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PRESENCE OF THE *CYP2B6 516G>T* POLYMORPHISM AND INCREASED PLASMA EFAVIRENZ CONCENTRATIONS IN SOUTH AFRICAN HIV INFECTED PATIENTS

by

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A research report submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in partial fulfilment of the requirements for the degree of Master of Medicine (Chemical Pathology)

Johannesburg October 2010

Declaration

I, Verena Gounden, declare that this research report is my own work. It is being submitted for the degree of Master of Medicine (Chemical Pathology) in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

…… (Signature of Candidate)

Signed on the …….. day of ……., 2010

Abstract

Introduction

The 516G>T polymorphism in exon 4 of the *CYP2B6* gene has been linked to increased plasma Efavirenz (EFV) levels. EFV levels can predict therapeutic efficacy and are related to the likelihood of developing adverse central nervous system (CNS) effects. The aims of this study were to a) determine the presence of the 516G>T and other possible *CYP2B6* exon 4 polymorphisms in a South African group of HIV-infected individuals and b) to investigate the relationship between the EFV plasma concentrations, the *CYP2B6 516G>T* polymorphism and the occurrence of CNS related side effects in this group of patients.

Methods

Data from 80 patients are presented. Genetic polymorphisms in exon 4 of the *CYP2B6* gene were identified using PCR amplification of this region followed by sequencing of the amplification products. EFV levels were analysed by Ultra performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS). Assessment of the presence of CNS related side effects following EFV initiation were elicited with the use of a questionnaire together with physical examination.

Results

Plasma EFV concentrations displayed high inter-individual variability amongst subjects with levels ranging from 94 µg /l to 23227 µg/l. 23 % of patients were TT homozygous for the 516G>T polymorphism. These patients had significantly higher EFV levels *vs*. those with the wild (GG) genotype ($p < 0.05$). Those who experienced no side effects had significantly lower EFV plasma concentrations *vs*. the group which experienced the most severe side effects ($p < 0.05$).

Conclusion

The significant association between the 516G>T polymorphism and plasma EFV concentrations has been demonstrated in a South African cohort. A rapid and sensitive method for the measurement of plasma EFV was developed and validated.

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Publications and presentations

Publications arising from this study

1. Publication in the journal "AIDS Research and Therapy "in 2010. Gounden et al.: Presence of the CYP2B6 516G> T polymorphism, increased plasma Efavirenz concentrations and early neuropsychiatric side effects in South African HIV-infected patients. AIDS Research and Therapy 2010 7:32. (Refer to Appendix D)

Presentations arising from this study:

1. Poster presentation
   a.Title: Correlation of Plasma Efavirenz levels with CNS related side effects in HIV-infected individuals.
   Authors: V Gounden, T Snyman and JA George
   Presented at: The International Federation of Clinical Chemistry Conference in Fortaleza, Brazil (28 September to 2 October, 2008). (Refer to Appendix E)

   b. Title: The Presence of the CYP2B6 516 G>T Polymorphism and Increased Plasma Efavirenz Concentrations in South African HIV Infected Patients.
   Authors: V Gounden, C van Niekerk, T Snyman and JA George
   Presented at: The Pathvine Congress (South African Pathology Congress), Stellenbosch. (2-5 September 2010)

2. Oral presentations
   a. Title: Plasma Efavirenz concentrations and CNS side effects in HIV-infected individuals.
   Authors: V Gounden, T Snyman and JA George
   Presented at: The South African Pathology Congress in Cape Town (19-21 July 2008).

   b. Title: A rapid method of measuring plasma Efavirenz levels in HIV infected individuals using LC–MS/MS methodology.
   Authors: V Gounden, T Snyman and JA George
   Presented at: The South African Pathology Congress in Cape Town (19-21 July 2008).
   (Refer to Appendix F for both abstracts)

Awards arising from this study:

1. Awarded the Roche Diagnostics Best Young Researcher prize for the above publication in AIDS Research and Therapy by the South African Association of Clinical Biochemists . September 2010

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Nomenclature and symbols

HIV- Human immunodeficiency virus

HAART- Highly active ante-retroviral therapy

ARVs- anti-retrovirals

ART- anti-retroviral therapy

NNRTIs- Non-nucleoside reverse transcriptase inhibitors

NRTIS- nucleoside reverse transcriptase inhibitors

HPLC – high performance liquid chromatography

UPLC – ultra performance liquid chromatography

MS- mass spectrometry

MRM- multiple reaction monitoring

PCR- polymerase chain reaction

µg/l- micrograms/l

EFV- Efavirenz

CNS- Central nervous system

˚C – degrees celcius

PI – protease inhibitor

CV – coefficient of variation

V- voltage

µA- micro Amperes

kV- kilovolts

µl/min – microlitres per minute

l/hour – litres per hour

g/mol – grams per mole

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Chapter 1: INTRODUCTION

1.1 General Introduction

The South African government introduced the national rollout program of treatment of HIV (Human immunodeficiency virus) infected patients with highly active anti-retroviral therapy (HAART) in April 2004. Currently over 400 000 patients receive anti-retroviral therapy (ART) at South African state hospitals (1). The first line therapy in treatment naïve patients consists of a combination of the non-nucleoside reverse transcriptase inhibitor (NNRTI) Efavirenz (EFV) and two nucleotide reverse transcriptase inhibitors (NRTI's) – Stavudine and Lamivudine.

In patients commencing therapy for the first time, the development of adverse effects may influence adherence and treatment success. Significant side effects may be associated with all groups of anti-retroviral drugs. Despite their recent introduction, in South Africa, ARV's have been shown to significantly reduce morbidity and mortality in patients with HIV infection (2). The ARV experience is relatively new to South Africa in comparison to many developed nations and studies looking at adverse effects of treatment and long-term treatment complications are only now beginning to emerge.

Patients often differ in their response to identical therapies. The reasons for this inter-individual variability in terms of drug related toxicity, drug levels and drug efficacy are multifactorial (3). It involves differences in gender, metabolism, drug compliance, presence of underlying diseases, use of concomitant medications as well as genetic factors (3). Genetic differences among individuals influence metabolism, distribution and elimination of drugs. Clinically important genetic

polymorphisms, which influence drug metabolism and drug response, have typically been discovered on the basis of phenotypic differences among individuals from different populations. Genotyping patients before drug therapy will help to better individualise therapy by enabling the identification of potential responders, non-responders, or patients at increased risk of toxicity (3).

Therapeutic drug monitoring (TDM) is useful to ensure that a patient has the appropriate blood levels of a particular drug to maximise therapeutic benefit and minimise toxicity. The relationship between plasma levels of NNRTI's as well as protease inhibitors has been shown to correlate with clinical antiviral effect in patients receiving HAART(4). Since the active tri-phosphate metabolite of NRTI's occurs intracellularly, the role of TDM for these agents is currently precluded (5).

In the first chapter the relevance of genetic polymorphisms on Efavirenz therapy will be discussed. The role of therapeutic drug monitoring in patients receiving Efavirenz therapy will also be reviewed.

1.2 Literature Review:

1.1.1 Pharmacokinetics and pharmacodynamics of Efavirenz

Efavirenz and its metabolism

Efavirenz belongs to the non-nucleoside reverse transcriptase inhibitor class of drugs utilised as part of HAART in the management of patients with HIV infection. It acts by binding to reverse transcriptase non-competitively thus altering the function of the enzyme and preventing conversion of viral RNA to DNA (6). Reverse transcriptase inhibitors are virustatic and thus do not eliminate HIV from the body. Although human DNA polymerase is less susceptible than the viral DNA polymerase to the pharmacologic effects of Efavirenz, the drug's action may still account for some of its associated toxicity.

Efavirenz is chemically described as (S)-6-chloro-4-(cyclopropylethynyl)-1,4-dihydro-4-(trifluoromethyl)-2H-3,1-benzoxazin-2-one and has a molecular weight of 315.675 g/mol. Its empirical formula is $C_{14}H_9ClF_3NO_2$ and its structural formula is illustrated in Figure 1.1 (7).

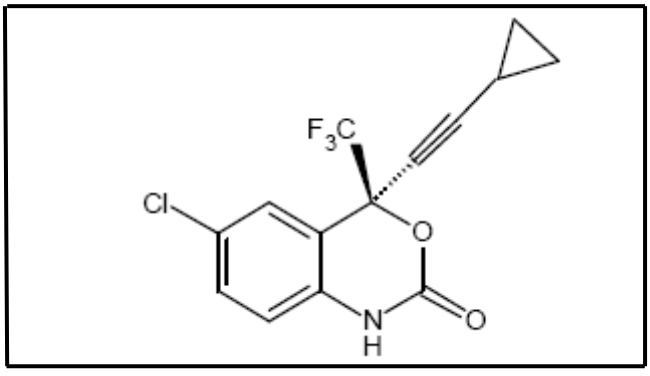


Figure 1.1. Chemical structure of Efavirenz

The drug forms part of the triple therapy regimen and is taken orally as a single dose of 600mg at night. Night time dosing is preferred in order to minimise the day time consequences of side effects such as hallucinations common in patients receiving Efavirenz therapy (7).

Pharmacokinetics

Efavirenz is absorbed orally, and is highly bound (> 99%) to plasma proteins, predominantly albumin. The drug is also widely distributed in the central nervous system (CNS) (7). It is primarily metabolised by the cytochrome P450 isoenzyme, CYB2B6 in the liver (6) and to a lesser extent by the CYP3A4 enzyme (7).

The *CYP2B6* gene has been mapped to chromosome 19. It is 28 kb long and consists of 9 exons (8). The presence of several polymorphisms present in the gene coding for the enzyme may influence drug metabolism. CYP2B6 is primarily responsible for the hydroxylation of efavirenz to 8-hydroxyefavirenz and 8, 14-hydroxyefavirenz (6). Efavirenz has a half-life of 52-76 hours after a single dose and 40-55 hours after multiple doses(7), thus allowing for once daily dosing. EFV and its metabolites are largely (60%) eliminated via bile through faeces (7). Few studies have examined the influence of renal impairment on Efavirenz levels. However the finding that less than 1% of Efavirenz is excreted unchanged in the urine suggests that the presence of renal impairment is unlikely to have an effect on EFV elimination (7).

Adverse effects

Efavirenz is associated with several neuropsychiatric side effects such as hallucinations, mood changes, insomnia and headaches (Refer to Table 1.1) which are usually mild and generally short lived (weeks) (7, 9), however, cases have been reported of individuals who have experienced significant depression or psychosis following initiation of Efavirenz which necessitate changing therapy (7).

Table 1.1. Reported side effects associated with Efavirenz (7)

System	Side effects experienced
Central and Peripheral Nervous System	abnormal coordination, ataxia, cerebellar coordination and balance disturbances, convulsions, hypoesthesia, paresthesia, neuropathy, tremor
Endocrine	gynaecomastia
Gastrointestinal	constipation, malabsorption
Cardiovascular	flushing, palpitations
Liver and Biliary System	hepatic enzyme increase, hepatic failure, hepatitis
Metabolic and Nutritional	hypercholesterolemia, hypertriglyceridemia
Musculoskeletal	arthralgia, myalgia, myopathy
Psychiatric	aggressive reactions, agitation, delusions, emotional lability, mania, neurosis, paranoia, psychosis, suicide
Respiratory	dyspnea
Skin and Appendages	erythema multiforme, photoallergic dermatitis, Stevens-Johnson syndrome
Special Senses	abnormal vision, tinnitus

Although previous centres (10) have reported central nervous system (CNS) side effects in >50% of patients following commencement of EFV therapy, no studies in South Africa have investigated EFV plasma concentrations and the incidence of CNS related side effects.

In patients commencing therapy for the first time, the development of adverse effects may negatively influence adherence and treatment success. Previous studies have shown that plasma EFV concentrations show large inter subject variability with a CV (coefficient of variance) of up to 118% (10). Plasma concentrations of EFV predict therapeutic efficacy and are related to the likelihood of developing adverse CNS effects (9, 10). Patients with EFV concentrations of > 4000 µg/l may experience neurological adverse effects more frequently, whilst those with plasma concentrations < 1000 µg/l appear to have a greater risk for emergence of selective drug resistance and treatment failure (10)

Few studies have examined the adverse effects of long term EFV use. Lochet *et al* investigated the long term effects by analysis of questionnaire responses from 174 individuals after 6 months post initiation of EFV (11). Their findings indicated the continued presence of moderate to severe neuropsychiatric symptoms in 23% of patients after a 3-month treatment period. Their study also noted higher reporting of anxiety, depression and increased stress in those receiving EFV versus the control group. 6.3% of patients discontinued EFV therapy because of persistent or late onset neuropsychiatric symptoms (11). Fumaz *et al* assessed adverse events, EFV plasma levels, quality of life, psychological status and adherence in patients on an

EFV based regimen vs. those on a protease inhibitor (PI) based regimen (12). These patients were followed-up for one year. Quality of life was assessed using a questionnaire where each question was scored to give a final score from 0 to 100. EFV levels were also measured in these patients. This study showed no association with EFV plasma levels greater than 4000 µg/l and the presence of neuropsychiatric symptoms in patients receiving longer term therapy. Overall there was no statistically significant difference in quality of life, adherence and psychological status between both groups. There were no reports of irrational thoughts or hallucinations amongst both groups of patients. However on observation the patients on the PI regime showed decreased adherence with longer treatment periods (12). Despite more neuropsychiatric abnormalities reported in the first four weeks of therapy in the EFV group vs. the PI group (54% vs. 27%), during this phase there was no difference in adherence between the two groups (12).

