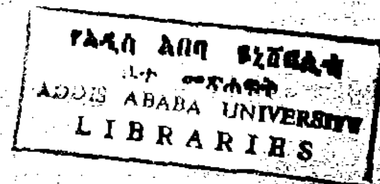
PHARMACOGENETIC AND MOLECULAR BIOLOGICAL ANALYSIS OF DRUG METABOLIZING ENZYMES, IN PARTICULAR, CYTOCHROMES P4502D6 (CYP2D6) AND P4502C19 (CYP2C19) IN AN ETHIOPIAN POPULATION

A THESIS PRESENTED TO
THE SCHOOL OF GRADUATE STUDIES
ADDIS ABABA UNIVERSITY



ADDIS ABABA UNIVERSITY
LIBRARIES

IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE
OF MASTER OF SCIENCE IN BIOCHEMISTRY

By

ELENI AKLILLU

1995

PHARMACOGENETIC AND MOLECULAR BIOLOGICAL ANALYSIS OF DRUG METABOLIZING ENZYMES, IN PARTICULAR, CYTOCHROMES P4502D6 (CYP2D6) AND P4502C19 (CYP2C19) IN AN ETHIOPIAN POPULATION

A THESIS PRESENTED TO
THE SCHOOL OF GRADUATE STUDIES
ADDIS ABABA UNIVERSITY

IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE
OF MASTER OF SCIENCE IN BIOCHEMISTRY

By

ELENI AKLILLU

1995

DEDICATED

To my family

ADDIS ABABA UNIVERSITY

ACKNOWLEDGEMENTS

The work presented in this thesis has been carried out at three places:

i. Department of Biochemistry, Medical Faculty, Addis Ababa University, Addis Ababa, Ethiopia.

ii. Berzelius laboratory–branch of the Department of Medical Biochemistry and Biophysics, Karolinska Institute, Stockholm, Sweden.

iii. Department of Medical Laboratory Sciences and Technology, Division of Clinical Pharmacology, Karolinska Institute, Huddinge University Hospital, Huddinge, Sweden.

None of the work would have been done without the support of all my teachers, colleagues, my family and all my friends. To these people I am greatly indebted.

My sincere gratitude goes to the Department of Biochemistry of the Medical Faculty, Addis Ababa University, for allowing me enrol on the Bio–Medical Research and Training Project (BRTP) and to Dr Yesehak Worku, Head, Department of Biochemistry for administratively facilitating everything my research work.

My heartfelt thanks is extended to my supervisors: 1. Dr. Frederick K. Rodrigues, WHO Consultant–Biochemist attached to the Faculty, for his valuable assistance in many ways. He put excellent facilities, including his personal computer, at my disposal for the preparation of the thesis. I am very grateful for his exciting and fruitful guidance of my research work and for his moral support and encouragement. 2. Professor Magnus Ingelman–Sundberg for his stimulating, enthusiastic discussions about the project and of the results of the research work. His broad knowledge in the field helped me in a constructive manner. He offered me an optimal research environment in his laboratory.

I am equally very much grateful to the following people: 1. Dr. Leif Bertilsson for allowing me do the phenotype analysis in his laboratory at Huddinge Hospital and for introducing me to the Gas Chromatography technique. He provided me with a fruitful discussion of my results. 2. Dr.Irene Persson, for introducing me to the molecular biology techniques. She was always willing and helpful both socially and academically. She is a real friend who made me feel at home while I was in Sweden. The pleasant time we had during the 1994 Christmas at Harnosand shall never be forgotten. 3. Laboratory technicians of the Department of Biochemistry, Ato Negash Tegene, Ato Mamo Fanta for helping me withdraw blood from volunteers. 4. Dr. Mikael Holst and Dr. Irene Persson for facilitating my work and providing me with pleasant friendly entertainments, especially, our ski training at Idre and Stockholm by night excursion which will always be remembered. 5. Staff of MIS laboratory for creating a friendly working atmosphere and for making my stay a pleasant and memorable one. The memory of the weekly Friday seminar at Magnus cave will last long.

I am equally grateful to W/o Tejetu Degefa, my mother and Ato Biratu Oljira, my husband, for all their love, moral support and especially for taking good care of the children for a long time. My daughters, Lenssa and Maria deserve special mention for their sweet love and for allowing me concentrate on my work at times that I normally would have been playing with them.

The laboratory staff and the Director of the Armauer Hansen Research Institute, Addis Ababa are not forgotten. They helped greatly by allowing me borrow chemicals and enzymes whenever the need arose. To them I say a big thank you.

Finally I would like to extend my thanks to all volunteers, staff of the Medical Faculty, my friends and colleagues for their cooperation. Without them only some of this would have been possible. I was sponsored by the Ministry of Heath, Ethiopia. This work was supported by grants from the Swedish Agency for Research Cooperation with Developing Countries (SAREC). I am indebted to them.

LIST OF FIGURES

Page

Figure 1. Schematic representation of the known variants of the *CYP2D6* locus detected by RFLP. E and X indicate the cleavage sites with the restriction endonuclease *Eco*RI and *Xba*I respectively 11

Figure 2. Diagrammatic representation of the major pathways of Debrisoquine metabolism by CYP2D6 14

Figure 3. Frequency distribution of debrisoquine metabolic ratio in Chinese and Swedish populations 18

Figure 4. Frequency distribution of debrisoquine metabolic ratio in Black populations and in Caucasians 20

Figure 5. Stereoselective metabolism of (S)– and (R)–mephenytoin by 4–hydroxylation and N–demethylation, respectively 22

Figure 6. Schematic representation of *CYP2D6* allele specific PCR–1 amplification of DNA fragments 34

Figure 7. Schematic illustration of strategy used to analyze *CYP2C19m1* and *CYP2C19m2* 39

Figure 8. Frequency distribution of debrisoquine UMR among 115 healthy Ethiopians 41

Figure 9. *Xba*I RFLP autoradiograph of the *CYP2D* locus of genomic DNA from some Ethiopian subjects showing the different haplotypes 43

Figure 10 Frequency distribution of debrisoquine urinary MR for the various *CYP2D6* haplotypes as detected by PCR and RFLP 47

Figure 11 *CYP2D6A* allele specific amplification of DNA (PCR–2) 49

LIST OF TABLES

Page

Table 1. List of drugs metabolized by CYP2D6 7

Table 2. Percentage of CYP2D6 Poor Metabolisers (PMs) among different populations 16

Table 3. List of drugs metabolized by CYP2C19 25

Table 4a. Oligonucleotide sequence of primers used in PCR for the analysis of ***CYP2D6*** genotype 32

Table 4b. Oligonucleotide sequence of primers used in PCR for the analysis of ***CYP2C19*** genotypes 33

Table 5. Frequency of ***CYP2D6*** haplotypes detected by *Xba*I RFLP and verified by *Eco*RI RFLP 44

Table 6. *Xba*I RFLP genotype of individuals with metabolic ratio less than 0.2 45

Table 7. Summary of *Xba*I RFLP genotypes in Ethiopian EMs 48

Table 8. Frequency of ***CYP2D6*** mutant alleles detected by PCR 53

Table 9. Summary of ***CYP2C19m1*** genotype analysis 56

Figure 12 *CYP2D6B* allele specific amplification of DNA (PCR-2) 50

Figure 13 Debrisoquine MR frequency distribution with respect to *CYP2D6* exon 1 mutation 52

Figure 14 Frequency distribution of *CYP2C19* genotype within the Ethiopian population in relation to S/R-mephenytoin ratio 54

Figure 15 *Sma*I and *Bam*HI Cleavage pattern of PCR amplified DNA from the 6 poor metabolizers of S-Mephenytoin for the analysis of *CYP2C19m1* and *CYP2C19m2* respectively 57

ABBREVIATIONS

AcO_2H	:Acetic acid.
$AgNO_3$	:Silver nitrate
AmAc	:Ammonium acetate
APS	:Ammonium persulphate
CS_2	:Carbon disulfide
CYP2D6	:Cytochrome P4502D6
CYP2C19	:Cytochrome P4502C19
DNA	:Deoxyribonucleic acid
dNTP	:Deoxynucleotide triphosphate
EDTA	:Ethylene diamine tetraacetate
~~EM~~	~~:Extensive metabolizer~~
EtOH	:Ethanol
ER	:Endoplasmic reticulum
GC	:Gas chromatography
GT	:Guanidine-isothiocynate
HCl	:Hydrochloric acid
$MgCl_2$	:Magnesium chloride
MeOH	:Methanol
ml	:Milliliter
μl	:Microliter
NaAc	:Sodium acetate
NaCl	:Sodium chloride
$NaHCO_3$	:Sodium bicarbonate
NaOH	:Sodium hydroxide
nM	:Nanomolar
ng	:Nanogram
PCR	:Polymerase Chain Reaction
PEH	:5-phenyl-5-ethylhydantoin
PM	:Poor metabolizer
RFLP	:Restriction Fragment Length Polymorphism
SDS	:Sodium Dodecyl Sulphate
S-MP	:S-Mephenytoin
SSC	:Standard Saline Citrate
SSCP	:Single Stranded Conformation Polymorphism.
ssDNA	:Salmon sperm DNA
TAE	:Tris Acetate EDTA
TBE	:Tris Borate EDTA
TE	:Tris EDTA solution
TEMED	:N,N,N',N'-tetra-methylenediamine.
UV	:Ultra violet light
$Na_2S_2O_3$	:Sodium thiosulphate
Na_2CO_3	:Sodium carbonate

LIST OF APPENDICES

Page

I. Information to volunteers 66

II. Isolation and purification of genomic DNA from whole blood 68

III. Cleavage of genomic DNA with restriction endonucleases *Eco*RI or *Xba*I. 70

IV. Southern blotting of DNA. 71

V. Preparation and ^{32}P-labelling of ***CYP2D6*** cDNA probe. 73

VI. SSCP analysis of *CYP2D6C* 74

- Solutions used for silver staining
- Silver staining of polyacrylamide gel

VII. Extraction of debrisoquine and 4-OH-debrisoquine from urine samples. 77

ABSTRACT

Debrisoquine and S-mephenytoin hydroxylation polymorphism was studied in 115 healthy unrelated Ethiopian volunteers after coadministration of debrisoquine (10mg) with S-mephenytoin (100mg). The ratio of the S to the R enantiomers of mephenytoin and of debrisoquine to 4-OH debrisoquine in 0-8 hr urine samples were determined using GC. Debrisoquine and S-mephenytoin are metabolized by CYP2D6 and CYP2C19 respectively. *CYP2D6* and *CYP2C19* show genetic polymorphism, dividing the population into Extensive metabolizers (EMs) and Poor metabolizers (PMs). The frequency of the PM phenotype is a subject of a pronounced inter-ethnic difference.

Two subjects (1.7%) are classified as a PMs (MR >12.6) with respect to CYP2D6. The debrisoquine MRs are trimodally distributed with a greater part of the population located in the MR interval of 1 - 10. The low prevalence of PMs is due to a lower frequency of a defective *CYP2D6* allele. Using a combination of RFLP, SSCP and PCR, subjects were genotyped for the known mutations in the *CYP2D6* locus. Using *Xba*I and *Eco*RI RFLP, 11.5kb, 44kb and 42kb haplotypes associated with gene deletion, defective *CYP2D* locus and gene duplication respectively were detected. Defective *CYP2D6A*, *CYP2D6B* and exon 1 mutations were detected using PCR. SSCP and PCR were used to genotype the *CYP2D6C* mutations.

No individual had either the *CYP2D6A* or the *CYP2D6C* allele. The allele frequencies for *CYP2D6B*, *CYP2D6D* and exon 1 mutation was 1.3%, 3% and 9.8% respectively. The allele frequency of the duplicated *CYP2D6* gene (42 kb allele) is significantly higher (12.9%) among Ethiopians than in any other population studied. Furthermore, the RFLP analysis indicated the existence of alleles carrying 3, 4 and even 5 active *CYP2D6* genes in tandem.

For S-mephenytoin hydroxylase (CYP2C19), six subjects (5.3%) with S/R ratio greater than 0.9 phenotyped as PMs. Three of them were homozygous (*CYP2C19m1/CYP2C19m1*) and the rest heterozygous (*CYP2C19m1/CYP2C19m2*) for mutated allele of *CYP2C19*. *CYP2C19m1* and *CYP2C19m2* account for 75% and 25% of defective alleles respectively in PMs. These results indicate that *CYP2C19* genotyping analysis for both *CYP2C19m1* and *CYP2C19m2*, enables one to apparently predict about 100% of the phenotypes.

TABLE OF CONTENTS.

PAGE

DEDICATION . . . . *i*

ACKNOWLEDGEMENT . . . . *ii*

LIST OF TABLES . . . . *iv*

LIST OF FIGURES . . . . *v*

LIST OF ABBREVIATIONS . . . . *vii*

LIST OF APPENDICES . . . . *viii*

ABSTRACT . . . . *ix*

1. INTRODUCTION AND LITERATURE REVIEW 1

1.1. THE CYTOCHROME P450 SYSTEM 2

1.1.1. STRUCTURE OF CYTOCHROME P450 3
1.1.2. NOMENCLATURE OF CYTOCHROME P450 4

1.2. DEBRISOQUINE HYDROXYLASE (CYP2D6) 6

1.2.1. *CYP2D6* GENE STRUCTURE 6
1.2.2. MUTANT ALLELES OF *CYP2D6* 8
1.2.3. *CYP2D6* GENETIC POLYMORPHISM 13

1.3. S-MEPHENYTOIN HYDROXYLASE (CYP2C19) 19

1.3.1. MUTANT ALLELES OF *CYP2C19* 23
1.3.2. *CYP2C19* GENETIC POLYMORPHISM 24

2. AIMS OF THE STUDY 27

3. SIGNIFICANCE OF THE STUDY 27

4. MATERIALS AND METHODS 29

4.1. SUBJECTS 29

4.2. CHEMICALS 30

TABLE OF CONTENTS.

PAGE

4.3. METHODS 30

4.3.1 GENOTYPING OF *CYP2D6* 30

(i) Extraction of genomic DNA from whole blood 30
(ii) Restriction Fragment Length Polymorphism (RFLP) analysis 30
(iii) Polymerase Chain Reaction (PCR)-based allele specific analysis 31

(a) Identification of *CYP2D6A* and *CYP2D6B* alleles 31
(b) Identification of C_{188}-->T mutation in exon 1 35
(c) Identification of the *CYP2D6C* mutation 36

4.3.2 CYP2D6 Phenotype analysis 36
4.3.3 GENOTYPING OF *CYP2C19* 37
4.3.4 PHENOTYPING OF CYP2C19 38

5. RESULTS 40

5.1. CYP2D6 PHENOTYPE 40
5.2. *CYP2D6* GENOTYPE 42

(i) RFLP analysis of *CYP2D6* 42
(ii) PCR-based analysis of *CYP2D6A* and *CYP2D6B* 46
(iii) PCR and SSCP analysis of *CYP2D6C* 46

5.3. CYP2C19 PHENOTYPE 51
5.4. *CYP2C19* GENOTYPE 55

6. DISCUSSION 55

7. CONCLUSION 64

8. APPENDICES: DETAILS OF METHODOLOGICAL PROCEDURES . 66

1. INTRODUCTION AND LITERATURE REVIEW

In animals drug metabolizing enzymes are responsible for the detoxification and excretion of foreign chemicals. Many foreign compounds, such as drugs, plant metabolites and environmental pollutants are non polar, dissolve readily in lipids but remain essentially insoluble in water. The principal function of drug metabolizing enzymes is to convert these chemicals into water soluble products that could easily be excreted in the urine or bile.

This biotransformation process occurs in two steps: In the first step, (also called phase I), chemical modification(s) of the parent compounds take(s) place. The modification(s) usually involving insertion of polar groups onto non polar parent compounds by such mechanisms as oxidation, hydroxylation, reduction, hydrolysis...etc, take place. In other cases, it may mean splitting off of alkyl groups from the parent compounds. Invariably, this results in increased hydrophilicity and facilitates excretion of the resulting products. Phase I reactions are catalyzed by different forms of cytochrome P450, having unique but, sometimes, overlapping substrate specificities that enable them to metabolize many different chemicals.

In the second step, (phase II), the polar groups of the intermediate metabolites arising from phase I are conjugated to highly water soluble molecules such as glucuronic acid, cysteine... etc, rendering the whole compound water soluble and consequently amenable to renal excretion. The latter process involves the action of various types of conjugating enzyme systems.

1.1. THE CYTOCHROME P450 SYSTEM.

Cytochrome P450 as we know it today was first described by Klingenberg and Garfinkle as a pigment found in the liver microsome (Klingenberg, 1958; Garfinkle, 1958). It was given the name cytochrome 'P450' by Omura and Sato, who observed that the reduced CO–bound pigment complex had an unusual absorption maximum at a wavelength of 450 nm (Omura and Sato, 1962).

Mammalian cytochrome P450 can be divided into two major categories: 1. P450s that are involved in the oxidative metabolism of endogenous substances such as fatty acids, prostaglandins, steroids ..etc, and 2. P450s, that are responsible for the metabolism of exogenous substances such as drugs, carcinogens environmental pollutants ..etc. The former class of P450 shows very high affinities for their substrates and are very well conserved within species. In contrast, members of the later category have a much lower substrate affinities and exhibit a relatively poor species structural conservation.

In general derivatives arising from biotransformation are pharmacodynamically less effective than the parent compounds from which they are derived (detoxification). Although cytochrome P450 enzymes play an important role in the detoxification of many drugs, chemical carcinogens and other toxic agents, it should be noted that they are also often responsible for catalyzing the metabolic activation of some substrates to highly reactive intermediates, which then attack cellular biomolecules, eventually initiating carcinogenic events. Numerous procarcinogens are known to require P450–dependent metabolic activation before they become carcinogenic (Nebert *et al.*, 1987).

1.1.1 STRUCTURE OF CYTOCHROME P450

Cytochrome P450s are membrane-bound heme containing enzymes containing about 500 amino acid residues. The mammalian liver microsomal enzyme system consists of two main components: cytochrome P450 itself and an NADPH-dependent cytochrome P450 reductase. It requires both molecular oxygen and the reduced form of the pyridine nucleotide, NADPH for its catalytic activity. Structurally all P450s are similar in the region around the heme cysteine forming the heme-thiolate. This accounts for the characteristic absorbance of the reduced CO-bound state of P450. Additionally the sequence, Phe-X-X-Gly-X-X-X-Cys-X-Gly (X denote any amino acid) close to the carboxy terminal is present in 202 out of 205 P450 enzymes sequenced (Nelson *et al.*, 1993). Cytochrome P450 functions as a monoxygenase, peroxidase, oxidase and reductase. It plays a critical role of binding substrate, activating oxygen and then catalyzing the insertion of the activated oxygen into the substrate. It thus determines the substrate specificity of the overall system as well as the structure of the product formed during metabolism.

