as column temperature, wavelength, flow rates and gradient or isocratic elution etc. The results are displayed as a chromatogram from which concentrations are determined(84).

JUSTIFICATION OF THE STUDY

- Patients admitted in MICU frequently develop hypoalbuminemia.
- Individualizing phenytoin dose in hypoalbuminaemic patients is a challenge as total measured phenytoin concentration is an overestimate.
- However, the important component in a patient with low albumin is the FREE drug concentration.
- At present the only way to estimate free concentration in these patients is Total phenytoin concentration calculated from Sheiner Tozer divided by 10.
- Currently, in our hospital the clinician depends on the corrected total phenytoin concentration (using Sheiner Tozer equation). This value obtained is used as a guide for dosing patients on phenytoin.
- Literature has reported that the directly measured free phenytoin concentration may not correlate with the Sheiner Tozer calculated total or free phenytoin concentration
- This highlights that directly measuring free phenytoin concentration in patients with hypoalbuminaemia is important in the critically ill.
- But the measurement of free phenytoin concentration is challenging because of the extra step of ultrafiltration and the additional expense may not be possible as a routine practice. We aim to look at the possibility to predict the measured free phenytoin concentration using total phenytoin concentration in consideration with other laboratory parameters.

METHODOLGY

The study was approved by the Institutional Review Board of the Christian Medical College, Vellore (IRB number-10321, dated-12.10.2016)

Study design:

This was an open label, prospective cohort observational study in a critical care setting. The study was done in the Clinical Pharmacology Unit (Department of Pharmacology and Clinical Pharmacology), Christian Medical College in collaboration with the Medical Intensive Care Unit (MICU)-Division of Critical Care, Christian Medical College.

A total of 56 patients who satisfied the inclusion criteria were included in the study. Patients were enrolled from December 15, 2016 to March 12, 2018 from the MICU, Christian Medical College, Vellore.

Setting:

Patients who develop a seizure in the ward or presented to the emergency department with a clinical history suggestive of a seizure are routinely initiated with a loading dose of phenytoin followed by a maintenance dose of Phenytoin. Those patients who were admitted into MICU were observed from the day of admission. Serum albumin is routinely measured twice a week in MICU. These results were collected from the clinical workstation, which is a database of the Christian Medical College Hospital where all the patient data is recorded.

Considering the half life of phenytoin to be 14 hours (average half life 7-29 hours) we estimated that steady state would be reached on the 4th to 5th day (5-7 half lives). When the serum albumin levels are below < 3.5 gm% and steady state of phenytoin is reached, the patient would be recruited into the study if the following inclusion criteria were met.

Inclusion criteria:

1. Patients of the age of 18 years or older
2. Patients on phenytoin
3. Serum albumin was < 3.5 mg%
4. Patients who would sign the written informed consent

Exclusion criteria:

Pregnant mothers

After obtaining written informed consent (Annexure 1 and 2) from the first degree relative in the presence of an MICU staff. A trough sample (5ml of blood) will be collected 30 -60 minutes prior to the next dose of phenytoin.

Data Collection:

Probable diagnosis and concurrent illness were noted in the case report form (Annexure3). Laboratory parameters such as Hemoglobin, liver Function Tests, renal function tests was collected from the clinical workstation and noted in the case report form. Dosing, route of administration of phenytoin and concomitant

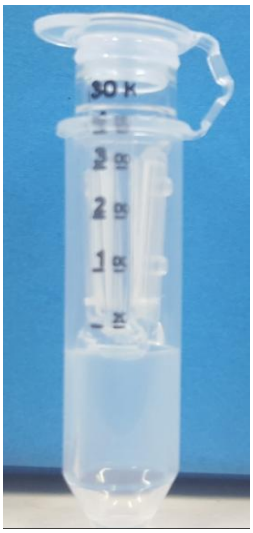
medication was also noted in the case report form. The sample was transported immediately to the Clinical Pharmacology unit for analysis.

The blood sample was centrifuged at room temperature for 5 min at 1400 rpm. Approximately 600 μL of serum samples were transferred and stored at −20°C temperature until final assay which were performed within a week, for **total phenytoin** measurement. The remaining 500 μL was used for estimating free phenytoin concentration, the serum samples were filtered through an ultrafilter (Centrifree cartridge) (Figure: 4). Molecular cutoff filters, such as the Amicon Centrifree micro partition (amicon, cut off = 5000 dalton) system was used(73,85,86). This was centrifuged at 13000rpm for 30 minutes(87) in a temperature controlled centrifuge(20˚C) using a Rota 4R-V/F M (Plastocrafts) and the filtrate **(free phenytoin**) was separated into a clean eppendorf and stored in -20 degrees C for the estimation of free phenytoin.

The total and free phenytoin assay was done on the HPLC (Figure: 3) in the Clinical Pharmacology Unit and the drug concentrations were recorded as and when the assay was completed.

Figure 3: High performance liquid chromatography (Shimadzu).

Figure 4 Centrifree cartridge



METHODOLOGY FOR ASSAY DEVELOPMENT AND VALIDATION

Chemicals and reagents-

Phenytoin pure powder (5,5 diphenyl hydantoin sodium salt - >99%) was obtained from Sigma Aldrich Incorporated. Pure nevirapine powder used as internal standard was obtained from CIPLA technologies, Goa. Acetonitrile, methanol, di-potassium hydrogen phosphate and potassium di hydrogen orthophosphate were of HPLC grade and were supplied by Thermo Fischer Scientific Private Limited Mumbai, India. Deionised water purified using the Millipore Milli-Q apparatus was used for the assay.

Equipment-

The assay was developed and validated using an Automated Injector High Performance Liquid Chromatography, LC-2010CHT with UV detector [Shimadzu Analytical (India) Pvt. Ltd]

HPLC Conditions

Instrument-Automated Injector Shimadzu LC-2010CHT with UV detector.

Column-Shiseido C_{18} column, (4.6 X 250mm, 5µm particle size, 120 A^0 pore size)

Buffer-20 mM Phosphate buffer (PH-3.5)

Mobile phase-29%acetonitrile and 71% Phosphate buffer by an isocratic method.

Flow rate-1.3ml/minute

Wavelength-214nm (UV detector)

Volume for injection - 20µL

Temperature-Ambient

(i) Total phenytoin

Preparation of Total drug(phenytoin) stock solution and QC preparation-

Phenytoin sodium is soluble in water.

Phenytoin sodium molecular weight (C15H11N2NaO2)	274.25gm/mol
Phenytoin molecular weight (C15H12N2O2)	252.273gm/mol

Phenytoin pure powder = (252.273/274.25) *100 =92%

The concentration of phenytoin stock prepared was 0.92µg/µL. This was prepared by dissolving one part of phenytoin powder in one part of water.

Two sets of stock and working solutions were prepared, one for the standards and the other for Quality controls (QC).

The Standard stocks prepared were as follows:

Primary Standard Stock (1µg/µL of phenytoin sodium= 0.92 µg/µl of phenytoin)- This was prepared by dissolving 1part of phenytoin pure powder (3.1mg) in 1part (3.1 ml) of water.

Secondary Standard Stock (0.1µg/µLof phenytoin sodium= 0.092 µg/µl of phenytoin)- This was prepared by mixing 100 µL of Primary Standard (1µg/µL) Stock and 900 µl of water.

Tertiary Standard Stock (0.01µg/µLof phenytoin sodium= 0.0092 µg/µl of phenytoin)- Prepared by mixing 100 µL of Secondary Standard (1µg/µL) Stock and 900 µL of water.

Preparation of Serum Standards and Quality Control

Two Stocks are made (1 µg/µl) – one is the standard stock and second is the quality control stock

To prepare standards

Two ml of the following working standards were made: 2.3, 4.6, 9.2, 18.4, 27.6 µg/ mL

Std 2.3 µg/ ml = 50 µL of secondary standard stock + 1950 µL of calf serum

Std 4.6 µg/ ml = 100 µL of secondary standard stock + 1900 µL of calf serum

Std 9.2 µg/ ml = 20 µL of primary standard stock + 1980 µL of calf serum

Std 18.4 µg/ ml = 40 µL of primary standard stock + 1960 µL of calf serum

Std 27.6 µg/ ml = 60 µL of primary standard stock + 1940 µL of calf serum

To prepare the quality controls:

Low QC 2.76 µg/ ml = 60 µL of secondary stock prepared for Quality Controls + 1940 µL of calf serum

Medium QC 13.8 µg/ ml = 30 µL of primary stock prepared for Quality controls+ 1970 µL of calf serum

High QC 23 µg/ ml = 50 µL of primary stock prepared for Quality controls+ 1950 µL of calf serum

Each standard/quality control is thoroughly mixed in a cyclomixer for 2 minutes.

Preparation of Internal Standard for extraction from serum

Nevirapine- was used as the internal standard. Nevirapine is soluble in methanol. A stock solution of nevirapine (1µg/1µL) was prepared. The final concentration used was120mcgm/ml.

Extraction of total phenytoin from serum

At the time of analysis, the samples were thawed. From the serum, 200 µL was taken and mixed with 40 µL of nevirapine. This mixture was vortexed (cyclomixed) at high speed for 30 seconds. For protein precipitation 400 µL of acetonitrile was added. This was vortexed for one minute. The specimen was then centrifuged at 13000 rpm for 8 minutes. The clear supernatant was transferred to a polypropylene vial and 20 µLwas injected into the HPLC for quantification.

(ii)Free phenytoin

The Chemicals, reagents, HPLC equipment and conditions remain the same. The standards and QC were prepared in saline.

Two primary stocks were made (1 µg/µl of phenytoin sodium = 0.92 µg/µl of phenytoin) in normal saline, one was used to make standards and the second was used for quality controls.

Primary stock = 0.92 µg/µl of phenytoin

Secondary Stock = 0.092 µg/µl of phenytoin

Tertiary stock = 0.0092 µg/µl of phenytoin

Two ml of the following working standards 3.68, 2.76, 1.84, 0.92, 0.46, 0.23, 0.09µg/ mL were prepared.

Std 0.09µg/ ml = 20 µL of tertiary standard stock + 1980 µL of saline

Std 0.23µg/ ml = 50 µL of tertiary standard stock + 1950 µL of saline

Std 0.46 µg/ ml = 100 µL of tertiary standard stock + 1900 µL of saline

Std 0.92µg/ ml = 20 µL of secondary standard stock + 1980 µL of saline

Std 1.84µg/ ml = 40 µL of secondary standard stock + 1960 µL of saline

Std 2.76µg/ ml = 60 µL of secondary standard stock + 1940 µL of saline

Std 3.68µg/ ml = 80 µL of secondary standard stock + 1920 µL of saline

Three quality controls were prepared as given below:

Low QC 0.28µg/ ml = 60 µL of tertiary standard stock + 1940 µL of saline

Medium QC 0.64µg/ ml = 140 µL of tertiary standard stock + 1860 µL of saline

High QC 2.3 µg/ ml = 50 µL of secondary standard stock + 1950 µL of saline

Each standard/quality control is thoroughly mixed in a cyclomixer for 2 minutes.

Preparation of Internal Standard for extraction from serum

Nevirapine- was used as the internal standard. The final concentration used was 20mcgm/ml.

Extraction of Free phenytoin from serum

To an eppendorf 20 µL of nevirapine was added and the 200µL of the above sample was added. This mixture was vortexed for 60 seconds in a cyclomixer. The clear filtrate was transferred to an eppendorf and 50 µL of sample was injected for quantification.

PRINCIPLES OF ASSAY VALIDATION

Validation of the analytical method of phenytoin is preformed to confirm that the methodology used is reliable and reproducible when used to quantitatively measure the analyte (phenytoin) in serum.

- **Selectivity** – is the capacity of the developed and validated method to identify and quantitate phenytoin (analyte) in the presence of other components in the biological matrix (serum).
- **Accuracy**: is the closeness of the value obtained from the developed method to the true value of phenytoin (analyte).
- **Precision**: is the closeness of individual measures of phenytoin (analyte) to each other on performing the developed method multiple times on the same biological matrix. Intra-batch and inter-batch precision is established for interbatch and intrabatch samples.
- **Reproducibility**: Phenytoin assay is validated for its reproducibility.
- **Recovery**: compares the detector response obtained when the same concentration of phenytoin (analyte) is added to a biological specimen versus the response of the detector when added to a pure solvent. The matrix affect is also checked.

- **Calibration curve**: is the relationship between the instrument response and the known concentration of phenytoin (analyte).
- **Stability**: of phenytoin (analyte) in the biological specimen in different storage conditions (at room temperature, transportation, at -20° Celsius, in a freezer) is also checked.

