DEVELOPMENT OF A HPLC METHOD FOR THE DETECTION OF LEVETIRACETAM IN BLOOD OF PATIENTS WITH EPILEPSY

Research Proposal submitted for the degree
MagisterTechnologiae

in the Faculty of Applied & Computer Sciences

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Date: May 2016

To my Lord, Saviour and Friend,

Jesus Christ

ABSTRACT

Approximately 1% of the world's population has epilepsy, the second most common neurological disorder after stroke. In South Africa almost 1 in every 100 people has epilepsy, affecting all ages. Levetiracetam (LEV), marketed as Keppra® is an anticonvulsant drug used in the treatment of epilepsy. The daily dosage is 500 mg twice daily with a maximum of 3000 mg. The therapeutic range of LEV is between 12-46 µg/ml. Therapeutic drug monitoring (TDM) should be considered for LEV in patients with poor seizure control or long term treatment. TDM depends on accurate drug concentration measurements. In order to provide an accurate measurement, the High performance liquid chromatography (HPLC) method was developed, compared with a commercially available kit, and the stability of the samples was investigated.

Ethical approval was obtained from the Human Research Ethics Committee (Medical), VUT (Ethics reference number: 2015024.4). The study was conducted from January to October 2015. This study involved three groups of volunteers who gave written consent. The first group were fifteen healthy MTech students in the Biomedical Technology Department at the Vaal University of Technology (VUT). Their blood samples were used for the analytical validation of the method and for the stability studies over a 4 weeks period. The second group were six patients from Pathcare Laboratories in Potchefstroom, Klerksdorp and Vereeniging who used Levetiracetam. Their blood samples were used to investigate the influence of different collection tubes as well as the handling and storage of samples on the LEV concentration. The third group were forty four patients from Pathcare Laboratories, Cape Town. Their blood samples were transported to Clinical Pharmacokinetic Laboratory (CPL) for routine therapeutic drug monitoring analysis of LEV and used to compare the newly developed HPLC method and the Commercial kit.

The HPLC method was successfully developed and validated to determine LEV in human plasma/serum samples. The calibration curves showed good linearity (r^2 = 0,999) over the concentration range of 1 – 60 µg/ml. Accuracy, mean extraction recovery, lower limit of detection (LLOD) and lower limit of quantification (LLOQ) were 98-112%, 97,15% (±1,57), 0,5 and 1,0 µg/ml respectively, in plasma standards.

The method was shown to be simple and fast, reproducible and effective for routine laboratory analyses in the future.

The agreement between the newly developed method and the ClinRep® HPLC complete commercial kit was the same and there was a statistical significant correlation between the two methods (average r=0.999; p-value < 0.0001, F-test with a true value =0). The method was much cheaper than the commercial kit, used less sample (100 µl) and had a longer running time (15 minutes) to ensure no endogenous interference. The costs of the developed method was 71-82% lower than the three commercial kits available in South Africa.

Stability experiments were performed to evaluate the stability of LEV in human plasma/serum, simulating the same conditions which occurred during study samples' analyses. The % RSD was lower than 5% under all the conditions: freeze, fridge, room temperature and auto sampler over the 4 week period. The results showed that both LEV and the I.S (internal standard) were stable in human serum/plasma under all these conditions.

The influence of five different collection tubes, Gold (SST Gel), Red, Purple (EDTA), Green (Heparin) and Blue (Sodium Citrate) was investigated. In two patients, decreased levels were observed in tubes containing blue (sodium citrate) and Green (Heparin). The decrease was not statistically significant. This is an important observation and is an indication that anticoagulants may cause some problems due to drug-protein binding and interference in the matrix effect.

A cost effective and reliable HPLC-method with minimal sample preparation time for the routine determination of LEV in plasma/serum samples was developed. It was also shown that the plasma/serum samples were stable at different temperatures over a time period. The only collection tubes that may interfere with the concentrations were the Green (Heparin) and Blue (Sodium Citrate) tubes.

Keywords: *Levetiracetam, HPLC, ClinRep® HPLC Complete kit, Stability, Collection Tubes*.

ACKNOWLEDGEMENTS

I wish to express my sincere appreciation to the following people:

- My supervisor, Dr Christa Grobler, for her invaluable guidance and encouragement throughout this.

- My co-supervisor, Dr Malie Rheeders, for her availability, advice and invaluable guidance and encouragement throughout the study. The sharing of her experience and knowledge is greatly appreciated.

- Mr Francois Viljoen for his advice, support and availability when needed.

- Prof Linda Brand, for the permission to use the HPLC of the North West University, Department Pharmacology at CPL.

- Ingrid Howes from Pathcare Laboratories – Cape Town (N1), for her support and contribution to the study regarding the recruitment of patients to participate in the study. I am grateful to work with you. You're a Star!!

- All the personnel from Pathcare Laboratories, Vereeninging, Klerksdorp and the Free State for your support and contribution to this study.

- My husband and children, Riaan and René, for their continuous motivation, patience and unconditional love and readiness to listen. Thank you for your support over the past few years.

- My fellow M-Tech students, especially Anita for all your support and motivation to finish my study.

- Hester de Beer, my special friend who helped me with the final finishing.

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LIST OF ABBREVIATIONS

%	Percentage
ACN	Acetonitrile
AED	Anti-epileptic drug
AFRO	Regional Office for Africa
AGNP	Arbeitsgemeinschaft für Neuropsychopharmakologie und Pharmakopsychiatrie
ANOVA	Analysis of Variance
ATL	Analytical Technical Laboratory
^0C	Grade Celsius
$C_{(max)}$	Peak plasma concentration
Ca^2+	Calcium
CDC	Centre for Disease Control
CNS	Central nervous system
CPL	Clinical Pharmacokinetic Laboratory
CV	Coefficient Variation
D2 lamp	Deuterium discharge lamp
DAD	Diode-array detector
EDTA	Ethylenediaminetetraacetic acid
e.g.	for example
EMA	European Medicines Agency
EMIT	Enzyme multiplied immunoassay technique
f-calc	Statically significance
FDA	Food and Drug Administration
FL	Fluorescence lamp
g	Gram
g	Gravitation (g-Force)
GABA	Gamma-amino butyric acid
GBP	Gabapentin
GCMS	Gas chromatography-mass spectrometry
GLP	Good Laboratory Practice
HPCSA	Health Professions Council of South Africa
HPLC	High Performance Liquid Chromatography
IBE	International Bureau for Epilepsy
ICC	Intra-class correlation
ICH	International Conference on Harmonization
ILAE	International League Against Epilepsy
INSTAND	External quality control program, Germany
IS	Internal Standard
ISO	International Organization for Standardization
Kg	Kilogram
KH_2PO_4	Potassium dihydogen phosphate
LCMS	Liquid chromatography mass spectrometry
LEV	Levetiracetam
L/kg	Litre per kilogram

LLE	Liquid-liquid extraction
LLOD	Lower limit of detection
LLOQ	Lower limit of quantification
LOD	Limit of detection
LOQ	Limit of quantification
MCM	Major congenital malformations
MEC	Minimum effective concentration
MeOH	Methanol
Mg	Magnesium
mg/kg/day	Milligram per kilogram per day
mg/ml	Milligram per millilitre
min	Minutes
ml/day	Millilitre per day
ml	Millilitre
ml/min	Millilitre per minute
ml/min/kg	Millilitre per minute per kilogram
mm	Millimetre
mM	Milli Molar
MRC	Medical Research Council
MRM	Multiple reaction monitoring
MS	Mass Spectrometry
MSC	Maximum safe concentration
Mw	Molecular weight
Na^+	Sodium
NaOH	Sodium hydroxide
nm	Nano meter
NWU	North West University
PP	Protein Precipitation
(Pty)Ltd	Proprietary limited company (
p-test	Statically significance
QC	Quality Control
QM-system	Quality Management-system
R^2	Regression
RBC	Red blood cells
Rev	Revision
RI	Refractive Index
RP	Reverse Phase
rpm	Revolutions per minute
%RSD	Percentage relative standard deviation
r-value	Statically significance
(s)-α	Subunit alpha
S.A	South Africa
SANAS	South African National Accreditation of Standardization
SD	Standard deviation
SPE	Solid phase Extraction
SST	Serum separator tube
SV_2A	Synaptic vesicle protein

TDM	Therapeutic drug monitoring
TG	Treatment Gap
t-test	Statically significance
UHPLC	Ultra-High Performance Liquid Chromatography
UCB	Union Chimique Belge (Biopharmaceutical Company in Brussels Belgium)
µg/ml	Micro gram per millilitre
µl	Microliter
µm	Micro metre
USP	United States Pharmacopeia-
UV	Ultra violet
UV-Vis	Ultra violet visible light
v/v	Volume per volume
VUT	Vaal University of Technology
WHO	World Health Organization
WMA	World Medical Association

CHAPTER 1

PROBLEM SETTINGS

1.1 INTRODUCTION

Approximately 1% of the world's population suffers from epilepsy, the second most common neurological disorder after stroke. Seizures are a disruption of the electrical communication between neurons, and epilepsy is characterized and defined by the presence of two or more unprovoked seizures (WHO, 2012).

Epilepsy can affect anyone at any age and is more common in young people due to complications at birth, infections or accidents in childhood (WHO, 2010; Centre for Disease Control (CDC), 2011). Epilepsy patients are in need of understanding and acceptance from the general public, due to the fact that they and their families suffer from humiliation and discrimination in many parts of the world. Epilepsy is treated with anti-epileptic drugs (AEDs), which control seizures but do not cure epilepsy (WHO, 2012).

1.2 PREVALENCE OF EPILEPSY

Prevalence is the proportion of a population found to have a certain condition, in this instance, epilepsy (WHO, 2015). It is calculated by comparing the number of patients with this condition to the total number of people in a specific population, and is usually expressed as a fraction, percentage or as the number of cases per 10,000 or 100,000 people.

1.2.1 Global prevalence and incidence of epilepsy

Approximately 50 million people are affected by epilepsy worldwide and 80% of these affected people live in the developing world such as Africa (Paul et al, 2012; WHO, 2015). 250 million people will experience at least one seizure in their lifetime and 2.4 million new cases of epilepsy arise each year and occur between 30 and 50/100 000 people in the general population (WHO, 2015). The estimated portion of the public with epilepsy at a given time is between 4 and 10/1000 people and in low and middle income between 7 and 14/1000 people (WHO, 2015).

There is a huge global discrepancy in the care of patients with untreated epilepsy between high and low income countries and between rural and urban areas (WHO, 2012). The medical infrastructure is much more advanced in urban areas and that leads to a large discrepancy in treatment. It was documented in the literature that the treatment gap for epilepsy in low-income countries was more than 75% in comparison with high-income countries (Meyer et al, 2010). The high incidence of people with epilepsy lead to the acknowledgement of epilepsy as a major public health concern by the World Health Organization (WHO) (Kassie et al, 2014). Figure 1 represents the prevalence of epilepsy in different countries of the world (Shakirullah et al, 2014). It is important to mention that the highest prevalence was found in Africa with an average of 15.83 per 1000 people.

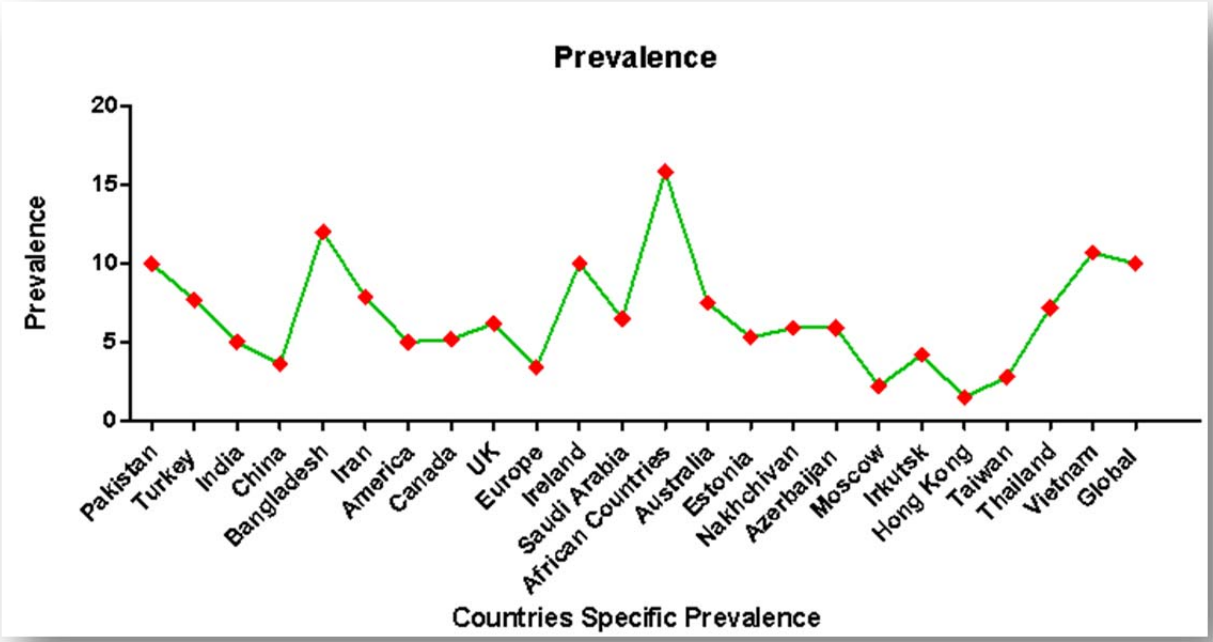


Figure 1 Prevalence of epilepsy in different countries of the World (Adapted from Shakirullah et al, 2014).

1.2.2 Epilepsy prevalence in Africa

In the WHO African region, 47 countries represent sub-Saharan Africa. Approximately 680 million people live in sub-Sahara and most of these populations lived in rural areas (Preux & Druet-Cabanac, 2005; Chin, 2012; Wagner et al, 2014).

The prevalence rate of epilepsy in Africa is reported in Table 1 and varied between 3.4 - 58/1000. The highest prevalence rates were reported in Cameroon (58/1000) and the lowest in South Africa (3.4/1000) (Ba-Diop et al, 2014).

Table 1 Reported prevalence of epilepsy in sub-Saharan African countries (adapted from Ba-Diop et al, 2014).

Country/Region	Population size	Prevalence (per 1000)	Area
Cameroon	1900	58.0	R
Congo	1000	20.0	R
Ethiopia	1154	29.5	U,R
Ghana	129 812	4.9	R
Kenya	2960	18.2	R
Liberia	4436	28.0	R
Malawi	90 000	5.2	U
Mali	5243	13.3	R
Nigeria	18 954	5.3	U
Rwanda	6757	7.0	U,R
Senegal	4500	14.2	R
South Africa	82818	3.4	R
Swaziland	8800	11.0	R
Tanzania	104889	7.2	R
Togo	9155	18.6	R
Uganda	4743	13.0	R
Zimbabwe	17 500	7.4	R

U, Urban; R, Rural.

1.2.3 Epilepsy prevalence in South Africa

Almost 1 in every 100 people is diagnosed with epilepsy in South Africa (S.A) (Epilepsy South Africa, 2013). The overall prevalence rate of 3.4/1000 as reported in Figure 1 and Table 1 is confirmed in a study by Eastman (2005). The data on epilepsy prevalence in specific populations and regions in S.A is limited. The prevalence of active epilepsy in adults in the Republic of Transkei located in the Eastern Cape Province was as high as 13.8 /1000 (Foyaca-Sidat et al, 2004). These

results were confirmed with a study done in Sidwadweni, Nkalukeni, Ngqwala, Kwandugwane and Makaula locations showing a prevalence between 13.7/1000 and 18.3/1000 in the Transkei (Foyaca-Sidat et al, 2004; Igumbor et al, 2011). The low use of AED's (only 14.7% of patients were on medication) and cultural misconceptions and attitudes were given as reasons for the higher than average prevalence rate (Foyaca-Sidat et al, 2004; Igumbor et al, 2011).

1.2.4 Challenges in Africa

An important factor that should be taken into account is that three quarters of the people in Africa diagnosed with epilepsy, have no access to healthcare practitioners and are therefore left untreated (WHO, 2012). More shocking is that most of the epilepsy patients are treated ineffectively due to the lack of general information regarding epilepsy, and insufficient resources such as trained healthcare workers, diagnostic facilities, surgeries and AEDs (Paul et al, 2012; Ba-Diop et al, 2014). Furthermore, most of these patients live in rural areas, refuse treatment because of cultural reasons or cannot afford appropriate treatment. Most people in Africa believe in spiritual causes and traditional healing, making treatment a difficult challenge. Many epilepsy patients do not know that the disorder can be controlled with medical treatment (Paul et al, 2012; Ba-Diop et al, 2014). Moreover, previous studies indicated that Africa did not receive acceptable attention in national health plans for epilepsy treatment (WHO, 2010).

The Global Campaign against Epilepsy of the World Health Organization (WHO), International League against Epilepsy (ILAE) and the International Bureau for Epilepsy (IBE) are working to overcome some of the challenges mentioned above (Ba-Diop et al, 2014). In 1997 - 2000, the WHO, ILAE and the IBE's aim was to bridge the treatment gap (TG) and bring epilepsy out of the shadows so that the physical and socio-economic burdens of epilepsy on individuals and society as a whole were reduced (WHO, 2004; WHO, 2010; Ba-Diop et al, 2014).

The Campaign assisted Governments worldwide to promote the understanding of epilepsy, education, health service, treatment and prevention of epilepsy nationwide (WHO, 2010).

Pilot projects have been officially launched by the Campaign responsible and the activities took place in 50% of Regional Offices for African (AFRO) countries. Numerous projects in Africa have also shown that intervention models are effective if there are adequate resources and commitment (Scott et al, 2001; WHO, 2004). The goal of the pilot projects was to develop a success model to control epilepsy and that can be integrated into the healthcare systems of the countries that took part and will then be applied on a global level (WHO, 2003).

The prevalence of epilepsy in Zimbabwe was unknown up to 2003 (WHO, 2010). However, a pilot project, managed by the Zimbabwe Committee of the Global Campaign Against Epilepsy Training, studied the diagnosing of epilepsy by primary health care workers and they observed that no patients with epilepsy had been treated inappropriately (WHO, 2010). After this study, recommendations were made to the National Drug and Therapeutic Committee as well as the Ministry of Health and Child Welfare to approve a national policy of primary health care workers trained to diagnose and treat certain types of epilepsy (WHO, 2010).

Another pilot project on epilepsy in Senegal investigated the outcomes for epilepsy patients after healthcare workers received training and education on the treatment of these patients (WHO, 2010). The Senegal Committee revealed that the public health methodology applied was effective, despite the difficulties related to the context, and that these methodology procedures could be extended to the rest of Senegal (WHO, 2010). The methodology could further be proposed as a model that could be suitably adapted for use by other countries of the Region, taking into account local specificity.

It is evident that the relationship between IBE, ILAE and WHO has given the Campaign the opportunity to build a framework for concerted action on a global, regional and national level to raise awareness and reduce the treatment gap. Recently, many countries have undertaken the initiatives to decrease the epilepsy treatment gap, particularly the pilot projects such as Global Campaign Against Epilepsy, directed by IBE, ILAE and WHO. These projects demonstrated that epilepsy can be treated inexpensively and effectively with AEDs at a community level by primary health care workers with basic training (Meyer et al, 2010).

1.3 TREATMENT OF EPILEPSY

The majority of patients diagnosed with epilepsy, have shown a reduction in seizures when treated with an appropriate AED (WHO, 2003; Ba-Diop et al, 2014). Epilepsy is a controllable disorder in the majority of affected individuals. Approximately 60–70% of patients will become seizure-free with AED's. Cost of treatment can be reduced when blood levels of AED's are monitored on a routine basis (WHO, 2005; Ba-Diop et al, 2014, Gilani et al, 2015).

In general, the choice of medication used in epilepsy depends on the type of the seizure, with partial and generalized seizures the most common (Epilepsy South Africa, 2013; Ba-Diop et al, 2014). Partial seizures refer to seizures where a specific locus in the brain can be affected and are normally treated with the older drugs such as carbamazepine, phenytoin and valproate (WHO, 2005; Mbuba et al, 2008; Krasowski, 2010). However, partial seizures also respond well to the newer drugs such as gabapentin, lamotrigine, levetiracetam, tiagabine and topiramate. The advantages of treating patients with the newer drugs include fewer side effects and these drugs are normally well tolerated in the patients (Krasowski, 2010). However, the newer drugs are more expensive and for that reason not so readily available in the public sector of poorer countries (WHO, 2005; Ba-Diop et al, 2014). Only lamotrigine, of the newer drugs, is present on the essential medicine list of Standard Treatment Guidelines and Essential Medicines List for South Africa, 2012.

Generalized seizures are typically characterized by no evidence of localized onset (Krasowski, 2010). These seizures are also treated with the older and newer drugs such as valproate, carbamazepine, phenytoin, lamotrigine and topiramate (Krasowski, 2010; French & Gazzola, 2011).

A very important factor in the treatment of epilepsy is therapeutic drug monitoring, which plays a vital role in the management of epilepsy (Neels et al, 2004; Krasowski, 2010; Patsalos & Berry, 2013). Some of the older drugs have a narrow therapeutic range and blood level monitoring is essential to maintain the level in the therapeutic range. The newer generation of drugs, such as levetiracetam, have a wider therapeutic range but blood level monitoring is still important to measure compliance and to sustain therapeutic levels (Patsalos et al, 2008).

1.4 BACKGROUND OF LEVETIRACETAM

Levetiracetam (LEV), came on the market as Keppra ® in 2000 and was approved for the treatment of partial onset seizures and as adjunctive therapy for myoclonic seizures (Contin et al, 2008). It has many therapeutic advantages for patients with epilepsy, including favourable pharmacokinetic characteristics such as good bioavailability, linear pharmacokinetics, insignificant protein binding, and minimum hepatic metabolism (two thirds of the drug is excreted unchanged in the urine) (Johannessen et al, 2003; Patsalos, 2004; Food and Drug Administration (FDA), 2015). It is rapidly absorbed after oral administration, with peak plasma /serum levels at 1 – 1.5 hours (half-life is 6 to 8 hours) and a low potential for drug interactions (Patsalos et al, 2008; Zufia et al, 2010).

Furthermore, it has a favourable safety profile with minimal adverse effects, with the most common being somnolence, fatigue and dizziness. Uncommon side effects include behavioural effects like hallucinations and psychosis. The daily recommended dosage is 500mg twice a day (1000mg daily) with a daily maximum of 3000mg (Johannessen et al, 2003; Pucci et al, 2004, Patsalos, 2004; FDA, 2015).

The therapeutic range (effective therapeutic concentration) of LEV is between 12-46 µg/ml (Krasowski, 2010; Stepanova & Beran, 2014). This means that if the concentration of LEV is below 12 µg/ml in blood plasma, it will be therapeutically insufficient and the patient may still have epileptic seizures whereas, if the concentration is above 46 µg/ml in blood plasma it will be too high and unsafe and the patient may experience serious side effects such as irritability, agitation, aggressive behaviour and anger, depressed level of consciousness, respiratory depression and coma observed with overdoses in postmarking use (Krasowski, 2010; Wright et al, 2013).

Therapeutic drug monitoring (TDM) enables clinicians to adjust the dosage of each individual patient to maintain drug concentration levels within the therapeutic range. TDM should thus be taken into consideration for LEV when seizure control is poor and to determine the patient's compliance to the treatment (Krasowski, 2010; Stepanova & Beran, 2014).

1.5 CONTEXT OF THE STUDY

This study was conducted in the Clinical Pharmacokinetic Laboratory (CPL) in the department of Pharmacology at the North West University (NWU). The CPL is a laboratory where blood concentrations of various drugs for TDM are analysed on a routine daily basis and carried out to the highest standards of quality and reliability. CPL operates as a referral laboratory to service numerous pathology laboratories in S.A.

1.6 PROBLEM STATEMENT

Biomedical sciences and technology need to create new and better analytical methods to detect and quantify drugs in human serum/plasma samples. In the treatment of epilepsy it is crucial that anticonvulsants are used continuously and that it is well monitored to ensure effectiveness and safety in the long-term therapy of these patients. This Clinical Pharmacokinetic Laboratory (CPL) received numerous requests to analyse newer anti-epileptic medicines especially levetiracetam for therapeutic drug monitoring over the last couple of years. Currently only high cost commercial kits are available and the need to develop and validate more cost effective methods has increased. One of the factors influencing the quality of results, is the stability of samples prior to testing, therefore investigations with different blood collection tubes will be carried out as part of the study. Only reliable results can be used in routine therapeutic drug monitoring.

This study will fill a gap in the service rendered by this CPL where plasma/serum concentrations of various drugs are being analysed in patients on anticonvulsants. The availability of newer drugs creates a need for the accurate quantification and validation of these drugs for TDM.

1.7 SIGNIFICANCE OF THE STUDY

Therapeutic drug monitoring (TDM) is the clinical practice of measuring specific drugs in plasma/serum at designated intervals and interpretation of these results to maintain a constant concentration in a patient's bloodstream, thereby optimizing individual dosage regimens. The accurate determination of plasma levels is vitally important in TDM and is therefore a significant outcome in this study.

In order to develop an accurate and validated method knowledge of sample preparation techniques, the analytical instrument available, previous developed methods and sample collection techniques are required.

It is also important that the method is cost effective with minimal sample preparation that meets the criteria of the European Medicines Agency (EMA) (2011); FDA agency of Guidance for Industry: Bioanalytical Method Validation (2013); International Conference on Harmonization (ICH) (2015) and the general requirements of International Organization for Standardization (ISO) 17025 (2005) will be discussed below.

The CPL received numerous requests to provide a cost effective service to patients on anticonvulsant therapy. The implementation of this method will add value to the TDM service in S.A.

1.8 AIM AND OBJECTIVES

1.8.1 Aim

The aim of this study was to develop and optimize a new High Performance Liquid Chromatography (HPLC) method for the analysis of LEV concentrations in plasma/serum and to evaluate the stability of plasma/serum samples over time in different blood collection tubes.

1.8.2 Objectives

The research design was structured in order to achieve the following objectives:

1. To develop a new HPLC method to detect LEV levels in the plasma/serum.
2. To standardize and validate the method under the criteria of EMA, 2011; FDA, 2013; ICH, 2015 and ISO 17025 (2005) .
3. To compare the newly developed HPLC method with an available commercial HPLC reagent kit as follows:
    - Correlate the agreement of the plasma/serum level results obtained between the two methods

- Compare the operational costs
- Determine minimal sample preparation for reliable results in newly develop HPLC method

4. To optimize the stability of LEV under different temperatures, time periods and blood collection tubes as per ICH guidelines (ICH, 2015) in blood.

1.9 STRUCTURE OF THE DISSERTATION

Here follows a short description of the different chapters of the dissertation:

Chapter 1 described the background, introduction and problem setting of the study. The lack of treatment and support for epilepsy patients in poor countries and the rationale to develop more cost effective accurate methods for the determination of blood levels were highlighted.

Chapter 2 consisted of a clear literature review on LEV and the available methods for the accurate determination of LEV blood levels within the laboratory.

In **Chapter 3** all the methods of the study were discussed. These methods included study design, both analytical and statistical. The statistical programs Microsoft Excel and Prism version 5 software were used.

The results and statistical analyses were reported and discussed in **Chapter 4.** Conclusions and recommendations were reported in **Chapter 5.**

A **Reference** list was included at the end of the dissertation. The Harvard style was used as requested by VUT.

1.10 CONCLUSION

A vast majority of patients with epilepsy in developing countries do not receive adequate medical treatment and 80-90% are without any treatment (Ba-Diop et al, 2014; Gilani et al, 2015.

CHAPTER 2

LITERATURE REVIEW

2.1 INTRODUCTION

In this literature review the following will be discussed:

- Epilepsy, with the emphasis on the treatment of epilepsy with LEV.
- The importance of TDM in the management of epilepsy.
- The background of HPLC methods for the determination of LEV and the comparison with commercially available kits.
- Methods to investigate the stability of LEV blood samples in the laboratory.

2.2 EPILEPSY

2.2.1 Pathogenesis of Epilepsy

Epilepsy is defined as the occurrence of one or two unpredictable seizures (Fischer et al, 2014). These seizures are thought to arise from the cerebral cortex and involve the abnormal "firing" discharge of cerebral neurons (McNamara, 2011). The current drug treatment of epilepsy reduces these "firing" discharges of the cerebral neurons. These drugs normally work on one of the following mechanisms:

(1) To limit the repetitive firing of neurons by inactivating the voltage activated Na^+ channels (i.e. phenytoin).

(2) By increasing the gamma-amino butyric acid (GABA), an inhibitory neurotransmitter (i.e. gabapentin).

However, newer drugs act on different targets, such as the synapse of the neuron in order to lower "firing". One such drug, Levetiracetam (LEV), binds to the synaptic vesicular protein (SV_2A) which modifies the release of GABA and glutamate (an excitatory neurotransmitter. The treatment of epilepsy depends on the classification of the seizure type or epileptic syndrome and the choice of AED with the safest profile for the specific patient (Abou-Khalil, 2008; Goldenberg, 2010).

2.2.2 Classification of epileptic seizures

Epilepsy can be classified either by cause or by type of seizure. Firstly, epilepsy is classified according to the underlying causes: idiopathic (epilepsy with no underlying cause other than possible hereditary), symptomatic (epilepsy caused by known underlying causes) and cryptogenic (epilepsy with presumed symptomatic but not identified causes). Epileptic syndrome is defined by a cluster or group of features usually occurring together (McNamara, 2011; Smith, 2014).

Secondly, since 1981, the ILAE used the classification of seizures as a standard for the management and treatment of epilepsy worldwide. Under this classification, there are two main seizure types: partial and generalized seizures. A modified version of the ILAE is presented in Table 2. In Table 2, the features of each seizure type as well as the more conventional and recent drug treatments are described. LEV, the drug studied in this dissertation, is one of the newer drugs mentioned in the table. Partial seizures accounted for about 60% of all the epilepsies (Table 2) and consist of a lesion in some part of the cortex, developmental malformation or damage due to a stroke or trauma. Generalized seizures accounted for about 40% of all the epilepsies and these seizures are usually genetic. The generalised seizures are divided into myoclonic, tonic-clonic and absence seizures (McNamara, 2011; Smith, 2014).

Table 2 Classification of Epileptic Seizures (adapted from McNamara, 2011).

Seizure type	Features	Conventional Anti-seizure drugs	Recently developed Anti-seizure drugs
Partial Seizures			
Simple partial	Diverse manifestations determined by the region of cortex activated by the seizure(e.g. if motor cortex representing left thumb, clonic jerking of left thumb results; if somatosensory cortex representing left thumb, paraesthesia of left thumb results), lasting approximating 20-60 seconds. Key feature is preservation of consciousness.	Carbamazepine, phenytoin, valproate	Gabapentin, lacosamide, lamotrigine,
Complex partial	Impaired consciousness lasting 30 seconds to 2 minutes, often associated with purposeless move-ments such as lip smac-king or hand wringing.		levetiracetam, rufinamide, tiagabine, topiramate, zonisamide
Partial with secondarily generalized tonic-clonic seizure	Simple or complex partial seizure evolves into a tonic-clonic seizure with loss of consciousness and sustained contractions (tonic) of muscles through-out the body followed by periods of muscle contrac-tions alternating with periods of relaxation (clonic), typically lasting 1-2 minutes.	Carbamazepine, phenobarbital, phenytoin, primidone, valproate	

Table 2 Continued

Seizure type	Features	Conventional Anti-seizure drugs	Recently developed Anti-seizure drugs
Generalized Seizures			
Absence seizure	Abrupt onset of impaired consciousness associated with staring and cessation of on-going activities typically lasting less than 30 seconds.	Ethosuximide, valproate, clonzapam	Lamotrigine
Myoclonic seizure	A brief (perhaps a second), shock-like contraction of muscles that may be restricted to part of one extremity or may be generalized.	Valproate, clonazepam	Levetiracetam
Tonic-clonic seizure	As described earlier in table for partial with secondarily generalized tonic-clonic seizures except that it is not preceded by a partial seizure.	Carbamazepine, phenobarbital, phenytoin, primidone, valproate	Lamotrigine, levetiracetam, topiramate

2.2.3 Pathophysiology

Epileptic seizures characteristically involve excessive rapid firing of action potentials and synchronisation of neurons. The bursting activity is caused by long lasting depolarization of the neuronal membrane due to influx of extracellular calcium (Ca^{2}+) and sodium (Na+), membrane receptor response, messenger systems and gene transcriptions. An imbalance between excitatory (glumate) and inhibitory (GABA) neurotransmitters as well as excessive acetylcholine, norepinephrine and serotonin levels may also precipitate the processes in the brain which may cause seizures. These excitatory (glutamate) and inhibitory (GABA) neurotransmitters are primarily mediated by the acidic amino acid glutamate, and by different channels including voltage- and ligand-gated channels which include calcium (Ca^{2+}) and sodium (Na+)

channels as well as glutamate and GABA. Most of the AEDs act by affecting one or more of these processes, such as targeting different channels (Ha & Bellanger, 2013).

2.2.4 Etiologic classification of epilepsy

The etiologic classification of epilepsy can be divided into 5 categories. They include:

- In 60-70% of patients, no specific cause for their seizures can be identified. Epilepsy in these patients is referred to as being idiopathic (no definite cause).
- Infants/children: Congenital malformations, perinatal injuries or hypoxia, developmental neurologic disorders, metabolic defects, injury, and infection are common causes of seizures.
- Young/adults: Head trauma, brain tumours, infection, and arterio-venous malformations are common causes of seizures.
- Elderly: Cerebrovascular disease, central nervous system (CNS) degenerative diseases, and brain tumours are common causes.
- Genetic - Risk increased 2-3 times in individuals with first degree relative with epilepsy.
  (Goldenberg, 2010; Shorvon, 2011).

2.3 LEVETIRACETAM

In December 1999, Levetiracetam, with a unique chemical structure and mechanism of action, was approved by the FDA and the EMA (Ulloa et al, 2009; Adams et al, 2009) for the treatment of epilepsy. Levetiracetam is marketed as Keppra XR ™; Union Chimique Belge (UCB) Pharma (Adams et al, 2009; Goldenberg, 2010).

2.3.1 Chemical properties of levetiracetam

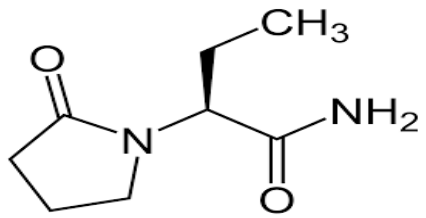
Levetiracetam is a (S)-α ethyl 2-oxo-1-pyrrolidineacetamide (Figure 2). This compound (Figure 2) is chemically similar to nootropic, a structural analogue of

piracetam, which is considered to be “pharmacologically safe” (Patsalos, 2004; Contin et al, 2008; Wright et al, 2013).

Levetiracetam is a white to off-white crystalline powder with a faint odour and a bitter taste (Moffat et al, 2011).

- **Molecular Formula:** ($C_8H_{14}N_2O_2$)
- **CAS registry number:** [102767-28-2]
- **Additional name(s):** Keppra
- **Molecular weight (Mw)**: 170.21
- **Melting point:** 118-119 °C
- **Solubility:** Soluble in water (104.0 g/100 ml). It is freely soluble in chloroform (65.3 g/100 ml) and in methanol (53.6 g/100 ml), soluble in ethanol (16.5 g/100 ml), sparingly soluble in acetonitrile (5.7 g/100 ml) and practically insoluble in n-hexane (Solubility limits are expressed as g/100 ml)
- **pKa:** 16.09
- **Storage:** Temperature: Store at room temperature

(Moffat et al, 2011)



Levetiracetam

Figure 2 The chemical structure of levetiracetam ($C_8H_{14}N_2O_2$) (USP24) (Contin et al, 2008).

2.3.2 Pharmacokinetics and metabolism of LEV

Levetiracetam has a favourable pharmacokinetic profile, which includes rapid absorption, excellent bioavailability, linear pharmacokinetics, minimal plasma protein

binding and excretion by the kidneys. The pharmacokinetic profile is comparable between healthy volunteers and patients with epilepsy *(*Wright et al, 2013).

- Absorption: LEV is rapidly and almost completely absorbed (>95%) after oral administration. The oral absolute bioavailability is close to 100% and the peak plasma concentrations (C_{max}) is reached 1 hour after administration. Steady-state is achieved after two days. Peak plasma concentrations (C_{max}) of 31 and 43 µg/ml are achieved after a single 1000mg dose or repeated 1000mg twice daily dose, respectively. The extent of absorption is dose-independent and when taken with food, the extent of absorption of LEV was not affected.

- Distribution: The volume distribution of LEV was 0.5 to 0.7 L/kg; a value close to the volume of distribution of intracellular and extracellular water. Neither LEV nor its metabolite, ucb L057, is significantly bound to plasma proteins (<10%) and the risk for protein-binding interactions negligible.

- Metabolism: LEV is minimal metabolized by the liver. The major metabolic pathway (24% of the dose) is an enzymatic hydrolysis of the acetamide group.

- Elimination: The major route of excretion is via urine. Approximately 93% of the dose is excreted within 24 hours, with 66% of the dose found unchanged in the urine and 24% is excreted in urine as its major metabolites. Excretion via faeces accounted for 0.3% of the dose. The total body clearance of LEV is 0.96 ml/min/kg and the renal clearance is 0.6ml/min/kg.

  (McNamara, 2011; Wright et al, 2013)

2.3.3 Role of levetiracetam in epilepsy

Levetiracetam is approved for the treatment of partial onset seizures in patients 4 years and older. LEV is also used in combination with other AEDs to treat myoclonic, partial onset, or tonic clonic seizures in children and adults (Contin et al, 2008).

There is also increasing evidence that LEV has potential benefits for other psychiatric and neurologic conditions such as Tourette syndrome, Lennox - Gastaut syndrome, autism, bipolar disorder and anxiety disorders (Farooq et al, 2009).

2.3.4 Pharmacology of levetiracetam

2.3.4.1 Mechanism of action

LEV is different in its mechanism of action than all the other AEDs, illustrated in Figure 3. The unique mechanism of action is an infection of synaptic neurotransmitters, released through binding to the synaptic vesicle protein (SV_2A) in the brain. The SV2A binding affinity of LEV derivatives correlates strongly with their binding affinity in the brain, including their ability to protect against seizures (Lynch et al, 2004; Deshpande & DeLorenzo, 2014). The specific effect of LEV binding to SV2A appears to be a reduction in the rate of vesicle release. LEV has other mechanisms of action that likely play a comparatively smaller role, such as: (1) reversing the inhibition of neuronal GABA- and glycine-gated currents by the negative allosteric modulators zinc and ß-carbolines and (2) the partial depression of the N calcium current. The attachment of LEV to the SV2A molecules may help reduce the abnormal spread of signals that could lead to a seizure (Yang et al, 2007; Abou-Khalil, 2008). (Figure 3 accessed from Wiffen et al, 2014).

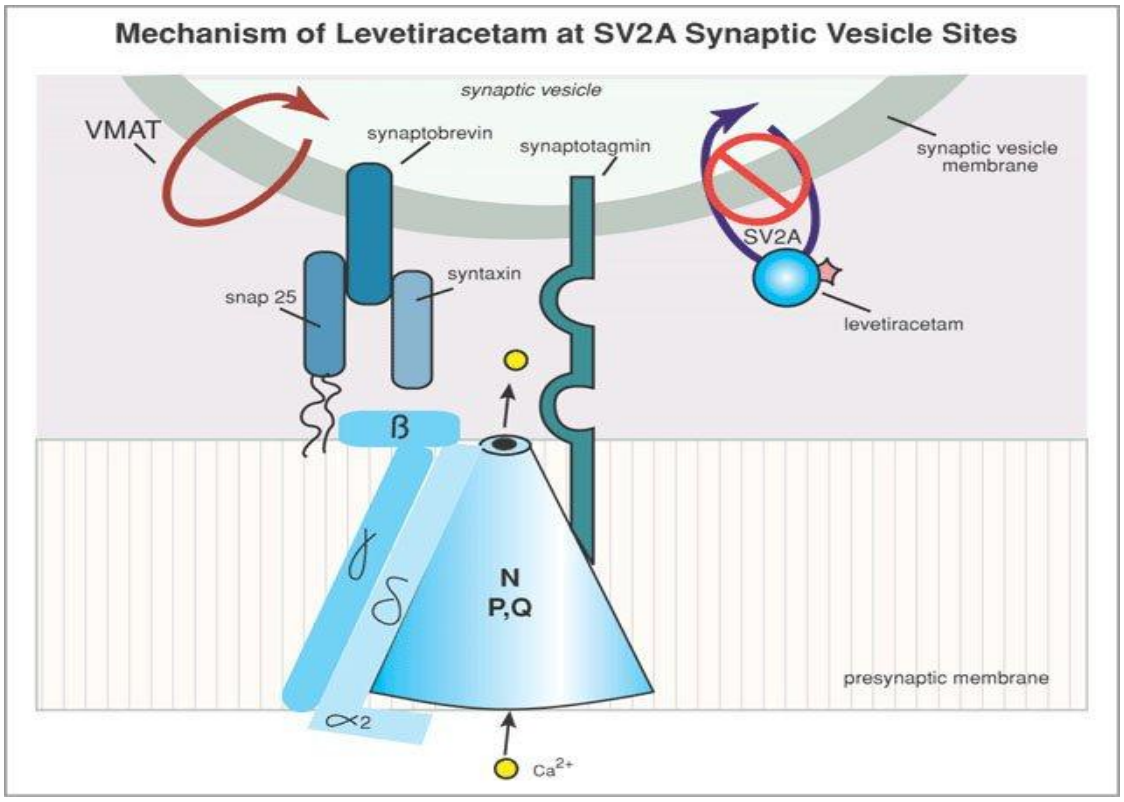


Figure 3 Mechanism of action of levetiracetam (Figure 3 accessed from Wiffen et al, 2014).

2.3.4.2 Adverse effects

Levetiracetam was generally well tolerated. The main adverse effects were CNS related and include: somnolence, asthenia, and dizziness. Other CNS effects reported were irritability, agitation, aggressive behaviour, depressed level of consciousness, respiratory depression and coma in overdoses (Abou-Khalil, 2008; Wright et al, 2013; Halma et al, 2014). The same effects were reported in children (Abou-Khalil, 2008; Adams et al, 2009). Behaviour problems occur often in patients who have underlying behaviour or cognitive problems and were not dose related (Adams et al, 2009; Halma et al, 2014).

A small number of patients also experienced a decrease in red blood cell counts (RBC), haemoglobin, haematocrit values and an increase in eosinophil counts. Few cases of agranulocytosis were reported (Abou-Khalil, 2008; Verrotti et al, 2014).

Skin rashes were rare but in a few patients serious dermatological reactions were reported after 4 months of treatment (Abou-Khalil, 2008; Alkhotani & McLachlan, 2012).

2.3.4.3 Pregnancy

The FDA uses a pregnancy category system to classify the possible risks to a foetus when a specific medicine is taken during pregnancy. Category C is given to medications that have not been studied in pregnant humans, but do appear to cause harm to the foetus in animal studies. Also, medications that have not been studied in any pregnant women or animals are automatically given a pregnancy Category C rating (Adams et al, 2009).

According to Mawhinney et al, (2013), a meaningful study indicated that LEV confirms a low risk for major congenital malformations (MCM) when it's used as monotherapy in pregnancy. A higher MCM occur when LEV is taken as part of a polytherapy treatment. LEV can be considered a safer alternative than valproate for women with epilepsy of childbearing age (Mawhinney et al, 2013).

In an article by Adams et al. (2009), a higher elimination rate in 12 pregnant women was reported in the third trimester. This decline in drug levels may require additional therapeutic drug monitoring.

2.3.4.4 Dosage

Dosing regimens of LEV depend on the indication, age group, dosage form (tablet or oral solution) and renal function.

Based on clinical studies the effective starting dose is 500mg LEV twice daily in adults. The dose can be increased with 1000mg increments to 3000mg daily with or without second line AED's (Wright et al, 2013). The appropriate paediatric dose is calculated as follows (FDA, 2015):

Total daily dose (ml/day) = Daily dose (mg/kg/day) x patient weight (kg) / 100mg/ml

The FDA has approved a LEV injection 500mg/5ml (100mg/ml) in 2006, as an alternative when oral administration is temporarily not possible. In patients with renal impairments, dose adjustments and supplemental doses should be administered after dialysis to compensate for loss during dialysis (Adams et al, 2009; Wright et al, 2013).

2.3.4.5 Over dosage

Clinical trials reported adverse effects at doses higher than 6000mg/day. These adverse effects correspond with the ones mentioned in 2.3.4.2 (Adams et al, 2009; Farooq et al, 2009; Larkin et al, 2013).

2.3.4.6 Drug Interactions profile of levetiracetam

Levetiracetam is not metabolized by the hepatic CYP450 system and therefore shows no interactions with other AED's metabolized in the liver (Pucci et al, 2004; Adams et al, 2009; Farooq et al, 2009).

According to the literature of Neels et al, (2004), the serum levels/dose ratio of LEV decrease in co-medication with phenytoin, carbamazepine and oxcarbazepine. The levels stay unchanged when used in combination with valproate, phenobarbital and lamotrigine (Wright et al, 2013).

No interactions could be found between LEV, Dioxin and Warfarin (Ulloa et al, 2008).

2.3.4.7 Clinical efficacy

Studies to investigate the effectiveness of LEV are summarized in Table 3. These studies evaluate LEV as adjunctive and monotherapy, the influence of long term therapy and in paediatric use. The designs of the studies were parallel, crossover, pooled and open.

Betts et al. (2000); Cereghino et al. (2000); Shorvon et al. (2000) and Boon et al. (2002) studied LEV as adjunctive therapy. In all these studies a reduction in seizures were observed after 14 weeks.

All the studies shown in Table 3 conducted on LEV as monotherapy showed a reduction in seizures and a small number of adverse effects (Adams et al, 2009; Farooq et al, 2009).

Additional studies (Krakow, 2001; Ben-Menachem & Gilland, 2003; Kumar & Smith, 2004) evaluated long-term efficacy of LEV and came to the conclusion that LEV is a safe and effective long-term treatment in Table 3 (Adams et al, 2009; Farooq et al, 2009).

The role of LEV in paediatric migraine disorders was investigated by Pakalnis et al. (2007) and the outcome of the study was that 20 patients had a positive response to LEV treatment with a 50% reduction in monthly migraine disorders.

The conclusion of all these clinical studies in Table 3 is that LEV is a safe, effective and tolerable AED as an add-on and monotherapy for epilepsy (Ulloa et al, 2008; Adams et al, 2009; Farooq et al, 2009).

Table 3 Results of clinical trials and efficacy of LEV in neurologic disorders (Farooq et al, 2009).