Cytochrome P450 enzymes are synthesized on ribosomes associated with the endoplasmic reticulum (ER) and are co-translationally inserted into the ER membrane (Bar-Nun *et al.*, 1980). All microsomal P450 structures contain a very hydrophobic stretch of about 20 amino acid residues at their N-terminal, followed by one or two basic amino acid residues (Nelson *et al.*, 1988). This conserved character of the N-terminal serves as the signal sequence for the membrane insertion and halt-transfer which causes the rest of the P450 molecule to face the cytoplasmic side of the ER membrane. The

active site of P450s is part of a large cytoplasmic domain that may have one or two additional peripheral contacts (Black., 1992). The substrate binding site is not shielded by lipids, allowing only the substrate in the lipid phase to gain access to the binding site. Most substrates of P450 enzyme are largely hydrophobic (Imai *et al.*, 1967).

The active site of P450s contains an iron protoporphyrin IX moiety in a large relatively open hydrophobic cleft or depression in the surface of the protein. The heme is bound, apparently somewhat loosely, by a combination of hydrophobic forces and coordinate covalent bonds to the central metal ion. The iron is always penta– or hexa–coordinated, with four of the ligands contributed by the planar pyrrole nitrogens in the heme ring (White *et al.*, 1980). The 5th ligand, appears to be a thiolate anion contributed by a cysteine residue of the polypeptide chain while the 6th coordination position of the iron is occupied by an easily exchangeable ligand, perhaps water, in the native substrate free ferric state. Upon reduction of the iron, the 6th position becomes the site of dioxygene binding. Other diatomic ligands, such as carbon monoxide, nitric oxide and cyanide, alternatively occupy the 6th position under appropriate conditions.

1.1.2 NOMENCLATURE OF CYTOCHROME P450

Cytochrome P450s are considered as belonging to a super family. According to amino acid alignment studies, multiple isozymes of P450 are believed to have been derived from a common ancestral gene via divergent evolution (Gonzalez *et al.*, 1990). Several molecular mechanisms (gene duplication, gene conversion, gene amplification and

mutations) have been suggested to be involved in the evolution of P450 genes. This apparently resulted from co-evolution with plants that produced toxins in order to avoid being digested by animals. The development of new P450 genes under the conditions served therefore as a molecular defence mechanism.

Cytochrome P450s have been divided into gene families based on the amino acid sequence similarities of the protein molecules. P450 proteins having 40% or less amino acid sequence similarity are considered to belong to different families. Thus within a single gene family members have more than 40% amino acid sequence similarity. Each gene family is further subdivided into gene subfamilies. Generally, in mammals all members of the gene subfamily exhibit more than 55 % amino acid sequence similarity. It should be noted though that sequence similarity does not necessarily imply functional similarity. To date some 200 P450 genes and 7 putative pseudogenes have been identified in 27 gene families (Nebert *et al.*, 1991) among which 12 are found in mammals (Ingelman-Sundberg and Johansson, 1995).

Following the latest form of nomenclature (Nebert *et al.*, 1991) every P450 is named starting with the 'root' name CYP in the case of human (cyp for mouse), followed by an Arabic number denoting the family, a capital letter designating the subfamily (if any), and finally an Arabic number representing the individual gene within the subfamily. Accordingly CYP2D6 represents the human gene 6 belonging to family 2 and subfamily D. Under the nomenclature, italicized names, denote genes and cDNA and non-italicized names indicate the corresponding mRNA or protein. In most cases, all genes within a given family have the same number of exons and similar intron-exon boundaries. Also

genes within the same subfamily have been found to lie within the same cluster on the chromosome (Simmons *et al.*. 1983). P450 genes encoding proteins having greater than 97 % amino acid sequence similarity are considered as allelic variants of the same gene (Nebert *et al.*, 1987), except when there are differences in catalytic activity or when non-translated regions are clearly divergent: situations indicating distinct genes.

1.2. DEBRISOQUINE HYDROXYLASE (CYP2D6)

The inter-individual variability in drug metabolism has been shown to be caused by inter-individual variations in amount and/or activity of specific drug metabolizing enzymes. One important drug metabolizing enzyme which has specially pronounced variations in amount and activity between individuals is cytochrome P4502D6. The variations are governed mainly by genetic factors and are not significantly affected by environmental factors (Steiner *et al.*, 1985). CYP2D6 alone is responsible for the metabolism of more than 30 important drugs, including ß-receptor blockers, neuroleptics, tricyclic antidepressants, antiarrhythmics and some analgesics (table 1).

1.2.1 *CYP2D6* GENE STRUCTURE

CYP2D6 is localized on the long arm of chromosome 22 (Gonzalez, *et al.*, 1988). It contains 9 exons and has been completely sequenced (Gonzalez *et al.*, 1988). Two to three additional highly homologous pseudogenes are located upstream of ***CYP2D6*** within the *CYP2D* locus. Two of these have been sequenced and designated as ***CYP2D7P*** and ***CYP2D8P*** (Kimura *et al.*, 1989) with ***CYP2D7P*** located downstream of ***CYP2D8P***.

Table 1. List of selected drugs the metabolism, of which, is catalyzed by the **CYP2D6** enzyme. (Extracted from Dahl and Bertlisson., 1993; Kim Brosen., 1993).

BETA BLOCKERS	NEUROLEPTICS
Metoprolol	Haloperidol
Propranolol	Perphenazine
Timolol	Thioridazine
Bufarolol	Zuclopenthixol
Alprenolol	Remoxipride
ANTIARRYTHMICS	**ANTIDEPRESSANTS**
Encaimide	Amitriptyline
Flecainide	Clomipramine
Propafenone	Desipramine
Mexiletine	Fluoxetine
	Imipramine
	Nortriptyline
	Paroxetine
	Trimipramine
	Fluvoxamine
MISCELLANEOUS	**OPIATES**
Amiflamine	Codeine
Perhexiline	Dextromethorphan
Phenformin	Ethylmorphin
Tomoxetine	
Sparteine	
Guanoxan	

CYP2D8P contains several gene disrupting insertions, deletions and termination codons within its exons, while *CYP2D7P* is apparently normal except only for the presence in the first exon of an insertion that disrupts the reading frame. The three *CYP2D* genes display 92–97% nucleotide sequence similarities with each other across their introns and exons.

1.2.2 MUTANT ALLELES OF *CYP2D6*.

Mutations in *CYP2D6* cause enzyme variants with higher, lower or no activity. They may even lead to total absence of the enzyme molecule. Two molecular biological techniques are currently used to detect the different mutant alleles:

(a). RFLP detectable CYP2D6 variant alleles.

Restriction endonucleases recognize and cleave at specific nucleotide sequences. Since mutations affect the order of such sequences, some mutations are likely to create new restriction sites or eliminate existing ones. When the sizes of restriction fragments are altered this way it gives rise to Restriction Fragment Length Polymorphism (**RFLP**).

RFLP analysis of the *CYP2D* locus with restriction endonuclease *Xba*I frequently produces a 29kb fragment (either normal: D6–wt, or mutated) and two mutated *Xba*I alleles of 44kb, 11.5kb respectively (Skoda *et al.*, 1988). A 29kb *Xba*I fragment indicates the presence a normal locus with one copy of *CYP2D6* and two pseudogenes (*CYP2D7P* and *CYP2D8P*). The 44kb *Xba*I fragment has a gene insertion and contains 4 instead of three *CYP2D* genes (Heim and Meyer, 1992). In Caucasians, the 44kb allele contains 3

pseudogenes (*CYP2D8, CYP2D7A*, and *CYP2D7B*) and one *CYP2D6* gene containing a B type mutation, it is associated with the poor metabolizer (PM) phenotype. In contrast to Caucasians, the Chinese 44kb allele contains the two pseudogenes and two functional *CYP2D6* genes called, *CYP2D6ch1* and *CYP2D6ch2* (Johansson *et al.*,1994) and is not associated with the PM phenotype.

The allelic variant characterized by the 11.5kb *Xba*I fragment lacks the entire *CYP2D6* gene (*CYP2D6D*). It consists only of the two pseudogenes *CYP2D7P* and *CYP2D8P* (Gaedigk *et al.*, 1991). A *Xba*I 16 plus 9 kb DNA restriction fragment also identifies a mutant allele for debrisoquine hydroxylase that is associated with the PM phenotype (Evans *et al.*, 1990). Another allele of *CYP2D6* characterized by a 42kb *Xba*I fragment contains the two pseudogenes and two functionally active *CYP2D6* genes (*CYP2D6L1* and *CYP2D6L2*) in the *CYP2D* locus (Bertilsson *et al.*, 1993). The L allele contains a silent mutation in exon 3 and two point mutations; one in exon 6 yielding a Arg_{296}-->Cys exchange and the other in exon 9 causing Ser_{486}-->Thr (Johansson *et al.*, 1994). The resulting expression and catalytic properties of the encoded enzyme remains the same as that in the wild type gene, however it appears as if *CYP2D6L* has an intrinsic capacity for amplification.

Presence of two or more copies of an active gene in the *CYP2D6* locus, causing the expression of a higher amount of active enzyme is associated with ultrarapid metabolism (UM) of debrisoquine (Johansson *et al.*, 1993). The allele frequency of the 42kb *Xba*I is about 1% in the Swedish (Dahl *et al.*, 1995) but 7% in spanish populations (Agùndez *et al.*, 1995). Recently, a 54kb *Xba*I fragment indicating the presence of two

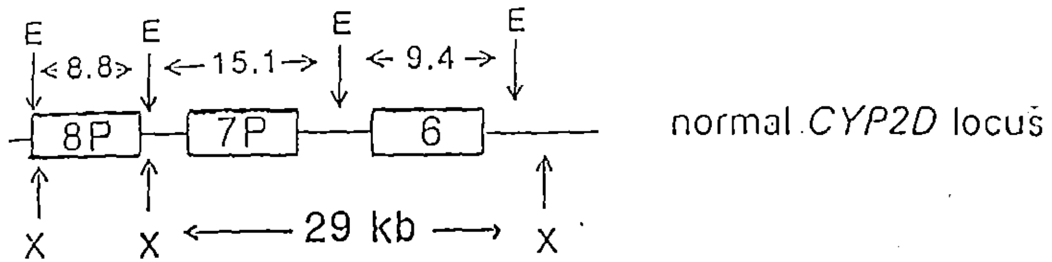
extra genes in the ***CYP2D6*** locus and associated with the ultra rapid metabolizer phenotype has been reported (Dhal *et al.*, 1995). Figure 1 shows the *Xba*I and *Eco*RI restriction sites for the known variants of the ***CYP2D6*** locus.

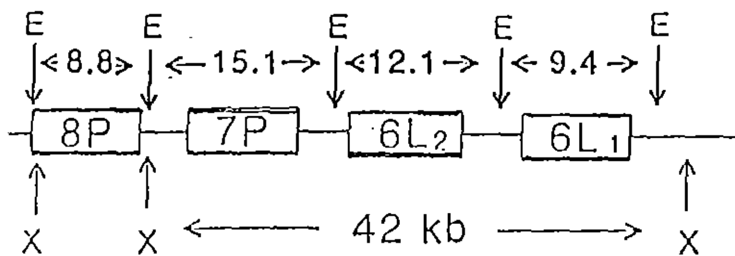
*Eco*RI digestion of the genomic DNA corresponding to the 29kb allele yields a 8.8 kb fragment (***CYP2D8P***), a 9.4kb fragment (***CYP2D6***) and a 15.1kb fragment (***CYP2D7P***). The 44kb allele contains 4 ***CYP2D*** genes in the ***CYP2D*** locus and its *Eco*RI digestion results in 4 fragment: 15.1 kb (***CYP2D7AP***), 13.7kb (***CYP2D7BP***), 9.4kb and 8.8kb. Similarly the 42kb allele contains 4 genes and upon *Eco*RI digestion gives 4 fragments: 15.1kb, 12.1kb (***CYP2D6L2***), 9.4kb (***CYP2D6L1***), and 8.8kb. The 12.1kb and 9.4kb fragments correspond to the two functional ***CYP2D6L*** genes. The allelic variant ***CYP2D6*** gene cluster characterized by the 11.5kb *Xba*I fragments lacking the entire ***CYP2D6*** gene gives *Eco*RI fragments of slightly shorter sizes: 13kb (***CYP2D7***) and 8.8 kb.

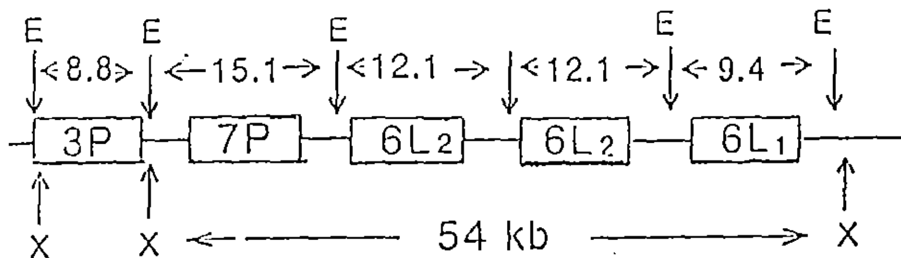
(b). PCR detectable *CYP2D6* alleles.

Allelic variants of ***CYP2D6*** that do not experience any significant change in fragment length are indistinguishable from the wild type gene by RFLP analysis. They include: ***CYP2DA*** which has a single base pair deletion (A_{2637}) in exon 5. The result is a reading frame disruption and premature termination of protein synthesis (Kagimoto *et al.*, 1990) and ***CYP2D6B*** that contains multiple mutations, notably four base changes in exons 1, 2, and, 9 causing amino acid substitutions, two silent mutations and more importantly a point mutation, G_{1934}->A, at the consensus sequence of the splice site of

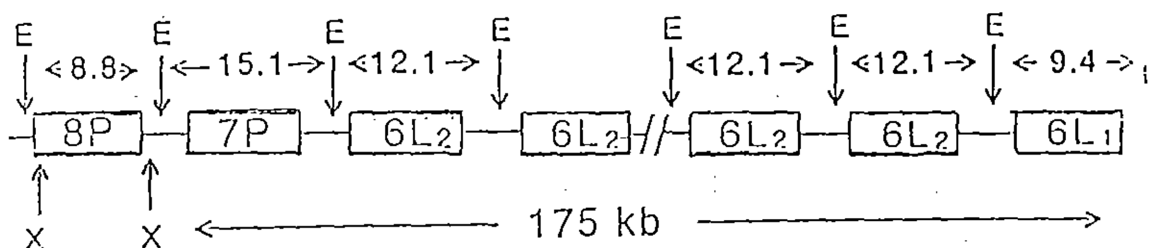
Figure 1. SCHEMATIC REPRESENTATION OF SOME KNOWN VARIANTS OF THE *CYP2D6* LOCUS DETECTED BY RFLP. E AND X DENOTE CLEAVAGE SITES OF RESTRICTION ENDONUCLEASES *Eco*RI AND *Xba*I RESPECTIVELY







Totally 12 2D6L$_2$



the 3rd intron and exon 4 (Hanioka *et al.*, 1990). This single base pair change alters the 3' consensus (AG) splice site in the 3rd intron of ***CYP2D6*** leading to an incorrectly spliced primary transcript and a mRNA having a single base deletion. The deletion presumably disrupts the reading frame and causes a prematurely terminated polypeptide. The aberrant splicing of 2D6 mRNA explains the absence of P4502D6 enzyme in the livers of poor metabolizers (Kagimoto *et al.*, 1990).

CYP2D6C contains a 3 base pair deletion between positions 2702 and 2706 which corresponds to the 3' end of the ***CYP2D6*** exon 5; the result is a mRNA that lacks a single codon and a protein lacking a single amino acid, Lys_{281} (Tyndale *et al.*, 1991). ***CYP2D6C*** is capable of producing an intact protein that has a slightly lower activity than the corresponding wild type normal enzyme, suggesting that ***CYP2D6*** does not cause a PM phenotype.

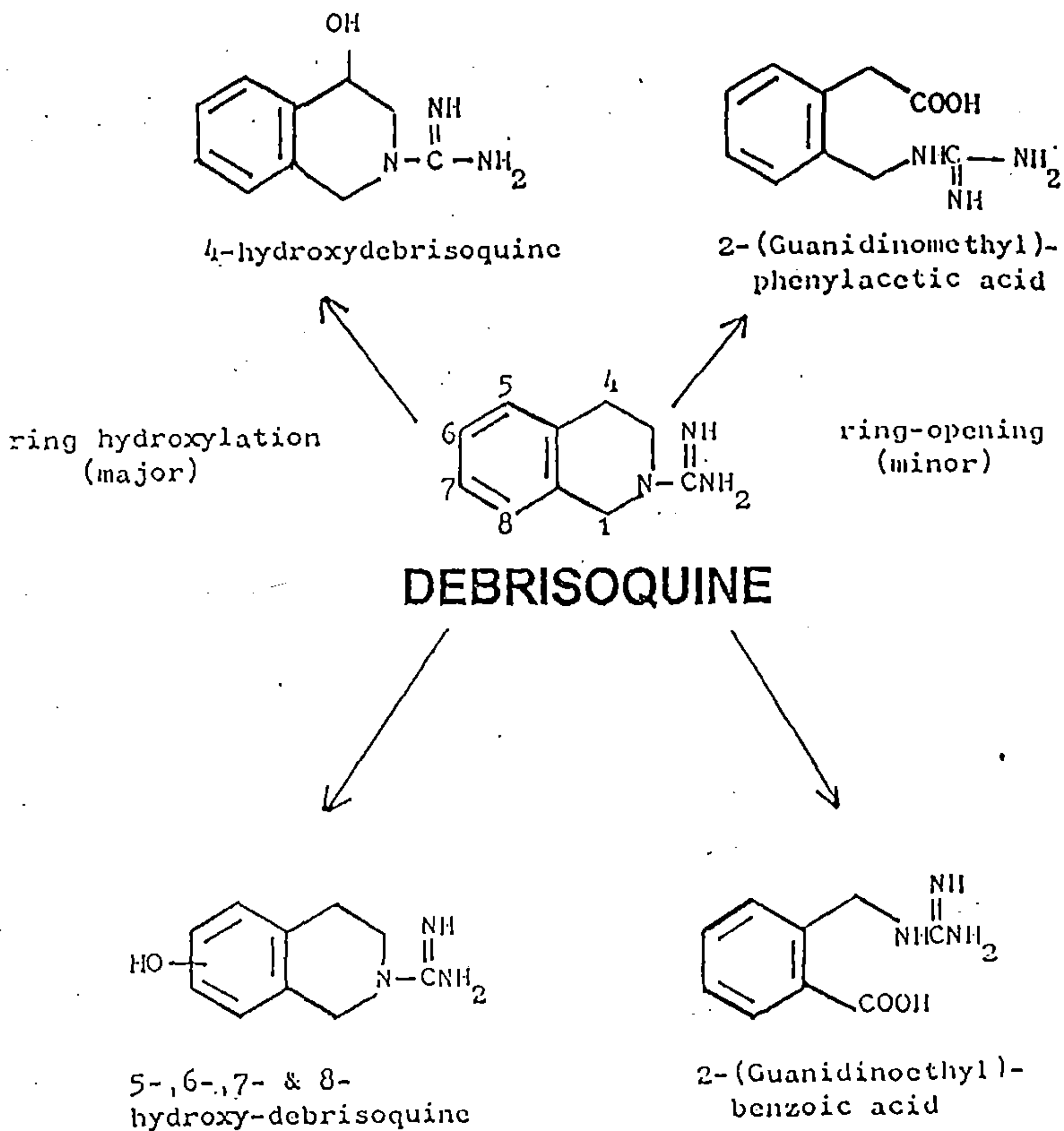
The **exon 1 mutation** is associated with a base change (C_{188}-->T) in exon 1. This causes, a Pro ---> Ser amino acid substitution in the highly conserved region consisting of Pro-Gly-Pro sequence of all P450s belonging to gene families 1 and 2 (Nelson *et al.*,1988.). The result of such amino acid substitution is the expression of an unstable gene product, a detrimental mutation responsible for the reduced activity of the ***CYP2D6ch*** genes (Johansson *et al.*, 1994).