Sample size calculation

The required sample size to show a relationship between the measured free and Scheiner – Tozer equation was found to be 67 subjects with 90% power and 5% level of significance where the anticipated relationship was considered as 0.8 in accordance to the article: *BMC Medical Informatics and Decision Making 2012.*

Regression methods - Sample size for correlation coefficient analysis (testing against population value)							
Population correlation coefficient	0.8	0.8	0.8	**0.8**	0.8	0.8	0.7
Sample correlation coefficient	0.5	0.55	0.6	**0.6**	0.7	0.7	0.5
Power (1-beta) %	80	80	80	**90**	80	90	80
Alpha error (%)	5	5	5	**5**	5	5	5
1 or 2 Sided	2	2	2	**2**	2	2	2
Required sample size	29	37	51	**67**	150	199	81

Formula:

$$n = \frac{\left(Z_{1-\frac{\alpha}{2}} + Z_{1-\beta}\right)^2}{[FZ(\rho_1) - FZ(\rho_0)]^2} + 3$$

$$FZ(\rho_1) = \frac{1}{2}\ln\left[\frac{1+\rho_1}{1-\rho_1}\right] \qquad FZ(\rho_0) = \frac{1}{2}\ln\left[\frac{1+\rho_0}{1-\rho_0}\right]$$

Where,

ρ_0 : Population correlation coefficient

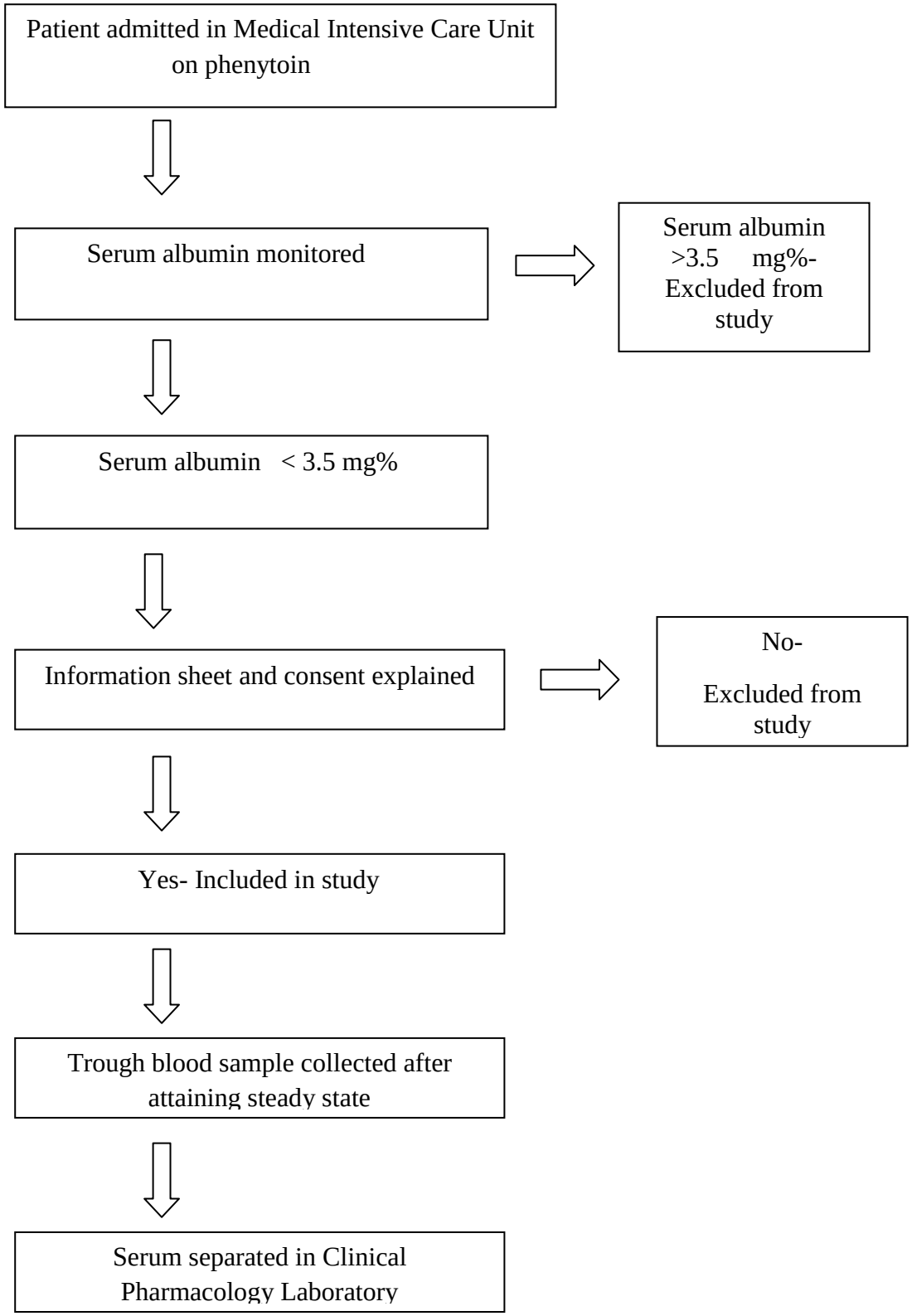
ρ_1 : Sample correlation coefficient

$Z_{1-\alpha/2}$: Desired confidence level

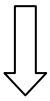
1-β : Power

Reference for the above formula: Machin D, Campbell MJ, Fayers MP and Pinal APY. Sample size tables for clinical studies. Blackwell science Ltd. Second Edition, 1997.

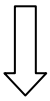
Detailed diagrammatic algorithm of the study



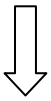
Measurement of phenytoin concentration by HPLC
Total and Free phenytoin concentration
(Direct method)



Calculate free fraction (10%)
(Routine method)



Calculate total and free phenytoin concentration using Sheiner Tozer equation.
(Sheiner Tozer calculated method)



Compare and assess agreement of different methods used to estimate total and free phenytoin concentration

Statistical Analysis

The trough concentration of serum phenytoin was noted by visual inspection of the data. The R software version 3·2·1 was used for data analysis. Free phenytoin concentration will be correlated with other parameters recorded. Agreement between the two methods used for total concentrations and three methods used for free concentrations will be analysed.

A multivariate linear regression was performed to predict the effect of age, total protein, albumin, creatinine, bilirubin and comedications (valproate and levetiracetam) on the free concentration of phenytoin. We will attempt to propose a better model to predict free phenytoin concentration from the total phenytoin concentration.

RESULTS OF VALIDATION

TOTAL PHENYTOIN VALIDATION RESULTS:

The aqueous standards were run for both the analyte (phenytoin) and the internal standard (nevirapine) to check their retention time. The retention time was as follows (see *figure 5*):

Phenytoin (analyte)-18.2 minutes

Nevirapine (internal standard)-5.9minutes

1. Selectivity:

A specimen of serum without the analyte (phenytoin) is known as a blank. Six blank specimens were obtained (after informed consent) from a similar cohort of patients admitted in MICU who have not been prescribed the drug phenytoin or the IS (nevirapine). These six blanks were run to confirm that the assay is *selective*, and that no interference is present at the retention time of the drug or the internal standard. (*Figure 5*)

Figure: 5: Chromatogram traces showing the analyte (total phenytoin) and IS (nevirapine) peaks (retention time).

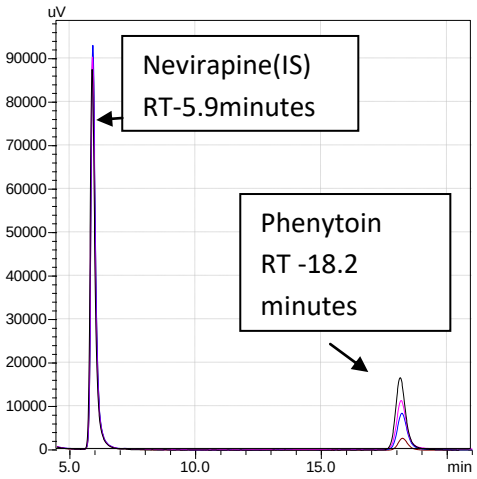
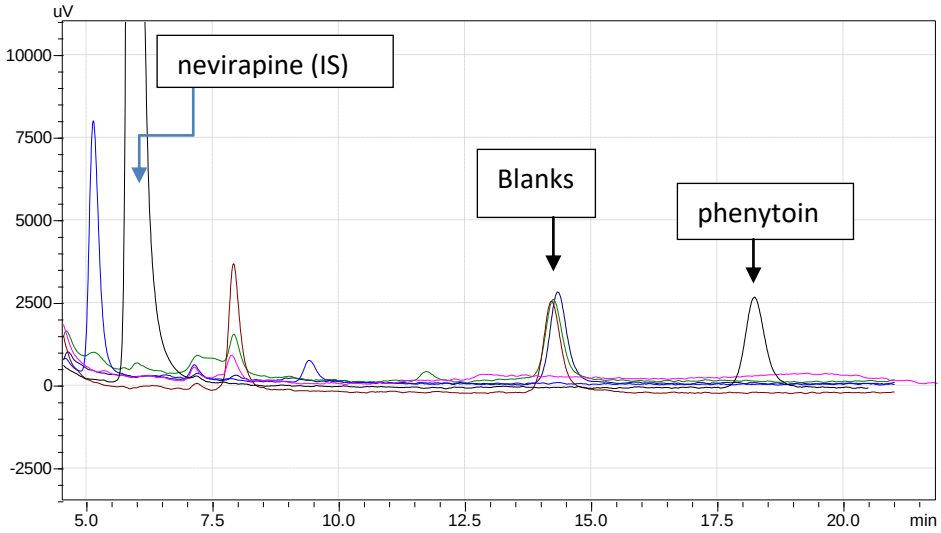


Figure: 5: Chromatogram traces showing blank specimens (No interference of IS at the retention time of phenytoin)



The next step in this process was to run 6 zero standard samples which contained the internal standard (nevirapine) and not the drug of interest (phenytoin). This was to confirm that there was no interference of the internal standard at the retention time of the drug (*Table3*).

Table 3: Zero standard samples -Retention time and area under the curve of IS for total phenytoin

Blanks with IS	RT in minute	IS area	RT in minute	Drug Area	Ratio	concentration
Zero standard 1	5.326	571664	18.2	No peak	0.00	0.00
Zero standard 2	5.331	556443	18.2	No peak	0.00	0.00
Zero standard 3	5.329	568492	18.2	No peak	0.00	0.00
Zero standard 4	5.332	611588	18.2	No peak	0.00	0.00
Zero standard 5	5.383	573874	18.2	No peak	0.00	0.00
Zero standard 6	5.358	614583	18.2	No peak	0.00	0.00

Next, we analysed patients on the drug phenytoin. This was obtained from 10 patients attending the epilepsy clinic in our hospital. This confirmed the drug peak, retention time and the expected range of study drug (phenytoin).

Finally, 4 samples were obtained from a similar cohort of patients (admitted in MICU) on the drug phenytoin. They were analysed to determine the expected range of phenytoin concentration in MICU. The expected range was found to be between 5μg/ml -30 μg/ml.

2. Accuracy: A measure of the closeness of the results obtained to the true value. The acceptable limit of accuracy is within 20% of the nominal value for the LLOQ (Lower limit of quantification)and within 15% of the nominal value for the other concentrations.

3. Precision: Is the degree of agreement among individual test results obtained when the method is applied to multiple injected samples of the same concentration. The acceptable limit of precision is within 20% of the nominal value for LLOQ and within 15% of the nominal value for higher concentrations.

Accuracy and precision was done in two different steps:

a. Same day, Same batch

b. Inter batch, 2 different days

a. Accuracy and precision -Intra-day variability (same batch, same day)

On the same day, this was performed by extracting three different *concentrations (high, medium and low concentration), each five times and analysed using HPLC(Table 4).* For calculating precision, the results are expressed in terms of %CV (coefficient of variation). The %CV was calculated for each concentration. %CV of less than 10% is acceptable. In addition, an accuracy of within 10 % of the original value is acceptable. But for the lowest concentration used, an accuracy and precision of up to 15% is considered acceptable. %CV=SD/Mean*100

Table: 4 Intraday variability of analyte (total phenytoin)

Known concentration µg/mL	Measured mean concentration µg/mL(SD)	Bias	%CV
2.76	2.744 (0.1431)	-0.5797101	5.21
13.8	12.61 (0.4218)	-8.6231884	3.35
23	21.388(0.2988)	-7.008696	1.4

Accuracy and precision -Intra-day variability (same batch, same day) for low QC

	RT (mins)	Area	Ratio	Concentration (µg/ml)
Low QC 1	5.823	613881		
	17.714	51682	0.08	3
Low QC 2	5.806	628707		
	17.92	46808	0.07	2.68
Low QC 3	5.802	630047		
	18.019	42722	0.07	2.68
Low QC 4	5.806	634472		
	18.062	44586	0.07	2.68
Low QC5	5.804	615892		
	18.066	43756	0.07	2.68

Accuracy and precision -Intra-day variability (same batch, same day) for Medium QC

	RT (mins)	Area	Ratio	Concentration (µg/ml)
Medium QC 1	5.838	626443		
	17.817	247910	0.4	13.32
Medium QC 2	5.818	627490		
	18.103	232728	0.37	12.35
Medium QC 3	5.819	634870		
	18.148	231891	0.37	12.35
Medium QC 4	5.816	630591		
	18.201	241932	0.38	12.68
Medium QC 5	5.821	648743		
	18.245	242316	0.37	12.35

Accuracy and precision -Intra-day variability (same batch, same day) for High QC

	RT (mins)	Area	ratio	Concentration (μg/ml)
High QC 1	5.816	620310		
	17.895	408767	0.66	21.71
High QC 2	5.848	626788		
	18.233	406386	0.65	21.39
High QC 3	5.828	619993		
	18.174	400867	0.65	21.39
High QC 4	5.823	620572		
	18.216	402229	0.65	21.39
High QC 5	5.837	632434		
	18.27	403448	0.64	21.06

b.Inter Day variability (Same batch, different days)

This was determined by using three different concentrations (high, medium and low concentration).

On day 1: Three extractions for the 3 different concentrations were performed and analysed by HPLC.