Author, year	N	Study type	Seizure type	Dose	Findings	Response rate	Number reporting side effect N (%)	Drop outs due to side effects
Levetiracetam use for adjunctive therapy								
Randomized studies								
Cereghino et al. (2000)	294	MC/R/PC/ DB/parallel	Refractory partial± generalization	1000mg 3000mg placebo	Decrease in mean# of partial seizures over 14weeks Response rate	1000mg-20.9% 3000mg- 27.7% (p<0.001) 1000mg-33% 3000mg-39.97% Placebo-10.8% (p<0.001)	1000mg –87 (88.8) 3000mg-90 (89.1) Placebo-84 (88.4)	18
Betts et al. (2000)	86	MC/R/DB/ parallel	Refractory	2000mg 4000mg placebo	Decrease in mean# of partial seizures over 14weeks Response rate	2000mg-48.1% (p<0.01) 4000mg- 28.6% Placebo-16.1% 2000mg-44.1% 4000mg-33.3% Placebo-19.4% (p<0.05	2000mg –35 (83.3) 4000mg-32 (84.2) Placebo-33(84.6)	33

Shorvon et al. (2000)	324	R/DB/PC/ parallel	Refractory partial± generalization	1000mg 2000mg placebo	Decrease in mean# of partial seizures per week Response rate	1000mg-16.4% (p=0.006) 2000mg- 17.7% (p=0.003) 1000mg-22.8% (p=0.019) 2000mg- 31.6% (p<0.001)	1000mg –70.8% 2000mg-75.5% 3000mg-73.2%	29
Boon et al. (2002)	324	12 week cross-over	Refractory partial± generalization	1000mg 2000mg placebo	Reduction in seizure frequency	1000mg-16.9% 2000mg- 18.5% Compare to placebo (p<0.001)	1000mg –14 2000mg-26 Placebo-16	
Pooled-data analysis								
Privitera (2001)	899	Pooled data analysis	Partial onset± generalization	1000mg 2000mg 3000mg placebo	Median reduction in seizure activity Response rate	31.3% compared to placebo (p<0.001) 1000mg-28.6% 2000mg-35.2% 3000mg-39.5%	Side effect data not analysed	
Open-label studies								
Morrell et al. (2003)	1030	Open-label	Partial onset± generalization	500mg BID-3000mg daily	Median reduction in seizure frequency Response rate	62.3% 57.9%	38.3%	133

Steinhoff et al. (2007)	1541	Open-label	Treatment resistant partial	500mg BID-3000mg daily	Median reduction in seizure frequency Response rate	50.2% 50,1%	936(60.7)	116
Levetiracetam use as monotherapy								
Ben-Menachem & Falter (2000)	86	MC/R/DB/ Parallel/responder selected	Refractory complex partial	1500mg BID mono-therapy	Decrease in median seizure frequency Responder rate	73.8% (p<0.037) 59.2%	LEV-55% Placebo-53%	21
Alsaadi et al. (2004)	14	Retrospec-tive	Partial onset ± generalization	Median 1839.2mg	Seizure free	8pts	1	0
Rocamora et al. (2006)	8	Retrospec-tive	Refractory IGE	1000mg to 3000mg	Seizure freedom Response rate	5pts 7pts	1	0
Labate et al. (2006)	35	Open-label Obser-vational	Generalized, myoclonic	2000mg to 3000mg	Seizure freedom Response rate	43% 83%	Side effects data not analysed	
Long-term use of Levetiracetam								
Krakow et al. (2001)	1325	Long-term follow-up for 1 year	Adjunct therapy	250-5000mg (max)	Seizure freedom Estimated continuation rate	4.5% from day 1 60% - I year 37% - 3 years 32% - 5years	Not reported	225
Ben-Menachem & Gilland (2003)	98	SC/ prospective, open-label 1 year	Adjunct therapy Monotherapy	1900mg ± 900mg	Seizure reduction (>50%)	57 patients	U	19

Kumar & Smith (2004)	25	Follow-up 1 year	Adjunct therapy	1000mg 2000mg	Seizure free Seizure reduction from baseline	16% 68%	11	4
Alsaadi et al. (2005)	35	Follow-up 1 year	Monotherapy	U	Seizure free for 6 months Seizure free for 6 month follow-up Decrease in seizure frequency	19 pts 18 pts 34 pts	10	3
Bauer et al. (2006)	505	Long-term, Open-label Follow-up 3 year median	Adjunct therapy Monotherapy	3000mg	Seizure freedom	15% - 12 weeks 8.6% - 48 weeks 6.6% - 156 weeks	168	39
Role of Levetiracetam in Paediatric Disorders								
Pakalnis et al. (2007)	20	Pilot, open-label	Paediatric migraine(6-17yrs)	40mg/kg/ day in divide dose 4 weeks	Reduction in headache Frequency	≥ 50%	4 pts migraine free	0

2.4 THERAPEUTIC DRUG MONITORING (TDM)

Therapeutic drug monitoring (TDM) is the process of measuring AED's in plasma/serum at designated intervals to maintain a constant concentration in a patient's bloodstream, in order to optimize individual dosage regimens (Kang & Lee, 2009).

Measuring plasma/serum concentrations is helpful and allows physicians to monitor the compliance and the consequences of dosage adjustments and drug interactions. Anti-epileptic treatment is usually prophylactic and lifelong, thus, TDM is justified to avoid the risks of acute and chronic adverse effects (Neels et al, 2004; St Louis, 2009). Monitoring serum concentration during pregnancy can also help with the correct recommended dose adjustments (Adams et al, 2009; Wright et al, 2013).

For a drug to be effective it needs to reach a specific therapeutic concentration in the blood. The minimum concentration that is necessary to obtain a therapeutic effect is known as the minimum effective concentration (MEC) indicated in Figure 4. Too high concentrations will increase side effects and could be toxic to the patient. The concentration at which toxicity starts is known as the maximum safe concentration (MSC) indicated in Figure 4. The therapeutic goal is to maintain the drug concentrations within the therapeutic range. A curve depicting the concentration of drug in plasma against time after drug intake can be seen in Figure 4 (Ashford, 2007). A therapeutic range is defined as the concentration of drug at which the patient will experience the desired clinical effect without a sub-therapeutic level or adverse reactions (Kang et al, 2011).

TDM of AED's faces challenges such as:

- Seizures occur irregularly. Long term observation of any therapy may be needed to assess clinical benefit.
- Adverse effects that may be difficult to distinguish from the underlying neurologic disease.
- There are no simple laboratory tests or diagnostic procedures that can evaluate the clinical efficacy of AED's.

- Clinical observation to identify inadequate adherence as a cause of poor treatment response.
  (Krasowski, 2010).

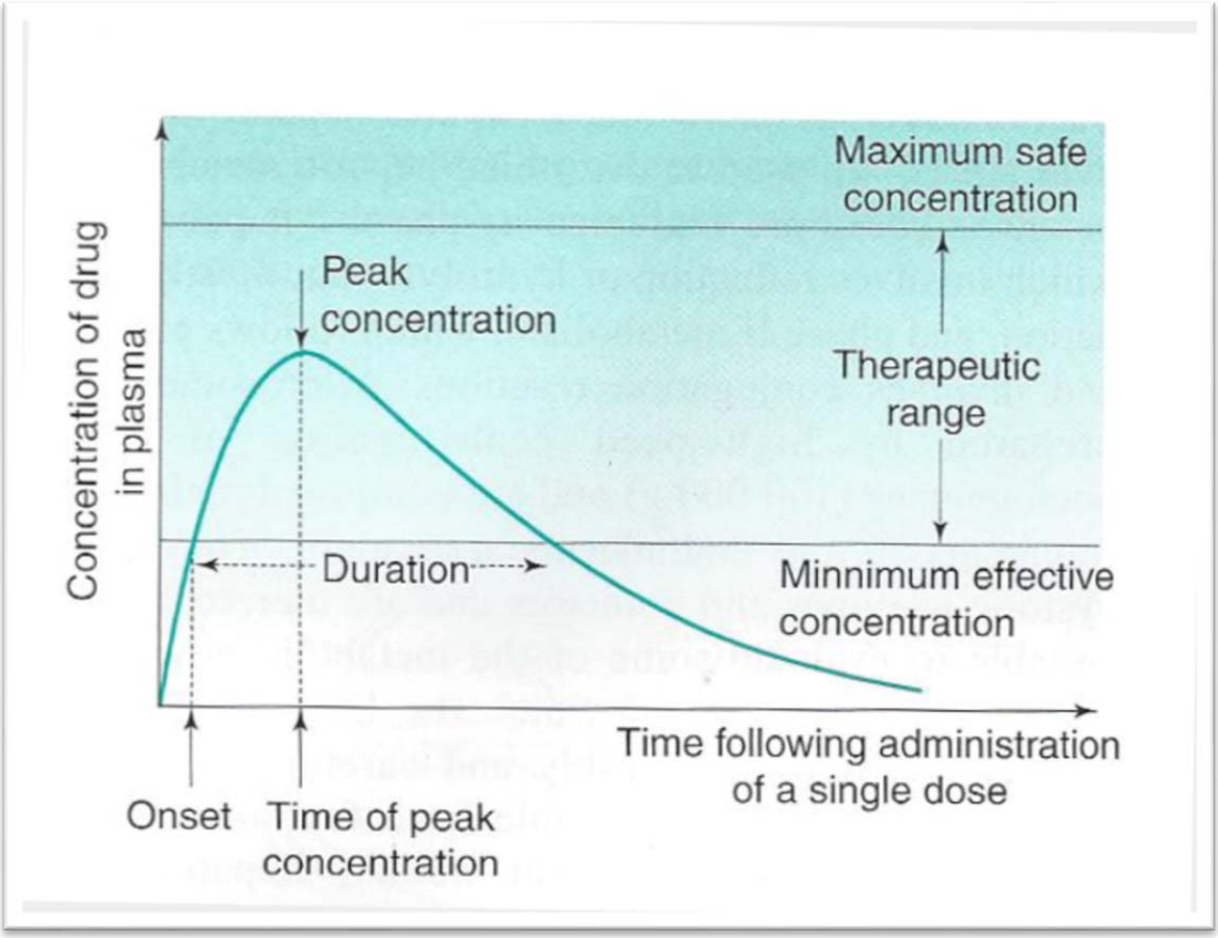


Figure 4 A curve depicting the concentration of drug in plasma against time after drug administration (Ashford, 2007).

2.4.1 Therapeutic drug monitoring for levetiracetam

Levetiracetam has linear kinetics; this means that in any individual the serum concentration is proportional to the dose. However, the relationship between LEV serum concentrations and clinical effective serum level for LEV is unknown. As for its favourable therapeutic index, low plasma protein binding and minimal side effects, it appears that routine drug monitoring is not necessary for the safe use of the drug, but can be used to assess compliance (Neels et al, 2004; Patsalos et al, 2008; Kang et al, 2011).

The therapeutic range of LEV is between 12-46 µg/ml. This means that if the concentration of LEV is below 12 µg/ml blood plasma, it will be sub-therapeutic and the patient may still have epileptic seizures. The concentration of LEV above 46 µg/ml blood plasma is unsafe and the patient may experience serious side effects (Krasowski, 2010).

It is very important to review the LEV plasma / serum levels in patients with epilepsy, in order to adjust the dose to decrease adverse effects and to optimize treatment effectiveness in the patient (Brodtkorb et al, 2004; Kang et al, 2011). A simple, rapid and cost effective method is needed for TDM of LEV to increase the effectiveness of treatment and to improve the safety of the patient with epilepsy (Contin et al, 2008; Kang et al, 2011; Poongothai et al, 2011).

2.4.2 Other methods available for therapeutic drug monitoring for levetiracetam

Only a few published studies were found where other alternative methods for TDM. According to the study of Bianchi et al. (2014), a HPLC method was compared with an immunoassay method for TDM purposes in serum, and found that the immunoassay method was acceptable (maximum allowable error 15%) and may be used routinely. The HPLC and the immunoassay method displayed comparable values. LEV can also be measured in saliva as an alternative method for TDM (Kang et al, 2011). Numerous methods have been reported (Contin et al, 2008; Kang et al, 2011; Poongothai et al, 2011; Jiménez Moreno et al, 2014) for the determination of LEV in human plasma and serum with different HPLC methods different detectors and sample preparations. The availability of the HPLC methods should be simple, accurate, reliable and cost effective analytical assays which are essential for TDM to be successful. Other methods such as GCMS reported to have a lack of sensitivity, selectivity, reliability and be time-consuming for sample preparation when a high sample volume is involved. LCMS is considered a gold standard to utilize analysis for a more sensitive and selective method for the detection of LEV for TDM such as to verify compliance or management of overdoses (Kang et al, 2011; Jiménez Moreno et al, 2014).

2.5 THE SAMPLE MATRIX

Human blood, plasma and serum, is normally used in chromatographic analysis (Jiménez Moreno et al, 2014). Usually AED's are monitored in venous blood (plasma/serum) and that is the most important matrix for the interpretations of the drug concentrations (Jiménez Moreno et al, 2014). In general the results in plasma and serum are comparable. Serum is an appropriate sample for monitoring AED's, however, current literature suggests that serum tubes with the gel separators should be avoided for some AED drugs, especially Phenytoin, Phenobarbitone and Carbamazepine (Bowen & Remaley, 2014). These authors showed a 10% decrease in concentration which can be clinical significant.

Plasma is a useful alternative but lithium, heparin and sodium citrate anticoagulants should be avoided because they can interfere with drug-protein binding and it can contribute to matrix effect problems (Jiménez Moreno et al, 2014). These anticoagulants substantially decreased the total concentration of Phenytoin and Valproic acid levels by 20 – 50% (Bowen & Remaley, 2014).

2.5.1 Handling of blood samples

Care must be taken in the handling of blood samples to prevent haemolysis, and that changed the colour of the plasma or serum from straw yellow to red. Haemolysis can have an impact on the accuracy of laboratory tests (Tuck et al, 2010; Hansen et al, 2012). LEV can *in vitro* hydrolyse and that may decrease the serum concentration (Patsalos et al, 2006; Kang et al, 2011).

Numerous analytical chromatographic methods for the quantitation of LEV in blood and other biological fluids were reported in the literature (Pucci et al, 2004; Patsalos et al, 2006; Krasowski, 2010). However, only a few published studies used other matrixes such as saliva, urine, cerebrospinal fluids and hair (Pucci et al, 2004; Patsalos et al, 2006; Krasowski, 2010). The monitoring of concentrations in saliva is promising because it is easier to collect the samples, especially in children and elderly patients, and therefore less traumatic and invasive (Krasowski, 2010). Monitoring of drug levels is more difficult due to half-life in saliva compared to plasma/serum. Other challenges include difficulty to analyze little or viscous saliva of patients and problems with pipette accuracy (Krasowski, 2010).

Ideally measurements for LEV should be done in serum of plasma drawn at steady-state which is at least 4-5 half-lives after the first dose. The sample should be taken just before the administration of a dose to ensure trough levels that can be comparable where the distribution phase is formerly completed (Patsalos et al, 2006; Kang et al, 2011).

2.6 HIGH PERFORMANCE LIQUID CHROMOTOGRAPHY (HPLC) ANALYTICAL METHODS

After years of research, the HPLC-system is demonstrated as a remarkable technique and suitable for routine work, especially for analyses with high sample volumes. One of the main advantages of HPLC is its high specificity, reproducibility and reliability results in comparison with other methods, such as immunoassays, which face major problems with cross reactivity that can result in false negative or positive results. The HPLC technique also achieves precise and rapid results for the determination of different drug levels and creates chromatographic profiles to identify different analytes (Burghardt, 2006). HPLC technique is not costly, particularly when a method is developed where multiple AEDs' concentration levels can be measured simultaneously. Most of the authors found HPLC method development to be simple, rapid reproducible, specific and cost efficient. Using the HPLC system is demonstrated to be more cost-effective to maintain than Liquid chromatography mass spectrometry (LCMS), Gas chromatography-mass spectrometry (GCMS) and other technologies available in other pathology laboratories (Contin et al, 2008; Poongothai et al, 2011; Jiménez Moreno et al, 2014).

2.6.1 Definition of HPLC

High-performance liquid chromatography (**HPLC**; formerly referred to as **high-pressure liquid chromatography**), is a technique in analytical chemistry used to separate, identify, and quantify each component in a mixture. It relies on pumps to pass a pressurized liquid solvent containing the sample mixture through a column filled with a solid adsorbent material. Each component in the sample interacts slightly differently with the adsorbent material, causing different flow rates for the different components and leading to the separation of the components as they flow out of the column (Kazakevich & Lobrutto, 2007).

2.6.2 History of HPLC

Chromatography was discovered by a Russian botanist, Mikhail S. Tswett in the early 1900's (Tswett, 1906). Tswett's experiments were based on the separation of plant pigments from extracted plants using a solvent in a column packed with particles. As the sample passed through the column by gravity, different colours bands could be seen as certain compounds were moving faster than other compounds (Kazakevich & Lobrutto, 2007; Arsenault & McDonald, 2009).

Tswett's studies were focused on the separation of complex mixtures and described as a new form of phenomena of adsorption-based separation of complex mixtures. He later called it "chromatography" from the Greek words "colour writing". (Interestingly, Tswett in Russian means *colour*).

The chromatographic method was not appreciated at the time of discovery and nearly 10 years later L.S. Palmer and C Dhere published a similar separation process. In 1931, Lederer with Kuhn and Winterstein published a paper on purification of xantophylls on $CaCO_3$ absorption column (Kuhn et al, 1931; Kazakevich & Lobrutto, 2007).

A.J.P Martin and R.L.M Synge at Cambridge University of United Kingdom discovered partition chromatography in 1941 and were awarded the Noble Prize in 1952 (Kazakevich & Lobrutto, 2007).

In the mid 1960 Prof. Csaba Horváth developed a porous column layer open tubular column for gas chromatography columns packed with beads. Columns packed with small glass beads developed a significant resistance to liquid flow and forced to build an instrument that allowed a continuous flow of the liquid through the column. The real HPLC for a separation method was introduced by Prof. Csaba Horváth in 1970. In 2001, Prof. Csaba Horváth defined the meaning of the world's "performance" as "collective of the efficiency parameters" (Kazakevich & Lobrutto, 2007; Arsenault & McDonald, 2009).

Liquid chromatography has come a long way to understand the practical development of the HPLC system and the theoretical understanding of the different parameters involved (Kazakevich & Lobrutto, 2007).

2.6.3 HPLC System

A typical HPLC system consists of the following main components (Figure: 5, Typical HPLC system adapted from Levin (2010).

Degasser – Degassing is necessary to prevent bubbles of a sufficient amount of solvents (mobile phase) for continuous operation of the system in the detector cell which can cause noise in the detector signal (Kazakevich & Lobrutto, 2007, McMaster, 2007).

Pump – The pump delivers the mobile phase at a constant flow rate. It forces the eluent through the column and detector. The pump provides constant flow regardless of the back-pressure of the column up to 400 bar (Kazakevich & Lobrutto, 2007; Arsenault & McDonald, 2009).

Column – The column is the heart of the HPLC system. The C18 column is made from stainless steel and is built to withstand high pressure. The inside is packed with small particles of porous silica, alumina or organic resin that contains the stationary phase. It separates the sample compounds by the interaction between the surface of sample compounds and the materials (silica gel with fractional group and resin) in the column (Kazakevich & Lobrutto, 2007; Arsenault & McDonald, 2009).

Injector – This allowed an injection of the analyte mixture (sample) into the mobile phase before it enters the column. Most of the injectors are autosamplers and programmed to inject different volumes (1-100 µl) of samples from the vials in autosampler tray (Kazakevich & Lobrutto, 2007; Arsenault & McDonald, 2009).

Detector - Identifies the presence of the specific compounds of interest in the eluent from the column**.** The type of detector depends on the characteristics and concentrations of the compounds that are needed to be separated and analysed (Arsenault & McDonald, 2009). In this study the UV detector will be used and will be discussed in more detail in section 2.6.5. (Kazakevich & Lobrutto, 2007; Arsenault & McDonald, 2009).

Data processor: - Is connected to a computer with a software program that translates the analogue signal from the detector to a digital signal in order to give an electronic interpretation of the data found. The output is in the form of a

chromatogram. The solvents, mobile phase, stationary phase, pH and temperature play an important role in the outcome of the chromatogram with all the different analytes separated into individual peaks, as shown in the final picture of the chromatogram (Kazakevich & Lobrutto, 2007; Arsenault & McDonald, 2009; Levin, 2010).

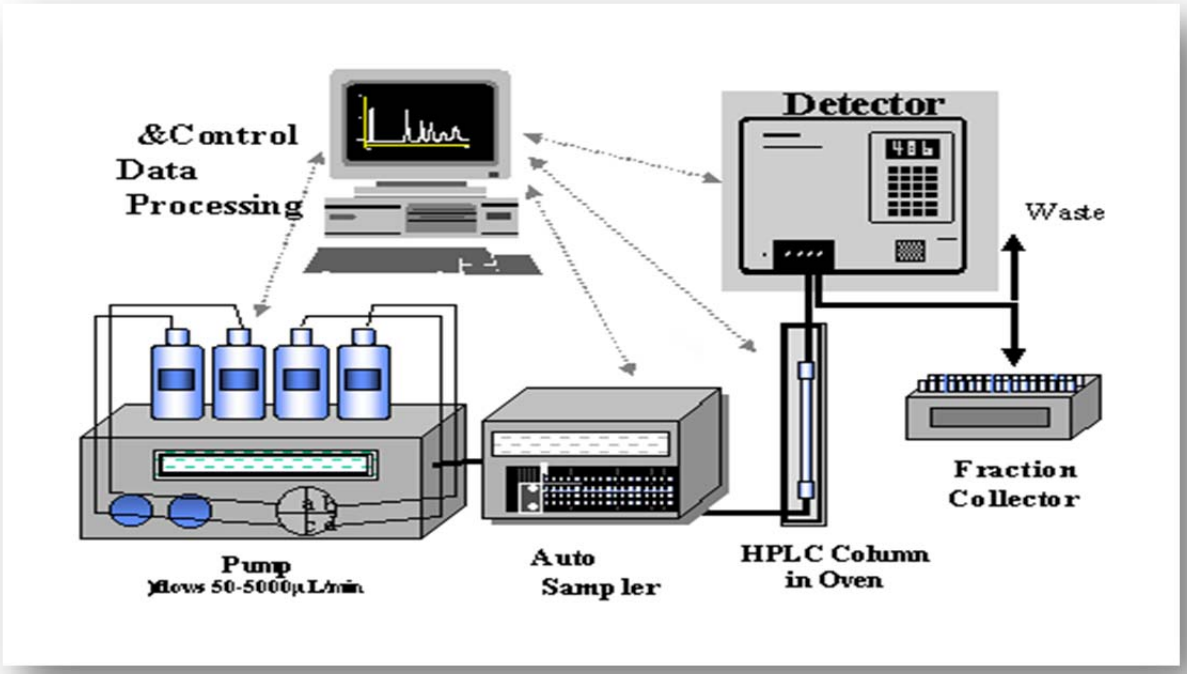


Figure 5 Typical HPLC system adapted form Levin (2010).

2.6.4 HPLC separation methods

There are two basic separation methods used in HPLC:

1) **Isocratic elution:** Where the mobile phase is a pure solvent or a mixture (Buffer and solvent). The mobile phase remains in one bottle throughout the run and only one pump is used. The mobile phase can also be recycled (Kazakevich & Lobrutto, 2007; Arsenault & McDonald, 2009).

2) **Gradient elution:** Where the mobile phase configuration changes during the separation of the compound. This method is useful for samples that have a span range of chromatography polarity. Two bottles of solvents and two pumps are involved and a predetermined timetable. Solvent A contains a higher concentration of a weaker solution (Buffer) over a certain time and Solvent B a

stronger (Arsenault & McDonald, 2009). The two streams are combined in the mixer to create the actual mobile phase composition that is delivered in the column over time. As the separation continues, the elution strength of the mobile phase is increased to elute the more strongly retained sample compounds where each of the pumps' speed is managed by the gradient controller to deliver more or less of the solvents for the separation of the compound (Kazakevich & Lobrutto, 2007; McMaster, 2007; Levin, 2010).

2.6.5 Ultra violet (UV) Detector

The Ultra Violet (UV) detector is suitable for most compounds of interest. The UV detector can use many solvents including the ones useful for reverse-phase separations and transparent to UV.

There are three major types of UV detectors: the fixed wavelength, variable wavelength and diode array (major detectors with their advantages that are summarized in Table 4 (Accessed from Hitachi-high technologies, 2014). The UV detectors connected to the HPLC is based on absorption of the analytes of the UV light. The wavelength ranges from 190 – 400nm. The lower limit of detection is not necessarily sensitive enough to the analysis of low concentrations of drugs in biological materials. A UV detector contains a deuterium discharge lamp (D2 lamp) as a light source to produce a broad spectrum of wavelengths that are separated by a diffraction grating. A diffraction grating consists of a large number of very fine grooves engraved into a highly polished surface. The grating works like a prism where the light from the grating is reflected to a barrier covering a small slit. The instrument is adjusted so that only the wavelength of interest passes through the slit. The selected wavelength is passed through the sample. Some of the light is absorbed by the sample and the amount passing through the sample is measured and is proportional to the concentration of the absorbing compound. Sensitivity of the substances should be measured at their maximum UV absorbance. These UV detectors can select a single or dual wavelength of light to pass through the sample (Hansen et al, 2012).

Table 4 Major types of detector (Accessed from Hitachi-High Technologies, 2014).

Types of Detector	Description of detector
UV detector	The light source is a D2 lamp. This detector is used mainly to detect components having an absorption wavelength of 400 nm or less in the ultraviolet region.
UV-VIS detector	A D2 lamp and a W lamp are used as light source. This detector is effective in the detection of colouring components such as dyes and stains because of coverage of the visible light region.
Diode array detector (DAD)	Data on the spectrum from the ultraviolet to visible light range is also collected.
Fluorescence (FL) detector	Fluorescent substances can be detected specifically with high sensitivity.
Differential refractive index (RI) detector	Change in the refractive index is detected. Components absorbing no ultraviolet light can also be detected despite low sensitivity.
Conductivity detector	Mainly inorganic ions are detected by monitoring the conductivity.

Another type of detector is the Diode Array detector (DAD) that will be utilised in this study. The DAD offers more possibilities to measure the intensity of the array of the UV radiation. Figure 6 shows an illustration of a Diode Array Detector (Agilent Technologies, 2015). The photodiode array detector (DAD) passes a wide UV spectrum of light through the sample and then the light is separated into individual wavelengths after passing through the sample. The spectrum of light is directed to an array of photosensitive diodes. Each diode can measure a different wavelength which allows for the monitoring of many wavelengths at once. Only one or two wavelengths are monitored during a chromatographic run on the UV detector. Monitoring two peaks instead of one can provide information on the purity of the peak, or it can be used to quantify a peak when an interfering peak is present. A photodiode array can also be used to monitor two compounds that have different spectra. It is also possible to obtain a complete spectrum of a molecule and to identify the compound of interest, to choose selected wavelengths where different substances in the sample can be detected at an optimal wavelength. (Arsenault & McDonald, 2009).

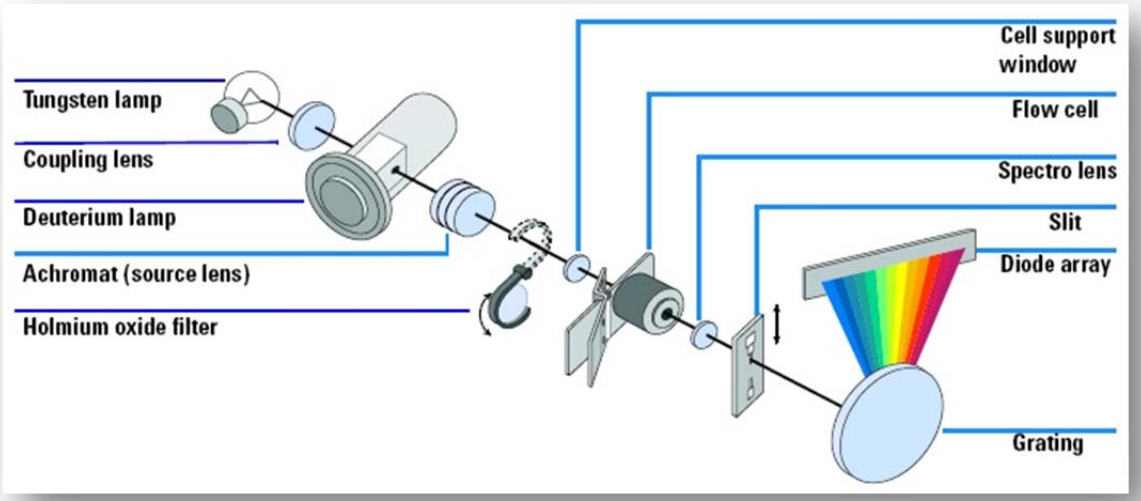


Figure 6 Illustration of the Diode Array detector (DAD) (Accessed from Agilent Technologies, 2015).

2.7 OPTIMISATION OF AN HPLC METHOD

Development and validation of an analytical method are the key elements to identify and quantify the compound of interest (Singh, 2013). Various steps for the development of an HPLC method are given in Figure 7. These include sampling, sample preparations, separation, detection, identification, calibration and quantification (Hansen et al, 2012).

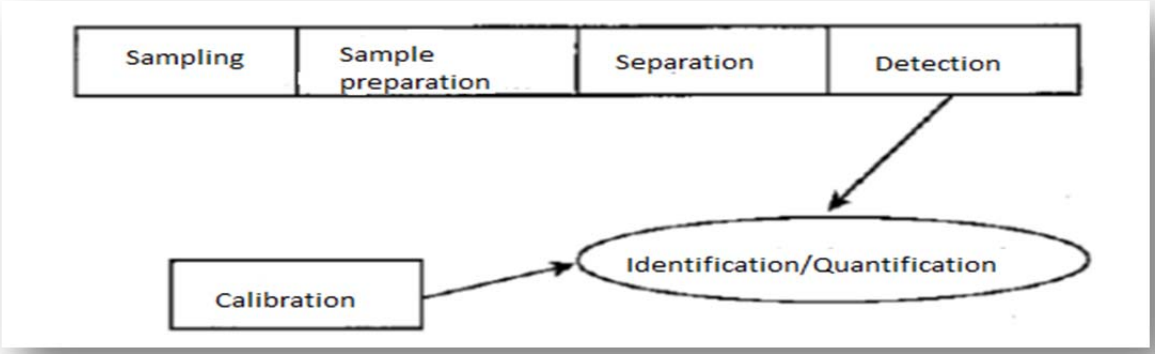


Figure 7 Steps involved in HPLC method development (adapted from Hansen et al, 2012).

Development of a method is usually based on existing literature or to improve the current method by modifying existing methods or instruments to current requirements of the methods (Prathap et al, 2013). Development of a new method

on HPLC comprises the following factors to be taken into account during the process of optimising the method (Prathap et al, 2013; Singh, 2013; Arora & Gangadharappa, 2016):

- Best detector.
- The composition of the mobile phase.
- The pH of the mobile phase.
- The analytical column.
- Internal standard for the column.
- The flow rate of the mobile phase through the HPLC.
- The optimal injection volume of the standards and samples.
- The sample preparation.
- The calibration range for the standards.
- The lower limit of detection (LLOD).

2.7.1 Detection wavelength

The UV detector is based on the absorption of UV light. For the highest sensitivity to detect the sample components that contain chromophore there should be at least one double bond in the molecule of the analyte (Hansen et al, 2012; Prathap et al, 2013). UV below 200nm should be avoided because detector noise increases in this area. Higher wavelengths give better selectivity. It is important to use reagents and solvents of high purity to minimise detection limits for optimal sensitivity. Organic and buffer salts absorb in the UV range and the detection limit is related to wavelength (Prathap et al, 2013; Arora & Gangadharappa, 2016).

2.7.2 Optimal mobile phase and pH

The optimization of the mobile phase composition (i.e. buffer type and concentration of the organic solvent and the optimization of the pH) is the most powerful way of optimizing selectivity (Prathap et al, 2013). The selectivity varies on changes in the pH of the mobile phase and should be tested to achieve the successful separation of the chromatographic process. The two most widely organic solvents to be used are

acetonitrile and methanol which are less viscous and UV transparent (Prathap et al, 2013).

Many buffer salts have been used in ion-exchange chromatography. It is helpful to choose a buffering salt that has a functional group that is similar to the analyte e.g. dihydrogen phosphate (KH_2PO_4) to elute nucleotides which are organophosphates. The buffer should be chosen to be undetectable by the detector that will be used (Arora & Gangadharappa, 2016).

pH plays an important role in completing the chromatography separations and controls elution properties by controlling the ionization characteristics (Prathap et al, 2013). Three main factors play an important role on the different pH ranges for a better chromatographic resolution between two or more peaks of the analyte column's efficiency, selectivity and retention time. To achieve optimum results for better separations, one should start to investigate at low pH and progress then to higher pH (Singh, 2013; Arora & Gangadharappa, 2016).

Changing the pH of the mobile phase can improve column efficiency because it alters the ionization of the analyte (Singh, 2013). It is important to keep the pH of the mobile phase in a range of 2.0 – 8.0 (Prathap et al, 2013). To get a sharp peak, low tailing factor and the base line separation of the components, a number of experiments were carried out by variable compositions of a variety of solvents and flow rate. Mixtures of solvents like methanol, distilled water and acetonitrile with or without buffers and various combinations were tested in a C18 column. In this case, a mobile phase that contained phosphate salts was suitable for this research project set at pH close to neutrality and was more volatile (Prathap et al, 2013; Singh, 2013).

2.7.3 Optimal column

The HPLC column stationary phase is where the separation occurs and is the most important part of the system. Different types of analyses are classified based on the type of stationary phase and mechanism behind the separation in the column.

Three main characteristics of chemical compounds can be used to create HPLC separations modes:

- **Polar Interactions:** Differences in polarity between the sample components and the bonding entities on the stationary phase result in preferential retention.

    - Reverse Phase HPLC Separations

      Stationary phases mostly comprise of non-polar alkyl hydrocarbons such as C-8 or C-18 chains bound to silica or other inert supports. Mobile phase is polar and the elusion order is polar followed by less polar and weakly polar or non-polar compounds in the end. Disadvantage of producing a relatively highly viscous mixture in methanol with water, giving rise to much higher pressures than other mobile phases (Arsenault & McDonald, 2009; Bhanot, 2014).

    - Normal Phase HPLC Separations

      Normal Phase separations are the opposite of reverse phase separations. The stationary phases are polar, having either plain silica or organic compounds such as amino, cyano propyl, Diol and N-propylethylenediamine groups bound to silica based supports. Mobile phases are non-polar such as hexane and heptane with small quantity of polar modifiers such as methanol, ethanol and isopropanol. The elution order is non-polar molecules followed by weakly polar and polar molecules at the end. The main disadvantages of normal phase chromatography lack of selectivity of the stationary phase. All compounds are eluted in the same order regardless of the stationary phase selected. The mobile phase is therefore used to achieve any change in selectivity. (Arsenault & McDonald, 2009; Bhanot, 2014).

- **Ionic Interactions:** Separation based on charge properties of sample molecules. Analyte ions have affinity for oppositely charged ionic centres on the stationary phase.

    - Ion Exchange Chromatography

      Synthetic organic resins are normally working for separation or water soluble ionisable compounds. Anion exchangers have positive centres on surface and used to separate compounds having sulfonate, phosphate or carboxylate groups. Cation exchangers have negative centres on the surface and are

used to separate positively charged ions on a negative surface basic substances such as amines. Cross-linked styrene divinylbenzene is typical base material with charged groups linked to phenyl rings. Charges on packing material attract oppositely charged molecules from mobile phase and release them in inverse order of the attraction forces. Separation of components can be controlled by control of pH of mobile phase, temperature, ionic composition and addition of modifiers. One of the main disadvantages of ion exchange chromatography is its buffer because binding to the resins dependent on electrostatic interactions between proteins of interest and the stationary phase. These columns must be loaded in low-salt buffers (Arsenault & McDonald, 2009; Bhanot, 2014).

- **Molecular Size -** Separation takes place through the entrapment of small molecules in the stationary phase pores. Large molecules pass through first followed by elution of smaller trapped molecules.

  - Size Exclusion Chromatography

    Separation takes place on basis of molecular size of molecules. Small molecules penetrated more of the stationary phase pores and exit after the large molecules. Larger molecules only penetrated pores above a certain size and spend less time in the column. There are no chemical or ionic forces involved in the separation process. Such phases are available with silica or zirconium backbones with heavily cross-linked polymers and are used for separation of large molecules such as polysaccharides, peptides, proteins and polymers (Bhanot, 2014).

After an understanding of stationary phase it is necessary to understand mobile phases which primarily serve to carry the sample through the HPLC system (Bhanot, 2014). Column length with the same stationary phase has significant effect on separation. Long and wide columns can take higher sample loads and provide higher resolution where the shorter columns reduce analysis time resulting in lower mobile phase consumption (Bhanot, 2014).

In HPLC, column selection makes all the difference. It could significantly increase the reliability and reproducibility of separation results as well as saving time and

costs. The most important factors to consider are the sample's content and the goal to develop an analytical method (Agilent Technologies, 2015).

The main advantage of column chromatography is the relatively low cost and disposability of the stationary phase used in the process. The most prominent advantage is its applicability to diverse analyte types, from small organic molecules and ions to large biomolecules and polymers. The successful coupling of HPLC to mass spectrometry (MS) gave it an invincible edge as "the perfect analytical tool" combining excellent separation capability with the unsurpassed sensitivity and specificity of MS. HPLC-MS is rapidly becoming the standard platform technology for bioanalytical testing (drugs in biological fluids), trace analysis for residues in food, forensic and environmental samples, and life science research. The excellent precision and robustness of HPLC with UV detection makes it an indispensable tool for quality control (QC) (Agilent Technologies, 2015).

To select a column depends on the nature of the solute and the information of the analyte regarding which is the most important step in method development and to make sure the columns are stable and reproducible to avoid problems from the sample retention during method development, and cost effectiveness (Prathap et al, 2013).

To select the right column, detailed information about the sample components is important. Where a sample contains a high number of analytes, a reversed-phase HPLC mode is most suitable. Analyte pKa values determine what pH should be used. The concentration range limits the injection or requires larger columns. In general, no more than 10% of the column volume should be injected, and the concentration should not exceed about 5 mg/g, if a high number of theoretical plates are desired. Finally, pore size should suit the analytes' molecular weight (Arora & Gangadharappa, 2016; Merck Millipore, 2016).

Column chemistry depends on:

- Structure of sample components
- Solubility
- Log P value
- Number of compounds present

- pKa values of sample components
- Concentration range
- Sample matrix
- Molecular weight range
- Other relevant data

(Merck Millipore, 2016)

Columns should offer reasonable resolution in the initial method, short running time and acceptable pressure drop for different mobile phases (Prathap et al, 2013).

C18 columns are common and specifically designed for the separation of basic compounds and suitable for a variety of samples (Prathap et al, 2013). Columns with a 5µm particle size gave the best compromise of efficiency, reproducibility and reliability.

In HPLC, a solvent containing the sample is injected at one end of the column. The affinity of the sample for the column packing as well as the mobile phase will help separate the molecules in the solvent. C18 columns actually can handle more than 60% of the applications in most HPLC labs. Mobile phase is polar and the elution order is polar followed by less polar and weakly polar or non-polar compounds in the end (Bhanot, 2014).

C18 columns are bonded to the maximum density to have the lowest polarity and the lowest interaction of analytes with silanol. It also has to have a high stability at high pH and the widest pH range. For most samples, a short column is recommended to reduce the method's running time (Levin, 2010; Prathap et al, 2013).

2.7.4 Flow rate

Relative minor publications have been concentrating on the effects of other variables such as temperature and flow rate on HPLC chromatography recently. According to McCalley (2000) the optimum flow rate for highest efficiency is often lower for basic compounds than neutrals, where high flow rates appeared to be damaging the basic compounds in chromatography. The flow rate is the volume of the mobile phase passing through the column in unit time and is expressed in ml/min (volume/time). The flow rate is important to the liquid chromatography techniques especially in

separation of the compounds where retention times are the key to identify the analyte. Precision and reproducibility of the flow rate is important to many HPLC techniques, where the retention time are the key to identify the analyte (Arsenault & McDonald, 2009).

2.7.5 Temperature

Sample separations can be improved by choosing the correct column temperature. A higher column temperature decreases system backpressure by reducing the mobile phase viscosity (Hansen et al, 2012; Prathap et al, 2013). This is obtained when a longer column with higher separation efficiency is used. Selecting the best optimal temperature depends on the nature of the combination of the components and the strength of the solvent of the mobile phase (Singh, 2013).

2.7.6 Retention time

The time taken for a particular compound to travel through the column to the detector is known as **retention time**. This time is measured from the time at which the sample is injected to the point at which the display shows a maximum peak height for that compound. Different compounds have different retention times. For a particular compound, the retention time will vary depending on many factors such as:

- the pressure used (because that affects the flow rate of the solvent).
- the nature of the stationary phase (not only what material it is made of, but also particle size).
- the exact composition of the solvent.
- the temperature of the column.

(Moldoveanu & David, 2013)

The conditions, therefore, have to be carefully controlled if one wants to use retention times as a way of identifying compounds.

Retention time can be improved by changing the pH of the mobile phase for better separation of the analyte and internal standard (IS) to improve column efficiency. Although the retention time depends on many factors it has still no effect on the compound, IS, method and the chromatogram (Singh, 2013).

2.7.7 Volume of injection

The solution injected into the HPLC should be compatible with the mobile phase and exchange of the solvent from the sample (Hansen et al, 2012).

2.8 SAMPLE PREPARATION

There are several reasons why the analysis of drug substance in biological samples can be complicated (Hansen et al, 2012; Prabu & Suriyaprakash, 2012; Jiménez Moreno et al, 2014).

- Good sample preparation is the key to successful analytical results and has the impact on accuracy, precision and quantification limits (Prabu & Suriyaprakash, 2012).
- Sample preparation is required to reduce the complex mixtures in blood samples and to make them more compatible for chromatographic methods (Hansen et al, 2012; Jiménez Moreno et al, 2014). There are several reasons why the analysis of drug substance in biological samples can be complicated (Hansen et al, 2012; Prathap et al, 2013).
- Samples (complex mixtures) can be a risk when they contain a substance that can give a response interference). The substance can sometimes not be separated from the analyte during the analytical measurement.
- The presence of proteins in blood samples can also destroy the analytical instrument and can clog the HPLC column (Jiménez Moreno et al, 2014).
- Samples can be incompatible with the type of analytical instruments.
- The drug concentrations can be too low to be detected; below the LOQ (lower limit of quantification) (Jiménez Moreno et al, 2014; Arora & Gangadharappa, 2016).

2.8.1 Methods for sample preparations

Sample preparation is the matrix clean-up procedures usually for chromatographic analysis to remove as much endogenous material as possible from the drug samples

(Prabu & Suriyaprakash, 2012; Arora & Gangadharappa, 2016). Sample preparation is carried out by the following three methods that will be discussed briefly:

- Protein precipitation(PP)
- Liquid-liquid extraction (LLE)
- Solid-phase extraction(SPE)

Protein precipitation (PP) is used for serum and plasma samples that contain proteins (Hansen et al, 2012). The main purpose of PP is to eliminate the proteins that can cause problems, as the proteins can clog the column of the HPLC. PP is a popular sample preparation method and the procedure is very simple, fast and cost effective and fits into the daily routine (Pucci et al, 2004). Plasma or serum samples are mixed with acetonitrile (ACN) (polar solvent) and the proteins start to precipitate (see Figure 8). The sample is centrifuged and the clear supernatant (containing the analyte) is collected for the final analysis and ready for injection into the HPLC/LCMS (Hansen et al, 2012). ACN gave better results in terms of % recovery, the sensitivity and linearity obtained used for monitoring drug levels, and the supernatant is almost free of proteins (Jiménez Moreno et al, 2014). Acetone and methanol (MeOH) are less efficient than ACN (Pucci et al, 2004; Hansen et al, 2012). Trichloroacetic acid, perchloric acid and other metal ions can also be used as protein precipitation (Pucci et al, 2004; Hansen et al, 2012). The protein can be precipitated by addition of 10-20% trichloroacetic acid or five volumes of a water-miscible solvent like ACN (Prabu & Suriyaprakash, 2012). One of the disadvantages of PP is that the back pressure of the HPLC system may increase. The components of plasma which are soluble in diluting solvent that bound to the mobile phase will then affect the column performance (Prabu & Suriyaprakash, 2012).

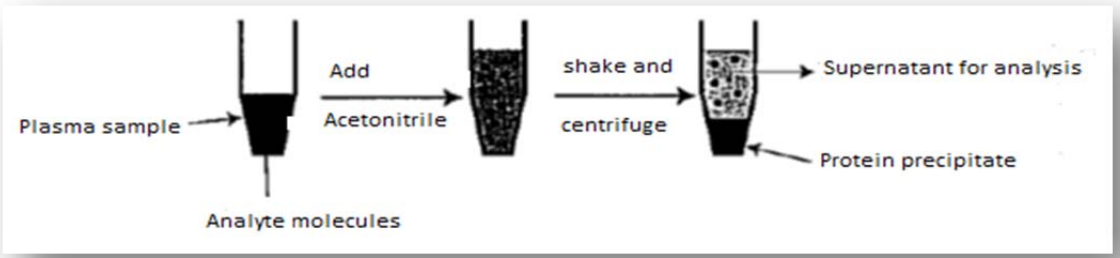


Figure 8 Principle of protein precipitation (adapted from Hansen et al, 2012).

Liquid-liquid extraction (LLE) is used for plasma or serum blood samples, urine and other pharmaceutical products like solutions and ointments (Pucci et al, 2004; Hansen et al, 2012; Jiménez Moreno et al, 2014). The aqueous sample is mixed with an organic solvent that is immiscible with water, resulting in a two phase system (Figure 9). The second phase is shaken to ensure that the small droplets of the organic phase mix with the water phase and the analytes are transferred from the aqueous sample to the organic liquid. The organic phase is called the extract. After shaking the mixture is centrifuged where the two phases separate again. The extract which is the organic phase contains the analyte and is used for the final analysis (Pucci et al, 2004; Hansen et al, 2012). Chloroform gave good results in the LLE procedure. LLE is associated with many drawbacks such as long procedures to be done during the extraction process where much of the analyte will often be lost and the use of toxic organic solvents (Prabu & Suriyaprakash, 2012; Jiménez Moreno et al, 2014). Disadvantages in LLE procedure is that it is time consuming, large solvent consumption is needed for extraction and two immiscible phases were used for extraction procedures. LLE technique is not suitable for several analytes. An evaporation step is needed to remove excess organic solvents (Prabu & Suriyaprakash, 2012).

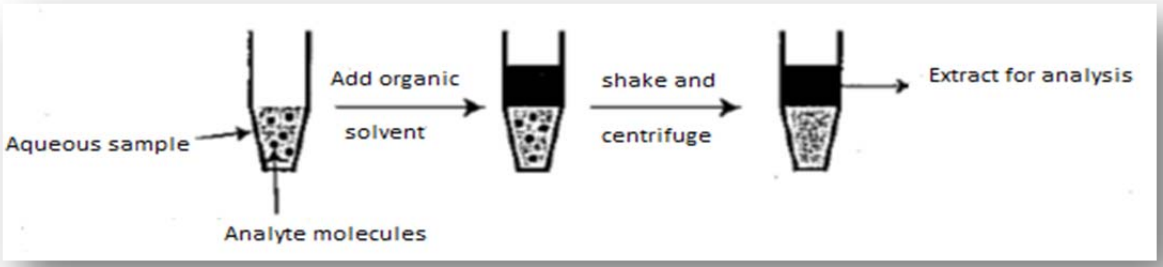


Figure 9 Principle of liquid-liquid extraction (adapted from Hansen et al, 2012).

