DRUG METABOLISM AND ITS IMPACT ON DRUG DOSING: TOWARDS A MORE COST-EFFECTIVE DRUG THERAPY

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INTRODUCTION

Ancient Malays originated from Yunnan in China. Their expansion from the coasts of Borneo to the Malay Peninsula and much of South East Asia, and their evolution into Modern Malays, both resulted from their trading and seafaring ways. They traded with and married their early trading partners, the Arabs, Indians, Chinese and Thais. This could thus make them differ from Chinese in their genetic make up and such a difference would be expected in the genetic polymorphism of CYP2D6 that is highly polymorphic. The polymorphic cytochrome P450 CYP2D6 is involved in the metabolism of various drugs of wide therapeutic use and is a presumed susceptibility factor for certain environmentally-induced diseases (Marez et al., 1997). The polymorphism shows pronounced interethnic variations (Munoz et al., 1998; McLellan et al., 1997; Bertilsson et al., 1992; Johansson et al., 1994; Dahl et al., 1995). A report by Lee on 97 Malay students estimated that 2.06% of Malays were poor metabolisers (PM) of debrisoquine (Lee et al., 1988). The authors in this study did not attest too much importance to the figure but this figure was higher than most Chinese and Asian figures. It was for this reason that we decided to study the problem especially in view of the potential importance in the metabolism of various drugs of wide therapeutic use and susceptibility for certain environmentally induced diseases.

MATERIALS AND METHODS

HPLC Determination of DEB and 4OHDEB In Human Urine and Metoprolol in Plasma

Component	DEB and 4OHDEB	Metoprolol
Drug Standards	Debrisoquine; 4-Hydroxydebrisoquine	Metoprolol
Internal Standard	Phenacetin	Phenacetin
Solvents and Reagents	Methanol HPLC; Acetonitrile HPLC; Na-Dihydrogenphosohate; Demonized distilled water Filtered three times through membrane filter 0.45 μm; Triethylamine (TEA)	Acetonitrile HPLC; K-Dihydrogenphosohate; Diethyl ether; TEA; HCl; NaOH; Demonized distilled water Filtered three times through membrane filter 0.45 μm.
Chromatography Equipment	Gilson 305 Model Pump; Hewlett Packard 1040 A/M DAD set at 262 nm Detector; Gilson Injection valve 7161 model Injector; Hewlett Packard, HP 3329 A; Chart speed 0.2 cm/min; Threshold 2; Peak width 0.16 min.; Attenuation 1 Integrator	Gilson 305 Model Pump; Gilson 115 UV/VIS Detector set at 230 nm; Hewlett Packard, HP 3329 A; Chart speed 0.2 cm/min; Threshold 2; Peak width 0.16 min.; Attenuation 1 Integrator; Gilson Injection valve 7161 model Injector.
Analytical Column	Bondapak- C18, 10 μm, (3.9 x 300 mm), Waters, U. S. A.	Supelcosil LC-PCN, 15 cm X 4.6 mm, Supelco, USA.
Extraction Column	Silica gel-based, bonded phase packing (2 ml capacity, containing CN functional group), SYVA ®, USA	NONE
Mobile Phase	Acetonitrile 20% (filtered through membrane filter 0.45μm); Phosphate Buffer, 80 % (0.008M) (pH - 5.52); TEA 100 μl for each 500 ml mobile phase; Flow Rate 1 ml/min	Acetonitrile 15% (filtered through membrane filter 0.45μm); Phosphate Buffer, 85 % (0.008M) (pH - 5.52); TEA 100 μl for each 1000 ml mobile phase; Flow Rate 0.5 ml/min
Injection Volume	20 μl	20 μl
Analysis Time	Maximum analysis time is approximately 20 min due to the lateness of the appearance of the internal standard	Maximum analysis time is approximately 10 min

Table 1. HPLC Analyses of DEB and 4-OHDEB in Urine and Metoprolol in Plasma

Clinical Methods

Determination of DEB Metabolic Ratios

Subjects

Fifty-one unrelated healthy Malay medical students aged 21 to 25 years from Universiti Sains Malaysia, in Kelantan volunteered for this study. Baseline screening included urine for Full Examination and Microscopic Examination (FEME), liver function test, and renal function test. Before the study, the volunteers were not allowed to take any drugs for two weeks. Written informed consent was obtained from each subject and the Universiti Sains Malaysia ethical committee approved the study.