1.2.3 *CYP2D6* GENETIC POLYMORPHISM.

The term genetic polymorphism defines a monogenic trait resulting in at least two phenotypes, neither of which is rare, that exist in a normal population. The rarest phenotype still occurs at a frequency of more than 1% (Vogel *et al.*, 1979).
Genetic polymorphism in drug metabolism gives rise to distinct populations differing in their abilities to perform a certain drug biotransformation reaction. Individuals who are able to metabolize the drug relatively rapidly are designated as extensive metabolizers (EM) while those who are deficient in metabolic capabilities are referred to as poor metabolizers (PM) (Eichelbaum, 1982).

Strikingly exaggerated responses or lack of effectiveness of drugs administered in the usual dosage have been observed as manifestations of inherited genetic polymorphism with respect to drug metabolism. The Debrisoquine/Sparteine type polymorphism probably represents the best-studied example of a genetic deficiency of a drug-metabolizing enzyme. This polymorphism was independently discovered by two groups (Mahgoub *et al.*, 1977: Eichelbaum *et al*, 1979). Figure 2 shows a diagrammatic representation of the major pathway of debrisoquine metabolism by CYP2D6. The PM phenotype which is monogenically inherited as an autosomal recessive trait (Evans *et al.*, 1980; Idle *et al.*, 1979) is due to complete absence or marked decrease in the amount or activity of the CYP2D6 enzyme in the liver (Zanger., 1988; Matsunaga., 1989). Clinical studies have indicated that the PMs are at a greater risk of developing concentration dependent adverse reactions to drugs administered at a routine dose (Eichelbaum *et al.*, 1990). It was also suggested that EMs may be more prone than PMs to develop cancer

Figure 2. DIAGRAMMATIC REPRESENTATION OF THE MAJOR AND MINOR PATHWAYS FOR THE METABOLIC DISPOSITION OF DEBRISOQUINE IN MAN BY CYP2D6



because they are better able to activate procarcinogens into active carcinogens by oxidation (Idle *et al*., 1981; Hirvonen *et al*., 1993), although this remains a controversial issue (Duche *et al*., 1991; Horsmans *et al*., 1991), inviting further investigation. Studies with different ethnic groups have demonstrated pronounced inter ethnic variations in the incidence of the PM phenotype (see table 2).

The capacity to 4-hydroxylate debrisoquine, an antiadrenergic hypotensive drug, is bimodally distributed in Caucasian populations in which 5–10 % are poor metabolizer (PM) (Johansson *et al*., 1991; Nakamura *et al*., 1985). Three main types of defective alleles have been found in this population: The most common mutant allele, ***CYP2D6B*** accounts for 75% of the defective alleles, the rest being due to ***CYP2D6A*** (5%) and ***CYP2D6D*** (11%)(Hirvonen *et al*., 1993). Among Caucasians about 1–7% belong to a newly classified group, the ultrarapid metabolizers (UM). These individuals possess more than one copy of the active gene in the ***CYP2D*** locus. Indeed 2 up to 13 active ***CYP2D6*** gene copies appearing in tandem has been reported in one Swedish family (Johansson *et al*., 1993). Using RFLP and PCR analysis, it is possible to correctly identify over 95% of PMs among Caucasians (Heim, *et al*., 1990; Broley *et al*., 1991; Dahl *et al*., 1992).

Among Orientals the corresponding frequency of PMs as defined for Caucasians is very low (<1%) (Lou *et al*., 1987; Nakamura *et al*., 1985). The molecular basis of the large inter-ethnic difference between Chinese and Caucasian populations with respect to the frequency of PMs and the mutant alleles has been investigated (Yue *et al*., 1989); Johansson *et al*., 1991). Apparently, the deleterious mutations in the *CYP2D6* gene, common among Caucasians, are absent in Orientals. The low frequency of PMs reported

Table 2. INCIDENCE OF CYP2D6 AND CYP2C19 POOR METABOLISER PHENOTYPES AMONG DIFFERENT ETHNIC GROUPS.

ETHNIC GROUP	% PM OF DEBRISOQUINE	%PM OF MEPHENYTOIN
CAUCASIANS	5 - 10	2 - 5
ORIENTALS	<2	18 - 23
BLACK AMERICANS	<2	18.5
ZIMBABWEANS	1.5	4.0
ETHIOPIANS	1.7	5.2

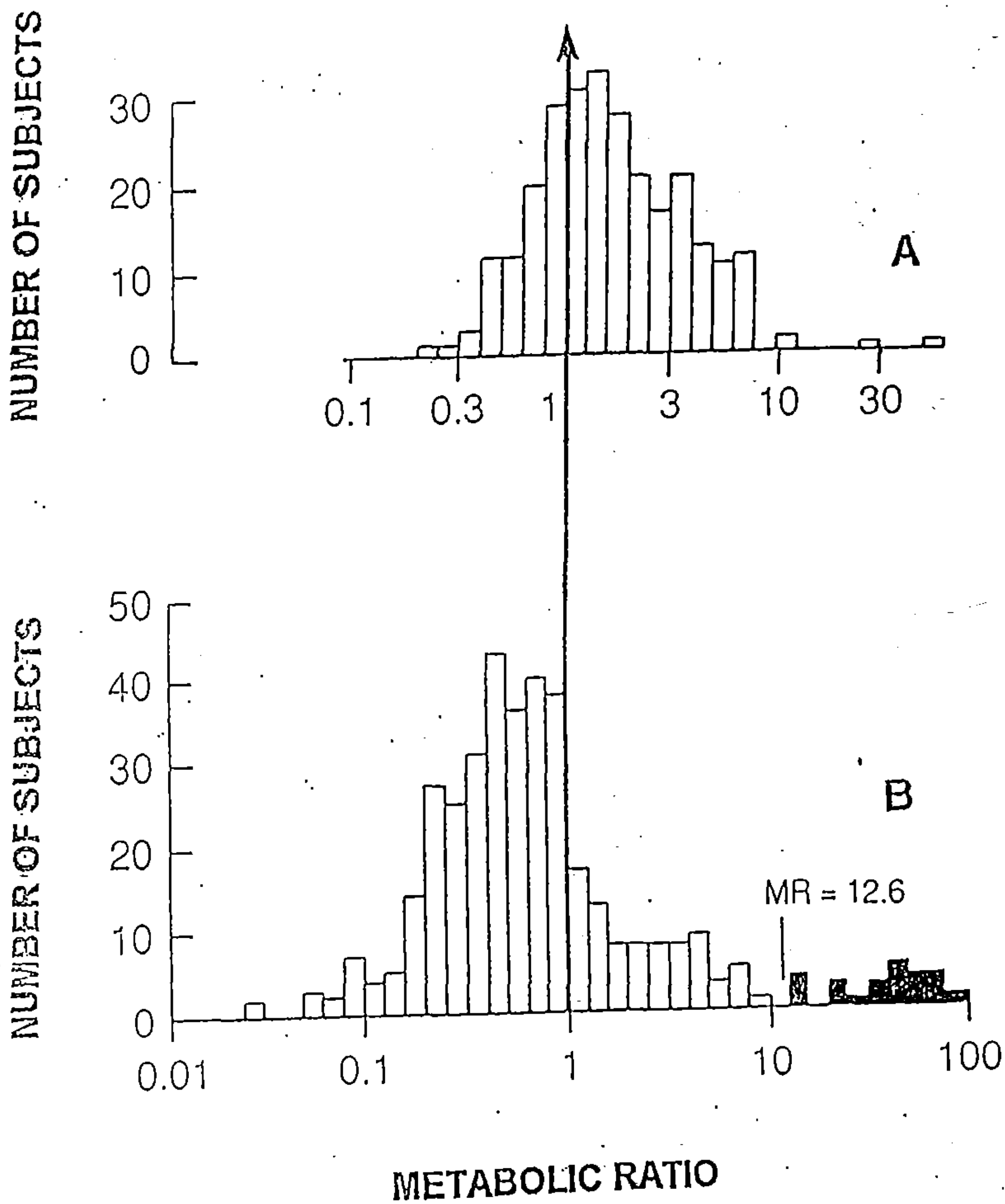
PM: Poor Metabolizers

among Chinese appears to be exclusively inherent in gene deletions (***CYP2D6D***). Another interesting inter-ethnic difference is that the mutant *Xba*I 44kb allele is more common in the Chinese populations (Yue *et al.*, (1989). In contrast to Caucasian *Xba*I 44kb allele which contains the B type mutation (***CYP2D6B***), the Chinese *Xba*I 44kb allele is functional but with a reduced capacity to metabolize drugs that constitute substrates for ***CYP2D6*** (Johansson *et al.*, 1991).

The frequency distribution of debrisoquine metabolic ratios (MRs) is skewed towards higher values in Chinese EMs compared to Caucasian EMs, indicating a lower metabolic capacity among Chinese (see figure 3). Within the Chinese EM subjects those with 44/44 kb have significantly higher MR than those with the 29/44 pattern (Wang *et al.*, 1993). Sequence analysis of the ***CYP2D*** locus from the Chinese 44kb allele has revealed the presence of two slightly defective ***CYP2D6*** genes designated as ***CYP2D6ch1*** and ***CYP2D6ch2*** that cause 2 and 8 amino acid substitutions respectively (Johansson *et al.*,1994). The detrimental mutation of the ***CYP2D6ch*** genes is due to the presence of a C_{188}-->T transition in exon 1 which results in a Pro_{34}-->Ser amino acid substitution. This, apparently, is responsible for the expression of a relatively unstable gene product, probably accounting for the reduced activity associated with this allele (Johansson *et al.*, 1994).

Significantly lower frequency of PMs (1.9%) has been reported among Black Americans as compared to Whites (7.7%) (Relling *et al.*, 1991). Such a low prevalence of PMs in Blacks is the result of significantly lower frequency of the ***CYP2D6*** mutant genes that are common among Caucasians (Evans *et al.*, 1993). Also, the prevalence of

Figure 3: FREQUENCY DISTRIBUTION OF DEBRISOQUINE METABOLIC RATIOS IN TWO POPULATIONS : (A) CHINESE (B) CAUCASIANS (Reprinted from Kalow, 1991)

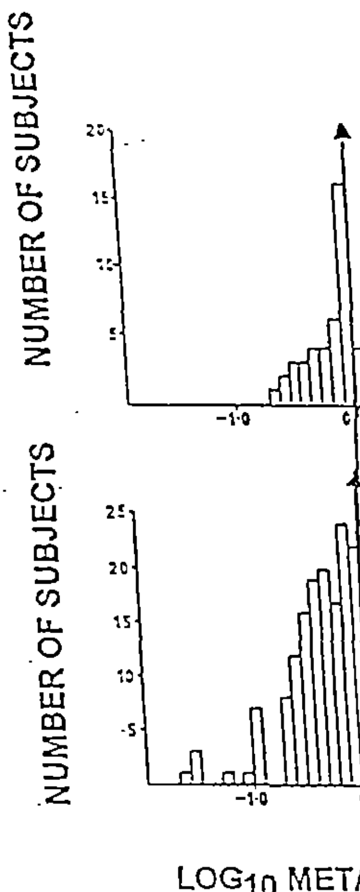


PMs in Zimbabweans is reported to be 1.5% (Masimirembwa *et al.*, 1995). No individual was found to be homozygous for the defective alleles among the 114 Zimbabwean subjects analyzed (Masimirembwa *et al.*, 1993). The prevalence of PMs of debrisoquine among Ghanaians was reported to be 6% (Woolhouse *et al.*, 1979). No PMs were observed for the Nigerian population studied (Iyun *et al.*, 1986). The molecular genetic basis for these observations has however, not been established, since only phenotype analysis were conducted. In contrast to this extremely high prevalence of PMs (18%) was found within African vendas, a South African tribe (Sommers *et al.*, 1988), suggesting the heterogeneity of the African population. Studies on African populations, similar to the Chinese, have revealed a right shift in the distribution histogram of debrisoquine MRs (see figure 4) although the cause of the slow debrisoquine hydroxylation may be different in the different cases. In many black populations studied: Nigerians, African Venda, and Ghanaians a strong tendency of right shift and trimodality is observed although the frequency of the described mutations are expected to be low.

1.3. S-MEPHENYTOIN HYDROXYLASE (CYP2C19).

Inter-ethnic and Inter-individual variability with respect to the disposition of drugs has been well characterized in human. Hydroxylation polymorphism of the anti-convalescent drug Mephenytoin is also a reasonably well studied variation. Mephenytoin exists as a 1:1 racemate mixture of the S and R enantiomers. The drug is extensively biotransformed in man although small amounts (<5%) of orally administered dose is excreted unchanged in the urine irrespective of phenotypic status (Wedlund *et al.*, 1985).

Figure 4. FREQUEN
CAUCASI
1986 ; Ka



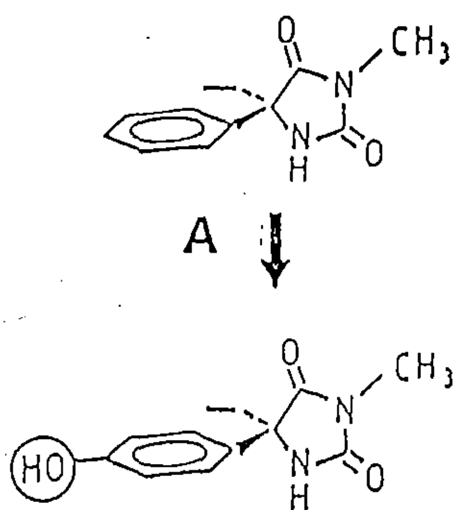
Two principal routes (see figure 5) have been described for the metabolism of mephenytoin (Kupfer *et al.*, 1980). One of them is the slow oxidative N-demethylation of both enantiomers to S and R -PEH (5-phenyl-5-ethylhydantoin) also called Nirvanol, a pharmacologically active metabolite (Troupin *et al.*, 1976). Urinary elimination of PEH is slower than its formation. Chronic administration of mephenytoin therefore results in accumulation of PEH in the body. Alternatively, mephenytoin is hydroxylated at the para position of the aromatic ring to 4-hydroxy-mephenytoin (4-OH-M). This aromatic hydroxylation is highly stereoselective and occurs exclusively with S-mephenytoin (Kupfer, *et al* 1981). S-mephenytoin is thus quickly excreted as 4-OH mephenytoin with a high metabolic clearance while the R- enantiomer is slowly eliminated as R-PEH, the major contributor to the therapeutic response (Kupfer *et al.*, 1982).

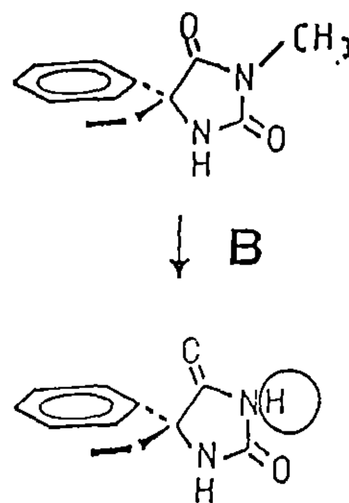
Only stereoselective S-mephenytoin 4-hydroxylation is subjected to genetic polymorphism (Jacqz *et al.*, 1986), dividing the population into PMs and EMs. Since the renal clearance of the two enantiomers is similar (Jacqz *et al.*, 1986) and S-mephenytoin is rapidly eliminated as 4-OH-mephenytoin, the enantiomeric ratio is not equal to unity. On the other hand, the pharmacokinetics of the two enantiomers are more similar if 4-hydroxylation is impaired, giving rise to a urinary ratio (S/R) close to unity. As the ability to 4-hydroxylate mephenytoin increases, the S/R ratio progressively becomes less and less than unity. The S/R ratio in a 0-8 hrs urine sample after oral administration of racemic mephenytoin is therefore used as a phenotype determinant (Jacqz, *et al.*, 1986).

Figure 5. STEREOSELECTIVE METABOLISM OF (S)- AND (R)- MEPHENYTOIN:
(A) 4-HYDROXYLATION
(B) N-DEMETHYLATION

S- MEPHENYTOIN

R- MEPHENYTOIN





4-OH-MEPHENYTOIN

NIRVANOL
(PEH)

Note. Thickness of arrow indicates differential extent of reactions

An acid labile metabolite of S–mephenytoin, excreted in the urine of EM but not found in PM has been reported (Wedlund *et al.*, 1987). Storage of urine in the unfrozen state, and acidification secondary to bacterial growth cause the conversion of this acid labile metabolite back to S–mephenytoin. As the storage time of frozen urine samples increases the S/R ratio in EMs urine increases (Tybring *et al.*, 1992), so the presence of this acid labile metabolite in urine may lead to an artificially increased concentration of S–mephenytoin and misclassification of EM to PM. It is thus necessary to reconfirm the S/R value before and after acid treatment of urine samples (Tybring *et al.*, 1992)

1.3.1 MUTANT ALLELES OF *CYP2C19.*

Two major genetic defects in ***CYP2C19***, responsible for the poor metabolizer phenotype have recently been described. The first defect (*CYP2C19m1*) consists of a single base pair (G-->A) transition within the coding sequence of exon 5 creating an aberrant splice site (deMorais *et al.*, 1994a). The aberrantly spliced mRNA lacks the first 40 base pairs of exon 5. The reading frame of such a mRNA is altered starting with amino acid 215 and leading to a premature stop codon 20 amino acids residues downstream. The consequence of this is a truncated, non functional protein. ***CYP2C19m1*** accounts for 75% of the defective alleles among both Caucasians and Orientals (deMorais *et al.*, 1994b) and also within the Zimbabwean population (Masimirembwa *et al.*, 1994).

The second genetic defect (*CYP2C19m2*) consists of a single base pair mutation (G-->A) in exon 4 of *CYP2C19* (deMorais *et al.*, 1994b). It introduces a frame shift that creates a premature stop codon and results in a truncated polypeptide product. *CYP2C19m2* accounts for the remaining 25% of the defective alleles in Orientals. No mutation of this kind has so far been reported in Caucasians and in Black populations.