1.3.2 The role of therapeutic drug monitoring in Efavirenz measurement

Current consensus is that there is a definite role for TDM in the management of specific groups of patients receiving anti-retroviral therapy (4). This is particularly so in the case of PIs and NNRTIs. NRTI's which are phosphorylated to their active metabolite intracellularly show poor correlation between plasma concentrations of parent drug and intracellular biologically active metabolites (5). TDM has a role to play in monitoring drug related toxicity, patient adherence, drug-drug interactions and in patients with co-infections and hepatic disease. In addition TDM may be useful in specific populations such as in paediatric and pregnant patients. These populations undergo various metabolic, physiological and, in the case of children, also

developmental changes which may make prediction of drug concentrations difficult. TDM would allow for a more evidence based manner of adjusting dosage and regimens during these periods of transition. Current PENTA (Paediatric European Network for the treatment of AIDS) guidelines recommend the use of TDM in young children receiving HAART (13).

Increased plasma levels of Efavirenz have been associated with serious CNS side effects in those taking the drug and thus may affect patient compliance. As previously stated, plasma EFV concentrations < 1000 µg/l have been associated with increased risk for emergence of drug resistance (10).

The concomitant use of drugs such as Flucanazole and traditional medicines (e.g. herbs such as St Johns Wort) can lead to significant drug interactions, affecting EFV levels (7). In South Africa, with the high rate of co-infection with TB and HIV, the concomitant use of EFV and Rifampicin is common. Rifampicin is a potent inducer of the CYP450 enzymes (14). Plasma Efavirenz levels have been reported to be reduced by Rifampicin. One study showed a decrease of 20-25% in area under the curve (AUC) levels for Efavirenz and no significant effect on the pharmacokinetics of Rifampicin in patients receiving both drugs (15). Patients receiving anti-TB therapy and ART, who were less than 50 kg, had similar EFV concentrations as those patients receiving only HAART whilst EFV levels in patients > 50 kg were 50% less than levels found in patients receiving only HAART (15). Another study demonstrated a very large inter-subject variation (CV 157%) in plasma EFV levels in patients receiving concurrent rifampicin therapy but reported excellent clinical outcomes despite these variations in EFV levels (16).

TDM would be useful in this scenario to assess the effect of these drug-drug interactions on plasma EFV levels and to assist with dosage adjustment of Efavirenz. These factors make monitoring of ART a useful tool.

A common argument used against therapeutic monitoring of NNRTI's is the fact that these drugs have a long half-life (40-50 hours) (7). Thus it is thought that plasma concentration of NNRTI's would be adequate in most of these patients; however, high inter-individual variability in both concentration and patient response shown in various studies with NNRTI's suggests a prominent role for TDM (17).

Efavirenz measurement

Various chromatographic methods have been utilized in the measurement of EFV. These include high performance liquid chromatography (HPLC), reverse- phase HPLC and liquid chromatography (LC) coupled to mass spectrometer (MS) or tandem MS (9, 10, 18). These methods allow for simultaneous measurement of the parent drug, significant metabolites and other ARV's. The use of HPLC or LC-MS allows for multiple drugs to be measured. This feature is of benefit in HIV infected patients receiving concomitant medication which can affect EFV levels such as for those receiving concomitant Rifampicin therapy. Liquid chromatography refers to the separation of a specific material based on its affinity or interaction with the moving solvent, called the mobile phase, and the particles, called the stationary phase. The stationary phase may be in a planar form or column form (where the column is packed with different types of chromatographic packing material which allows for separation of the compound of interest) (19). High pressure or high performance LC

originally indicated that high pressure was used to generate the flow required for liquid chromatography in packed columns. Ultra performance LC is a progression on HPLC, which uses smaller particles (sub-2-µm versus standard HPLC 5µm particles) as column packing material. The use of smaller particles allows for significant increases in resolution, speed, and sensitivity in liquid chromatography (20).
The tandem MS serves as the "detector system" for the UPLC. The MS converts the molecules that enter into it into charged ions via various ionisation processes (for example chemical, electron spray ionisation). The ions are separated, identified, detected and measured according to their mass-to-charge ratios (m/z). The relative abundance of each ion is plotted as a function of its m/z ratio and this is represented as a mass spectrum. The ion with the highest abundance in the mass spectrum is assigned a value of 100%. Tandem MS allows for increased sensitivity and very high selectivity (19).

However, these methods are often technically demanding, requiring extractions to be performed on samples prior to analysis, are only available in tertiary institutions and are not currently standardized. In spite of initial capital costs of instruments consumables are inexpensive and extraction procedures can be modified to increase sample throughput. LC-MS has a very low interference rate which makes it highly suitable for identifying a specific drug in a plasma sample of a patient taking concomitant drug therapy. HPLC-MS is considered a reference methodology for TDM due to its increased sensitivity and specificity.

1.3.3 The role of genetic polymorphisms and methods for their detection

Cytochrome P450- B6 (CYP2B6) alleles

CYP2B6 belongs to the enzyme family of cytochrome P450 mono-oxygenases. Like most other members of the CYP family its function is to catalyse the oxidation of various organic substances including xenobiotics (drugs) (21). The enzyme CYP2B6 is involved in the metabolism of a variety of clinically important drugs such as Efavirenz and Nevaripine.

Due to its extensive genetic polymorphism, *CYP2B6* is characterized by wide inter-individual variability in hepatic expression and activity (6). The presence of several polymorphisms present in the gene coding for the enzyme may influence drug metabolism. Therefore in patients with polymorphisms causing decreased enzyme function, increased plasma levels and toxicity may occur .In patients with polymorphisms resulting in increased enzymatic activity, decreased drug levels and reduced therapeutic benefit may occur. Previous studies have shown that the allelic variant *516G>T* (located in exon 4) is associated with increased plasma drug levels (9, 22). The allelic variant *516G>T* (*CYP2B6*6*) was also shown to have increased prevalence amongst African Americans with studies quoting the frequency of this allele as 30-38% (9, 23). Studies in African populations indicate a prevalence ranging between 36-60% (Table 2). Klein *et al* showed a prevalence of close to 50% for the *516G>T* allele amongst a study population of Ghanaians (23). A similar prevalence for this single nucleotide polymorphism (SNP), ranging from 36% (Sierra Leone) to 60% (Senegal) was reported by Mehlotra *et al (*24*)*. In a Zimbabwean

cohort Nyakutira *et al* demonstrated a frequency of 49% for the *CYP2B6 516G>T* variant (25). A study in Zanzibar examining the effect of the *CYP2B6 516G>T* polymorphism on artemisinin (an anti-malarial drug) demonstrated a 9.7% frequency for the *TT* homozygotes (26).

Other polymorphisms influencing CYP2B6 enzyme activity have been described. A number of polymorphisms have been located in exon 9 of the *CYP2B6* gene (22). Twenty-four non-synonymous SNPs and several SNPs, in the promoter region of the *CYP2B6* gene have been reported (25). Rotger et al recently described the presence of a polymorphism causing a premature stop codon resulting in a truncated CYP2B6 protein. The *CYP2B6*16* allele is defined by the presence of the *983T>C*, together with the *785A>G* polymorphism. These SNPs are associated with reduced CYP2B6 protein expression. The presence of polymorphisms may have an additive effect for example, *516G>T* and *983T>C* are associated with a five fold greater mean plasma EFV concentration (18). See Table 1.2 below for summary of important *CYP2B6* SNPs.

The relationship between a patient's sex and their ability to tolerate antiretroviral drugs is increasingly being examined (27). Differences in terms of incidence of side effects (22, 28) and response to treatment (29) have been demonstrated in various studies. Mechanisms proposed to explain the above differences between males and females include weight, fat distribution, glomerular filtration rate (GFR) and the effect of sex hormones (27).

However evidence suggests that these differences between the genders may also be explained by different occurrences at the molecular level such as the presence of SNPs. One study looking at *CYP2B6* expression in liver tissue showed a significant difference between the two sexes. They showed that CYP2B6 activity was below quantifiable limits in 7.1% of the female study population versus 20% of the male liver microsomes (30).

Table 1.2. Summary of studies evaluating the prevalence of CYP2B6 516 G>T and other polymorphisms

Authors	**Population group**	**Polymorphism**	**Prevalence**	**Effect on EFV level**
Klein *et al* (23)	Ghana	*CYP2B6 516G>T* variant	50%	Increased
Mehlotra *et al* (24)	Sierra Leone	*CYP2B6 516G>T* variant	36%	Increased
	Senegal	*CYP2B6 516G>T* variant	60%	Increased
Nyakutira *et al*(25)	Zimbabwe	*CYP2B6 516G>T* variant	49%	Increased
Rotger *et al* (18)	Various population groups in North America	*a)CYP2B6 917 C>G (exon 6)* *b) CYP2B6 983 T>C (exon7)* *c)CYP2B6 1132 C>T (exon7)* *d) CYP2B6 1172 T>A (exon8)* *e) CYP2B6 1459 C>T (exon9)*	a) allele CG in black patients 1.3% b) allele TC in black patients 18.75% c) allele CT in black patients 1.3% d) polymorphism not observed in black population e) allele CT in black patients 6.25 %	
Haas *et al* (9), Klein *et al* (23)	African American	*1459C>T* (exon 9)	1%	Increased

This suggests that differences in plasma drug concentrations between the sexes may also be attributed to molecular differences in genetic expression and not only factors like body mass index (BMI).

Methods for assessing enzyme and gene activity

Possible differences in drug metabolizing enzyme activity may be investigated by either examining phenotype with the use of probe drugs or with genotype analyses of the enzyme of interest. Bupropion is the probe drug commonly used to assess CYP2B6 activity (31). The use of probe drugs is time consuming and tedious for the patient.

Genotypic analysis of the *CYP2B6* gene typically involves polymerase chain reaction (PCR) with either restriction fragment length polymorphism or sequencing analysis of PCR products**.** PCR utilises the ability of DNA polymerase to synthesize a new strand of DNA complementary to a specifically chosen template strand. As DNA polymerase can add a nucleotide only onto a pre-existing 3'-OH group, it requires a primer (reverse and forward) to which it can add the first nucleotide. Nucleotides (dNTPs or deoxynucleotide triphosphates**)** – single units of the bases A, T, G, and C are essentially "building blocks" for new DNA strands that are added to the master mix. The use of primers allows one to choose a specific genomic sequence to be amplified, in this case exon 4 of the *CYP2B6* gene. The PCR process involves the following steps: denaturation (the separation of complementary strands), annealing (attachment of primer to target sequence) and elongation (addition of base pairs to

form complete strands). PCR allows for exponential amplification of initial DNA templates (32).

Polymorphism detection using sequencing

The initial Sanger method used for DNA sequencing is based on the principle of DNA replication. The Sanger method utilises the ability of DNA polymerase to incorporate 2' and 3" di-deoxynucleotides, nucleotide base analogues that lack the 3' hydroxyl group. The Sanger procedure requires a DNA template, a sequencing primer, DNA polymerase, nucleotides (dNTPs), di-deoxynucleotides (ddNTPs) and a reaction buffer (33).

Four separate reactions are set up, each containing labelled nucleotides (fluorescent labels or radioactive labels) and either ddA, ddC, ddG, or ddT. Addition of ddNTP results in chain termination whilst addition of a dNTP results in chain extension by DNA polymerase. This process results in the formation of extension products of various lengths terminated with ddNTPS at their respective ends. These extension products can be separated and analysed by various methods including electrophoresis and mass spectrometry. The ABI 3130XL sequencer used in this study utilises capillary electrophoresis, a process whereby the DNA fragments are separated in a narrow bore silica tube by size and charge under the force of an electrical field. The sequencing process consists of five integral steps which are; 1). Template preparation 2). Cycle sequencing 3). Purification after cycle sequencing 4). Capillary electrophoresis and 5). Data analysis (33).