On day 2: Three extractions for the 3 different concentrations were performed and analysed by HPLC. The accuracy and precision were calculated in relation to day 1. The %CV was calculated for each concentration.*(Table 5)*

Table:5 Inter-day variability of analyte (total phenytoin)

Concentration	Mean measured concentration	%CV	Bias
High QC	22.85	4.35	-0.6521
Medium QC	13.55	0	-1.81159
Low QC	3.03	0	9.7826

Accuracy and precision-Interday variability (same batch, different days) for High QC(23.0g/mL)

	RT(minutes)	Area	ratio	Concentration (µg/ml)
High QC-1	5.469	471441		
	16.11	372929	0.79	21.71
High QC-2	5.469	474098		
	16.122	407392	0.86	23.55
High QC-3	5.471	474936		
	16.135	405012	0.85	23.29

Accuracy and precision-Interday variability (same batch, different days) for Medium QC(13.8µg/mL)

	RT (minutes)	Area	ratio	Concentration (µg/ml)
Medium QC-1	5.467	493333		
	16.094	238510	0.48	13.55
Medium QC 2	5.471	488538		
	16.1	234146	0.48	13.55
Medium QC 3	5.465	523819		
	16.053	249021	0.48	13.55

Accuracy and precision-Interday variability (same batch, different days) for low QC(2.76)

	RT(minutes)	Area	ratio	concentration (µg/ml)
Low QC 1	5.468	504560		
	16.093	40071	0.08	3.03
Low QC 2	5.469	498313		
	16.085	42060	0.08	3.03
Low QC 3	5.469	515597		
	16.035	43250	0.08	3.03

4. Reproducibility:

This was determined by using 3 concentrations- a high, medium and low concentration. After performing one extraction, the specimen is placed in an auto sampler and reinjected 3times. This tests the reliability of the sample as they are in the autosampler for a period of time. In addition, any other influences of system fluctuation which may alter the readings of the assay may also be observed. The %CV was calculated for each concentration. *(Table 6)*

Table: 6 Reproducibility of total phenytoin

Known concentration	Measured mean concentration(SD)	%CV	Mean bias (%)
Low QC	2.5	0	-9.420289
Medium QC	13.98(0.155)	1.11	1.3224637
High QC	21.98(0.179)	0.81	-4.420289

Repeat injections of High QC from one extract

High QC	RT(mins)	Area	ratio	concentration (µg/ml)
High QC-1	5.36	553344		
	16.392	390091	0.7	21.88
High QC-2	5.34	551438		
	16.538	387437	0.7	21.88
High QC-3	5.329	553346		
	16.708	392458	0.71	22.19

Repeat injections of Medium QC from one extract

Medium QC	RT(minutes)	Area	ratio	Concentration (µg/ml)
Medium QC-1	5.357	564232		
	16.35	246043	0.44	13.75
Medium QC-2	5.326	552786		
	16.683	246849	0.45	14.06
Medium QC-3	5.345	547754		
	16.499	246085	0.45	14.06

Repeat injections of Low QC from one extract

Low QC	RT(minutes)	Area	ratio	Concentration (µg/ml)
Low QC-1	5.361	561245		
	16.32	44926	0.08	2.5
Low QC-2	5.349	543903		
	16.479	45970	0.08	2.5
Low QC-3	5.331	549385		
	16.657	46530	0.08	2.5

5. **To check the precision for LLOQ and ULOQ**

The peak area of the lower limit of quantification (LLOQ) has a response greater than 10 times that of the blank response. Likewise, the upper limit of quantification (ULOQ) is selected based on the uppermost value that may be required in relation to the values expected in the patients on phenytoin. *(Table 7)*

Table: 7 Precision of total phenytoin

	Bias (%)	Precision (%)
LLOQ	_-1.316	4.35
ULOQ	0	20

Precision of LLOQ and ULOQ for total phenytion

Total phenytoin	RT(mins)	Area	ratio	Concentration (µg/ml)	Bias%
ULOQ-1	5.457	510202			
	15.933	530437	1.04	28.29	2.5
ULOQ-2	5.474	472213			
	16.128	468619	0.99	26.97	-2.28260
ULOQ-3	5.473	487616			
	16.16	471759	0.97	26.45	-4.16666
LLOQ-1	5.473	485232			
	16.169	36179	0.07	2.76	20
LLOQ-2	5.465	515942			
	16.022	38153	0.07	2.76	20
LLOQ-3	5.47	477736			
	16.193	35684	0.07	2.76	20

6. Sensitivity:

Sensitivity is the lowest concentration of analyte (phenytoin) which can be measured within the acceptable limits of accuracy and precision. The lowest standard used was *2.3µg/ml.*

7. Recovery (Extraction efficiency)

Extraction efficiency for low and high concentration of total phenytoin was analysed and the extraction efficiency was 100% and 96.92% respectively. (*Table 8*)

Table 8: Extraction efficiency of low and high QC

Based on Ratio	Low QC	High QC
% Recovery	114%	100%
% of Process efficiency	100%	96.92%

Pre-extraction, post extraction and aqueous extraction of low QC:

Pre- extraction-total phenytoin (Low QC)			
	RT(mins)	Area	ratio
Low QC-1	5.47	556964	
	15.753	47959	0.09
Low QC-2	5.475	564695	
	15.811	46498	0.08
Low QC-3	5.449	565171	
	15.922	46599	0.08
Post extraction-total phenytoin(Low QC)			
Low QC-1	5.418	514875	
	16.252	36389	0.07
Low QC-2	5.381	521350	
	16.49	38763	0.07
Low QC-3	5.382	523013	
	16.508	38710	0.07

Aqueous extraction total phenytoin(Low QC)			
Low QC-1	5.357	509591	
	16.674	39510	0.08
Low QC-2	5.355	505689	
	16.702	38061	0.08
Low QC-3	5.361	509521	
	16.728	38661	0.08

Pre-extraction, post extraction and aqueous extraction of High QC

Pre- extraction-total phenytoin (High QC)			
	RT(mins)	Area	ratio
High QC-1	5.476	569445	
	15.787	367359	0.65
High QC-2	5.429	586263	
	16.034	365421	0.62
High QC-3	5.412	582591	
	16.147	365879	0.63
Post extraction total phenytoin(High QC)			
High QC-1	5.375	516982	
	16.596	324127	0.63
High QC-2	5.371	519421	
	16.633	326398	0.63
High QC-3	5.37	513412	
	16.657	319667	0.62
Aqueous extraction total phenytoin(High QC)			
High QC-1	5.356	503824	
	16.759	334157	0.66
High QC-2	5.359	512715	
	16.789	331212	0.65
High QC-3	5.359	514383	
	16.799	332375	0.65

8. Stability:

Using a sample of a patient on phenytoin we checked stability. This sample was immediately extracted and analysed. This was compared with the same sample, which had been left on the bench top without extraction for six and a half hours. (*Table 9*)

Table 9: Stability of total phenytoin

	Fresh extracted QC s			
	RT	Area	Ratio	Concentration
Low QC	5.47	556964		
	15.753	47959	0.09	2.68
Medium QC	5.47	545257		
	15.771	233655	0.43	12.99
High QC	5.476	569445		
	15.787	374908	0.66	19.96
	After 6.5hr Extraction			
Low QC	5.349	476895		
	16.819	44870	0.09	2.68
Medium QC	5.339	478950		
	16.866	224587	0.47	14.2
High QC	5.343	485018		
	16.871	362673	0.75	22.68

The sample was stable when stored at -20℃. The stability of *serum standards* and *stock solution* was also checked. The serum standards of phenytoin, QC and stock solution were found to be stable for 83 days.

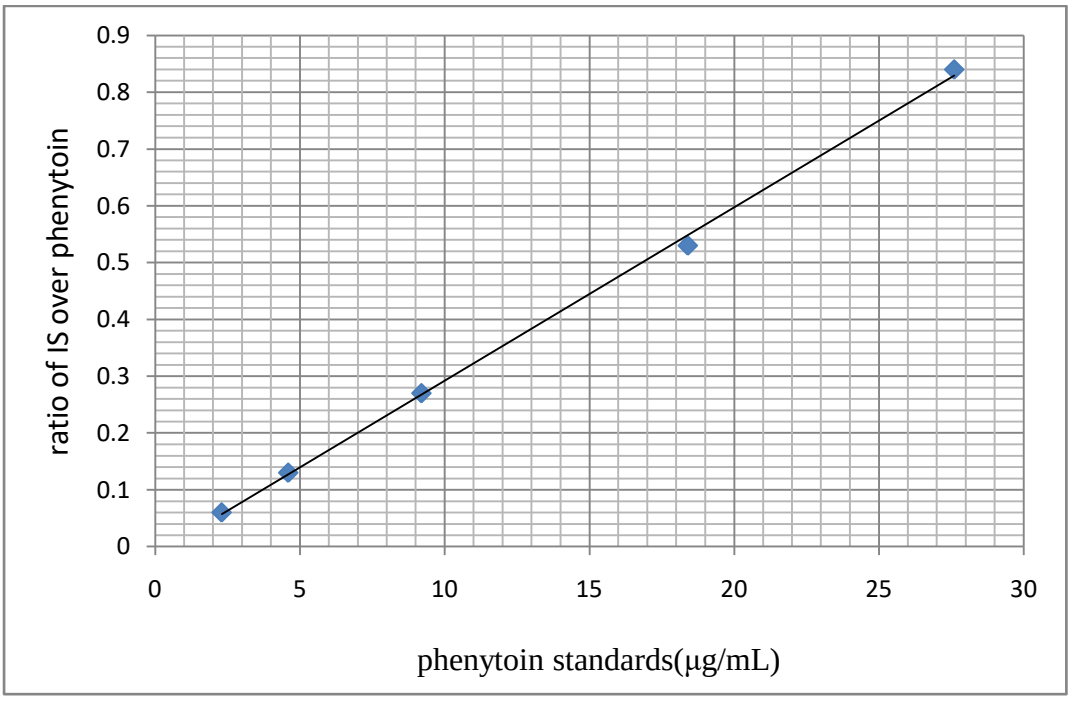
Calibration curve –

This is drawn to confirm the *linearity* of the phenytoin assay. A total of 5 calibrators and 3 Quality controls (QCs) were used. The standards prepared *were* 27.6 µg/ml, 18.4µg/ml, 9.2µg/ml, 4.6 µg/ml, 2.3 µg/ml and the QC's were 23µg/ml, 13.8µg/ml, 2.76µg/ml. The standards were checked for linearity using the equation: y=m x +c

In this equation 'm' is the slope and 'c' is the y-intercept.

To confirm linearity, the same calibrators and QC's were run on several days using freshly prepared standards. The results showed a linear calibration curve from 2.3µg/mL to 27.6µg/mL.*(Figure 6)*

Figure: 6: Linear calibration curve of total phenytoin (2.3µg/mL to 27.6µg/mL)



Retention time and AUC of IS and total phenytoin

Standard	RT(mins) nevirapine	AUC	RT(mins) phenytoin	AUC	Ratio of area of IS/phenytoin
27.6	5.941	591187	17.207	495848	0.84
18.4	5.917	625327	17.268	330086	0.53
9.2	5.871	61303	17.49	170598	0.27
4.6	5.847	61898	17.563	79062	0.13
2.3	5.824	619144	17.675	38803	0.06

Data for the analyte (total phenytoin) depicting a linear calibration curve

Analyte	Slope(m)	Intercept(y)	Correlation
Phenytoin	0.030508	-0.01291	0.999418

Calculation of the unknown concentration of total phenytoin

The ratio of the area of the drug (phenytoin) peak with that of internal standard (nevirapine) is calculated. This is done for all standards and QCs. With concentration on the x axis and the ratios on the y axis, a linear regression line is drawn. From the regression line the slope, intercept and correlation coefficient are calculated.