Phenotyping Procedures

All volunteers were phenotyped with DEB. The volunteers presented themselves at 9.00 p.m. to the medical ward in HUSM and their blood pressure and weight were checked . After emptying the bladder, each subject took a single oral dose of 10 mg of debrisoquine (Declinax, Hoffmann-La Roche Inc., Nutley, NJ) and slept overnight at the hospital. Urine specimen was collected from 0 to 8 hours after drug intake. Urine volume was measured and aliquots were analyzed by High Performance Liquid Chromatography for DEB and 4OHDEB.

Debrisoquine hydroxylation phenotype was assigned by the debrisoquine MR, calculated as follows-

$$MR = \frac{\text{Percentage of dose excreted as debrisoquine}}{\text{Percentage of dose excreted as 4-hydroxydebrisoquine}}$$

in 0-8 h urine (Westward et al.. 1986)

Where, PM described as deficient hydroxylation of debrisoquine and the MR is greater than 12.6 and EM classified as fasted hydroxylation of debrisoquine with MR between 0.03 to 12.6 (Wanwilmolruk et al. 1990).

CYP2D6 GENOTYPING

Preparation of Human Serum for DNA Collection

The subjects included the healthy volunteers who previously participated in the DEB phenotyping study and 97 Malay blood donors who were enrolled later.

Venous blood, 8 to 10 ml, was collected into sterile 10-ml centrifuge tubes containing Na- EDTA. After collection, the tubes were left to stand at an angled position of 450 at room temperature for not more than 2 hours. They were stored at 40C to enable maximum plasma yield and to minimize contamination. After several hours at 40C, the sample was brought down to room temperature and spun at 2500 rpm for 8 min. Plasma was removed using a sterile pipette and pooled into another tube while the buffy coats remaining with the blood were left in the tube. The blood with buffy coat was used to obtain purified DNA.

Extraction Of DNA Template From Blood

Preparation of the STE Buffer

STE buffer was prepared by adding 5.8g of 10 mM Sodium Chloride (Sigma Chemical, GRM), 3.1g of 20 mM Tris base (Enzyme grade, Gibco BRL), and 3.7g of 10 mM ethylene diamine tetra acetic acid (EDTA, Stratagene), into a beaker containing 900 ml of sterile distilled water. The mixture was completely dissolved by stirring with an electric stirrer and dH2O water was then added to make up a 1 liter solution. The buffer was stored at room temperature before use.

Preparation of the 10% SDS Buffer

A 10% SDS buffer was prepared by adding 100 g of Sodium dodecyl sulphate (SDS) to 1 litter of distilled water. The buffer was stored at room temperature before use.

Preparation of the TAE Buffer

Tris Acetate (TAE) buffer was prepared by adding 4.8 g of Tris base (Enzyme grade, Gibco BRL), 1.6 g of Sodium Acetate (Ultra pure, Gibco BRL), and 0.7g of ethylene diamine tetra acetic acid (EDTA, Stratagene), into a beaker containing 900 ml of sterile distilled water. The mixture was completely dissolved by stirring with an electric stirrer while more dH2O was added to make up a 1 litter solution. The buffer was stored at room temperature before use.