1.3.2 *CYP2C19* GENETIC POLYMORPHISM

Impairment in mephenytoin hydroxylation implicating a genetic cause was first recognized in a family study (Kupfer *et al* 1979). Subsequent studies revealed that S-mephenytoin hydroxylation exhibited genetic polymorphism (Jacqz *et al* 1986). The enzyme responsible for the 4-hydroxylation of S-mephenytoin (S-mephenytoin hydroxylase) was found to be **CYP2C19** (Wrighton *et al.*, 1993: Goldstein *et al.*, 1994). Deficient metabolism of S-mephenytoin arises from partial or complete absence of S-mephenytoin hydroxylase in the liver of PMs (Meier *et al.*, 1985). It has also been demonstrated that there is a significant correlation between the amount of **CYP2C19** elaborated and the ability to 4-hydroxylate S-mephenytoin (Wrighton *et al.*, 1993).

Besides S-mephenytoin **CYP2C19** metabolizes several commonly used drugs (see table 3). Family studies have indicated that S- mephenytoin hydroxylation is under a monogenic control, that the PM phenotype is inherited as an autosomal recessive trait (Inaba *et al.*, 1986: Ward *et al.*, 1987), and that it is determined by at least two alleles at a single gene locus. Accordingly, the PM phenotype represents the homozygous

Table 3. LIST OF SELECTED DRUGS METABOLIZED BY CYP2C19.

DRUGS	REFERENCES
PROGUANIL	Ward *et al.,* 1991.
PROPRANOLOL	Ward *et al.,* 1989.
OMEPRAZOLE	Chiba *et al.,* 1993.
IMIPRAMINE	Skjelbo *et al.,* 1993.
HEXOBARBITAL	Adedoyin *et al.,* 1994.
DIAZEPAM	Andersson *et al.,* 1994.

recessive genotype while the EM population is a mixed distribution of heterozygous and homozygous dominant individuals.

Expression of S–mephenytoin hydroxylase displays pronounced inter–ethnic variation. About 2–5% of Caucasians are known to be PMs with respect to **CYP2C19** (Sanz *et al.*, 1989: Alvan *et al.*, 1990) whereas significantly higher proportion of the Oriental populations (18–23%) demonstrate complete deficiency of **CYP2C19** activity. (Nakamura *et al.*, 1985: Horai *et al.*, 1989).

Incidence of PM phenotype in Black populations seems to be variable. The prevalence of PM phenotype in a limited sample (n = 27) of American Black population was found to be 18.5 % (Pollock *et al.*, 1991), the corresponding incidence of PMs among Zimbabweans was found to be 4% (Masimirembwa *et al.*, 1994). Significantly higher incidence of poor metabolizer phenotype (35 %) has been reported in Kenyans using proguanil as probe drug (Watkins, *et al.*, 1990). A study done on Nigerians indicated absence of bimodality in the distribution of S–mephenytoin hydroxylation (Iyun *et al.*, 1990).

The impression is entertained in some circles that blacks are evolutionarily distinct from Caucasians and Orientals. Also, no extensive study relating to genetic polymorphism of **CYP2C19**, in particular ***CYP2C19m2***, has ever been carried out in a black population. It is therefore imperative to start an investigation regarding the **CYP2C19** status in Black populations. The present study has as one of its aims to explore the situation with the view to identifying both the genotypes (***CYP2C19m1*** and ***CYP2C19m2***) as well as the phenotypes of S–mephenytoin hydroxylase among Ethiopians.

2. AIMS OF THE STUDY.

Broadly, the aim of this study can be put as follows:

(A). To analyze the frequency of various *CYP2D6* and *CYP2C19* genotypes among Ethiopians, in relation to reported cases for other ethnic groups.

(B). To analyze the different phenotypes among Ethiopians with respect to **CYP2D6** and **CYP2C19** activities.

(C). To examine the relationship between *CYP2D6* and *CYP2C19* genotypes and phenotypes among Ethiopians, and to evaluate the predictive value of genotype analysis as a phenotype determinant within the Ethiopian population.

3. SIGNIFICANCE OF THE STUDY

(i). Therapeutic relevance.

Administration of drugs subject to genetic polymorphism to poor metabolisers may result in accumulation of the parent drug and hence increased responsiveness with several side effects even at doses considered 'normal' or 'standard'.

When the pharmacological effect of a drug is inherent in the metabolite form, as is the case with codeine (Mikus *et al.*, 1994), the therapy for poor metabolisers will

inevitably be insufficient and possibly ineffective. On the other hand, it is unlikely that therapeutic plasma concentration of administered drugs will be attained in highly extensive metabolisers at doses normally considered routine. Knowledge of a patient's phenotype and/or genotype prior to treatment with drugs metabolized by the **CYP2D6** and **CYP2C19** systems (or for that matter any drug the metabolism of which is subject to genetic polymorphism), will be of immense practical value for dose adjustment purposes, particularly, for those drugs that have narrow interval of therapeutic plasma concentration.

(ii). Toxicological importance

The principal function of drug metabolizing enzymes is to facilitate excretion of administered drug by way of chemical modification of the parent drug, rendering it more water soluble. Toxic reactions to drugs are quite common. Study of the relationship between genetically determined differences in the activity of drug metabolizing enzymes and the risk of adverse drug reactions has immense value. With regard to susceptibility of the two phenotypes, drug mediated toxicity can be anticipated in two ways:

(a). Where drug toxicity is caused by the parent drug compound and the elimination of the drug proceeds exclusively via polymorphic enzyme, the PM phenotype is more likely to be prone to such type of toxicity. The drug will accumulate due to impaired metabolism.

(b). When toxicity is mediated by a reactive intermediate, generated by a polymorphic enzyme, EMs are at much higher risk than PMs to develop toxicity or cancer. Knowledge of ones phenotype therefore will be of great value in avoiding toxicity caused by administered drugs.

(iii). Implication for drug development.

In the development of new drugs, where the metabolism of a particular drug under investigation is subject to different degrees of genetic polymorphism within different ethnic groups, it may be inappropriate to extrapolate pharmacokinetics and metabolic data obtained from studies conducted solely on one such ethnic group to another. The discovery of important inter-ethnic differences in drug metabolizing ability with reference to dose would require that this phenomenon be given serious consideration in international test protocols for new drugs by pharmaceutical companies.

4. MATERIALS AND METHODS

4.1. SUBJECTS

One hundred and fifteen healthy Ethiopians (54 females and 61 males), aged between 18 and 35, mostly recruited from among medical students of Addis Ababa University (originally various regions of the country), and staff members of Black Lion Hospital, Addis Ababa volunteered as subjects for this study. The study had been approved by the ethics committees of Addis Ababa University Medical Faculty and of Karolinska Institute of Sweden. Subjects were given a brief information about the study including any possible side effects that might arise from administration of Debrisoquine and Mephenytoin (see appendix I). All subjects had no recent illness as confirmed by physical examination and were not receiving any other drugs during their period of participation in the study.

4.2. CHEMICALS

S-mephenytoin (Mesantoin) and debrisoquine tablets were obtained from Sandoz and Declinax, Roche respectively. Restriction enzymes, *XbaI*, *Eco*RI, *Bam*HI and *SamI* were purchased from Boehringer Mannheim, Germany and heat stable DNA polymerase with buffer from KEBO, Sweden. One for all buffer was obtained from Pharmacia. cDNA was kindly provided by U.A. Meyer, Basel, Switzerland and F. Gonzalez, Bethesda, USA. Multiprime DNA labelling kit Code RPN 1601 Y and ^{32}P dCTP were purchased from Amersham, UK. Agarose, NuSieve GTG and SeaKem LE, were from SIGMA. Primers were bought from Scandinavian Gene Synthesis AB.

4.3. METHODS

4.3.1. GENOTYPING OF *CYP2D6*

(i). Extraction of genomic DNA from whole blood:

Ten ml of blood was taken from each randomly selected Ethiopian volunteer in EDTA-containing vacutainer tube. leukocyte DNA was extracted by the Guanidine isothiocynate method (Sambrook *et al.*, 1989). (see appendix II for details).

(ii). Restriction Fragment Length Polymorphism (RFLP) Analysis.

Seven μg of genomic DNA was digested with restriction endonuclease *XbaI* or *Eco*RI to completion (see appendix III for details). The resulting DNA fragments were subsequently subjected to agarose gel electrophoresis (0.55% or 0.85% agarose at 0.5 V/cm for 5 days or 1 V/cm for 72 hrs respectively. After Southern blotting using

Quiabrane membranes (see appendix IV for details), the membrane was prehybridized for 2 hrs at 61°C. Hybridization was done with full length radioactively labelled CYP2D6 cDNA which recognizes ***CYP2D6*** as well as the pseudogenes ***CYP2D7*** and ***CYP2D8***. The probe was prepared using Multiprime DNA labelling kit and labelled with ^{32}P dCTP, as recommended by the manufacturer (see appendix V.). The membrane was then washed 3 times and the different restriction fragment length patterns were analyzed after exposing the film for 2-7 days at 70°C.

(iii). <u>Polymerase Chain Reaction (PCR)-based allele specific analysis.</u>

(a). Identification of *CYP2D6A* and *CYP2D6B*.

CYP2D6A and ***CYP2D6B*** mutations were studied using PCR-based allele specific amplification of parts of the genes covering the specific mutation as described by Heim and Mayer (1990). In brief the method consists of two consecutive steps: In the first step, (PCR 1), DNA fragment of the ***CYP2D6*** gene was amplified specifically using oligonucleotide primers that are complimentary to unique ***CYP2D6*** intronic sequences on either side of the mutation of interest. Primers 1+2 and primers 3+4 (See table 4a for primer sequences) were used to identify ***CYP2D6B*** and ***CYP2D6A***, to give fragment B and fragment A respectively (See figure 6).

In the second step, (PCR 2), 1 µl of the amplified product from PCR 1 was used as a template. Fragment B was reamplified with primer 1 and wild type specific primer 7, and in a separate reaction tube with primer 1 and mutation specific primer 8. Similarly, fragment A was reamplified with primer 4 and wild type specific primer 5, and also with primer 4 and mutation specific primer 6. Typically, six reactions were carried out with each isolated DNA sample to identify each mutation type.

Table 4a. Sequence of oligonucleotide primers used in the Polymerase Chain Reaction (PCR) for the analysis of *CYP2D6A*, *CYP2D6B* and Exon 1 (C_{188} -->T).

PRIMER No.	OLIGONUCLEOTIDE SEQUENCE	POSITION
Primer 1	5'-(ATTTCCCAGCTGGAATCC)-3	1385 - 1402
Primer 2	5'-(GAGACTCCTCGGTCTCTC)-3'	2122 - 2139
Primer 3	5'-(GCGGAGCGAGAGACCGAGGA)-3'	2098 - 2117
Primer 4	5'-(CCGGCCCTGACACTCCTTCT)-3'	3200 - 3181
Primer 5	5'-(GCTAACTGAGCACA)-3'	2624 - 2637
Primer 6	5'-(GCTAACTGAGCACG)-3'	2624 - 2637
Primer 7	5'-(CGAAAGGGGCGTCC)-3'	1947 - 1934
Primer 8	5'-(CGAAAGGGGCGTCT)-3'	1947 - 1934
Primer 9	5'-(ACTAGGCCCCTCCACCGG)-3'	196 - 179
Primer 10	5'-(TCTGTAGGGGAGTCTCAGC)-3'	321 - 302
Primer 10B	5'-(GTGGTGGGGCATCCTCAGG)-3'	302 - 333
Primer 11	5'-(AGGGGGCCTGGTGG)-3'	201 - G_{188}
Primer 12	5'-(AGGGGGCCTGGTGC)-3'	201 - C188

Table 4b. Sequence of oligonucleotide primers used in the Polymerase Chain Reaction (PCR) for genotyping the population for *CYP2C19m1* and *CYP2C19m2*

PRIMERS	OLIGONUCLEOTIDE SEQUENCE
CYP2C19m1F*	5'-(AATTACAACCAGAGCTTGGC)-3'
CYP2C19m1R*	5'-(TATCACTTTCCATAAAAGCAAG)-3'
CYP2C19m2F*	5"-(TATTATTATCTGTTAACTAATATGA)-3'
CYP2C19m2R*	5'-(ACTTCAGGGCTTGGTCAATA)-3'

F* : FORWARD PRIMER

R* : REVERSE PRIMER

Note. The *CYP2C19* gene is not yet fully sequenced

FIGURE 6

PRIMERS

Typically, about 200 ng genomic DNA was amplified by PCR in a total of 25 μl reaction mixture containing 200 μM of dNTP, 1.0 mM $MgCl_2$, 2.5 μl of 10X reaction buffer, 0.25 μM primers. In PCR 1, after an initial denaturation at 94°C for 1.5 min, the PCR reaction was carried out for 35 cycles under the following conditions: denaturation at 94°C for 1 min; annealing temperature, 52°C for 1 min; extension period at 72°C for 1 min and finally 7 min elongation at 72°C. PCR 2 was performed for 15 cycles under the following conditions; 94°C 1 min, 50°C 1 min, 72°C 1 min, with final extension time of 7 minutes at 72°C after the last cycle.

(b). Identification of C_{188}-->T Mutation in Exon 1

Amplification and detection of exon 1 mutation was performed in two steps according to Johansson *et al.*,(1994), using primers 9 and 10 in PCR 1 and for PCR 2, primers 9+11 for the wt and primers 9+12 for the mutated alleles. Because of the possibility of having an exon 1 mutation in ***CYP2D6L*** (where a gene conversion event had occurred in intron 1 as a result of part of the ***CYP2D7P*** gene being introduced into the intron), amplification of exon 1 in PCR 1 using the usual primers for the 2D6wt gene becomes impossible. Amplification in such cases was carried out using primer 9 and 10B. The exon 1 mutation was then determined by a second PCR on the amplified products of PCR 1 using primer 9+11 and primer 9+12. In all cases 10 μl of the different reaction products was analyzed by 1.2% agarose gel electrophoresis in 1 X TBE buffer. The gene band pattern was visualized with ethidium bromide, fluoresced under a UV transilluminator and photographed on a polaroid instant pack film for examination and subsequent statistical analysis.

(c). Identification of *CYP2D6C* mutation

Presence of *CYP2D6C* allele (a deletion of the triplet nucleotide that codes for Lys_{281}, Tyndale *et al.*,1991), was screened by single stranded conformational polymorphism (SSCP). The relevant DNA fragment containing exons 5 and 6, and obtained in PCR 1 for the *CYP2D6A* allele using primers 3+4 as templates for PCR 2. In the second PCR, a 320bp fragment was amplified using primers 2D8ex5F and 2D6ex5R. 10 µl PCR 2 product was analyzed on 1.5% agarose gel electrophoresis. Five µl of PCR 2 product was denatured by heating at 95°C for 5 min and subjected to a non-denaturing polyacrylamide gel. DNA fragments were visualized by silver staining according to the method of Blum *et al.*,(1987) (see appendix VI). As a positive control for the C-type mutation, DNA from an individual who had been genotyped as ***CY2D6C*** by DNA sequence analysis was used. Presence of C-mutation could clearly be confirmed from a fragment with a higher mobility.

4.3.2. CYP2D6 PHENOTYPE ANALYSIS.

Ten mg of Debrisoquine (Declinax, Roche) was orally co-administered with 100mg of racemic mephenytoin (Mesantoin, Sandoz) to volunteers together with a glass of water, after they had emptied their bladder, preferably before bedtime. It had been established that these two drugs are metabolized by two different P450 enzyme systems so the question of metabolic interference between the two drugs does not arise.

Concentrations of the parent drug (Debrisoquine) and its metabolite (4–OH–Debrisoquine) was analyzed by gas chromatography according to Lennard *et al* (1977). The procedure requires derivatization of the debrisoquine and 4–OH–debrisoquine to pyrimidine derivatives by overnight incubation of the urine samples with acetyl acetone at 50°C followed by organic solvent extraction of the derivatives (see appendix VII). Urinary metabolic ratio (MR) was calculated as the ratio of debrisoquine recovered in urine to that of the corresponding derivative 4–OH debrisoquine.

$$\textbf{MR Debrisoquine} = \frac{\textbf{Debrisoquine recovery in urine}}{\textbf{4–OH Debrisoquine recovery in urine}}$$

Log_{10} MR values were used in the graphs and phenotype assignment was made according to previous reports on Caucasians: PMs and ultra rapid metabolisers (UMs) were defined as subjects with MR >12.6 and <0.2 respectively.

4.3.3. *CYP2C19* GENOTYPING

Two mutations recently found in the *CYP2C19* gene: *CYP2C19m1* and *CYP2C19m2*, that account for deficient metabolism of drugs metabolized by this enzyme were analyzed according to deMorais *et al.* (1994a, 1994b). About 200ng of genomic DNA was amplified by polymerase chain reaction in a total of 25 µl reaction mixture containing 200 µM dNTP, 1.0mM $MgCl_2$, 2.5 µl of 10X reaction buffer, 0.25µM primers (CYP2C19m1F + CYP2C19m1R and CYP2C19m2F + CYP2C19m2R for the ***CYP2C19m1*** and *CYP2C19m2* mutations respectively). PCR reaction cycles of

denaturation, annealing and extension temperature was as described in section 4.3.1. for *CYP2D6* genotyping.

Eight µl of each PCR product was electrophoresed on a 3% agarose gel and stained with ethidium bromide. For ***CYP2C19m1*** analysis, 12µl of PCR product were digested with *Sma*I in a total of 23µl reaction mixture containing 0.3U of *Sma*I, 6µl of buffer and 6µl of water. After an overnight incubation at 25°C, the volume of the mixture was reduced to half by vacuum drying and the sample was analyzed on 3% agarose gel. Analysis of ***CYP2C19m2*** was carried out as follows: 8µl of PCR product was digested with 10 U of *Bam*HI, 5µl of One phor all buffer and water to make a total reaction mixture of 50µl. After an overnight incubation at 37°C and vacuum drying to half the original volume, the digested DNA was subjected to electrophoresis on a 3% agarose gel in 1 X TBE buffer.

Figure 7. shows the strategy used to genotype ***CYP2C19m1*** and ***CYP2C19m2*** after digestion of the amplified gene product with *Sma*I and *Bam*HI respectively. Presence of ***CYP2C19m1*** and ***CYP2C19m2*** eliminates the restriction cleavage site for *Sma*I and *Bam*HI respectively.

4.3.4. CYP2C19 PHENOTYPE

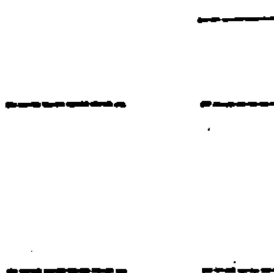
The concentration of S- and R-mephenytoin in urine was measured by stereoselective gas chromatography according to the technique of Wedlund *et al*. (1984), as modified by Sanz *et al* (1989). 1 ml of thawed urine was placed in a glass tube to

Figure 7.