Newer genotyping methods include genotyping by matrix-assisted laser desorption/ionization–time of flight mass spectrometry (MALDI-TOF/MS). MALDI-TOF/MS utilises a soft ionization technique which allows for the analysis of various large organic molecules, proteins & polypeptides to be analysed. This technique combines high throughput with high accuracy. This method may be used for rapid screening for SNPs, quantitative allele studies and for discovery of new polymorphisms (34).

1.3 Objectives

The aims of our study were three-fold 1) to investigate and describe polymorphisms present in exon 4 of the *CYP2B6* gene in black HIV infected individuals, 2) to develop and validate a rapid method for determination of EFV in plasma to enable monitoring of drug levels in HIV-infected patients and 3) to investigate the relationship between the EFV plasma concentrations and the presence of *CYP2B6* exon 4 SNPs with the occurrence of CNS related side effects in this group of patients.

Chapter 2 : MATERIAL AND METHODS

2.1 Sample collection

Specimens were collected from 100 Black South African patients attending the ARV clinic at the Charlotte Maxeke Johannesburg Academic Hospital. Samples from 80 patients were used. Twenty patients were excluded due to insufficient plasma volumes for UPLC-MS/MS analysis, poor DNA yields following extraction or technical problems with regards to sequencing. Informed consent was obtained from all participants involved with the study (see consent form in Appendix B). Ethical approval for the study was obtained from the Research Ethics Committee, Faculty of Health Sciences, University of the Witwatersrand (refer to Appendix G). 10 ml of blood in EDTA tubes were collected from each patient. These samples were collected from patients 2- 4 weeks after initiation of Efavirenz therapy. This time interval was chosen as plasma EFV levels take 6-10 days to achieve steady state concentrations (7). It was also to ensure better recall of side effects experienced by patients following initiation of ARVs. All patients received the same dosage of 600mg EFV nightly. The samples were separated by centrifugation at 5000 g for 10 minutes (immediately) after collection. Buffy coats were stored at -20^0 C until DNA extraction and plasma samples were stored at –70^0 C until the analysis for EFV levels was performed. Time of last dose was obtained by patient report. Patients who had not taken their EFV the night before or those who had missed more than two doses were excluded from the study. (Refer to Table 2.1 for further exclusion criteria).

The use of concomitant drugs and herbal medications, which are known to influence plasma EFV levels, were excluded with the aid of a questionnaire administered to all participants (refer to Appendix C). The presence of abnormalities on a patient's liver function test (LFT) (refer to Table 3) was also an exclusion factor because significant liver dysfunction can affect CYP 450 enzyme function (6).

Table 2.1. Exclusion criteria for study

Pregnancy or breast feeding
Previous or current psychiatric disease being treated by a medical practioner
Non compliance (missed more than 2 doses in one month)
Alcohol intake >4 units/day for male and > 3 units/day in females (1unit = 8g of alcohol) (35)
Patients taking drugs that potentially may interact with Efavirenz metabolism (as stated in drug manufacturer's information sheet)
Liver affectation as indicated by : a) Transaminases > 5-10x the upper limit of normal b) ALP > 5-10x the upper limit of normal c) Total bilirubin > 2.5-5x the upper limit of normal (36)

LFTS, CD4 and viral load

The LFTs, viral load and CD4 analyses are performed routinely on all patients commencing ARV therapy at the clinic. LFTs were carried out by the NHLS Chemistry laboratory. Samples were analysed on the Roche Modular automated

chemistry analyser. All analytes contained in the LFT panel were measured by spectrophotometric methodology.

Viral loads were measured in the Department of Haematology using the Roche COBAS AMPLICOR HIV-1 MONITOR version 1.5 Test and the CD4 counts were determined by using the PLG (PanLeucogating) methodology flow cytometry (Beckman Coulter) in the Department of Haematology Johannesburg Hospital. PanLeucogating involves the use of a combination of antibodies to CD45 and CD4 or CD8 to identify the total leucocyte population on the fluorescence flow cytometer. This method developed by Drs Glencross, Janossy and Jani allows for a more accurate CD4 cell count to be determined by a dual-platform method (37).

On their initial visit to the clinic each patient was weighed and their height recorded using standard methodologies. These were then used to calculate BMI for each patient in the study. Case files were analysed with regard to documentation of common presumptive Efavirenz side effects and a questionnaire (refer to Appendix C) was administered to patients at two weeks post treatment initiation. Treatment adherence was also assessed using patient files and patient disclosure.

2.2 DNA extraction

DNA was extracted from buffy coats using Invisorb Blood Mini Kit (Invitek, Germany).

Procedure:

1. Lysis of cells in buffy coat
   - 200ul of buffy coat sample was transferred to a 1.5 ml reaction tube.

- 200 µl of Lysis buffer A and 20 µl of proteinase K was added and the sample mixed and incubated at 56 ˚C for 10 minutes.

2. DNA binding

- 400 µl of binding buffer was to tube in step one and vortexed.
- The sample was loaded onto a spin filter, incubated for 1 minute, then centrifuged at 12000 g for 2 minutes.

3. Washing

- The spin filter was placed into a new receiver tube, washed with 500 µl of Wash buffer 1 and centrifuged for 1 min at 10000g.
- The filtrate was discarded and the spin filter placed in a new receiver tube. 800 µl of wash buffer 2 was added and the sample was centrifuged at 10000g for 1 minute.
- This filtrate was discarded and the spin filter placed in a new receiver tube and centrifuged at maximum speed for 4 minutes.

4. Elution of DNA

- The spin filter was placed into a new receiver tube, 200 µl of pre-warmed Elution Buffer was added to centre of the filter, incubated and centrifuged for 1 min at 10000 g

The concentration and purity of extracted DNA was determined on a Nanodrop spectrophotometer (Thermo Scientific, USA) by measuring the absorbance at a wavelength of 260 nm for concentration and 260/280 nm for purity.

2.3 Amplification of exon 4 of the *CYP2B6* gene

Exon 4 of the *CYP2B6* was amplified using conventional PCR.

Primer design

Forward and reverse primers for exon 4 were designed using the published gene sequence on GenBank (accession number NM 000767) together with the GeneRunner 3.05 software (Hastings Software Inc.). Primer selection was based on those primers with the most specific binding capacity, the lowest likelihood of primer dimer formation and non-specific binding, and similar melting temperatures. The Basic Local Alignment Search Tool (BLAST) accessed at www.ncbi.nlm.nih.gov/blast, was used to align primer sequences to the known gene sequence to confirm that the primers would amplify the desired gene region (refer to Table 2.2). The size of the amplicon was 518 base pairs (bp) long.

Table 2.2. Exon 4 primers

Name	**Sequence**	**Tm (melting temperature in ºC)**
Exon 4 forward	5'-TGTTGTAGTGAGAGTTCAATG-3'	56
Exon 4 reverse	5'-CTATCCCTGTCTCACCGTC-3'	62

The PCR reaction was carried out with a 12.05 µl master mix consisting of:

dNTPs (Bioline,US) 0.75 µl (Total concentration 10 mmol/l)

forward primer 0.25 µl (concentration 100 pmol/µl)

reverse primer 0.25 µl (concentration 100 pmol/µl)

Taq polymerase 0.15 µl (concentratioin 5 U/µl)
(SuperTherm Gold DNA polymerase, US)

Extracted DNA 2 µl (concentration above 30 (µg / µl)

Water 7.65 µl

Total volume 12.05 µl

PCR reactions were performed on the MyCycler thermal cycler (BioRad Laboratories, US) using the following programme:

Table 2.3 PCR programme

Temperature (˚C)	**Time**	**Number of cycles**
1. 94 (initial denaturation)	11 minutes	1
2. 94 (denaturation)	40 seconds	35
56 (annealing)	25 seconds	
70 (extension)	1 minute	
3. 70 (final extension)	10 minutes	1

PCR products were run on 1 % agarose gels together with a 50bp DNA molecular weight marker (Generuler; Fermentas, Lithuania) and a negative control to detect any possible contamination. 1 % agarose gels were made with 0.5g agarose tablets

(Bioline, UK) and 50 ml TBE (Tris, boric acid, EDTA) buffer (for composition refer to Appendix A). Ethidim bromide (2.5 µl) was added to the gel mixture in order to visualise the PCR products under UV light (Gel-doc viewer, BioRad laboratories, Italy). 5 µl of sample from each PCR reaction was mixed with 2 µl of loading buffer (refer Appendix A for constituents), and loaded into separate lanes of the gel, run at 100V for 30 minutes and visualised under UV light.

2.4 Polymorphism detection using sequencing

Amplicons were sequenced by Inqaba Biotech (South Africa). Sequencing was performed using the ABI 3130XL sequencer which is based on capillary electrophoresis methodology.

Sequences were analysed with the Sequencher 4.1.4 software (Genecodes, USA). The reference sequence used was that for the *CYP2B6* gene (accession number NM 000767). Each patient's forward and reverse sequences were aligned against the reference sequence and any SNPs in the patients. Sequences were reviewed by the investigator with the aid of the Sequencher software.

2.5 Measurement Plasma Efavirenz concentrations

Sample preparation for analysis

EFV was extracted using solid phase Weak Cation Exchange cartridges (WCX, Oasis-Microsep, Massachusetts, USA). Solid-phase extraction (SPE) is a separation

process that utilises physical and chemical properties of compounds to isolate them from a complex mixture and thus in itself can also be considered a type of chromatography (19). Knowledge regarding the chromatographic behaviour of the analyte of interest, and of other matrix components assists in the choice of proper sorbent and eluents. The mechanism of separation employed by the WCX cartridge used, is based on ion-exchange principles and thus best suited to isolate compounds that are easily ionisable. Cartridges can be either cation or anion exchangers and are further subdivided into weak or strong exchangers, depending upon the type of ionic group on their surface. Weak cation exchangers possess an acidic surface moiety such as a carboxylic acid that is ionised (negatively charged) at high pH but neutral at low pH whereas strong cation exchangers possess a moiety that remains ionised over the whole pH range (20). The pK_a of Efavirenz was found to be 10.2 thus allowing for adequate recovery on the WCX cartridge (38).

Preparation of solutions and standards

Solutions required for sample extraction, and analysis on UPLC- MS/MS were prepared using commercially available HPLC reagent grade materials.

Extraction procedure:

- 200µl of plasma was used for analysis of the drug levels. WCX cartridges were conditioned with 1 ml Hydrochloric acid (HCl) solution (concentration 1%) and 1 ml methanol (concentration 100%) before sample loading. The HCl solution was made by dilution of 100% HPLC reagent grade HCl with Grade 1 water.

- 1 ml of patient ‘s plasma was then loaded onto the cartridge
- Vacuum pressure was exerted at a pressure of 10 kPa.
- 1ml of HCL (concentration 1%) used to wash each cartridge
- Collection tubes were discarded and new tubes were placed under each WCX cartridge
- The specimens were then eluted with 1ml solution of 30% acetonitrile (which was made up by dilution of 100% HPLC reagent grade Acetonitrile with Grade 1 water)

Instrumentation and UPLC- MS/MS conditions

EFV plasma levels were measured by Ultra Performance Liquid Chromatography Quatro micro (UPLC-MS/MS), (Waters, Massachusetts, USA).
Chromatographic separation was performed on an Acquity, (Waters, Massachusetts, USA) phenyl column 1.7µm (diameter of packing material) [2.1 (diameter of column) x 50mm (column length)]. The phenyl columns have a wide pH range and are suitable for separation of polar compounds (such as EFV) (29). A guard column was used to protect the phenyl column from any particulate matter thus prolonging the life of the phenyl column. The chromatographic column used was stable for > 200 injections. This stability allows for cost reduction in running the assay.
The mobile phase consisted of A: B at 30:70 (2 mM ammonium acetate with 1% formic acid: 100% Acetonitrile).
The mobile phase was made up in the following manner:
A: 1. 2mM ammonium acetate (molecular weight 77.08 g/mol) solution-
0.308 g of ammonium acetate dissolved in 2 litres of Grade 1 water.

2. 1% formic acid – 100% (concentration w/v) was diluted with Grade 1 water to give a final solution of 1% formic acid.

B: 100% HPLC reagent grade acetonitrile was used

The mobile phase was run on an isocratic gradient with the analyte eluting within 1.5min. The column temperature was maintained at 50ºC throughout the runs. Injection volume for each sample was 10 µl. The instrument was operated in Electron spray ionization positive (ESI+) mode. The multiple reaction monitoring (MRM) transition used for EFV was *m/z* (mass to charge ratio) 357.7>316.3. Refer to the table 6 below for further details of UPLC–MS/MS settings.