FREE PHENYTOIN VALIDATION RESULTS

Water Standards

Water standards were prepared for determining the retention time of the analyte (free phenytoin) and the IS (nevirapine*).(Table 10)*

1. Selectivity

Fig :7: Chromatogram traces of blank and free phenytoin

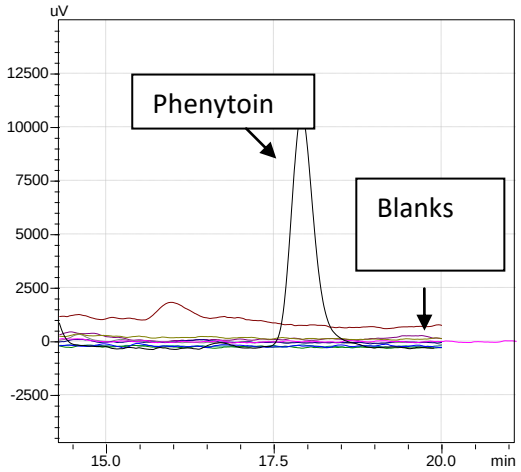


Table: 10 Zero standard samples -Retention time and area under the curve of IS for free phenytoin

	RT	IS Area	RT	Drug Area	Ratio	concentration
Zero Standard 1	6.03	404057	18.2	No peak	0.000	0.000
Zero Standard 2	6.06	345074	18.2	No peak	0.000	0.000
Zero Standard 3	6.039	379364	18.2	No peak	0.000	0.000
Zero Standard 4	6.034	383468	18.2	No peak	0.000	0.000
Zero Standard 5	6.067	387181	18.2	No peak	0.000	0.000
Zero Standard 6	6.06	373161	18.2	No peak	0.000	0.000

2. **Accuracy and precision, same day, same batch**

For low QC and high QC, five different extractions were taken from the same sample. One injection was given for each extract. The results are expressed in terms of % CV (coefficient of variation) for calculating precision.(*Table 11*)

Table: 11 Intraday variability of free phenytoin

Concentration	%CV	Mean Bias
0.28	6.07	-1.27306
0.66	2.58	3.125
2.36	4.45	2.6086

Accuracy and precision -Intra-day variability (same batch, same day) :

	RT (minutes)	Area	Ratio	concentration(µg/mL)
Low QC 1	5.944	330528		
	17.898	31322	0.09	0.26
Low QC 2	5.962	342431		
	17.967	33174	0.1	0.29
Low QC 3	5.965	344961		
	18.031	32861	0.1	0.29
Med QC 1	5.976	334004		
	18.074	72315	0.22	0.65
Med QC 2	5.984	316033		
	18.121	70933	0.22	0.65
Med QC 3	5.99	324816		
	18.146	74121	0.23	0.68
High QC 1	5.998	342699		
	17.868	260356	0.76	2.26
High QC 2	5.998	334226		
	17.756	264122	0.79	2.35
High QC 3	6.006	320010		
	17.717	264365	0.83	2.47

3. Reproducibility

After one extraction, the specimen is placed in an auto sampler and reinjected 3 times. The results are seen in *table 12*

Table: 12 The mean and % CV of free phenytoin concentrations

Measured concentration	Bias	%CV
2.32	6.402	0.73
0.94	3.998	1.81
0.2	0	0

Repeat injections of high, medium and low QC from one extract:

	Repeat injections of high QC from one extract				
	RT minutes	**Area**	**ratio**	**concentration µg/ml**	**Accuracy**
Sample1	6.053	343482			
	18.229	284648	0.83	2.34	0.862068966
Sample1	6.049	346798			
	18.233	283313	0.82	2.31	-0.431034483
Sample1	6.061	349362			
	18.277	285487	0.82	2.31	-0.431034483
	Repeat injections of medium QC from one extract				
Sample2	6.029	346604			
	18.268	112000	0.32	0.92	-2.127659574
Sample2	6.049	341599			
	18.25	111677	0.33	0.95	1.063829787
Sample2	6.067	338322			
	18.292	111342	0.33	0.95	1.063829787
	Repeat injections of low QC from one extract				
Sample3	6.032	341206			
	18.215	21136	0.06	0.2	0
Sample3	6.059	337915			
	18.254	19942	0.06	0.2	0
Sample3	6.039	338439			
	18.218	21660	0.06	0.2	0

4. To check the precision for LLOQ and ULOQ for free phenytoin

Four different extractions of the LLOQ and ULOQ were analysed for free phenytoin precision (*Table 13*).

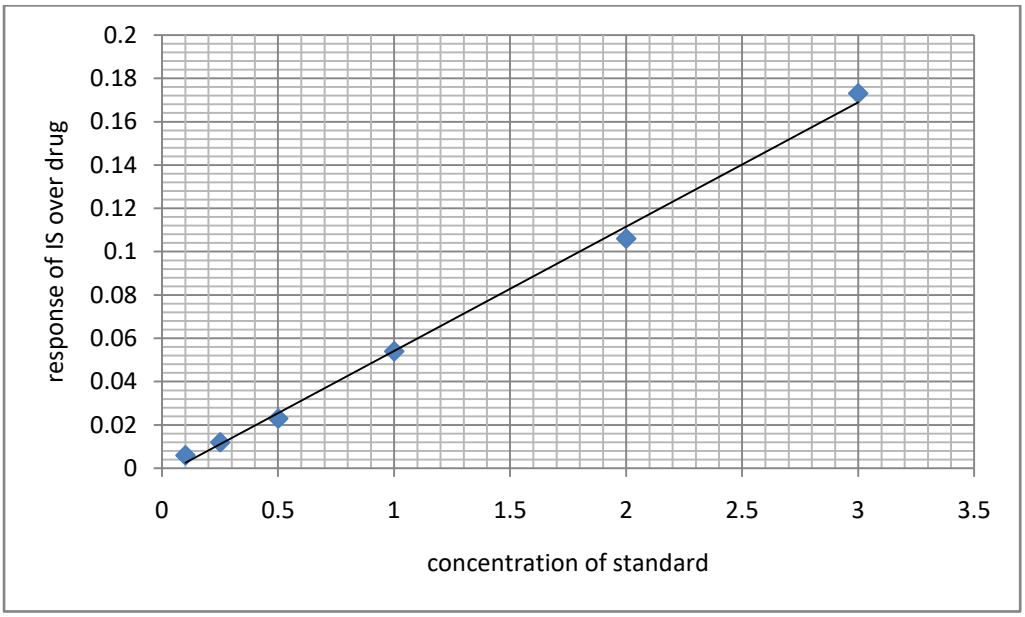
Table 13: Precision for LLOQ AND ULOQ for free phenytoin

LLOQ std 0.09	RT (minutes)	Area	ratio	concentration (µg/ml)	%Accuracy
1	6.101	345616			
	18.16	12090	0.03	0.06	-33.3
2	6.084	357496			
	18.226	12317	0.03	0.06	-33.3
3	6.099	359791			
	18.227	11758	0.03	0.06	-33.3
4	6.089	355070			
	18.21	10968	0.03	0.06	-33.3
ULOQstd2.76					
1	6.071	353385			
	18.134	293485	0.83	2.55	-7.60869
2	6.09	356746			
	18.171	304780	0.85	2.62	-5.07246
3	6.065	358590			
	18.162	308580	0.86	2.65	-3.98550
4	6.059	354357			
	18.179	309443	0.87	2.68	-2.8985

Calibration curve

A total of six Standards and 3 QC were used as calibrators. The concentrations for standards were free phenytoin concentrations 0.09µg/ml, 0.23µg/ml, 0.46 µg/ml, 0.92µg/ml, 1.84µg/ml, 2.76µg/ml and QC of 2.3 µg/ml, 0.64µg/ml, 0.28 µg/ml. The calibration curve of phenytoin was linear from 0.09 to 3.0µg/ml.

Figure:8 : Linear calibration curve of free phenytoin (0.09µg/ml to 2.76 µg/ml)



Statistical data for the analyte (phenytoin) depicting a linear calibration curve

Analyte	Slope(m)	Intercept(y)	Correlation
Free phenytoin	0.35951	-0.0108	0.99962

Standards	retention time	area	concentration
2.76	6.039	325855	
	17.952	323437	0.99
1.84	6.048	336723	
	17.956	214648	0.64

0.92	6.024	340013	
	18.168	51195	0.14
0.23	6.025	328997	
	18.218	25925	0.08
0.09	6.02	333642	
	18.213	9888	0.03

Sensitivity:

Sensitivity is the lowest concentration of analyte (phenytoin) which can be measured within the acceptable limits of accuracy and precision. The lowest standard used was 0.09µg/mL.

Stability:

Using a sample of a patient on phenytoin, we checked stability. This sample was immediately extracted and analysed. This was compared with the same sample, which had been left on the bench top without extraction for five hours.

The stability of *serum standards* and *stock solution* was also checked. The serum standards of phenytoin, QC and stock solution were found to be stable for 83 days. Accuracy of low, medium and high QCs were 96%, 100% and 97 % respectively.

The sample was stable when stored at -20°C.

This completed the validation of total and free phenytoin. This was applied to study samples collected from MICU.

RESULTS

Baseline characteristics of all study participants

A total of 57 patients admitted in MICU were recruited into my study. Up to 58% of patients were diagnosed to have either meningoencephalitis or intracranial bleed/ infarct/cortical venous thrombosis (CVT)) as shown in table

The baseline demographic characteristics of the patients included in the study are summarized in Table 14.

Table14: Characteristics of the patients at baseline values are n (%) or median (IQR)

Characteristic	
Age(years) Median ±IQR	37 (29.0-56.0)
Sex - Male-Number (%)	36 (63.2)
Female-Number (%)	21(36.8)
Biochemistry Parameters	**Median± IQR**
Creatinine (mg/dl) Median± IQR	0.83(0.59-1.48)
Total Bilirubin (mg/dl)Median± IQR	0.54(0.38-0.72)
Direct Bilirubin (mg/dl)Median± IQR	0.26(0.15-0.40)
Total Protein (gm/dl)Median± IQR	5.70(5.30-6.30)
Total Albumin(gm/dl) Median± IQR	2.50(2.20-2.80)
Co medications	**Number**
Monotherapy with phenytoin(n)	30

One additional antiepileptic drug(n)	10
Two additional antiepileptic drug(n)	8
Three or more additional antiepileptic drug(n)	9
MICU admission diagnosis	**Number of patients (%)**
Meningoencephalitis	17(29.8)
Intracranial bleed/infarct /CVT	16(28.1)
Poisoning	5(8.77)
Epilepsy	4(7.02)
Systemic Lupus Erythematosis	2(3.51)
Others (Malignancy, Infections)	13

TOTAL PHENYTOIN CONCENTRATION

Two methods: Direct measured total versus Sheiner Tozer calculated total phenytoin

For all the 57 patients, the median and interquartile range for direct measured total and Sheiner Tozer calculated total phenytoin concentration was 9.82(6.02-13.85) and 17.14(10.63-24.53) respectively (*Fig: 9).* The interpatient variability in direct measured total and Sheiner Tozer total phenytoin concentration was 63.74% and 62.46% respectively (*Table 15).*

(i) Comparison of subtherapeutic, therapeutic and supratherapeutic phenytoin concentration by the two methods:

With respect to the direct measured total phenytoin concentration, twenty four patients (42.1%) were observed to have their phenytoin concentration within the therapeutic range (10-20 ug/dl). Whilst 30 patients (52.6%) were found to have serum phenytoin concentration which was subtherapeutic (<10 ug/ml) and only three patients (5.3%) were supratherapeutic(>20ug/ml). In contrast, the Sheiner Tozer calculated total phenytoin concentration was found to be therapeutic in 21(36.8%), supratherapeutic in 23(40.4%) and subtherapeutic in 13 (22.8%) patients. *(Figure 10*)

(ii) Correlation between the two methods

To estimate the overall concordance between the direct measured total phenytoin concentration and the Sheiner Tozer calculated total concentration the Pearson correlation coefficient (R^2) was found to be 0.90 *(Figure 11*). However, this correlation was not satisfactory when analysed separately as patients who were subtherapeutic, therapeutic and supratherapeutic with respect to direct measured total concentration of phenytoin .(R^2=0.82, 0.37 and 0.74 respectively).

The level of agreement between direct measured total phenytoin concentration and corresponding Sheiner Tozer total phenytoin concentration was stratified with respect to therapeutic category. This was done by constructing three by three contingency tables, grouping direct measured phenytoin concentration into below, within and above therapeutic range. Concomitant Sheiner Tozer calculated total phenytoin concentrations were compared. (*Figure 12*)

As seen in the three bar graphs *(Figure 12),* the concordance between the two methods in subtherapeutic, therapeutic and supratherapeutic was 100%, 29% and 13% respectively.

Figure 9: Boxplot comparing median concentration of total phenytoin by 2 methods:direct measured total and Scheiner Tozer calculated total

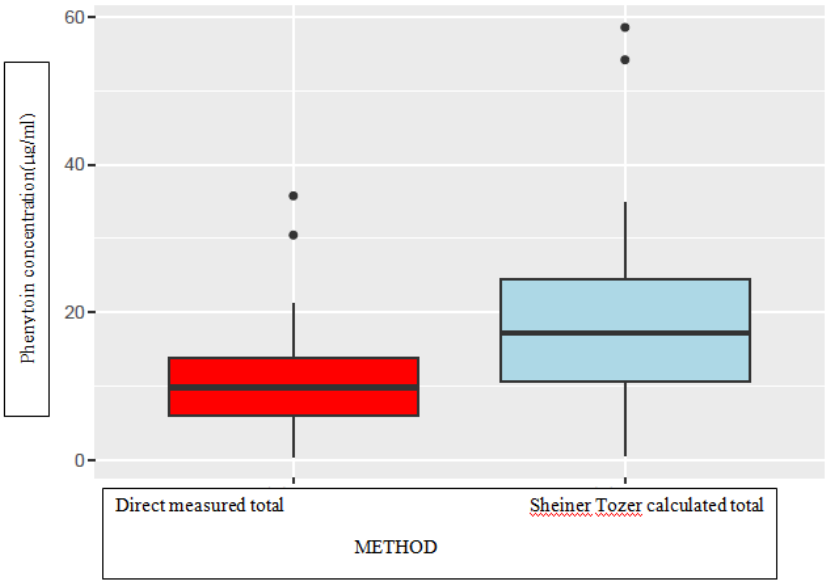


Fig:10 Beeswarm plot comparing concentrations of total phenytoin by 2 methods: direct measured total phenytoin and Sheiner Tozer calculated total phenytoin concentration.