CY

Sma

wt/wt wt/n



which was added 200 μl of chloroform. This was shaken vigorously for 15 min followed by bench centrifugation at 3000 RPM for 10 min. One μl of the organic phase was injected to the chiral gas chromatography. All urine samples giving S/R ratios greater than 0.6 were reanalyzed after acid treatment by adding 25μl of concentrated HCl according to Tybring *et al.* (1992). Subjects with S/R ratio greater than 0.9 and whose value did not increase after acid treatment were classified as PM.

5. RESULTS

5.1. CYP2D6 PHENOTYPE

The frequency distribution of $\log_{10}$MR in 0–8 hr urine samples of the 115 subjects is shown in figure 8. Debrisoquine MRs appear trimodally distributed in the population with a large part of them located within the range 1 – 10. The distribution histogram is skewed to the right when compared to the corresponding situation for Caucasians. Two subjects with UMR >12.6 (#E89 female, MR = 13.2 and #E11 male, MR = 45.12 respectively) are assigned PM status. The prevalence of CYP2D6 PM among Ethiopians as revealed by this study is 1.74%. This is within the range (0–2%) indicated for Black populations (Relling *et al.*, 1991; Evans *et al.*, 1993). Six subjects (5%) had UMR < 0.2 and are classified as Ultrarapid metabolizers (UMs).

NUMBER OF SUBJECTS

Fig

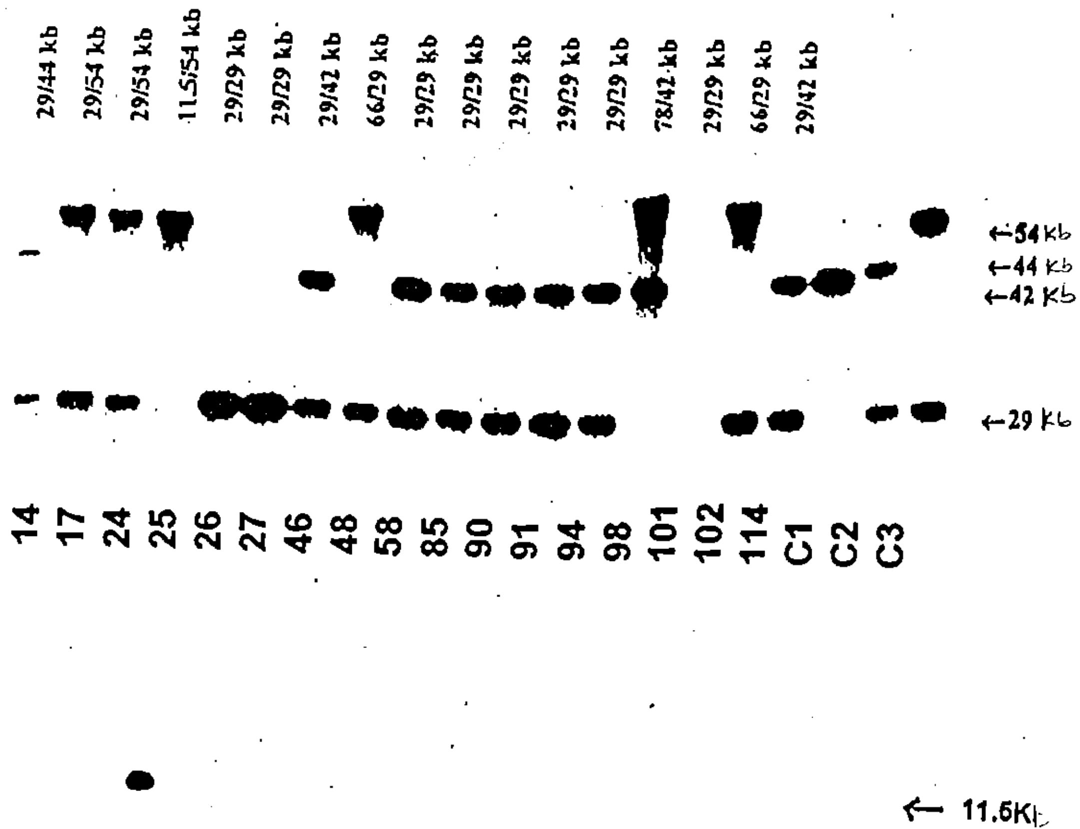
5.2. *CYP2D6* GENOTYPE

(i). RFLP analysis of *CYP2D6*:

Genomic DNA from all subjects were analyzed for the known variants of ***CYP2D6*** by RFLP using both *Xba*I and *Eco*RI to detect the 11.5kb, 44kb and 42kb haplotypes associated with gene deletion, defect ***CYP2D*** locus and duplication respectively. These ***CYP2D6*** haplotypes (see figure 9) as well as new multiduplicated types (54kb, 66kb and 78kb), indicative of three, four and five ***CYP2D6*** genes respectively have been shown by this study to be present in the Ethiopian population. It should be added here that the 66kb and 78kb *Xba*I haplotypes are new variants of ***CYP2D6*** identified by this study. The frequencies of these, together with other variant alleles as detected by RFLP among Ethiopians are summarized in table 5. For all subjects with MRs lower than 0.2 (n=6) RFLP analysis of genomic DNA revealed the presence of extra genes as indicated by the *Xba*I 42kb or 54kb fragment containing in all two and three functionally active ***CYP2D6*** genes respectively (Table 6).

Interestingly the allele frequency of the *Xba*I 42 kb haplotype found in Ethiopians is 12.9%, considerably higher compared to any other groups studied. Three subjects were found to be heterozygous for the 54 kb allele (E#17 (54/29, MR=0.05), E#24 (54/29, MR = 0.27) and E#25 (54/11.5, MR = 0.28). Two individuals were heterozygous for *Xba*I 66kb, and one with 78kb (MR = 0.2). In all these subjects, the *Eco*RI 12.1kb fragment had a higher intensity when compared to the other fragments. The RFLP data are consistent with the presence of alleles carrying two (42kb), three

FIGURE 9. AUTORADIOGRAM FROM *XbaI* RFLP ANALYSIS OF THE *CYP2D6* LOCUS OF GENOMIC DNA OF SOME ETHIOPIAN SUBJECTS. NOTE THE PRESENCE OF ALL THE DIFFERENT KNOWN *XbaI* HAPLOTYPES (11.5Kb, 29Kb, 42Kb, 44Kb, 54Kb) AS WELL AS THE NEWLY IDENTIFIED (66Kb, 78Kb) IN THE ETHIOPIAN POPULATION



NUMBERS AT THE BASE INDICATE DIFFERENT SAMPLES

C: CONTROL DNA

TABLE 5.

HAPLOTYP

ALLELE FREQUENC

*: VERIFIED

●: 116 SUBJ

Table 6. *XbaI* HAPLOTYPES OF SUBJECTS WITH DEBRISOQUINE METABOLIC RATIO LESS THAN 0.2

DAN SAMPLE	*XbaI* HAPLOTYPE	DEBRISOQUINE METABOLIC RATIO
E5	42/29	0.07
E17	54/29	0.05
E37	42/29	0.11
E85	42/29	0.13
E93	42/29	0.12
E116	42/29	0.18

E: ETHIOPIAN

(54kb), four (66kb) and even five (78kb) active *CYP2D6* genes in tandem. Figure 10. shows the frequency distribution of debrisoquine MR with respect to the different *CYP2D6* haplotypes as detected by RFLP and PCR analysis.

From the distribution histogram it can be inferred that more than one copy of functionally active *CYP2D6* genes are present and responsible for the expression of higher amounts of the CYP2D6 enzyme. This probably is the reason for the lower MR. Three Subjects (44/29) haplotype with relatively higher debrisoquine MR were found: E#14, E#51 and E#89 with MR of 1.29, 0.87 and 13.2 respectively. All of them are heterozygous for the exon 1 C_{188}-->T mutation. Table 7 summarizes the *Xba*I RFLP results for the 113 Ethiopian EMs.

(ii). PCR-based analysis of *CYP2D6A* and *CYP2D6B*.

Using PCR all subjects were investigated for *CYP2D6A* and *CYP2D6B*. No *CYP2D6A* allele was found (figure 11) and only three subjects were heterozygous for *CYP2D6B* (figure 12) one of the PMs, #E11 and two EMs, #E9 and #E87 with UMR 1.9 and 1.13 respectively. The allele frequency of *CYP2D6B* is thus 1.3 %.

(iii). PCR and SSCP analysis of *CYP2D6C*:

All subjects that have got MR > 1 were investigated further for the presence of *CYP2D6C* allele, using PCR and SSCP. This was to see if the presence of *CYP2D6C* was responsible for the right shift of the frequency distribution histogram. None of them contained the *CYP2D6C* allele.

Figure 10, FREQUENCY DISTRIBUTION OF DEBRISOQUINE URINARY METABOLIC RATIO WITH RESPECT TO VARIOUS HAPLOTYPES AS DETECTED BY PCR AND RFLP

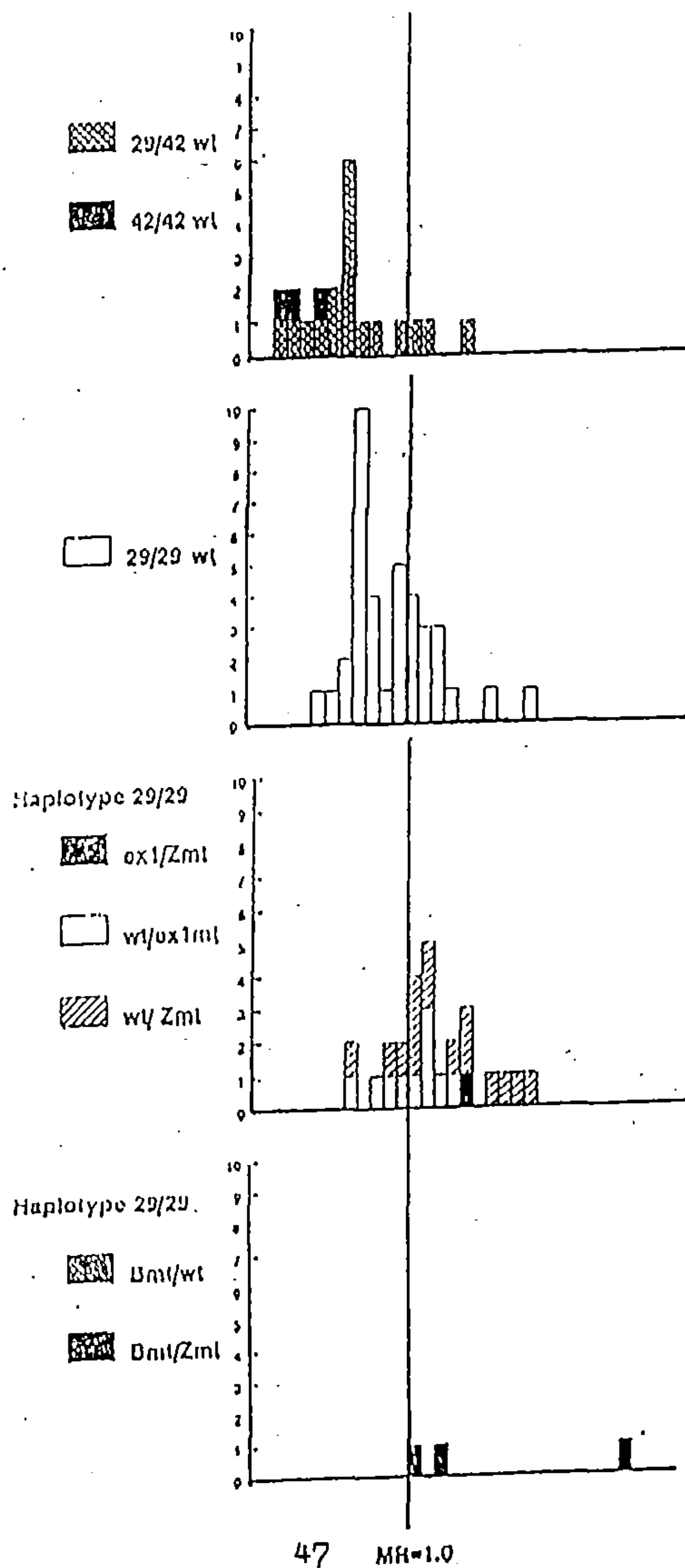
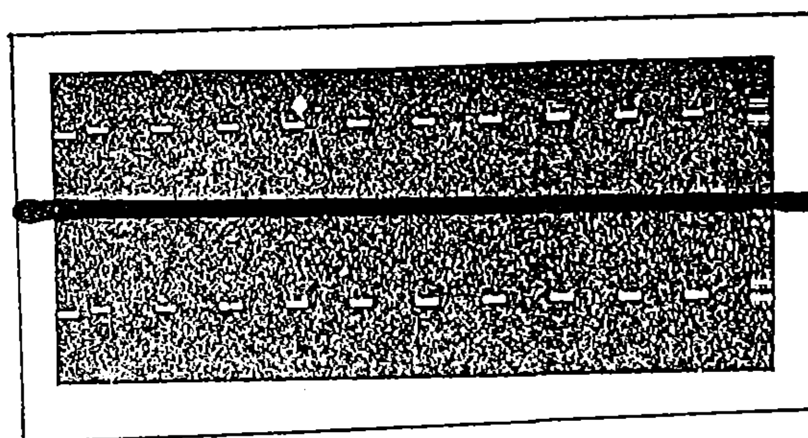


Table 7. SUMMARY OF THE *XbaI* RFLP RESULTS FOR THE 113 ETHIOPIAN EXTENSIVE METABOLISERS (EMs) OF DEBRISOQUINE

XbaI HAPLOTYPES	NUMBER OF SUBJECTS	FREQUENCY (%)	MEDIAN MR	MEAN MR
29/29	75	66.4	0.95	1.47
42/29	22	19.5	0.37	0.52
42/42	3	2.7	0.13	0.16
44/29	2	1.8	1.08	1.08
54/29	2	1.8	0.16	0.16
66/29	1	0.9	0.24	0.24
78/29	1	0.9	0.20	0.20
11.5/29	5	4.4	1.55	5.19
11.5/42	1	0.9	0.25	0.25
11.5/54	1	0.9	0.28	0.28

MR: METABOLIC RATIO

FIGURE 11. ***CYP2D6A*** **ALLELE-SPECIFIC AMPLIFICATION OF DNA (PCR-2). NO** ***CYP2D6A*** **ALLELE WAS FOUND IN THE ETHIOPIAN POPULATION STUDIED AS SHOWN BY CORRESPONDING REGIONS WITHOUT BANDS. EXTREME END LANES REPRESENT MOLECULAR WEIGHT MARKER VIII. UPPER AND LOWER PANELS REPRESENT DIFFERENT DNA SAMPLES**



(iv). Analysis of exon 1 (C_{188}-->T):

The exon 1 (C_{188}-->T) mutation commonly found among Orientals, and responsible for the higher MR in Oriental EMs is also found in the Ethiopian population. This was analyzed using two sets of primers in the PCR 1 (see section 4.3.1.). In addition to the 115 subjects that were phenotyped for debrisoquine, 7 additional subjects were also analyzed for exon 1 mutation but not phenotyped. Twenty two subjects were found heterozygous for this mutation. One individual was found to be homozygous mutated but we were unable to trace him for phenotyping. The allele frequency of the exon 1 mutation in the Ethiopian population is 9.8 %. Figure 13 shows the debrisoquine MR frequency distribution with respect to *CYP2D6* exon 1 mutation. Table 8 summarizes the frequencies of PCR detectable *CYP2D6* mutant alleles as compared to those of other ethnic groups in which the mutation is more common.

5.3. *CYP2C19* PHENOTYPE

S-mephenytoin hydroxylase polymorphism was analyzed in 114 Ethiopians (for some unknown reason, it was impossible to measure S-MP concentration from the urine sample of the remaining one subject) using racemic mephenytoin as a probe drug. The frequency distribution of the urinary S/R ratio obtained is shown in figure 14. The distribution curve seems to be trimodal dividing the population in three groups with S/R values as follows: 0.05 - 0.25, 0.25 - 0.75 and 0.9 - 1.2. Eight subjects had S/R ratio > 0.9. After acid treatment and reanalysis of these urine samples two of them showed an increase in S/R ratio (from 1.066 and 0.972 to 6.176 and 2.178 respectively), a characteristic associated with EMs. They are therefore assigned as EM phenotype. Three female and three male subjects are assigned as PM. The incidence of poor metabolisers of S-mephenytoin is 5.3% (6 out of 114). The lowest S/R ratio detected was 0.004.

Figure 13. FREQ
MET
MUT

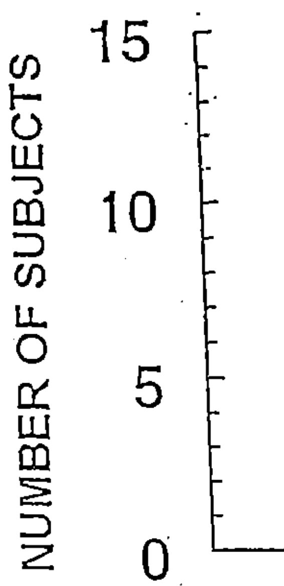


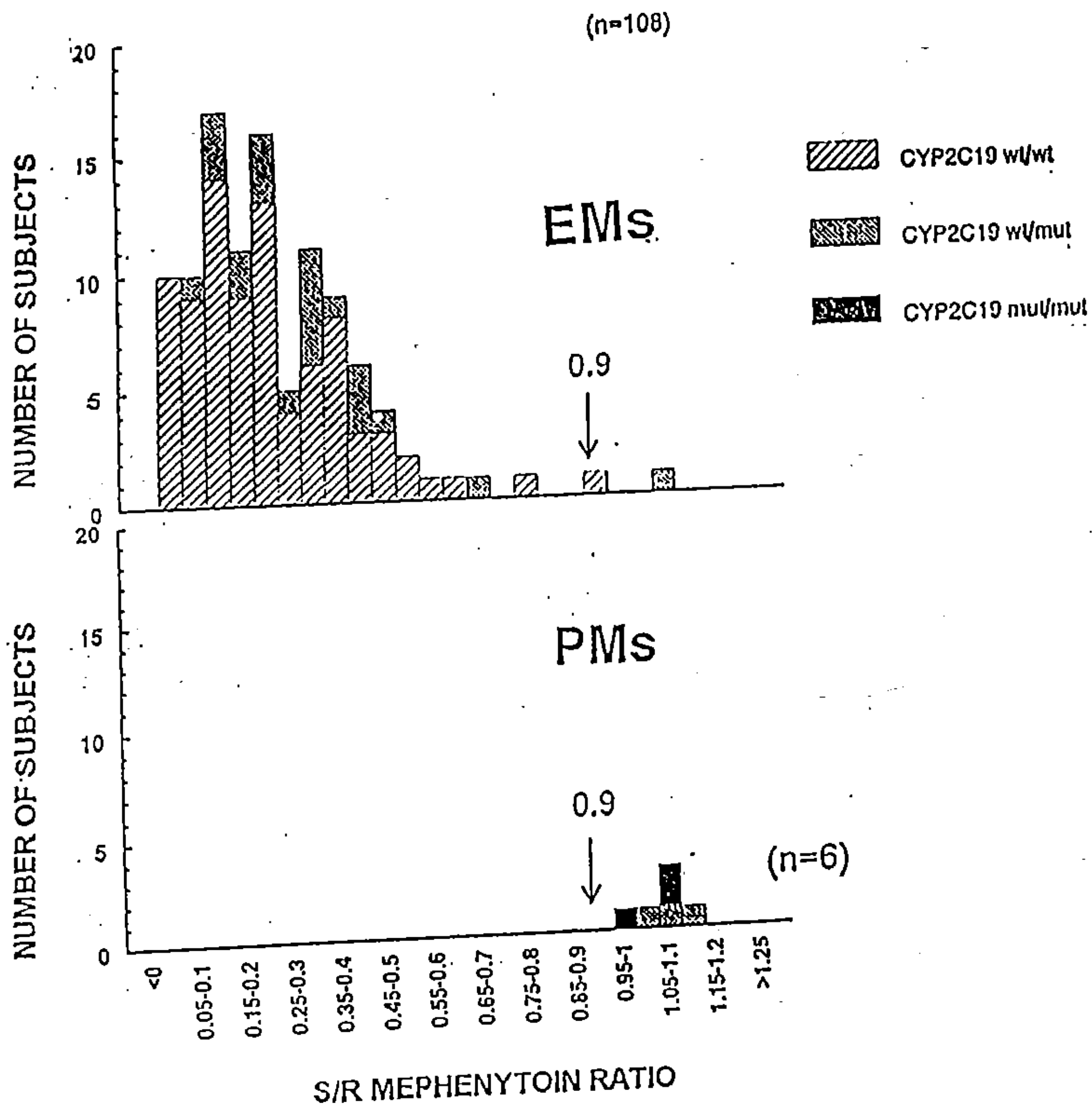
Table 8.