Solid phase extraction (SPE) is to be implemented for the clean-up of the plasma, serum and urine samples. SPE is based on separation of the analyte between the aqueous biological fluid and a solid phase (Pucci et al, 2004; Hansen et al, 2012; Jiménez Moreno et al, 2014). SPE is a small column that is packed with a stationary phase and the sample is drawn through the column. This is called the loading step (See figure 10). The analytes are retained in the column by different types of

interactions with the stationary phase. The second step is called the washing step and is the step where the column is washed to remove the matrix components and the analytes remain in the column (Pucci et al, 2004; Hansen et al, 2012). The final step is the elution where the analytes are eluted from the column with a solvent that breaks the bond between the analytes and the stationary phase. The eluate is collected and used for the final analysis. SPE may not always be the choice, depending on the nature of the analyte (Prabu & Suriyaprakash, 2012).

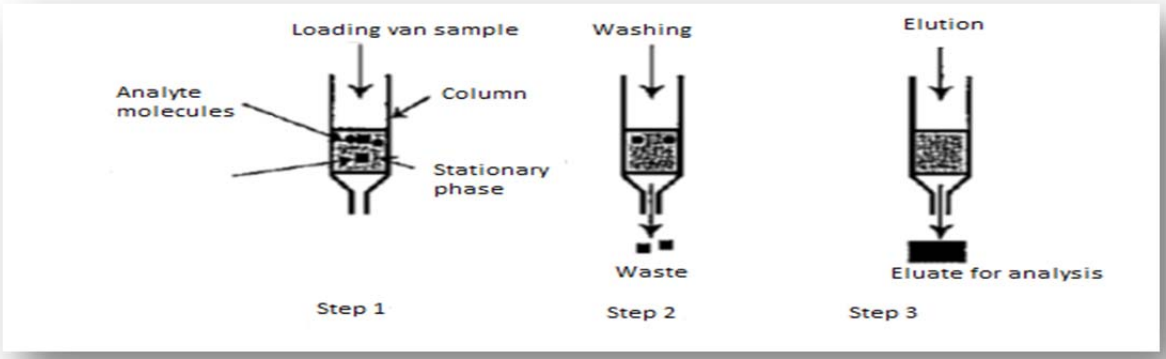


Figure 10 Principal of solid phase extraction (adapted from Hansen et al, 2012).

2.9 INTERNAL STANDARD (IS)

The Internal standard (IS) is important to improve the precision and accuracy of the results for various analytical errors due to sample losses and variable injection volumes (Hansen et al, 2012; Usher et al, 2015). The IS is a known compound of fixed concentration which is added to known concentrations of standard solutions and to the sample test before sample treatment. The ratio of the peak area/height of the target compound in the sample or sample extract to the peak area/height of the internal standard in the sample or sample extract, is compared to a similar ratio derived for each calibration standard (Hansen et al, 2012; Usher et al, 2015). The internal standard can improve precision when the dominant sources of error are related to sample preparation or injection. With these errors the internal standard and the sample (analyte) peak/height ratio become unaffected. To choose a suitable internal standard, the following requirements are needed (Hansen et al, 2012; Usher et al, 2015):

- It must have a clear peak with no interferences and separated from all the other substances in the sample.
- It must be corresponding to the analyte of interest in the sample preparation procedure.
- It must be used in a concentration that will give a peak area/height ratio in the same range as the analyte.
- It must have the same detector response as the analyte.
- It must not be present in the original sample.
- It must be stable, unreactive with sample components, column packing and the mobile phase.

To be able to recalculate the concentration of a sample component in the original sample, one has to determine the response factor first (Hansen et al, 2012). The peak area of the analyte and the peak area of the IS were determined from the chromatogram where the ratio of the analyte/IS of each solution were calculated and plotted as a function of concentration. The calibration should be a straight line (Hansen et al, 2012; Usher et al, 2015).

2.10 METHODS FOR THE DETERMINATION OF LEV IN HUMAN PLASMA/ SERUM

Different methods and equipment for the determination of LEV were reported in the literature: HPLC, Ultra High performance liquid chromatography (UHPLC), GCMS and LCMS (Valamathy et al, 2008; Poongothai et al, 2011; Shah et al, 2012). The literature also discussed a number of HPLC methods where a UV detector was used for the detection of LEV in human biological samples. A summary of these methods can be seen in Table 5.

Table 5 Previous results from literature.

Author	Method	Comment
Martens-Lobenhoffer & Bode-Böger, 2005	Method for the determination of LEV in human plasma with minimal sample preparation on HPLC.	This method described as easy, cheap, and quick, making it suitable for routine TDM. This method has also been successfully applied to a large number of patient samples.
Contin **et al**, 2008	A simple, fast, validated method, using HPLC with UV detection to determine LEV in deproteinized plasma of patients with epilepsy.	The validation is reliable and required minimal sample preparation, which allows large quantities of human samples to be analysed in a short period of time, making it very practical in TDM.
Poongothai **et al**, 2011	HPLC methods using UV detection in human biological samples.	Method for LEV is specific, sensitive and reliable; seen as an improvement of quality in human plasma / serum of patients using LEV.
Guo **et al**, 2006	A simple and rapid LCMS method for the determination of LEV in human matrix (plasma, serum or saliva.	Method required as little as 50 µl of patient sample with minimal sample preparation, making it specifically suitable for a high throughput environment such as clinical or reference laboratories.
Matar, 2008	A sensitive LCMS method for the determination of LEV in plasma.	Most of the LCMS methods reported lack sensitivity for LEV in routine monitoring laboratories.
Valamathy **et al**, 2008	The determination of LEV in pure drug, pharmaceutical dosage formulas and included a RP (reverse phase) – HPLC.	Method was found to be simple, accurate, precise and rapid and could be used for routine analysis
Pucci **et al**, 2004	HPLC determination of LEV in human plasma: comparison of different sample clean-up procedures.	Reliable HPLC method for LEV in human plasma is described. .SPE procedure is best suited, but it is preferable to use protein precipitation with zinc sulphate or Methanol, because it is faster and simpler.
Shah **et a**l, 2012	Developed and validated a stable RP-HPLC method for the quantitative determination of LEV in tablet dosage forms	Method has also been successfully applied for chemical kinetics. A stress testing procedure also indicated that this method was selective and stable. Method is simple, accurate, precise and has the ability to separate the drug from degradation products and excipients found in the dosage forms.

For this study, the principles of the methods described by Pucci et al. (2004); Contin et al. (2008) and Poongothai et al. (2011) were adapted and modified to develop and validate a method to be used in the CPL.

It can therefore be hypothesized that this method for LEV in human plasma/serum will be specific, sensitive and reliable; seen as an improvement of quality in patients using LEV. These methods will also provide minimal sample preparation and will be cost effective for a routine monitoring laboratory.

2.11 ANALYTICAL METHOD VALIDATION

The main objective of an analytical method validation is to demonstrate that the method is suitable for its intended purpose before implementing it into routine use (Singh, 2013).

If any changes were made to suit the requirements of the laboratory, from previously validated or published methods, it should be ensured that these modifications meet with the current validation criteria or re-validation will be required to ensure the method is specific, sensitive and reliable.

The fundamental parameters to be validated include the following: EMA, 2011; FDA, 2013; ICH, 2015 and the general requirements of ISO 17025 (2005) will be discussed below.

2.11.1 Specificity

Specificity refers to accurately measuring the analyte in the presence of compounds that may be expected to be present, which may include impurities, degradation products and matrix compounds. The specificity of an assay is the capability of the assay to differentiate similar analytes or other interferences from the matrix that could have a positive of negative effect on the assay value. Peak purity should be used to demonstrate that the analyte's chromatographic peak is not recognised as more than one compound. These peak purities are founded on spectra which are record on the UV detector (FDA, 2013; Arora & Gangadharappa, 2016).

Specificity has the following implications:

- to ensure the identity of an analyte

- to ensure that all analytical procedures performed allow an accurate statement of the content of impurities of the analyte

- to provide an exact result in the assay allowed and accurate statement of the content of potency of the analyte in sample
  (FDA, 2013)

2.11.2 Selectivity

Selectivity means that the method can be used to determine specific analyte and IS in mixtures or matrices without interference from other components. Selectivity is the ability of an analytical method to differentiate and quantify the analyte in the presence of other compounds in the sample. Indication should be provided that the substance to be quantified is intended to be the specific analyte. At least six sources of blank samples from the appropriate matrix (plasma/serum) should be obtained and each blank sample should be tested for interference and the selectivity be ensured at lower limits of quantification (LLOQ) (FDA, 2013).

According to the EMA, (2011) any absence of interfering compounds is accepted where the response is < 20%of the LLOQ for the analyte and 5% for the IS.

2.11.3 Accuracy

Accuracy describes the closeness of mean test results to the true value (concentration) of the analyte of interest. Accuracy is determined by replicate analyses of samples containing a known concentration of the analyte. Inter- and intra-day determinations are important.

Intra-day accuracy is calculated by a minimum of five (5) determinations per concentration and a minimum of three (3) concentrations in the calibration range of expected concentrations, and should be analysed on the same day (EMA, 2011; FDA, 2013).

Inter-day accuracy is calculated in the same way as intra-day accuracy, but the 5 determinations should be done on 5 different days. The % RSD (percentage relative standard deviation) of the mean value should be less than 15% of the actual value except for values at LLOQ, where it should be less than 20%. The measurement of

accuracy is expressed in the deviation of the mean from the true value (Singh et al, 2008; EMA, 2011).

2.11.4 Precision (Reproducibility)

Precision is the ability to measure the closest of individual measurements of an analyte where the analytical procedure is applied repeatedly to multiple preparations of the same homogeneous sample on the same HPLC instrument. Precision will be calculated by measuring a minimum of 5 determinations per concentration and a minimum of 3 concentrations in the calibration range of expected concentrations. The % RSD determined at each concentration level should be less than 15% of the actual value except for values at LLOQ, where it should be less than 20%. Precision measures the repeatability of the whole analytical procedure (EMA, 2011; FDA, 2013).

2.11.5 Lower limit of detection (LLOD)

The LLOD is the lowest concentration that can be detected in the analyte under the analytical procedures. It is not necessarily to be quantified but only to be notable from the baseline above the noise level of the system, typically three times the noise level with a certain degree of certainty. For example an LLOD criteria is that at the 0.05% level, an impurity will have S/N ≥3. LLOD should be determined theoretically by using regression data (EMA, 2011; FDA, 2013).

2.11.6 Lower limit of quantification (LLOQ)

The LLOQ is the lowest concentrate of an analyte that can be quantified with suitable accuracy and precision measured. The LLOQ should be the lowest concentration value of the calibration curve range. The LLOQ value should be determine by the presence of a baseline, background noise (accuracy) and the precision of the analytical procedure (reproducibility) and should be determined theoretically by using regression data (EMA, 2011; FDA, 2013).

2.11.7 Calibration curve / Linearity

Linearity is the ability to obtain test results that are directly in correlation to the sample concentration over a range (FDA, 2013). For the HPLC methods, the

relationship between the detector response (peak area or height) and the known concentrations (amount) of the analyte of interest is to be expressed in the calibration curve (standards). Each analyte should have its own calibration curve. Depending on the monitoring value of the study a minimum of six to seven concentrations (standards) should be enough to generate a calibration curve to cover the therapeutic concentration range (FDA, 2013). It should cover 50% below the lower therapeutic range and about 50% above the therapeutic concentration range. The calibration curve data should contain a blank sample (sample analysed without an internal standard (IS) or a zero sample (sample with IS) to improve the error in the intercept (FDA, 2013). Acceptability of linearity is usually examining the correlation coefficient (r). The importance of the calibration curve is to form a linear curve with an r^2-value not less than 0.998. Least square analysis should be carried out for the correlation coefficient and the linearity curve of LEV should be within a concentration range of 2-60 µg/ml (Krasowski, 2010). The curve should be used on a daily basis to calculate the concentration levels of LEV in the sample in the study and from the patients. For a more accurate evaluation of the linearity, analysis of variance (ANOVA) could be used with the correlation coefficient and the plot of the residual values (Boqué et al, 2002). If the r-value is in doubt a t-test or f-calc can be carried out to prove the significant linearity and meet with the current validation criteria (EMA, 2011; FDA, 2013; Arora & Gangadharappa, 2016). Chromatograms will be obtained from water, plasma and serum spiked with a mixture of LEV and IS.

2.11.8 Range

The working range of an analytical method is the interval over which the method provides results with an acceptable uncertainty. The lower analytical concentration of an analyte is bounded by the LLOQ. The upper analytical concentration of the working range is defined by the concentrations at which important differences in the analytical sensitivity are observed (FDA, 2013). The working range of an analytical method is the interval between the upper and lower analytical concentrations that has demonstrated acceptable levels of accuracy, precision and linearity obtained (Donga, 2006). The range that is used is usually the same as for the calibration curve. The minimum specified ranges are from 80 – 120% of the test concentration (ICH, 2015). A typical range criterion could include a precision of < 2.0 % RSD (Arora & Gangadharappa, 2016).

2.11.9 Robustness

Robustness measures the analyte of interest's capacity to remain unaffected by small but deliberate variations in the analytic method's parameters. Ruggedness provides an indication of the method's reliability during normal usage (FDA, 2013). The analytical method's parameters that can change are the flow rate of the mobile phase, the pH of the mobile phase, the percentage of the inorganic phase in the mobile phase and the column's temperature (FDA, 2013; Arora & Gangadharappa, 2016).

2.11.10 Percentage recovery (% recovery)

The percentage recovery of the analyte must be reliable, precise and also reproducible. Experiments to determine % recovery should be done at least on 3 concentrations of the standard range (Calibration curve). The recovery on the water, plasma and serum standards after preparation, will be determined and the same concentrations will be used in the validation of the analytical method (FDA, 2013).

To calculate the recovery the next equation will be used:

$$\text{Recovery (\%)} = \frac{\text{Area or Height of extracted sample}}{\text{Area or Height of unextracted sample}} \times 100$$

2.11.11 Stability

Stability of the stock and working solution should be tested with appropriate dilutions, taking in consideration the linearity and the measuring range of the detector. Stability studies should be investigated under different storage conditions over a time of period that equals or exceeds that of the actual study. It is not needed to study the stability of the internal standard and may be needed on case by case basis (FDA, 2013; Arora & Gangadharappa, 2016). Attention should be paid to the stability of the analyte in matrix and will be discussed in section 2.10: Stability in blood samples.

Minimum acceptance criteria

The first step in method development and validation should be the determination of the required minimum acceptance specification for the method.

List of criteria to be followed (Kazakevich & Lobrutto, 2007):

1. Statistical validation of accuracy over a certain range.

2. An instrument precision (% RSD) < 1.0%

3. An intra-assay precision (% RSD) < 2.0%

4. A regression precision (% RSD) < 2.5%

5. LLOD

6. LLOQ

Regression precision is important because it is a measurement of error when unknown concentrations are determined, especially in instrumental analysis (Kazakevich & Lobrutto, 2007).

2.12 HPLC METHOD VERSUS COMMERCIAL HPLC KIT

One of the objectives of the study was to compare the HPLC method of the laboratory in question, with a commercially available kit for the determination of LEV.

Manufacturers have developed commercial HPLC kits for the newer anticonvulsants such as lamotrigine, gabapentin, zonisamide and levetiracetam in the global environment. Only three commercial HPLC kits for the determination of LEV in human plasma/serum are available in South Africa. These commercial kits are standardised and therefore produce reliable HPLC methods that are fully certified according to ISO 13485 standards. The manufacturers provided all the reagents which are developed, produced and validated in accordance with the FDA regulatory guidelines (Burghardt, 2006). These methods enable technicians with little or no relevant experience to analyse samples in clinical routine laboratories (Burghardt, 2006). According to Fritzler et al. (2003), there are limitations and concerns in the use and clinical application of test results derived from commercial kits:

- Upgrading and use of equipment to standard

- Follow the manufacturer's protocols and standard operating procedures

- Utilize normal controls to assess performance of kit before adopting into laboratory
- Training and maintenance of competence
- Awareness of physician capabilities before ordering Kits
- Attention to the quality of samples provided for ongoing quality assurance programs
- The costs of the kit can be easily calculated, very little is known what the actual costs incurred through inappropriate laboratory testing

The commercial HPLC reagent kit that is provided for the research is designed for the quantitative determination of LEV in human serum or plasma for *in vitro* diagnostic use and will be standardized according to the described method. This method permits a fast, reliable and specific quantification of LEV in plasma and serum. The quality of the test reagents and reference materials is guaranteed by the internal Quality management-system (QM-system) (certified according to ISO 9001 and ISO 13485) and by the external quality control programs of Germany e.g. INSTAND. (INSTAND e.V. - Gesellschaft zur Förderung der Qualitätssicherung in medizinischen Laboratorien e.V.)

2.12.1 Validation of the commercial kit

If a commercial kit is used the FDA makes the following recommendations: (FDA, 2013).

- Specificity, accuracy, precision and stability conditions should be demonstrated under real conditions of use
- Calibration standards (supply usually one or two point calibration curve). In-house validations should be supplied with sufficient number of standards across for significant calibration ranges
- Quality controls (QC) with known concentrations should be prepared and used as provided by the manufacturer

- Standards and QC's should be prepared in the same matrix as the actual samples otherwise appropriate cross-validation should be performed

- If the reference standards (analyte) in the kit differ from the actual samples, testing should evaluate the differences in the immunological activity with the kit reagents

2.12.2 Comparison of different methods in the literature study

No comparison between the commercial kit and normal HPLC method for the determination of LEV could be found in the literature. According to Bianchi et al. (2014) the goal was to replace the HPLC method with an Immunoassay method which is easy to use, requires less training and produces quick results for LEV dose adjustments, especially for patients in an Intensive care unit. The comparison between the two methods shows no deviation within predetermined quality specifications and the two methods do not differ more than the allowable total error.

The need to develop new methods quicker was also to reduce the costs in new analytical instrumentation and new technologies. (Hansen et al, 2012). AEDs for TDM usage is one of the various techniques that can be reasonable cost effective, and HPLC techniques are not as expensive as LCMS, particularly if the patient is unstable or taking several AEDs whose concentrations must be analysed concurrently (Kang et al, 2011). These advantages lead to better control of patients with epilepsy and will probably result in improved patient care. A big part of the cost may lie in the blood collections, handling and transport of the samples (Tiwari et al, 2015).

The cost-effectiveness of the whole process can be improved if more of the clinical laboratories use techniques that are more cost effective to assist poor patients. World Health Organization (WHO) stated that the cost of the medication (AEDs) as well as testing should be cost-effective, to ensure that effective epilepsy care is available to all who need it for a better quality of life (WHO, 2012) and to control their epilepsy with AEDs.

2.13 STABILITY OF BLOODSAMPLES

It is apparent from the lack of modern literature that the stability of LEV in human plasma/serum samples, used for TDM purposes, have not been studied yet in recently years. This has serves as a motivation for this study to both address and inspect this research including the influences it might have on the HPLC method development. The handling of samples, sample processing, sample transport and storage all play a role in the stability of samples (Peters, 2007). Most of the blood samples are often taken in rural regions and sent to the laboratories for drug measurements. These blood samples may not be processed and handled in the same manner as the private laboratories. In these poor regions, blood is often collected in red top blood tubes (no additive) to separate the serum from the red blood cells. The blood sample should be placed on ice or in a cooled place but instead it is left on the laboratory bench for a period of time (may be up to 24 h or more) before packed and shipped to the laboratory to be analysed. Because of the hot climates, especially during summer, these samples may be exposed to high temperatures and direct sunlight for an unknown amount of time (Bennetto et al, 2004; Tiwari et al, 2015). Data showed that the majority of drugs are stable in plasma or serum under normal conditions in a clinical laboratory. Variability in stability occurs in drugs with ester and sulphur atoms or other easily oxidized or reduced structures. The acetamide group on the LEV molecule is especially prone to hydrolysis by β-esterase enzymes present in whole blood (Patsalos et al, 2006; Kang et al, 2011). Guidelines to maintain the stability of TDM samples during handling, transport and storage are generalized and non-specific. Relatively little is known about the stability of LEV under these various conditions (Saravanan et al, 2008, Kang et al, 2011). The effect of long distance transport of blood samples, especially during summer, has not been established for LEV but only in various other anti-epileptic drugs (Shazi et al, 2010).

2.13.1 Stability testing requirements

The investigation of stability should be carried out to ensure that every step taken during sample preparation, sample analysis as well as the storage conditions of the analyte in the matrix, does not disrupt the concentrations of the analyte (Singh et al, 2008; FDA, 2013). Stability of the analyte in the studied matrix should be evaluated

in low, medium and high concentrations (blank matrix spiked with analyte with three different concentrations) and prepared after the applied storage conditions to be evaluated (FDA, 2013; ICH, 2015). The mean concentration at each level should be 15% of the nominal concentration.

In the Guidance for Industry a number of requirements were stipulated for stability testing of a Bioanalytical validation method (FDA, 2013). That includes storage conditions, time frames and also the stability of different tubes.

2.13.2 Stability of different blood collection tubes

There are various blood test tubes for collection of plasma or serum and it may contain different substances (anticoagulants) that preserve the blood. Various types of anticoagulants are frequently added e.g. heparin (green top tube); potassium oxalate (brown top tube), Ethylenediaminetetraacetic acid (EDTA), thepurple top tube and sodium citrate (blue top tube). A clot activator or gel (refer yellow top tube to SST) or red top tube without gel may also be used as vacutainer tubes to separate serum from the blood cells (Hansen et al, 2012; Bowen & Remaley, 2014).

According to Bowen and Remaley (2014), serum collected in tubes without gel separators is preferred for the analysis of AED for TDM. Sodium citrate anticoagulants (blue blood tube) should be avoided; it is documented that they gave false higher concentration levels of AEDs. Heparin anticoagulant (green blood tube) may activate lipoprotein lipase, which may increase the concentration of free fatty acids and the transfer of the AEDs from albumin protein binding resulting in an increase in the free fraction of the drug. EDTA does not influence the sample components in any way (Neels et al, 2004; Hansen et al, 2012; Bowen & Remaley, 2014).

2.14 CONCLUSION

The literature demonstrated that LEV belongs to a valuable class of medications called AED's and is unique in its mechanism of action. Numerous studies have demonstrated LEV to be effective in the management of partial seizures. It also increased the treatment options available to patients with refractory epilepsy as an effective AED with added potential benefits in neurological and psychiatric disorders.

A large number of patients with epilepsy receiving this new generation anti-epileptic drug could now be monitored. Thus, to develop and validate a method that is cost effective will definitely add value to the TDM of AEDs in patients with epilepsy.

Literature demonstrated that an HPLC method with UV detection is an accurate method for the determination of LEV in plasma.

The Guidance for Industry for bio-analytical method validation (FDA, 2013) and ICH (2015) gave valuable instructions as how to determine the stability of samples under different conditions and in different tubes.

CHAPTER 3

RESEACH DESIGN & METHODOLOGY

3.1 INTRODUCTION

In this chapter the following will be discussed:

(1) Ethical approval.

(2) Study design.

(3) Sample population.

(4) Analytical and statistical methods.

3.2 ETHICAL APPROVAL

This study was conducted within the boundaries of the Constitutional Law of The Republic of South Africa by holding Section 12(2)(c) of the Constitution of South Act, No 108 of 1996, which states: 'Everyone has the right to bodily and psychological integrity, which includes the right not to be subjected to medical or scientific experiments without their informed consent'.

The study was approved by the Vaal University of Technology's (VUT) ethical committee (2015024.4) attached as Annexure A**.** Guidelines and principles prescribed by the Belmont Report (1978) and Helsinki Declaration (World Medical Association (WMA), 2013) were followed during the execution of this study. To ensure that the study was carried out in alignment with the prescribed principles, the Medical Research Council (MRC) of South Africa adopted ethics (from Helsinki Declaration) guidelines were also observed. The adapted principles promoted by MRC of South Africa are: autonomy, beneficence, non-maleficence and justice (Belmont report, 1978). Informed consent was obtained from all the participants.

To effectively incorporate the above mentioned principles into study, a code of conduct guided by the Health Professions Council of South Africa's (HPCSA) guidelines for good practice (2008) was also observed.

In compliance with this law and principles of the Belmont report and the previously mentioned Act of South African constitution, the following principles of biomedical ethics defined by the MRC were practiced;

- *Autonomy*- respect for the participants and their human dignity. All study participants were given an informed consent to sign at free will (Annexure B)
- *Beneficence*- the benefit to the study participants. Scientific knowledge gained in this study provides a more cost effective, reliable method development and TDM services to patients with epilepsy for more accurate dose regimen
- *Non-maleficence*- the study should be harm-free for participants. A qualified phlebotomist collected blood samples to ensure that the procedure is carried out without adverse aftermath on the participants
- *Justice*-risk and benefits of the study should be equally distributed amongst communities

3.3 STUDY DESIGN

This research project was designed in 4 phases:

Phase 1 – Development of a new HPLC method to detect LEV in human serum/ plasma samples.

Phase 2 – Standardisation and validation of the new HPLC method according to the Bioanalytical Method Validation criteria (EMA, 2011; FDA, 2013; ICH, 2015 and ISO 17025, 2005).

Phase 3 – Comparison between the newly developed and validated HPLC method with the Commercial available HPLC reagent kit. This was done as follows:

- Correlate the agreement of plasma/serum level concentrations between the two methods
- Compare the operational costs between the two methods

- Compare minimal sample (< 50 µl) preparation for reliable results in the new HPLC method

Phase 4 - Stability of LEV under various conditions as required by FDA (2013) and ICH (2015) in spiked plasma and serum. Investigation of the influence of LEV in five different collecting blood tubes.

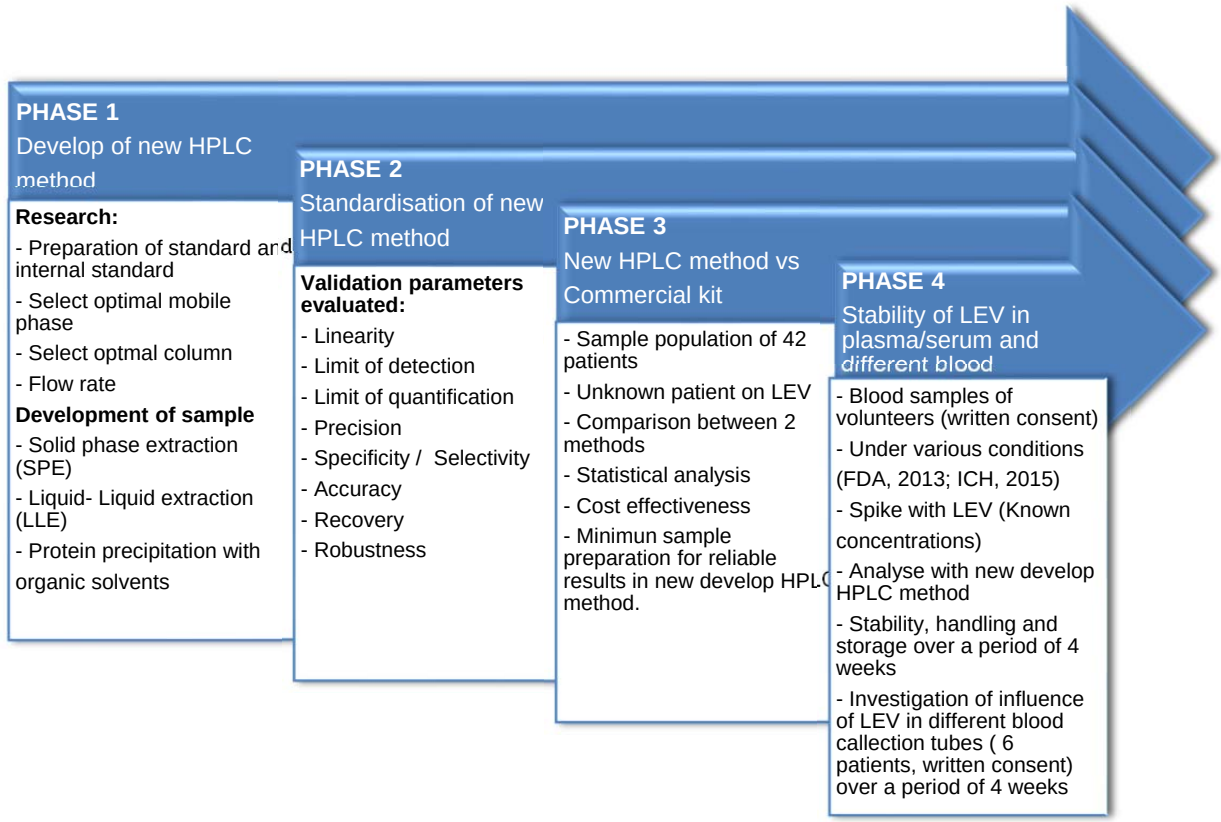


Figure 11 Overview of study design.

3.4 SAMPLE POPULATION

3.4.1 Participants used in this study

The blood samples were collected from 3 groups of study participants:

Group 1 was used for method development and validation. Blood samples, serum and plasma, were collected voluntarily from fifteen healthy MTech students in the Biomedical Technology Department at VUT. A fully qualified phlebotomist of the Biomedical Technology Department at VUT drew 10 ml of venous blood (1 x Serum separating tube (SST) with gel barrier and 1x Ethylenediaminetetraacetic acid

(EDTA) blood tube) from the students at one stage. They gave written consent that their blood samples could be used for analytical validation of the developed method and for the stability studies over a period of 4 weeks. All the blood tubes were centrifuged and the plasma or serum transferred to Eppendorf tubes and stored for further study. Nine of the students' blood samples were used for the development and validation of the HPLC method to determine LEV. Six of the blood samples, (serum and plasma), were pooled and spiked with known concentrations for the stability studies.

Group 2 was six volunteer patients on LEV. Blood samples were collected from Pathcare Laboratories in Potchefstroom, Klerksdorp and Vereeniging. These patients gave written consent that their blood could be used for the investigation of the influence of LEV in different collection blood tubes as well as the handling and storage of these blood samples. The following blood collection tubes were investigated:

- Serum separating tube (SST) without the gel barrier (red top)
- Serum separating tube (SST) with the gel (yellow top)
- Ethylenediaminetetraacetic acid (EDTA - purple top)
- Heparin (green top)
- Sodium citrate (blue top) was used on individual patients all at the same time

Six different blood samples were collected from Pathcare Laboratories and transferred to CPL. The blood was centrifuged and ± 2 ml of serum/plasma were transferred to Eppendorf tubes. Samples were analysed immediately (duplicate) and stored at 4 ^{o}C in a refrigerator and re-analysed weekly for four weeks. These blood samples were treated anonymously by using a numerical system.

Group 3 Forty four plasma/serum samples were received of patients from Pathcare Laboratories, Cape Town, and transported to CPL for routine therapeutic drug monitoring analysis of LEV. Written consent was given. These samples were used in the comparison study where the newly developed HPLC method and the Commercial kit were compared. The blood samples were treated anonymously after

routine therapeutic drug monitoring and results were sent to Pathcare Laboratories. Minimum of 500 µl of plasma/serum samples were stored at -20 ^{o}C until analysis.

3.4.2 Sample sizes

3.4.2.1 Method development and validation

For the method development, the sample size was kept to a minimum due to difficulty in obtaining plasma/blood samples that conform to selection criteria. It was also a priority to collect enough samples to obtain meaningful data, but to not oversample due to resource (both time and economical) constraints.

The study population consisted of patients' already in distress and from an ethical standpoint obtaining plasma/blood samples without causing further harm was the first priority. Therefor it was decided to collect more samples from willing donors and do analysis/method development on these samples. The blood of nine volunteers was used to develop and validate the method.

3.4.2.2 Sample size to determine the difference between the two methods

The power and sample size needed are determined by the type of data and distribution. Several types of sample size estimation should be performed to improve the precision of the final results (Jones et al, 2003; Charan & Biswas, 2013). A priori power analysis for a correlation analysis was conducted using the following formula and demonstrated in Figure 12, using the software package G*Power 3.1.9.2 (Faul et al, 2007):

The following equation for the correlation analysis for the distribution of the sample was used:

$$n = \left[\frac{Z\alpha + Z\beta}{c}\right]^2 + 3$$

Z_α = Standard normal deviate

Z_α = 1,960 (type 1 error = level of statistical significance or regulator's error, also known as a 95% significance)

α=0,05 (probability value and was conventionally set at 5%)

Z_β= Standard normal deviate

Z_β= 0,842 (Type 2 error not finding a difference when it could actually exist or investigator's error)

β=80% (the (statistical power of the study was conventionally set at 20%)

$C = 0.5 \times \ln\left[\frac{1+r}{1-r}\right]$ *r = 0,42 (effect on correlation coefficient)*

$$n = \left[\frac{1.960+0.842}{C=0.5 \times \ln\left[\frac{1+0.42}{1-0.42}\right]}\right]^2 + 3$$

n=42

A sample size of 42 would be sufficient to detect an effect of 0.4 with a power of 80% and an alpha of 0.05 to obtain good results.

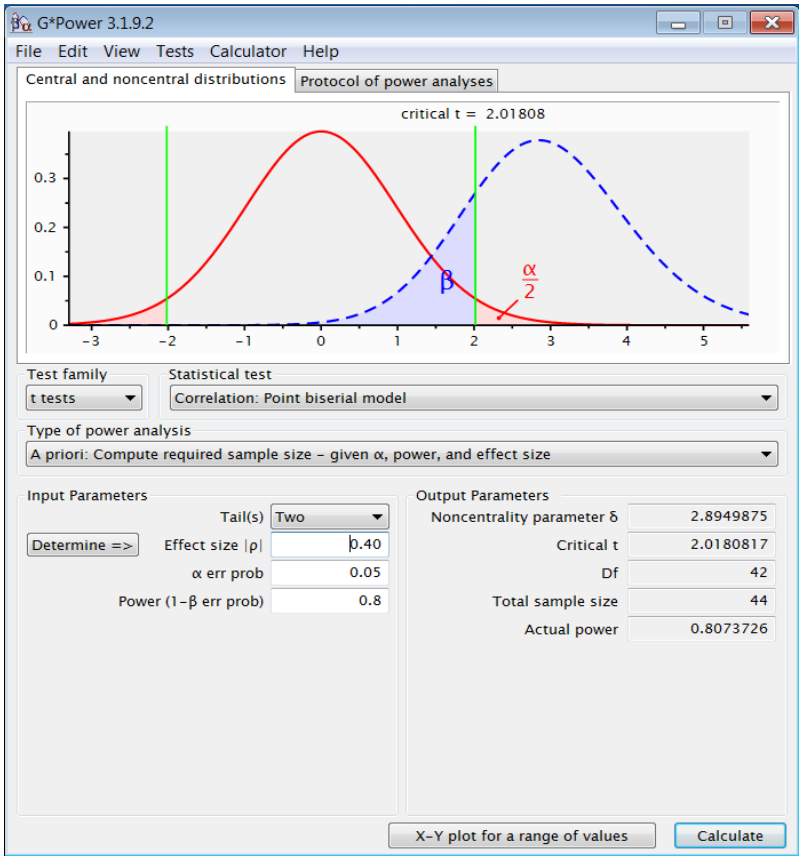


Figure 12 Sample size demonstrated the power and effect size to be sufficient for the study on software package G*Power 3.1.9.2 (Faul et al, 2007).

3.4.2.3 Sample size for stability testing

The estimation of the minimum sample size required for this study was a crucial aspect of the study design. A random sample has the advantage that it allows the study both a small population, time and money (Ellis & Steyn, 2003; Ellis, 2012; Sullivan & Feinn, 2012). Due to circumstances the researcher used a convenience sampling method for which the data is considered as a small population. The small p-value obtained from a small population does not necessarily imply insignificance of results (Ellis & Steyn, 2003; Sullivan & Feinn, 2012). Convenience sampling was the sampling method of choice and the blood of six patients using LEV was used to investigate the stability in five different collecting tubes.

3.5 MEASURING INSTRUMENT (PHASE 1)

The analytical HPLC method was optimized to comply with all the required validation criteria as stated by the following: EMA (2011); FDA (2013) and ICH (2015).

3.5.1 Chromatographic conditions for the newly developed HPLC method

The HPLC instrumentation consists of an Agilent 1200 series containing a degasser, binary pump, thermostatic column oven and a diode-array detector (DAD) with variable wavelengths (Table 6). Data was collected and analysed by Agilent Chemstation software package, Revision A.10.02. The chromatography was obtained on a Venusil XBP C18, 250 x 4.6 mm, a 5 µm particle size column protected by a security guard precolumn with a graphite filter was used. The mobile phase was a mixture of 50 mM KH_2PO_4 buffer with ACN (90:10) with a flow rate of 1ml/min, an injection volume of 10 µl and a detection wavelength of 205 nm. (See Table 6).

The commercial HPLC kit was designed for the quantitative determination of LEV in human plasma/serum for *in vitro* use and was standardized on the same HPLC Agilent 1200 series instrument; the data was also collected on the same Chemstation software package, Revision A.10.02. Validation and chromatographic conditions were done as described in the manual of the commercial kit with all the materials contained the HPLC commercial kit (Table 10).

3.5.2 Materials and consumables for HPLC method

The ClinRep® HPLC complete kit for levetiracetam (Keppra®) in serum/plasma (Order no. 15500), standard for levetiracetam, reagents and consumables needed for the study were supplied by the VUT representing in Table 7.

All the materials and equipment used for this study were available in the Analytical Technology Laboratory (ATL), CPL of the School of Pharmacy at the NWU, Potchefstroom Campus (Table 8).

Table 6 Chromatographic conditions on HPLC system for the newly developed HPLC method.

Parameters	New Develop HPLC Method
Analytical Instrument	Agilent 1200 series, HPLC-instrumentation, which consists of a degasser, diode array detector, auto sampler, binary pump and thermo-regulated column compartment. Data were collected and analysed by an Agilent Chemstation software package, Revision A.10.02.
Column	Venusil XBP C18, 250 X 4.6 mm, 5 µm particle size, 100 Å pores, 18% carbon load, end capped, Bonna-Agela Technologies, USA. (BN-QR-8.2-18-VX-c18-075)
Column guard	SecurityGuard™, HPLC Guard Cartridge System, with SecurityGuard Cartridges, C18, 4.0 x 3.0 mm, Phenomenex, Torrance, CA.
Mobile Phase	50 mM KH_2PO_4 buffer (6,8045 g/l) with ACN (90:10). The pH of the mobile phase was set at ± pH 5.5 with NaOH.
Pump mode	Isocratic mode
Flow rate (ml/min)	1,0 ml/min
Run time (min)	15 min
Column temperature (°C)	Ambient
Volume of injection (µl)	10 µl
Detection wavelength (nm)	205 nm
Retention time: **Levetiracetam** **Internal Standard (Gabapentin)**	 ± 8.59 min ± 5.9 min

Table 7 Materials and consumables used in HPLC method.

Materials & Consumables	Brand name
Levetiracetam by HPLC, Reagent Kit, 100 Tests. (Ref 195-6690)	ClinRep® HPLC complete kit for Levetiracetam (Keppra®) provided by Microsep
LEV Compound 10 mg	Industrial Analytical
Internal Standard (Gabapentin)	Supplied by CPL
Precolumn cartridge holder	Separations
Precolumn cartridge (Separations)	Separations
Venusil C18, 250 X 4.6 mm, 5 µm **Venusil C18,150 x 4 mm, 5 µm**	Stargate Scientific
Methanol (HPLC grade) 2 x 2.5 L	Romil
Acetonitrile (HPLC grade) 2 x 2.5 L	Romil
2 ml Eppendorf tubes	Merck
Pipette tips (yellow and blue)	Merck
Inserts, Vials, seals and bottles	Separations
Gloves (Powder free)	CPL

Table 8 Equipment and Instrumentation used in the HPLC method.

Equipment and Instrumentation	Brand name	Location
Volumetric glassware	Blue brand	CPL
Centrifuges	Eppendorf	CPL
Fridge	G E C Gala eleven	CPL
Freezer	G E C Gala eleven	CPL
Vortex	Gemini	CPL
HPLC	Agilent 1200 Series: binary pump and auto sampler	CPL
Detector	DAD	CPL
Column	Venusil C18, 250 X 4.6 mm, 5 µm Venusil C18,150 x 4 mm, 5 µm	Stargate Scientific
Data Acquisition & Analytical Software on computer connected to HPLC	Agilent Chemstation Rev.A.06.02. Data Acquisition & Analytical Software	CPL
UV lamp	Agilent	Agilent
Balance	Sartorius	ATL
Distilled H_2O	Milli-Q® reagents water system	ATL
UV lamp	Agilent	Agilent

3.5.3 Maintenance and validation of the HPLC system

An HPLC maintenance program was followed every week. The column was rinsed with 10% ACN and 70% ACN to prevent deterioration. Operational protocol and criteria for the HPLC system were also followed every 6 -12 months. This maintenance inspection related to method validation and system suitability tests to ensure that the injection valves, autosampler, pump, and detectors work at maximum efficiency. As a result, it expanded the HPLC system's hours of continuous operation, decreased downtime, and increased productivity. Hardware validation was also a precondition since the performance of the HPLC changes with age and replacement of consumables.

3.5.4 Standards

3.5.4.1 Preparation of standards, calibration ranges and internal standard

Standard solution: levetiracetam = Mw 170.218 (UCB (S.A) Pharmaceuticals) from company Industrial Analytical (Pty) Ltd).

1 mg LEV of the standard solution was dissolved in 10 ml distilled water using a standard volumetric flask to get a concentration of 100 µg/ml. The solution was sonicated for 5 min and stored at 4 ^{o}C. The stock solution is stable for six months.

From the stock solution, calibrations for water standards and spiked human serum (n=10, blank included) were prepared in a concentration range of 1 to 60 µg/ml (see Table 9). This calibration range covers the therapeutic concentration range of LEV (12 – 46 µg/ml) in the patient samples.

The accuracy of the LEV stock solution was verified by the comparison with the water and serum calibration solutions. Working range of 2; 7,5; 25 and 50 µg/ml were respectively run as a calibration curve, control samples with known values were run before running any patient samples.

Table 9 Dilution table for preparation of water and serum standards.

Standard level	Concentration µg/ml	Dilution Volume of Stock solution (SS)		Distilled H20 Plasma Serum		Total Volume	Therapeutic range of LEV
1	0	0		0		0	12 – 46 µg/ml
2	1	20 µl	+	1980 µl	=	2 ml	
3	2	40 µl	+	1960 µl	=	2 ml	
4	5	100 µl	+	1900 µl	=	2 ml	
5	7.5	150 µl	+	1850 µl	=	2 ml	
6	15	300 µl	+	1700 µl	=	2 ml	
7	25	500 µl	+	1500 µl	=	2 ml	
8	35	700 µl	+	1300 µl	=	2 ml	
9	50	1000 µl	+	1000 µl	=	2 ml	
10	60	1200 µl	+	800 µl	=	2 ml	

3.5.4.2 Preparation of Internal standard

Internal Standard solution: Gabapentin = 171.237 Mw (Sigma-Aldrich, Aston Manor).

Gabapentin was chosen as internal standard because it elutes before LEV and is well separated from the analyte and minimal disturbance in patients' blood was observed (See Section 4.2.2; Figures 21 & 22).

The internal standard was prepared by dissolving 20 mg of gabapentin in 20 ml of MeOH to a final concentration of 1 mg/ml. The internal standard was freshly prepared every week for more stability conditions or when needed and was used as a protein precipitation.

3.5.4.3 Quality control samples

The quality controls from Microsep, Clin Chek® Controls, Level I (Lot no: 509) and Level II (Lot no: 509), were used in this study. The same control samples were used for the development of the HPLC and the Commercial kit methods.

Clin Chek® Controls (Level I and II; Lot no 509) were lyophilised and were reconstituted before used (Annexure D). The concentrations of the Clin Chek® Controls of Level I was 13,5 µg/ml and Level II was 46,2 µg/ml. Clin Chek® Controls were treated the same as the standards and patient samples. See 3.6.1.1 for sample preparation.

3.6 SAMPLES

3.6.1 Samples collection

The collection and preparation of samples for phase 1, 2 and 3 were discussed under section 3.4.1. As soon as the samples reached the CPL it was numbered and centrifuged. The blood was stored in a refrigerator (4 ^{o}C) and kept for analysing in a batch.

3.6.1.1 Sample preparations

The following steps were taken to prepare a sample:

1. Deproteinized (100 µl) serum/plasma samples, standards and the control samples were placed in 1.5 ml Eppendorf tubes.

2. Add 300 µl MeOH spiked with internal standard.

3. The samples were vortexed for 60 seconds and the precipitated proteins were separated by centrifugation at 14000 rpm for 10 min.

4. Transfer 100 µl of the upper layer into auto sampler vials with inserts and placed into the sample tray of the Agilent 1200 HPLC system auto sampler ready to be injected.

5. The Agilent 1200 HPLC systems parameters that were set as LEV method were programmed to inject 10 µl of the sample into the column.

6. Calibration curve is to form a linear curve with an r^2-value not less than 0.998. The curve was used on a daily basis to calculate the concentration levels of the sample in the study and from the patients.

7. The results were expressed in µg/ml.

3.7 ANALYTICAL METHOD VALIDATION (PHASE 2)

The main objective of this validation was to provide a method that was accurate, specific, sensitive, repeatable and reliable to determine LEV in human plasma/serum in patients.

The parameters used to validate the new optimised method were the following steps.

3.7.1 Validation of analytical parameters

The method was validated to meet the general requirements of EMA (2011); FDA (2013); ICH (2015) and also the general requirements of the ISO 17025 (2005). The following were the validation parameters as discussed below.

3.7.1.1 Selectivity / Specificity

Selectivity: Six different blank drug free plasma/serum samples were used. The samples were prepared as described in 3.6.1.1. Samples were injected to determine the extent to which endogenous plasma/serum components may contribute and interfere with the analyte or internal standard at the retention times on the chromatogram.

Specificity: The specificity was confirmed by analysing standards of the most common AED's like lamotrigine, oxcarbazepine, phenobarbitone, phenytoin, carbamazepine and clonazepam. Blank plasma was spiked with these drugs and the IS and analysed to make sure no interference was observed on the same method to ensure that the method was specific for LEV and IS.

3.7.1.2 Accuracy and Precision (Reproducibility)

According to the guidelines, (EMA, 2011; FDA, 2013), it is necessary to analyse three (3) concentrations in five replicates. For this study six replicates were performed at five (5) different concentrations.

Intra-day accuracy and precision were determined by six replicates of the water and plasma standards at lower, low, medium and high concentrations (2; 7,5; 15; 25 and 50 µg/ml respectively. Inter-day accuracy and precision were determined of six

replicates by water and plasma standards at very low (2 µg/ml), low (7,5 µg/ml), medium (15 µg/ml) and extremely high concentration (50 µg/ml) over six days. The standard deviation (SD), percentage relative standard deviation (% RSD) and percentage of accuracy were calculated for each concentration respectively. Accuracy is expressed as the percentage of recovery. The mean values were also reported. These samples were treated the same as in section 3.6.1.1.

3.7.1.3 Lower limit of detection (LLOD)

The LLOD was determined by measuring the lowest concentration that can be detected in a sample by the DAD detector (EMA, 2011; FDA, 2013). The LLOD was determined by measuring the lowest concentration of 0,25; 0,5 and 1.0 µg/ml ten times and was distinguished from the baseline and background noise with a certain degree of confidence. The average, SD and % RSD were calculated for each concentration for the lower limit of detection. These samples were prepared the same as in section 3.6.1.1.