Isolation of Genomic DNA

Eight to ten ml of fresh whole blood of the 51 phenotyped subjects was taken in a 15 ml tube. 0.2% NaCl was then added to make 15 ml. The mixture was centrifuged at 7000 g for 5 min. The supernatant was discarded and 200 µl of STE buffer, 20 µl of 10% SDS, 5 µl of 10 mg/ml Proteinase- K was added and thoroughly mixed. The mixture was incubated at 370C for 3 hours. Subsequently 3 µl of 10U/µl RNAase was added and the mixture was incubated at 370C for 15 min. Five M NaCl were then added and the mixture centrifuged at 7000 rpm for 5 min. The upper layer was then separated and 1 ml of absolute ethanol was added to the tube. This mixture was then centrifuged at 7000 rpm for 5 min. and the supernatant was discarded. After adding 1 ml of absolute ethanol the residue was kept overnight at -200C. The mixture was later centrifuged at 7000 g for 5 min. and the supernatant was again discarded. The pellet will form and is air dried for 30 min.

After adding 1 ml of 70% cold ethanol, the mixture was centrifuged at 7000 g for 5 min. The supernatant was again discarded and the pellets were dried in the air for 30 min. 500 µl of TE buffer was then added to dissolve the DNA that was stored at -200C until analysis.

For the 97 subsequent subjects, a slight modification was made to the DNA extraction procedures. Five ml of blood was withdrawn from blood donor's bag into a sodium citrate containing tube and stored at -70° C until DNA extraction. Genomic DNA was extracted from the leukocytes obtained from the thawed blood that was diluted with a lysis buffer (0.64 M Sucrose, 0.02 M Tris Hl, 2% Triton-X 100) and centrifuged at 3500 rpm for 15 minutes. The pellet obtained was rinsed with Tris-EDTA (TE) and again centrifuged at the same speed. The pellet was then resuspended in saline-EDTA to which a 20% SDS solution was added. RNAase A was added and the sample was incubated at 37°C for 1 hour followed by the addition of Proteinase K and overnight incubation at 37°C.

Genomic DNA was precipitated with 2 M KCl followed by cold absolute ethanol. The DNA precipitate was spooled up and then washed with cold 70% ethanol and left to air-dry for 5 minutes. The clean DNA was reconstituted with TE buffer and left to dissolve in cold room overnight. DNA amount was quantified using absorbance at 260 nm and purity was estimated using absorbance ratios at 260 nm and 280 nm with a UV spectrophotometer. The DNA was stored at -70C until PCR analysis.

Estimation Of DNA Content and Purity

DNA concentration was estimated by ultraviolet absorbance spectrophotometry. The amount of ultraviolet radiation absorbed by a solution of DNA is directly proportional to the amount of DNA in the sample. The absorbance was measured at 260 where 1.0 OD is equivalent to 50 µg/ml of double stranded DNA or to 30 µg/ml of single stranded DNA. Ultraviolet absorbance was used to check the purity of the DNA preparation. With a pure sample of DNA, the ratios of the absorbance at 260 nm and 280 nm (A260/ A280) is 1.8, ratio of less than 1.8 indicates that the preparation is contaminated either with protein or with phenol.

PCR- genotyping

A modification of the nested PCR assay of Heim and Meyer (1990) was followed in this experiment. It involved a double amplification reaction in which the product of the first reaction (nest one) was used as the DNA template for the second reaction (nest two). Details of the primers used in the first and second nested PCR is described by Kimura et al. (1990). The primers were all based on the CYP2D6 genes. Two specific primers, primer 1 - (ATT TCC CAG CTG GAA TCC) and primer 4 - (CCG GCC CTG ACA CTC CTT CT) which were used in the first amplification reaction to amplify the large portion of the CYP450IID6 gene, were also used in the second amplification reaction as a common primer. All primers were purchased from an established local agent. These were resuspended in commercial dH20 to a stock concentration of 50 ppmol.

First Nest Amplification Reaction (Nest One)

From the storage at - 200C freezer, all 'working stock' reagents for the PCR assay, except water and mineral oil, were kept on ice ready for the 'master mix' preparation on a bench at which no other CYP450IID6 DNA or PCR products had been handled (the 'clean bench').

A 'master mix' containing all the reaction components in their appropriate volumes was prepared for a specific number of reactions or samples, including positive and negative controls, based on a total reaction volume of 50 µl. This was made in a sterile 1.5 ml Eppendrof microcentrifuge tube by using a set of Eppendrof pipetteman with sterilized plugged micropipette tips. The 'master mixes' containing all the

necessary reaction reagents in their appropriate concentrations was thoroughly mixed by pipetting up and down.