CYP2D6 ALLELE	FRE WH CO
CYP2D6A	CAU
CYP2D6B	CAU
EXON 1 MUTATION	ORI
CYP2D6Z	ZIM

wt: Wild Type

mt: Mutated

Figure 14. *CYP2C19* GENOTYPES IN EXTENSIVE METABOLISERS AND POOR METABOLISERS OF S-MEPHENYTOIN



5.4. *CYP2C19* GENOTYPE

All subjects were investigated for ***CYP2C19m1***. The result of this genotype analysis is summarized in table 9. The S/R ratio median value for homozygous and heterozygous EMs excluding those that had S/R ratio > 0.9 is 0.19 and 0.32 respectively. The heterozygous EMs had S/R ratio higher than the homozygous subjects. There is a statistically significant difference between them ($P < 0.04$) using Mann-Whitney two-tailed t-test, indicating gene dose-dependent hydroxylation of the drug. The allele frequency of ***CYP2C19m1*** is 13.6%. Three out of the 6 PMs were heterozygous (***CYP2C19m1/CYP2C19wt***) and the remaining three homozygous mutated (***CYP2C19m1/CYP2C19m1***). Only these 6 PMs were investigated for ***CYP2C19m2*** (see figure 15). Those who were heterozygous wt for ***CYP2C19m1*** were also found to be heterozygous for ***CYP2C19m2***, whereas those homozygous mt for ***CYP2C19m1*** were found to be homozygous wt for ***CYP1C19m2***. Thus three of the PMs contain a combination of the two ***CYP2C19*** defective alleles (***CYP2C19m1/CYP2C19m2***) and the remaining three are homozygous ***CYP2C19m1*** mutated (***CYP2C19m1/CYP2C19m1***).

6. DISCUSSION

CYP2D6

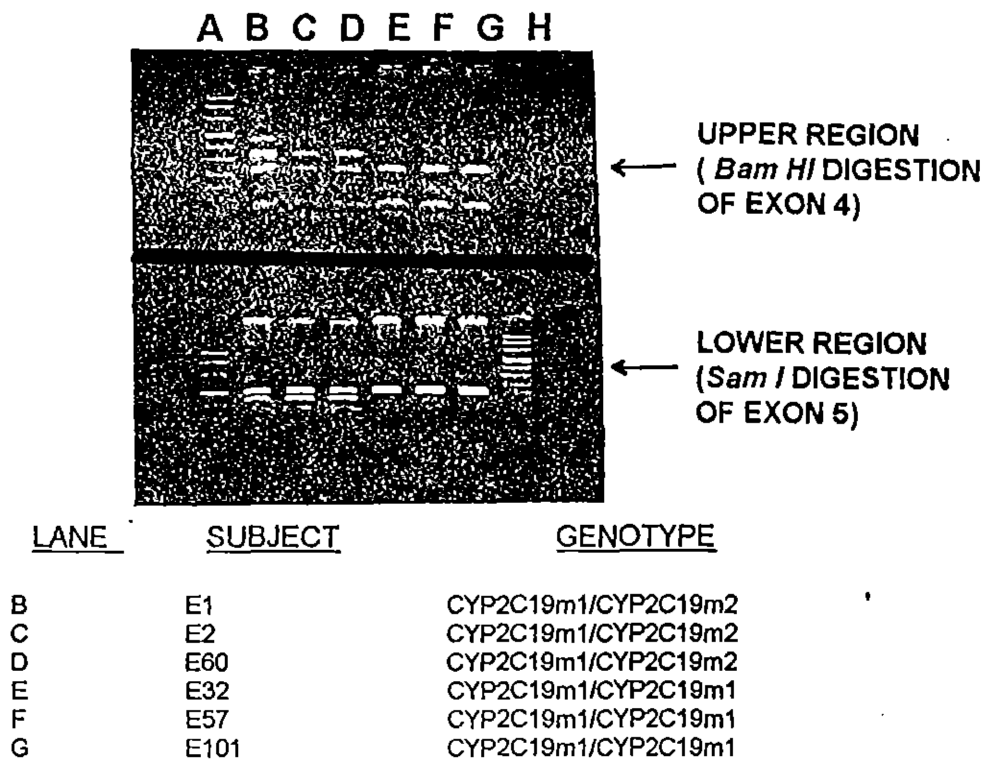
Two out of 115 subjects were identified as PMs of debrisoquine since they had metabolic ratios greater than 12.6. The prevalence of CYP2D6 PM phenotype is thus 1.74%. Figure 8 shows the frequency distribution of the $\log_{10}$ metabolic ratio among

Table 9. SUMMARY OF *CYP2C19m1* GENOTYPING RESULTS FOR 114 ETHIOPIANS

GENOTYPES	NUMBER OF SUBJECTS	NUMBER OF EMs	NUMBER OF PMs
wt/wt	86	86	0
wt/mt	25	22	3
mt/mt	3	0	3
TOTAL	114	108	6

wt: wild type
mt: mutated
EMs: Extensive metabolisers
PMs: Poor metabolisers

FIGURE 11. GENOTYPE ANALYSIS OF *CYP2C19m1* AND *CYP2C19m2* OF SIX ETHIOPIAN PMs OF S-MEPHENYTOIN.
THE CORRESPONDING PCR PRODUCT WAS DIGESTED WITH *Smal* AND *Bam*HI RESPECTIVELY. UPPER PANEL SHOWS CLEAVAGE PATTERN OF BamHI DIGESTED PCR AMPLIFIED DNA (*CYP2C19m2* ANALYSIS) AFTER AGAROSE GEL ELECTROPHORESIS. LOWER SHOWS CLEAVAGE PATTERN OF SamI DIGESTED PCR AMPLIFIED DNA (*CYP2C19m1* ANALYSIS) FROM THE SAME SUBJECTS. LANES (A) AND (H) MOLECULAR WEIGHT MARKERS.



LANE	SUBJECT	GENOTYPE
B	E1	CYP2C19m1/CYP2C19m2
C	E2	CYP2C19m1/CYP2C19m2
D	E60	CYP2C19m1/CYP2C19m2
E	E32	CYP2C19m1/CYP2C19m1
F	E57	CYP2C19m1/CYP2C19m1
G	E101	CYP2C19m1/CYP2C19m1

Ethiopians. The debrisoquine MRs are trimodally distributed with the larger part of the population located in the MR interval 1 – 10. Apparently, the distribution histogram is skewed to the right indicating that most of the Ethiopian EMs have a higher MR compared to the case with Caucasian populations. The genotypes of the two PMs, E#11 (MR = 45.12; 29/29), is B/wt and E#89 (MR = 13.2, 44/29) exon 1 mut/wt. These genotypes are however not considered to be responsible for the PM status of these individuals. It is therefore proposed that another as yet unidentified mutation could be responsible for this status in the Ethiopian population.

The genetics of ***CYP2D6*** among Ethiopians appear to be significantly different from that of Caucasians. Apart from the significantly lower frequency of PMs, the 44kb *Xba*I RFLP fragment appears not to be associated with the B type mutation and therefore not linked with the PM phenotype status. The low prevalence of PMs of debrisoquine among Ethiopians is within the range (0–2%) of PM phenotype observed in other Black populations studied to date (Relling *et al.*, 1991; Masimirembwa *et al.*, 1995). As in other African and American Black populations, the low prevalence of PMs among Ethiopians is due to the lower frequency of ***CYP2D6*** mutant allele that predict the PM phenotype. The allele frequencies of ***CYP2D6B*** and ***CYP2D6A*** alleles was found to be 1.3% and 0% respectively.

The exon 1 (C_{188}-->T) mutation which is very common among Orientals (Lee *et al.*, 1994), is strongly associated with a higher MR in EMs. This mutation was found to be the determinant for the reduced activity of CYP2D6ch and explains the right shift of the frequency distribution histogram in Orientals (Johansson *et al.*, 1994). There has

been no reported case of exon 1 mutation in a Black population. It is reported here, on the basis of this investigation, that the exon 1 mutation does occur in the Ethiopian population with an allele frequency of 9.8%. Unlike in Orientals, the exon 1 mutation seems to have a wider spread among the Ethiopian EMs (see figure 9). It appears as if this mutation is not responsible for the right shift in the frequency distribution histogram observed among Ethiopians. This however is yet to be fully determined.

The *Xba*I 44kb allele is very common (37%) in Oriental EMs and is associated with higher debrisoquine MR (Johansson *et al.*, 1991). The Chinese 44kb allele contains two ***CYP2D6*** genes designated ***CYP2D6ch1*** and ***CYP2D6ch2*** with reduced enzyme activity. Genetic analysis of the Chinese *CYP2D6* locus implicated the exon 1 C_{188}-->T mutation as the cause for the diminished capacity for debrisoquine hydroxylation (Johansson *et al.*, 1994). Three Ethiopian subjects were identified as having the 44kb allele. Although, as in Orientals, the Ethiopian 44kb haplotype is associated with higher MR and also contains an exon 1 mutation, the two populations differ significantly in the frequency of this allele being higher in Chinese (37%) compared to 1.3% in Ethiopians.

The ***CYP2D6C*** allele which has a codon deletion in exon 5, has been identified in a Caucasian liver with deficient metabolism. It is estimated to account for about 1.5% of ***CYP2D6*** alleles in Caucasians. No corresponding frequency of the ***CYP2D6C*** allele has as yet been reported in any African population. ***CYP2D6C***, like exon 1 mutation has a reduced activity, but it is not associated with the PM phenotype (Tyndale *et al.*, 1991; Lee *et al.*, 1994). All subjects that had MR > 1 were analyzed for ***CYP2D6C*** with the

view to seeing if this allele is the cause for the higher MR among the Ethiopian EMs. No such allele was found among the Ethiopians investigated.

Another interesting result arising from this study is the demonstration of a very high prevalence among Ethiopians of a duplicated *CYP26D6* gene as confirmed by the *Xba*I 42 kb allele. This occurs to a significantly higher frequency (12.9%) than in any other populations studied to date. The 42 kb allele frequency in Caucasians is between 1–7% (Dahl *et al.*, 1994; Agùndez *et al.*, 1995). Among Zimbabweans it is 2.2% (Masimirembwa *et al.*, 1993). In Caucasians the allele contains two duplicated or amplified functional ***CYP2D6*** genes which predicts the ultra rapid metabolizer phenotype. In the Zimbabwean black subjects studied, the ***CYP2D6B*** mutation is associated with the 42 *Xba*I haplotype. It has been suggested that the Zimbabwean 42kb *Xba*I allele may contain a duplicated non functional ***CYP2D6*** gene (Masimirembwa *et al.*, 1993). In contrast to the Zimbabwean experience the Ethiopian 42kb allele is not associated with the ***CYP2D6B*** mutation. It is found to contain two functional ***CYP2D6*** genes. All the three ***CYP2DB*** alleles found in this study are associated with the 29/29 *Xba*I Haplotype.

Figure 10 shows that subjects carrying the 42/42 genotype are associated with lower debrisoquine MR compared to those with 29/42 and 29/29 genotypes. The implication is that the duplication or amplification of a functional ***CYP2D6*** gene is responsible for the higher hydroxylation capacity of the enzyme which then predicts the rapid metabolizer phenotypes. Surprisingly, the MR of Ethiopian subjects carrying the 42kb *Xba*I allele and those subjects carrying multiduplicated copies of the *CYP2D6* gene, is not as low as that for Caucasians. The Caucasian 42kb haplotype is associated with

extremely high CYP2D6 activity as indicated by the very low debrisoquine MRs (<0.2) (Dahl *et al.*, 1995). The indications are that the Ethiopians 42kb *XbaI* may be somehow different from that of Zimbabweans and Caucasians. It was also possible to identify a new ***CYP2D6*** allele in the Ethiopian population carrying four and even five active ***CYP2D6*** genes.

From the distribution histogram of debrisoquine, obtained for Caucasians and Chinese (see figure 3), the right shift of the curve in the case of Chinese EMs is rather obvious. Chinese EM subjects metabolize CYP2D6 substrates more slowly than do Caucasians. This is supported by the fact that they generally have higher debrisoquine MRs. Genetic analysis of the Chinese ***CYP2D6*** locus as revealed by the frequency distribution of the exon 1 mutation C_{188}-->T explained the lower capacity among them to metabolize drugs that are substrate of CYP2D6 (Johansson *et al.*, 1994). Similarly the distribution curve obtained for Ethiopians and for some other Black populations studied (see figures 4 and 7) is shifted to the right indicating that these EMs have higher MR.

Recently, the presence of a ***CYP2D6*** mutation, the ***CYP2D6Z*** allele (frequency> 40%) has been reported among Zimbabweans. ***CYP2D6Z*** has a mutation in exon 2 causing a Thr_{107} ---> Ile amino acid substitution that gives rise to an unstable enzyme (Masimirembwa *et al.*, 1995). This was reported to be responsible for the right shift of the Zimbabwean distribution curve and explains the slow metabolism of substrates of CYP2D6 (Masimirembwa *et al.*, 1995). The allele frequency of ***CYP2D6Z*** among Ethiopians is 11.2% meaning again that the presence of ***CYP2D6Z*** in Ethiopians cannot alone explain the right shift of the distribution curve for Ethiopians. It is therefore

suggested that another, as yet unidentified, mutation among Ethiopians could be responsible for the trimodality and right shift of the distribution curve. This calls for further investigation including sequence analysis of the Ethiopians *CYP2D6* locus. This study has demonstrated new *CYP2D6* variant alleles among Ethiopians namely the 66kb and 78kb *Xba*I haplotypes. These suggest the presence of four and five active copies of the ***CYP2D6*** gene in tandem and indicate that duplicated (42kb) and multiduplicated (54kb, 66kb, and 78kb) ***CYP2D6*** genes are of common occurrence among Ethiopians. These observations may have given a clue to the genetic heterogeneity of African Black populations with respect to ***CYP2D6*** pointing to the fact that Blacks should not be viewed as one homogeneous entity, and that extrapolation and application of results obtained in one Black population to another need to be seriously discouraged.

CYP2C19

In this study 114 healthy unrelated Ethiopian subjects were phenotyped for CYP2C19 activity using S-mephenytoin as the probe drug. Figure 14 shows the frequency distribution histogram with respect to S/R ratio. The EM status had earlier been confirmed by the increased S/R value after acid treatment of urine samples. The prevalence of **CYP2C19** PMs in the population is 5.3%. This compares with the result obtained with Caucasians (2–5%) and some Black population studies (Zimbabweans, 4%) and is in contrast to the Oriental (18–23%). The result of this study is inconsistent with the higher incidence (18%) of PMs found in Black Americans (Pollock *et al.*, 1991) as well as in Kenyans (35%) (Watkins *et al.*, 1990) and the lack of bimodality in Nigerians

(Iyun *et al.*, 1990). This stresses the heterogeneous nature of the different African populations.

Impaired metabolism of S-mephenytoin and other drugs whose metabolism co-segregates with S-mephenytoin is caused by the genetic defect in **CYP2C19**. So far two mutant alleles of *CYP2C19* that account for the PM phenotype are described. ***CYP2C19m1*** is a G-->A transition in exon 5 of *CYP2C19* that produce an aberrant splice site resulting in the production of a non-functional protein. The ***CYP2C19m1*** mutant allele accounts for 75% of the defective alleles in both Caucasians and Orientals (deMories *et al.*, 1994a) as well as in Zimbabweans (Masimirembwa *et al.*, 1994). ***CYP2C19m1*** also accounts for the 9 out of 12 (75%) defective alleles in Ethiopian PMs. This suggests that this mutation is the major *CYP2C19* gene defect responsible for the PM phenotype in many ethnic groups.

The second *CYP2C19* gene defect responsible for the PM phenotype is *CYP2C19m2*. It is a single base pair mutation (G-->A) at position 636 of exon 4 of *CYP2C19* which creates a premature stop codon. *CYP2C19m2* has not been detected in Caucasian and Zimbabweans PMs. It has however been found in Orientals where it accounts for the remaining 25% of defective alleles in the PMs (deMorais *et al.*, 1995b). As in Oriental populations *CYP2C19m2* exists in Ethiopian populations and accounts for the 25% (3 out of 12 alleles) of the defective alleles in the poor metabolizers. ***CYP2C19m1*** and *CYP2C19m2* thus apparently account for about 100% of the available PMs among Ethiopians. The relative proportions between the frequencies of the defect alleles m1 and m2 is similar among Ethiopians and Orientals, but the frequency of PMs

is about threefold higher in Orientals. This study supports the view that predictive value of *CYP2C19* genotype analysis as a phenotype determinant among Ethiopians is strongly suggestive.

Although the sample size may not have been large enough and might not be representative of all the different tribes in Ethiopia, this study nevertheless provides a clue to the genetic status of the two important and genetically polymorphic drug metabolizing enzymes, CYP2D6 and CYP2C19 among Ethiopians.