3.7.1.4 Lower limit of quantification (LLOQ)

The LLOQ is the lowest concentrate of an analyte that can be quantified with suitable accuracy and precision measured (EMA, 2011; FDA, 2013). The signal of the blank at the retention time of LEV must have an area no greater than 20% of the area corresponding to the LLOQ.

The lowest concentration of 1,0 µg/ml on the calibration curve was measured ten times. The average and % RSD were calculated for the lowest limit of quantification. These samples were prepared the same as in section 3.6.1.1.

3.7.1.5 Calibration curve / Linearity

The importance of the calibration curve is to form a linear curve with r^2 – value not less than 0.998 (FDA, 2013). Linearity was done on the following concentration range: 0; 1; 2; 5; 7,5; 15; 25; 35; 50 and 60 µg/ml on the water and plasma standards spiked with LEV and IS. The concentration ranges were prepared in six replicates and were treated the same as in section 3.6.1.1.

Daily standard calibration curves (5; 15; 25 and 50 µg/ml) were used to calculate the LEV concentration levels of both the Clin Chek® Controls (Level I and II) and the sample concentrations. Data were captured on Excel, and the evaluation was done on Prism, version 5 to determine the correlation coefficient. If the r-value is in doubt a t-test or f-calc can be carried out to prove the significant linearity on Analysis of variance (ANOVA).

3.7.1.6 Range

For this study, the calibration curve was constructed from 1 - 60 µg/ml. That is important to cover the whole range (from when a patient is non-compliant to toxic levels). The therapeutic range of LEV is between 12-46 µg/ml in the literature (Krasowski, 2010).

3.7.1.7 Robustness

The analytical method's parameters of the flow rate of the mobile phase, the pH of the mobile phase, the percentage of the inorganic phase in the mobile phase and the column's temperature were changed slightly to see if there was any effect on the method.

3.7.1.8 % Recovery (percentage recovery)

The recovery for both water and plasma standards after it had been prepared, was determined. The same concentration ranges of 2; 7,5; 15; 25 and 50 µg/ml respectively were used.

To calculate the recovery the following equation was used:

$$\text{Recovery (\%)} = \frac{\text{Area or Height of extracted sample}}{\text{Area or Height of unextracted sample}} \times 100$$

3.7.1.9 Stability

The stability of the LEV as a standard was tested over a period of 6 months under the following conditions: The stock solution for LEV (concentration of 100 µg/ml) was tested for short-term storage (Bench-top and auto sampler stability) and long-term storage (Freeze - thaw (unfreezing)) cycles and kept in the fridge by 4 ^{o}C).

3.8 HPLC METHOD OF THE COMMERCIAL HPLC KIT (PHASE 3)

3.8.1 Methodology of the Commercial kit

The commercial HPLC reagent kit was designed to quantitatively determine the LEV levels in human serum/plasma for *in vitro* diagnostic use. This method was standardized according to the described method. The ClinRep® HPLC Complete Kit for levetiracetam (Keppra®) in serum/plasma (Order no. 15500) was obtained from Microsep (See Annexure D). A ClinCal® Serum Calibrator (Order no. 15513) was provided for quantification (See Annexure D). ClinChek® Serum Controls (different concentrations, Order no. 15582) were used to perform quality control (See Annexure D). The quality of the test reagents and reference materials were guaranteed by the internal QM-system (certified according to ISO 9001 and ISO 13485) and by the external quality control programs (INSTAND, Germany) supplied from the commercial kit. Analytical Colum (Order no 1 x 14030) was designed for the complete kit to perform chromatographic separations.

3.8.2 Components of the complete kit

ClinRep® HPLC Complete Kit for Levetiracetam in plasma and serum (Order no 15500) for 100 assays contains (Annexure D):

- Mobile phase (Order no 1 X 15510)
- Standard solution (Order no 1 X 15511)
- IS Internal standard (Order no 1 X 15512)
- Serum calibrator, lyophil (Order no 1 X 15513)
- Sample Preparation Columns (Order no 2 X 5520)

- Conditioning Reagent (Order no 1 X 15521)
- W Washing solution 1 (Order no 3 X 15522)
- W Washing solution 2 (Order no 1 X 15523)
- E Eluting Reagent (Order no 1 X 15524)
- Manual
- Quick Reference
- Analytical Column (Order no 1 x 14030)

3.8.3 Equipment and Instruments

The ClinRep® HPLC Complete Kit required an HPLC System with UV detector and Optional pulse damper (See section 3.5.1).

3.8.4 Sample preparation equipment and consumables

The HPLC instrumentation, an Agilent 1200 system containing a degasser, quaternary pump, thermostatic column and a diode-array detector (DAD) with variable wavelengths was used for both the commercial kit and developed HPLC method for comparison. Data was collected and analysed by Agilent Chemstation software. The commercial HPLC reagent kit was standardized according to the described method (see section 3.11.6).

3.8.5 Optimised chromatographic conditions and parameters required for the Commercial Kit.

Table 10 provided the conditions for the HPLC system according to the kit manual.

Table 10 HPLC parameters provided by ClinRep® HPLC Complete Kit, Levetiracetam (Keppra®) in serum/plasma.

Parameters	ClinRep® HPLC Complete Kit
HPLC pump:	Flow rate: 1.4 ml/min
Mobile phase:	Make sure the bottle is closed well to avoid alteration of the retention times through evaporation of components of the mobile phase. Recycling: The mobile phase may be circulated through the system for 100 analyses. After 100 analyses a new bottle of mobile phase has to be used.
Autosampler:	Injection volume: 20 µl Injection interval: 6 min For minimum sample carry over use the needle wash settings recommended by the autosampler supplier. The mobile phase has to be used as a washing solution for the autosampler.
Column heater:	30 ^{o}C
Column:	Unknown Analytical column (Supply by the kit) was installed with the column heater (30 ^{o}C).
UV detector;	Was set to 205 nm.
Evaluation unit:	Integration stop has to be set at 6 min. Retention times: Levetiracetam: 2.49 min Internal Standard: 4.89 min

3.8.6 Reagents

3.8.6.1 Reconstitution of the ClinCal® Calibrator and the Clin Chek® Controls.

The ClinCal® Calibrators were used for the calibration on the HPLC system. The calibrator was a lyophilised matrix and after reconstitution. The calibrator has to be prepared like a patient sample. The ClinCal® Calibrators were stable for at least 36 months (Annexure D).

The Clin Chek® Controls (Level I and II) were used as the quality assurance of the method and were a lyophilised matrix and should be reconstituted before used

(Annexure D). The concentrations of Level I was 13,5 µg/ml and Level II was 46,2 µg/ml. The Clin Chek® Controls were stable for 36 months indicated in the appropriate product data sheet (refer to Annexure D).

3.8.7 Sample preparations

Workflow diagram (Figure 13) depicting preparation steps for calibration, controls and samples.

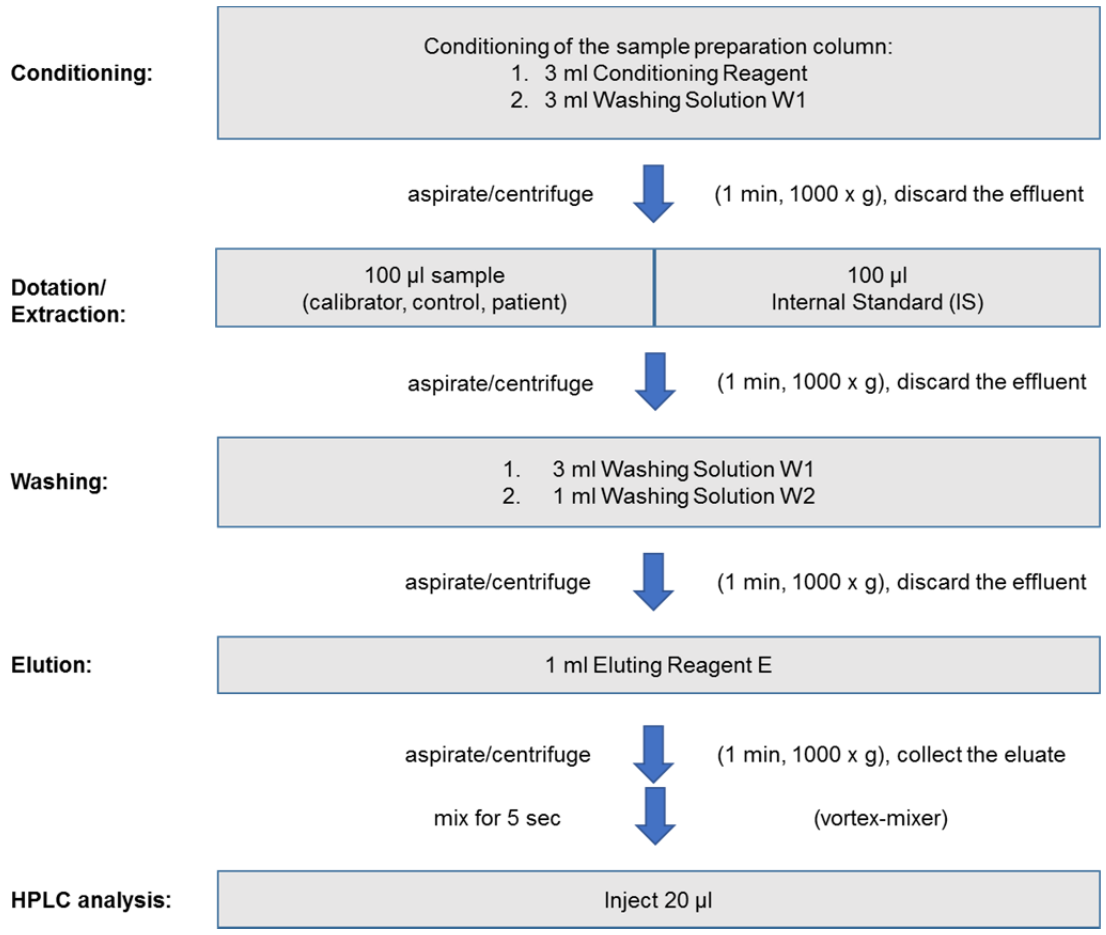


Figure 13 Flow diagram for sample preparation (adapted from the ClinRep® HPLC Complete kit manual).

3.8.8 Chromatographic results of ClinRep® HPLC Complete kit

3.8.8.1 Test Run

Before running samples, the HPLC system was checked with the ClinTest® Standards solution to ensure the chromatogram was identical regarding retention times and peak resolution (Annexure D). The integration parameters (e.g. run time,

peak identification, marks for the peak start and end) had been corrected once for verification.

3.8.8.2 Calibration

Serum calibrator was injected several times as single point-calibration average. The mean value of the ClinCal ® calibrator was 33,0 µg/ml as mentioned in the manual and prepared the same as sample preparation mentioned in Figure 13. The reason was to evaluate the conditions of the HPLC system and shifted retention times without repeating samples.

A four point calibration concentration from the newly developed method was also used to prove the linearity of the Commercial Kit. The concentrations for the calibration range were 5; 15; 25 and 50 µg/ml respectively. The standards were treated the same as sample preparation adapted from the ClinRep® HPLC Complete kit manual (Annexure D).

3.8.8.3 Accuracy control

The Clin Chek® Controls (Level I and II) were used to ensure quality of the method and the condition of the HPLC system before analysing patient samples. These quality controls were treated as a patient sample. It was also necessary to run the quality controls when large batches of samples were running till the end of the series. The concentration of the Clin Chek® Controls of Level I was 13,5 µg/ml and Level II was 46,2 µg/ml.

3.8.9 Validation of the ClinRep® HPLC Complete kit

The HPLC system was validated under the criteria of the FDA and according to the ClinRep® HPLC Complete kit's manual to ensure the kit was suitable for its intended purpose and to provide that it was accurate, specific, sensitive, and reliable to determine LEV in human plasma and serum with epilepsy.

3.8.9.1 Specificity, accuracy and precision

The specificity of the ClinRep® HPLC Complete kit's manual was confirmed: no interfering peaks were observed at the retention times of either the analyte or the IS (refer to Annexure D).

Six replicates of four different concentrations: 5; 15; 25 and 50 µg/ml (standards from the calibration curve) were injected to ensure reproducibility. Precision (repeatability) is a measure of the closeness between multiple measurements of the same homogenous sample. It is important to note that the samples should all be taken through the whole sample preparation process, and not only injected multiple times. The intra-assay precision was obtained by measuring four different concentration ranges: 5; 15; 25 and 50 µg/ml (standards from the calibration curve) six times which had been prepared independently and then % RSD was calculated for each sample by dividing the SD of a set of measurements by the set mean, and multiplying by 100. The inter-assay was measured by four different samples in duplicate to monitor the precision of results between different assays.

3.8.9.2 Linearity, lower limit of detection and lower limit of quantification

The linearity was verified where the concentration range corresponded with the linearly proportional concentration (one point calibration curve) of the ClinRep® HPLC Complete kit manual (refer to Annexure D). The following standard concentration ranges: 5; 15; 25 and 50 µg/ml of the newly developed method were analysed. Least square analysis was carried out for the correlation coefficient and the linearity of LEV was within a concentration range of 1 - 60 µg/ml.

Lower limit of detection and lower limit of quantification were used as indicated in the ClinRep® HPLC Complete kit manual (Annexure D).

3.8.9.3 Recovery

Any losses during sample preparation were determined by calculating the recovery. The IS of the sample (peak area) and IS of the calibration (peak area) were used to calculate the recovery. According to the manual of the kit the recovery was between 97 – 105% (Annexure D).

3.8.9.4 General evaluation

The stability of the HPLC system was checked regularly and to avoid stable HLPC conditions during analysing samples, measured samples were compared with the serum calibrator to correlate the peaks on the chromatogram and retention times.

Temperature variations may lead to retention time shifting and false peak identification during the measurements of the standards, controls and patients' samples to correlate the peaks and the retention times of the serum calibrator.

3.9 NEWLY DEVELOPED HPLC METHOD VERSUS THE COMMERCIAL KIT (PHASE 3)

3.9.1 Sample preparation procedures between two methods.

Blood samples were received from the pathology laboratories and kept frozen (-20 ^{o}C) until analysed. Plasma/serum was thawed at room temperature and sample preparations on the new developed HPLC analytical method were analysed (See section 3.6.1.1) during the first day. The following day the same plasma/serum samples (used for the new method) were done on solid phase extraction (SPE) on the ClinRep® HPLC Complete Kit (See section 3.8.6. for sample preparation). Analyses were done on both methods and all the results were interpreted.

3.9.2 Interpretation on the results between the two methods

Microsoft Excel software was used for data capturing and statistical analysis. Descriptive statistical analyses (with the assistance of a statistician from NWU Department Statistics) between the newly developed method and the ClinRep® HPLC Complete Kit were performed using *p*-tests, Bland-Altman plots and Intra-class correlation (ICC).

3.9.3 Statistical methods to correlate agreement between two methods

Mean values and standard deviation values were computed for each variable using Microsoft Excel. Linear regression analysis and r^2 were determined to confirm linearity and range. Correlation coefficients were also used to examine the correlation between the study variables. To determine if a statistical difference exists between the two methods, *t*-tests, Bland-Altman plots (Bland & Altman, 1986) and Intra-class correlation (ICC) were calculated. It is very important to implement these methods to make unbiased decision on the merits of the two methods.

3.9.4 Operational cost

The availability of commercial HPLC kit in South Africa was examined and only three representatives were traced. The three suppliers in South Africa were:

- Microsep provides ClinRep® HPLC Complete Kit.
- Bio-Rad provides Bio-Rad Laboratories (Pty) Ltd.
- Separations provide Chromsystems.

A quote from the manufacturers (Annexure C) was obtained and the cost was evaluated between these manufactures including the newly validated HPLC method.

3.9.5 Minimal sample preparation for reliable results in newly developed HPLC method

Clin Chek® Controls (Level I and II; Lot no 509) provided by Microsep were used to determine the minimum sample volume needed between 10 - 50 µl in the newly developed HPLC method. Sample adjustments were made between 10 - 50 µl instead of using a 100 µl sample. See 3.6.1.1 for sample preparation.

3.10 STABILITY OF LEV BLOODSAMPLES (PHASE 4)

3.10.1 Stability testing at different storage conditions

The samples were centrifuged and separated. The drug-free blank plasma and serum samples were pooled as A (plasma) and B (serum). Samples A and B were spiked with LEV with known concentration levels of 2; 7,5; 15; 25 and 50 µg/ml respectively. The spiked samples were prepared freshly and at least 3 replicates of each of the concentrations were assessed under different storage conditions over a period of 4 weeks as follows:

- Freeze – thaw stability and long term stability were performed by analysing the spiked samples A and B after a minimum of 3 cycles of thawing and after being stored for 4 weeks. These evaluations were imitating the intended sample handling conditions to be used through sample analysis
- Fridge – spiked samples A and B were analysed after a minimum 3 cycles in the fridge and then analysed over for 4 weeks in fridge. The evaluation of results was representing possible sample handling conditions in the laboratory
- Bench-top stability was performed by analysing the spiked samples A and B after they stood on a laboratory bench at room temperature for longer than 1 week

until needed. Bench top stability investigation was conducted to cover handling conditions that are expected for the stability of the samples and analysis

- Auto sampler stability – The study of auto sampler stability was performed by analysing the spiked plasma samples immediately and then repeated after 24 hours at room temperature (20 - 24 ^{o}C)

- UV (direct sunlight) - was performed by analysing the spiked samples A and B over 4 weeks until analysing was needed

3.10.2 Stability of LEV in different blood collection tubes

The blood samples of six volunteer patients (n=6) were freshly prepared at room temperature and analysed immediately. The samples were stored in the fridge over a period of 4 weeks in the 5 different tubes: SST (Gel), Red, Purple (EDTA), Green (Heparin) and Blue (Sodium Citrate). The samples were analysed weekly to determine if a change happened in the specific tube over time.

3.11 CONCLUSION

In this chapter the ethical considerations and study design were discussed. The steps to develop the HPLC method and how this method will be compared to the commercial kit were also described. The chapter also includes how the prices between these two methods will be compared. Finally, the methods to determine the LEV stability under different conditions and in different tubes were investigated.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 INTRODUCTION

In this chapter the results of the study will be presented and discussed. These results included:

- The development, validation and correlation between the newly developed and ClinRep® HPLC Complete Kit HPLC methods for the determination of LEV
- A comparison of the operational costs between the two methods
- The optimal sample amount for reliable results with the new HPLC method
- The stability of LEV under different conditions and in different blood tubes

4.2 LEV METHOD DEVELOPMENT (PHASE 1)

The factors mentioned in Chapter 3, Table 6, were taken into account to optimise the HPLC method. They included the parameters of the Analytical instrument, column and column guard, mobile phase, pump mode, flow rate of the mobile phase, running time of each sample, column temperature (^{o}C), volume of injection (µl), retention time of both LEV and the IS as well as sample preparation technique used.

4.2.1 Optimization of chromatographic results

Optimal conditions for separation of LEV were established as follows:

- HPLC column: In order to optimize the column, different column lengths (150 and 250 mm) and models (Venusil and Phenomenex Luna C18) were compared. The columns produced equally satisfactory results, but the Phenomenex Luna C18 column was more expensive and for that reason the Venusil C18, supplied by Bonna-Agela Technologies, was chosen for this study. The C18, 150 mm column was recommended in most studies (Martens-Lobenhoffer & Bode-Böger, 2005; Contin et al, 2008; Poongothai et al, 2011), to reduce the total running time per sample (Figure 14) to under 10 min. However, the 250 mm Venusil C18 was preferred for this study due to the presence of endogenous interferences in

patient's blood. The running time was 15 min and the peaks were well separated on the chromatogram (Figures 14 and 15)

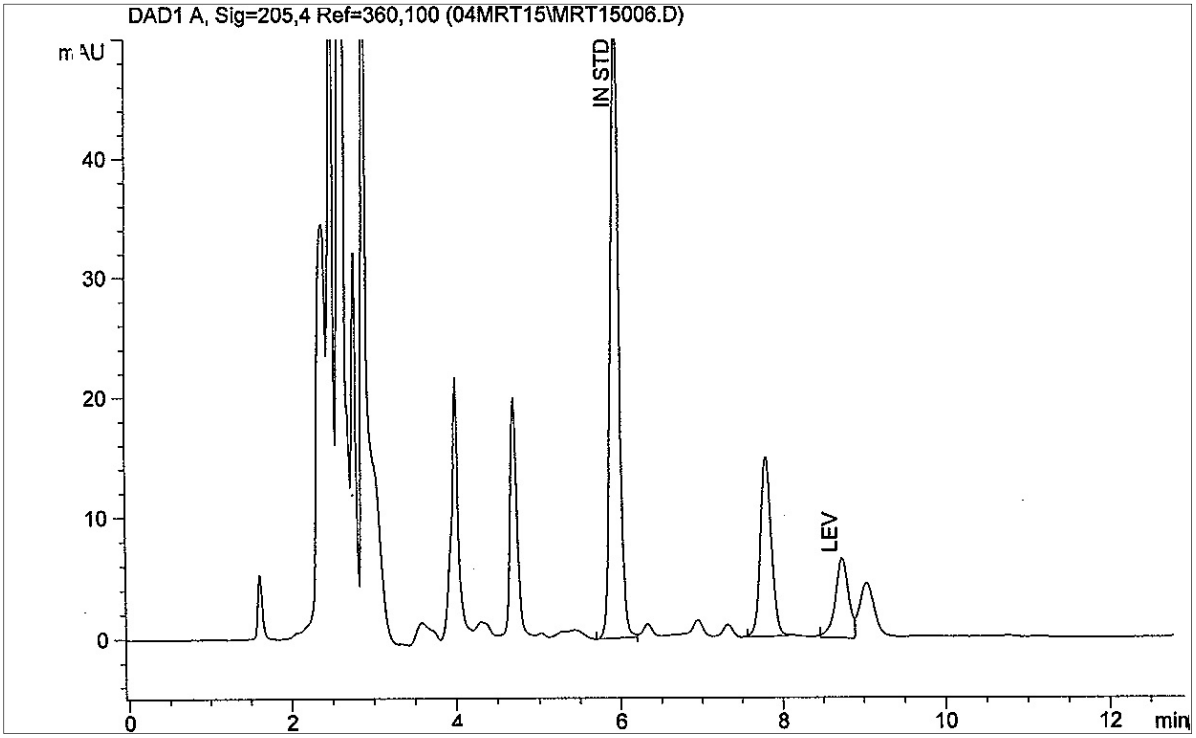


Figure 14 Chromatogram of 150 mm column with running time of 10 min.

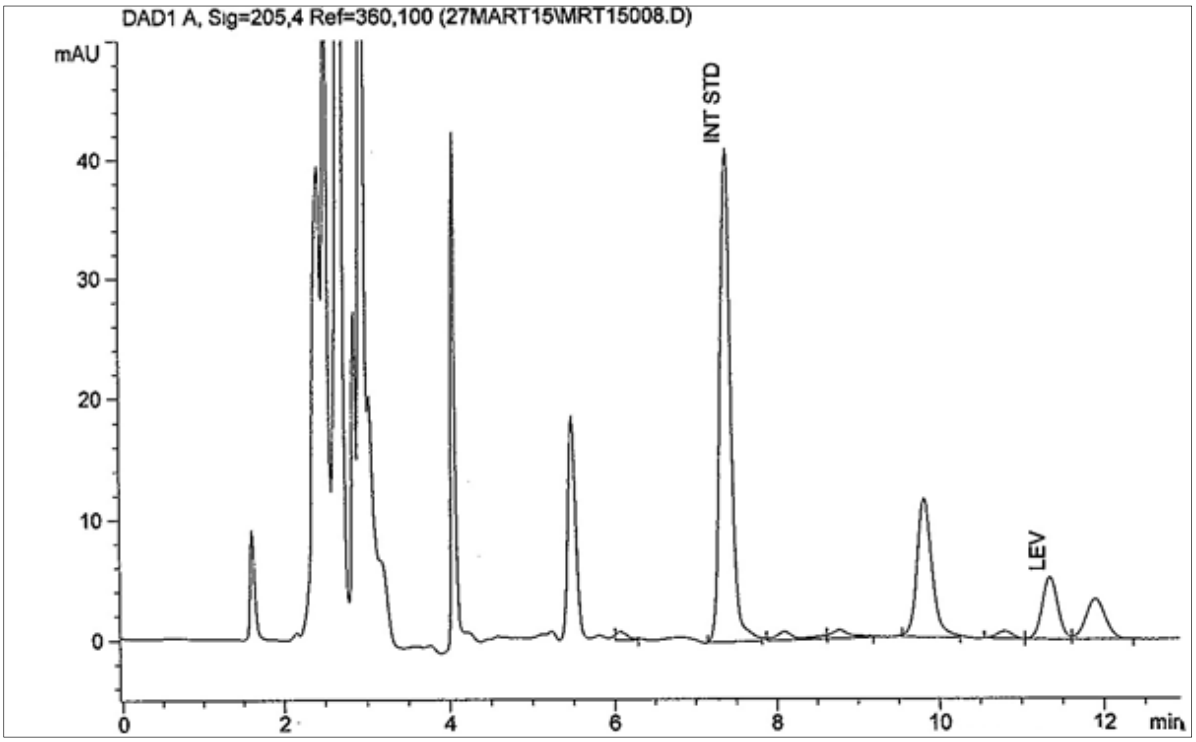


Figure 15 Chromatogram of 250 mm column with a running time of 15 min.

- Mobile phase: Different combinations of buffer-, MeOH-ACN mixtures were tested. Figure 16 shows a plasma chromatogram with a mobile phase containing MeOH and a buffer. It is clear from figure 16 that the peaks were not sharp enough. The best results were obtained with a mobile phase containing phosphate salts and a pH set close to neutrality, and which became more volatile (Figure 17). The pH of the buffer (KH_2PO_4) was adjusted with sodium hydroxide (NaOH) to pH 5.5 which proved to be the most suitable separation of LEV under isocratic conditions with ACN as solvent. Under these conditions LEV and IS gave a neater chromatogram where the peaks were narrow and sharp and steadiness of the baseline was observed

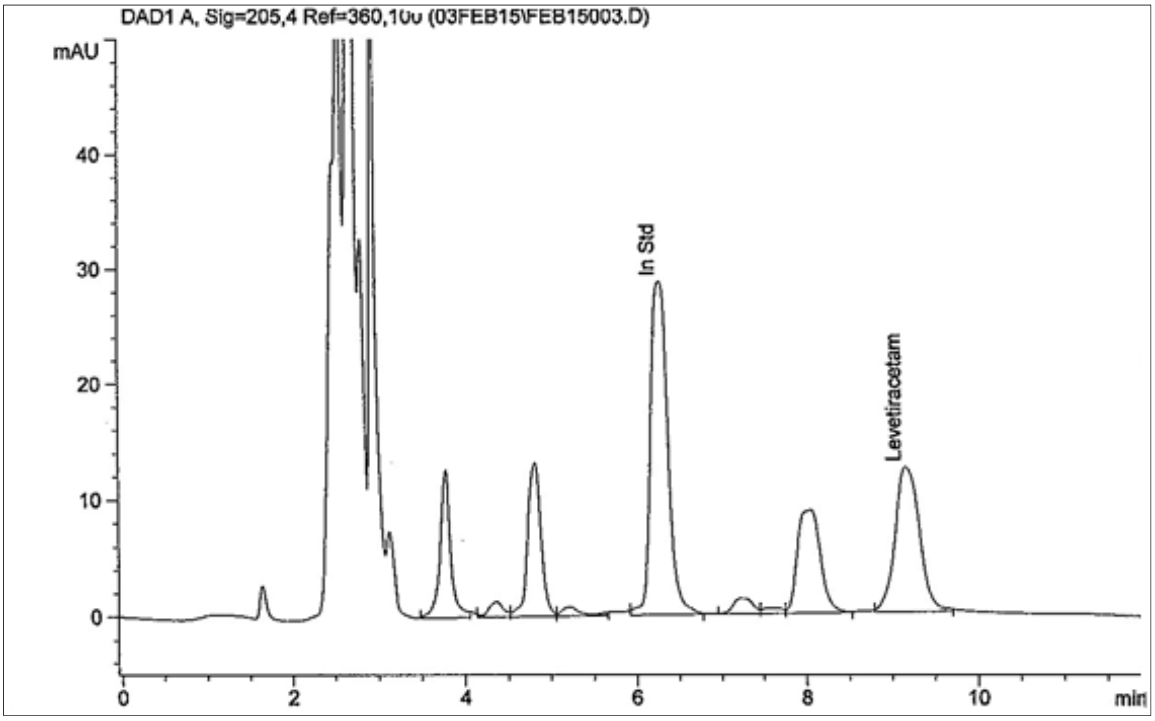


Figure16 Chromatogram with methanol as mobile phase.

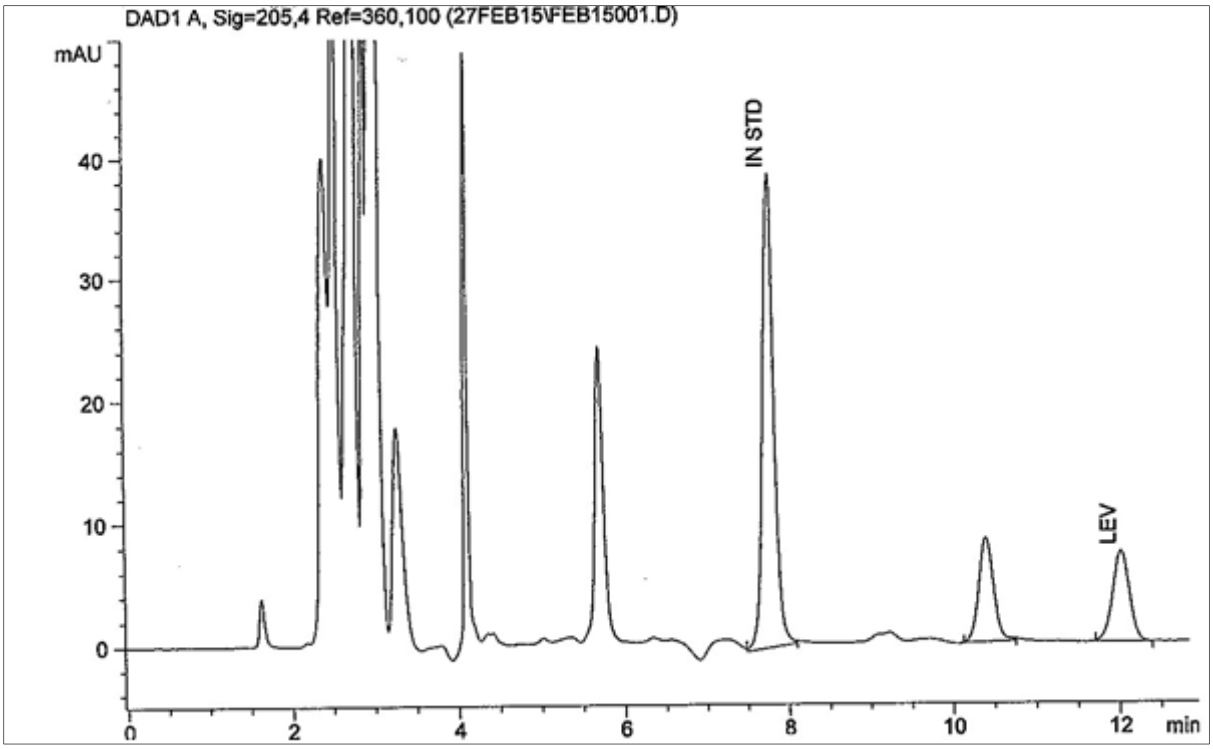


Figure 17 A neat chromatogram with well separated peaks on KH_2PO_4 buffer and ACN (90:10).

- Flow rate - Flow rate of the mobile phase was evaluated at 0.8; 1.0 and 1.5 ml/min for optimum separations. This was based on existing literature (Contin et al, 2008) and from studies performed in the laboratory that showed that 1.0 ml/min flow rate was needed for the successful elution of LEV and IS to maintain a separation and reasonable retention times

- Temperature - Sample separations improved by choosing the optimum column temperature. Studies were performed at temperatures, 25 ^{o}C – 40 ^{o}C. The column temperature was set at ambient (room) temperature

- Injection – A volume of 10 µl was found to compatible with the mobile phase and exchange of the solvent from the sample

- Wavelength – 205 nm was selected as the optimum wavelength to detect LEV. The maximum absorbance on the DAD detector was determined by scanning the LEV standard solution and LEV in plasma/serum of patients with epilepsy over wavelengths of 200 to 400 nm as depicted in Figure: 18

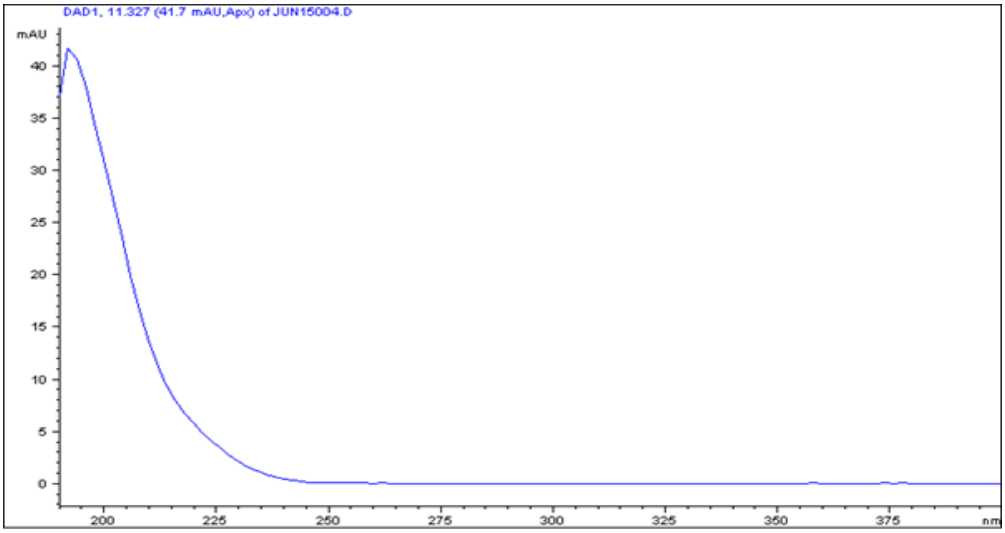


Figure 18 UV spectra of LEV between 200 and 400 nm by DAD spectrophotometer.

4.2.2 Chromatographic results

Adenosine, caffeine and gabapentin were tested as potential internal standards. The retention time of Adenosine (Figure 19) eluted too soon or overlaid with the chromatogram when running patients' samples. Caffeine eluted after levetiracetam (Figure 20) but resulted in false values with caffeine intake by the patients. Gabapentin (1mg/ml) was therefore selected as the IS. The drug eluted before LEV and was well separated from the analyte (Figure 21) with a retention time of ± 5.9 min. The samples' running time on the HPLC was set on 15 min. Figure 22 is an example of the internal standard in a blank serum sample.

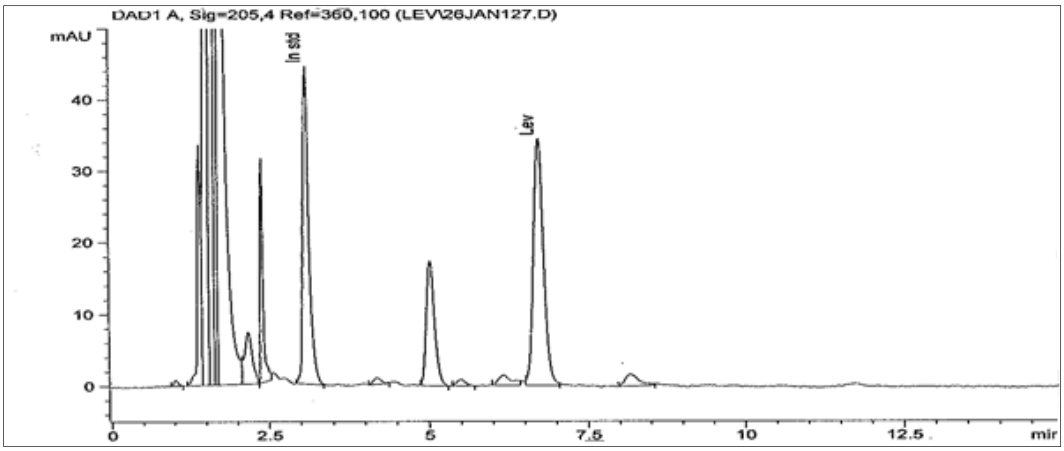


Figure 19 Chromatogram of adenosine as internal standard (10 µg/ml) was set on 3.090 min and LEV in patient sample (54.248 µg/ml) was set 6.73 min.

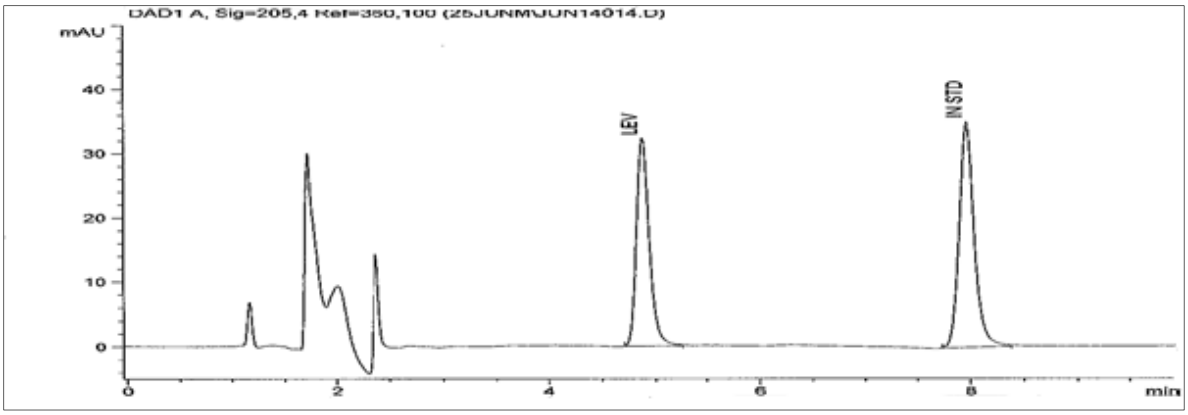


Figure 20 Chromatogram of caffeine as IS (1 µg/ml) was set on 7.964 min and LEV standard (50 µg/ml) was set on 4.848 min.

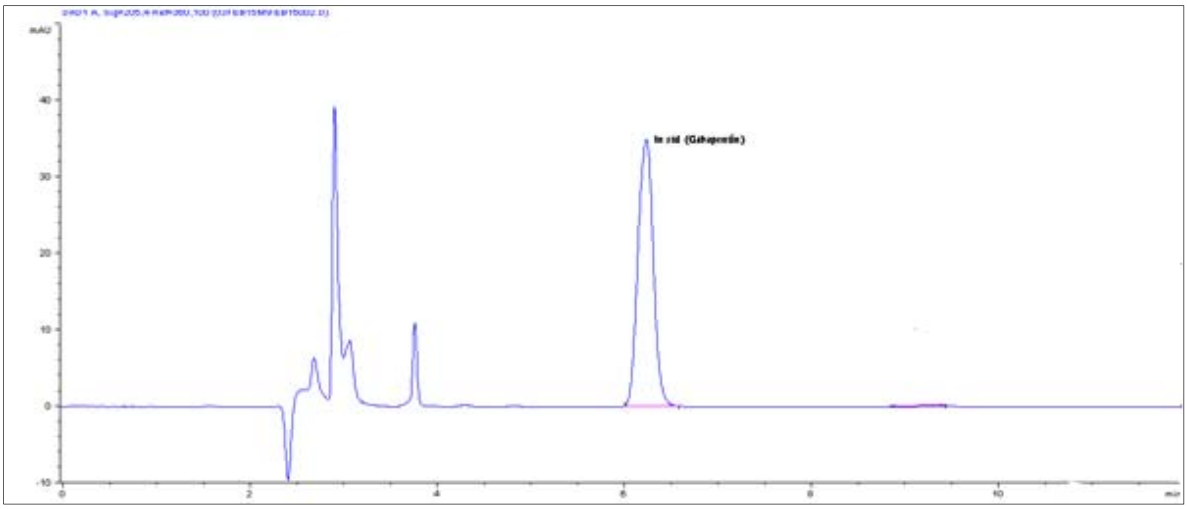


Figure 21 Chromatogram of gabapentin as IS (1 mg/ml) was set on 5.91 min.

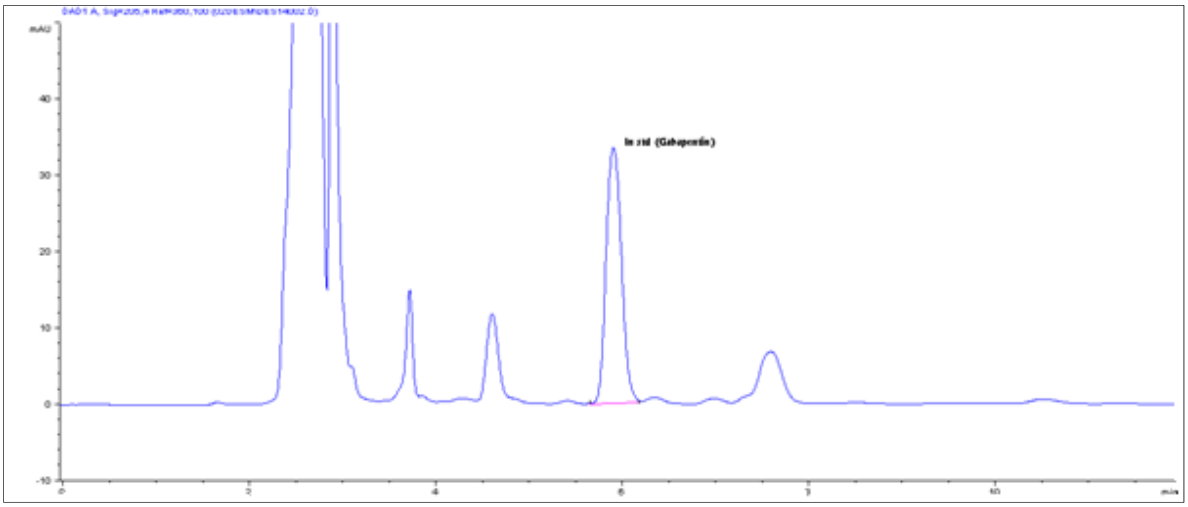


Figure 22 Chromatogram of a blank serum sample with IS (1 mg/ml) was set on 5.91 min.

The following figures show typical chromatograms at different drug concentrations (5; 15 and 50 µg/ml) in water (Figures 23; 24; 25) and serum (Figures 26; 27; 28) after protein precipitation (PP). The retention time of LEV was ± 8.5 min, the column (250 mm) and the mobile phase at a pH of 5,5.

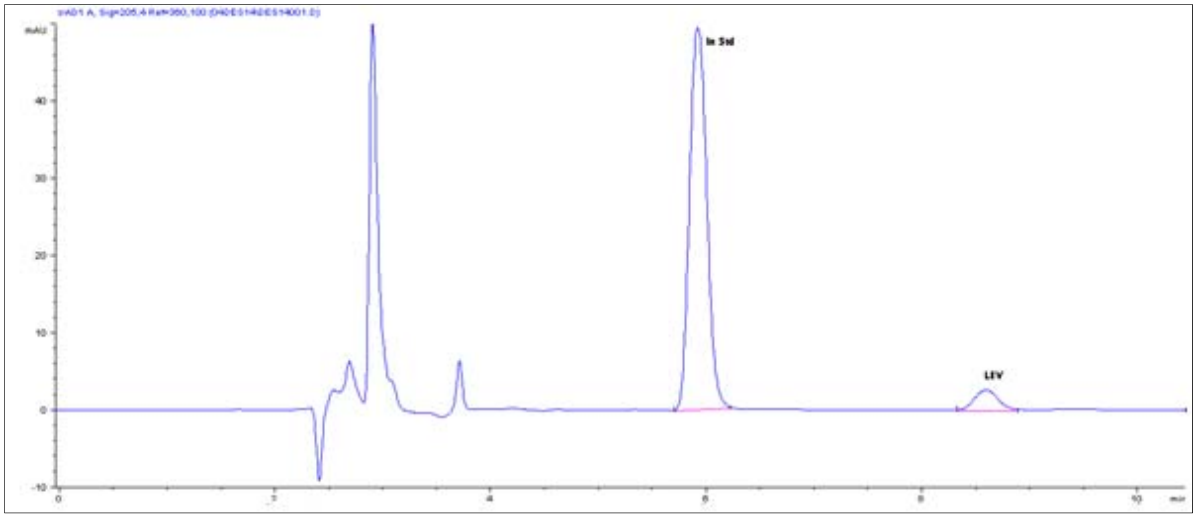


Figure 23 Chromatogram of a LEV water sample with a concentration of 5 µg/ml with IS (1 mg/ml). Retention time of LEV was 8.59 min and IS was 5.91 min.

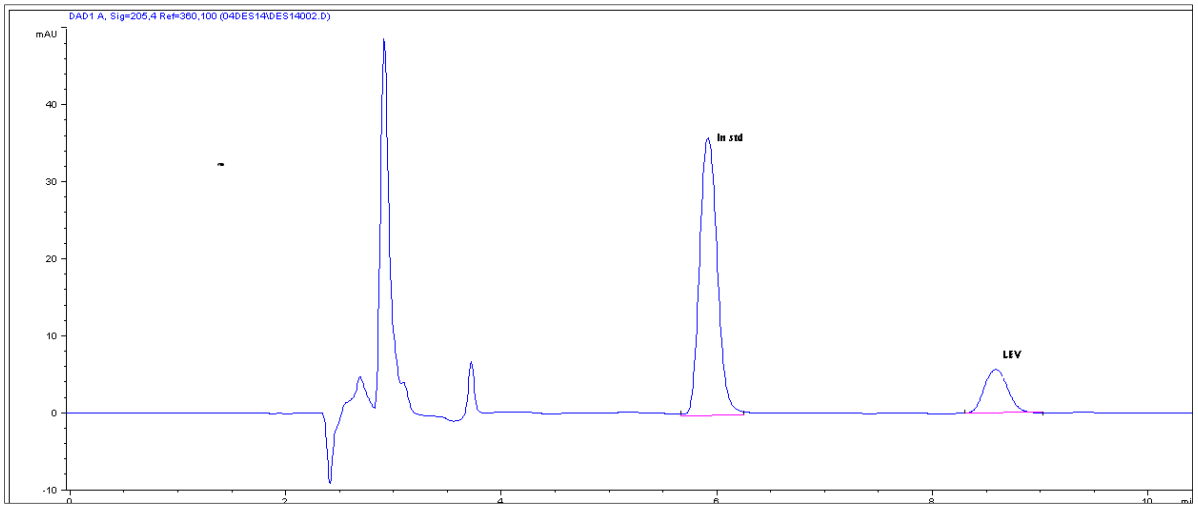


Figure 24 Chromatogram of a LEV water sample with a concentration of 15 µg/ml with IS (1 mg/ml). Retention time of LEV was 8.597 min and IS was 5.91 min.