Table 2.4 . UPLC–MS/MS settings for EFV analysis

Source (ESCi+)	**Settings**
Capillary (kV)	2.40
Corona (µA)	14.00
ES cone (V)	25.00
APCI cone (V)	30.00
Extractor (V)	3.00
RF lens (V)	0.0
Source temperature (˚C)	120
Desolvation temperature (˚C)	400
Cone gas flow (l/hour)	50
Desolvation gas flow (l/hour)	650

Table 2.4 . UPLC–MS/MS settings for EFV analysis continued

Analyser	Settings
LM 1 Resolution	10.0
HM1 Resolution	8.0
Ion energy 1	0.5
Entrance	2
Collision	15
LM 2 Resolution	10.0
HM 2 Resolution	8.0
Ion energy 2	3.0
Multiplier (V)	650
Syringe pump flow (µl/min)	10.0

Calibrators and controls.

Commercially available calibrator standards and controls were used. (Chromosystems Instruments and Chemicals GmbH, Munich, Germany). 3 standards of the following values: standard 1 -1415 µg/l, standard 2 - 2830 µg/l and standard 3- 5661 µg/l were used for calibration. The lypholised controls and the calibrators were made up to the designated manufacturer volumes using Grade 1 reagent water. Calibration curves and controls were run with every batch of patient specimens. The correlation coefficient of the standard curves obtained on multiple days was consistently ≥0.98 (n=18).

Assay procedure

Following sample extraction, 200µl of sample was placed in sampling vials which were then placed in the UPLC sampling system. Standards were run initially to establish the calibration curve. Once the curve was acceptable, quality control materials (Chromogen systems) at two levels i.e. one within the therapeutic reference range and a high value were run before patient samples. Retention time for EFV was 0.72 min with total run time of 2 min.

Method characteristics: Limit of quantification, Recovery, Linearity, Precision and accuracy

An evaluation [Clinical and Laboratory Standards Institute (CLSI) protocol EP10] to assess recovery, assay precision and linearity was performed for validation. Three levels of QC material at a low, mid (1:1 mixture of the low and high controls) and a high value were run in a specific sequence over 5 days. This protocol examined specific performance parameters such as linearity, carryover, bias, recovery (39).

Specimen stability

Specimens were assessed for stability by comparing initial values to values following storage at room temperature, 2 to 8 degrees Celsius, at -20 degrees Celsius and at -70 ˚C.

Specificity

To assess the effect of Bactrim (a commonly used co- medication in patient with Acquired Immunodeficiency Syndrome) three patients' plasma samples were spiked with Bactrim (concentration 1mg/ml) and then analysed on the UP-LCMS/MS.

2.6 Assessment of EFV-related side effects

A questionnaire adapted from one used in the AIDS Clinical Trials Group study A5095 (40) was administered to all participants. Responses were scored in terms of frequency of side effects experienced and severity in terms of effect on daily activities. The maximum score that could be obtained was 72 points. Based on their questionnaires, subjects were grouped into those with no side effects (Group1), those with mild symptoms (1-12 points - Group2), with moderate symptoms (13-48 points - Group3) and with severe side effects (> 48 points or presence of hallucinations or psychotic episodes - Group 4). All subjects were also seen by the same doctor during this visit to assess for the presence of neuropsychological side effects.

2.7 Data analysis

The Online Encylcopedia for Genetic Epidemiology Studies (http://www.genes.org.uk/software/hardy-weinberg.shtml) was used to assess test whether the SNPs were in Hardy-Weinberg equilibrium. The Hardy-Weinberg equilibrium states that over serial generations the genetic variation in a population will remain constant if there are no disturbing factors such as mutations, natural

selection and non random mating. Thus genotype and allele frequencies will remain constant in a large population where there are no disruptive factors (41).

Statistical analyses were conducted using Statistica 8 (Statsoft, Tulsa, USA). Plasma EFV levels were analysed across genotype groups using nonparametric analysis of variance (ANCOVA) using appropriate post hoc test for paired means. Regression analysis was performed to assess the influence of other possible confounders such as age, gender & BMI.

Chapter 3: RESULTS

3.1 Patient results

Data for 80 consecutive patients were analysed. Twenty patients from the initial 100 recruited were excluded due to insufficient plasma volumes for UPLC-MS/MS analysis, poor DNA yields following extraction or technical problems with regards to sequencing. The main characteristics of the study cohort are summarised in Table 3.1.

Table 3.1. Demographic details of subjects

	Male	***Female***	***Total***
Number	20	60	80
Age (years)	38.5 ±8.9	37.2±9.1	37.5±9.0 (n=80)
BMI (kg/m2)	21.4±2.3	23.9±3.8	23.3±3.6 (n=80)
CD4 cell count (x106 /l)	162 (19-378)	115 (2-293)	128 (3-378) (n=80)
Viral load (copies/ml)	$7.25x10^4$ ($150-3x10^6$) (n=20)	$8.9x10^3$ ($39-3x10^6$) (n=56)	$8.63\ x10^3$ ($39-3x10^6$) (n=76)
Side Effects (Yes %)	25 (n=5)	13 (n=8)	33 (n=13)

*Figures stated as mean±1SD, or as median (range). Reference range for CD4 count (500-1500x 10^6/l)

SNP results for *CYP2B6* exon 4

The genotype distribution and EFV levels were as follows: 36% (n=29) of patients were homozygous GG for the *CYP2B6 516G>T* polymorphism with); 23 % (n=18) of patients were characterised as homozygous TT, and 41% (n=33) of patients were

heterozygous GT. The frequency of the T allele was 43%. (Refer to figure 2 for the PCR gel run and refer to figure 3 for sequencing chromatograms). A previously unreported SNP was identified in one patient in the non-coding region of the CYP2B6 gene. This SNP was a G>C point mutation 54 bases upstream of the *CYP2B6 516G>T* SNP in exon 4. The patient was homozygous for the G allele *CYP2B6 516G>T* polymorphism, had a plasma EFV level of 6812 µg/l (therapeutic range 1000-4000 µg/l) and was characterised as having mild side effects following ARV initiation. This SNP was not observed in other patients in this study. The significance of this is currently, unknown.

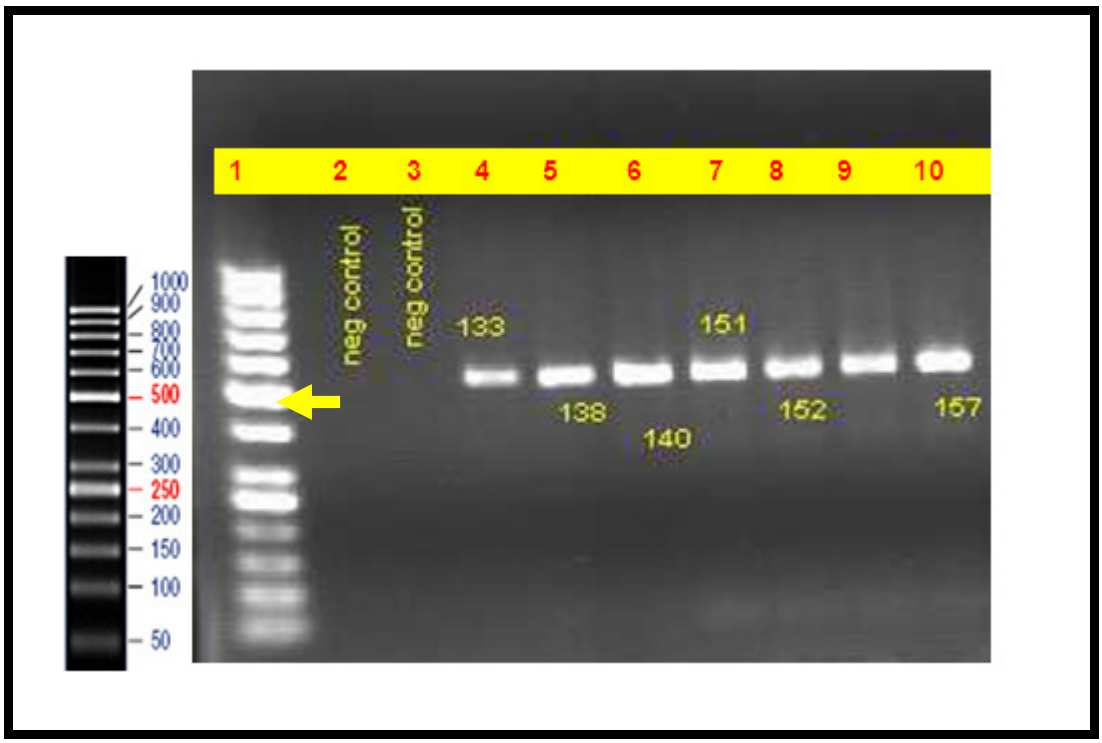


Figure 3.1 Amplicons for exon 4 of the CYP2B6 gene (of patients' samples) run on a agarose gel. **Arrow indicates 500 base pair marker of the molecular ladder. In lane 1 is the molecular ladder, lanes 2 and 3 are the negative controls and in lanes 4-10 are patient samples.*

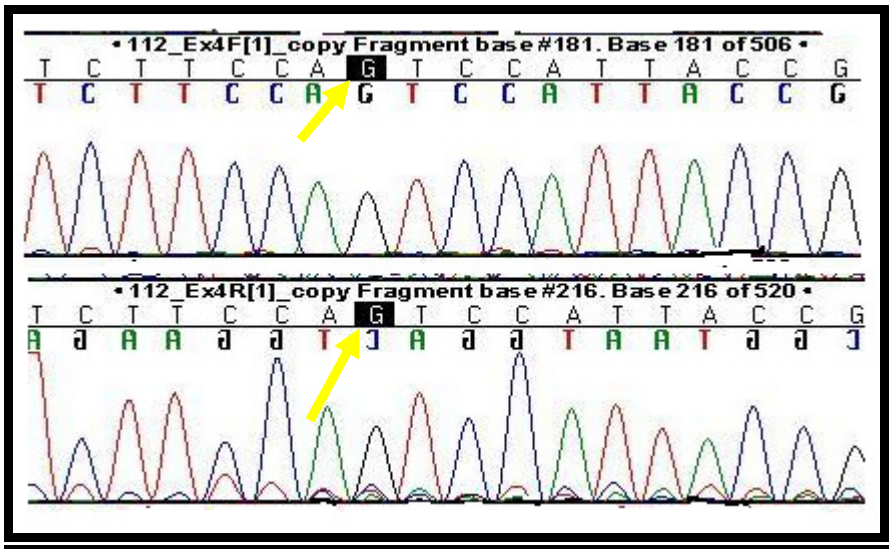


Figure 3.2a Electrophoretogram showing a GG homozygous patient at the 516G position of exon 4 in the *CYP2B6* gene (as indicated by the yellow arrow*s*)

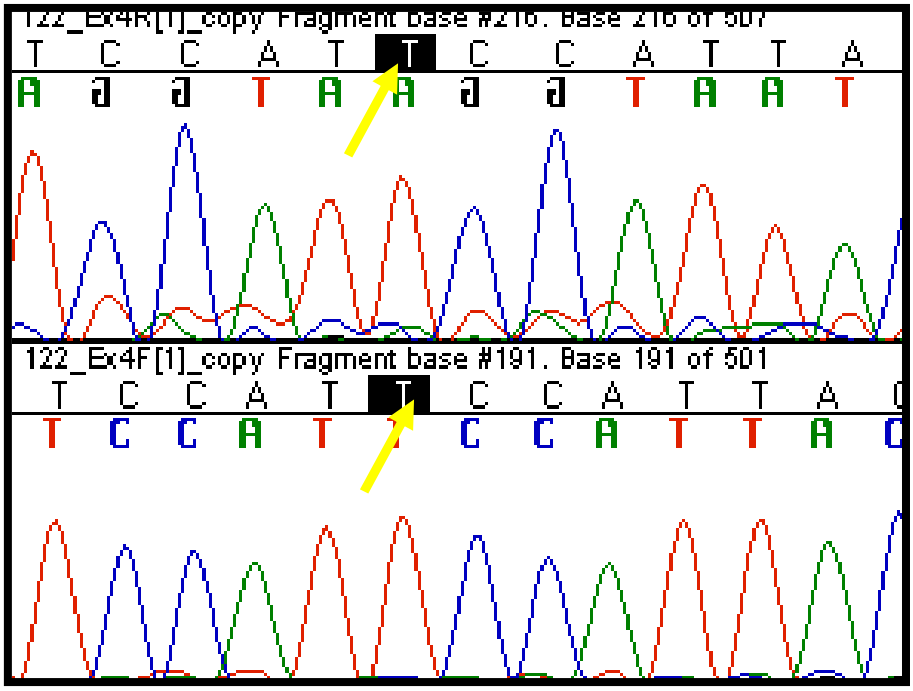


Figure 3.2b Electrophoretogram showing a TT homozygous patient at the 516G position of exon 4 in the *CYP2B6* gene (as indicated by the yellow arrow*s*)

The frequency of the allele, in the study population, was in Hardy-Weinberg equilibrium. These results are similar to those of Nyakutira *et al*. who studied this particular SNP amongst a Sub-Saharan African population (25).