7. CONCLUSION.

Expression of **CYP2D6** and **CYP2C19** is polymorphic, displaying large inter-individual variation among Ethiopians. The prevalence of the poor metabolizer phenotype of **CYP2D6** is lower compared to Caucasians, it is within the range (0–2 %) as found among Orientals, American Blacks and some other Black Africans. The low incidence of PM among Ethiopians is the result of lower prevalence of *CYP2D6* defective alleles. *CYP2D6* genotype analysis of Ethiopian PMs indicated that they are not homozygous for the known *CYP2D6* defective alleles that predict the PM phenotype. It is likely that there is another Ethiopian mutation, yet to be identified, in the *CYP2D6* locus. Also the cause for the majority of the EMs having a higher MR remains to be investigated.

In conclusion it has been shown that Ethiopians differ in a pronounced manner compared to other ethnic groups in the constitution of the *CYP2D* locus. In particular, an extremely high frequency of subjects carrying duplicated or multiduplicated active *CYP2D6* genes was found, indicative of very rapid drug metabolism. This fact has to be considered during drug therapy using substrates for CYP2D6, since therapeutic drug levels

will not be reached at ordinary doses in these subjects. Furthermore, the mean metabolic ratio for debrisoquine in the major part of the population is higher than in Caucassians, indicating lower capability for drug metabolism among the majority of subjects

The prevalence of PM phenotype of **CYP2C19** among Ethiopians is comparable to that of Caucasians and Zimbabweans but is in contrast to other Black populations and Orientals. There is a good correlation between the genotype and the phenotype analysis of **CYP2C19.** The incidence of PMs among Kenyans and American Blacks is higher. Lack of bimodality has been shown in Nigerian populations, confirming the heterogeneity of Blacks.

8. APPENDICES

APPENDIX I. INFORMATION TO VOLUNTEERS

Determination of the metabolic capacity of Debrisoquine and S-mephenytoin

Many drugs are metabolized in the body, preferentially in the liver, before excretion in urine. The metabolic capacity for a number of drugs is subject to a high degree of interindividual variation which will influence the rate of elimination of the drugs. There are pronounced interethnic differences in the relative number of people that have increased or, alternatively, very diminished ability to metabolize drugs.

Debrisoquine, an antihypertensive drug, and S-mephenytoin, an anticonvulsant drug are metabolized and the products eliminated in the urine. The capacity to metabolize these two drugs is genetically determined and can be measured by a simple test procedures. About 7% of Caucasians belong to the group of poor metabolizers (PM) debrisoquine. The corresponding percentage of poor metabolizers among Africans is low. Studies on the metabolism of these drugs have been carried out in a number of countries, it is our intention to investigate the metabolism of these drugs among Ethiopians.

Debrisoquine (10 mg), a Roche product and S-mephenytoin (100 mg), are swallowed together with a glass of water just before bed time. The urinary bladder is emptied before the tablets are taken. All urine will be collected from 0 to 8 hours after intake for phenotype analysis. Ten ml of blood will be also taken by a qualified personnel for the determination of genetic characteristics of individuals to metabolize the drugs.

Extensive research work done by many groups revealed that there is very little side effect associated with the use of debrisoquine and S-mephenytoin. In very rare cases however, debrisoquine may cause dizziness when a person rises too quickly, this side effect disappears within a couple of hours. The puncture of the vein during the blood drawing operation is associated with very little pain lasting only a few seconds.

Your participation in this study is voluntary and you can interrupt the trial at your own volition whenever you feel like it and without having any reason whatsoever. Your contribution towards this exercise is deeply appreciated. Without Your cooperation only some of this study would have been possible.

RECEIVE OUR SINCEREST THANKS

APPENDIX II. ISOLATION AND PURIFICATION OF GENOMIC DNA.

1. 10 ml blood sample was transferred to a falcon tube and 15 ml of Solution A (see below) was added. The resultant mixture was shaken and centrifuged at 4K for 10 min.

2. The supernatant was discarded, the pellet homogenized and 10 ml of solution B (washing buffer) was added. The mixture was shaken and centrifuged at 2.5K, for 10 min.

3. Supernatant was discarded and the pellet (nuclei fraction) washed again with 5 ml of solution B and homogeneously suspended by a whirlimixer.

4. 10 ml Guanidium isothiocynate (GT) solution was added to the above homogenate which was incubated at 37°C for 15 min. 0.7 ml of 7.5 M Ammonium acetate (AmAc) was added, the mixture shaken and incubation continued for 2 hrs. The tubes were shaken once each half hour.

5. The DNA was precipitated with 10 ml of 95% cold Ethanol (EtOH), by gently turning the tube upside down. The precipitate was washed with 70% cold EtOH and the DNA dissolved overnight in 2 ml of TE-buffer.

6. One ml of 5 M NaCl solution was added followed by shaking in water bath for 30 min then cooled on ice for 30 minutes. After centrifugation at 2.5K for 15 min, the supernatant was poured into a new tube and 300 µl of 3M sodium acetate (NaAc), pH 5.2 was added with gentle mixing.

7. The DNA was precipitated with 5 ml of 95% cold EtOH, washed with 70% cold EtOH and allowed to dissolve in 0.7 ml TE-buffer at room temperature overnight. DNA concentration was measured by reading absorbance at 260 nm using a UV spectrophotometry.

Solution A (lysis buffer)

0.32 M sucrose

10 mM TRIS-HCl, pH 7.6

5 mM EDTA

5 % Triton X-100

Solution B (washing buffer)

0.32 M sucrose

10 mM TRIS-HCl, pH 7.6

5 mM EDTA

GT solution

4.2 M Guanidium-isothiocynate

10 mM TRIS-HCl, pH 8.0

10 mM EDTA

2 % (w/v) Sarcosyl.

APPENDIX III. CLEAVAGE OF GENOMIC DNA WITH RESTRICTION ENDONUCLEASE *Eco*RI or *Xba*I.

1. 7 μg of DNA, 10 μl of 10X–H buffer, 28 unit of the restriction enzyme and sterile water to 100 μl was mixed gently in an eppendorf tube and incubated at 37°C overnight.

2. The volume was then reduced to 15–20 μl with SpeedVac and 5 μl of loading buffer was added, the digested DNA was subjected to agarose gel electrophoresis (0.5% or 0.85% agarose at o.5 V/cm for 5 days or 1V/cm for 72hrs. respectively) in 1 X TAE–buffer.

TAE–Buffer 50X

1.6M TRIS–BASE
0.8M $Na(CH_3COO).3H_2O$
40mM $EDTA{-}Na_2.2H_2O$
pH adjusted to 7.2 with glacial Acetic acid

APPENDIX IV. SOUTHERN BLOTTING OF DNA.

1. Alkaline blotting of the gel was effected in 0.4 M NaOH and 0.6 M NaCl overnight using Quiabrane membrane. The membrane was neutralized in 0.5 M TRIS-HCl, pH 7 and 1M NaCl and baked at 80°C in an oven in order to fix DNA to the membrane.

2. The membrane was subsequently prehybridyzed in a prehybridization buffer containing the following final concentrations: 6X SSC, 10X Denhardt's solution, 1% SDS, 100μl/ml of ssDNA (ssDNA was boiled 10 min just before use) and water to a total volume of 20 ml for hybridizing one membrane. The prehybridization was performed in a Hybridization mini oven at 61°C for 2hr.

3. The membrane was further hybridized with ^{32}P-labelled **CYP2D6** cDNA probe in a hybridization buffer containing final concentration of 6X SSC, 1% SDS, 5% Dextran Sulfate and water to a volume of 15 ml for one membrane. Hybridization performed at 61°C over night. The probe was denatured by heating at 95°C for 5 min prior to addition to the hybridization buffer.

4. After hybridization the membrane was washed once in 2X SSC, 0.1% SDS, twice in 0.1X SSC, 0.1% SDS, and once in 0.1X SSC each time at 61°C for 30 min.

5. The different restriction fragment length patterns obtained were analyzed after autoradiography. The films were exposed for 2-7 days at 70°C.

SSC 25X

3 M NaCl

0.3 M Na_3.citrate.2H2O

Water to make 1000 ml.

PH adjusted with 1 m HCL to 7.0

Denhardt's solution 50X

5 gm Ficoll 400 (type 400, Pharmacia)

5 gm polyvinyl pyrolidone

5 gm bovine serum albumen

Water to make 500 ml.

APPENDIX V. PREPARATION AND ^{32}P-LABELLING OF CYP2D6 cDNA PROBE

1. CYP2D6 cDNA was diluted to a concentration of 5 ng/µl in 10mM TE buffer.

2. Primers (5µl) were added to 5µl of cDNA in a microcentrifuge tube. The mixture was denatured by heating to 95–100°C for 5 minutes in a boiling water bath.

3. The tube was briefly spun down and kept at room temperature for 10 minutes.

4. 10µl of labelling buffer, 2µl of enzyme and 5µl of α–^{32}P–dCTP was added and the mixture incubated at 37°C for 10 minutes.

5. The reaction was stopped by the addition of 5µl of 0.2 M EDTA and the labelled cDNA purified using Sephadex G–50 column chromatography

6. The labelled cDNA was denatured by heating to 95–100°C for 5 minutes before use.

APPENDIX VI. SSCP ANALYSIS OF *CYP2D6C*

1. Preparation of Polyacrylamide Gel. To a 250ml Vacuum bottle, 25.2 ml of acrylamide-bisacrylamide (49:1) solution, 11 ml of glycerol-water (1:1), 12 ml of 10X TBE and 11.5 ml of water were mixed and degassed for 10 min
2. 42µl of TEMED and 420µl of 10% freshly prepared APS was added and mixed. The mixture was quickly casted on a two dimensional gel electrophoresis apparatus (protein slab gel,Bio-Rad).
3. After polymerization the gel was equilibrated by keeping in the cold room (4°C) overnight. 5µl of the second PCR product was mixed with 5µl of the stop solution (9.6 ml 95% formamide, 5ml 0.05% bromophenol blue, 5ml xylene cynanol FF and 0.4ml of 0.5M EDTA) in an eppendorf tube. The mixture was heated to 95–100°C in a boiling water bath and immediately the tube was transferred and kept on ice. 8µl of it was applied on the gel for electrophoresis in the cold room at 270 V for 4 hrs.

TBE-buffer 10X

Tris base 0.5M

Boric acid 0.5M

EDTA.Na_2.H2O 10mM

Solutions used for Silver staining

The following solutions were prepared just before use.

1. **Solution 1. (0.5 litre)**

   Methanol.................250 ml

   Glacial acetic acid.......60 ml

   37% formaldehyde.........250 µl

   Water.................189.75 ml

2. **50% EtOH**

3. **$Na_2S_2O_3.5H_20$ 200 mg/l**

4. **Solution 2.(200ml)**

   $AgNO_3$.............0.4 gm

   37% Formaldehyde...150 µl

5. **Solution 3. (0.5 litre)**

   Na_2CO_3.............30 gm

   37% Formaldehyde...250 µl

   $Na_2S_2O_3.5H_20$.........2 mg

6. **50% MeOH**

7. **Solution 4.**

   MeOH...............50%

   AcO_2H.............12%

Silver staining of polyacrylamide gel

1. The gel was fixed in solution 1 overnight

2. Solution 1 was discarded and the gel was rinsed twice with 200 ml 50% EtOH for 20 min and then with 30% EtOH on a shaker.

3. The 30% EtOH solution was discarded. 200 ml $Na_2S_2O_3$ solution was added and the gel shaken for 1 min. The $Na_2S_2O_3$ solution was discarded and the gel was carefully washed three times with 200 ml of distilled water for 20 sec.

4. 100 ml of solution 2 was added to the gel and it was shaken 20 min.

5. The gel was washed with 200 ml of distilled water for 20 sec, 200 ml of solution 3 was added and it was shaken for 10 min.

6. The reaction was stopped by the addition of 200 ml of solution 4 followed by further shaking for 10 min.

7. Finally the gel was incubated with 50% MeOH on a shaker for 20 min, sealed with a transparent sheet and vacuum dried.

APPENDIX VII. EXTRACTION OF DEBRISOQUINE AND 4-OH DEBRISOQUINE FROM URINE SAMPLES.

1. Urine was allowed to thaw and 1 ml of it was placed in glass tube.

2. 0.5 ml of $NaHCO_3$, 0.5 ml of methanol, 50µl of internal standard (0.1 µg/µl guanoxanehemisulfate) and 0.5 ml of acetyl acetone were added.

3. The mixture was shaken and incubated in a 50°C water bath for 16 hrs.

4. The reaction mixture was extracted with 6 ml of diethylether for 5 min.

5. The ether phase was carefully separated and back-extracted with 0.3 ml 4M HCl by vortex mixing for 15 sec.

6. The water phase was transferred to a new glass tube and shaken in a 56°C water bath in order to allow the organic phase evaporate.

7. 0.4 ml of 4M NaOH was added and vortex-mixed.

8. 150 µl of CS_2 was added. The mixture was vortex mixed for 15 sec. and centrifuged at 3000 RPM for 5 min.

9. 2µl of the CS_2 phase was injected for gas chromatographic analysis.

10. The peak area ratio of debrisoquine to guanoxan or of 4-OH debrisoquine to guanoxan was calculated and the concentration ratio of debrisoquine to 4-OH debrisoquine obtained.

REFERENCES:

Alvan, G., Bechtel, P., Iselius, L. and Gundert-Remy, U. (1990). Hydroxylation polymorphism of debrisoquine and mephenytoin in European populations. Eur. J. Clin. Pharmacol. **39**; 533–537.

Adedoyin, A., Prakash, C., O'shea, D., Bilair, A. and Wilkinson, G. R. (1994). Stereoselective disposition of hexobarbital and its metabolites: relationship to the S-mephenytoin polymorphism in Caucasians and Chinese subjects. Pharmacogenetics. **4**; 27–38.

Agùndez, J. A. G., Ledesma, M. C., Ladero, J. M., and Benitez, J. (1995) Prevalence of CPY2D6 gene duplication and its repercussion on the oxidative phenotype in a white population. Clin. Pharmocol. Ther. **57**; 265–269.

Andersson, T., Miners,J. O., Veronese, M. E. and Birkett, D. J., (1994) Diazepam metabolism by human liver microsomes is mediated by both S-mephenytoin hydroxylase and CYP3A isoforms. J. Clin. Pharmacol. **38**; 131–137.

Bar-Nun, S., Kreibich, J., Adesnik, M., Alterman, L., Negishi, M. and Sabatini, D.D.(1980) Synthesis and insertion of cytochrome P-450 into endoplasmic reticulum membranes. Proc. Natl. Acad. Sci. U.S.A. **77**; 965–969

Bertilsson, L., Dahl, M. L., Sjoqvist, F., Aberg-Wistedt, A., Humble, M., Johansson, I., Lundqvist, E. and Ingelman-Sundberg, M. (1993) Molecular basis for rational megaprescribing in ultrarapid hydroxylators of debrisoquine. Lancet. **341**; 63.

Black, S.D.(1992) Membrane topology of the mammalian P450 cytochromes. The FASEB J. **6**; 680–685.

Blum, H., Beier, H. and Gross, H.J. (1987) Improved silver staining of plant proteins, RNA, and DNA in polyacrylamide gels. Electrophoresis **8**; 93–99.

Broly, F., Gaedik A., Heim, M., Eichelbaum, M., Morike, K. and Meyer, U. A. (1991) Debrisoquine/Sparteine hydroxylation genotype and phenotype: analysis of common mutations and alleles of CYP2D6 in a European population. DNA Cell Biol. **10**; 545–558.

Brosen, K. (1993) Isozyme specific drug oxidation:genetic polymorphism and drug-drug interactions. Nord. J. Psychiatry.**47**; Suppl 30: 21–26.

Chiba, K., Kobayashi, K., Manabe, K., Tani, M., Kamataki, T. and Ishizan, T.(1993) Oxidative metabolism of omeprazole in human liver microsomes. co-segregation with S-mephenytoin 4-hydroxylation. J. Pharmacol. Exp. Ther. **266**; 52-59.

Dahl, M. L., Johansson, I., Palmeretz, M. P., Ingelman-Sundberg, M., Sjöqvist, F. (1992) Analysis of the CYP2D6 gene in relation to debrisoquine and desipramine hydroxylation in a swedish population. Clin. Pharmocol. Ther. **51**; 12-17.

Dahl, M. L. and Bertilsson, L. (1993) Genetically variable metabolism of antidepressants and neuroleptic drugs in man. Pharmacogenetics. **3**; 61-70.

Dahl, M. L., Bertilsson, L., Ingelman-Sundberg, M., Lundqvist, E. and Sjoqvist, F. (1993) Molecular basis of drug oxidation polymorphisms. Nord. J. Psychiatry. **47**; suppl **30**: 27-31.

Dahl, M. L., Johansson, I., Bertilsson, L., Ingelman-sundberg, M. and Sjoqvist, F. (1995) Ultra rapid hydroxylation of debrisoquine in a Swedish population. Analysis of the molecular genetic basis. J.Pharmacol. Exp. Thr. (In press).

deMorais, S.M.F., Wilkinson, G.R., Blaisdell, J., Nakamura, K., Meyer, U.M. and Goldstein, J.A. (1994 a) The major genetic defect responsible for the polymorphism of S-mephenytoin metabolism in humans. J. Biol. Chem. **269**; 15419-15422.

deMorais, S.M.F., Wilkinson, G.R., Blaisdell, J., Meyer. U.M., Nakamura, K. and Goldstein, J.A. (1994b) Identification of a new genetic defect responsible for the polymorphism of (S)-mephenytoin in Japanese. Mol. Phar. **46**; 594-598.

Duche, J. C., Joanne, C., Barre, J., deCremoux, H., Dalphin, A., Brochard, P., Tillement, J. P. and Bechtel, P. (1991) Lack of a relationship between the polymorphism of debrisoquine oxidation and lung cancer. Br. J. Clin. Pharmac. **31**; 533-536.

Eichelbaum, M. (1982) Defective oxidation of drugs. Clin. Pharmacokin **7**; 1-22.

Eichelbaum, M. and Gross, A. S. (1990) The genetic polymorphism of debrisoquine/sparteine metabolism-clinical aspects. Pharmac. Ther. **46**; 377-394

Eichelbaum, M., Spannbrucker, N., Steincke, B. and Dengler, J. J.(1979) Defective N-oxidation of sparteine in man: A new pharmacogenetic defect. Eur. J. Clin. Pharmac. **16**; 183-187.

Evans, D. A., Mahgoub , A., Sloan, T. P., Idle, J. R. and Smith, R. L. (1980) A

family study of the genetic polymorphism of debrisoquine oxidation in a British population. J. Med. Genet. **17**; 102–105.

Evans, W. E., Relling, M. V. (1990) XbaI 16– plus 9–Kilobase DNA restriction fragments identify a mutant allele for Debrisoquine hydroxylase: report of a family study. Mol. Pharmacol. **37**; 639–642.