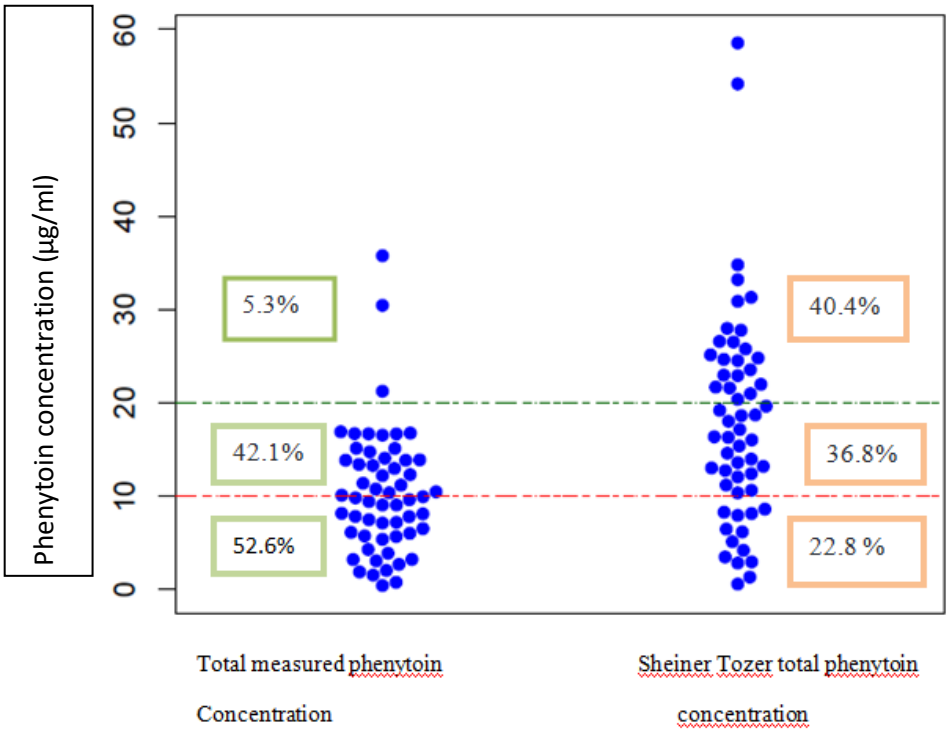


Figure11 Correlation of direct measured total and Sheiner Tozer phenytoin plasma concentrations.

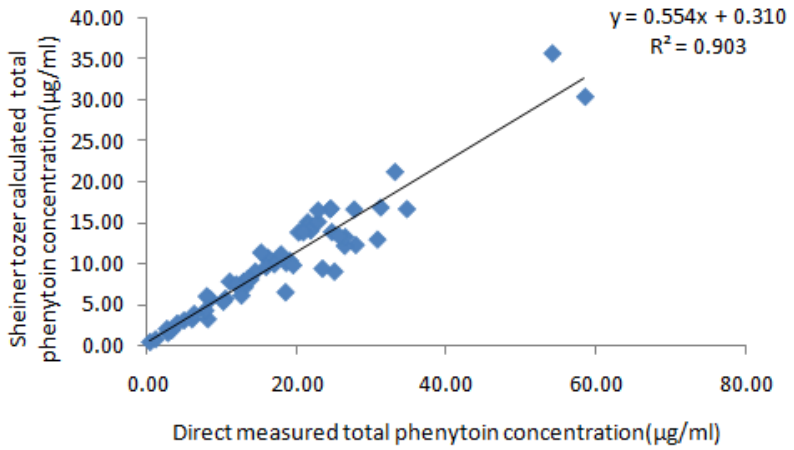
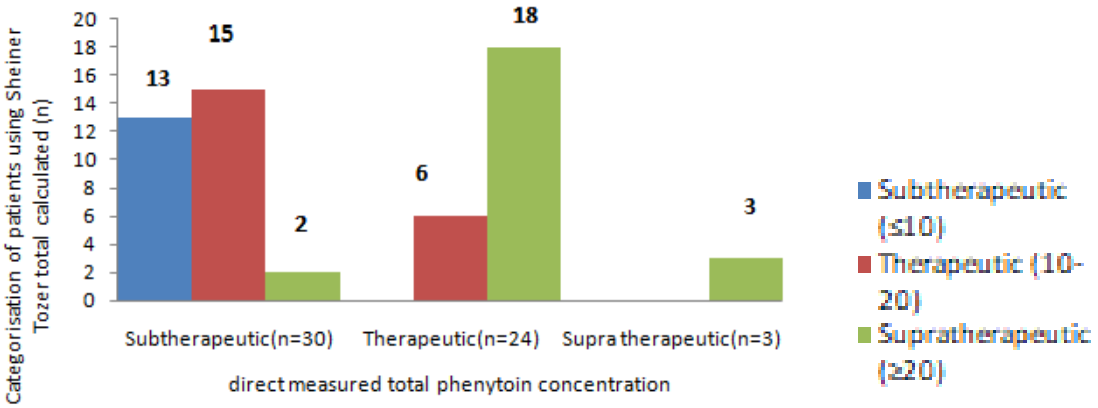


Figure12 Concordance data comparing direct measured total and Sheiner Tozer calculated total phenytoin concentration

	Direct measured total phenytoin concentration		
Sheiner Tozer calculated total phenytoin concentration	Subtherapeutic (<10 µg/mL) (n=30)	Therapeutic (10 - 20µg/mL) (n=24)	Supratherapeutic (>20 µg/mL) (n=3)
Subtherapeutic (n=13)	13	0	0
Therapeutic (n=21)	15	6	0
Supratherapeutic(n=23)	2	18	3

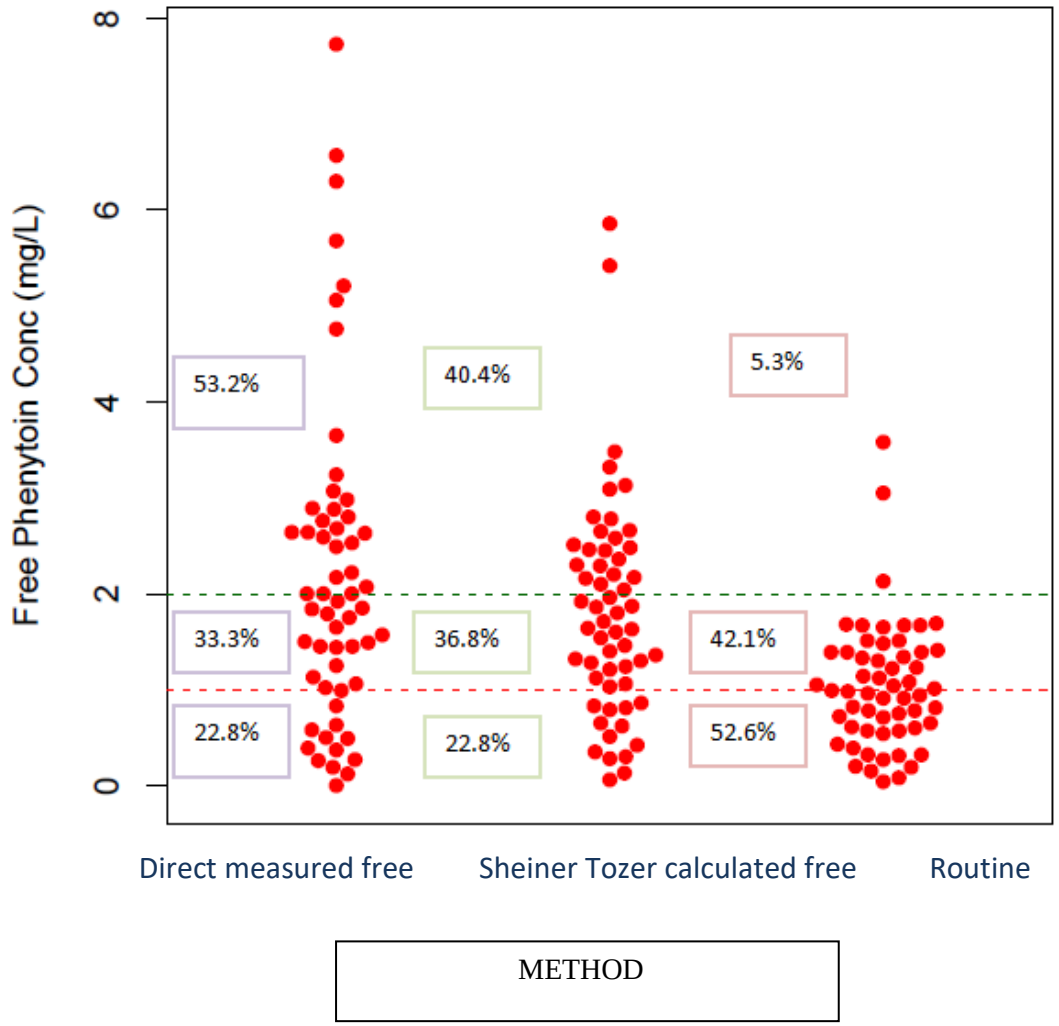


FREE PHENYTOIN CONCENTRATION

Three methods: Routine, Direct measured and Sheiner Tozer calculated free phenytoin-

The median (IQR) for routine, direct measured and Sheiner Tozer free phenytoin was 0.98 (0.60-1.39), 1.92 (1.06-2.76) and 1.71 (1.06-2.45) respectively. The interpatient variability in direct measured free phenytoin concentration was 76.5% *(Table 15)*

Figure: 13: Beeswarm plot comparing free phenytoin concentration by 3 methods: direct measured, routine and Sheiner Tozer calculated free method



(i) Direct measured versus routine free phenytoin

By using the direct measured free phenytoin method, the patients in the subtherapeutic (<1µg/ml), therapeutic (1-3µg/ml) and supratherapeutic(>2µg/ml) category were 13 (22.8%), 19(33.3%) and 25(53.2%) respectively*(Figure 13)*

Amongst the patients who were subtherapeutic, all 13 continued to remain in the subtherapeutic category when analysed by the routine free phenytoin method. Of the 19 in the therapeutic range category, 11 remained therapeutic whereas eight patients would be reported as subtherapeutic by routine free method. Amongst the 25 patients in the supra therapeutic category, only three patients were in agreement with the routine free phenytoin measurement, whereas 8/25 and 14/25 patients were in the subtherapeutic and therapeutic category. (*Figure 15)*

The Pearson correlation coefficient (R^2) for direct measured and routine free phenytoin concentration was found to be 0.63 *(Figure 14)*

Figure14: Correlation of direct measured free and routine free phenytoin concentrations

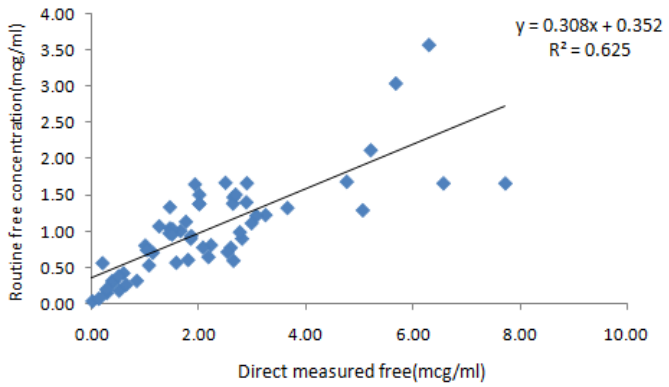
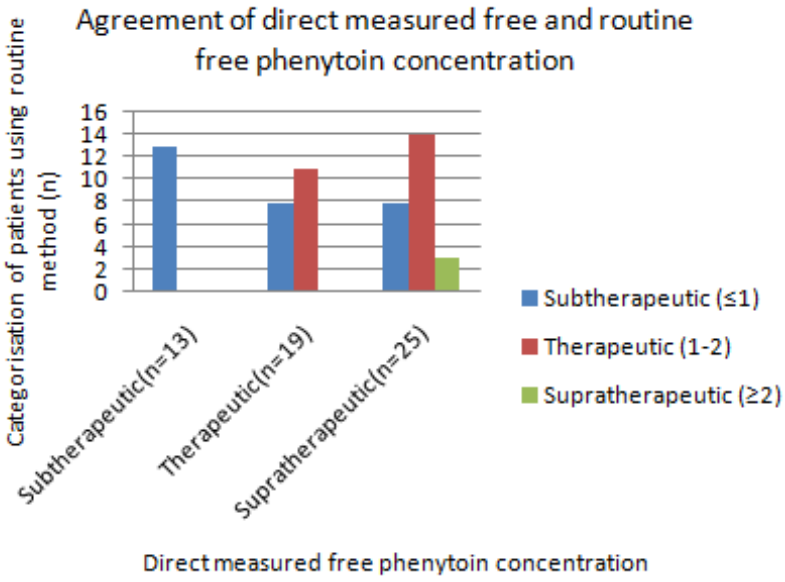


Fig:15 Three by three contingency tables comparing grouping of free measured phenytoin and routine free phenytoin concentration into sub-therapeutic, therapeutic and supratherapeutic range

	Direct measured free phenytoin concentration		
Routine free phenytoin concentration	Subtherapeutic (<1µg/mL) (n=13)	Therapeutic (1 -2µg/mL) (n=19)	Supratherapeutic (>2 µg/mL) (n=25)
Subtherapeutic (n=29)	13	8	8
Therapeutic (n=25)	0	11	14
Supratherapeutic(n=3)	0	0	3



(ii) Direct measured free versus Sheiner Tozer calculated free phenytoin concentration

On categorising data, all patients except one would continue to remain subtherapeutic using the Sheiner Tozer calculated free. The category grouping for the Sheiner Tozer free against the therapeutic and supratherapeutic category for direct measured free is represented in the *figure 17.* Correlation for direct measured free and Sheiner Tozer calculated free concentrations R^2 was 0.637 (*Figure 16).*

Figure 16: Correlation of direct measured free and Sheiner Tozer calculated free phenytoin concentrations.