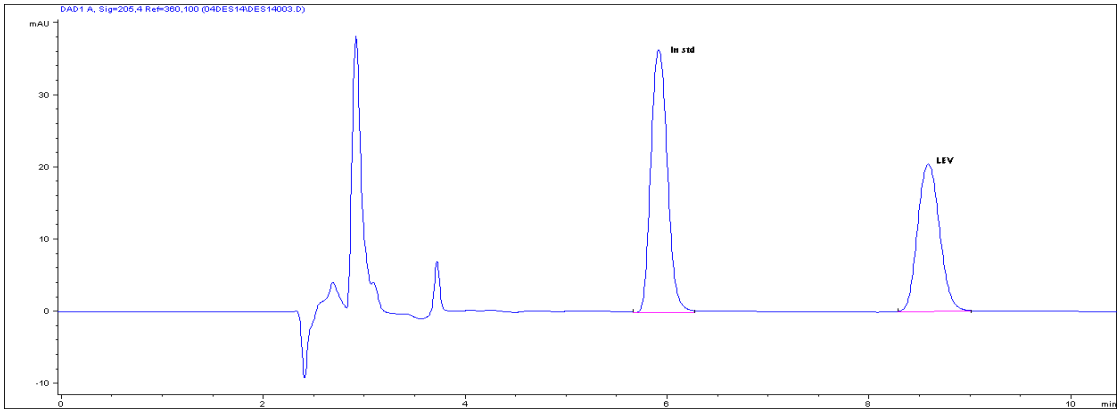


Figure 25 Chromatogram of a LEV water sample with a concentration of 50 µg/ml with IS (1 mg/ml). Retention time of LEV was 8.59 min and IS was 5.91 min.

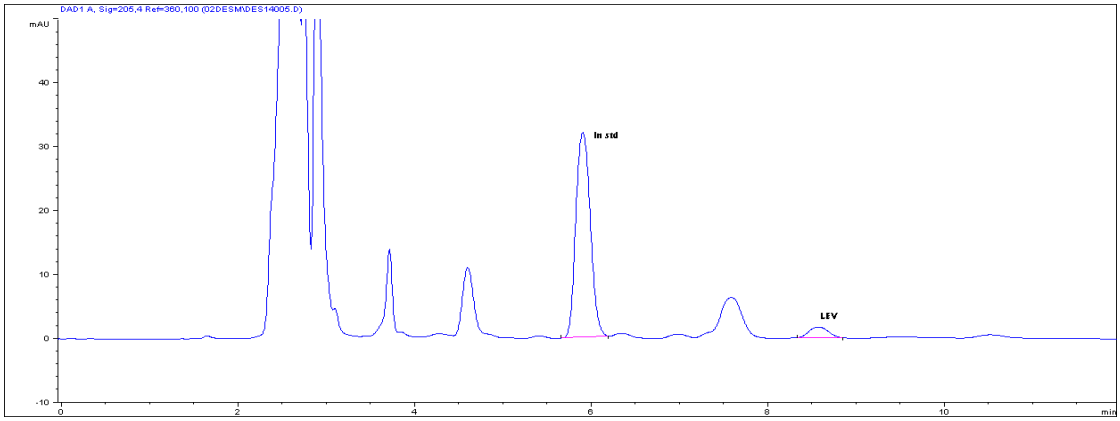


Figure 26 Chromatogram of a LEV serum sample with a concentration of 5 µg/ml with IS (1 mg/ml). Retention time of LEV was 8.57 min and IS was 5.90 min.

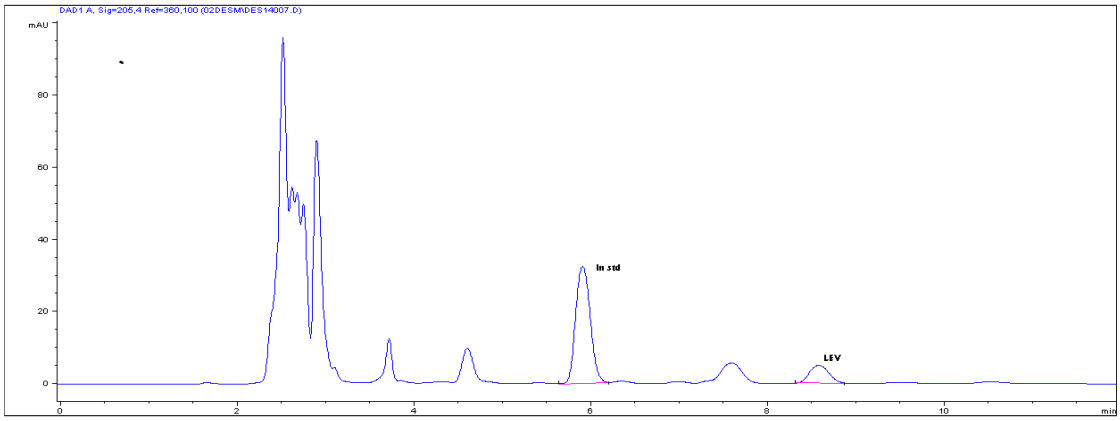


Figure 27 Chromatogram of a LEV serum sample with a concentration of 15 µg/ml with IS (1 mg/ml). Retention time of LEV was 8.57 min and IS was 5.90 min.

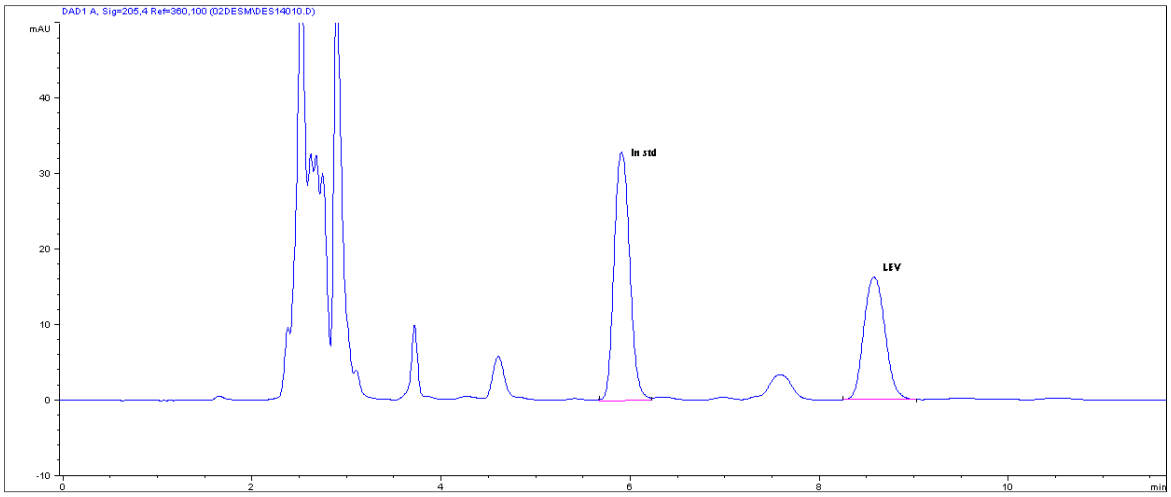


Figure 28 Chromatogram of a LEV serum sample with a concentration of 50 µg/ml with IS (1 mg/ml). Retention time of LEV was 8.57 min and IS was 5.90 min.

4.3 ANALYTICAL METHOD VALIDATION (PHASE 2)

The main objective of the analytical HPLC method validation was to ensure reliable and reproducible results.

4.3.1 Specificity / Selectivity

The chromatograms to ensure selectivity can be seen in figures 29 and 30. Figure 29 shows a blank plasma chromatogram sample; no potentially interfering peaks from endogenous components were present with the LEV or internal standard (IS) at the retention times over 15 min. No endogenous interferences were found in any of the six healthy voluntary MTech students' blank blood samples analysed in the presence with the LEV and IS. A representative chromatogram from one of the volunteers can be seen in figure 30. The selectivity of the method was therefore proven.

Assay specificity was assessed by analysing the most common AED drugs found in the laboratory: lamotrigine, oxcarbazepine, phenobarbitone, phenytoin, carbamazepine and clonazepam (Figure 31A and Figure 31B). Blank plasma was spiked with these drugs, and no interfering peaks were observed at the retention times of either the analyte (LEV) or internal standard (IS) which is an indication of specificity.

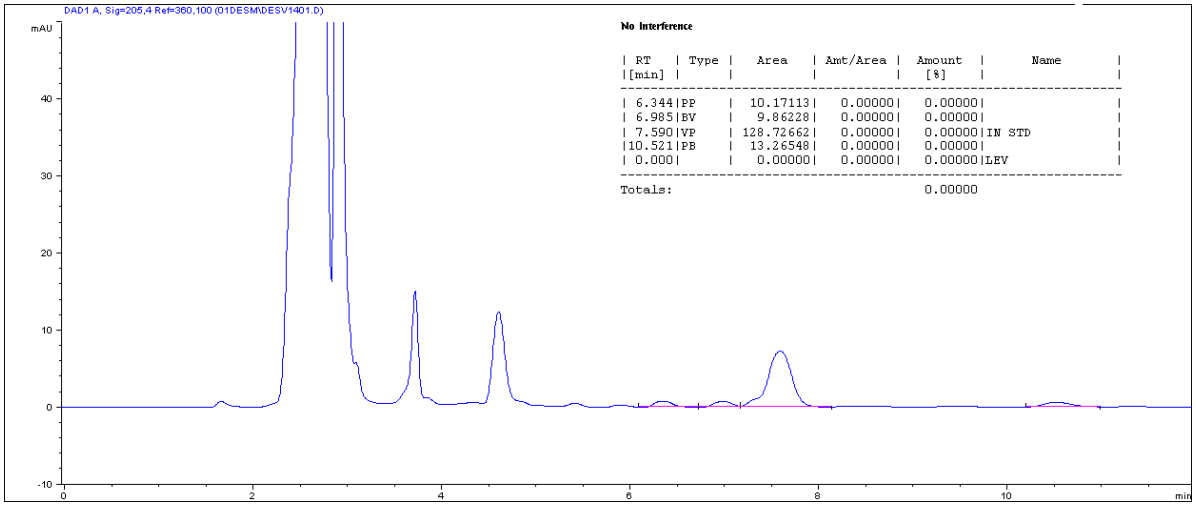


Figure 29 Chromatogram of blank plasma patient sample.

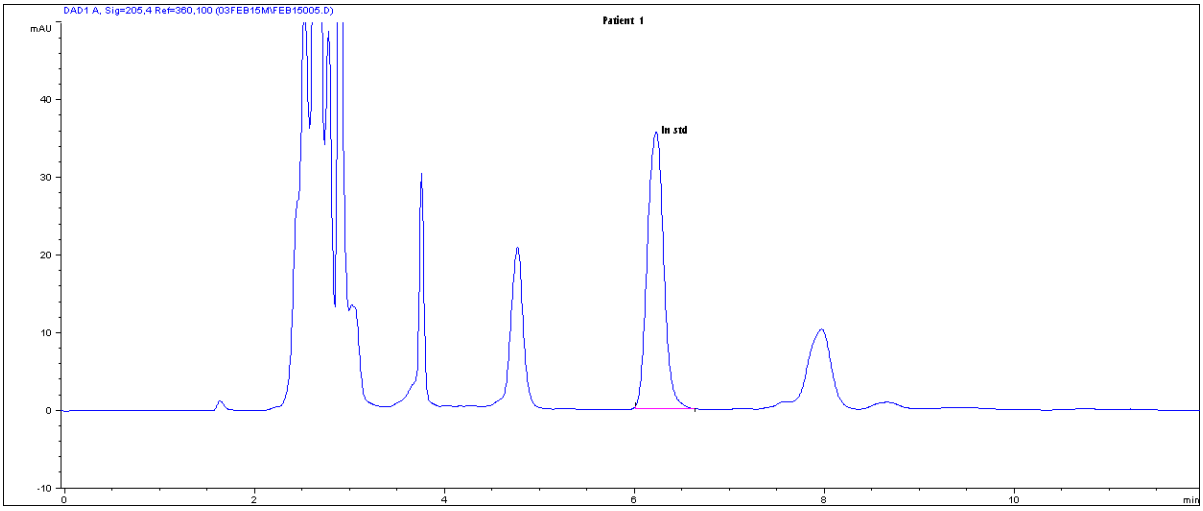
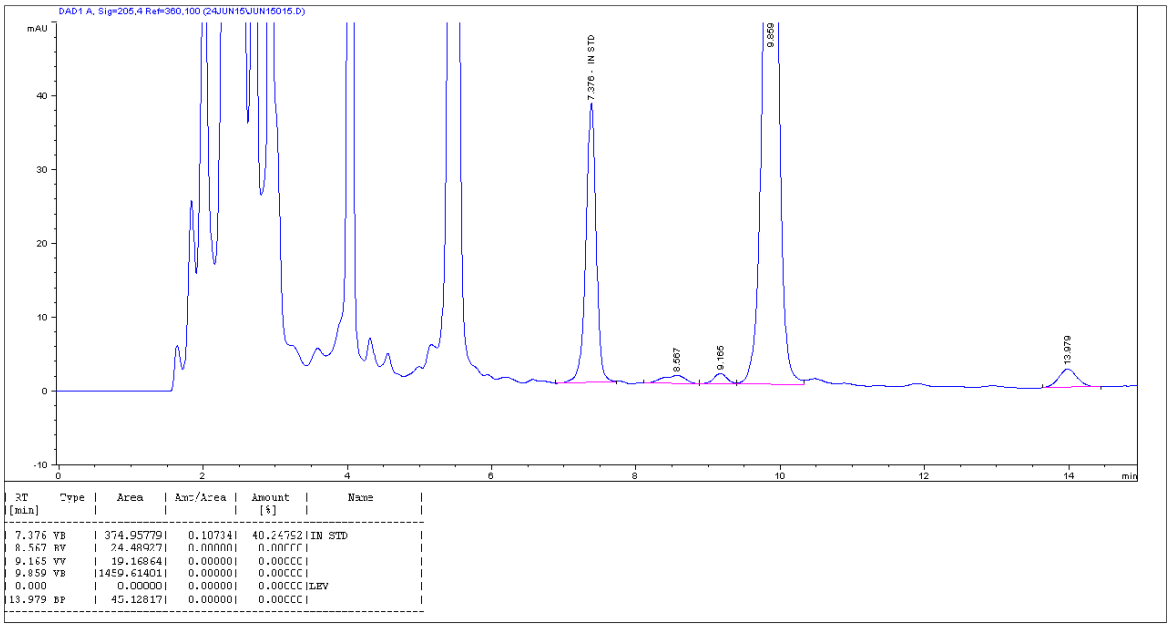


Figure 30 Chromatogram of blank endogenous plasma of Patient 1 spiked with gabapentin as internal standard.



RT [min]	Type	Area	Amt/Area	Amount [%]	Name
7.376	VB	374.957791	0.10734	40.24752	IN STD
8.567	BV	24.489271	0.000000	0.00000	
9.165	VV	19.168641	0.00000	0.00000	
9.859	VB	1459.614011	0.00000	0.00000	
0.000		0.000001	0.00000	0.00000	LEV
13.979	BP	45.128171	0.00000	0.00000	

Figure 31A Chromatogram of other AEDs (Lamotrigine, Oxcarbazepine, Phenobarbitone, Phenytoin, and Carbamazepine) spiked in plasma shows no interferences with LEV (8,57 min) and IS (5,9 min).

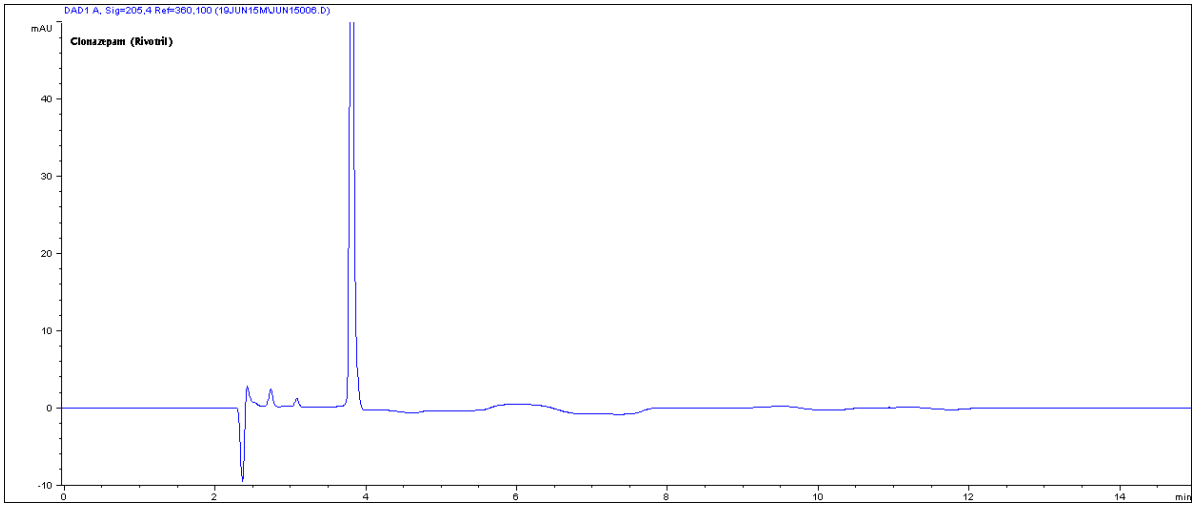


Figure 31B Chromatogram of blank plasma spike with clonazepam shows no interferences with LEV (8,57 min) and IS (5,9 min).

4.3.2 Accuracy and precision

The accuracy and precision results for inter- and intraday variability of the LEV standards in water and plasma at lower, low, medium and higher concentrations (2; 7,5; 15; 25 and 50 µg/ml respectively) are provided in Table 11. The relative standard deviation (% RSD) was below 3,5% for the inter- and intra-day (6 days)

results for both the water and plasma standards and therefore in the acceptable range of < 5%. The accuracy for the intra-day results was between 99 and 103% for the water standards and between 98 and 110% for the plasma standards. The accuracy for the inter-day results showed the same trend viz. 99 to 107% for the water standards and 99 to 110% for the plasma standards. These results were in the acceptable range of 95 – 110% and accuracy was proven.

Table 11 Precision and accuracy of the studies for LEV in water and plasma standards (n=6).

Nominal Concentration (µg/ml)	Intra-day studies (n=6)			Inter-day studies (n=6)		
Water standards	Measured Conc. mean ±S.D (µg/ml)	Precision % RSD	Accuracy (%)	Measured Conc. mean ±S.D (µg/ml)	Precision % RSD	Accuracy (%)
2	2,03 ± 0,03	1,94	101,52	2,14 ± 0,04	2,22	107,15
7,5	7,61 ± 0,03	0,47	101,49	7,67 ± 0,17	2,22	102,36
15	15,59 ± 0,06	0,16	103,96	15,12 ± 0,38	2,56	100,82
25	25,53 ± 0,03	0,12	102,13	25,05 ± 0,50	2,00	100,22
50	49,93 ± 0,09	0,18	99,86	49,97 ± 0,11	0,22	99,93
Plasma standards	Measured Conc. mean ±S.D (µg/ml)	Precision % RSD	Accuracy (%)	Measured Conc. mean ±S.D (µg/ml)	Precision % RSD	Accuracy (%)
2	2,25 ± 0,07	3,33	110,39	2,23 ± 0,01	0,87	110,26
7,5	7,36 ± 0,08	1,12	98,15	7,75 ± 0,11	1,43	103,35
15	15,19 ± 0,16	1,05	101,31	15,05 ± 0,16	1,09	100,34
25	25,67 ± 0,46	1,82	102,69	25,23 ± 0,13	0,522	100,93
50	50,40 ± 0,08	0,17	100,80	49,59 ± 0,41	0,83	99,19

4.3.3 Lower limit of detection (LLOD)

The LLOD is defined as the lowest concentration that gives a reliable, detectable but not necessary quantified as an exact, value. In this study, 0,25; 0.5 and 1.0 µg/ml were measured ten times and were distinguished from the baseline and background noise with a certain degree of confidence. A clearly observed peak of 0,25 µg/ml appeared as the lowest concentration and the % RSD was < 5,0 on the water

standards. A clearly observed peak in plasma was found at 0,5 µg/ml as the lowest concentration and the % RSD was < 5,0%.

4.3.4 Lower limit of quantification (LLOQ)

The LLOQ was set as 1,0 µg/ml in the water and plasma samples. Ten replicates were performed at 1,0 µg/ml and determined as the lowest quantifiable concentration with precision and accuracy. The SD was 0,097 and % RSD was 2,96% for the water standards, therefore in the acceptable range of < 5%. The SD was 0,091 and % RSD was 2,59% for the plasma standards, therefore in the acceptable range of < 5%. One µg/ml was also the lowest concentration on the calibration curve.

4.3.5 Calibration curve and Linearity

The calibration curve was constructed from the following concentrations: 0; 1; 2; 5; 7,5; 15; 25; 35; 50 and 60 µg/ml. Each standard was prepared and analysed six times (n=6). The calibration graph was plotted using the peak area of LEV standards divided by the peak area of that specific standard's IS. Least square analysis was carried out to determine the correlation coefficient. The data was analysed using ANOVA from MS-Excel, version 2013 and Prism, version 5.

Table 12 Data of the calibration curve over a concentration range of 1 – 60 µg/ml (n=6).

	Avg Water Std/I.S	Avg Plasma Std/I.S
Relative Conc. (µg/ml)	**Response**	**Response**
0	0	0
1	0,014434335	0,016525223
2	0,028057157	0,032929157
5	0,070658455	0,07187851
7,5	0,112072371	0,10999442
15	0,217721703	0,21161581
25	0,366904902	0,35338678
35	0,506791608	0,489454019
50	0,720553263	0,692634776
60	0,865982955	0,833105903

The linear regression (r^2) values were calculated as 0,9999 for the water and 1,000 for the plasma standards over the concentration range of 1 - 60 µg/ml. (Table 12 and figure 32).

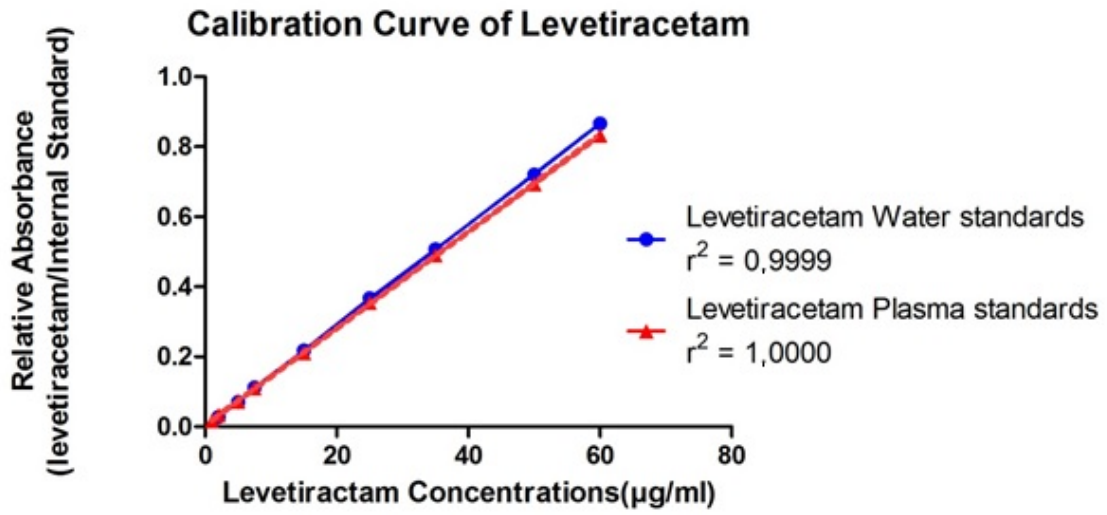


Figure 32 Calibration curve of LEV in water and plasma standards.

Statistical data (n=6) of the linearity of the calibration curve of LEV are given in Table 13. The regression line equation for the water standards was: y= 0,01443x + 0,00113 (r^2= 0,99) and for the plasma standards: y= 0,013806x + 0,00481 (r^2= 1). The calibration curve was done on a daily basis to ensure accuracy.

Table 13 Regression parameters of LEV in water and plasma (n=6).

Compound	*r^2*	*slope*	*intercept*
Levetiracetam (water std)	0,9999	0,01443	0,00113
Levetiracetam (plasma std)	1,0000	0,013806	0,00481

4.3.6 Robustness

Figure 33 shows the representative chromatograms of LEV and IS at different retention times. The difference in retention times was due to back pressure of the

column, variations in the mobile phase composition or an older than one month circulating mobile phase.

No effects on the chromatogram and the size and the shape of the peak were detected with the changes in retention times.

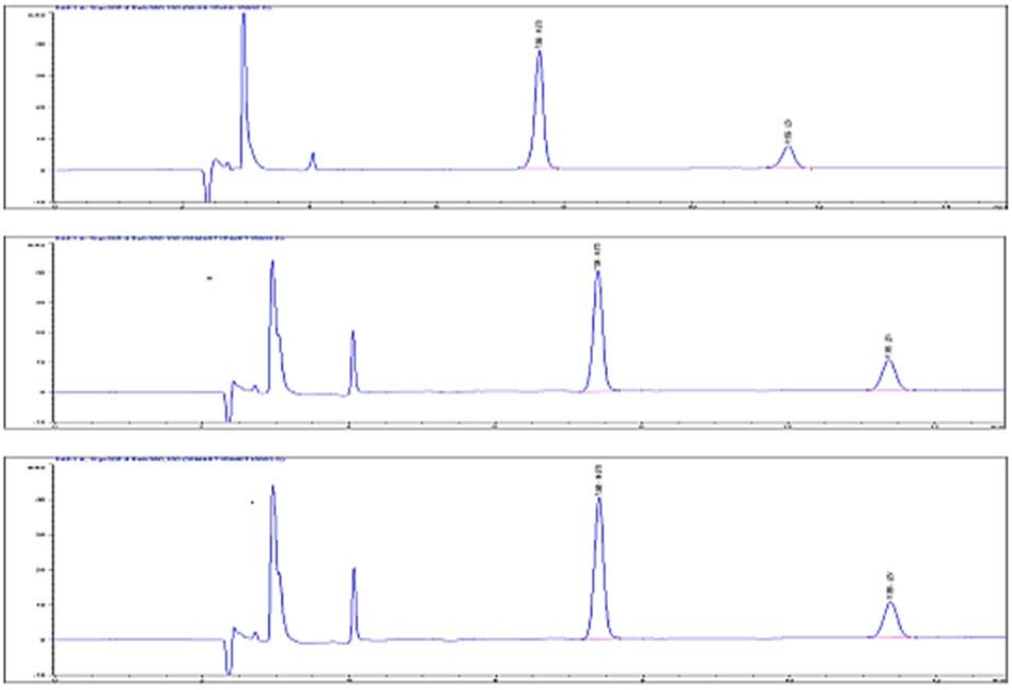


Figure 33 Change in retention time have no effect on the method and chromatogram in the three different chromatograms.

4.3.8 Recovery of the method

The recovery was calculated as a percentage of the plasma standards' concentration for LEV and the concentrations in water standards. The absolute recovery for LEV (2; 7,5; 15; 25 and 50 µg/ml respectively) varied from 94,89 to 100,31% with an average value of 97,15 ± 1,97%. The results were in the acceptable criteria, more than 95% recovery and can be seen in table 14.

Table 14 Recovery of extraction for the analysis of LEV and I.S (n=6).

Water Standards		Plasma Standards		% Recovery
Conc. (µg/ml)	**Peak Area**	**Conc. (µg/ml)**	**Peak Area**	
2	11,3878	2	11,0313	**96,87**
7,5	41,2526	7,5	39,8248	**96,54**
15	83,1976	15	83,4577	**100,31**
25	138,1821	25	134,2027	**97,12**
50	271,3088	50	257,4305	**94,89**
Average				**97,15**
SD				**1,97**

4.3.9 Stability

The standard stock solution of LEV (100 µg/ml) was tested for long term stability at the following temperatures and time periods: fridge (4 ^{o}C), freezer (-20 ^{o}C) with 5 cycles of freeze thaw, bench top (7days) and Room temperature (4 weeks).

The results are presented in Table 15. The % RSD was acceptable and below 2% that indicated that the stock solution was stable over a period of 6 months under all these conditions.

Table 15 Data of the stability of LEV standard under different conditions.

Type of stability	Compound	Measured Concentration (µg/ml)	S.D (µg/ml)	% RSD
Freeze – thaw (5 cycles)	LEV Standard	100,08	1,10	1,10
Fridge (4 weeks)	LEV Standard	100,39	0,84	0,84
Bench top (1 week)	LEV Standard	100,27	0,0013	0,0013
(Room temperature) (4 weeks)	LEV Standard	100,51	0,769	0,76

Summary of the results of the validation parameters for the water and plasma standards was illustrated in Tables 16 and 17. The linearity range was the same for both the water and plasma standards over a concentration range of 1 – 60 (µg/ml). The LLOQ was both the same and was set on 1,0 µg/ml for water and plasma standards.

Table 16 Validation parameters for water standards.

Parameters	Results	
Linearity range (µg/ml)	1 – 60 (µg/ml)	
Standard Regression equation	y= 0,01443x + 0,00113	
Correlation coefficient	0,9999	
LLOD (µg/ml)	0,25 µg/ml	
LLOQ (µg/ml)	1,0 µg/ml	
Precision (at 50 µg/ml)	Intra-day (% RSD)	Inter-day (% RSD)
	0,18	0,22

Table 17 Validation parameters for plasma standards.

Parameters	Results	
Linearity range (µg/ml)	1 – 60 (µg/ml)	
Standard Regression equation	y= 0,013806x + 0,00481	
Correlation coefficient	1,0000	
LLOD (µg/ml)	0,5 µg/ml	
LLOQ (µg/ml)	1,0 µg/ml	
Precision (at 50 µg/ml)	Intra-day (% RSD)	Inter-day (% RSD)
	0,17	0,83

4.4 HPLC METHOD VERSUS COMMERCIAL KIT (PHASE 3)

4.4.1 Optimised chromatographic conditions and parameters required for the ClinRep® HPLC Complete kit to detect LEV

The same HPLC system was used for the ClinRep® HPLC Complete Kit. In table 18 a comparison between the parameters for the newly developed HPLC method and the ClinRep® HPLC kit can be seen. The run time of the ClinRep® HPLC kit was considerably shorter (6 min versus 15 min) than the newly developed method. It is also important to mention that the volume of injection was smaller (10 µl) for the new method in comparison with the 20 µl of the ClinRep® HPLC kit.

Table 18 Optimised chromatographic conditions of both HPLC methods to detect LEV.

Parameters	New Develop and validated HPLC Method to detected LEV	ClinRep® HPLC Complete Kit for Levetiracetam (Keppra®)
Sample preparation	Protein precipitation(PP)	Solid phase extraction (SPE)
Column	Venusil C18, 250 X 4.6mm, 5 µm	Unknown Analytical column
Mobile Phase	50 mM KH2PO4 buffer with ACN (90:10). pH 5,5 with NaOH.	Mobile Phase unknown
Pump mode	Isocratic mode	Isocratic mode
Flow rate (ml/min)	1.0 ml/min	1.4 ml/min
Run time (min)	15 min	6 min
Column temperature (°C)	Ambient	30 °C ± 1 °C
Volume of injection (µl)	10 µl	20 µl
Detection wavelength (nm)	205 nm	205 nm
Retention times **Levetiracetam** **Internal standard**	 8.57 min 5.90 min	 2.67 min 5.07 min

4.4.2 Chromatographic results of ClinRep® HPLC Complete Kit

4.4.2.1 Test Run

A ClinTest® Standard solution is included in the kit. The chromatogram can be seen in figure 34 and is used as a standard reference. LEV concentration was 2 µg/ml at a RT of 2.67 min and the IS concentration was 1 µg/ml at RT of 5.07 min.

The HPLC system was checked with the ClinTest® Standards solution to ensure that the chromatogram was identical to the retention times and peak resolution, and the integration parameters (e.g. run time, peak identification, marks for the peak start and end) had been corrected once for verification as described in Figure 34. No extraction needed and injected directly to the HPLC system. LEV concentration were 2 µg/ml at RT of 2.67 min and the IS concentration was 1 µg/ml at RT of 5.07 min.

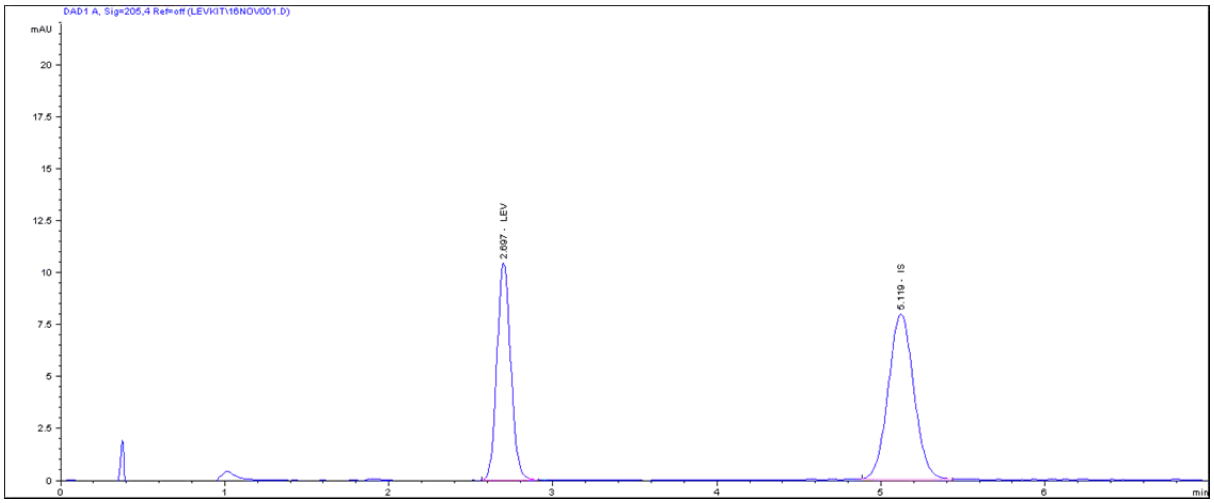


Figure 34 Chromatogram of ClinTest® Standards solution for LEV at 2.67 min and IS at 5.07 min.

4.4.2.2 Calibration

Serum calibrator, ClinCal ® was injected five times as single point-calibration with a concentration of 33,0 µg/ml (see Table 19) where the average (n=5) of the peak area of the LEV (analyte), IS and analyte / IS was evaluated.

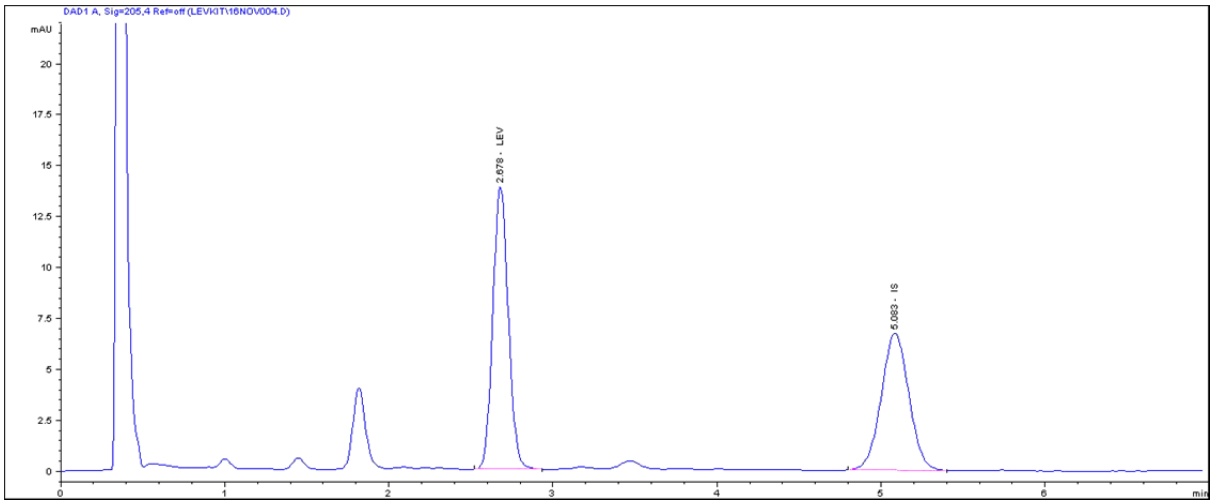


Figure 35 Chromatogram of ClinCal ® with a concentration of 33,0 µg/ml for LEV at 2.67 min and IS with a concentration of 1,0 µg/ml at of 5.07 min.

Table 19 Data of single point calibration (n=5).

	Peak Area		
LEV std concentration	**STD**	**IS**	**STD/IS**
LEV std 33,0	90,76161	79,65765	1,13940
LEV std 33,0	90,57961	790,42427	1,14045
LEV std 33,0	90,47581	79,58198	1,13689
LEV std 33,0	90,41027	79,32826	1,13970
LEV std 33,0	90,17116	79,25934	1,13767
Average (n=5)	**90,479692**	**79,4503**	**1.13882**

The concentrations of unknown samples (analyte) were calculated manually by using the internal standard method via peak areas illustrated in Table 20. These results also demonstrated linearity significance (4.5.2) and the % recovery (4.5.3) of the ClinRep® HPLC Complete Kit.

Calculation of the analyte concentration (C):

C (analyte, sample) [µg/ml] = (Sample peak area/IS peak area) / (STD/IS peak area) C (analyte, calibrator) [µg/ml*].

Table 20 Calculation of the concentrations of unknown samples illustrated.

Samples	Lev Peak Area	IS Peak Area	Sample peak area / IS peak area	Concentration (µg/ml)
Control 1	37,21985	78,94347	0,47	13,66208
Control 11	121,95542	77,97169	1,56	45,32340
Sample 5	12,76389	80,84661	0,16	4,57488
	12,83025	80,34582	0,16	4,62733
Sample 15	35,4266	78,71245	0,45	13,04200
	35,73064	78,58099	0,45	13,17594
Sample 25	58,42634	79,22356	0,74	21,37040
	58,88395	79,91677	0,74	21,35096
Sample 50	129,1572	80,85747	1,60	46,28676
	129,91026	80,6186	1,61	46,69458

4.4.2.3 Accuracy control

The ClinChek® Quality controls (level I and II) were included and analysed in each batch of patients' samples. The chromatograms of the level I and II controls can be seen in Figure 36 and 37. The mean concentrations were 13,5 µg/ml and the control range between 11,1 and 16,7 µg/ml for level I. The mean concentrations were 46,2 µg/ml and the control range between 37,0 and 55,4 µg/ml respectively for II. The retention times were ± 2.67 min for levetiracetam and ± 5.07 min for the IS with a running time of 7 min in both figures.

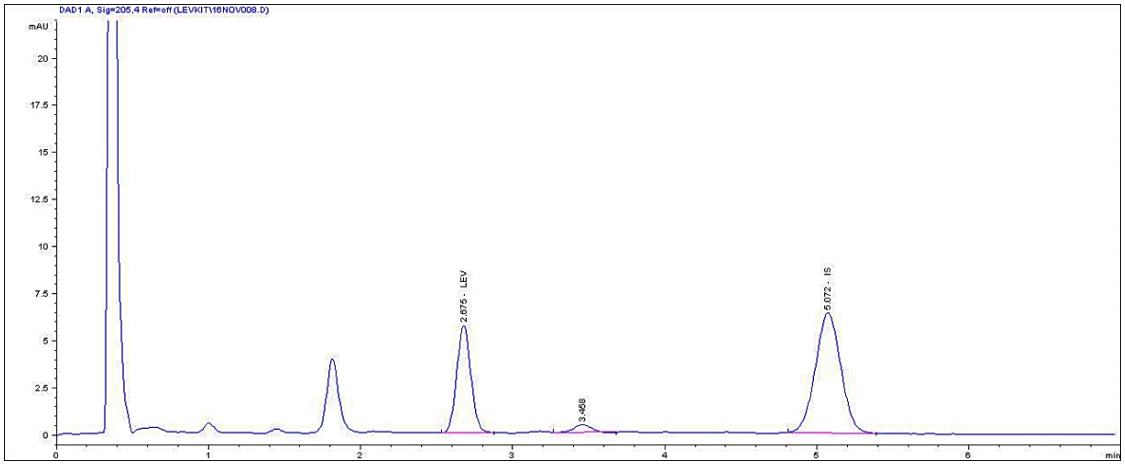


Figure 36 Chromatogram of ClinChek quality control level I with a concentration of 13,5 µg/ml with IS (1 µg/ml). Retention time of LEV was 2.67 min and IS was 5.07 min.

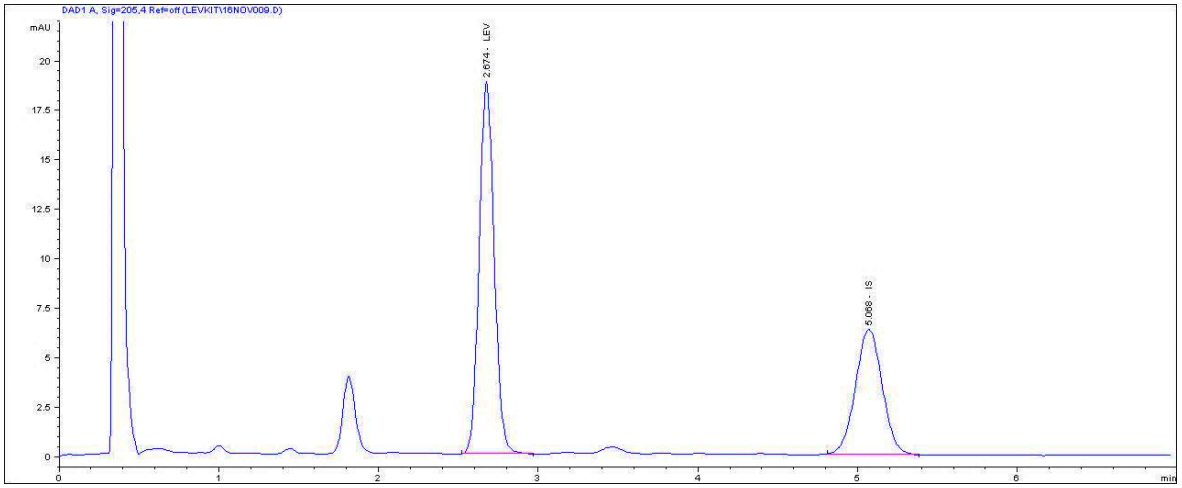


Figure 37 Chromatogram of ClinChek quality control level II with a concentration of 46,2 µg/ml with IS (1 µg/ml). Retention time of LEV was 2.67 min and IS was 5.07 min.

4.5 VALIDATION OF CLINREP® HPLC COMPLETE KIT

The results of the following parameters for validation of the ClinRep® HPLC Complete were followed:

4.5.1 Specificity, accuracy and precision

The specificity of the ClinRep® HPLC Complete Kit was addressed in the manual. They guaranteed specificity in the presence of 16 AED drugs and their metabolites:

lamotrigine, oxcarbazepine, phenobarbitone, phenytoin, carbamazepine and clonazepam and the results can be seen in Annexure D.

The intra- and inter-assay precision were determined by three different concentrations in the therapeutic range (10 – 40 µg/ml). The intra-assay precision was obtained by injecting the three concentration six times. The % RSD was then calculated for each sample. Finally, the average of the individual SD was denoted as intra-assay SD. The inter-assay precision was determined by analysing the three different concentrations in triplicate and also expressed as SD (Table 21).

Table 21 Precision and accuracy of the studies of the ClinRep® HPLC Complete kit (n=6).

Spiked plasma concentration (µg/ml)	Measured Conc. mean ±S.D (µg/ml)	Precision % RSD	Accuracy (%)	Measured Conc. mean ±S.D (µg/ml)	Precision % RSD	Accuracy (%)
5	4,56 ± 0,07	1,54	91,15	4,65 ± 0,07	1,59	93,00
15	12,97 ± 0,16	1,31	86,43	13,28 ± 0,14	1,07	88,53
25	21,50 ± 0,22	1,06	86,00	21,62 ± 0,34	1,59	86,48
50	46,62 ± 0,77	1,66	93,23	47,23 ± 0,54	1,57	94,46

4.5.2 Linearity, lower limit of detection and lower limit of quantification

Four calibration curve concentrations from the newly developed method were used to prove linearity of the Commercial Kit. The concentration range for the calibration range were 5; 15; 25 and 50 µg/ml respectively. Statistical data (n=6) of the linearity of the calibration curve of LEV was demonstrated in Figure 38, the regression parameter of LEV standards were r^2=0,999 and the regression line equation was: y= 0,0295x – 0.0009.

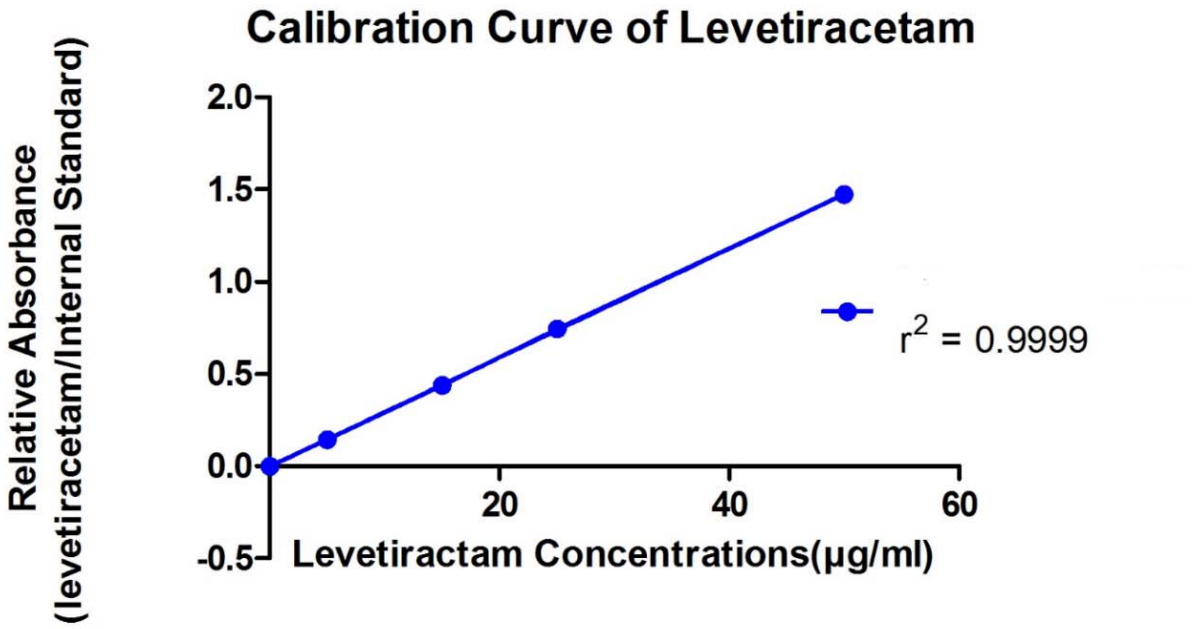


Figure 38 Calibration curve of LEV (Standards from the newly developed method) analysed on the ClinRep® HPLC Complete kit method (n=6).

The results of the LLOD and the LLOQ were 0,14 µg/ml and 0,46 µg/ml respectively (refer to Annexure D).

4.5.3 Recovery

Any losses during the sample preparation were determined by calculating the recovery. Calculation of the recovery rate (REC):

REC = Peak area IS (sample) / Peak area IS (calibrator)

The mean absolute for LEV measured 5; 15; 25 & 50 µg/ml respectively where a mean recovery of 100, 33% is shown in Table 22.

Table 22 Recovery of plasma standards for the analysis of LEV and I.S on the ClinRep® HPLC Complete kit method (n=2).