Subsequently, 48 µl aliquots of the 'master mix' were transferred into 0.5 ml microcentrifuge tubes, pre-labeled with inedible ink marker pens, using sterile micropipette tips. Fifty µl of mineral oil (Molecular Grade, Sigma Chemical Company) was then added to the top of the 'master mix' in each tube. The tubes were tightly closed and transferred to a separate bench where addition of DNA templates was performed.

Collected DNA samples, stored at 40C, were used as DNA templates for the first nest amplification reaction. A different set of micropipettes, designated especially for this purpose, and sterile plugged micropipette tips (Costar Corporation) were used to prevent cross-contamination. Each sample was opened individually while each 600 ng of DNA template was transferred into its corresponding 'master mix' tube to produce a total reaction volume 50 µl. This was accomplished by carefully inserting the micropipette tip through the oil layer before pipetting the suspension into the 'master mix' layer. Finally, the tubes were tightly closed and transferred to the PCR machine for the first amplification reaction.

The PCR assays were performed using an Eppendrof Master Cycler 5330. The cycling parameters were pre-programmed into the Master Cycler as follows:

Step 1:	Initial Denaturation	at	940C	for	4 minutes
Step 2:	Loop<= 2(35 cycle)				
Step 3:	Denaturation	at	940C	for	60 seconds
Step 4:	Annealing	at	520C	for	90 seconds
Step 5:	extension	at	720C	for	90 seconds
Step 6:	Next =>2				
Step 7:	Final Extension at	720C	for	7 minutes	
Step 8:	End				

Upon termination, the sample was brought to room temperature and the PCR products obtained were either used immediately for the second nest amplification or kept at 40C for later use.

Second Nest Amplification reaction (Nest two)

The procedures for the preparation of the 'master mix', the addition of DNA templates and the running of the reactions on the PCR machine were done in exactly the same fashion as in nest one, taking great care to avoid cross - contamination. A total reaction volume of 50 µl was also used for nest two reactions. The second amplification reaction also involved the use of 4 sets of specific CYP450IID6 primers.

Unlike in nest one, where 600 ng of DNA template were used, only one µl of the 50 µl nest one product was used as DNA template for nest two. The annealing temperature was decreased from 52 to 500C and the duration for the annealing and extension period was also decreased from 90 seconds to 60 seconds. The number of amplification cycles was decreased from 35 to 15. The resultant product obtained in nest two was stored at 40C or analyzed immediately to visualize the amplified product.

Preparation of 20X TBE Buffer

A 20X TBE buffer was prepared by adding 121 g of Tris base (Enzyme grade, Gibco BRL), 61.7 g of boric acid (Ultra pure, Gibco BRL) and 7.44 g of ethylene diamine tetra acetic acid (EDTA) (Stratagene) into a beaker containing 1000 ml of sterile dH20. The mixture was completely dissolved by stirring with an electric stirrer while more dH20 was added to make up a 1 liter solution. It was then aliquoted into 500 ml bottles and stored at room temperature until use.

Preparation of 1 X TBE Buffer

100 ml of 20X TBE buffer was diluted into 1000 ml of dH20 to make 1X TBE buffer. This buffer was used for the preparation of agarose gel and for running the gel electrophoresis.

Preparation of Loading Buffer

Either orange G (Molecular Grade, Sigma Chemical Company) or bromophenol blue (ACS Reagent, Sigma Chemical Company) was used in the loading buffer as a tracking dye. Electrophoresis loading buffer was prepared by adding 0.25% (w/v) of the tracking dye and 40% (w/v) sucrose (Analytical Reagent, Ajax Chemicals PTY Limited, Australia) in sterile dH20 and stored at 40C.