Results for plasma EFV concentrations

Plasma EFV concentrations in patients ranged from 94 µg/l to 23227 µg/l (median 3980 µg/l), confirming the high inter-individual variability previously noted (10, 25) in patients receiving EFV therapy. (Refer to figure 3.3 for MRM chromatogram)

However, only 51% of patients had EFV levels within the recommended concentration range of 1000 µg/l to 4000 µg/l (10). The average time between last dose of EFV taken by patients and sample collection was 14.6±1.5 hours. Efavirenz plasma levels were not significantly affected by sampling times. Multivariate regression analysis demonstrated that gender, age, BMI and time of dosage did not significantly affect EFV levels with $p > 0.1$.

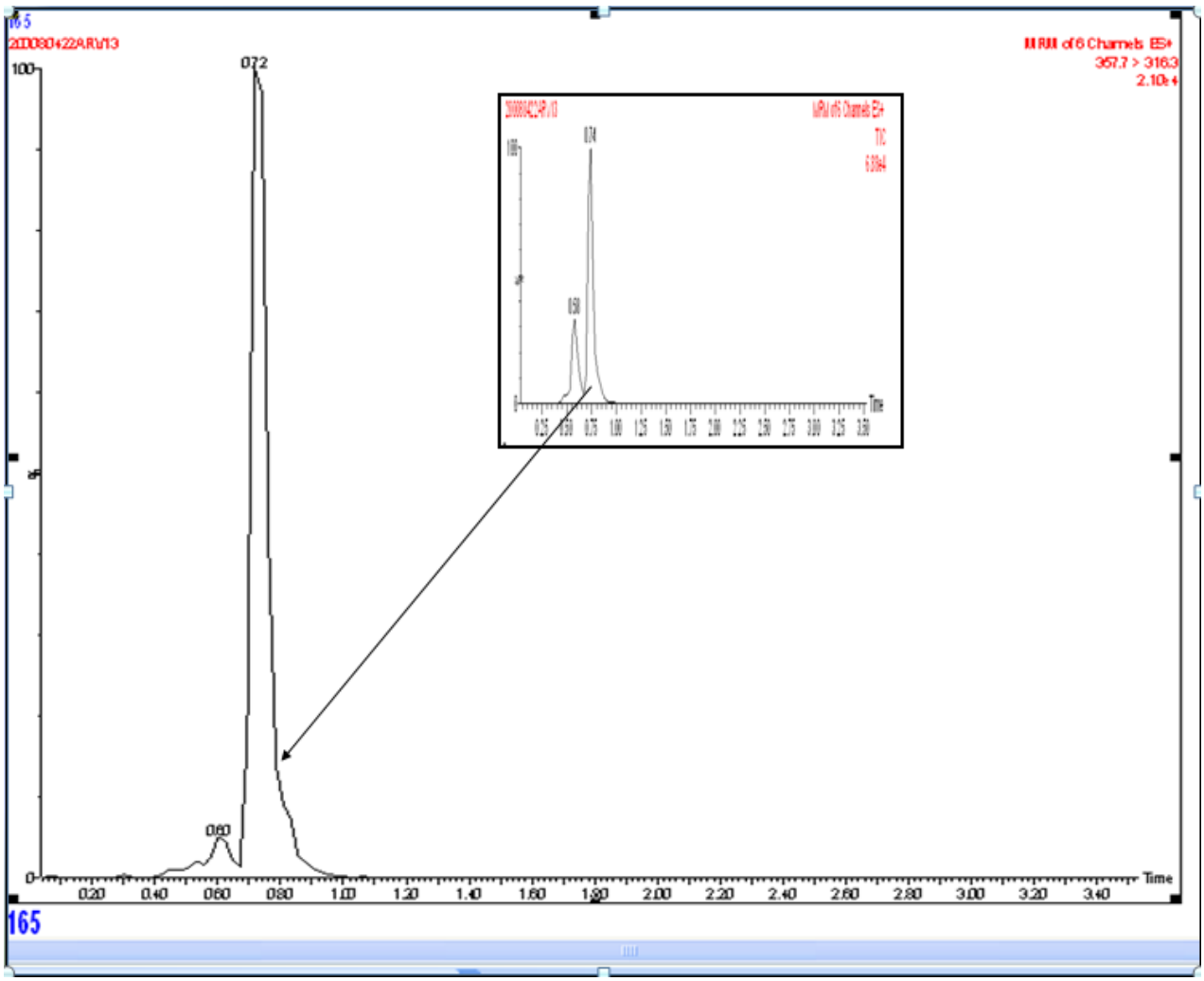


Figure 3.3 The MRM chromatogram of EFV for a patient sample. *The MRM chromatogram for the transition 357.7>316.3 is shown in the larger square. The chromatogram in the smaller square illustrates the total ion chromatogram for that specimen run.*

Findings of relationship between 516G>T SNP and EFV concentrations

The homozygous GG group had a median EFV plasma level of 2260 µg/l (range 94 µg/l to 12957 µg/l); the homozygous TT, had a median EFV level of 7136 µg/l (range

1334 µg/l to 23227); and the patients that were heterozygous GT for the polymorphism had a median EFV level of 3857 µg/l (range 184 µg /l to 15581 µg/l).

Interestingly, most (61%) of patients who were homozygous GG for the 516G>T polymorphism had EFV levels within the therapeutic range, whilst only 16% of those with the TT genotype had levels within this range. Further analysis demonstrated that patients who were homozygous TT for the 516G>T polymorphism had significantly higher EFV levels than those with the GG genotype ($p < 0.05$) (Refer to Figure 3.4).

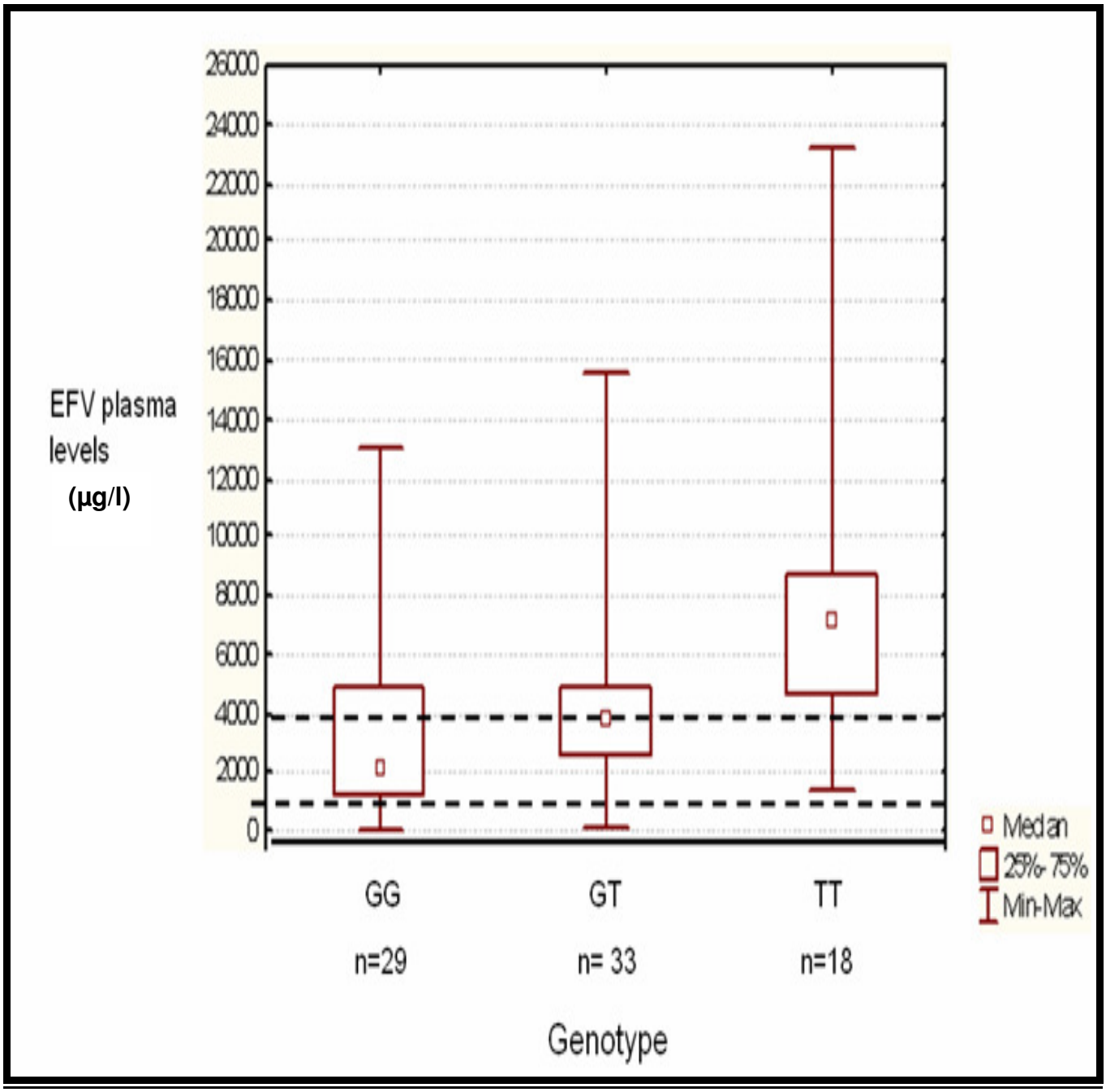


Figure 3.4. Box and whiskers plot of median Efavirenz (EFV) plasma concentrations by *CYP2B6-516* genotype

**small boxes*, median values; *bars,* interquartile ranges; *whiskers*, full range. *GG*, homozygous genotype*; GT,* heterozygous genotype*, TT* homozygous genotype; --- *Value between dashed lines represents therapeutic range*

Results of Questionnaire

85% of patients experienced some form of EFV-related side effects. The majority of patients (56%) experienced mild symptoms. Those who experienced no side effects had a significantly lower median EFV plasma concentration of 2666 µg/l (concentrations ranged from 102.3 to 4839.7 µg/l) compared to the group which experienced the most severe side effects with a median EFV plasma concentration of 14882 µg/l (concentrations ranged from 9825 µg/l to 23227 µg/l) ($p < 0.05$). (Refer to Figure 3.5)

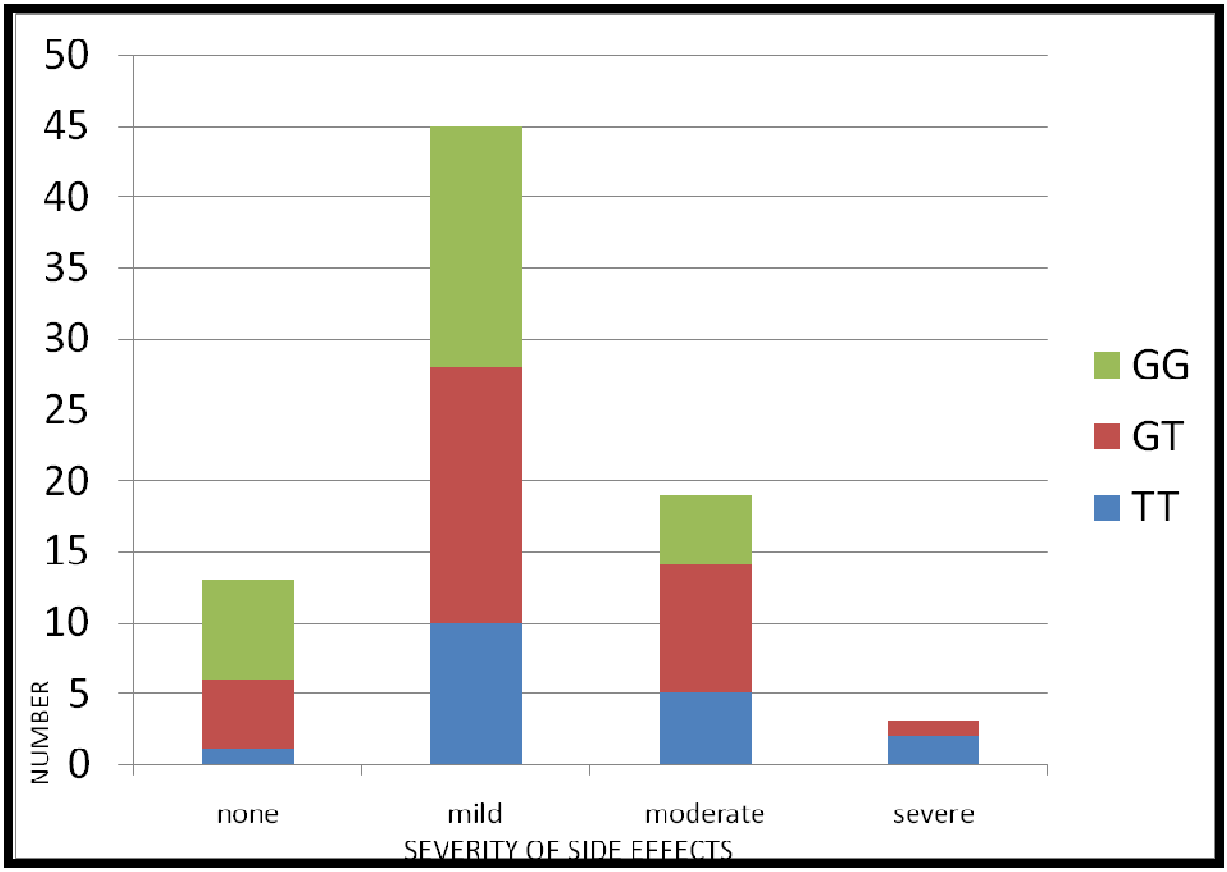


Figure 3.5. Bar graph illustrating the distribution of the severity of EFV related side effects amongst the different genotypes

The majority of patients, who did experience side effects following initiation of Efavirenz therapy, had mild symptoms with dizziness (55%) and headache (45%) as the two most frequent complaints. No patients reported suicidal ideation whilst only 5 % of patients reported having experienced hallucinations following initiation of EFV therapy. These findings are in concordance with other studies reporting the side effect profile of EFV therapy (7, 9, and 10). Figure 3.6 below shows the distribution for side effect distribution experienced by patients in the study.