Samples	IS Peak Are (Sample)a	IS Peak Area (Calibrator)	% Recovery
Control 1	78,94347	**79,4503**	99,3
Control 11	77,97169	**79,4503**	98,1
Sample 5	80,84661	**79,4503**	101,7
	80,34582	**79,4503**	101.1
Sample 15	78,71245	**79,4503**	99.0
	78,58099	**79,4503**	98.9
Sample 25	79,22356	**79,4503**	99.7
	79,91677	**79,4503**	100.5
Sample 50	80,85747	**79,4503**	101.7
	80,6186	**79,4503**	101.4
Average			**100.33**
SD			**1.144**

The recoveries were recorded as 89 – 102% respectively.

4.5.4 Reference range

The therapeutic reference range for LEV in the kit was 10 – 40 µg/ml according to the Arbeitsgemeinschaft für Neuropsychopharmakologie und Pharmakopsychiatrie (AGNP) as indicated in the ClinRep® HPLC Complete kit's manual.

4.6 THE LEV BLOOD LEVEL RESULTS

According to the priori power analysis a sample size of 42 was needed to detect any difference between the two methods. In this study 44 samples were analysed. The consent forms were obtained from the 44 patients (See example of consent in Annexure B*).* One sample was below the limit of detection with a result of 2.65 µg/ml and another sample was above the maximum therapeutic level with a result of 72.61 µg/ml, indicated in Table 23.

Table 23 Results of 44 samples between the newly developed method and the commercial kit on serum/plasma concentrations on LEV.

	New Validation Method Protein Precipitation (PP)			ClinRep® HPLC Complete kit Solid Phase Extraction		
No of samples	**Result 1**	**Result2**	**Average**	**Result 1**	**Result2**	**Average**
LEV Control I	13,83	14,00	13,915	14,67	14,32	14,495
LEV Control II	46,69	46,71	46,70	47,14	46,91	47,025
1	8,27	8,39	8,33	8,36	8,50	8,43
2	4,63	4,61	4,62	4,45	4,48	4,47
3	10,11	10,30	10,21	9,96	10,09	10,03
4	19,57	19,52	19,54	19,52	19,20	19,36
5	20,96	21,02	20,99	20,43	20,30	20,37
6	17,72	71,49	17,60	17,32	17,34	17,33
7	2,68	2,65	2,67	2,75	2,78	2,77
8	16,60	16,66	16,63	16,04	16,34	16,19
9	14,00	14,15	14,08	14,28	14,12	14,20
10	18,57	19,00	18,79	18,99	18,37	18,68
11	17,38	17,48	17,43	17,26	17,43	17,35
12	7,29	7,36	7,33	7,81	7,73	7,77
13	7,73	7,69	7,71	7,79	7,79	7,79
14	19,53	19,56	19,54	19,07	19,15	19,11
15	12,31	12,36	12,34	12,55	12,33	12,44
16	13,96	13,98	13,97	14,00	14,06	14,03
17	8,85	8,87	8,86	8,74	8,81	8,78
18	15,99	15,67	15,83	14,69	14,61	14,65
19	24,48	24,72	24,60	25,16	25,43	25,30
20	25,48	25,46	25,47	25,36	25,08	25,22
21	8,43	8,53	8,48	8,23	7,95	8,09
22	18,67	18,70	18,68	18,29	18,33	18,31
23	12,60	12,70	12,65	12,84	12,73	12,79
24	12,06	1,92	11,99	12,96	13,18	13,07
25	27,27	27,41	27,34	27,44	27,33	27,39
26	8,42	8,31	8,37	8,12	8,22	8,17
27	10,05	10,14	10,09	10,2	10,32	10,27
28	8,01	8,15	8,08	8,08	8,60	8,34
29	21,12	20,96	21,04	21,09	21,37	21,23
30	37,91	37,93	37,92	37,61	37,14	37,38
31	16,05	46,22	16,13	15,91	16,05	15,98
32	23,07	22,93	23,00	22,37	22,27	22,32
33	13,85	13,85	13,85	13,77	13,90	13,84
34	14,17	14,22	14,20	14,03	13,84	13,94
35	9,53	9,45	9,49	9,59	9,24	9,42
36	28,11	28,36	28,24	28,34	28,22	28,15
37	32,33	32,51	32,42	32,80	32,65	32,73
38	25,09	25,10	25,08	25,11	24,74	24,93
39	24,56	24,72	24,64	24,29	24,58	24,44
40	18,72	18,54	18,63	19,71	19,92	19,82
41	72,41	72,61	72,51	71,24	71,39	71,32
42	21,66	21,66	21,66	21,24	21,34	21,29
43	18,20	18,32	18,26	18,47	18,17	18,32
44	17,51	17,58	17,54	17,52	17,95	17,74
	Results below the therapeutic range < 12,0 µg/ml Results above the Therapeutic range > 47,0 µg/ml					

4.6.1 Statistical methods to correlate agreement between two methods

Firstly, the results of the 44 samples presented in Table 23, were plotted to establish the degree of agreement between the two methods (Figure 39). The correlation coefficient (r^2= 0.99); p, 0.0001 was obtained as indicated in Table 25. In Figure 39, the points lie along the straight line of equality which indicated perfect agreement and perfect correlation.

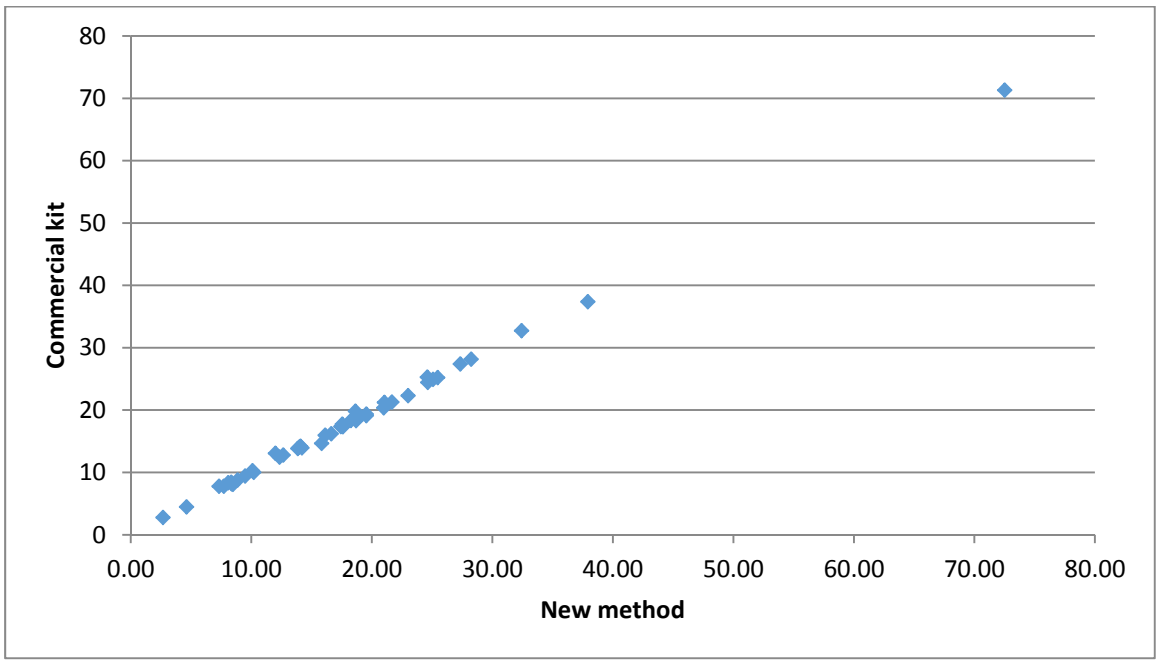


Figure 39 Data plotted the newly developed method and the commercial kit on serum/plasma concentrations on LEV with line of equality.

The Bland-Altman plot of agreement method was used to determine any differences between the two methods and can be seen in Figures 40 and 41. Thus method is an alternative method to investigate the agreement in clinical studies. A total of 44 paired measurements were used in the calculations. Horizontal lines were drawn at the mean difference (yellow line) and the limits of agreements which were defined as the mean difference plus and minus 2 times (red lines) which were the SD of the differences. In Figure 40 and Figure 41, there was a constant scatter of points around the mean difference line which indicating agreement. In Figure 40, the results 1, showed the mean difference that was equal -0,05 and in the results 2 (Figure 41), there was a slight mean difference that was equal -0,11.

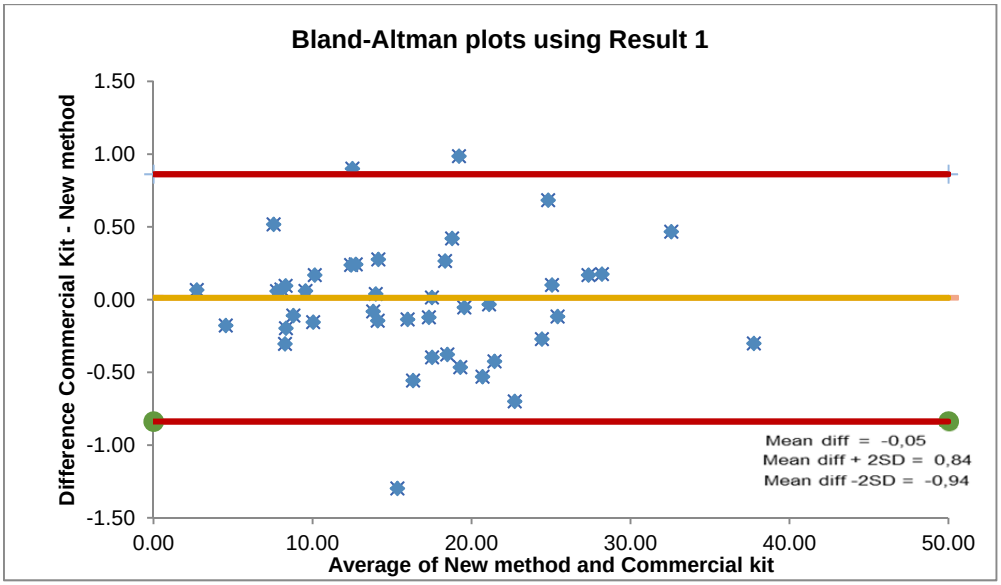


Figure 40 Bland-Altman plot of result 1: Difference between the newly developed method and the commercial kit on serum/plasma concentrations on LEV.

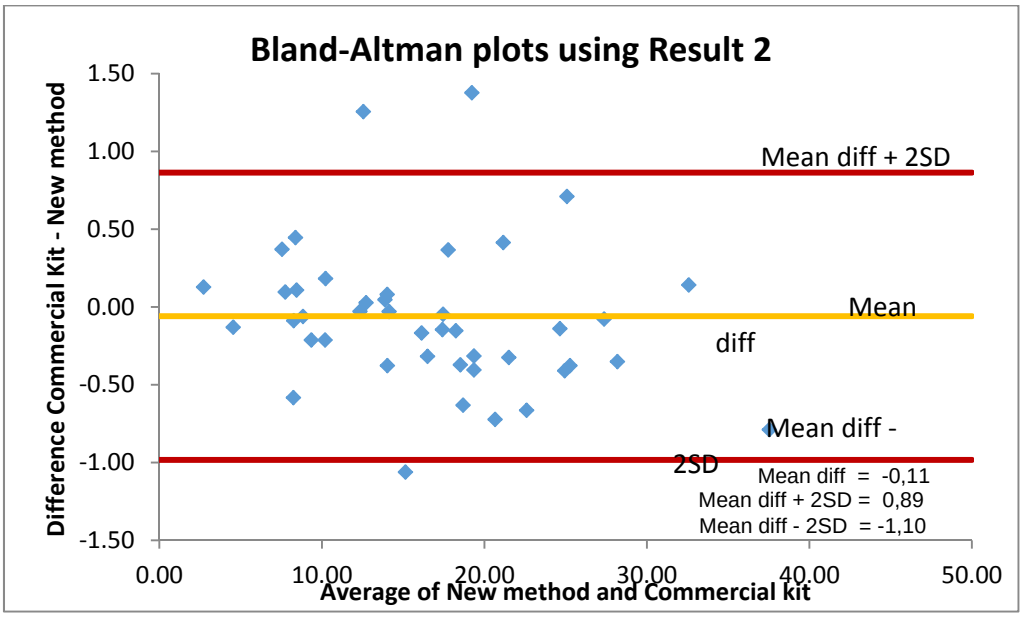


Figure 41 Bland-Altman plot of result 2: Difference between the newly developed method and the commercial kit on serum/plasma concentrations on LEV.

The results of the difference between the newly developed method and the commercial kit on serum/plasma concentrations on LEV (n=2) were presented in duplicate (result1 and result 2) in Table 24. The average difference of -0,05 was not clinically important, the two methods may be used interchangeably.

Table 24 Difference between the newly developed method and the commercial kit on serum/plasma concentrations on LEV (n=2).

	Result 1	Result 2
Bias (95% CI)	-0,05 (-0,18;0,09)	-0,11 (-0,26;0,04)
Lower limit of agreement (95% CI)	-0,94 (-1.17;0,07)	-1,10 (-1,36;0,07)
Upper limit of agreement (95% CI)	0,84 (0,61;1,07)	0,89 (0,63;1,15)

It can be concluded that the agreement between the two methods (newly developed method and the commercial kit) in the measurement of LEV was an almost perfect agreement (average r=0.999; p-value < 0.0001, F-test with a true value =0) presented in Table 25.

Table 25 The reliability between the two methods with Intraclass Correlation Coefficient (ICC) was measured in LEV serum/plasma concentrations measured by HPLC.

	Intraclass Correlation[b] (ICC)	95% Confidence Interval		F Test with True Value 0			
		Lower Bound	Upper Bound	Value	df1	df2	Sig
Single Measure	0,999	0,999	1,000	2866,969	47	47	< 0,001
Average Measure	1.000[c]	0,999	1,000	2866,969	47	47	< 0,001

Two-way mixed effects model where people effects were random and measured effects were fixed.

a. The estimator was the same, whether the interaction effect was present or not.

b. Type A intraclass correlation coefficients were used with an absolute agreement definition.

c. This estimate was computed assuming the interaction effect was absent, because it was not estimable otherwise.

4.6.2 Operational costs

The operational costs between the optimised and validated method and three commercially available HPLC reagent kits were compared and can be seen in table 26 and 27. The three companies were Microsep, Chromsystems and Bio-Rad. The total cost per sample was: Microsep, R177.55-, Chromsystems supplied by Separations, R206.65 and Bio-Rad® from Bio-Rad Laboratories, R278.66

respectively as indicated in Table 26. The newly developed HPLC method was much cheaper, at R50.48 per sample as indicated in Table 27. It is also important to mention that the commercial kit price excluded costs for consumables per sample (pipette tips, extra tubes, column, column guard and cartridge filters) and the quality controls. These costs were also included in the total costing of Table 26.

It can be concluded that the commercial kits (refer to the quotes in Annexure C) were much more expensive than the newly developed method. The costs of the newly developed method was 71 - 82% lower than the three commercial kits supplied in South Africa.

Table 26 The operational cost between three HPLC commercial kits from different manufacturers.

Commercial Kits		Clin Rep® HPLC Complete kit for Levetiracetam (Keppra®) in plasma/serum	Reagent kit for HPLC analysis for Levetiracetam (Keppra®) in plasma/serum	Levetiracetam by HPLC reagent kit
South Africa Suppliers		**Recipe by Microsep**	**Chromsystems by Separations**	**Bio-Rad® by Bio-Rad laboratories (Pty) Ltd**
1. Cost of Kit per 100 samples		R10 474,00	R13 350,00	R17 777,80
2. Cost of controls per 100 samples		R 4 748,00	R 4 360,00	R 6 114,46
3. # of injections per column		**1000**	**1000**	**500**
4. Cost per column		R11 826,36	R13 050,00	R13 757, 54
5. Cost per pre-column filters 10 pk		R 4 284,12	R 4 284,12	Supply with column
Costing	Cost of kit per extraction	R 101,74	R 133,50	R 177,78
	Cost of Controls per sample	R 47,48	R 43,60	R 61,14
	Cost of column per sample	R 11,83	R 13,05	R 27,52
	Cost of pre-column per sample	R 4,28	R 4,28	----------
	Cost of consuma-bles per sample	R 12,22	R 12,22	R 12,22
	TOTAL COST per SAMPLE	**R 177,55**	**R 206,65**	**R 278,66**

Table 27 Operational cost of the newly validated HPLC method.

Validated New HPLC Method		
Pricing		**HPLC**
1. Drug compound for Standards (110 mg) 2. Control I+II supplied by Microsep 3. 2000 µl required for 4 standards 4. Internal Standard (Gabapentin) 5. Control I+II required 6. Methanol (2,5 L) 7. # of injection per column 8. Average cost per column 9. Cost of pre-column filters (10pk)		R2 303,80 R4 748,00 R 4,19 Gifted by CPL R 2,54 R 6,00 1000 R3 420,00 R4 284,12
TOTAL COST OF STANDARD PREPARATION		**R 12.73**
Method		
1. Mobile phase 2. Venusil XBP C18 Column 3. Flow rate (ml/min) 4. Injection per volume **5.** Running time per injection (min**)**		Isocratic C18 1,000 10 µl 15 min
Mobile Phase Consumption		
• Volume prepared • KH_2PO_4 Buffer (500 g) • Acetonitrile (2,5 L)		**1000 ml** **R 8,22** **R 12,90**
TOTAL COST OF MOBILE PHASE		**R 21,12**
Volume (ml) of Solvent used in run		15 ml
Costing	Cost of injection per sample	R 3,42
	Cost of pre-column filter per sample	R 2,86
	Cost of column per injection	R 2,28
TOTAL COST PER INJECTION PER SAMPLE		**R 8,56**
Extra cost for Consumables per sample		
Costing	1. Blue and Yellow tips	R 1,57
	2. Eppendorf (Eppi's 2ml)	R 0,91
	3. Inserts and Vials	R 5,59
TOTAL COST FOR COMSUMABLES PER SAMPLE		**R 8,07**
TOTAL COST PER SAMPLE		**R50,48**

4.6.3 Sample amounts for reliable results in new HPLC method

It was important to establish the minimum sample requirements for the new HPLC method. Samples, ranging from 10 – 50 µl**,** were prepared and analysed at two different QC (Quality control) concentrations. Sample amounts' of 10 µl, 20 µl, 30 µl, 40 µl and 50 µl respectively results showed little difference between 98 – 101%. When samples less than 50 µl were used, there was a slight increase of 1,27% in comparison to the low QC. A slight decrease of 1,7% in comparison with the high QC was found (Table 28).

Table 28 Minimal sample preparation (10 – 50 µl) for reliable results in the newly developed HPLC method to detect LEV.

Control I	**Sample preparation < 50 µl**					
	QC Control I (100 µl)	QC Control I (50 µl)	QC Control I (40 µl)	QC Control I (30 µl)	QC Control I (20 µl)	QC Control I (10 µl)
Day 1	13,85421	13,41166	13,596575	13,29457	13,88000	13,78770
Day 2	13,59581	13,76300	13,500400	13,33323	13,32175	13,25910
Day 3	13,79201	13,56316	13,451600	13,24733	13,99015	13,76270
Day 4	13,77173	13,85440	13,747325	13,78533	13,47380	13,31990
Day 5	13,70473	13,87982	13,614625	13,53996	13,38870	13,58980
Average	13,74369	13,69440	13,582105	13,44008	13,61088	13,54384
SD	0,09838	0,20115	0,11431	0,22299	0,303324	0,245308
%		**99,64136**	**99,179935**	**98,95438**	**101,2706**	**99,50745**
Control II	**Sample preparation < 50 µl**					
	QC Control II (100 µl)	QC Control II (50 µl)	QC Control II (40 µl)	QC Control II (30 µl)	QC Control II (20 µl)	QC Control II (10 µl)
Day 1	44,27798	44,22232	43,08675	43,23790	43,76755	43,78440
Day 2	44,40985	44,34304	43,21222	43,41546	43,76940	43,99350
Day 3	44,45480	44,43510	4339517	44,20116	43,48010	44,19511
Day 4	44,27931	44,33206	44,45572	44,29260	44,35581	44,50061
Day 5	44,38267	44,87040	44,32047	44,54893	44,12615	44,22700
Average	44,36092	44,44391	43,69407	43,93921	43,89980	44,14012
SD	0,07940	0,25895	0,64475	0,57693	0,34271	0,26858
%		**100,1870**	**98,31283**	**100,5610**	**99,91030**	**100,5474**

4.7 STABILITY OF BLOODSAMPLES (PHASE 4)

4.7.1 Stability testing under different storage conditions

Spiked samples (pooled plasma and serum) were prepared freshly and at least a duplicate of each of the concentrations was assessed under different storage conditions over a period of 4 weeks. All the stability results of LEV in human plasma and serum are summarized in Table 29. The % RSD was lower than 5% under all the conditions viz. freeze, fridge, room temperature and auto sampler. It is however important to mention that the % RSD was in most instances higher for plasma than serum. The highest difference could be seen with the 5 µg/ml. Under freezing conditions the % RSD was 1,342 for plasma and 1,171 for serum. In the fridge 1,625 versus 1,082 and at room temperature 1,647 versus 1,416. It seems that serum is more stable at lower concentrations. The opposite was documented for the auto sampler, the serum samples were more stable than the plasma. Testing the stability of LEV in the auto sampler (Table 29) indicated that LEV was stable when kept in an auto sampler for up to one week.

Table 29 Stability of levetiracetam in human plasma and serum (n=2).

Type of stability	Type of specimen	Ave Conc. found (µg/ml)	S.D (µg/ml)	% RSD
Freeze – thaw (3 cycles)	**Plasma(EDTA)**			
	5 µg/ml	5,053	0,067	1,342
	25 µg/ml	25,074	0,131	0,526
	50 µg/ml	50,066	0,093	0,186
	Serum			
	5 µg/ml	5,078	0,059	1,171
	25 µg/ml	25,109	0,098	0,392
	50 µg/ml	50,066	0,187	0,375
Fridge (4 weeks)	**Plasma(EDTA)**			
	5 µg/ml	5,099	0,082	1,625
	25 µg/ml	25,005	0,179	0,718
	50 µg/ml	50,011	0,089	0,179
	Serum			
	5 µg/ml	5,097	0,055	1,082
	25 µg/ml	25,055	0,076	0,304
	50 µg/ml	50,045	0,085	0,170

Table 29 Continued

Type of stability	Type of specimen	Ave Conc. found (µg/ml)	S.D (µg/ml)	% RSD
Bench top (Room temperature) (4 weeks)	**Plasma(EDTA)** 5 µg/ml 25 µg/ml 50 µg/ml	 5,122 25,108 50,080	 0,084 0,064 0,136	 1,647 0,257 0,272
	Serum 5 µg/ml 25 µg/ml 50 µg/ml	 5,087 25,082 50,049	 0,072 0,048 0,147	 1,416 0,191 0,294
Auto sampler (1 week)	**Plasma(EDTA)** 5 µg/ml 25 µg/ml 50 µg/ml	 5,125 25,284 50,343	 0,085 0,421 0,406	 1,667 1,665 0,294
	Serum 5 µg/ml 25 µg/ml 50 µg/ml	 5,074 25,236 50,319	 0,060 0,330 0,374	 1,190 1,310 0,691
Internal standard (gabapentin)	1 µg/ml	374,556	7,333	1,957

4.7.2 Stability of blood in different collection tubes

The data on the stability of LEV in the 5 different collection tubes of SST (Gel), Red, Purple (EDTA), Green (Heparin) and Blue (Sodium Citrate), can be seen in Table 30 and Figure 42. The stability was tested in 6 patients on LEV over a period of 4 weeks. In both patients 4 and 6 the concentration was lower in the blue (Sodium Citrate) tubes during all the weeks. The concentration was also lower for patient 6 in the green (Heparin) tube. The average concentration over the 4 weeks period for each tube can be seen in Figure 42.

Table 30 Data of the stability of LEV in different blood collection tubes stored in fridge over a period of 4 weeks.

Stability of LEV in different blood tubes									
	Different Blood tubes	**Week 0**	**Week 1**	**Week 2**	**Week 3**	**Week 4**	**Ave**	**SD**	**% RSD**
Patient 1	SST	8,7750	8,6890	8,6729	8,4370	8,5193	8,618	0,12	1,42
	Red	8,6624	8,6820	8,5893	8,3417	8,3444	8,526	0,15	1,79
	Purple	8,6945	8,6932	8,6870	8,4959	8,5270	8,619	0,08	1,03
	Green	8,2477	8,1980	8,6590	8,4832	8,6464	8,446	0,19	2,29
	Blue	8,3914	8,5097	8,6896	8,41940	8,2011	8,442	0,15	1,88
Patient 2	SST	8,5390	8,6200	8,3267	8,39319	8,3047	8,436	0,13	1,62
	Red	8,5701	8,6419	8,5817	8,52522	8,4747	8,558	0,06	0,73
	Purple	8,5443	8,4171	8,4518	8,20969	8,4332	8,411	0,12	1,46
	Green	8,2914	8,4179	8,3749	8,40368	8,3573	8,369	0,04	0,59
	Blue	8,41783	8,4888	8,3992	8,3139	8,4822	8,420	0,07	0,84
Patient 3	SST	21,3039	21,4236	21,195	21,4351	21,3701	21,345	0,09	0,46
	Red	21,5367	20,9850	20,822	21,4382	21,2346	21,203	0,30	1,41
	Purple	21,0685	21,2522	21,471	21,2204	21,0538	21,213	0,16	0,79
	Green	21,5367	21,4500	21,170	21,2341	21,4243	21,363	0,15	0,72
	Blue	21,5792	22,6099	22,7535	22,4622	22,5684	22,394	0,46	2,08
Patient 4	SST	17,7690	17,8240	17,8248	17,9760	17,7024	17,819	0,09	0,50
	Red	17,4760	17,5570	17,5571	17,2828	18,0218	17,579	0,24	1,38
	Purple	17,5411	17,5351	17,1164	17,0641	18,2888	17,509	0,43	2,50
	Green	17,8540	17,2273	17,6259	17,8736	17,2784	17,571	0,27	1,56
	Blue	14,5855	14,3383	14,3559	15,9360	16,2128	15,085	0,81	5,412
Patient 5	SST	31,7450	31,2874	30,8794	31,4643	31,4037	31,355	0,31	1,00
	Red	31,1056	31,0954	30,9365	31,1879	31,2808	31,121	0,12	0,40
	Purple	31,3888	31,1888	30,7830	30,7527	31,1217	31,047	0,27	0,88
	Green	30,8937	30,8802	30,8230	30,8346	30,9832	30,882	0,06	0,20
	Blue	31,8156	30,5602	30,2693	30,8402	31,7648	31,050	0,70	2,27
Patient 6	SST	45,8759	15,8631	15,7856	15,7856	15,7438	15,822	0,05	0,35
	Red	15,7976	15,7651	15,7697	15,7300	15,7383	15758	0,02	0,15
	Purple	15,7657	15,7401	15,7280	15,7280	15,7333	15,740	0,01	0,09
	Green	12,5941	12,3438	12,3384	12,3384	12,9982	12,655	0,33	2,62
	Blue	41,6310	11,1643	11,4822	11,4822	11,3275	11,361	0,19	1,71

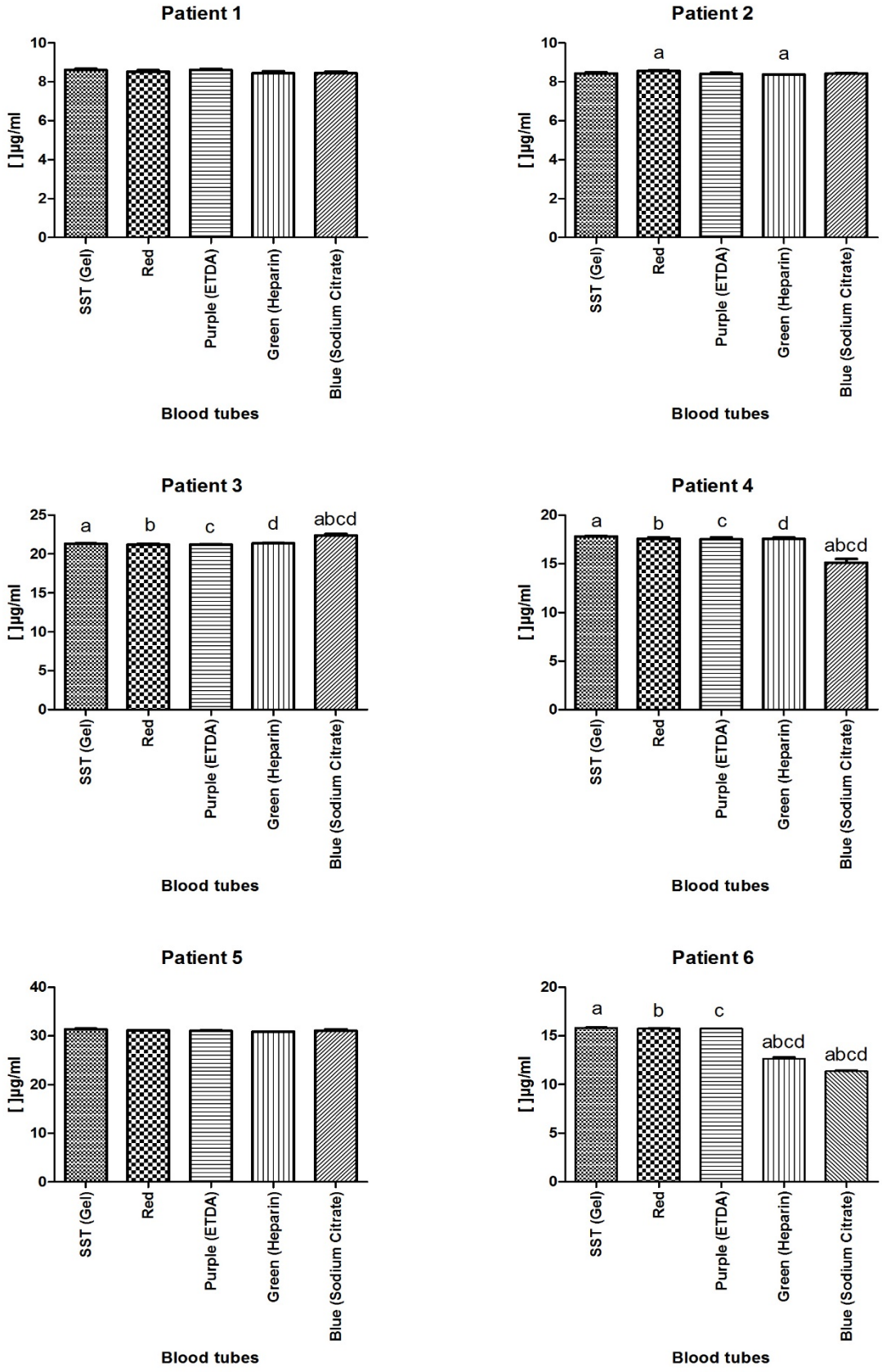


Figure 42 Average blood concentration of six patients in five different blood collecting tubes over a period of 4 weeks.

Two-way repeated measures ANOVA (Analysis of Variance) was used to test for statistical significance between the different blood tubes over a period of 4 weeks. No statistically significant differences could be found between the tubes over the time period in Table 31. (The p-value =0,246 over a period of 4 weeks and the p-value=0,402 for the tubes)

Table 31 Two-way ANOVA to prove the significance differences between the different tubes over a period of 4 weeks.

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	42458,914	1	42458,914	22,879	0,005
Error	9279,069	5	1855,814		

4.8 DISCUSSION

The study was aimed to develop a simple, fast, highly sensitive, accurate and precise HPLC analytical method for the determination of LEV in human plasma/serum in the laboratory in question, according to ICH and FDA. Additionally the study aimed to compare the new method to a commercially available HPLC kit and to investigate the stability of the LEV samples over time and in different collection tubes. It is important to mention that the study was conducted on actual patient samples and not samples spiked with the drug. The study is therefore a true representation of the clinical setting.

The method was developed and adapted from methods described in the literature by Contin et a*l*. (2008) and Poongothai et al. (2011). The sample preparation and clean up procedures were based on the studies reported by Pucci et al*. (*2004) and Olàh et al, (2012). The following adaptations and improvements to the published methods are:

- According to Jiménez Moreno et al. (2014), the two most important parameters are the composition and pH of the mobile phase. Contin et al*.* (2008) evaluated different buffer and ACN ratios and documented 50 mM KH_2PO_4 with ACN (94:6) adjusted to a pH 4.5 as optimal for the separation of LEV and IS. In this study

experiments were performed with MeOH and mixtures of water and buffer concentrations to find the ideal mobile phase. Broad peaks were recorded until MeOH was replaced with ACN. Different ratios of buffer and ACN were tested and 50 mM KH_2PO_4 with ACN (90:10) adjusted to a pH 5.5 with NaOH gave the most reliable results. In our method the pH was therefore set at 5.5 instead of 4.5 as reported by Contin et al. (2008). The combination of the above mentioned mobile phase and the reverse-phase C18 column also provided sharp and distinctive peaks

- Within the South African context it is important to consider costs. The Phenomenex Luna C18 column produced excellent chromatograms but was more expensive than the Venusil C18 column. Studies were performed to compare the two columns and when they produced the same results, it was decided to use the Venusil C18 column. The longer 250 mm column was decided on to prevent endogenous interferences in patients' blood. The type of column lead to better retention time, very sharp and symmetrical peaks and demonstrated a very good selectivity for LEV. Martens-Lobenhoffer & Bode-Böger (2005) and Contin et al. (2008) used a porous graphitic carbon column (150 mm) that can cause endogenous interference

- Two different methods for sample preparation were used in this study: i.e. the protein precipitation (PP) extraction method for the developed method and the solid phase extraction (SPE) method for the commercial kit. It was evident from the results of this study that the PP method is simpler, quicker and more cost effective than the SPE method. The same observations were reported in the literature (Pucci et al, 2004; Hansen *et al*, 2012). Oláh et al. (2012) also compared three different sample preparation methods: Liquid-liquid extraction (LLE), SPE with cartridges and PP with organic solvents. The authors also concluded that PP is the easiest method with the best recovery. By applying the developed method instead of the SPE in the commercial kit, the PP method can be used which will save time and is easier

- All the standardisation and validation criteria requirements set by the FDA (2013) were met for both the ClinRep® HPLC Complete Kit and the newly developed method. The new method validation was as follows:

1. The linear regression (r^2) values were 0,9999 and 1,0 respectively for the water and plasma standards over the concentration range of 1 - 60 µg/ml. These results compared favourably with results reported in the literature, especially with Contin et al. (2008) (r^2= 0,9999) over a range of 4-80 µg/ml.

2. The working range was wide enough (1 - 60 µg/ml) and implied a good range of standards in the new validated method. The therapeutic range of LEV is between 12-46 µg/ml (Krasowski, 2010). It is important to have the working range larger than the therapeutic range to include sub-therapeutic and toxic plasma levels.

3. The lowest calculated concentration that the method could detect was 0,25 µg/ml in water standards. Plasma can be detected at the lowest concentration of 0.5 µg/ml with this method compared to the detection limit of 0,1 µg/ml (Martens-Lobenhoffer & Bode-Böger, 2005).
4. The lowest calculated concentration that the method could quantify was 1,000 µg/ml both in water and plasma standards. Compared to other studies, quantitation limit was 2 µg/ml (Contin et al, 2008).

5. The method is selective for levetiracetam and gabapentin for IS. No interference of other AED's and endogenous peaks was observed.

6. In this study the intra and inter-assay precision and accuracy were < 5%. That was better than values reported in the literature. Contin et al. (2008) reported intra and inter-assay precision and accuracy lower than 7,5% and Martens-Lobenhoffer & Bode-Böger (2005) reported < 5.

7. The recovery was acceptable 97,15% compared to an absolute recovery >90% for LEV (Contin et al, 2008).

8. Stability study was an integral part of the development and the outcome of LEV standard was described as stable. The % RSD below 2% indicated that the stock solution was stable over a period of 6 months.

The development of a cost effective method was important to be able to determine accurate blood concentrations that can be used in the management of patients with epilepsy. In order to compare this method with a commercially available method,

forty four samples were analysed by both methods. The samples of 44 patients were detectable. The correlation coefficient between the two methods r^2= 0.99; p, 0.0001 was obtained and the points lie along the straight line of equality which was a perfect agreement and perfect correlation. The Bland-Altman plot was used to show agreement between the two methods. At the 95% limits of agreement more the 95% of the samples were included in both the analyses and it could be concluded that the two methods were the same.

This is the first study to our knowledge where a developed HPLC method was compared to a commercial available kit. In a study by Akgül et al. (2005), three different HPLC methods were compared and at the end they chose the one that worked out the cheapest. In an article by Tesfaye et al. (2014) an HPLC method was compared with an Enzyme immunoassay technique (EMIT) method; they also concluded that if two methods are the same the cost plays an important role.

The costs of the newly developed method was 71 - 82% lower than the three commercial kits supplied in South Africa. Thus it is very important in our country where resources are limited. It has been established in general that commercial kits are more expensive per analysis compared to developed HPLC methods (Antunes et al, 2009). Costs are significantly reduced by preparing the reagents in the laboratory at moderately low prices compared to the purchase price of commercial kits (Antunes et al, 2009).

Another advantage of the newly developed method is the fact that a smaller sample volume of 10 µl can be used. According to Yeap & Lo (2014), a small volume is a big advantage, especially in a clinical setting.

Running time of both the Recipe (Microsep) and Chromsystems (Separations) methods was very quick, with a total running time of 6 min. The running time of the newly developed and validated method was longer with a total running time of 15 min which was more acceptable. A longer running time ensures no endogenous interferences from patients' blood as shown in the results.

Stability studies with LEV in human plasma/serum are needed for Good Laboratory Practice (GLP) and must be carried out under different conditions (ICH, 2015). A variety of factors may influence the stability of blood samples such as: handling of

samples before analysis (sample not centrifuged), transport and packaging of the blood samples (kept samples cool, no request forms or proper information of the intake of the drug) (Siddiqui et al, 2014). Guidelines to maintain the stability of TDM samples during handling, transport and storage are generalized and non-specific. Relatively little is known about the stability of LEV under these various conditions in South Africa. The effect of long distance transport of blood samples, especially in hot summer months, was documented for other AED drugs but not for LEV (Shazi et al, 2010).

Stability experiments were performed to evaluate the stability of LEV in human plasma and serum under various conditions, simulating the same conditions which occurred during study samples' analysis: room temperature stability, freeze/thaw stability, long term stability, and fridge stability for plasma and serum samples over a period of 4 weeks. The results obtained were within the acceptable limit and it was found that LEV was stable in human plasma and serum with the IS under all these conditions. Normally the human plasma and serum samples were stored in the fridge for at least one month for routine laboratory analysis if there were any inquiries. The results indicated less degradation which suggested that LEV had a good stability in human plasma and serum, either short term or long term stability test. The relative standard deviation (% RSD) was below 5.0% over a period of 4 weeks. It is important to mention that the % RSD was lower for plasma than serum in our results. In Matar (2008), data on the stability of LEV in human plasma under various conditions (room temperature stability, freeze/thaw stability, long term stability fridge) over a period of 4 weeks were stable. The % RSD was below 10% over a period of 4 weeks.

Investigation into the effect that collection blood tubes may have on laboratory results, emphasized the importance of understanding the different collection blood tube limitations. Improper use of blood collection tubes could affect the accuracy of the results. Components from blood collection tubes such as separator gel and additives can alter the stability of the analyte. Bowen & Remaley (2014), gave an in depth review on blood collection tubes' additives and their components and the strategies to minimize their effects on assays. They reported on several studies where the SST tube (gel) affected the concentrations of phenytoin, phenobarbitone

and carbamazepine. A decrease in concentration with as much as 20% after 4 hours at 4 ^{o}C was documented.

Whereas serum was used in most of the assays, plasma was a useful alternative to analyse analytes. It is important to remember that plasma contained fibrinogen and other preservatives which had a higher viscosity and total protein content as serum and that anticoagulants used to preserve analytes may then further interfere with AED's determination in plasma. The most common anticoagulants used are: Ethylenediaminetetraactic acid (EDTA), heparin and sodium citrate.

In this study, decreased levels were observed in both blue (sodium citrate) and Green (Heparin) tubes. The same observations were documented in the literature found in Prabu & Suriyaprakash (2012). The authors reported that lithium heparin (green top tube) and sodium citrate (blue top tube) cause some problems due to drug-protein binding and interference in the matrix effect (Prabu & Suriyaprakash, 2012). The results of the study showed that the green top tube (heparin) and blue top tube (sodium citrate) should be avoided for plasma samples due to the possibility of decrease in concentration levels.

4.7 CONCLUSION

The newly developed and validated RP-HPLC method produced very good resolution to determine LEV in human plasma/serum samples. The method was shown to be simple and fast, reproducible and effective for routine laboratory analyses. The agreement between the newly developed method and the commercial kit was the same and there were satisfactory correlations between the two methods. The method was much cheaper than the commercial kit, used less sample and had a longer running time to ensure no endogenous interference.

The samples were stable under freezing, fridge and on the bench temperatures. It seems that the plasma/serum samples were stable at different temperatures over a time period. Both the Green (Heparin) and Blue (Sodium Citrate) collection tubes may interfere with the concentration levels.

CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

5.1 INTRODUCTION

A vast majority of patients with epilepsy in developing countries do not receive adequate medical treatment. In the treatment of epilepsy it is crucial that anticonvulsants are used continuously and that it is well monitored to ensure effectiveness and safety in the long-term therapy of these patients. It is important to develop more cost effective methods for the measurement of antiepileptic drugs. The aim of this study was to develop and validate a method to determine LEV in serum/plasma levels that can be used for therapeutic drug monitoring in patients with epilepsy. The study was therefore, firstly, designed to develop an accurate method and secondly to compare the method with commercial kits on the market.

One of the factors influencing the quality of results, is the stability of samples prior testing. The study was also designed to investigate the stability of LEV under different temperatures, time periods and in different blood collecting tubes.

This chapter also includes the main findings and limitations of the study and recommendation for future studies.

5.2 RESEARCHER'S CONTRIBUTION

The contributions the researcher herself made to this study are summarized under the following headings: planning and design of the study, literature review, the ethical clearance application, arranging and assessing of blood collection, design and validation of the method, analyses of the data and assembly of dissertation.

5.2.1 Planning and design of the study

It was mentioned in the introduction that the researcher identified a need for the development of an accurate HPLC method for the determination of LEV blood levels. The researcher works in the CPL laboratory at NWU, Potchefstroom and is therefore continuously aware of the need in the health care community and also about challenges experienced during the transport and storage of samples.

The researcher had done an in depth literature research (see 5.2.2) to familiarize herself with the methods available and the pitfalls regarding the stability of the samples before the study was planned.

The researcher decided to compare the developed method with a commercial kit on samples received by the laboratory on a routine basis. The design of the stability study also includes blood samples of patients on LEV. The choice to use routinely collected samples made the study design unique but contributed that the study took much longer than anticipated.

After ethical approval for the study was obtained the researcher purchased the active drug compound, all materials and consumables. A time frame for the purchasing of the HPLC commercial kit, standardizing and validation of the method, and blood samples' collection was scheduled.

5.2.2 Literature review

The researcher was responsible for the literature review as reported in Chapter 2. The literature review consists of a comprehensive overview of epilepsy, treatment of epilepsy with specific emphasis on LEV. The pharmacodynamics and pharmacokinetics of LEV were also discussed as well as the role of TDM in the treatment of epilepsy. The researcher investigated the background of HPLC methods, different methods that were available in the literature and the validation of these methods. The advantages and disadvantages of the commercial kit were also discussed. The final part of the literature review dealt with the different factors which can influence the stability of LEV. These factors included temperature, time and different tubes.

5.2.3 Ethical clearance application

In order to perform the study it was necessary to obtain ethical approval from the Human Research Ethics Committee (Medical), Vaal University of Technology. The researcher had to submit the ethical clearance form to the committee. An example of the form can be seen in Annexure A. The study was approved on the 27th February 2015 with the following Ethical clearance number: **2015024.4.**

As soon as the clearance number was obtained, the researcher applied for approval at the Pathcare Laboratories Ethics committee. It was necessary to obtain ethical approval from the Pathcare laboratory before the blood samples of the patients who participated in the study, could be collected. Written informed consent was obtained from each participant in the study. The researcher had to liaise with the nursing personnel in the different laboratories to obtain the written consent. Parental or guardian informed consent was sought in case of children or patients with cognitive impairment.

5.2.4 Arranging and assessing of blood collection

The researcher had to contact the personnel at the different departments where the samples were collected. The researcher also had to arrange for transport and the phlebotomist. The study involved 3 groups of patients who had to be organized by the researcher.

A letter was sent by the researcher to the nursing personnel of Pathcare Laboratories that indicated to follow a protocol to collect, store and transport blood samples. The protocol included the amount of blood to be drawn, the collection tubes to be used and storage conditions. It was also important to document the time it would take the sample to reach the laboratory. The researcher checked these transport times to ensure stability and quality.

Their blood samples with the written consent were transported to Clinical Pharmacokinetic Laboratory (CPL) for routine therapeutic drug monitoring analysis on a daily basis, with Pathcare Laboratories' overnight couriers.

5.2.5 Design and validation of the method

All the phases and aspects of the design and validation of the method were executed by the researcher herself. They included:

Phase 1 – The development of the HPLC method to detect LEV in human serum/plasma samples was implemented in the following steps:

- Preparation of standard solutions and internal standard

- The selection for the optimal mobile phase, chosen by the researcher to achieve this goal, was based on the optimisation of the mobile phase composition [i.e. buffer type and concentration of the organic solvent and the optimisation of the pH]. The selectivity varies on varying the pH of the mobile phase and should be tested to achieve the successful separation of the chromatographic process

- The optimal column for this method was chosen by the researcher for best separation of the analyte and internal standard

- Flow rate - The exact time each molecule takes to flow through the column was measured by a detector and recorded as retention time. By comparing the retention time of the sample to the internal standard, the molecule (or drug) could be identified. The concentration of the molecule could also be determined from the peaks produced during the run. The peak size used to calculate the quantity of the drug in the specimen, was chosen by the researcher

- Different sample preparations were optimised. Protein precipitations (PP) with methanol were the simplest and used the lowest possible amount with organic solvent with a ratio 1:3.

Phase 2 – The standardisation and validation of the developed HPLC method according to EMA (2011); FDA (2013); ICH (2015); and ISO 17025 (2005).

The following parameters were validated on water and plasma standards:

- Concentration range
- Calibration curve / Linearity
- Lower limit of detection
- Lower limit of quantification
- Accuracy and Precision – repeatability, reproducibility
- Specificity / Selectivity
- Robustness

- % Recovery on both the water standards and plasma standards
- Stability of LEV and IS was tested over a period of 6 months under different conditions (ICH, 2015).

Phase 3 – The comparison of the plasma/serum concentrations between the newly developed and validated HPLC method with the commercially available HPLC reagent kit. This was done as follows:

- Implementation and optimisation of the commercial ClinRep® HPLC Complete Kit on the HPLC was as described in the manual. The following criteria were used in the process:
- Calibration
- Accuracy and precision
- Specificity
- Linearity, lower limit of detection and lower limit of quantification
- Recovery
- Correlation between the plasma/serum concentration levels of the two methods was determined by analysing the samples of 44 patients in duplicate
- Comparison of the operational costs between the two methods was also done by the researcher

Determining the minimal sample (< 50 µl) amount for the preparation of reliable results in the newly developed HPLC method was also the researcher's responsibilities where adjustments were made in duplicate to test the validity and reliability of the results of the newly developed method. The researcher was also responsible for the calculation of the outcome of the experiment.