Preparation of 1.2% agarose Gel

Materials and Equipment

Agarose powder-ultra pure molecular biology grade for DNA
Gel electrophoresis equipment
Conical flask
Ethidium bromide (10 mg/ml)
Microwave oven
DNA molecular weight marker (1 kb ladder)
Polaroid film

Methods

A gel mould was prepared according to the manufacturer's specification.
For the 1.2 % agarose gel, the composition was as follows:

Ultra pure agarose 0.60 gm
1 X TBE buffer 50 ml

The agarose was dissolved in a microwave oven and swirled in a conical flask to mix the dissolved agar. It was left to stand at about 650C. Two microlitre µl of 10 mg/ml ethidium bromide solution was added and the mixture was swilled before being molded. The gel was kept at between 3-5 mm thick and it was poured into the checked to see that there were no air bubbles under or between the teeth of the comb. After the gel was completely set (30-40 min. at room temperature). The comb was carefully removed and the gel was transferred to an electrophoresis tank. Just enough electrophoresis buffer to cover the gel to a depth of about 1 mm was then added. The samples of DNA were mixed with the gel loading buffer and slowly loaded into the slots of the submerged gel using micropipette. The lid of the gel tank was closed and electrical leads were attached so that the DNA would migrate towards the anode. The electrophoresis was carried out at 100 volt for 60 min.

Preparation, Loading and Running of Samples and Size Markers.

For each sample to be analyzed, 2 µl of loading buffer and 10 µl of the nested two amplified PCR product was added and mixed thoroughly. Using sterile micropipettes tips the 12 µl of the product were set up into the slots of the agarose gel. A 'molecular size marker' sample tube was also made up by adding 0.5 µl of 100 base pair DNA marker ladder (1µg/µl), Gibco BRL, with 2 µl of loading buffer and 9.5 µl of sterile distilled water.

The gel was transferred into the gel electrophoresis chamber and 1XTBE buffer as running buffer was poured into the apparatus to cover the gel. The samples were thoroughly mixed with the loading buffer and were individually loaded into each well with their positions noted. A separate micropipette tip was used for each sample.

Once loading of samples and the size marker had been completed, the lid of the electrophoresis apparatus and the attached electrodes were connected both to the chamber and the power pack. Electrophoresis was carried out at 100 volts for the 1 hour until the migrating samples moved out of the wells into the gel.

Visualization of Amplified Products

The DNA products in the agarose gel were visualized under ultraviolet (UV) illumination. This was achieved by the utilization of ethidium bromide (Gibco BRL) as the staining agent. Ethidium bromide intercalates with DNA in the gel and fluoresces as an orange band under UV excitation.

The gel containing the samples was submerged in 1XTBE buffer and 2 µl of ultra pure ethidium bromide (10 mg/ml) was pipetted into the buffer. To obtain a uniformed staining gel, it was shaken on an orbital shaker for at least 30 to 35 minutes before being taken to the dark room for visualization of the PCR products (DNA bands). If excess ethidium bromide produces background fluorescence, the gel was de-stained by submerging it in 1 mM MgS04 for about 20 minutes at room temperature. The gel image was captured using an Image Analyzer system (IS-1000 Digital imaging system). After documentation, the gel was re-submerged in fresh 1XTBE buffer.

For the 97 donors' DNA, genotyping for the CYP2D6*3 and CYP2D6*4 was similarly done using specific primers according to the methods of Heim and Meyer (Heim & Meyer, 1990) with slight modifications. All the reactions were carried out in 25 µL in a buffer (10mM Tris-HCl pH 8.0, 50 mM KCl, 1mM EDTA, 0.1%Triton X-100, 50% glycerol v/v) on a Perkin-Elmer GeneAmp PCR System 9700®. One Unit of Biotool Taq® was used for the first PCR of CYP2D6*3 and 0.5 U for the first PCR of CYP2D6*4 in the presence of 1.5mM MgCl2, 0.2 mmol/L dNTPs, 0.25 µmol/L of each primers and 200 ng DNA. Hot start was carried out by subjecting the DNA to 94°C for 2 min followed by 35 cycles at 94°C for 1 min. Annealing was carried out at 58°C for 90 sec and 72°C for 90 sec with final extension at 72°C for 7 min. The second PCR was performed using 0.25 U Biotool Taq®, 1.3 mM MgCl2, 0.2 mmol/L dNTPs, 0.25 µmol/L of each primers. 1.5 µL of the diluted first PCR product (1in 10) was used as template for this second PCR of 15 cycles of 45 seconds at 94°C, 50°C and 72°C.