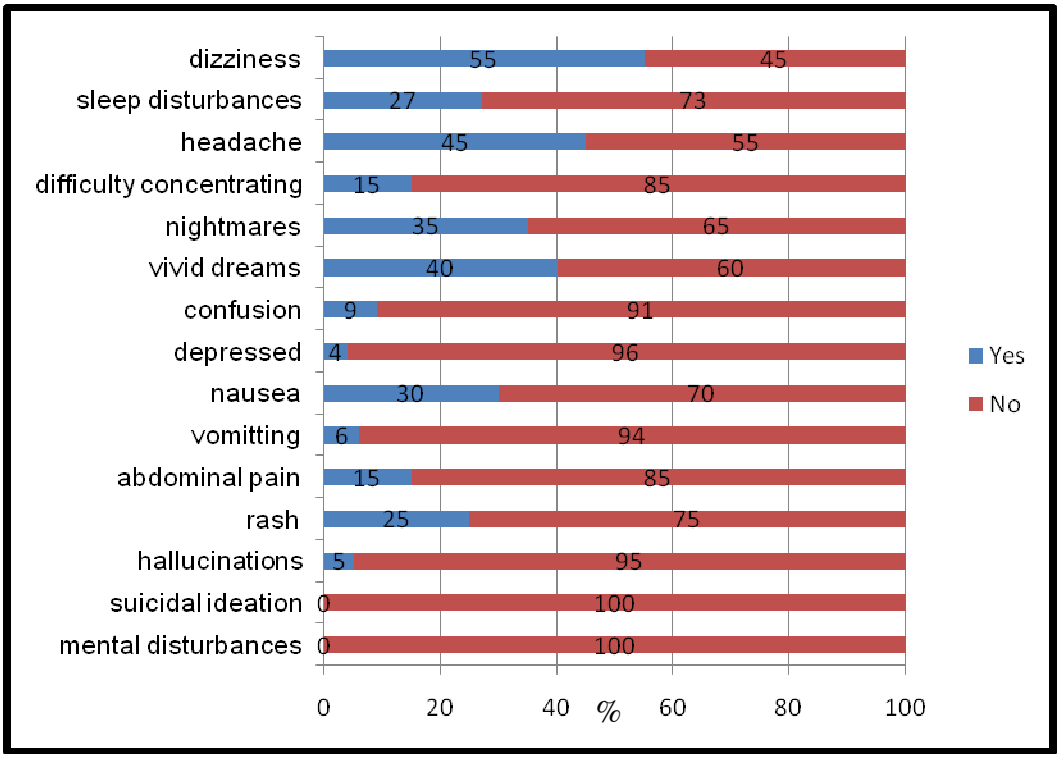


Figure 3.6 Bar graph illustrating side effect profile of patients on Efavirenz therapy

At the1-month follow-up visit following initiation of therapy, the specific EFV-related side effects had resolved for all patients involved in the study. 33% (7 of 21) of all patients who reported severe and moderate EFV related side effects carried the TT

genotype. Patients homozygous for the TT genotype did show increased overall side effects as compared to those displaying the GG genotype, however this difference did not reach statistical significance (p=0.08)

3.2 Method characteristics: Limit of quantification, Recovery, Linearity, Precision and accuracy

Intra-assay and inter-assay precision CV's ranged from 2.8 to 10%, and 8 - 8.9%, respectively. Overall bias was ≤ 5% with the mid level control (level at therapeutic range) displaying the best accuracy. Guidelines for TDM have recommended a between day coefficient of variation between 5% and 10% (42). Refer to Table 3.2 below for the assay performance data for this study

The extraction efficiency/recovery ranged from 83-118% (acceptable recovery 80 – 120%), with mean recovery following extraction of 101%. The assay was linear up to a concentration of 30630 µg/l. The limit of detection for the assay was 85 µg/l. No significant carryover or drift was observed.

Table 3.2 Assay performance data (EP 10 evaluation)

RUN	**REPLICATE**	**LOW** (units µg/l) Target value 1105 µg/l.	**MID** (units µg/l) Target value 3306 µg/l.	**HIGH** (units µg/l) Target value 5507 µg/l.
DAY 1	1	929	3660	5419
	2	1136	3209	5721
	3	1052	3802	5660
	4		3434	
	5		2914	
Mean Concentration		***1039***	***3404***	***5600***
DAY2	1	1082	3321	6020
	2	1091	3783	6155
	3	1211	3505	6483
	4		3150	
	5		3062	
Mean Concentration		***1128***	***3364***	***6219***
DAY3	1	1173	2816	6461
	2	1032	3022	6511
	3	1027	3652	6104
	4		3117	
	5		3107	
Mean Concentration		***1077***	***3143***	***6359***
DAY4	1	1000	3240	5662
	2	938	3224	5744
	3	975	3648	5261
	4		3434	
	5		3533	
Mean Concentration		***971***	***3416***	***5556***
DAY 5	1	1199	3150	5529
	2	1118	3145	5263
	3	1083	3069	5068
	4		3146	
	5		2747	
Mean Concentration		***1133***	***3051***	***5286***
Mean concentration (all days)		***1070***	***3276***	***5804***

Table 3.2 Assay performance data continued

Within run CV for day 1 (%)		LOW 10	MID 10	HIGH 2.8
Within run CV for day 2 (%)		6.3	8.5	3.8
Within run CV for day 3 (%)		7.68	9.8	3.5
Within run CV for day 4 (%)		3.165	5.3	4.65
Within run CV for day 5 (%)		5.27	5.6	4.37
Mean inter-assay precision (CV)		8.25	8.9	8
Average bias		35 µg/l. (-3%)	30 µg/l. (1%)	297 µg/l. (+5%)

3.3 Specimen stability

No significant changes in Efavirenz levels were observed in plasma that had been subjected to two freeze-thaw cycles. See Table 3.3 for details on stability experiment.

Table 3.3. Stability of plasma Efavirenz samples

Storage condition	**Plasma EFV values (units : µg/l)**	**Recovery (%)**
Initial EFV plasma values	1) 4957 µg/l 2) 5821 µg/l 3) 11594 µg/l	
Stored at room temperature for 3 hours	1) 5271 µg/l 2) 5419 µg/l 3) 9864 µg/l	1)106 % 2) 93 % 3) 85 %
Stored at fridge temperature (2-8 C) for 24 hours	1) 4691 µg/l 2) 5584 µg/l 3) 10625 µg/l	1) 95 % 2) 96 % 3) 92 %
Stored at -20 C for 1 week	1) 4350 µg/l 2) 5402 µg/l 3) 11055 µg/l	1) 88% 2) 93 % 3) 95 %
Stored at -70 C for 3 months	1) 5097 µg/l 2) 5712 µg/l 3) 11405 µg/l	1) 102 % 2) 98 % 3) 98 %

3.3 Specificity

The results obtained after the addition of Bactrim did not display any significant change with regards to the original Efavirenz concentrations.

Chapter 4: DISCUSSION

The current interest in pharmacogenomics and "personalised medicine" has gone from research to providing real clinical solutions for physicians and their patients. The increasing list of ARVs available together with the issues of drug-drug interactions, the presence of concomitant diseases, unpleasant and dangerous side effects of certain ARVs and patient non-compliance makes the treatment of HIV-infected patients highly complex. Plasma EFV levels have been shown to be predictive of treatment success and the occurrence of adverse effects (9, 10). The relationships between drug efficacy and lower virological failure rates when optimal drug concentrations are achieved have been demonstrated in a number of studies (10, 22, 23).

The utilization of TDM together with relevant genotype analysis can potentially make the treatment of HIV infected individuals less complex and more effective. The detection of polymorphisms affecting EFV concentrations may be used to direct therapy and dosage, thus minimizing toxicities. Repeated exposure to sub-therapeutic levels of EFV also increases the chance for the development of resistant viral strains and thus treatment failure (43). Differences in metabolism of EFV play a role in determining effective plasma concentrations (9, 10). Therefore SNPs in the CYP2B6 metabolizing enzyme resulting in decreased drug levels may select out for EFV resistance in certain patients whilst the expression of allelic variants such as the *CYP2B6 516G>T* polymorphism may lead to higher plasma levels, toxicity and eventually treatment failure. The long half-life of EFV (42) suggests that treatment

interruption in patients carrying the *TT* genotype also selects for EFV resistance due to sub-therapeutic levels for extended periods.

This study revealed the prevalence of the allelic variant *CYP2B6 TT* (poor metabolisers) to be 23% amongst our study population. The percentage is very similar to the Adult AIDS Clinical Trials Group study by Haas *et al* (9), which reported a 20% prevalence of the *TT* genotype amongst their African–American cohort. Both the prevalence of the SNP and correlating EFV plasma concentrations as reported in this study was similar to the published results of Nyakutira *et al* in Zimbabwe (25).

The findings of this study echoed those of previous studies (10, 25) by demonstrating the association of significantly greater EFV plasma levels during ARV therapy with the *CYP2B6 TT* genotype. In this study the statistically significant relationship between the occurrences of severe Efavirenz related side effects and increased plasma concentrations of the drug was observed.

These findings although significant, do not allow for immediate use of this information in the prescribing of antiretrovirals nor the adjustment of dosage due to side effects. It must be remembered that this polymorphism does not act independently and the presence of other polymorphisms and factors such as concomitant illnesses, use of polypharmacy and viral genotype also influence drug levels (43, 44). Most of the pharmacogenomic studies investigating polymorphisms affecting the efficacy of EFV therapy have been performed on fairly small cohorts and larger studies are required to confirm their findings before these can be used to guide ARV therapy. In nations like South Africa where the goal of adequate access

to antiretroviral therapy for all HIV-infected patients is still to be achieved, the added expense of pharmacogenomic genotyping may seem unrealistic. However, TDM, which can be used to monitor patient adherence, manage ARV-related toxicities and detect treatment failure due to sub-therapeutic drug levels, appears to be fairly accessible, convenient and affordable method. TDM for EFV using a LC-MS/MS method like that which is described in this study, allows for the accurate measurement of EFV and high throughput with a run time of only two minutes.

To date this study has been the first to describe the presence of the *CYP2B6 516 G>T* polymorphism in the South African black population and the effect this genotype has on EFV levels in South African black HIV infected individuals.

Study limitations

This was a small pilot study and larger studies in the South African population are needed to confirm these findings. Pharmacokinetics shows that trough levels of drugs are the most useful in assessing efficacy and toxicity of the drug. The nighttime dosing of EFV results in difficulty obtaining trough doses. The suggested therapeutic range of 1000 – 4000 µg/l is not based on trough levels but on levels 8-20 hours post dosing (10). Another limitation of this study is that pharmacokinetic modeling was not done to assess the possibility of reducing EFV dosage in those patients with the *TT* genotype. Long term follow up of these patients to determine any correlation between the presence of the polymorphism and its influence on continued virological suppression in HIV–infected patients as well as obtaining personalized pharmacokinetic parameters will be useful

CONCLUSION

This study has confirmed the presence of the *CYP2B6 516G>T* polymorphism in Black South African HIV patients. The findings also show a significant relationship (p value <0.05) between the presence of the above polymorphism and elevated EFV plasma levels in HIV-infected patients receiving HAART. Information gathered from studies like this one and those to follow will ideally assist in the formulation of protocols for the role of pharmacogenetics and monitoring of ARV levels for HAART in South Africa in HIV infected patients. The use of pharmacogenetics and TDM to assist in the management of complex drug therapies such as HAART is an attractive option. Particularly as this study has revealed that a significant proportion of patients have EFV concentrations below the therapeutic range emphasizing that the measurement of EFV does have a role to play in their management in an environment of increasing incidences of treatment failure.