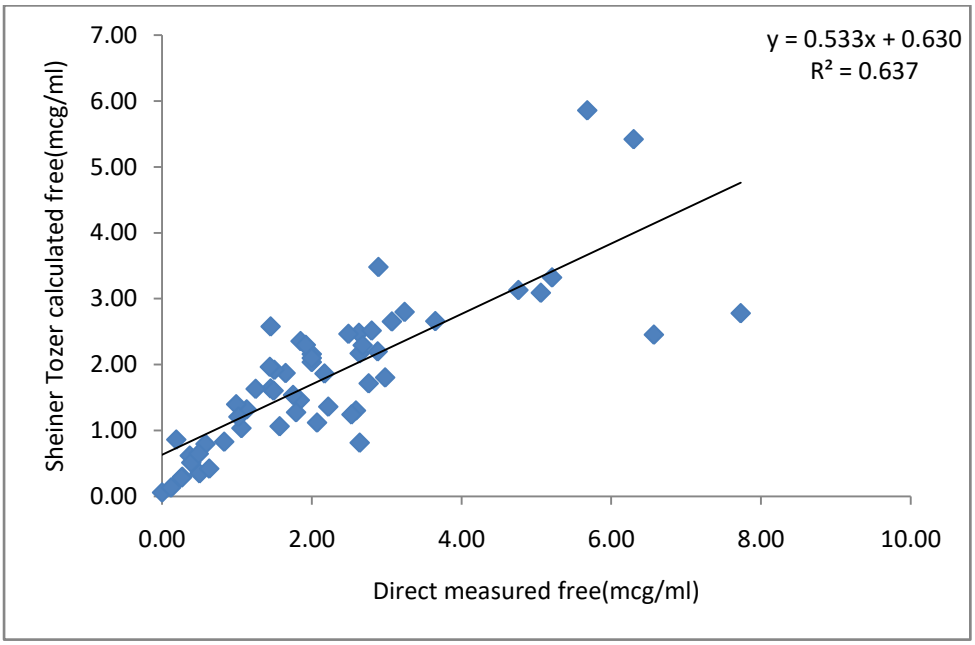
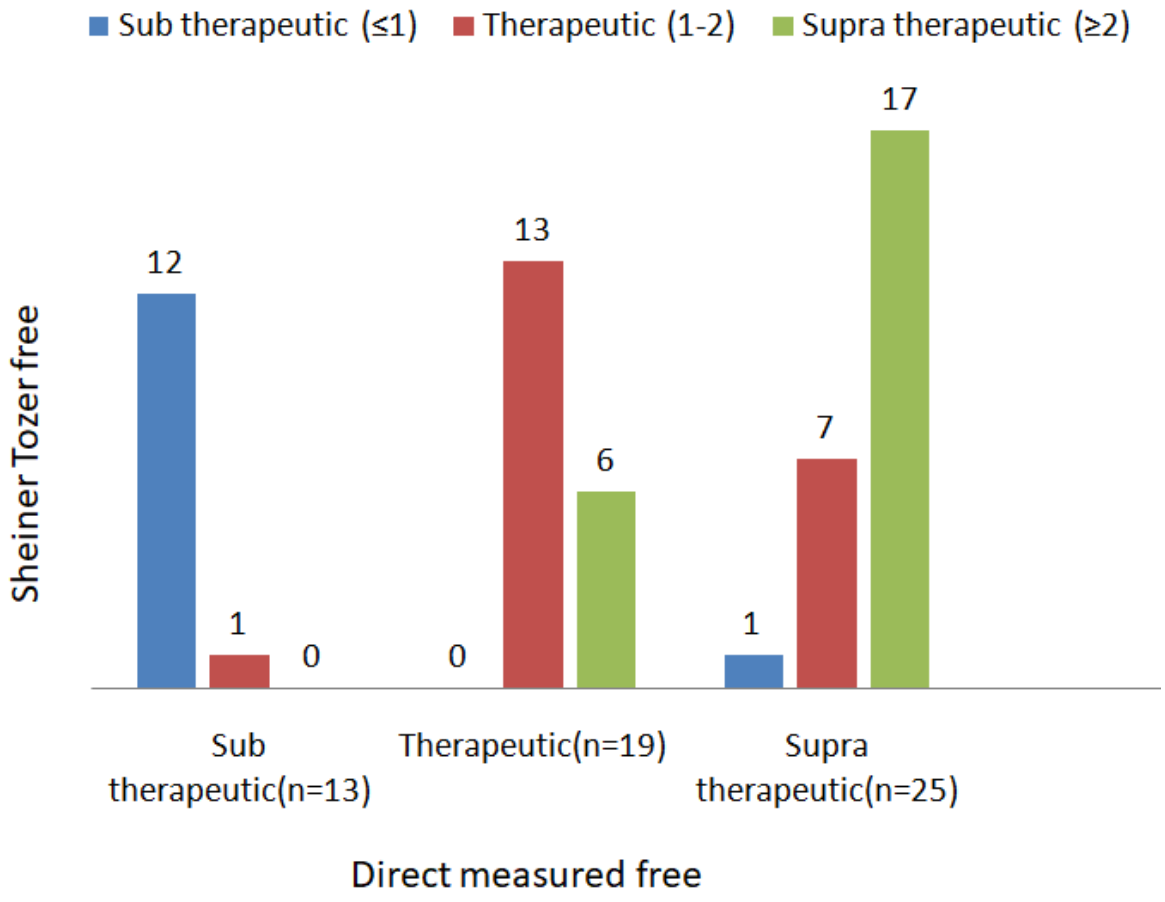


Figure:17 Three by three contingency tables comparing grouping of direct measured free phenytoin and Sheiner Tozer calculated free phenytoin concentration

	Direct measured free phenytoin concentration		
SheinerTozer calculated free Phenytoin concentration	Subtherapeutic (<1µg/mL) (n=13)	Therapeutic (1 -2µg/mL) (n=19)	Supratherapeutic (>2 µg/mL) (n=25)
Subtherapeutic (n=13)	12	0	1
Therapeutic (n=21)	1	13	7
Supratherapeutic(n=23)	0	6	17



(iii) Direct measured free phenytoin concentration with respect to direct measured total phenytoin concentration:

The median (IQR) free phenytoin concentration and direct total phenytoin concentrations was 1.92 (1.06 -2.76) and 9.82(6.02-13.85) with a correlation of (R^2=0.63) *(Table15*)

Table 15: Total and free phenytoin concentrations by different methods:

Parameter	Concentration (mcg/ml)	Coefficient of variation
	Total phenytoin	
Direct measured	9.82(6.02-13.85)	63.7%
Sheiner Tozer calculated	17.14(10.63-24.53)	62.5%
	Free phenytoin	
Direct measured	1.92 (1.06-2.76)	76.5%
Sheiner Tozer calculated	1.71 (1.06 - 2.45)	62.5%
Routine	0.98 (0.60 - 1.39)	63.7%

On stratifying patients, 26/57 patients (45.7%) had phenytoin concentration of direct measured total and direct measured free in agreement. As seen in the beeswarm below *(Figure 18*), approximately (30/57) 53% of patients total measured phenytoin concentration were subtherapeutic. Whereas, only (13/57) 23% patients direct measured free phenytoin concentration were subtherapeutic. Similarly, (24/57) 42% patients were in the therapeutic range when total serum phenytoin was measured directly, whilst (10/57) 17.5% were therapeutic when free concentration was measured directly as seen in the table below.

Figure 18: Beeswarm plot comparing direct measured total and direct measured free phenytoin concentration

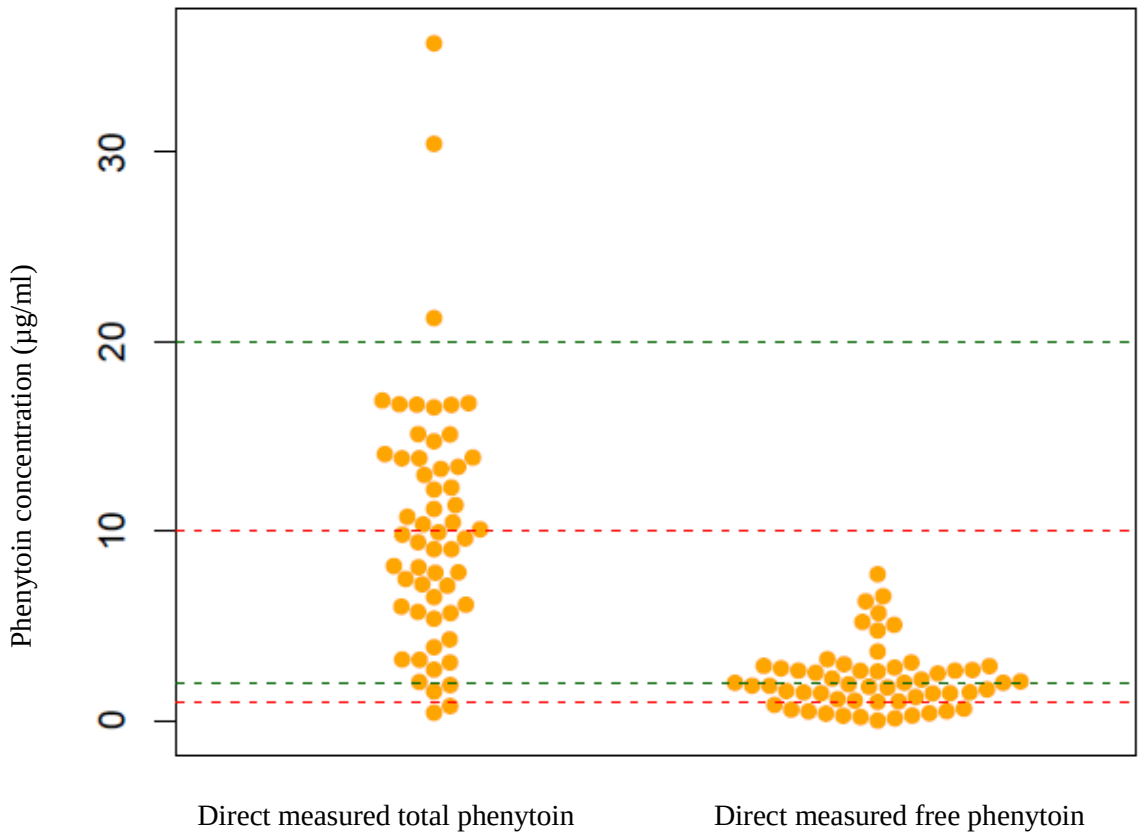


Table 16: Concordance data of direct measured total and direct measured free phenytoin concentration

	Direct measured total phenytoin concentration		
Direct measured free phenytoin concentration	Subtherapeutic (<10 µg/mL) (n=30)	Therapeutic (10 -20 µg/mL) (n=24)	Supratherapeutic (>20 µg/mL) (n=3)
Subtherapeutic (<1µg/mL) (n=13)	13	0	0
Therapeutic (1-2µg/mL) (n=19)	9	10	0
Supra therapeutic (>2 µg/mL)(n=25)	8	14	3

(iv) Direct measured free phenytoin concentration with respect to Sheiner Tozer calculated total phenytoin concentration:

The median (IQR) free phenytoin concentration and Sheiner Tozer calculated total phenytoin concentrations was 1.92 (1.06 -2.76) and 17.14(10.63-24.53) (R^2=0.64). On stratifying patients (as seen in the table below), 42/57 patients (73.7%) had phenytoin concentration of Sheiner Tozer total and direct measured free in agreement. However, the level of agreement of the above two methods was found to be 68% (17/25 and 13/19 patients respectively) in supratherapeutic and therapeutic concentrations. And the concordance in the subtherapeutic category was found to be 92 %(12/13).

Table 17: *Concordance data of Sheiner Tozer total and direct measured free phenytoin concentration*

	Sheiner Tozer calculated total phenytoin concentration		
Direct measured free phenytoin concentration	Subtherapeutic (<10 μg/mL) (n=13)	Therapeutic (10 -20μg/mL) (n=21)	Supratherapeutic (>20 μg/mL) (n=23)
Subtherapeutic (<1 μg/mL) n=13	12	1	0
Therapeutic (1-2 μg/mL)n=19	0	13	6
Supratherapeutic (>2 μg/mL) n=25	1	7	17

(v) Free phenytoin concentration with respect to albumin concentration

The plasma albumin ranged from 1.3 to 3.3 g/dl with a median (IQR) of 2.50 (2.20-2.80). A total of 48 (84%) patients were found to have a very low albumin (<2gm %). The correlation between free fraction (direct measured free/direct measured total of phenytoin) and serum albumin was 0.04. The correlation between free fraction (direct measured free/Sheiner Tozer calculated total phenytoin) and serum albumin was 0.02.

The severity of albumin with respect to the phenytoin concentrations was analysed by categorizing patients into severe-(<2g/dl) and moderate hypoalbuminemia (>2g/dl*)*. The median (IQR) for free phenytoin concentration with severe hypoalbuminemia was 2.89 (1.85-3.24) and for moderate hypoalbuminemia was 1.80 (0.95- 2.63).