To genotype for the CYP2D6*10 and *17, specific primers of McLellan (McLellan et al., 1997) were employed in the first PCR. Specific primers of Johansson (Johansson et al., 1994) were used for the second PCR for *10 and specific primers of Masimirembwa (Masimirembwa et al., 1996) for *17. In the amplification of the first fragment spanning exon 1 and 2, the PCR was performed in 25 µL reaction volumes. They contained 1 x XL PCR reaction buffer, 200 ng of genomic DNA, 200 µmol/L of each dNTP, 0.25 µmol/L of each primer, 1 mmol/L Mg (OAc) 2, and 1 U of rTth DNA polymerase® (Perkin Elmer). Hot start was performed at 93°C for 2 minutes followed by 35 cycles at 93°C for 45 sec. Annealing was carried out at 66°C for 45 sec and 72°C for 3 minutes and final extension at 72°C for 10 min. The second PCR consisted of 15 cycles of 1 min at 94°C and 54°C and 2.5 min at 72°C. The reaction mixture consists of 0.5U Biotool Taq®, 2.6 mM MgCl2, 0.2 mmol/L of each dNTPs, 0.25 µmol/L of each primers. 1.5 µL of diluted first PCR product (1in 10) was used as the template in two parallel allele-specific multiplex reactions.

For the duplicated gene, allele-specific PCR methods of Lundqvist were used (Lundqvist, Johansson & Ingelman-Sundberg, 1999). The long PCR methods of Steen was used with slight modifications for the determination the deleted gene (Steen et al., 1995) using a reaction volume of 25 µl on a Perkin-Elmer GeneAmp PCR System 9700® in 0.2 ml thin-walled tubes. One U rTth DNA polymerase (Gene Amp XL®, Perkin-Elmer), 1.3 mM Mg (OAc) 2, 0.3 mmol/L of each dNTPs, 0.35 µmol/L of each primers and 50 to 100ng of human genomic DNA were used in this two-step PCR. The PCR consists of 12 sec denaturing at 94°C and 5 min annealing for 35 cycles.

Positive controls for CYP2D6* 5 and *10 was courtesy of Dr. Jennie Wong (National University of Singapore), for the duplicated gene of Prof. Inger Johansson (Karolinska Institutet, Sweden) and for

CYP2D6*3, *4 of Dr. rer. nat. Ulrich Griese (Dr. Margarete Fischer-Bosch - Institut fuer Klinische Pharmakologie Auerbachstr). A negative control of autoclaved distilled miliQ water was also used.

Calculations of Allele Frequency

The frequency of a determined CYP2D*X allele in a sample of n individuals was estimated from (2nx/x + nx/-) ÷2n, where nx/x is the number of individuals homozygous for X and nx/- heterozygous for X (Garcia-Barcelo et al., 2000). Only results for the 97 donors were used in the calculations.

Investigation of Patients Treated with Metoprolol

Selection of patients

Consecutive adult patients attending specialty nephrology, hypertension, endocrinology and cardiology clinics at Hospital University Sains Malaysia (HUSM), in Kubang Kerian, Kelantan between June and September 1997 were screened. They were subsequently enrolled into the study if they were prescribed long-term metoprolol and were able and willing to sign a written informed consent. They were however excluded if they were receiving concurrent CYP2D6 drugs or had a recent exposure (less than one month) to them, had a history of liver disease or a previous hypersensitivity to metoprolol.