Phase 4 –The investigation into the stability of LEV under various conditions as required by the EMA (2011), FDA (2013) and ICH (2015). The plasma and serum was spiked in pooled plasma and serum and was also analysed by the researcher in

duplicate over a period of 4 weeks. The influence of five different collecting blood tubes on LEV concentration was investigated. 6 Patients were analysed over a period of 4 weeks for each blood tube and the results were evaluated.

5.2.6 Analyses of data

The results of the study were presented and discussed in Chapter 4. These results included:

- The development, validation and correlation between the newly developed and ClinRep® HPLC Complete Kit methods for the determination of LEV
- A comparison of the operational costs between the two methods
- The optimal sample amount for reliable results with the new HPLC method
- The stability of LEV under different conditions and in different blood tubes

All the data of the results were captured by the researcher. Microsoft Excel software was used for data capturing and statistical analysis. The basic descriptive statistical analyses of the study and linear regression of the calibration curve were performed by the student herself. The Bland-Altman plots, Two-way ANOVA and Intra-class correlations (ICC) were done with the assistance of a statistician from North West University, Department Statistics.

5.2.7 Assemble dissertation

The whole dissertation was assembled by the researcher and consists of 5 chapters. In chapter 1 the setting and problem were discussed. The literature was discussed in chapter 2 and the materials and methods in chapter 3. The results were discussed in chapter 4 and a synopsis of the study was given in chapter 5.

5.3 LIMITATIONS

This study presented with the following limitations:

- The literature on the pharmacokinetics on LEV is older than 5 years in most instances and it was difficult to obtain recent articles

- Challenges in the collection of blood samples. LEV is not used on a regular basis in the public sector and we had to rely on the private sector. Pathcare Laboratories was the only private pathology laboratory prepared to fill in the patient consent forms and didn't provide all the necessary information to do therapeutic drug monitoring. Valuable knowledge regarding LEV pharmacokinetics and compliance of patients could be explored if more information was provided

- The sample preparation of the commercial HPLC kit method was time consuming due to the SPE columns. To prepare these columns an extra 30 minutes was added to complete sample preparation. The CPL is not equipped or automated to prepare SPE samples in large batches

- Only 100 samples could be analysed with the kit which included setting the method of the commercial kit on the HPLC. Validation of the method as well as sample preparation could not be done in duplicate

- The stability of the samples was only tested over a 4 week period. Samples are sometimes frozen for longer and it could be of value to test stability after 3 to 6 months. In the laboratory in question most of the samples are tested in 5 – 7 days and for that reason a 4 week period was stipulated in the protocol

- The high cost of instruments and the technical demands of this technology limit its application in many clinical laboratories

5.4 MAIN FINDINGS

5.4.1 Problem settings

Chapter 1 showed that an accurate and fast method for the determination of LEV by HPLC was needed in the CPL laboratory. It was also noted that the laboratory lacks information on the stability of these samples under different conditions.

5.4.2 Literature review

The literature demonstrated that LEV belongs to a valuable class of anticonvulsant medications with a unique mechanism of action. Numerous studies have demonstrated LEV to be effective in the management of partial seizures. It also increased the treatment options available to patients with refractory epilepsy as an effective AED with added potential benefits in neurological and psychiatric disorders.

Literature demonstrated that the HPLC method with UV detection to determine LEV in plasma/serum for TDM is a simple, fast, cost effective, reliable, and accurate method with minimal sample preparation for daily routine. The researcher found the review articles by Pucci et al, (2004) and Contin et al, (2008) well written and very informative to assist in the develop and standardization of a new HPLC method that is simple and cost effective to provide a TDM service to patients with epilepsy.

5.4.3 Objectives

The objectives of this study were achieved, and were:

1. An HPLC method to determine LEV levels in human plasma and serum was developed and optimized successfully

2. The HPLC method was validated and standardized according to the criteria of EMA (2011), FDA (2013), ICH (2015), and ISO 17025 (2005).

3. The newly developed HPLC method was compared with a commercial kit to determine LEV in human plasma or serum.

    - The agreement between the newly developed method and the ClinRep® HPLC complete commercial kit was the same and statistically significant (average r=0.999; p-value < 0.0001, F-test with a true value =0)

    - Operational costs were compared between the newly developed HPLC methods with 3 available commercial HPLC kits. Costs for the newly developed method were between 71% - 82% lower than for the three commercial kits available in South Africa

4. The minimum amount of blood needed for accurate results in the newly developed method was determined. There were no statistical differences between the 10 µl, 20 µl, 30 µl, 40 µl and 50 µl respectively

5. The stability of LEV in human plasma and serum under various conditions, simulating the same conditions which occurred during study samples analyses was evaluated: room temperature stability, freeze/thaw stability, long term fridge stability for plasma and serum samples over a period of 4 weeks. The results obtained were within the acceptable limit and it was found that LEV was stable in human plasma and serum with the IS under all these conditions. Investigation on the stability of LEV in 5 different collection blood tubes was done. It was found that the blood tubes containing sodium citrate and heparin interfere with the concentrations in patients and should be avoided in therapeutic drug monitoring.

5.5 CONCLUSION

A new HPLC method was successfully developed, validated and standardized. The method was proven to be specific, sensitive and reliable for the determination of LEV in human plasma/serum. The HPLC method is simple and cost effective with minimal sample preparation time, especially for daily routine work. The concentration range was between 1 and 60 ug/ml with the LLOQ = 0,5 µg/ml. The calibration showed good linearity (r^2 was 0.9999). This method was as accurate as the commercially available kit, cheaper and used less plasma sample.

In conclusion, a new method was developed in the study that adds value to the CPL laboratory. The study also gave new insight into the stability of LEV under different conditions. The results of this study can add value to the therapeutic drug monitoring of epileptic patients.

5.6 RECOMMENDATION

A protocol can be drafted to improve quality and stability in blood samples. The importance of the correct collection tube, storage and transport conditions must be emphasized.

It is also important to educate the personnel at the depots regarding temperature control and vital information of the patients. The temperature control is necessary to avoid errors and for quality. The information of the patients can improve the TDM report. A more informative TDM report can lead to a better quality life of the patient and cost effectiveness.

To draw blood can be problematic in paediatrics and the elderly, therefore another project for the determination of LEV in saliva or dried blood spots is recommended.

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ANNEXURES A

Annex A. 1 Ethics Clearance Certificate

VAAL UNIVERSITY OF TECHNOLOGY

RESEARCH & INNOVATION ETHICAL CLEARANCE CERTIFICATE

Applicant:	Ms Lynette Engelbrecht
Project:	Development of a HPLC method for the detection of levetiracetam in blood in patients with epilepsy.
Institution:	Vaal University of Technology
Date Approved:	9 February 2015
Ethical Clearance Number:	2015024.4
Approved: Yes/No	Yes

Approved by: _____ Date: 27/2/15

Dr BJ Johnson
Chairperson: Research & Innovation Ethics Committee

ANNEXURES B

Annex B. 1 Consent forms MTech students

Annex B. 2 Consent forms Patients

Annex B. 3 Letter Path Care Option 1

Annex B. 4 Letter Pathcare Option 1 & 2

Annex B. 5 Approval of research study by PathCare research committee

INFORMATION LEAFLET AND INFORMED CONSENT FOR BLOOD SAMPLING FROM VOLUNTEERS

VALIDATION OF A HPLC METHOD FOR THE DETECTION OF LEVETIRACETAM IN BLOOD IN PATIENTS WITH EPILEPSY

Clinical Pharmacokinetic Laboratory (CPL), Department of Pharmacology, School of Pharmacy, North-West University, Potchefstroom

Principal investigators:	Mrs Lynette Engelbrecht	Contact No: 018 2992242
Supervisors:	Mrs C J Grobler	Contact No: 016 9509210 / 0827753192
	Dr M Rheeders	Contact No: 0828525547

DATE OF INFORMED CONSENT (***BLOOD***):

		20
day	month	Year

To my MTech student colleague's
Biomedical Department
VUT

INTRODUCTION:

You are hereby being invited to take part in a research project where your volunteering blood sample will be used for:

1. Analytical validation of a HPLC method for the detection of Levetiracetam
2. Testing the stability of LEV under various conditions over a period of 4 weeks.

The research is important to maintain our laboratory at the highest international standards for Good Laboratory Practice (GLP).

The study site is The Clinical Pharmacokinetic Laboratory (CPL) at the North-West University, Potchefstroom Campus and it forms part of my M-Tech research project at VUT.

You can contact the following 24-hour telephone number: **Cell:** 082 3354107 for any study related information.

Your participation in this study is entirely voluntary and you are free to decline to participate. If you say no, this will not affect you negatively in any way whatsoever.

This information leaflet is to help you to decide if you would want to take part. If you decide to take part in this study, you will be asked to sign this document to confirm that you understand the study. You will be given a copy to keep.

BLOOD SAMPLING; HANDLING AND STORAGE:

Your blood sample is will be drawn by a qualified nurse or Phlebotomist and will be collected and sends to CPL, North-West University, Potchefstroom. Your blood will be spiked with LEV and used for analytical validation purposes.

2 x EDTA and 2x STT (serum) blood tubes will be drawn at any one stage. Your blood (unknown) will also be spike with LEV and will be used to investigate the stability of LEV stored and handling over a period of 4 weeks.

RISKS AND CONFIDENTIALITY:

We will do everything we can to protect your privacy. Your name will not be shown on the sample. It is important to understand that your blood samples will **only** be used for validation of the analytical method and will be spiked with a known concentration LEV drug and for the stability studies.

POTENTIAL BENEFITS:

There are no direct benefits to you. The information gathered from this study will be used to keep improving the high standards in the laboratory

.

ETHICAL APPROVAL:

The study protocol has been submitted to the Human Research Ethics Committee (Medical), Vaal University of Technology. These committees are established to protect the rights of people taking part in research studies. The protocol has been granted approval by committees on …….. (Date) with the following reference number …….

INFORMED CONSENT and ASSENT (participants):

..… …. … ….. ….. …… ….. …. … . *(NURSE / PHLEBOTOMIST)* has provided me with a copy of this Participant Information Leaflet and Consent regarding the study, **protocol number……..:…..**and has fully explained to me the nature, risks, benefits and purpose of the study.

- the study investigators have given me the opportunity to ask any questions concerning the study procedures, the potential benefits and risks.
- It has been explained to me that I'm under no obligation to sign the form

PARTICIPANT:

Printed Name	Signature / Mark or Thumbprint	Date and Time

TRANSLATOR / OTHER PERSON EXPLAINING INFORMED CONSENT: ……..…….

Printed Name	Signature	Date and Time

WITNESS (If applicable):

Printed Name	Signature	Date and Time

INFORMATION LEAFLET AND INFORMED CONSENT FOR BLOOD SAMPLING FOR PARTICIPANT

Project: VALIDATION OF A HPLC METHOD FOR THE DETECTION OF LEVETIRACETAM IN BLOOD IN PATIENTS WITH EPILEPSY

Clinical Pharmacokinetic Laboratory (CPL), Department of Pharmacology, School of Pharmacy, North-West University, Potchefstroom

Principal investigators:	Mrs Lynette Engelbrecht	Contact No: 018 2992242
Supervisors:	Mrs C J Grobler	Contact No: 016 9509210 / 0827753192
	Dr M Rheeders	Contact No: 0828525547

DATE OF INFORMED CONSENT (***BLOOD***):

		20
day	month	Year

INTRODUCTION:

Good day,

You are hereby being invited to take part in a research project where your blood sample(s) will be used to compare two analytical methods in our laboratory and to investigate the influence of different blood collection tubes in patients using Levetiracetam. The research is important to maintain our laboratory at the highest international standards for Good Laboratory Practice (GLP).

The study institution is The Clinical Pharmacokinetic Laboratory (CPL) at the North-West University, Potchefstroom Campus and it forms part of my M-Tech research project at VUT.

You can contact the following 24-hour telephone number: **Cell:** 082 3354107 for any study related information.

Your participation in this study is entirely voluntary and you are free to decline to participate. If you say no, this will not affect you negatively in any way whatsoever.

This information leaflet is to help you to decide if you would want to take part. If you decide to take part in this study, you will be asked to sign this document to confirm that you understand the study. You will be given a copy to keep.

BLOOD SAMPLING; HANDLING AND STORAGE:

☐ Your blood sample will be drawn and sends to our laboratory for the routinely monitoring of your Levetiracetam blood concentration. The laboratory wants to compare two methods for the determination of levetiracetam with each other. It is important to understand no extra blood will be drawn from you, the sample send to the laboratory will be used and all the data will be de-identified (anonymised) and will be kept confidential.

OR

☐ Need at least 6 volunteer patients. These blood samples will be used for investigating the influence of levetiracetam in five different blood collection tubes. Not more than 10 ml blood will be drawn at any one stage.

It is important to understand that blood will be drawn from you, the samples will be sending to the laboratory and be used and all the data will be de-identified (anonymised) and will be kept confidential.

The results will be stored in a safe dedicated locked filing cabinet with limited access, under the direct supervision of Mrs L Engelbrecht at the Department of Pharmacology, North-West University, Potchefstroom Campus.

RISKS AND CONFIDENTIALITY:

We will do everything we can to protect your privacy. Your name will not be shown on the sample. A code will be used to identify the samples. No additional blood or needle pricking will take place as the same blood will be used for your levetiracetam testing.

POTENTIAL BENEFITS:

There are no direct benefits to you. The information gathered from this study will be used to keep improving the high standards in the laboratory and no feedback to you will be provided other than the levetiracetam drug concentration.

ETHICAL APPROVAL:

The study protocol has been submitted to the Human Research Ethics Committee (Medical), Vaal University of Technology. These committees are established to protect the rights of people taking part in research studies. The protocol has been granted approval by committees at the Vaal University of Technology on the 27 February 2015 (date) with the following approved Ethnical clearance number: **2015024.4**…….

IMPORTANT CONTACT DETAILS:

If you have consulted your doctor or the ethics committee and if they have not provided you with answers to your satisfaction, you should write to the Medicines Control Council (MCC) South Africa at: The Registrar, Medicine Control Council SA, Department of Health, Private Bag X828, Pretoria, 0001.

INFORMED CONSENT (parents/legal guardians) and ASSENT (participants):

..… …. … ….. ….. …… ….. …. … . *(NURSE / PHLEBOTOMIST)* has provided me with a copy of this Participant Information Leaflet and Consent regarding the study, **protocol number…2015024.4. and** has fully explained to me the nature, risks, benefits and purpose of the study.

- The study investigators have given me the opportunity to ask any questions concerning the study procedures, the potential benefits and risks.
- It has been explained to me that I'm under no obligation to sign the form

PARENT/LEGAL GUARDIAN:

Printed Name	Signature / Mark or Thumbprint	Date and Time

PARTICIPANT ASSENT: (7 years old and above)

Printed Name	Signature / Mark or Thumbprint	Date and Time

TRANSLATOR / OTHER PERSON EXPLAINING INFORMED CONSENT: ……..……

Printed Name	Signature	Date and Time

WITNESS (If applicable):

Printed Name	Signature	Date and Time

NORTH-WEST UNIVERSITY
YUNIBESITI YA BOKONE-BOPHIRIMA
NOORDWES-UNIVERSITEIT
POTCHEFSTROOM CAMPUS

Private Bag X6001, Potchefstroom
South Africa 2520

Tel: 018 299-1111/2222
Web: http://www.nwu.ac.za

CPL
School for Pharmacy
Department of Pharmacology

Tel: 0182992242
Email:lynette.engelbrecht@nwu.ac.za
17 March 2015

Pathcare Laboratories

STUDY: LEVETIRACETAM (KEPPRA, REDILEV) Ethical no 2015024.4

Aim of the study: To compare the developed HPLC method for levetiracetam with an available commercial HPLC reagent kit. The CPL laboratory analysed the patient's levetiracetam sample from your laboratory. The investigator needs consent from the patient to use the sample to compare the two methods. It is important to understand that no additional blood will be drawn. It is however important that at least 500 µl plasma is available for testing.

Instructions:

- **The patient and phlebotomist must please sign the attached informed consent form.**
- At least 500 µl plasma.
- Attach the consent form to the sample.

Contact details:

Lynette Engelbrecht: 018 2992242 or 0823354107

Sendways:
Send all samples with consent forms to: CPL at the Northwest University, Pharmacology, Building G16, Room 122, Potchefstroom. Tel: 018 299 2242; Fax: 018 299 2225

E-mail: Lynette.Engelbrecht@nwu.ac.za

Find enclosed the informed consent form.

Thank you for your assistance and willingness to help me. It is highly appreciated!

Yours sincerely

Lynette Engelbrecht

Do not type here
Original details: (12022551) C:\Users\12022551\Desktop\To whom it may concern.docm
4 March 2015

NORTH-WEST UNIVERSITY
YUNIBESITI YA BOKONE-BOPHIRIMA
NOORDWES-UNIVERSITEIT
POTCHEFSTROOM CAMPUS

Private Bag X6001, Potchefstroom
South Africa 2520

Tel: 018 299-1111/2222
Web: http://www.nwu.ac.za

CPL
School for Pharmacy
Department of Pharmacology

Tel: 0182992242
Email:lynette.engelbrecht@nwu.ac.za
17 March 2015

Pathcare Laboratories

STUDY: LEVETIRACETAM (KEPPRA, REDILEV) Ethical no 2015024.4

Aim of the study:

The study can be divided into two parts:

(1) To compare the developed HPLC method for levetiracetam (LEV) with an available commercial HPLC reagent kit.

(2) To optimise the stability (influence) of LEV in plasma and serum samples from different blood collection tubes.

The investigator needs the participation of the patients in either part (1) or if willingly in both parts (1) and (2)

In part (1): the investigator needs consent from the patient to use the sample to compare the two methods. It is important to understand that no additional blood will be drawn. It is however important that at least 500 µl plasma is available for testing.

In part (2): 10 ml blood is additionally needed and collected in the following tubes:

- STT without the gel,
- STT with the gel,
- EDTA (purple top),
- Heparin (green top)
- Sodium citrate (blue top) will be used on individual patient all at the same time.

Instructions:

- **The patient and phlebotomist must please sign the attached informed consent form.**
- At least 500 µl plasma for part (1)
- 2 ml blood in each of the 5 tubes mentioned in part (2) if patient is willingly to participate in the second part of the study.
- Attach the consent form to the sample.

Contact details:

Lynette Engelbrecht: 018 2992242 or 0823354107

Sendways: Send all samples with consent forms to: CPL at the Northwest University, Pharmacology, Building G16, Room 122, Potchefstroom. Tel: 018 299 2242 Fax: 018 299 2225

E-mail: Lynette.Engelbrecht@nwu.ac.za

Find enclosed the informed consent form.

Thank you for your assistance and willingness to help me. It is highly appreciated!

Yours sincerely

Lynette Engelbrecht

From: "Madaleen Olivier" <m.olivier@pathcare.co.za>
To: <Lynette.Engelbrecht@nwu.ac.za>
CC: <ryan.soldin@pathcare.org>, <elizabeth.wasserman@pathcare.co.za>
Date: 2015/03/12 09:45 AM
Subject: Approval of research study by PathCare research committee
Attachments: image001.jpg

Dear Lynette,

I am writing on behalf of the PathCare research committee to inform you that your study "Development of an HPLC method for the detection Levetiracetam in blood in patients with epilepsy" have been approved. Thank you for submitting your study. Please acknowledge the assistance from PathCare in your study and in any publications that may come out of it.

I you have any further queries or need assistance, please contact me or dr Ryan Soldin. Dr Wasserman will be out of the office until the 16th of April.

We wish you all the best with your study.

Kind regards.
DR MADALEEN OLIVIER
Clinical Pathologist

Description: cid:image001.jpg@01C8E0FC.E7058C20

Telephone: +27 51 401 4633
Fax: +27 51 401 4649

Postal address:
Drs Voigt and Partners
PO Box 4266
Bloemfontein
9300
E-mail: <mailto:mvanvuuren@pathcare.co.za> mvanvuuren@pathcare.co.za

ANNEXURES C

Annex C. 1 Biorad Quote

Annex C. 2 Microsep Quote 1

Annex C. 3 Microsep Quote 2

Annex C. 4 Separations Quote

Bio-Rad Laboratories (Pty) Ltd
34 Bolton Road, Rosebank, 2196
PO Box 2884, Parklands, 2121
Tel: (011) 442 8508 Fax: (011) 442 8525
email:Safrica_Helpdesk@bio-rad.com

QUOTATION

RH140915-07

Date:	14 September 2015	**Pages:**	2 (including cover)
To:	Bongani Dube	**From:**	Roxanne Huysamen
Company:	VUT	**Company:**	Bio-Rad Laboratories
Fax:		**Fax:**	011 442 8525
Phone:	083 481 1715	**Phone:**	0861 246 723
E-Mail :	bonganid@vut.ac.za	**E-Mail:**	roxanne_huysamen@bio-rad.com

We thank you for your enquiry, and take pleasure in quoting you as follows:-

Item Code	Description	Quantity	Unit Price
195-6690	**Levetiracetam by HPLC Reagent Kit**	1	R 17 777.80
195-6206	**Antiepilep. by HPLC ANLT COL**	1	R 13 757.54
195-6695	**Levetira. by HPLC CONTROL SET**	1	R 6 114.46

Quote Reference No: RH140915-07_Bongani Dube_VUT_ Levetiracetam by HPLC Reagent Kit

Please state quotation reference number on your order.

Sole Supplier
Bio-Rad Laboratories (Pty) Ltd is the sole supplier of Bio-Rad products in South Africa.

Terms and Conditions
All orders placed with Bio-Rad Laboratories (Pty) Ltd are subject to the acceptance by the customer of the following terms and conditions, and imply full acceptance by the buyer of all these terms and conditions.

*** Prices quoted exclude VAT.**
*** This quotation is valid for a period of 30 days from date hereof.**
*** Freight charges are applicable on orders less than R1500.**
*** Delivery is approximately 4-6 weeks from receipt of order, unless items are ex-stock.**
*** A handling fee of 20% of the value of the order will be charged if the order is cancelled within the specified delivery time.**

Please fax or email your order on an official order form with the product code, price and your delivery address to:

- Fax: 011 442 8525
- Email: southafrica_orders@bio-rad.com

Please note that verbal orders and email orders without an official order form will not be accepted.

We trust that this quotation meets with your approval. If you have any further queries, please do not hesitate to contact me on 0861 246 723.

Assuring you of our best attention at all times.

Yours sincerely
Roxanne Huysamen
Sales Support Specialist

MICROSEP

RECIPE®

09 June 2014

Mrs Lynette Engelbrecht
North-West University
Main Campus - Department of Pharmacology
Private Bag X6001
Potchefstroom
2520

Dear Mrs Engelbrecht

Quote Number: 23914Q0208

RE: ClinRep® Complete Kit for Levetiracetam (Keppra®) in Serum / Plasma

With reference to your enquiry, we have pleasure in submitting our official quotation.

Part No.	Qty	Description	Unit Cost	Total (excl.VAT)	Total (incl.VAT)
15500	1	ClinRep® Complete Kit for Levetiracetam (Keppra®) in Serum / Plasma	10 174.00	10 174.00	11 598.36
14030	1	Analytical Column with test chromatogram	10 374.00	10 374.00	11 826.36
15580	1	Serum Control, lyophil., Level I (10x 3ml)	2 374.00	2 374.00	2 706.36
15581	1	Serum Control, lyophil., Level II (10x 3ml)	2 374.00	2 374.00	2 706.36
		(VAT amount R3 541.44)	**Grand Total**	**R 25 296.00**	**R 28 837.44**

Prices

1. Prices are net, include delivery
2. This quotation is valid for 30 days from date of quote.
3. When order is placed, we reserve the right to recalculate the price, taking the current Rand/Dollar Exchange Rate into account.

Delivery

Delivery is 3-4 weeks from receipt of an official order.

Warranty

All items are covered by the warranty as per the attached schedule.

Yours sincerely,

Marelize Ferreira
Waters Sales Representative
011 553 2300

Microsep Warranty

Microsep provides this limited product Warranty (the Warranty), to protect customers from non-conformity in product workmanship or materials. The Warranty covers all new products manufactured by Microsep and its subsidiaries for a period of one year.

The Warranty is as follows:

Microsep warrants that all products sold by them are of good quality and workmanship. The products are fit for their intended purpose(s) when used strictly in accordance with Microsep instructions of use during the applicable warranty period.

The foregoing Warranty is exclusive and in lieu of all other express and implied warranties, including but not limited to fitness or any other purpose(s).

In no event is Microsep liable for consequential, economic, or incidental damages of any nature. Microsep reserves the right not to honour this Warranty if the customer abuses the products.

The Warranty is not deemed to have failed of its essential purpose so long as Microsep is able and willing to repair or replace any non-conforming part or product.

MICROSEP

RECIPE®

22 April 2015

Mrs Lynette Engelbrecht
North-West University
Main Campus - Department of Pharmacology
Private Bag X6001
Potchefstroom
2520

Dear Mrs Engelbrecht

Quote Number: 23915Q0126

RE: Recipe Consumables

With reference to your enquiry, we have pleasure in submitting our official quotation.

Part No.	Qty	Description	Unit Cost	Total (excl.VAT)	Total (incl.VAT)
14030	1	Analytical Column with test chromatogram	10 374.00	10 374.00	11 826.36
		(VAT amount R1 452.36)	**Grand Total**	**R 10 374.00**	**R 11 826.36**

Prices

1. Prices are net, include delivery
2. This quotation is valid for 30 days from date of quote.
3. When order is placed, we reserve the right to recalculate the price, taking the current Rand/Dollar Exchange Rate into account.

Delivery

Delivery is 3-4 weeks from receipt of an official order.

Warranty

All items are covered by the warranty as per the attached schedule.

Yours sincerely,

Marelize Ferreira
Waters Sales Representative
011 553 2300

Microsep Warranty

Microsep provides this limited product Warranty (the Warranty), to protect customers from non-conformity in product workmanship or materials. The Warranty covers all new products manufactured by Microsep and its subsidiaries for a period of one year.

The Warranty is as follows:

Microsep warrants that all products sold by them are of good quality and workmanship. The products are fit for their intended purpose(s) when used strictly in accordance with Microsep instructions of use during the applicable warranty period.

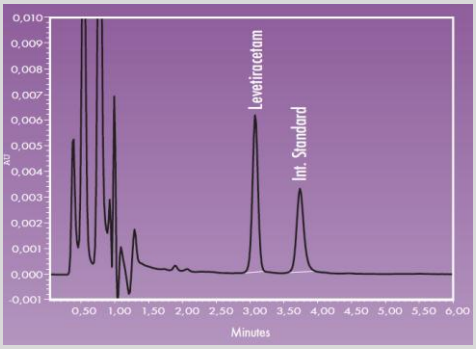
The foregoing Warranty is exclusive and in lieu of all other express and implied warranties, including but not limited to fitness or any other purpose(s).

In no event is Microsep liable for consequential, economic, or incidental damages of any nature. Microsep reserves the right not to honour this Warranty if the customer abuses the products.

The Warranty is not deemed to have failed of its essential purpose so long as Microsep is able and willing to repair or replace any non-conforming part or product.

Justine Daniels Separations +27119191000 +27(0)798998896 Justine@separations.co.za	SEPARATIONS Simply Spectacular 4 September 2015
Lynette Engelbrecht Univ North-West - Potchefstroom Campus: Pharmacology Private Bag X6001 Potchefstroom, North-West Province 2520 South Africa	DI1UNIVN20150904025 018 299 2242 018 299 2225 Lynette.Engelbrecht@nwu.ac.za

Qty	Item #	Description	Unit Price	Total
		HPLC ANALYSIS OF LEVETIRACETAM IN SERUM/PLASMA		
1	CS24000	**Reagent kit for HPLC analysis of Levetiracetam (Keppra®) in serum/plasma.** **For 100 analyses** **Contents of a kit:** Mobile phase (1 x 1000 ml) Levetiracetam serum calibration standard (lyoph.) (5 x 1 ml) Internal standard (1 x 10 ml) Equilibration buffer 1 (1 x 100 ml) Equilibration buffer 2 (1 x 100 ml) Wash buffer 1 (1 x 100 ml) Wash buffer 2 (1 x 100 ml) Elution buffer (1 x 50 ml) Sample clean up columns (2 x 50 pcs.)	R 13,350.00	R 13,350.00
1	CS24100	HPLC column Equilibrated, with test chromatogram	R 13,050.00	R 13,050.00
1	CS0086	Levetiracetam serum control, Bi-level (I + II), (2 x 5 x2ml)	R 4,360.00	R 4,360.00



PromoLab (Pty) Ltd T/A Separations
VAT # 4520105083
Company Reg No. 1988/002149/07

First National Bank Randburg
Account No: 62219447465
Branch Code: 254005\Swift Code: FIRNZAJJ

Terms and Conditions of Sales
Estimated Delivery Time: approximately 4-10 weeks from date of order, depending on Supplier Delivery Schedules
Warranty: As per Suppliers Warranty (usually 12 months from the date of MANUFACTURE)
Quotation validity: 1 month and within exchange rate limits.

1. PRICES
Our prices exclude VAT and delivery charges. We reserve the right to charge prices ruling on the date of dispatch.
2. QUOTATIONS
All quotations are subject to these Conditions of Sale, generally valid for 30 days but are also subject to exchange rate fluctuations. Separations reserves the right to nullify any quotes at any time. They are made in good faith and subject to correction of accidental errors or omissions as well as exchange rate fluctuations. Delivery statements are subject to prior sale or estimates without engagement.
3. DELIVERIES
Delivery will be free of charge for orders above R850 (ex VAT), unless otherwise specified. If part delivery is required, additional courier charges will apply. All orders will be dispatched via the most economical method possible. If urgent delivery is required, additional courier charges may be levied. A minimum processing fee of R75(ex VAT) will be levied for all orders under R850 for customers. Customers in regional areas will be advised of additional delivery charges. Prices for Dealers and Resellers **EXCLUDE** Delivery. All prices exclude delivery for customers outside South Africa.
4. TERMS OF PAYMENT
Standard Payment terms for orders over R500 000(incl. VAT) is 50% in advance and 50 %(balance) after invoice date. Alternative payment terms need to be stated in the quotation and agreed in writing with the Financial Director. A finance charge of 2% per month will be applied for each outstanding amount that is past due.
For approved account holders (within their credit limit) our terms are 30 days net from statement date(invoice date is used, NOT DELIVERY DATE) into our account PROMOLAB @ FNB Bank, Randburg Branch, 254005, Acc. No. 62219447465(for South African Customer, Investec Bank details for Foreign Exchange Receipts). Delivered goods remain the seller's property until full payment has been received. Full payment in advance has to be received before orders will be processed for COD customers. Special Payment terms may be specified for larger orders, also for approved account holders. Unless otherwise specified, customers outside South Africa need to pre-pay before orders will be processed. Separations reserve the right to change the payment terms before processing the order or due to any errors or omissions on behalf of any employee. Only Cash and EFT accepted, NO CHEQUES!!
5. ERRORS AND RETURNS
Discrepancies between delivery note and actual delivery must be claimed immediately and quantities, not checked at delivery are deemed to have been correct, unless the count discrepancy is reported within five working days from receipt. Duplications of items or entire orders, which are due to our error, will be collected by us or our agent. Orders, which are duplicated by the purchaser, are deemed to be valid and legally binding new orders. Returns for credit or re-supply cannot be accepted unless a valid Return Authorization Number has been issued by our Customer Service Department and is quoted on the purchaser's delivery note.
6. WARRANTIES AND LIABILITIES
All products are supplied under the manufacturers' guarantees. Warranties beyond manufacturer's liabilities will not be accepted, unless such promises were made in writing and signed by a director of the company. Contrary warranty statements in purchasers' documents are invalid. Warranty claims must be made in writing within one working day of detection of a defect of mal-performance, within the warranty period and receipt must be confirmed by the seller's Customer Service Department. The seller's total liability for any actual delivery is limited to the value of the goods. Compensations of consequential damages from use of products delayed or failed deliveries or any other reasons are explicitly ruled out. African customers: Separations accepts responsibility for damage to goods only as far as we are involved in the freight process. Customers are liable for any loss or damage to products being returned for service, repairs, calibration or other reasons not specified here. The seller only bears limited responsibility for customer products, whilst they are on the seller's property. All services of customer products are to be handled as ex-works. All warranties start from date of delivery.
7. PERMITS/LICENSES
Purchase orders for items falling under any kind of government supervision - especially for drugs of abuse or radioactivity, but not limited to these - is deemed to be the purchaser's irrevocable assurance to hold suitable permissions and licenses. Copies or relevant permits must be given to Separations on request without delay.
8. Cancellations
Official orders can only be cancelled with written approval and will be subject to special terms. Goods that have been ordered by Separations through their suppliers cannot be cancelled. Separations reserves the right to charge a cancellation fee for orders. The amount of the fee will be determined by Separations.

9. Technical data and documents
Technical documents such as drawings, descriptions, illustrations and data on dimensions, performance and weight are for information purposes only and shall not imply any warranties. SEPARATIONS reserves the right to make any necessary changes. All technical documentation and technical data remain the property of SEPARATIONS and may neither be used for production purposes nor be made available to third parties.
10. Copyright protection, patent and trademark rights
Trademarks, designs, and projects remain SEPARATIONS' property. Without SEPARATIONS' previous written consent, the reproduction, utilization, or handing over to third parties is prohibited. Particularly, SEPARATIONS equipment must not be made available directly or indirectly (through drawings, diagrams, schematics etc.) to any third party without SEPARATIONS' written authorization.
11. Delivery time
The delivery time of a system will be stated on each quotation but is subject to Supplier Delivery Schedules. The delivery time excludes full installation or software programming. The delivery time is reasonably extended if one of the cases applies: The information required by SEPARATIONS for performance of the contract is not received in time, or if the customer subsequently changes the contract thereby causing a delivery delay of the goods. All contract changes require revised Purchase Orders from the customer. Hindrances occur which prevent SEPARATIONS from performing the contract by force majeure. Hindrances include epidemics, mobilization, war, revolution, serious breakdowns in the works, accidents, labour conflicts, late or deficient delivery by subcontractors of raw materials, semi-finished or finished products, official actions or omissions by any state authorities or public bodies, and natural catastrophes. Any delayed delivery does not entitle the customer to any rights and claims.
12. Acceptance
Separations will at its own discretion accept or decline an order. Accepted orders may be cancelled by Separations before final payment has been received by the customer, without any liability. Unless otherwise agreed upon, acceptance shall be affected immediately after installation at the customer's premises. The customer shall inspect the goods within 5 days and shall immediately notify SEPARATIONS in writing of any deficiencies. If the customer fails to do so, the goods shall be deemed to have been accepted. In addition, acceptance shall also be deemed completed when: SEPARATIONS has satisfied its standard site acceptance conditions or exceptional conditions agreed upon in writing with the customer prior to acknowledgement of the Purchase Order by SEPARATIONS. As soon as the customer uses the goods in a non-acceptance test mode. If the customer delays acceptance, the outstanding amounts are due 30 days after the original acceptance.

13. Exclusion of further liability

All cases of breach of contract and the relevant consequences as well as all rights and claims on the part of the customer are exhaustively covered by these general conditions of supply. In no case whatsoever, shall the customer be entitled to claim damages other than compensation for costs of remedying defects of the goods. This, in particular, refers, but shall not be limited to, loss of production, loss of use, loss of orders, loss of profit, and other direct or indirect or consequential damage.

14. Patents

SEPARATIONS represents and warrants to Buyer that the manufacture, use or sale of HAMILTON ROBOTICS/SIEMENS/RADLEYS/MOLECULAR DEVICES/AB Sciex standard Products do not infringe on any patent, trademark or other intellectual property of any third party. If the standard products sold under the present conditions are produced or modified according to customer's specifications, customer agrees to indemnify and hold SEPARATIONS harmless from all lawsuits, judgments, claims, costs and expenses, including but not limited to attorney's and accountant's fees arising in connection with patent infringement.

15. License Agreement

The computer software programs remain the property of SEPARATIONS. SEPARATIONS grants to customer a non-exclusive license to use the proprietary software programs with the equipment specified in the purchase agreement and under the general terms and conditions specified herein. SEPARATIONS may declare parts of the software programs as PUBLIC and which parts will be free of restrictions. SEPARATIONS are not liable for the functionality of third party components. SEPARATIONS are not aware of the rights of any third parties that would oppose the utilization purposes of the licensed software programs. In the event that the software programs infringe on the rights of a third party SEPARATIONS will not be liable. SEPARATIONS guarantees the operability of the software programs with the equipment and for the purpose as set forth in the specifications of the purchase agreement under normal conditions of operation and that the software programs have been written following the accepted rules of programming. SEPARATIONS will not be liable for delays, errors or failures in performance due to causes beyond its control or operation by unqualified personal. SEPARATIONS ` warranty expires immediately if changes in the setup of the equipment or the exchange of equipment the licensed software programs are operating take place without written confirmation of SEPARATIONS. In case of software program defects limiting the usability of the software programs, SEPARATIONS will deliver corrections free-of-charge during the warranty period. After expiry of the warranty period SEPARATIONS will provide error maintenance and other support only if the customer agreed upon and entered into a maintenance and service contract. SEPARATIONS will provide the appropriate support during the installation and for the configuration of the software programs if the customer provides SEPARATIONS access to the hardware and software. After installation, SEPARATIONS will provide education and consulting services according to its current schedule of charges. The customer will perform backup operations to protect himself from loss of data due to any error conditions.

ANNEXURES D

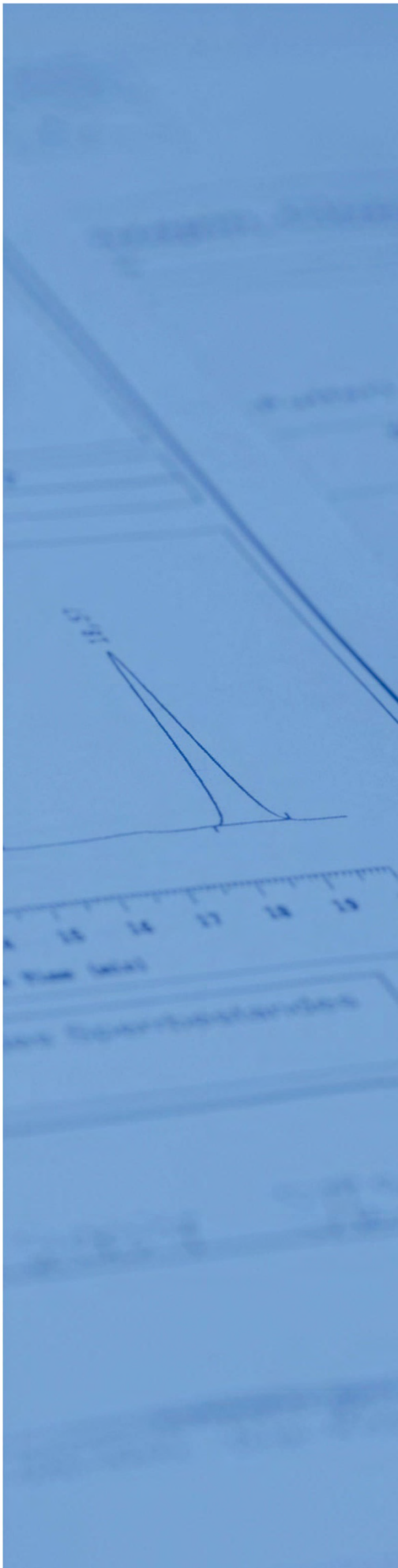
Annex D. 1 Instruction manual

Annex D. 2 Standard

Annex D. 3 Calibrator

Annex D. 4 Controls

RECIPE®



Instruction Manual

ClinRep® HPLC Complete Kit

Levetiracetam (Keppra®) in Serum / Plasma

REF 15500, 15600

IVD For in vitro diagnostic use

CE IVDD, 98/79/EC

RECIPE Chemicals + Instruments GmbH
Dessauerstraße 3, 80992 Munich / Germany
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REF 15500, 15600

IVD For in vitro diagnostic use

Document Version: 2.1
Date of revision: 22.07.2014
File name: 15500-15600_m_e.docx

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1 Introduction

1.1 Intended use

The present analytical procedure is intended for the determination of Levetiracetam (Keppra®) from serum and plasma with HPLC. The sample preparation can be performed manually (see section 5.3) or automated with the pipetting system Gilson® ASPEC™ (see section 5.4). For this purpose, two separate complete kits with order nos. 15500 and 15600 are available (see section 2).

The kit components have to be used in accordance with this user manual. The kit is not designed for combination with components from other manufacturers.

1.1.1 IVD symbols

Symbols according to EU directive 98/79/EC for in vitro diagnostic medical devices (IVDD), which are used on the product labels and in this user manual:

For in vitro diagnostic use


Manufacturer

Order number

Lot number


Upper temperature limit: … °C


Temperature limits: … °C to …°C


Expiry date


See instructions for use

1.2 Clinical background

Epilepsy is a chronic neurological disorder which is characterised by recurrent epileptic seizures. The seizures are characterised by partial or generalised convulsions and impaired consciousness. The frequency and the rhythm of seizures are mostly not predictable. Therefore, seizures are not only a considerable psychological and physical burden for the patient, but may also lead to grave and even life-threatening hurts (e.g. craniocerebral injuries in case of falls). Brain damages may result in case of a hypoxia [1].

With a prevalence of 0.5 - 1 % [2, 3] epilepsy is among the most common disorders of the central nervous system.

For the pharmacological therapy of epilepsy, a variety of antiepileptic drugs (AEDs) with different pharmacological properties are available today.

Levetiracetam (Keppra®) is a relatively new AED which, compared to the AEDs in current use, shows a different pharmacological mechanism of action. Thus, levetiracetam exhibits no relevant interactions with other anticonvulsants and is often used in co-medication with other AEDs (adjunctive therapy) [4, 5].

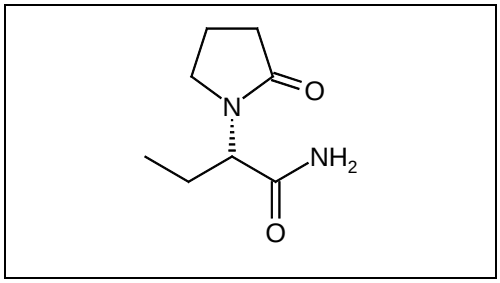


Figure 1: Chemical structure of levetiracetam

Most of the AEDs, including levetiracetam, exhibit a pronounced intra- and interindividual variability in pharmacokinetics. Thus, the therapeutic drug dose has to be ascertained for the individual patient and subsequently has to be controlled by measuring the drug concentration in blood (Therapeutic Drug Monitoring, TDM) [4, 6].

Irrespective of the properties of the monitored drug, TDM is helpful to clarify for possible influences on the patient's pharmacokinetics, especially in cases of co-medication and AED polytherapy [4].

The ClinRep® HPLC Complete Kits for levetiracetam with order nos. 15500 and 15600 complete the RECIPE kit-portfolio for AED analysis. By the use of the same analytical column, they can easily be combined with the ClinRep® HPLC Complete Kit with order no. 14000 which allows the quantification of further 16 different AEDs and clinical relevant metabolites (see annotation below and section 4.5).

For quantification a ClinCal® Serum Calibrator is provided. Quality control is performed by the use of ClinChek® Serum Controls.

The constantly high quality of test reagents and reference materials is guaranteed by our internal QM-system (certified according to ISO 9001 and ISO 13485) as well as by external quality control programs (INSTAND, Germany).

Note:

The present analytical procedure can easily be combined with the ClinRep® HPLC Complete Kit with order no. 14000* which allows the quantification of the following 16 AEDs and metabolites:

Carbamazepine, Carbamazepine-epoxide, N-Desmethylmethsuximide, Eslicarbazepine, Ethosuximide, Felbamate, 10-Hydroxy-Carbamazepine, Lacosamide, Lamotrigine, Oxcarbazepine, Phenobarbital, Phenytoin, Primidone, Rufinamide, Sulthiame, Zonisamide

The chromatographic separation is performed with the same analytical column. Thus, a time consuming change of the column and the equilibration of the analytical system is not required for switching these applications (see section 4.5).

*Order no. 14000: ClinRep® HPLC Complete Kit, *advanced*, for Antiepileptics in Serum / Plasma

1.3 General description of the analytical procedure

In this analytical method, levetiracetam is determined from plasma and serum by HPLC with UV detection.

Prior to HPLC analysis a sample clean-up is performed via solid phase extraction in order to remove the sample matrix and to spike with the internal standard (sample preparation, see section 5.3).

The sample preparation can be performed manually (see section 5.3) or automated with the pipetting system Gilson® ASPEC™ (see section 5.4).

Afterwards the samples are injected into the HPLC system. At this, the sample components are separated on the analytical column and levetiracetam is detected by the UV detector.

For the check-up of the analytical system a matrix-free ClinTest® Standard Solution is available (see section 5.6.1).

When the analytical system has been successfully tested, samples (calibrator, control, patient) are injected for calibration and measurement. The obtained chromatograms are evaluated by use of the internal standard method via peak areas. Alternatively, the evaluation may be performed via peak heights (see section 6).

For calibration a ClinCal® Serum Calibrator is provided (see section 5.6.2). Quality control is performed by the use of ClinChek® Serum Controls. These controls are available in two different concentrations (see section 5.6.3).

2 Components of the complete kit and accessories

2.1 Ordering information

2.1.1 Manual sample preparation

Order No.	Description	Quantity
15500	**ClinRep® Complete Kit for Levetiracetam in Serum / Plasma** for 100 assays	1 pce.
	Contents:	
	Mobile Phase	1 x 15510
	Standard Solution	1 x 15511
	IS Internal Standard	1 x 15512
	Serum Calibrator, lyophil.	1 x 15513
	Sample Preparation Columns	2 x 15520
	Conditioning Reagent	1 x 15521
	W Washing Solution 1	3 x 15522
	W Washing Solution 2	1 x 15523
	E Eluting Reagent	1 x 15524
	Manual	
	Quick Reference	
	Separately available components:	
15510	Mobile Phase	1000 ml
15511	Standard Solution	3 ml
15512	IS Internal Standard	10 ml
15513	Serum Calibrator, lyophil.	3 ml
15520	Sample Preparation Columns	50 pcs.
15521	Conditioning Reagent	300 ml
15522	W Washing Solution 1	200 ml
15523	W Washing Solution 2	100 ml
15524	E Eluting Reagent	100 ml
	Start Accessories:	
14030	Analytical Column with test chromatogram	1 pce.
	Accessory:	
FK5810	PEEK-tubing (connection of prefilter and column)	1 pce.
FK5820	Stainless steel prefilter holder	1 pce.
FK5821	Replacement filter for stainless steel prefilter holder	10 pcs.
	ClinChek® Controls:	
15580	Serum Control, lyophil. Level I	10 x 3 ml
15581	Serum Control, lyophil. Level II	10 x 3 ml
15582	Serum Control, lyophil. Level I, II	2 x 5 x 3 ml

2.1.2 Automated sample preparation

Order No.	Description	Quantity
15600	**ClinRep® Complete Kit for Levetiracetam in Serum / Plasma (automated sample preparation with Gilson® ASPEC™)** for 200 assays	1 pce.
	Contents:	
	Mobile Phase	2 x 15510
	Standard Solution	1 x 15511
	IS Internal Standard	1 x 15512
	Serum Calibrator, lyophil.	1 x 15513
	Sample Preparation Columns	2 x 15620
	Conditioning Reagent	1 x 15521
	W Washing Solution 1	2 x 15522
	W Washing Solution 2	1 x 15523
	E Eluting Reagent	1 x 15524
	Manual	
	Quick Reference	
	Separately available components:	
15510	Mobile Phase	1000 ml
15511	Standard Solution	3 ml
15512	IS Internal Standard	10 ml
15513	Serum Calibrator, lyophil.	3 ml
15620	Sample Preparation Columns	100 pcs.
15521	Conditioning Reagent	300 ml
15522	W Washing Solution 1	200 ml
15523	W Washing Solution 2	100 ml
15524	E Eluting Reagent	100 ml
	Start Accessories:	
14030	Analytical Column with test chromatogram	1 pce.
	Accessory:	
FK5810	PEEK-tubing (connection of prefilter and column)	1 pce.
FK5820	Stainless steel prefilter holder	1 pce.
FK5821	Replacement filter for stainless steel prefilter holder	10 pcs.
	ClinChek® Controls:	
15580	Serum Control, lyophil. Level I	10 x 3 ml
15581	Serum Control, lyophil. Level II	10 x 3 ml
15582	Serum Control, lyophil. Level I, II	2 x 5 x 3 ml

2.1.3 Safety information

Several of the kit components (e.g. mobile phases and reagents) are chemical preparations and thus may contain hazardous substances. For safety information, please consult the appropriate Material Safety Data Sheet (MSDS) for each component.