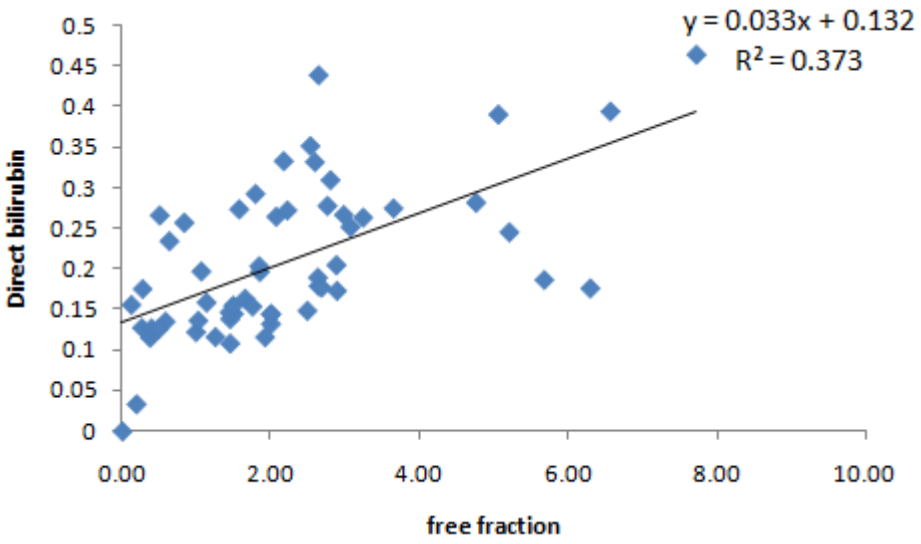
(vi) Free phenytoin concentration with respect to comedications

Of the 57 patients recruited, 10 received the antiepileptic drug valproic acid (known for displacing phenytoin and altering drug metabolism). Thirty patients (53%) received the drug levetiracetam (no effect on phenytoin pharmacokinetics) and 5/57 received folic acid (increases drug metabolism). The absolute total median (IQR) direct measured free phenytoin concentration was 1.92 (1.06 -2.76), whilst the median (IQR) of patients on valproate was found to be high [3.23 (2.67-5.45)]. As expected, the effect of free phenytoin concentration of patients treated with the drug levetiracetam was not remarkable [median (IQR) =1.57(0.81-2.70)].

(vii) Direct measured free phenytoin in relation to bilirubin:

Fifty four % (31/57) of patients were found to have a high bilirubin >2.0mg/dl. The median (IQR) of the free concentration of phenytoin in patients with raised bilirubin was 1.57(1.01-2.7). Though earlier studies had reported a high free fraction of phenytoin in patients with hyperbilirubinemia, we found a poor correlation (R^2=0.37) between the free fraction and bilirubin.

Fig 19: Correlation of free fraction and bilirubin



PREDICTING FREE PHENYTOIN CONCENTRATION FROM TOTAL PHENYTOIN CONCENTRATION

The correlation between direct measured free and direct measured total was poor ($R^{2=}0.63$). Using the stepwise multiple linear regression analysis, different variables such as total protein, serum albumin, and valproate (if present or absent) were added in a stepwise manner, to predict the free phenytoin concentration from the value of the direct measured total phenytoin.

Multiple linear regression equation obtained with direct measured total phenytoin was:

Predicted free phenytoin concentration = 1.179 + 0.204 X [Total measured phenytoin] – 0.44 X [Serum Albumin] ---- Eq. 1

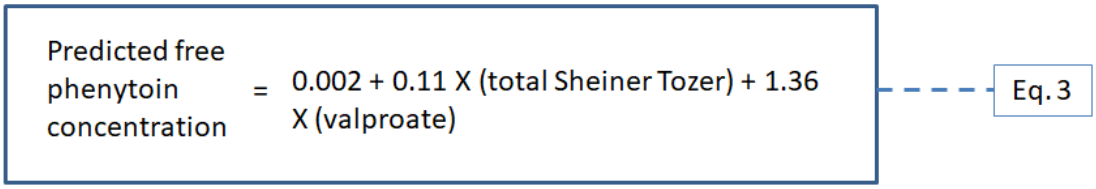
The predicted free phenytoin concentration had a poor correlation to the direct measured free phenytoin concentration (R^2=0.62), using equation (1)

Multiple linear regression incorporating comedication.

Predicted free phenytoin concentration = 0.19 X (total measured phenytoin) +1.49 X (valproate) + 0.04 X (levetiracetam) - 0.001 ---- Eq. 2

The predicted free concentration with covariates such as comedication improved (R2=0.72), using equation (2)

Similarly, stepwise multiple linear regression analysis using Sheiner Tozer calculated total.



The correlation of predicted free phenytoin concentration with Sheiner Tozer total phenytoin improved after introducing covariates like comedications. (R2=0.72), using equation (3)

Discussion

In a developing country, phenytoin remains the major first line treatment in the management of status epilepticus. To our knowledge, there have been very few studies done on Indian patients to elucidate a thorough understanding of measurement of free phenytoin in comparison to total phenytoin in low albumin patients admitted in the Critical Care Unit. We validated and developed a HPLC method for measurement of both, total and free phenytoin concentrations in serum.

However, the mathematical model "Sheiner Tozer equation" has been extensively used by clinicians to predict first the total phenytoin and then the free phenytoin concentration in low albumin patients. Despite being used worldwide, the model's accuracy has been questioned. Four reputed literature in this area of work is discussed below in relation to our findings.

In critically ill neurosurgical patients, Sadeghi et al (41) concluded that measurement of total phenytoin concentrations is not a reliable parameter for therapeutic drug monitoring in severely ill head trauma patients (n=40). The Pearson correlation analysis showed poor correlation between free and total measured concentrations(r = 0.28) which was lower to what we observed of (r = 0.79). However, categorising patients to below, within and above therapeutic range we also found a poor correlation of 0.60, 0.26 and 0.94 respectively.

However, a retrospective study on 756 patients by D Krasowski et al(38) revealed an improved Pearson correlation coefficient between routine (total phenytoin /10)

and direct measured free of 0.72. This is in agreement with our study results, where we got an r of 0.79 between the routine total phenytoin and direct measure free phenytoin.

They also reported a good correlation between the direct measured free phenytoin and Sheiner Tozer free (r=0.79). In our study the correlation between direct measured free phenytoin and Sheiner Tozer free was 0.798. The authors also further analysed the agreement between direct measured total and direct measured free phenytoin concentration and found only a 43.1% concordance in the therapeutic group. In our study we obtained a similar concordance of 41.6% in the same therapeutic category. They reported the concordance between Sheiner Tozer calculated total and free phenytoin concentration in the therapeutic group was 68.7% which was also similar to our study (68.4%).

Krowaskai and his colleagues concluded that a measured free phenytoin would be the most ideal approach to estimating free phenytoin concentration. And if not available, the Sheiner Tozer calculated free phenytoin may be used, but with a clear understanding of its limitations.

Hong et al(50) reported the correlation between the measured free and measured total phenytoin concentrations to be moderate (r=0.822, p<0.001), which was similar to our findings of (r= 0.79). The correlation between Sheiner Tozer calculated total and direct measured free phenytoin concentration did not show marked improvement in the correlation (Pearson r = 0.762,). Our results were similar with a correlation of (Pearson r = 0.80. The mean difference between the

direct measured and Sheiner Tozer calculated free phenytoin concentrations was high (0.65 ± 0.88 μg/mL; 95%confidence interval, -1.11to2.41). However, in our study the mean (sd) difference between measured and Sheiner Tozer free phenytoin concentration was 0.40 ±1.03 μg/mL, which was lower than that reported by Hong et al. The reason for this difference may be due to the serum albumin concentration which was 3.3 ± 0.8 in their study. The mean albumin in our study was much lower (2.45 ± 0.44).

A retrospective cohort conducted on 238 patients admitted in ICU by Buckley et al (77) revealed an absolute agreement of 77% between Sheiner Tozer calculated total (mean albumin 3.1 ± 0.6 g/dL) and direct measured free phenytoin concentration. However, they reported the concordance of Sheiner Tozer calculated total phenytoin and direct measured free phenytoin concentration in the sub-therapeutic, therapeutic, and supratherapeutic category as 67.2%, 80.8%, and 82.3%, respectively (P= 0.063). In our study we observed an absolute concordance of 73.7% and on stratifying a concordance of 92% in the sub therapeautic category and 68% in the supratherapeutic and therapeutic range.

Many hospitals, like ours depend on the Sheiner Tozer calculated free concentration for dosing a patient admitted in MICU.

Considering the findings in our study and earlier reported literature, the most ideal method for measuring free phenytoin concentration would be by direct measurement using HPLC. In India, measurement of free phenytoin concentration

may not be freely available. The cost of free concentration is more than twice that of total concentration because of the requirement of the centrifree cartridge for the ultrafiltration process and the time taken to process the sample may be longer. However in the unavailability of the facility to measure free phenytoin concentration, Sheiner tozer calculation may be used for dose adjustment. But it is crucial to keep in mind that 26.3 % of patients would be dosed differently with respect to the category of the phenytoin concentration. This is because, there are other factors apart from low albumin such as co administered drugs that alter protein binding, elevated bilirubin, sugar and free fatty acid that alter the actual free phenytoin concentration in serum. So, our recommendation to a clinician would be to measure the actual free phenytoin concentration in serum. The treating clinician should be aware of this while taking decisions regarding dose adjustment of this drug.

Therapeutic drug monitoring of free phenytoin is essential for optimizing therapy in low albumin patients.

CONCLUSION

- In patients with low albumin, there was a mean relative difference of 42% in the total phenytoin measured concentration compared to the Sheiner Tozer calculated total phenytoin.
- 75 % of patients with low albumin if reported using the measured concentration would have total phenytoin concentration within the therapeutic range, whereas these patients would have a supratherapeutic concentration by the Sheiner Tozer calculation. These patients would require a reduction in the phenytoin dose. However if patient is managed with only measured phenytoin concentration, a dose change may be unlikely. The role of monitoring total phenytoin concentration for purpose of dose adjustment will be undermined.
- The correlation between measured total and measured free phenytoin concentration was moderate. Measuring only total phenytoin concentration in patients with low albumin and extrapolating this concentration to estimate the free (by any simple calculation) is not reliable.
- Free phenytoin concentration has to be estimated by direct measurement, as the correlation of direct measured free with both calculated equations (Total phenytoin/10 and Sheiner Tozer calculation/10) was only moderate (R^2=0.63 and 0.64 respectively).
- Prediction of measured free phenytoin concentration using total phenytoin and additional variables did not give satisfactory results using the multivariate linear regression analysis. This reemphasizes the need to do real time monitoring of free phenytoin concentration, using HPLC, in patients with low albumin.

LIMITATIONS

1. We limited our study to those patients admitted in Medical Intensive Care unit. But patients with low albumin are also found in wards and general outpatient clinics. Future studies should be conducted on them to evaluate the pharmacokinetic variability of phenytoin.

2. We studied critically ill adult patients over the age of 17. This excluded the pediatric population. The pharmacokinetics of phenytoin in this age group needs to be studied.

3. Only a single trough sample was used for this study. Using multiple timed samples would have facilitated a more intense analysis and any possibility of developing an equation.

FUTURE SCOPE

- The comparison of total and free phenytoin concentration can be studied in the paediatric population.
- As free concentration is the active form of the drug, it will be useful to study the possibility of using noninvasive matrices like saliva to predict the free phenytoin concentration.
- Using multiple timed samples for total and free phenytoin and to study if it would be possible to develop a model to predict free using a population modeling approach. It will be good to recruit more patients with varied covariates for this purpose.

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Annexure 1

PATIENT INFORMATION SHEET

Study title: A study to evaluate the total and free concentration of phenytoin concentrations in low albumin patients admitted in Medical ICU

You or your relative is being requested to participate in a study as you are taking a medicine called “phenytoin”. We hope to include about 67 patients admitted in the Medical ICU from this hospital in this study.

1. *What is this study about?*

As you know you have been started with the drug phenytoin by your clinician for control of fits. Phenytoin is bound to blood protein (blood constituent). The binding of phenytoin to protein will decrease if the protein level in our body falls. Only the drug which is free [that is not bound to protein] is beneficial in controlling seizures. Phenytoin in the body is found both bound to blood proteins (normally around 90%), and unbound or "free" drug (normally around 10%, also known as the "free fraction"). Unbound drug can enter the brain and cause the harmful effects of phenytoin. However after 48 to 72 hours of admission in intensive care unit (ICU) the protein level in blood of your relative is likely to fall causing a rise in the free phenytoin fraction which can lead to toxic symptoms like roving of eyes, coma, delirium and may even cause seizures.

In the hospital, we only measure total phenytoin concentration. For patients with low protein, there are mathematical equations that can be used to calculate the corrected total blood concentration of phenytoin .This equation is useful for calculating total phenytoin concentration. The free phenytoin concentration is estimated by dividing the total phenytoin/10. This equation, which is referred to as - Sheiner-Tozer equation, is only reliable for patients with low protein. In critically ill patients, this equation to estimate free phenytoin, may not be accurate enough to guide dosing in patients admitted in a critical care.

Because of this, we wish to do a study, to measure the blood levels of free phenytoin (Direct Measurement)using a laboratory assay and compare it with that calculated from the mathematical equation (Calculated Method). Measurement of free phenytoin concentration by laboratory assay can be expensive. So in this study if the free phenytoin between the above two methods do not have a good agreement, we will look at the possibility of predicting free phenytoin from an equation using total phenytoin.

2. What do I have to do to take part in this study?

You **<u>will not</u>** have to do anything additional to the routine if you take part in this study.

3. Are there any other tests involved?

No. There are no other tests apart from the above.

4. *Will I have to pay anything?*

No. You do not have to pay anything, for this test.

5. *Is it compulsory to take part in the study?*

No. Participation is purely voluntary and based on your decision. Your treatment will not be affected in any way even if you do not participate. You can withdraw from the study at any time by informing us.

6. ***What are the potential risks to participating in this study?*** 5 ml of venous blood will be collected. There will be no extra risk other than sample collection from you.