Evans, W. F., Relling, M. V., Rahman, A., Mcleod, H. L., Scott, E. P. and Lin, J. (1993) Genetic basis for a lower prevalence of deficient CYP2D6 oxidative drug metabolism phenotype in Black Americans. J. Clin. Invest. **91**; 2150–2154

Gaedigk, A., Blum, M., Gaedigk, R., Eichelbaum, M. and Meyer, U. A. (1991) Deletion of the entire cytochrome P450 CYP2D6 gene as a cause of impaired drug metabolism in poor metabolizers of the debrisoquine/sparteine polymorphism. Am. J. Hum. Genet. **48**; 943–950.

Garfinkle, D. (1958) "Studies on pig liver microsomes. I. Enzyme and pigment composition of different microsomal fractions." Arch. Biochem. Biophys. **77**; 493–509.

Goldstein, J. A., Faletto, M.B. Romkes–Sparks, Sullivan, T., Kitareewan, S., Raucy, J. L., Lasker, J. M. and Ghanayem, B. I. (1994) Evidence that CYP2C19 is major (S)–mephenytoin 4–hydroxylase in humans. Biochemistry. **33**; 1743–1752.

Gonzalez, F. J. and Nebert, D. W. (1990)"Evolution of the P450 gene super family: animal plant 'warfare' drive and human genetic differences in drug oxidation". Trends. Genet. **6**; 182–186.

Gonzalez, F. G., Vibois, F., Hardwick, J. P., Mcbride, W., Nebert, D. W., Gelboin, H. V. and Meyer, U. A. (1988) Human debrisoquine 4–hydroxylase: cDNA and deduced amino acid sequence and assignment of the CYP2D locus to chromosome 22. Genomics. **2**; 174–179.

Hanioca, N., Kimura, S., Meyer, U. A. and Gonzalez, F. J. (1990) The human CYP2D6 locus associated with a common genetic defect in drug oxidation: a G>A base change in intron 3 of a mutant CYP2D6 allele results in aberrant 3' splice recognition site. Am J. Hum. Genet. **47**; 994–1001.

Heim, M. and Meyer, U.A. (1992) Evolution of highly polymorphic human cytochrome P450 gene cluster: CYP2D6. Genomics. **14**; 49–58.

Heim, H. and Meyer, U. A. (1990) Genotyping of poor metabolisers of debrisoquine by allele-specific PCR amplification. Lancet. **336**; 529-532.

Hirvonen, A., Husgafvel-Pursiainen, K., Anttila, S., Karjalainen, A., Pelkonen, O. and Vainio, H. (1993) PCR-based CYP2D6 genotyping for finnish lung cancer patients. Pharmacogenetics. **3**; 19-27.

Horai, Y., Nakano, M., Ishizaki, T., Ishikawa, K., Zhou, H., Zhou, B., Liao, C. and Zhang, L. (1989) Metoprolol and mephenytoin oxidation polymorphism in far eastern Oriental subjects: Japanese versus mainland Chinese. Clin. Pharmacol. Ther. **46**; 198-207.

Horsmans, Y., Desager, J. P. and Harvengt, C. (1991) Is there a link between debrisoquine oxidation phenotype and lung cancer susceptibility? Biomed & Pharmacother. 45; 359-362.

Idle, J. R., Mahgoub, A., Sloan, T. P., Smith, R. L., Mbanefo, C. O. and Bababunmi, E. A. (1981) Some observation on the oxidation phenotype status of Nigerian patients presenting with cancer. Cancer Lettres. **11**; 331-338.

Idle, J. R. and Smith, R. L. (1979) Polymorphism of oxidation at carbon centers of drugs and their clinical significance. Drug. Metabol. Rev. **9** **(2)**; 301- 317.

Imai, Y., and Sato, R. (1967) Studies on the substrate interactions with P450. J. Biochem. **62**; 239-249.

Inaba, T., Jurima. M. and Kalow, W. (1986) Family studies of mephenytoin hydroxylation deficiency. Am. J. Hum. Genet. **38**; 768-772.

Ingelman-Sundberg, M. and Johansson, I. (1995) The molecular genetics of the human drug metabolizing cytochrome P450s. Advances in drug metabolism. In press.

Iyun, A. O., Lennard, M. S., Tucker, G. T., Woods, H. F., (1986) Metoprolol and debrisoquine metabolism in Nigerians: Lack of evidence for polymorphic oxidation. Clin. Pharmacol. Ther. **40**; 387-394.

Iyun, A. O., Tucker, G. T., Woods, H. F. and Lennard, M. S. (1990) The 4-hydroxylation of (S)-mephenytoin in Nigerians: A population study. VIIIth International symposium:Microsomes and Drug Oxidations. Stockholm. Ed. Ingelman-Sundberg M, Gustafsson, J. A. and Orrenius, S. Abstr **373**.

Jacqz, E., Hall, S. D., Branch, R.A. and Wilkinson, G.R. (1986) Polymorphic metabolism of mephenytoin in man: Pharmacokinetic interaction with a co-regulated substrate, mephobarbital. Clin. Pharmac. Ther. **39**; 646-653

Johansson, I., Lundqvist, E., Bertilsson, L., Dahl, M, Sjoqvist, F. and Ingelman-Sundberg, M. (1993) Inherited amplification of an active gene in the cytochrome P450 CYP2D locus as a cause of ultrarapid metabolism of debrisoquine. Proc. Natl. Acad. Sci. USA. **90**; 11825-11829.

Johansson, I., Oscarson, M., Yue, Q. Y., Bertilsson, L., Sjoqvist, F. and Ingelman-Sdberg, M. (1994) Genetic analysis of the Chinese cytochrome P4502D locus: characterization of variant CYP2D genes present in subjects with diminished capacity for debrisoquine hydroxylation. Mol. Pharmacol. **46**; 452-459.

Johansson, I., Yue, Q. Y., Dahl, M. L., Heim, M., Sewe, J., Meyer, U. A. and Ingelman- Sundberg, M. (1991) Genetic analysis of the interethnic differences between Chinese and Caucasians in the polymorphic metabolism of debrisoquine and codein. Eur. J. Clin. Pharmacol. **40**; 553-556.

Kagimoto, M., Heim, M., Kagimoto, K., Zeugin, T. and Meyer, U. A. (1990) Multiple mutations of the human cytochrome P450IID6 gene (CYP2D6) in poor metabolizers of debrisoquine. J. Biol. Chem. **265**; 17209-17214.

Kalow, W. (1991) Interethnic variation of drug metabolism. TiPS. **12**; 102-107

Kimura, S., Umeno, M., Sköda, R. C., Meyer, U. A. and Gonzalez, F. J. (1989). The human debrisoquine 4-hydroxylase (CYP2D6) locus: Sequence and identification of polymorphic CYP2D6 gene, and pseudogene. Am. J. Hum. Genet. **45**; 889-904.

Klingenberg,M. (1958) "Pigments of rat liver microsomes." Arch. Biochem. Biophys. **75**; 376-386

Kupfer,A, Brilis, G.M., Watson, J. T. and Harris, T. M. (1980) A major pathway of mephenytoin metabolism in man. Aromatic hydroxylation to p-hydroxy mephenytoin. Drug Metab. Disp. **8**; 1-4

Kupfer, A., Desmond, P. V., Schenker, S. and Branch, R. A. (1979) Family study of a genetically determined deficiency of mephenytoin hydroxylation in man (abstr.) Pharmacologist **21**; 173.

Kupfer, A., Desmond, P. V., Schenker, S. and Branch, R. A. (1982) Stereoselective metabolism and disposition of the enantiomers of mephenytoin during chronic oral administration of the racemic drug in man. J. Pharmacol. Exp. Ther. **221**, 590–597.

Kupfer, A. Roberts, K., Schenker, S. and Branch, R.A.(1981) Stereoselective metabolism of mephenytoin in man. J. Pharmacol. Exp. Ther. **218**; 193–199.

Lee, E.J.D. and Jeyaseelan, K. (1994) Frequency of human CYP2D6 mutant alleles in a normal Chinese population. Br. J. Clin. Pharmac. **37**; 605–607.

Lennard, M.S., Silas, J.H., Smith, A.J. and Tucker, G.T. (1977) Determination of debrisoquine and its 4–hydroxy metabolite in biological fluids by gas chromatography with flame–ionization and nitrogen–selective detection. J. Chromatogr, **133**; 161–166.

Lennard, M. S., Silas, J. H., Freestone, S., Ramsay, L. E., Tucker, G. T., and Woods H. F. (1982). Oxidation phenotype : a major determinant of metoprolol metabolism and response. New Eng. J. Med. **307**; 1558–1560.

Lou, Y., Ying, L., Bertilsson, L. and Sjoqvist, F. (1987) Low frequency of slow debrisoquine hydroxylation in native Chinese population. Lancet. October 10, 852.

Mahgoub, A., Idle, J. R., Dring, L. G., Lancaster, R. and Smith, R. L. (1977) Polymorphic hydroxylation of debrisoquine in man. Lancet. 2; 584–586

Masimirembwa, C., Bertilsson, L., Johansson, I., Hasler, A. H. and Ingelman–Sundberg, M. (1994). Phenotyping and Genotyping of S–mephenytoin hydroxylase (cytochrome P4502C19) in a Shona population of Zimbabwe (in press).

Masimirenbwa, C., Bertilsson, L., Johansson, I., Hasler, A. H., and Ingelman–Sundberg (1995) Phenotype and genotype analysis of *CYP2D6* in a Shona population of Zimbabwe (to be submitted)

Masimirembwa, C. M., Johansson, I., Hasler, J. A., Ingelman–Sundberg, M. (1993) Genetic polymorphism of cytochrome P450 CYP2D6 in Zimbabwean population. Pharmacogenetics. **3**: 275–80.

Matsunaga, E., Ulrich, M., JAMES, P., Hardwick, Harry, V., Meyer, U. A. and Gonzalez, F. J. (1989) Analysis of the molecular basis of the debrisoquine 4–hydroxylasedeficiency in DA rats. Biochemistry. **28**; 7349– 7355.

Meier, U. T., Dayer, P., Male', P., Kronbach, T. and Meyer, U.A. (1985) Mephenytoin hydroxylation polymorphism: Characterization of the enzymatic deficiency in the liver microsomes of poor metabolisers phenotyped *in vivo*. Clin. Pharmacol. Ther. **38**: 488–494.

Mikus, G., Bochner, F., Eichelbaum, M., Horak, P., Somogyi, A. A. and Spector, S. (1994) Endogenous codeine and morphine in poor and extensive metabolisers of the CYP2D6 (debrisoquine/sparteine polymorphism. J. Pharmacol. and Exp. Ther. **268**; 546–551.

Nakamura, K., Goto, F., Ray, W. A., McAllister, C. B., Jacqz, E., Wilkinson, G.R. and Branch, R. A. (1985) Interethnic differences in genetic polymorphism of debrisoquine and mephenytoin hydroxylation between Japanese and Caucasian populations. Clin. Pharmacol. Ther. **38**; 402– 408.

Nebert, D. W. and Gonzalez, F. J. (1987) P450 Genes. structure, evolution and regulation. Ann. Rev. Biochem. **56**; 954–993

Nebert, D. W., Nelson, D. N., Coon, M. J., Estabrook, R. W., Feyereisen, R., Fujii-Kuriyama, Y., Gonzalez, F. J., Guengerisen, F. P., Gunsalus, I. C., Johnson, E. F., Loper, J. C., Sato, R., Waterman, M. R., and Waxman, D. J. (1991) The P450 superfamily: update on sequences, gene mapping, and recommended nomenclature. DNA and Cell Biol. **10**; 1–14.

Nelson, D.R., Kamataki, T., Waxman, D.J., Guengerich, F.P., Estabrook, R.W., Feyereisen, R., Gonzalez, F.J., Coon, M.J., Gunsalus, I.C., Gotoh, O., Okuda, K., and Nebert, D.W. (1993) The P450 superfamily–update on new sequences, gene mapping, accession numbers, early trivial names of enzymes, and nomenclature. DNA Cell Biol. **12** ; 1–15.

Nelson, D.R., Strobel,H.W.(1988) On the membrane topology of vertebrate cytochrome P–450 proteins. J. Biol. Chem. **263**; 6038–6050.

Omura, T. and Sato,R. (1962) A new cytochrome in liver microsomes. J. Biol. Chem. **237**; 1375–1376.

Pollock, B. G., Perel, J. M. Kirshnere, M., Altieri, L. P., Yeager, A.L. and Reynolds, C. F. (1991) S–mephenytoin 4–hydroxylation in older Americans. Eur. J. Clin. Pharmacol. **40**; 609–611.

Relling, M. V., Cherrie, J., Schell, M. J., Petros, W. P., Meyer, W. H. and Evans, W. E. (1991) Lower prevalence of the debrisoquine oxidative poor metabolizer phenotype in American Black versus White subjects. Clin. Pharmacol. Ther. **50**: 308-313

Sambrook, J., Fritach, E. F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Ed. 2. Cold Spring Harbour Laboratory, Cold Spring Harbor, NY, 9.16-9.18.

Sanz, E.J., Villen, T., Alm, C. and Bertilsson, L. (1989) S-mephenytoin hydroxylation in a Swedish population determined after coadministration with debrisoquine. Clin. Pharmacol. Ther. **45**; 495-499.

Simmons. D. L. and Kasper, C. B. (1983) Genetic polymorphism for a phenobarbital-inducible cytochrome P450 map to the coh locus in mice. J. Biol. Chem. **258**; 9585-9588.

Skjelbo, E., Gram, L. F. and Brosen, K.(1993) The N-demethylation of imipramine correlates with the oxidation of S-mephenytoin (S/R-ratio). A population study. Br. J. Clin. Pharmacol. **35**; 331-334.

Skoda, R. C., Gonzalez, F. G., Demierre, A. and Meyer, U. A. (1988). Two mutant alleles of the human cytochrome P450dbl gene associated with genetically deficient metabolism of debrisoquine and other drugs. Proc. Natl. Acad. Sci. USA. **85**, 5240-5343.

Sommers, D. K., Moncrieff, J. and Avenant, J. (1988) Polymorphism of the 4-hydroxylation of debrisoquine in the San Bushman of South Africa. Hum Toxic. **7**; 273-276.

Steiner, E., Iselius, L., Alvan, G., Lindsten, J. and Sjoqvist, F. (1985) A family study of genetic and environmental factors determining polymorphic hydroxylation of debrisoquine. Clin. Pharmacol. Ther. **38**; 394-401.

Troupin, A.S., Ojemann, L.M.and Dodrill, C.B. (1976) Mephenytoin: A reappraisal. Epilepsia **17**; 403-414.

Tucker, G. T., Jackson, P. R., Lennard, M. S. and Woods, H. F. (1986). The detection of polymorphic drug oxidation : Some theoretical and practical aspects. Ethnic differences in reactions to drugs and xenobiotics, pages 413-424. Allan R. Liss, Inc.

Tybring, G. and Bertilsson, L (1992) A methodological investigation on the S-mephenytoin hydroxylation phenotype using the urinary S/R ratio. Pharmacogenetics **2**; 241–243.

Tyndale, R., adyama, T., Broly, F., Matsunaga, T., Inoba, T., Kalow, W., Gelboin, H. V., Meyer, U. A., and Gonzalez, F. J. (1991) Identification of a new variant *CYP2D6* allele lacking the codon encoding Lys_{281}: possible association with the poor metabolizer phenotype. Pharmacogenetics **1**; 26–32.

Vogel, F. and Motulsky, A. G. (1979). "Human genetics". Springer Verlag, Berlin.

Wang, S., Huang, J., Lai, M., Liu, B. and Lai, M. (1993). Molecular basis of genetic variation in debrisoquine hydroxylation in Chinese subjects: Polymorphism in RFLP and DNA sequence of CYP2D6. Clin. Pharmacol. Ther. **53**; 410–418

Ward, S. A., Goto, F., Nakamura, k., Jacqz, E., Wilkinson, G. R. and Branch, R.A. (1987) S-mephenytoin 4-hydroxylase is inherited as an autosomal-recessive trait in Japanese families. Clin. Pharmacol. Ther. **42**; 96–99.

Ward, S.A., Helsby, N. A., Skjelbo, E., Brosen, K., Gram, L. F. and Breckenridge, A. M. (1991) The activation of the biguanide antimalarial proguanil co-segregate with the oxidation polymorphism – a panel study. Br. J. Clin. Pharmacol. **31**; 689-692.

Ward, S. A., Walle, T., Walle, K., Wilkinson, G. R. and Branch, R. A. (1989) Propranolol's metabolism is determined by both mephenytoin and debrisoquine hydroxylase activities. Clin. Pharmacol. Ther. **45**;72–79.

Watkins, W. M., Mberu, E. K ., Nevill, C. G., Ward, S. A., Breckenridge, A. M. and Koech, D. K. (1990). Variability in the metabolism of proguanil to its active metabolite cycloguanil in healthy Kenyan adults. Trans. Roy. Soc. Trop. Med. Hyg. **84**; 492–495.

Wedlund, P.J., Sweetman, B.J., McAllister, C.B., Branch, R.A. and Wilkinson, G.R. (1984) Direct enantiomer resolution of mephenytoin and its N-demethylated metabolite in plasma and blood using chiral capillary gas chromatography. J. Chromatogr, **307**; 121–127.

Wedlund, P. J., Sweetman, B. J., Wilkinson,, G. R and Branch, R. A. (1987) Pharmacogenetic association between the formation of 4-hydroxy-mephenytoin and a new metabolite of s-mephenytoin in man. Drug Metab Dispos. **15**; 277–279.

Wedlund, P. J., Aslanian, W. S., Jacqz, E, McAllister, C. B., Branch, R. A. and Wilkinson, G. R. (1985) Phenotypic differences in mephenytoin pharmacokinetics in normal subjects. J. Pharmacol & Exp. Ther. **234**; 662–669.

White, R.E. and Coon, M.J. (1980) Oxygen activation by cytochrome P450. Ann. Rev. Biochem. **49**; 315–56.

Woolhouse N. M., Andoh, B., Mahgoub, A., Sloan, T. P., Idle, J. R. and Smith, R. L. (1979) Debrisoquine hydroxylation polymorphism among Ghanaians and Caucasians. Clin. Pharmacol. Ther. 26; 584–91.

Wrighton, S. A., Stevens, J.C., Becker, G. W. and VandenBrande, M. (1993) Isolation and characterization of human liver cytochrome P4502C19: Correlation between 2C19 and S–mephenytoin 4–hydroxylation. Arch. Biochem. Biophy. **306**; 240–245.

Yue, Q. Y., Bertillson, L., Dahl–Puustinen, M. L., Johansson, I. and Ingelman–Sundberg, M. (1989) Disassociation between debrisoquine hydroxylation phenotype and genotype among Chinese. Lancet. October **7**; 870.

Zanger, U. M., Vilbois, F., Hardwick, J. P. and Meyer, U. A. (1988) Absence of hepatic cytochrome P450bufI causes genetically deficient debrisoquine oxidation in man. Biochemistry. **27**; 5447–5454