APPENDIX

Appendix A.

Constituents of loading buffer

0.025 grams bromophenol blue

0.025 grams xylene cyanol

3 ml glycerol

Make up to 10 ml with distilled water

Appendix B. CONSENT FORM

Good morning sir/madam (patient)

My name is Dr Verena Gounden and I am a doctor in the Department of Chemical Pathology. I would like to welcome you and request your participation in our study. The information below explains the study and what will be needed from you should you agree to participate.

Individuals infected with the human immunodeficiency virus (HIV) and receiving antiretroviral therapy (ART) may experience side effects which can make treatment unpleasant. It has been shown in studies carried out in other countries that some of these side effects are related to differences in genes which may affect the level of the ARV medication in your blood. We would like to look at the gene differences in the south African population and see if these affect levels of the drugs in blood and as well as side effects.

To do this we must carry out blood tests. Bloods will be drawn by placing a needle into your arm and about 5ml of blood (1 teaspoon) taken for each tube. If you agree to participate in the study I will carry out blood tests on you before you begin treatment and then 2 weeks after the start of treatment. The tests done on you will include a CD 4 count and a viral load. These help in assessing your immune status as well as your response to treatment. Genetic studies will include looking at the differences in the gene CYP2B6. This is a gene that is involved in metabolizing several drugs including Stocrin which you will be receiving. The blood taken will not be used for any tests other than those described.
If we noted any blood changes that require a change in your treatment we will inform your doctor of these changes.

Please note: Your participation in this study is voluntary and if you should choose not to participate this will in no way change your treatment at the clinic. Your identity will remain anonymous at all times and only your doctor will know then outcomes of the tests. You will be identified for the purposes of this study by a unique patient number.

I (Dr Verena Gounden) have described the tests we wish to carry out and have explained the reasons for it. I have also asked whether there were any questions about the tests and have answered them the best I can.

Date: Doctor:

Consent form (please sign this form)

I agree to take part in the above clinical study. The procedures to be carried out have been explained to me. The possible discomforts, risks and benefits involved in taking part in the test have also been described to me. I understand that I can stop the test at any point. I also understand that if I have any questions concerning the test then the clinician will explain these to me

Date: Patient:

Appendix C. PARTICIPANT QUESTIONNAIRE FOR EFAVIRENZ RELATED SIDE EFFECTS

Thank you for your participation. The purpose of this questionnaire is to help us evaluate the side effects you may have experienced since you started the ARV treatment.
Did you experience any of the side effects listed below, after starting the ARV treatment? Please circle your answer box.

1. Dizziness…… YES(1) NO (0)

a) How often did you experience this problem in one week? ONCE (0) TWICE (1) THRICE (2) MORE THAN THREE TIMES A WEEK(3)

b) Did it interfere with your daily activities/work? NEVER (0) SOMETIMES (1) MOST TIMES(2)

2. Difficulty sleeping…….. YES (1) NO(0)

a) How often did you experience this problem in one week? ONCE (0) TWICE (1) THRICE (2) MORE THAN THREE TIMES A WEEK(3)

b) Did it interfere with your daily activities/work? NEVER (0) SOMETIMES (1) MOST TIMES (2)

3. Headache…… YES(1) NO(0)

a) How often did you experience this problem in one week? ONCE (0) TWICE (1) THRICE (2) MORE THAN THREE TIMES A WEEK(3)

b) Did it interfere with your daily activities/work? NEVER (0) SOMETIMES (1) MOST TIMES (2)

4. Difficulty concentrating…….YES(1) NO(0)

a) How often did you experience this problem in one week? ONCE (0) TWICE (1) THRICE (2) MORE THAN THREE TIMES A WEEK(3)

b) Did it interfere with your daily activities/work? NEVER (0) SOMETIMES (1) MOST TIMES (2)

5. Scary dreams ……. YES(1) NO(0)

a) How often did you experience this problem in one week? ONCE (0) TWICE (1) THRICE (2) MORE THAN THREE TIMES A WEEK(3)

b) Did it interfere with your daily activities/work? NEVER (0) SOMETIMES (1) MOST TIMES(2)

6) Vivid dreams……..YES(1) NO(0)

a) How often did you experience this problem in one week? ONCE (0) TWICE (1) THRICE (2) MORE THAN THREE TIMES A WEEK(3)

b) Did it interfere with your daily activities/work? NEVER (0) SOMETIMES (1) MOST TIMES(2)

7) Confusion…… .. YES(1) NO (0)

a) How often did you experience this problem in one week? ONCE (0) TWICE (1) THRICE (2) MORE THAN THREE TIMES A WEEK(3)

b) Did it interfere with your daily activities/work? NEVER (0) SOMETIMES (1) MOST TIMES (2)

8) Did you feel sad/depressed (crying a lot, poor appetite, poor sleep) YES(1) NO(0)

a) How often did you experience this problem in one week? ONCE (0) TWICE (1) THRICE (2) MORE THAN THREE TIMES A WEEK(3)

b) Did it interfere with your daily activities/work? NEVER (0) SOMETIMES (1) MOST TIMES (2)

9) Nausea ……YES (1) NO(0)

a) How often did you experience this problem in one week? ONCE (0) TWICE (1) THRICE (2) MORE THAN THREE TIMES A WEEK(3)
b) Did it interfere with your daily activities/work? NEVER (0) SOMETIMES (1) MOST TIMES (2)

10) Vomitting……..YES(1) NO(0)

a) How often did you experience this problem in one week? ONCE (0) TWICE (1) THRICE (2) MORE THAN THREE TIMES A WEEK(3)

b) Did it interfere with your daily activities/work? NEVER (0) SOMETIMES (1) MOST TIMES (2)

11) Abdominal pain……..YES (1) NO(0)

a) How often did you experience this problem in one week? ONCE (0) TWICE (1) THRICE (2) MORE THAN THREE TIMES A WEEK(3)

b) Did it interfere with your daily activities/work? NEVER (0) SOMETIMES (1) MOST TIMES (2)

12) Rash……YES (1) NO(0)

15) Hallucinations (seeing things other people can't see) YES
NO

16) Suicidal tendencies requiring admission to hospital... YES
NO

17) Mental disturbances that required admission to hospital. YES
NO

18) Are you taking any other medication, herbal or traditional medicine YES
NO
If YES what are the names

19). Do you drink alcohol? YES NO
if yes how many drinks do you have a day 1 / 2 / 3 / 4/ more than 4

a"drink" is equivalent to…
• 340 ml beer (1 can of beer)
• 125 ml wine (half a normal glass, one wine glass)
• 25 ml spirits (whisky, brandy, etc.) (1 shot)
• 60 ml sherry
• 25 ml liqueur (1 shot)

Appendix to questionnaire
Points are allocated to each possible answer as indicated above
-No symptoms (0)
-Mild symptoms –with a total score of (1-12)
-Moderate symptoms- with a total score of (13-48)
-Severe symptoms-a score of >48 or the presence of hallucinations,
-suicidal tendencies or hospital admissions for mental disturbances

Appendix D: Article published in AIDS Research and Therapy

Appendix E: Poster presented at the IFCC conference –Brazil 2008

Correlation of Plasma Efavirenz levels with CNS related side effects in HIV-infected individuals

Email: verenagounden@yahoo.com

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[1]Department of Chemical Pathology,University of the Witatersrand Medical School, NHLS

Background

Efavirenz (EFV), a non- nucleoside reverse transcriptase inhibitor (NNRTI), forms part of the first line therapy for many HIV infected individuals in South Africa. Central nervous system (CNS) side effects have been reported in >50% of patients following commencement of EFV therapy. Therapeutic drug monitoring (TDM) has a role to play in monitoring drug related toxicity, patient adherance, drug-drug interactions and in patients with co-infections and hepatic disease. Previous studies have shown that plasma concentrations of EFV predict therapeutic efficacy and are related to the likelihood of developing adverse CNS effects. Patients with EFV concentrations of > 4000ug/l may experience neurological adverse effects more frequently whilst those with plasma concentrations < 1000ug/l appear to have a greater risk for emergence of selective drug resistance and treatment failure.

Aim

To investigate the relationship between the EFV plasma concentrations and the occurrence of CNS related side effects in HIV-infected patients.

Methods

Plasma samples from 100 patients attending the Anti-retroviral clinic at Johannesburg Hospital were used. EFV was analyzed using Ultra-performance liquid chromatography- tandem mass spectrometry (UPLC-MS/MS). A questionnaire eliciting the occurrence of CNS side effects was administered to all participating patients. Responses were scored in terms of number of episodes of side effects experienced and severity in terms of effect on daily activities. The presence of hallucinations or mental disturbances following initiation of EFV therapy was scored as the most severe side effects. Using their scores on the questionnaire patients were grouped into those with no side effects (Group1), with mild symptoms (Group2), with moderate symptoms (Group3) & with severe side effects (Group 4).

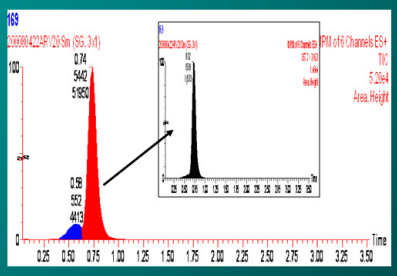


Figure 1. Total ion chromatogram of one of the samples run on UP LC-MS/MS

Results

Results	
Age (years)	Mean 36.9 ±8.4 (n=100)
Female	77
Male	23
CD4 count (x 10^6 l)	Mean 116±78
Viral load	Median 8300 (Range 0-3000000) (n=95)
Efavirenz levels	Median 3635 ug/l (Range 115 - 30222 ug/l) (n=100)

85% (n=100) experienced some form of adverse effect fg. initiation of HAART. Most individuals (53%) experienced mild symptoms

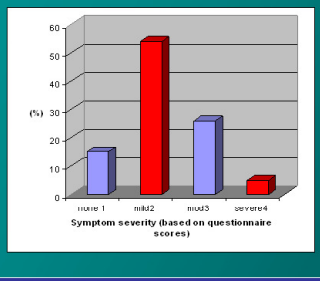


Figure 2. Graph illustrating the distribution of severity of EFV related side effects amongst study population

Those who experienced no side effects (Group 1) had a significantly lower mean plasma EFV concentration (2540.47 ug/l) vs the group which experienced the most severe side effects, Group 4 (mean EFV concentration 16874,89 ug/l), ($p < 0.05$).

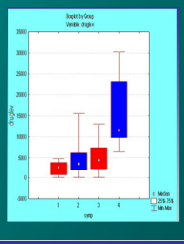


Figure 3. Illustrates the relationship between plasma EFV concentrations with the severity and incidence of EFV- related side effects

Conclusion

Higher EFV levels were associated with increased incidence & severity of CNS related side effects

References

1. Marzolini C, Telenti A et al. Efavirenz plasma levels can predict treatment failure and CNS side effects in HIV-1 infected patients. AIDS 2001; 15:71-75
2. Lopez-Cortez L, Alarcon A. Efavirenz plasma concentrations and efficacy. AIDS 2001;15:1192-1193
3. Hugen PWH, Burger DM, Aarnoutse RE, et al.. Therapeutic drug monitoring of HIV-Protease inhibitors to assess non-compliance. Therapeutic Drug Monitoring **2002**; 24: 259-287

Appendix F. Abstracts: South African Pathology Congress, Cape Town 19-21 July 2008

Abstract 1:
Plasma Efavirenz concentrations and CNS side effects in HIV-infected individuals.

V. Gounden[1], T. Snyman[1], J George[1]
[1]Department of Chemical Pathology, University of Witwatersrand, NHLS

Introduction: Efavirenz (EFV), a non- nucleoside reverse transcriptase inhibitor (NNRTI), forms part of the first line therapy for many HIV infected individuals in South Africa. Central nervous system side effects have been reported in >50% of patients following commencement of EFV. Therapeutic drug monitoring (TDM) has a role to play in monitoring drug related toxicity, patient adherence, drug-drug interactions and in patients with co-infections and hepatic disease. Studies have shown that plasma concentrations of Efavirenz predict therapeutic efficacy and are related to the likelihood of developing adverse CNS effects.
Aim: To investigate the relationship between the Efavirenz (EFV) plasma concentrations and the occurrence of CNS related side effects in patients that are compliant.
Methods: Plasma samples were collected from 100 patients at the Johannesburg Hospital ARV clinic. All patients received the standard dose of EFV 600 mg nightly. Specimens were collected 2-4 weeks after initiating EFV therapy. Samples were taken the morning after the evening dose of EFV. They were then stored at – 70^{0} C until analysis by UPLC-MS/MS. A questionnaire eliciting the occurrence of CNS side effects was administered to all participating patients. Responses were scored in terms of number of episodes of side effects experienced and severity in terms of effect on daily activities. Patients were also seen by a doctor during this visit to assess neuropsychological side effects.
Results: EFV concentration of samples ranged from 94,65 ug/l to 15581,5 ug/l. The mean plasma EFV concentration of the patient group with severe side effects was greater than the mean EFV levels in the patients with less severe side effects.
Conclusion: Higher plasma EFV levels are associated with increased incidence & severity of CNS side effects.