7. ***How will this study benefit me and others?***
Free phenytoin levels measured will be informed to the treating physician.

8. ***Will my confidentiality be maintained?***
Yes. We will maintain absolute confidentiality. In any publication resulting from the study your identity will not be revealed.

If you are willing to take part in the study I will ask you to sign an informed consent and then you will be enrolled for the study.

If you have any questions, please feel free to ask doubts to any of the treating doctors as well as the principal investigator.

Contact number and E-mail address are given below:

Dr. Premila. Wilfred

Contact no-xxxxxx

Dr. Sumith. Mathew

Contact no-xxxxxxxx

Annexure 2

INFORMED CONSENT

Study Title: A study to evaluate the total and free phenytoin concentration in patients admitted with low albumin in ICU.

Study Number: **Participant's name:**

Date of Birth / Age (in years):

I, Mr/Ms/Mrs ----------, relative of the patient ----------, have read the information sheet provided to me which describes about this study and its importance. I understood the details and had enough opportunity to discuss it with the research staff and clarify my doubts. I understand that I have the right to deny participation in this study and also know that denial does not affect the process of my medical care. Please tick boxes

(i) I confirm that I have read and understood the information sheet dated _____ for the above study and have had the opportunity to ask questions. [ ]

(ii) I understand that my participation in the study is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. [ ]

(iii) I understand that the investigators, the Ethics Committee and the regulatory authorities will not need my permission to look at my health records both in respect of the current study and any further research that may be conducted in relation to it, even if I withdraw from the trial. I agree to this access.

(iv)I understand that my identity will not be revealed in any information released to third parties or published. [ ]

(v) I agree not to restrict use of any data or results that arise from this study provided such a use is only for scientific purpose(s). [ ]

(vi) I agree to give 5 ml blood for the study purpose

(vii) I volunteer to take part in the above study. [ ]

Name of the Subject/Legally Acceptable _____
Signature (or Thumb impression) _____
Relation to participant: _____ Date_____

Name of Witness _____
Relation to participant
Signature / Thumb print_____ Date_____

Name of the person who has taken the informed consent: _____

Signature: _____ Date: _____

Annexure 3

CASE REPORT FORM

To determine the total and free phenytoin concentration in low albumin patients admitted in medical intensive care unit.

Study No: Patient I.D.: Address:

Hospital Number: Telephone number:

Diagnosis:

Age: Gender: M / F height= Weight (kg): Education-

Occupation

Significant medical history:

History of any chronic illness:

History of liver disease/gastrointestinal disorders/renal failure:

Brand name: Route of administration:

Date of loading dose Date of maintenance dose

History of dose of phenytoin received

Biochemistry

Total Bilirubin: S.Albumin: SGOT SGPT Alk Phosphatase

S. creatinine: Serum urea: Lipid profile

Date of blood specimen collection: Time of specimen collection:

Co-medications: Warfarin, phenobarbitone, valproate, aspirin, amiodarone

Albumin infusion

Principal investigator: Date:

Annexure 4: Institutional Review Board

OFFICE OF RESEARCH
INSTITUTIONAL REVIEW BOARD (IRB)
CHRISTIAN MEDICAL COLLEGE, VELLORE, INDIA

Dr. B.J. Prashantham, M.A., M.A., Dr. Min (Clinical)
Director, Christian Counseling Center,
Chairperson, Ethics Committee.

Dr. Anna Benjamin Pulimood, M.B.B.S., MD., Ph.D.,
Chairperson, Research Committee & Principal

Dr. Biju George, M.B.B.S., MD., DM.,
Deputy Chairperson,
Secretary, Ethics Committee, IRB
Additional Vice-Principal (Research)

November 25, 2016

Dr. Premila Magdalene. Wilfred,
PG Registrar,
Department of Pharmacology and Clinical Pharmacology,
Christian Medical College,
Vellore - 632 004.

Sub: **Fluid Research Grant NEW PROPOSAL:**
To measure total and free concentration of serum phenytoin in patients with hypoalbuminemia, in a critical care setting.
Dr. Premila Magdalene. Wilfred (Employment Number: 28480), Post Graduate Registrar, Pharmacology and Clinical Pharmacology, Dr. Binu Susan Mathew, Employment Number: 30442, Pharmacology and Clinical Pharmacology, Dr.Binila.Chacko, Employment no: 28471, Medical Intensive Care Unit, Dr. Sumith K Mathew, Employment no: 20758, Pharmacology and Clinical Pharmacology, Dr. J. V .Peter, Medical Intensive Care Unit, Dr Ratna. Prabha, Emp No: 20466, Pharmacology and Clinical Pharmacology, Dr. Denise H Fleming, Clinical Pharmacology Unit, Dr Visalakshi Jeyaseelan Employment number: 31093, Biostatistics.

Ref: IRB Min No: 10321 [OTHER] dated 12.10.2016

Dear Dr. Premila Magdalene. Wilfred,
The Institutional Review Board (Blue, Research and Ethics Committee) of the Christian Medical College, Vellore, reviewed and discussed your project titled "To measure total and free concentration of serum phenytoin in patients with hypoalbuminemia, in a critical care setting" on October 12th 2016.

The Committee reviewed the following documents:

1. IRB Application format
2. Patient information sheets and Consent forms
3. Cv's of Drs. Densie Fleming, Binila, Binu S Mathew, Peter, Ratna, Sumit K Mathew, Premi and Visalakshi.
4. No. of documents 1 – 3.

The following Institutional Review Board (Blue, Research & Ethics Committee) members were present at the meeting held on October 12th 2016 in the BRTC Conference Room, Christian Medical College, Bagayam, Vellore 632002.

Ethics Committee Blue, Office of Research, 1st Floor, Carman Block, Christian Medical College, Vellore, Tamil Nadu 632 002
Tel: 0416 – 2284294, 2284202 Fax: 0416 – 2262788, 2284481 E-mail: research@cmcvellore.ac.in

Dr. B.J. Prashantham, M.A., M.A., Dr. Min (Clinical)
Director, Christian Counseling Center,
Chairperson, Ethics Committee.

Dr. Anna Benjamin Pulimood, M.B.B.S., MD., Ph.D.,
Chairperson, Research Committee & Principal

Dr. Biju George, M.B.B.S., MD., DM.,
Deputy Chairperson,
Secretary, Ethics Committee, IRB
Additional Vice-Principal (Research)

Name	Qualification	Designation	Affiliation
Dr. Biju George	MBBS, MD, DM	Professor, Haematology, Research), Additional Vice Principal , Deputy Chairperson (Research Committee), Member Secretary (Ethics Committee), IRB, CMC,Vellore	Internal, Clinician
Dr. B. J. Prashantham	MA(Counseling Psychology), MA (Theology), Dr. Min (Clinical Counselling)	Chairperson, Ethics Committee, IRB. Director, Christian Counseling Centre, Vellore	External, Social Scientist
Dr. Ratna Prabha	MBBS, MD (Pharma)	Associate Professor, Clinical Pharmacology, CMC, Vellore	Internal, Pharmacologist
Dr. Rekha Pai	BSc, MSc, PhD	Associate Professor, Pathology, CMC, Vellore	Internal,Basic Medical Scientist
Rev. Joseph Devaraj	BSc, BD	Chaplaincy Department, CMC, Vellore	Internal, Social Scientist
Mr. C. Sampath	BSc, BL	Advocate, Vellore	External, Legal Expert
Dr. Santhanam Sridhar	MBBS, DCH, DNB	Professor, Neonatology, CMC, Vellore	Internal, Clinician
Mrs. Sheela Durai	MSc Nursing	Professor, Medical Surgical Nursing, CMC, Vellore	Internal, Nurse
Ms. Grace Rebekha	M.Sc., (Biostatistics)	Lecturer, Biostatistics, CMC, Vellore	Internal, Statistician
Mrs. Pattabiraman	BSc, DSSA	Social Worker, Vellore	External, Lay Person
Dr. Vivek Mathew	MD (Gen. Med.) DM (Neuro) Dip. NB (Neuro)	Professor, Neurology, CMC, Vellore	Internal, Clinician
Dr. Balamugesh	MBBS, MD(Int Med), DM, FCCP (USA)	Professor, Pulmonary Medicine, CMC, Vellore	Internal, Clinician

Ethics Committee Blue, Office of Research, 1st Floor, Carman Block, Christian Medical College, Vellore, Tamil Nadu 632 002
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Chairperson, Research Committee & Principal

Dr. Biju George, M.B.B.S., MD., DM.,
Deputy Chairperson,
Secretary, Ethics Committee, IRB
Additional Vice-Principal (Research)

Dr. Sneha Varkki	MBBS, DCH, DNB	Professor, Paediatrics, CMC, Vellore	Internal, Clinician
Mrs. Emily Daniel	MSc Nursing	Professor, Medical Surgical Nursing, CMC, Vellore	Internal, Nurse
Dr. Sathish Kumar	MBBS, MD, DCH	Professor, Child Health, CMC, Vellore	Internal, Clinician
Dr. Inian Samarasam	MS, FRCS, FRACS	Professor, Surgery, CMC, Vellore	Internal, Clinician
Dr. Thomas V Paul	MBBS, MD, DNB, PhD	Professor, Endocrinology, CMC, Vellore	Internal, Clinician

We approve the project to be conducted as presented.

Kindly provide the total number of patients enrolled in your study and the total number of withdrawals for the study entitled: "To measure total and free concentration of serum phenytoin in patients with hypoalbuminemia, in a critical care setting" on a monthly basis. Please send copies of this to the Research Office (research@cmcvellore.ac.in).

Fluid Grant Allocation:

A sum of 1,00,000/- INR (Rupees One Lakh Only) will be granted for 2 years. 50,000/- INR (Rupees Fifty Thousand only) will be granted for 12 months as an Ist Installment. The rest of the 50,000/- INR (Rupees Fifty Thousand only) each will be released at the end of the first year as 2 nd Installment.

Yours sincerely,

Dr. Biju George
Secretary (Ethics Committee)
Institutional Review Board

Dr. BIJU GEORGE
MBBS., MD., DM
SECRETARY - (ETHICS COMMITTEE)
Institutional Review Board,
Christian Medical College, Vellore - 632 002.

IRB Min No: 10321 [OTHER] dated 12.10.2016

Annexure 5: Raw Data of patients

Sl No	Age	Sex	Albumin	Creatinine	Measured_TOTAL	Measured_FREE
1	23	F	1.90	0.46	16.70	2.89
2	23	F	2.20	4.70	1.88	0.50
3	37	M	2.80	0.53	5.68	0.19
4	29	M	2.10	0.45	30.45	5.68
5	24	F	2.10	0.38	3.21	0.37
6	26	M	2.20	0.57	7.12	1.13
7	65	M	2.50	1.09	7.81	2.59
8	41	M	2.20	0.60	5.74	1.57
9	75	M	1.60	2.60	12.97	5.06
10	47	M	2.20	1.89	16.90	4.76
11	77	M	3.00	0.76	15.11	2.00
12	65	M	2.10	0.80	1.54	0.27
13	26	F	2.70	1.56	10.47	1.45
14	20	M	2.90	0.81	14.75	2.64
15	67	M	2.30	1.32	13.89	2.63
16	37	M	2.90	8.24	3.23	0.83
17	58	M	1.30	1.66	9.05	2.80
18	55	F	2.60	0.59	9.05	1.84
19	47	M	2.80	0.92	10.76	1.25
20	56	M	2.90	1.07	16.76	2.49
21	35	F	2.40	3.71	8.10	0.99
22	62	F	2.20	0.29	10.10	1.65
23	30	M	2.80	3.37	35.76	6.30
24	18	F	2.50	0.08	8.16	2.22
25	22	F	2.40	0.29	9.94	2.76
26	29	M	3.00	6.64	7.83	2.07
27	33	F	3.20	0.21	6.02	2.64
28	64	M	1.50	2.68	9.42	1.85
29	21	M	2.50	8.15	6.52	2.17
30	29	F	2.20	1.15	10.36	1.50
31	56	M	2.90	1.07	13.85	2.00
32	38	F	2.70	0.38	21.25	5.21
33	62	M	1.70	1.65	12.31	3.24
34	35	M	2.50	1.84	16.67	7.73
35	21	M	2.90	0.45	16.68	6.57
36	22	F	2.40	0.39	7.20	2.53
37	31	F	1.80	1.04	12.20	3.07
38	29	F	3.10	0.79	2.04	0.26
39	50	M	1.90	1.48	6.12	1.79
40	32	M	2.50	0.70	3.07	0.39
41	64	M	2.00	1.01	9.82	1.44
42	58	F	2.00	0.98	13.29	3.65
43	55	M	3.10	0.63	0.42	0.00
44	75	F	2.70	0.83	14.07	2.88
45	21	M	2.20	0.66	4.29	0.58
46	23	M	2.50	0.74	3.88	0.49
47	40	M	2.80	1.59	15.13	2.68
48	36	F	2.10	0.75	13.40	1.45
49	35	M	3.10	0.88	16.54	1.92
50	37	F	2.70	0.60	2.69	0.63
51	47	M	2.40	0.80	0.77	0.12
52	48	M	2.50	0.79	9.62	1.49
53	75	M	2.80	0.88	13.85	2.00
54	35	M	2.10	0.85	5.38	1.06
55	57	F	3.20	0.59	11.38	1.75
56	30	F	2.60	0.84	11.18	2.98
57	30	M	2.60	0.56	7.48	1.02