The calibrator- and control materials are prepared from human serum and plasma. Although the products are tested for the absence of common infection markers, they should still be considered as potentially infectious. For this reason we recommend the product to be handled with the same precautions as patient samples. Detailed safety information is indicated in the appropriate MSDS.

2.1.4 Storage conditions and lifetime of kit components

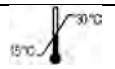
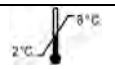
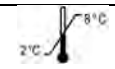
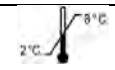
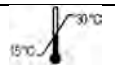
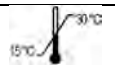
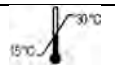
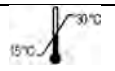
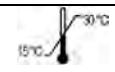
Please unpack the kit components from the transport packaging **immediately upon receipt** and follow the instructions for the storage conditions indicated on the product labels and in Table 1.

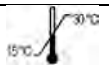
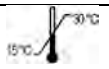
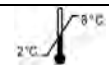
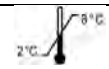
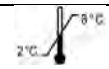
Unused components, stored under appropriate conditions can be used until the expiry date indicated on the product label.

After use of ClinRep® Reagents and the ClinRep® Mobile Phase, the bottles must be closed tightly and stored immediately under the required conditions. Provided proper use and storage procedures are followed, the lifetime of these reagents is the same as for the unused products.

For storage conditions and life times of the ClinTest® Standard Solution as well as for the ClinCal® Calibrator and ClinChek® Controls (lyophilised / after reconstitution) please also refer to the appropriate product data sheets.

Table 1: Storage conditions of kit components

Order no.		Product description	Storage conditions	
REF	15510	Mobile Phase		Store at 15 - 30 °C
REF	15511	Standard Solution		Store at 2 - 8 °C
REF	15512	IS Internal Standard		Store at 2 - 8 °C
REF	15513	Serum Calibrator, lyophil.		Store at 2 - 8 °C *
REF	15520	Sample Preparation Columns		Store at 15 - 30 °C
REF	15521	Conditioning Reagent		Store at 15 - 30 °C
REF	15522	W Washing Solution 1		Store at 15 - 30 °C
REF	15523	W Washing Solution 2		Store at 15 - 30 °C
REF	15524	E Eluting Reagent		Store at 15 - 30 °C

REF	15620	Sample Preparation Columns		Store at 15 - 30 °C
REF	14030	Analytical Column		Store at 15 - 30 °C
REF	FK5810	PEEK-tubing	Store at ambient temperature	
REF	FK5820	Stainless steel prefilter holder	Store at ambient temperature	
REF	FK5821	Replacement filter for stainless steel prefilter holder	Store at ambient temperature	
REF	15580	Serum Control, lyophil., Level I		Store at 2 - 8 °C *
REF	15581	Serum Control, lyophil., Level II		Store at 2 - 8 °C *
REF	15582	Serum Control, lyophil., Level I + II		Store at 2 - 8 °C *

*Refers to the lyophilised product. For storage conditions after reconstitution, please refer to the product data sheet.

2.1.5 Disposal of laboratory waste

For disposal, laboratory waste should be collected separately according to the different chemical properties. Recommendations for the disposal of the product and of the packaging are indicated in section 13 of the appropriate Material Safety Data Sheet.

3 Required instruments

This test kit requires a HPLC system with UV detector and evaluation software.

Required HPLC modules:

- Isocratic HPLC pump
- Autosampler
- Column heater (30 °C)
- UV detector (205 nm)
- optional: degasser
- optional: pulse damper

For sample preparation the following laboratory instruments are required:

- Pipettes, pipette tips
- Tabletop centrifuge
- Vortex-mixer

Required for automated sample preparation (complete kit with order no. 15600):

- Pipetting system from Gilson® (Gilson® ASPEC™)

4 Operation of the analytical system

4.1 Passivation of the HPLC system

If you run different applications on your HPLC system, we recommend to clean the system regularly by passivation. Otherwise problems may occur, like ghostpeaks (e.g. contaminated injection system) and/or baseline problems (e.g. contaminated detector cell). It is important, to passivate all fluidic components of the HPLC system, **with exception of the analytical column**.

The passivation is performed as follows:

- Connect pump, injection system, column heater, detector and all capillaries **with exception of the column**.
- Put the outlet-capillary into a safe waste container.
- Flush the system for 15 min at a flow of 1.5 ml/min with HPLC water.
- Then pump 2-propanol through the system for 10 min
- and afterwards HPLC water for 15 min.
- Flush the system for 30 min with half concentrated nitric acid (1 volume each of concentrated nitric acid (65 %) and HPLC water).
- Afterwards purge the system with HPLC water until the pH of the waste solution is neutral. Change the water in the eluent container several times to be sure that the nitric acid will be washed out of the frit.
- Finally equilibrate the system for about 15 min with the mobile phase at a flow rate of 1.0 ml/min.

4.2 Connection of the column and the detector

- Set the temperature of the column heater to 30 °C.
- Prior to connecting the ClinRep® Analytical Column, briefly equilibrate the HPLC system (pump feed, pump, injection system, column heater, capillaries, detector) with mobile phase; this should be done by pumping the mobile phase through the system for 15 min at a flow rate of 1.0 ml/min.
- Switch off the pump and install the column in the column heater, between injection system and detector. Connect the analytical column in flow direction and
- allow approximately 10 ml of the mobile phase to flow through the column (flow rate of 0.5 ml/min, 20 min). Then increase slowly to the intended value of the chromatographic separation, in this application 1.4 ml/min.
- Connect a 0.15 mm I.D. capillary to the outlet of the analytical column and the other end to the detector inlet.
- Wait until no more air bubbles exit from the detector outlet.
- Afterwards connect a 0.50 mm I.D. capillary to the detector outlet.

We recommend to continue circulating the mobile phase for further 30 min before injecting the first sample.

4.3 Starting the analytical system

The following table provides the parameters for the HPLC system. To ensure appropriate usage of the HPLC system, please consult the user manual of the instrument manufacturer. User trainings, provided by the manufacturer, may also be advisable.

For test run, calibration and measurement please refer to section 5.6.

Table 2: HPLC parameters

HPLC pump:	Flow rate: 1.4 ml/min
Mobile Phase:	Make sure that the bottle is closed well to avoid alteration of the retention times through evaporation of components of the mobile phase. Recycling: The mobile phase may be circulated through the system for 100 analyses. After 100 analyses a new bottle of mobile phase has to be used.
ASPEC™:	See section 5.4.2
Autosampler:	Injection volume: 20 µl Injection interval: 6 min For minimum sample carry over use the needle wash settings recommended by the autosampler supplier. The mobile phase has to be used as a washing solution for the autosampler.
Column heater:	30 °C
Column:	The analytical column* is installed within the column heater (30 °C), see section 4.2. The use of a prefilter (order nos. FK5820, FK5821, see section 2.1) is recommended. The backpressure of the analytical column should not exceed 220 bar. **For proper handling of the analytical column please also note section 4.3.1.**
UV detector:	Set the UV-detector to 205 nm. "Sensitivity" or "Range" has to be set appropriately, depending on the quality of the detector used.
Evaluation unit:	Integration stop has to be set at 6 min. Retention times: Levetiracetam: 2.49 min Internal Standard: 4.89 min Please note: Depending on the HPLC system used, differences to the indicated retention time may be observed. These differences do not influence the efficiency of the analytical procedure, however they must be considered with regard to the settings for autosampler and evaluation unit. Also, a shifting of retention times may occur due to an aging of the column or its improper use.

4.3.1 Instructions for proper handling of the analytical column

4.3.1.1 Installation and flushing for analysis

The analytical column has to be stored in a 1:1 methanol/water mixture. For installation, please flush the column with mobile phase for 20 min at a flow rate of 0.5 ml/min. Subsequently, increase slowly the flow rate to the intended value of 1.4 ml/min as described in section 4.2.

4.3.1.2 Regeneration of the analytical column

A regeneration of the column is generally recommended in case of a decreasing chromatographical performance (test run of the HPLC system, see section 5.6.1) and in case of an increasing back pressure of the analytical column (max. 220 bar).

For regeneration, please flush the column in reverse flow direction with 1:1 methanol/water and 2-propanol. Subsequently, please store the column for at least 12 hours and repeat the flushing procedure in reverse order (see table 3).

Table 3: Regeneration of the analytical column

Solvent	**Flow rate [ml/min]**	**Time [min]**
1:1 Methanol/water	0.5	20
2-Propanol	0.2	100
Store the column for at least 12 hours.		
2-Propanol	0.2	100
1:1 Methanol/water	0.5	20

For reinstallation, please flush the analytical column with mobile phase as described in section 4.3.1.1.

4.3.1.3 Deinstallation and storage

For deinstallation and storage of the analytical column, see section 4.4.

4.4 Standby mode

Should the system not be required for use within several days, the mobile phase may be left circulating at a reduced rate (0.2 ml/min).

For longer periods (i.e. longer than approx. 1 week), the analytical column should be disconnected and closed tightly. The column has to be stored in a 1:1 methanol/water mixture. For this purpose, flush the HPLC system before with 1:1 methanol/water with a flow rate of 0.5 ml/min for 20 min.

The UV detector should either be switched to "stand-by" or switched off, in order to preserve the lamp.

4.5 Application change for the determination of 16 further AEDs

The present analytical method can be easily combined with the ClinRep® HPLC Complete Kit with order no. 14000* which allows the quantification of the following 16 AEDs and metabolites:

Carbamazepine, Carbamazepine-epoxide, N-Desmethylmethsuximide, Eslicarbazepine, Ethosuximide, Felbamate, 10-Hydroxy-Carbamazepine, Lacosamide, Lamotrigine, Oxcarbazepine, Phenobarbital, Phenytoin, Primidone, Rufinamide, Sulthiame, Zonisamide

The chromatographic separation is performed with the same analytical column as being used for the present complete kit. A change of the analytical column and a time-consuming equilibration of the analytical system (see section 4.2) is therefore not required for switching these applications.

For switching the application, change the mobile phase and flush with the HPLC conditions of the desired application, i.e.:

- Levetiracetam (order no. 15500, 15600) → Antiepileptics (order no. 14000):

  Flush with the mobile phase for antiepileptics (order no. 14010) for 20 min and at a flow rate of 1.8 ml/min.

- Antiepileptics (order no. 14000) → Levetiracetam (order no. 15500, 15600):

  Flush with the mobile phase for levetiracetam (order no. 15510) for 20 min and at a flow rate of 1.4 ml/min.

Annotation:

The switching of the application can be automated by use of a controllable binary HPLC pump.

Please note:

While switching the application, the mobile phase must not be circulated through the system (no recycling!).

*Order no. 14000: ClinRep® HPLC Complete Kit, *advanced*, for Antiepileptics in Serum / Plasma

5 Implementation of the analytical procedure

5.1 Extraction and storage of samples

The determination of levetiracetam can be performed from serum or plasma.

Samples can be stored at least 7 days at room temperature (15 - 30 °C), at least 4 weeks at temperatures between 2 - 8 °C and at least 3 months at temperatures below -18 °C (multiple freeze-thaw cycles should be avoided).

Please note:
Serum extraction should not be performed by use of gel tubes. The use of certain gel tubes may cause interfering peaks within the chromatogram.

5.2 Reconstitution of the lyophilised serum calibrator and the serum controls

The ClinCal® Serum Calibrator and the ClinChek® Serum Controls (see section 2.1) are lyophilised and thus must be reconstituted before use. Information regarding reconstitution, along with analyte concentrations and information about storage and stability, is indicated in the appropriate product data sheets.

5.3 Manual sample preparation (order no. 15500)

5.3.1 Work flow

Sample preparation:

Conditioning:	Conditioning of the sample preparation column: 1.) 3 ml Conditioning Reagent 2.) 3 ml Washing Solution W1
	aspirate or centrifuge ⇩ (1 min, 1000 x g), discard the effluents
Dotation / Extraction:	100 µl sample (calibrator, control, patient) | 100 µl Internal Standard IS
	aspirate or centrifuge ⇩ (1 min, 1000 x g), discard the effluent
Washing:	1.) 3 ml Washing Solution W1 2.) 1 ml Washing Solution W2
	aspirate or centrifuge ⇩ (1 min, 1000 x g), discard the effluents
Elution:	1 ml Eluting Reagent E
	aspirate or centrifuge ⇩ (1 min, 1000 x g), collect the eluate mix for 5 sec ⇩ (vortex-mixer)
HPLC analysis:	inject 20 µl

5.3.1.1 Conditioning

Pipette 3 ml conditioning reagent on the sample preparation column (included within the kit, order no. 15520). Aspirate or centrifuge (1 min, 1000 x g) and discard the effluent. Afterwards pipette 3 ml washing solution W1 on the column and aspirate or centrifuge (1 min, 1000 x g). Discard the effluent.

5.3.1.2 Dotation / Extraction

Pipette 100 µl of the sample (calibrator, control, patient) and 100 µl internal standard IS on the sample preparation column. Aspirate or centrifuge (1 min, 1000 x g) and discard the effluent.

5.3.1.3 Washing

First wash with 3 ml washing solution W1, aspirate or centrifuge (1 min, 1000 x g) and discard the effluent. Then pipette 1 ml washing solution W2 on the column and aspirate or centrifuge (1 min, 1000 x g). Discard the effluent.

5.3.1.4 Elution

Pipette 1 ml eluting reagent E on the sample preparation column. Aspirate or centrifuge (1 min, 1000 x g) and collect the eluate (sample vials for collection are not included within the kit). Afterwards mix the eluate for 5 sec on a vortex-mixer.

For practical reasons the sample vials should be suitable for the autosampler in use.

5.3.1.5 HPLC analysis

Inject 20 µl of the eluate (see section 5.3.1.4) into the HPLC system.

5.4 Automated sample preparation with Gilson® ASPEC™ (order no. 15600)

5.4.1 Work flow

Sample preparation:

Conditioning:	Conditioning of the sample preparation column: 1.) 1 ml Conditioning Reagent 2.) 1 ml Washing Solution W1	
	⇩ discard the effluents	
Dotation / Extraction:	50 µl sample (calibrator, control, patient)	50 µl Internal Standard IS
	⇩ discard the effluent	
Washing:	1.) 1 ml Washing Solution W1 2.) 330 µl Washing Solution W2	
	⇩ discard the effluents	
Elution:	500 µl Eluting Reagent E	
	⇩ collect the eluate ⇩ mix	
HPLC analysis:	inject 20 µl	

Duration of the automated sample preparation: approx. 10 min.

5.4.2 Operation of the Gilson® ASPEC™

For the operation of the ASPEC™ the sample preparation steps have to be imported into the instrument software. The settings depend on the system in use and are available on request.

5.5 Stability of the prepared samples

The prepared samples can be stored for at least 3 days at room temperature (15 - 30 °C), for at least 1 week at temperatures between 2 - 8 °C (refrigerator) and for at least 3 months at temperatures below -18 °C.

5.6 HPLC Analysis

5.6.1 Test run

Prior to the injection of prepared samples (calibrator, control, patient), the HPLC system should be checked by the use of the ClinTest® Standard Solution (order no. 15511).

Repeatedly inject 20 µl of the standard solution until two consecutive chromatograms are identical in respect of retention times and peak resolution (see chromatograms in section 5.6.4). Now check the integration parameters (e.g. run time, peak identification, marks for peak start and end). Correct the parameters, if necessary and inject the standard solution once again for verification.

5.6.2 Calibration

For calibration, the ClinCal® Serum Calibrator (order no. 15513) has to be used. After reconstitution (see section 5.2), the calibrator must be prepared as described for the patient samples (see section 5.3 and 5.4).

Inject the serum calibrator several times; this enables a singlepoint-calibration with averaging. When carrying out large series of analyses, we recommend injecting the calibrator every tenth patient sample as well as at the end of the series. This allows checking the HPLC conditions and enables corrections without repeating the sample analyses, e.g. in the case of shifted retention times (see also section 6.1).

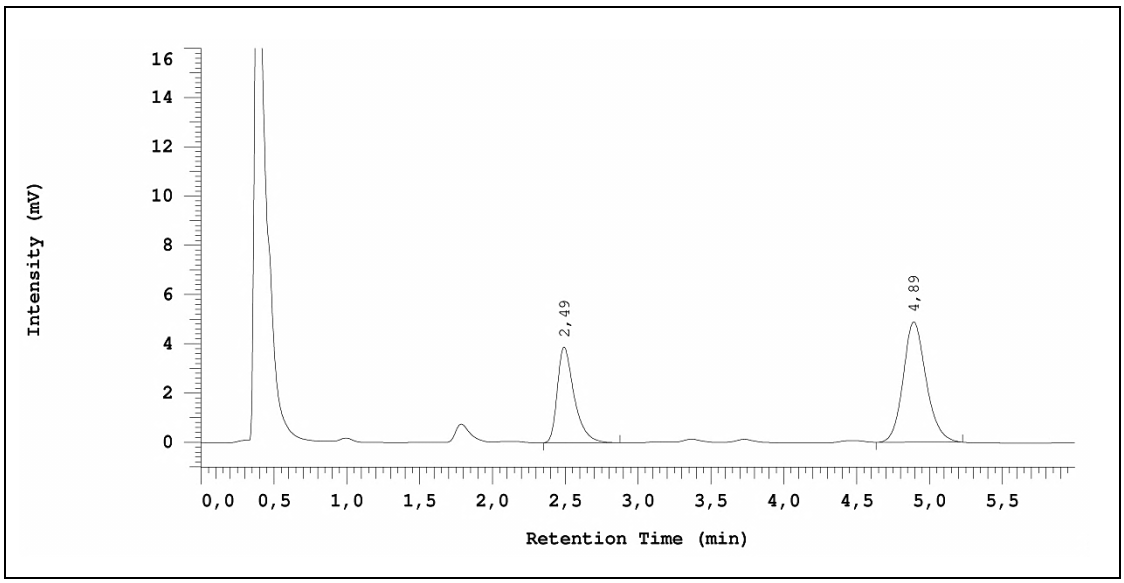
5.6.3 Accuracy control

For the quality control of the analytical measurements, ClinChek® Serum Controls are available in two different concentrations (level I, order no. 15580; level II, order no. 15581; level I + II, order no. 15582).

These controls are lyophilised and, subsequent to reconstitution, must be prepared as described for the patient samples (see section 5.3). The controls are analysed within the analytical series. In case of large analytical series, we recommend to insert control samples repeatedly within the series.

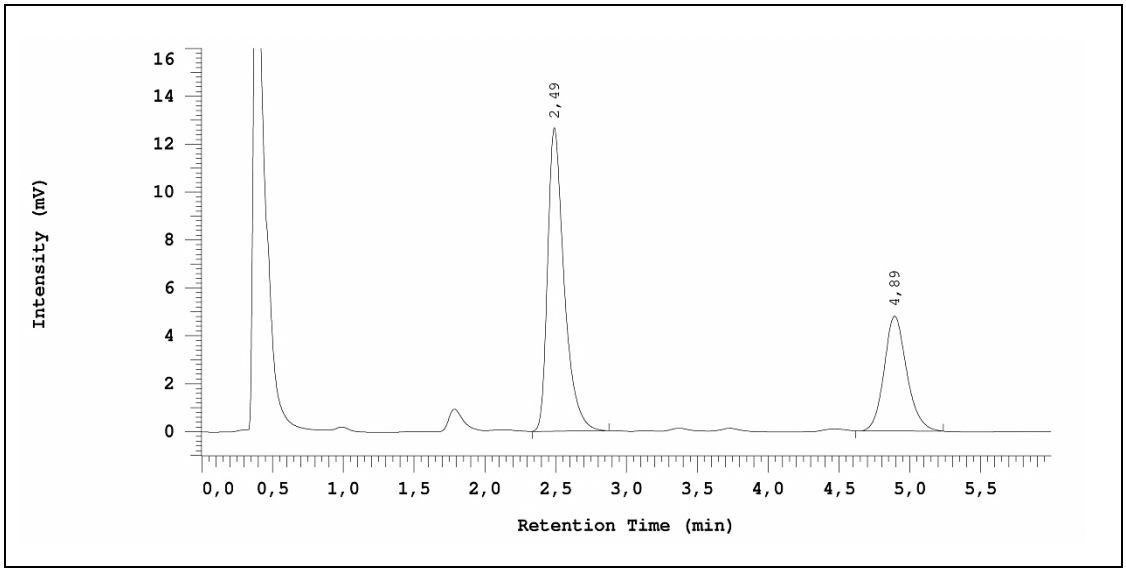
5.6.4 Example chromatograms

The figures below show chromatograms of the ClinChek® Serum Controls for levetiracetam, Level I (order no. 15580) and Level II (order no. 15581).



Levetiracetam: 2.49 min, internal standard IS: 4.89 min

Figure 2: Chromatogram of the ClinChek® Serum Control for levetiracetam, Level I (order no. 15580)



Levetiracetam: 2.49 min, internal standard IS: 4.89 min

Figure 3: Chromatogram of the ClinChek® Serum Control for levetiracetam, Level II (order no. 15581)

6 Evaluation

6.1 General

In order to check for stable HPLC conditions during an analytical series, the chromatograms of the measured samples (control, patient) are compared with those of the serum calibrator, particularly with regard to correlation of peaks and retention times. Temperature fluctuations may lead to shifted retention times and false peak identification. If calibrators have been run between samples, you are able to recalibrate without having to repeat the analysis (see also section 5.6.2).

6.2 Evaluation method

Calculation of unknown samples has to be done using the internal standard method via peak areas. Alternatively, the peak heights may be used.

According to the internal standard method, each sample is spiked with a so-called "internal standard" prior to the sample preparation. The internal standard is similar to the analytes in terms of behaviour during sample preparation and chromatography. Any losses during the sample preparation hence can be determined by calculating the recovery. Extrapolation to 100 % recovery allows establishing the concentration of the unknown substances in the sample.

Please consult the HPLC software manual of the instrument manufacturer in order to ensure correct evaluation of the results.

For the calculation of mass concentrations [mg/l] into molar concentrations [µmol/l], and vice versa, the analytical results should be multiplied with the factors shown in table 3.

Table 4: Conversion factors for levetiracetam

Molecular Weight [g/mol]	**Conversion: µmol/l --> mg/l**	**Conversion: mg/l --> µmol/l**
170.2	0.170	5.88

6.2.1 Manual calculation

Calculation of the recovery rate (REC):

$$REC = \frac{Area\ IS\ (sample)}{Area\ IS\ (calibrator)}$$

Calculation of the analyte concentration (C):

$$C\ (Analyte, sample)[\mathrm{mg/l}] = \frac{Area\ (Analyte, sample) \times C\ (Analyte, calibrator)\ [\mathrm{mg/l}]}{Area\ (Analyte, calibrator) \times REC}$$

7 Test data

7.1 Test performance

The results were obtained with a modular HPLC system. The test data are indicated for the measurement with manual sample preparation (order no. 15500, see section 2.1) and automated sample preparation with Gilson® ASPEC™ (order no. 15600, see section 2.2).

7.1.1 Linearity, detection limit, quantitation limit

The linearity and the lower limits of detection and quantitation are as follows.

Table 5: Linearity, lower limits of detection and quantitation for levetiracetam

	Order no. 15500	Order no. 15600
Linearity [mg/l]	0.46 - 1000	0.5 - 200
Linearity [µmol/l]	2.70 - 5880	2.94 - 1176
LLOD [mg/l]	0.14	0.15
LLOQ [mg/l]	0.46	0.50

LLOD: lower limit of detection, LLOQ: lower limit of quantitation

7.1.2 Recovery

The recovery is 97 - 105 % (using the complete kit with order no. 15500) and 90 - 102 % (using the complete kit with order no. 15600), respectively, referred to a directly injected external standard solution.

7.1.3 Precision

In order to determine the intra- and interassay precision samples with 3 different concentrations were used. The analyte concentrations were chosen according to the therapeutic reference range (see section 7.2) and are shown in Table 5 together with the precision results.

For the determination of the intraassay precision the samples were measured in 3 analytical series, each by 8-fold determination. For the interassay precision the samples were measured in 8 analytical series, each by 2-fold determination.

Table 6: Precision results for levetiracetam, determined for 3 concentrations

	Concentration [mg/l]	**Intraassay precision [%] (mean value)**		**Interassay precision [%]**	
		Order no. 15500	**Order no. 15600**	**Order no. 15500**	**Order no. 15600**
Sample 1	14	1.25	1.43	2.03	1.70
Sample 2	33	1.37	1.25	2.30	0.63
Sample 3	47	1.74	1.03	1.65	3.48

7.2 Reference Range

Therapeutic reference range for levetiracetam, according to the AGNP Consensus Guidelines 2011 [6]:

10 - 40 mg/l

Please note:

The indicated reference range is taken from thoroughly selected and current scientific literature. The actuality corresponds to the printing date of this document. Please note, that the range does not reflect any recommendations by the manufacturer of this product, but may be used as a guideline for the assessment of the patient's individual therapeutic concentration [6**] by the clinical laboratory.**

8 References

[1] W. Scheid, Lehrbuch der Neurologie, 5. Auflage, G. Thieme Verlag Stuttgart 1983, p. 466-483.

[2] C. Brand: Epilepsie in Zahlen (Stand: Januar 2008). Informationszentrum Epilepsie (ize) der Dt. Gesellschaft für Epileptologie e.V. http://www.izepilepsie.de (abgerufen am 19.06.2012)

[3] A.M. Arain: Medical therapy of epilepsy, Expert Opin. Ther. Patents 2007, 17(8), 955-964.

[4] P.N. Patsalos, D.J. Berry, B.F.D. Bourgeois, J.C. Cloyd, T.A. Glauser, S.I. Johannessen, I.E. Leppik, T. Tomson, E. Perucca: Antiepileptic drugs – best practice guidelines for therapeutic drug monitoring: A position paper by the subcommission on therapeutic drug monitoring, ILAE Commission on Therapeutic Strategies, Epilepsia 2008, 49(7), 1239-1276.

[5] K.A. Lyseng-Williamson: Spotlight on Levetiracetam in Epilepsy, CNS Drugs 2011, 25(10), 901-905.

[6] C. Hiemke , P. Baumann, N. Bergemann, A. Conca, O. Dietmaier, K. Egberts, M. Fric, M. Gerlach, C. Greiner, G. Gründer, E. Haen, U. Havemann-Reinecke, E. Jaquenoud Sirot, H. Kirchherr, G. Laux, U. C. Lutz, T. Messer, M. J. Müller, B. Pfuhlmann, B. Rambeck, P. Riederer, B. Schoppek, J. Stingl, M. Uhr, S. Ulrich, R. Waschgler, G. Zernig: AGNP Consensus Guidelines for Therapeutic Drug Monitoring in Psychiatry: Update 2011, Pharmacopsychiatry 2011, 44, 195-235.

9 Troubleshooting

Problem	**Possible Cause**	**Corrective Measure**
Pressure fluctuation	Air in the pump	Open the purge valve of the pump and aspirate the mobile phase by use of a syringe. Switch the pump for delivery at enhanced flow rate.
	Defective pump check valves	Clean the pump check valves (ultra sonic bath) or renew them (to be carried out by a service technician).
	Air in the pump, leakage	Check the pump.
Spikes on the baseline	Air bubbles in the detector cell	Disconnect the columns and flush the detector cell with mobile phase.
	Air bubbles in the mobile phase	Degas the mobile phase.
Baseline drift	The analytical system is not equilibrated	Equilibrate the analytical system (see section 4.2).
	Mobile phase contaminated	Renew the mobile phase (see section 4.3).
Noisy baseline	Detector cell contaminated	Flush the detector cell with a suitable solvent (to be performed by a service technician).
	Pressure fluctuation	See „problem caused by pressure fluctuation“.
	Contamination of the analytical column	Flush the analytical column with mobile phase (no recycling!). Renew prefilter.
Peak splitting	Defective analytical column (column packing)	Replace the analytical column.
	Defective injection valve	Maintenance and cleaning of the injection valve (to be carried out by a service technician).

Problem	**Possible Cause**	**Corrective Measure**
Broad peaks, tailing	The capability of the analytical column has been exhausted	Replace the analytical column.
	Overload of analytical column	Reduce the injection volume (see section 5.3.1.5).
	Dead volume within the analytical system	Check the analytical system.
Fluctuation of recovery	Defective pipettes	Check the pipettes.
	Injection volume not constant	Check the autosampler.
	Incorrect sample preparation	Take care of correct operation.
Interfering peaks within the chromatogram	Expiry of samples, reagents, etc. passed	See notes on storage and stability.
	Incorrect sample preparation	Take care of correct operation.
	Contamination of mobile phase or reagents for sample preparation	Renew the mobile phase and reagents.
	Prefilter exhausted, analytical column contaminated	Renew prefilter, replace the analytical column.
	Injection system contaminated	Clean the whole injection system (needle, washing station, etc.) with water, followed by isopropanol.
	Interfering peaks, despite of measures named above: Passivation of the HPLC system, see section 4.1	
High backpressure	Obstruction of components like capillaries, filters, columns, etc.	Localise obstructed components by successive disconnection.
Alteration of retention times	Temperature fluctuation	Check the column heater.
	Leakage within the HPLC system	Eliminate the leakage.
	Pressure fluctuation	See „problems by pressure fluctuation“.
Inappropriate detector sensitivity	Contamination of detector cell	Flush the detector cell with a suitable solvent (to be performed by a service technician).
	Detector lamp exhausted	Renew the detector lamp.

10 Appendix: EC-Declaration of Conformity

Declaration of Conformity

for in-vitro diagnostic medical devices, acc. to article 9 (1) of the directive 98/79/EC

The company

RECIPE Chemicals + Instruments GmbH
Dessauerstraße 3
D-80992 Munich / Germany

declares, that the CE labelled product

ClinRep® HPLC Complete Kit for Levetiracetam (Keppra®) (order no. 15500, 15600)

meets all applicable provisions of the directive on in vitro diagnostic medical devices 98/79/EC. The conformity assessment was performed according to annex III. The technical documentation is held according to annex III no. 3.

Munich, 22.07.2014

Alfred Bauer
General Manager

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Zertifiziert nach /
Certified acc. to
ISO 9001
ISO 13485

www.recipe.de

ClinTest® - Standard

Standard Solution for Levetiracetam (Keppra®) /
Standardlösung für Levetiracetam (Keppra®)

for HPLC Assay in Serum / Plasma
für HPLC Bestimmung im Serum / Plasma

REF 15511
LOT 233
2016-08

Analyte / Analyt	Concentration* / Konzentration *
Levetiracetam	2.0 mg/l (11.8 µmol/l)
Internal Standard / Interner Standard	1.0 mg/l (3.93 µmol/l)

*: Weighed analyte amounts; values not assayed / Analyteneinwaage; Werte nicht experimentell ermittelt.

Intended use:
ClinTest® Standard Solutions are intended to check the daily performance of your HPLC - system. This standard solution needs no preparation and can be injected directly into your analysing system (injection volume: 20 µl). After separation, please compare the retention times of the peaks with those of earlier analyses. If you run the standard solution for the first time, you can compare your results with the chromatogram on the back of this data sheet. Besides retention times you are also able to check peak shape, integration and baseline with your standard chromatogram. For calibration the ClinCal® Serum Calibrator, order no. 15513 has to be used.

Storage and stability:
Originally closed and stored at 2 - 8 °C, the ready-to-use standard solution is stable for 36 months, but not longer than the expiration date printed on the label. After opening the vial the standard solution is stable for at least 4 weeks, when stored at 2 - 8 °C.

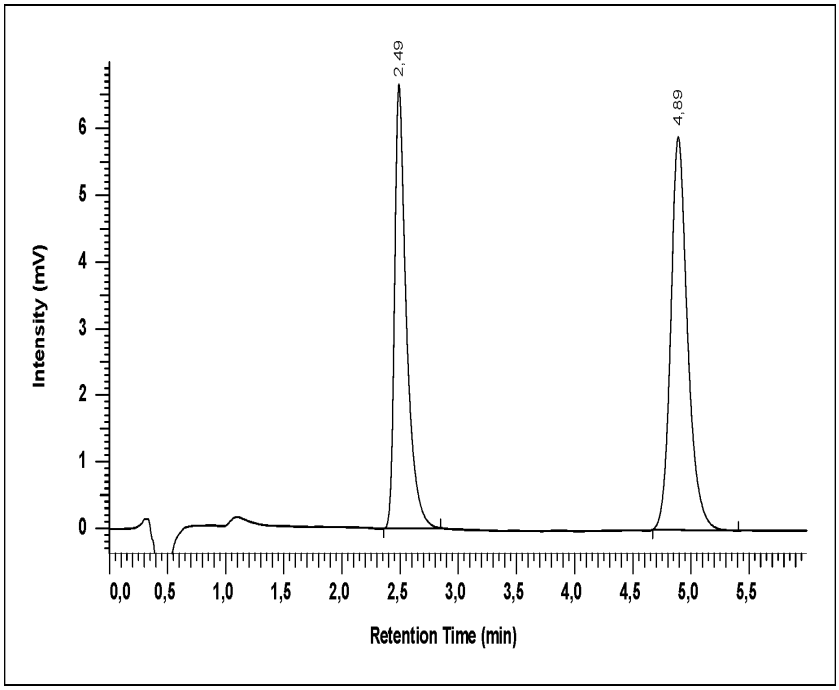
Pack size:
ClinTest® Standard Solution for Levetiracetam: 1 x 3 ml, **order no.: 15511**

Zweckbestimmung:
ClinTest® Standardlösungen dienen zur täglichen Überprüfung der Trennleistung Ihres HPLC-Systems. Diese Standardlösung ist gebrauchsfertig und kann direkt in Ihr Analysensystem injiziert werden (Injektionsvolumen: 20 µl). Nach erfolgter Auftrennung vergleichen Sie bitte die Retentionszeiten der Peaks mit denen der Analysen vergangener Tage. Falls Sie die Standardlösung das erste Mal analysieren, können Sie Ihre Ergebnisse mit dem auf der Rückseite dieses Datenblattes abgebildeten Musterchromatogramm vergleichen. Neben der Überprüfung der Retentionszeiten können Sie anhand des Standardchromatogramms auch die Peakform, Integration und Basislinie checken. Zur Kalibration verwenden Sie den ClinCal® Serum-Kalibrator, Best.- Nr. 15513.

Lagerung und Haltbarkeit:
Originalverschlossen und bei 2 - 8 °C aufbewahrt beträgt die Haltbarkeit der Standardlösung 36 Monate, jedoch nur bis zu dem auf der Packung angegebenen Verfallsdatum. Nach Öffnen der Flasche ist die Standardlösung bei 2 - 8 °C gelagert mindestens 4 Wochen haltbar.

Packungsgröße:
ClinTest® Standardlösung für Levetiracetam: 1 x 3 ml, **Best.-Nr.: 15511**

Chromatogram of the standard solution:
Musterchromatogramm der Standardlösung:



Levetiracetam	2.49 min
Internal Standard / Interner Standard	4.89 min

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CE

ClinCal® - Calibrator

Serum Calibrator lyophilised, for Levetiracetam (Keppra®) /
Serum-Kalibrator lyophilisiert, für Levetiracetam (Keppra®)

for HPLC Assay in Serum /
für HPLC Bestimmung im Serum

REF 15513
LOT 1026
2019-01

Analyte / Analyt	Mean Value / Sollwert
Levetiracetam	32.9 mg/l (193 µmol/l)

The mean value has been assigned with the ClinRep® Complete Kit for Levetiracetam.
Der Sollwert wurde mit dem ClinRep® - Komplettkit für Levetiracetam ermittelt.

Intended use:
ClinCal® Serum Calibrators are used for calibration of the HPLC-system. After reconstitution this lyophilised calibrator has to be prepared like a patient sample.

Reconstitution:
Add exactly 3.0 ml HPLC-water to the vial and mix for 15 min. When all material is dissolved, the solution is ready to use.

Storage and stability:
Originally closed and stored at 2 - 8 °C, the lyophilised serum calibrator is stable for 36 months, but not longer than the expiration date printed on the label. After reconstitution the stability of the analyte is:

at least 12 hours when stored at 15 - 30 °C
at least 7 days when stored at 2 - 8 °C
at least 3 months when stored below -18 °C
(avoid repeated freezing and thawing)

The variation of the filling volume (CV) is < 1 %. The average residual moisture of this lot is 1.02 %.

Pack size:
ClinCal® Serum Calibrator for Levetiracetam: 1 x 3 ml, **order no.: 15513**

Caution:
The human serum which was used for manufacturing the calibrator was tested for the following infectious markers and found negative: HIV1/2- and HCV-antibodies, hepatitis B-surface antigen, HIV1- and HCV-RNA (NAT). Nevertheless, the serum calibrator should be considered as potentially infectious and treated with appropriate care.

Zweckbestimmung:
ClinCal® Serum-Kalibratoren dienen zur Kalibrierung des HPLC-Systems. Nach Rekonstitution wird dieser Serum-Kalibrator wie eine Patientenprobe aufgearbeitet.

Rekonstitution:
Zum Inhalt eines Fläschchens werden exakt 3.0 ml HPLC-Wasser gegeben und die Lösung unter gelegentlichem Umschwenken für ca. 15 Minuten stehen gelassen. Nach erneuter sorgfältiger Durchmischung kann die Lösung verwendet werden.

Lagerung und Haltbarkeit:
Originalverschlossen und bei 2 - 8 °C aufbewahrt beträgt die Haltbarkeit des lyophilisierten Serum-Kalibrators 36 Monate, jedoch nur bis zu dem auf der Packung angegebenen Verfallsdatum. Die Haltbarkeit des Analyten in der rekonstituierten Lösung beträgt:

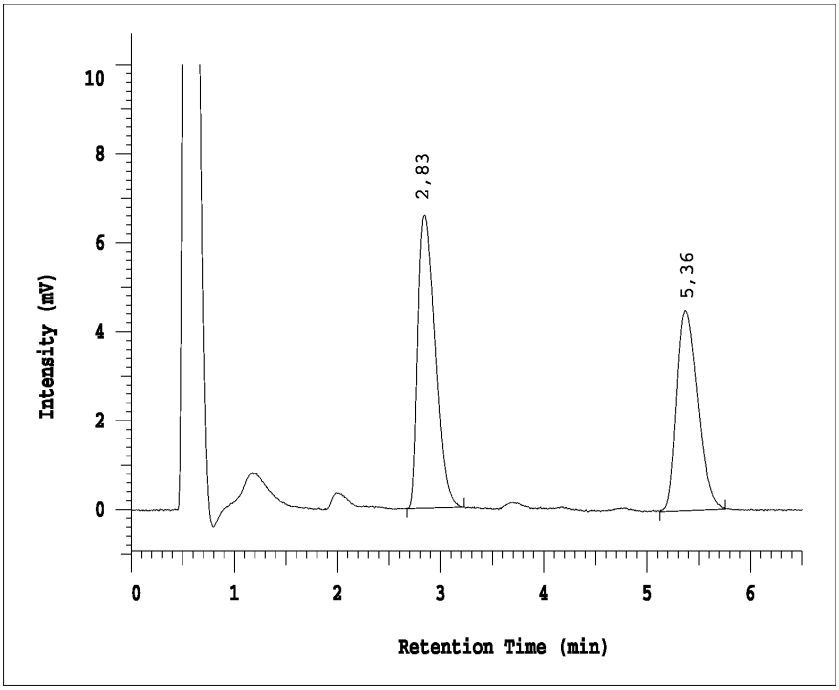
bei 15 - 30 °C mindestens 12 Stunden
bei 2 - 8 °C mindestens 7 Tage
bei < -18 °C mindestens 3 Monate
(Vermeiden Sie wiederholtes Einfrieren und Auftauen)

Die Abfüllpräzision (VK) ist < 1 %. Die durchschnittliche Restfeuchte dieser Charge beträgt 1.02 %.

Packungsgröße:
ClinCal® Serum-Kalibrator für Levetiracetam: 1 x 3 ml, **Best.-Nr.: 15513**

Achtung:
Das zur Herstellung des Kalibrators verwendete Humanserum wurde auf folgende Infektionsmarker untersucht und für negativ befunden: HIV1/2- und HCV-Antikörper, Hepatitis B-Oberflächenantigen, HIV1- und HCV-RNA (NAT). Unabhängig davon sollte der Serum-Kalibrator als potentiell infektiös angesehen und mit angemessener Sorgfalt behandelt werden.

Chromatogram of a prepared serum calibrator:
Musterchromatogramm eines aufgearbeiteten Serum-Kalibrators:



Levetiracetam	2.83 min
Internal Standard / Interner Standard	5.36 min

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CE

ClinChek® - Control

Serum Control lyophilised / Kontrollserum lyophilisiert

FOR LEVETIRACETAM (KEPPRA®) / FÜR LEVETIRACETAM (KEPPRA®)

Intended use:

ClinChek® serum controls are used for internal quality assurance in clinical-chemical laboratories. These lyophilised controls are based on human serum and are available with mean values in the therapeutical as well as in the elevated range. After reconstitution the controls have to be prepared like patient samples in one series of analyses.

Reconstitution:

Add exactly 3.0 ml of HPLC-water to the vial and mix for 15 min. When all material is dissolved, the solution is ready to use.

Storage and stability:

Originally closed and stored at 2 - 8 °C, the lyophilised serum control is stable for 36 months, but not longer than the expiration date printed on the label.

After reconstitution the stability of the analyte is:

- at least 12 hours
  when stored at 15 - 30 °C
- at least 7 days
  when stored at 2 - 8 °C
- at least 3 months
  when stored below -18 °C
  (avoid repeated freezing and thawing)

Notes:

The concentrations of the analyte are chosen in ranges where valid results can be obtained. According to quality assurance all ClinChek® controls have to pass strict quality control procedures during manufacturing. RECIPE guarantees the same stability and constitution for each vial of one lot. The variation of the filling volume (CV) is < 1 %. The average residual moisture of this lot is 1.12 %.

Mean values:

The mean values and confidence intervals have been established at RECIPE, according to the guideline of the German Medical Council for quality assurance (RiliBäk), with statistical methods.

Pack size:

ClinChek® Serum Control
Level I
10 x 3 ml, **order no.: 15580**
Level II
10 x 3 ml, **order no.: 15581**
Level I, II
2 x 5 x 3 ml, **order no.: 15582**

Precautions:

The human serum which was used for manufacturing the controls was tested for the following infectious markers and found negative: HIV1/2- and HCV-antibodies, hepatitis B-surface antigen, HIV1- and HCV-RNA (NAT). Nevertheless, the serum controls should be considered as potentially infectious and treated with appropriate care.

Zweckbestimmung:

ClinChek® Kontrollseren dienen der internen Qualitätssicherung im klinisch-chemischen Laboratorium. Es handelt sich um lyophilisierte Kontrollen auf Humanserumbasis mit Sollwerten im therapeutischen und erhöhten Bereich. Nach Rekonstitution werden die Kontrollproben analog zu den Patientenproben in einer Analysenserie aufgearbeitet.

Rekonstitution:

Zum Inhalt eines Fläschchens werden exakt 3.0 ml HPLC-Wasser gegeben und die Lösung unter gelegentlichem Umschwenken für ca. 15 Minuten stehen gelassen. Nach erneuter sorgfältiger Durchmischung kann die Lösung verwendet werden.

Lagerung und Haltbarkeit:

Originalverschlossen und bei 2 - 8 °C aufbewahrt beträgt die Haltbarkeit des lyophilisierten Kontrollserums 36 Monate, jedoch nur bis zu dem auf der Pakkung angegebenen Verfallsdatum.

Die Haltbarkeit des Analyten in der rekonstituierten Lösung beträgt:

- bei 15 - 30 °C
  mindestens 12 Stunden
- bei 2 - 8 °C
  mindestens 7 Tage
- bei < -18 °C
  mindestens 3 Monate
  (nur einmal auftauen)

Anmerkungen:

Die Analytkonzentrationen liegen im gut messbaren Bereich. Im Rahmen der Qualitätssicherung werden alle ClinChek® Kontrollen bei der Herstellung einer strengen Qualitätsprüfung unterzogen. RECIPE garantiert für jedes Fläschchen einer Charge gleiche Haltbarkeit und Zusammensetzung. Die Abfüllpräzision (VK) ist < 1 %. Die durchschnittliche Restfeuchte dieser Charge beträgt 1.12 %.

Sollwerte:

Die Sollwerte und Vertrauensbereiche wurden von RECIPE entsprechend der Richtlinie der Deutschen Bundesärztekammer zur Qualitätssicherung (RiliBäk) mit statistischen Methoden ermittelt.

Packungsgröße:

ClinChek® Kontrollserum
Level I
10 x 3 ml, **Best.Nr.: 15580**
Level II
10 x 3 ml, **Best.Nr.: 15581**
Level I, II
2 x 5 x 3 ml, **Best.Nr.: 15582**

Vorsichtsmaßnahmen:

Das zur Herstellung der Kontrollen verwendete Humanserum wurde auf folgende Infektionsmarker untersucht und für negativ befunden: HIV1/2- und HCV-Antikörper, Hepatitis B-Oberflächenantigen, HIV1- und HCV-RNA (NAT). Unabhängig davon sollten alle verwendeten Kontrollseren als potentiell infektiös angesehen und mit angemessener Sorgfalt behandelt werden.

ClinChek® - Control

Serum Control, Level I
Kontrollserum, Level I

REF 15580
LOT 509
2018-02

Analyte / Analyt	Method of Analysis / Analysenmethode	Unit / Einheit	Mean Value / Sollwert	Control Range / Kontrollbereich
Levetiracetam	RECIPE-HPLC	mg/l µmol/l	13.9 81.7	11.1 - 16.7 65.2 - 98.1

ClinChek® - Control

Serum Control, Level II
Kontrollserum, Level II

REF 15581
LOT 509
2018-02

Analyte / Analyt	Method of Analysis / Analysenmethode	Unit / Einheit	Mean Value / Sollwert	Control Range / Kontrollbereich
Levetiracetam	RECIPE-HPLC	mg/l µmol/l	46.2 271	37.0 - 55.4 217 - 325

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