Abstract 2:

A rapid method of measuring plasma Efavirenz levels in HIV- infected individuals using LC–MS/MS methodology

V. Gounden[1], J. George[1], T. Snyman[1]
[1]Department of Chemical Pathology, University of Witwatersrand, NHLS

Introduction: In many countries Therapeutic Drug Monitoring (TDM) of antiretrovirals have become part of the national guidelines in the management of HIV-infected individuals. Although not yet instituted in South Africa, TDM of ARVs will assist in both monitoring of drug toxicity and patient compliance. Increased plasma levels of Efavirenz (EFV) have been associated with serious CNS side effects. Patients with efavirenz concentrations of > 4000ug/l may experience neurological adverse effects more frequently whilst those with plasma concentrations < 1000ug/l appear to have a greater risk for emergence of selective drug resistance and treatment failure
Aim: To develop and validate a rapid method for determination of EFV in plasma to enable monitoring of drug levels in HIV-infected patients.
Methods: Plasma samples from 100 patients attending the ARV clinic were collected the morning after the last dose of EFV (2-4 weeks after initiating therapy). EFV was analyzed by UPLC-MS/MS. Samples were extracted using solid phase Weak Cation Exchange cartridges (WCX, Oasis-Microsep). The samples were run on a Acquity 1.7um 2.1 x 50mm phenyl column using a gradient of A: 2 mM ammonium acetate:100% acetonitrile(ACN) in a ratio of 70:30 B:100% ACN returning to A (70:30) after 1.5 min. The MRM transition used for EFV was *m/z* 357.7.>316.3. The retention time was 0.72 min with total run time of 2 min. Validation of the method was done by EP10 protocol & using specific performance parameters
Results: The limit of detection was 56 ug/l. Sample concentrations ranged from 94,65 ug/l to 15581,5 ug/l. Mean sample concentration was 4180.3 ug/l. The method has a CV of less than 15%.
Conclusion:UPLC-MS/MS is a rapid method for analysis of plasma EFV concentrations

Appendix G. Ethics Clearance Certificate

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 George

CLEARANCE CERTIFICATE	**PROTOCOL NUMBER M050824**
PROJECT	Cytochrome P450 and the Multidrug-resistance transporter (MDR1) as a Predicator of Toxicity and Clinical Response
INVESTIG ATORS	Dr J George
DEPARTMENT	Dept of Chemical Pathology
DATE CONSIDERED	05.08.26
DECISION OF THE COMMITTEE*	Approved unconditionally

Unless otherwise specified this ethical clearance is valid for 5 years and may be renewed upon application.

DATE 05.08.24 **CHAIRPERSON** .....
(Professor PE Cleaton-Jones)

*Guidelines for written 'informed consent' attached where applicable

cc: Supervisor :

DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and **ONE COPY** returned to the Secretary at Room 10005, 10th Floor, Senate House, University.
I/We fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee. **I agree to a completion of a yearly progress report.**

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

REFERENCES

1. UNAIDS 2008 Report on the global AIDS epidemic, August 2008. Available at: http://www.unaids.org/en/KnowledgeCentre/HIVData/GlobalReport/2008/2008_Global_report.asp. Accessed April 11 2009.

2. Coetzee D, Hildebrand K, Boulle A *et al.* Outcomes after two years of providing antiretroviral treatment in Khayelitsha, South Africa. *AIDS* 2004; 18: 887-895.

3. Flexner D. Advances in HIV Pharmacology: Protein Binding, Pharmacogenomics, and Therapeutic Drug Monitoring. *Top in HIV Med* 2003; 11: 40-44.

4. Website: John Hopkins University HIV Guide http://www.hopkinsaids.edu/management/antiretroviral_therapy/full_therapeutic_drug_monitoring.html. Accessed on 8 July 2009.

5. Aarnoutse RE, Schapiro JM, Boucher CA *et al***.** Therapeutic drug monitoring: an aid to optimising response to antiretroviral drugs? *Drugs* 2003; 63: 741-753**.**

6. Ward BA, Gorski JC, Jones DR, Hall SD *et al*. The cytochrome P450 2B6 (CYP2B6) is the main catalyst of efavirenz primary and secondary metabolism:

implication for HIV/AIDS therapy and utility of efavirenz as a substrate marker of CYP2B6 catalytic activity. *J Pharmacol Exp Therapy* 2003; 306: 287-300.

7. Website:http://www.rxlist.com/sustiva-drug.htm (Drug manufacturer's information sheet) Accessed 14 June 2009.

8. Yamano S, Nhambura PA *et al*. cDNA cloning and sequence and cDNA-directed expression of human P450 iiB1. *Biochemistr.* 1989; 28: 7340-7348.

9. Haas D, Ribaudo HJ, Kim RB, Camlin T *et al*. Pharmacogenetics of efavirenz and central nervous system side effects: an Adult AIDS Clinical Trials Group study. *AIDS*; 2004; 18: 2391-2400.

10. Marzolini C, Telenti A, Decosterd LA *et al*. Efavirenz plasma levels can predict treatment failure and central nervous system side effects in HIV-1 infected patients. *AIDS* 2001; 15: 71-75.

11. Lochet P, Peyriere H, Lotthe A *et al*. Long-term assessment of neuropsychiatric adverse reactions associated with efavirenz. *HIV Med* 2003; 4: 62-66.

12 . Fumaz CR, Munoz-Moreno BS, Molto J *et al*. Long-term Neuropsychiatric disorders on Efavirenz based approaches: Quality of life, Psychological issues and adherence. *JAIDS* 2005; 38: 560-565.

13. Welch S, Sharland M, Lyall EG *et al*. PENTA 2009 guidelines for the use of antiretroviral therapy in paediatric HIV-1 infection. *HIV Med* 2009; 10: 591-613.

14. Radner DB. Toxicologic and pharmacological aspects of rifampin. *Chest* 1973; 64: 2123-6.

15. López-Cortẽz LF, Ruiz-Valderas R, Viciana P *et al*. Pharmacokinetic interactions between efavirenz and rifampicin in HIV-infected patients with tuberculosis. *Clin Pharmacokinet* 2002; 41: 681-90.

16. Frieland G, Khoo S, Jack C *et al*. Administration of efavirenz (600 mg/day) with rifampicin results in highly variable levels but excellent clinical outcomes in patients treated for tuberculosis and HIV. *J of Antimicrob Chemother* 2006; 58: 1299-1302.

17. Back D, Gibbons S and Khoo S. Pharmacokinetic drug interactions with nevirapine. *JAIDS* 2003**;** 34: S8-14.

18. Rotger M, Tegude H, Colombo S *et al*. Predictive Value of Known and Novel Alleles of CYP2B6 for Efavirenz Plasma Concentrations in HIV-infected Individuals. *Clinl Pharm Therap* 2007; 81: 557-566.

19. Annesley T, Rockwood L and Sherman N. Mass spectrometry. In *Tietz Textbook of Clinical Chemistry and Molecular Diagnostics 4th edition*. Edited by Carl A Burtis. Philadelphia : Elsevier Saunders; 2006. pp 165-182

20. Website: www.waters.com/waters/nav.htm?locale=en_ZA&cid=10048920, Accessed on 12 Novermber 2009.

21. Website: www.ncbi.nlm.nih.gov/sites/entrez?Db=gene&Cmd , Accessed 15 November 2009.

22. Wang J, Sonnenerborg A, Rane A *et al*. Identification of a novel specific CYP2B6 allele in Africans causing impaired metabolism of the HIV drug efavirenz. *Pharmacogenet Genomics* 2006; 16:191-198.

23. Klein K, Lang T, Saussele T *et al*. Genetic variability of CYP2B6 in populations of African and Asian origin: allele frequencies, novel functional variants, and possible

implications for anti-HIV therapy efavirenz. *Pharmacogenet Genomics* 2005; 15: 861-8739.

24. Mehlotra RK, Ziats MN, Bockarie MJ *et al*. Prevalence of CYP2B6 alleles in malaria-endemic populations of West Africa and Papua New Guinea *Eur J Clin Pharm* 2006; 62: 267–275.

25. Nyakutira C, Röshammar D, Chigutsa E *et al*. High prevalence of the CYP2B6 516G→T(*6) variant and effect on the population pharmacokinetics of efavirenz in HIV/AIDS outpatients in Zimbabwe. *Eur J Clin Pharm* 2008; 64:357–365.

26. Ferrerira PE, Veiga MI, Cavaco I *et al*. Polymorphism of antimalaria drug metabolising nuclear receptor and drug transport genes among Malaria Patients in Zanzibar, East Africa. *Ther Drug Monit* 2008:30; 10-15.

27. Ofotokun I. Sex differences in the Pharmacologic Effects of Anteretroviral Drugs: Potential Roles of Drug Transporters and Phase 1 and 2 Metabolizing Enzymes. *AIDS* 2005;13:79-83.

28. Pernerstorfer-Schoen H, Jilma B, Perschler A *et al*. Sex differences in HAART-associated dyslipidaemia. *AIDS* 2001; 15: 725-734.

29. Moore AL, Mocroft A, Madge S *et al*. Gender differences in virologic response to treatment in HIV-positive population: a cohort study. *JAIDS* 2002; 29: 197-202.

30. Lamba V, Lamba J, Yasuda K *et al*. Hepatic CYP2B6 expression: Gender and ethnic differences and relationship to CYP2B6 genotype and CAR expression. *J Pharm Exper Therap* 2003; 307: 906-922.

31. Sheffield LJ and Phillimore HE. Clinical Use of Pharmacogenomic Tests in 2009. *Clin Biochem Rev* 2009; 30: 55-65.

32. Wittwer C and Kusukawa N. Nucleic acid Techniques. In *Tietz Textbook of Clinical Chemistry and Molecular Diagnostics 4th edition*. Edited by Carl A Burtis. Philadelphia : Elsevier Saunders; 2006. pp 1411-1417.

33. DNA Sequencing by Capillary Electrophoresis. Applied Biosystems Chemsitry Guide. 2nd edition. Website www.appliedbiosystems.com. Accessed on 26 November 2009.

34. Corona G and Toffoli G. High throughput screening of genetic polymorphisms by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. *Comb Chem High Throughput Screen*. 2004: 7:707-25.

35. The Royal College of Psychiatrists, Alcohol related disorders. Available at: www.rcpsych.ac.uk/default.aspx?page. Accessed 6 March 2007

36. American National Institute of Health, Division for Acquired Immune Deficiency Syndrome Toxicity Grading, August 2005. Available at: http://www.fda.gov/OHRMS/DOCKETS/dockets/05d0155/05D-0155_emc-000002-02.pdf Accessed on 6 June 2008.

37. Glencross D, Scott LE, Jani IV *et al*. CD45-assisted panleucogating for accurate, cost effective dual platform CD4+ T-cell enumeration. *Cytometry* 2002; 50: 69-77.

38. Rabel SR, Patel M, Sun S *et al*. Electronic and Resonance Effects on the Ionization of Structural Analogues of Efavirenz. *AAPS PharmSci*. 2001; 3 : article 28.

39. Krouwer JS, Preliminary Evaluation of Quantitative Clinical Laboratory Methods, Approved Guideline - EP10A3, 3rd edition , CLSI/NCCLS 2006.

Website: www.clsi.org. Accessed 10 May 2007.

40. Clifford D, Evans S, Yang Y *et al*. Impact of Efavirenz on Neuropsychiatric Performance and Symptoms in HIV-infected individuals. *Ann Intern Med* 2005; 143: 714-721.

41. Website : http://www.nature.com/scitable/definition/Hardy-Weinberg-equilibrium-. Accessed 11 March 2010.

42. Fernandez E, Pare L, Ajuria I *et al*. State of the art in therapeutic drug monitoring. Clin Chem Lab Med 2010; 48 *Printed ahead of press.*

43. Yeni P, Hammer S, Hirsch M *et al*. Treatment for adult HIV infection: 2004 recommendations of the International AIDS society – USA panel. *JAMA* 2004;292:251-265

44. Smith PF, DiCenzo R, and Morse GD. Clinical pharmacokinetics of nonnucleoside reverse transcriptase inhibitors. *Clin Pharmacokinet* 2001; 40:893-905.