Study design

Visit 1

Patients were screened for inclusion and exclusion criteria. A brief explanation of the study was made. Patients were asked to sign a written–informed consent. They were then asked to continue with their regular treatment from the specialty clinics for the whole of the study period. They were told to note the exact time of taking metoprolol on the day of subsequent visit. Results of laboratory investigations including renal function test, liver function test, cholesterol levels and random blood sugar were obtained from the patients' case notes. Patients were told to come for follow-up visits 1 month after the first visit.

Follow Up Visit

The enrolled patients were asked to come for a follow–up visit one month after the first visit. Data obtained at this visit were used as measures of outcomes for metoprolol therapy. At this visit the following was done.

Patients were asked to report on any side effects including headache, giddiness, and lethargy they experienced during the one-month prior. The exact time the patient took metoprolol on the day of the visit was recorded. Seven milliliters of blood was obtained with the exact time of sampling recorded: Five milliliter was used for DNA extraction and subsequent amplification to identify CYP2D6 allele(s) Two milliliter was used for the measurement of metoprolol and 4-hydroxy metoprolol concentrations. Three milliliter of blood was also obtained 4 hours after the morning metoprolol dose. Again the exact time of sampling was recorded. The blood was used for the measurement of metoprolol and 4-hydroxy metoprolol concentrations.

RESULTS

HPLC Determinations of DEB and 4OHDEB in Urine and Metoprolol in Plasma

Component	DEB and 4OHDEB	Metoprolol
Separation	The three compounds of interest DEB, 4OHDEB and PHE were adequately separated	The three compounds of interest Metoprolol and PHE were adequately separated
Retention times	respectively 8.78 min, 4.48 min and 16.10 min	respectively 9.1 min and 6.9 min
Quantification	Peak area ratios were plotted against spiked concentrations and the curves were linear within the working range of 0.1 to 20 μg/ml for DEB and 4OHDEB.	Peak area ratios were plotted against spiked concentrations and the curves were linear within the working range of 10 to 300 ng/ml.
Recovery	for DEB ranged from 80% at 0.2 μg/ml to 100% at 0.1μg/ml and for 4OHDEB from 87.5% at 0.2 μg/ml to 106.25 % at 1μg/ml	Averaged 79.54%
Assay error as described by standard deviation (SD) for the determinations	$SD = 0.0007X^3 - 0.022X^2 + 0.1988X + 0.0625$ for DEB and SD = 0.0022X3 -0.0774X2 + 0.7041 X+ 0.1889 for 4OHDEB.	$SD = -3 \times 10^{-6}X^3 - 0.0012X^2 - 0.1245X + 7.9292$
Interference from endogenous substance	None	None

Table 2. HPLC Results for DEB, 4-OHDEB and Metoprolol

Debrisoquine Oxidation Phenotyping.

Fifty-one subjects were phenotyped with DEB. All were males and their age ranged from 21 to 25 years.

Median urinary DEB concentration was 1.8 and mean was 3.3 μg/ml (range 0.12 to 19.18), 4OHDEB 1.4 and 2.7 μg/ml (range 0.10 to 15.29) contained in an average of 320 ml urine collected over 8 hours. The percentage of the dose recovered as DEB ranged from 0.57 to 57% (median 4.4%) and the percentage recovered as 4OHDEB ranged from 0.46 to 56% (median 4.12%). The calculated MR averaged 2.21 (median 1.03) with two subjects having MR values greater than 12.6, giving a rate of 3.9% for our PM incidence. One subject had an MR value of 0.03 and was classified as an ultrarapid metaboliser. The remaining 48 subjects were classified as EM and their MR ranged from 0.08 to 6.68. A summary of the findings is listed in Table 3. A probit plot of Log MR values is shown in Fig. 1 and our 2 PMs could clearly be identified.

	Mean	SD	Range
Volume Of Urine, ml	320.67	81.28	237.27-474.22
Urinary DEB Conc., mg/L	3.34	4.45	0.12-19.18
Micromoles of DEB excreted	5.46	7.61	0.33-32.89
Micromoles of OHDEB excreted	4.30	5.62	0.26-32.02
Molar Metabolic Ratio	2.21	2.88	0.03-13.67

Table 3.Summary of Phenotyping Study Results

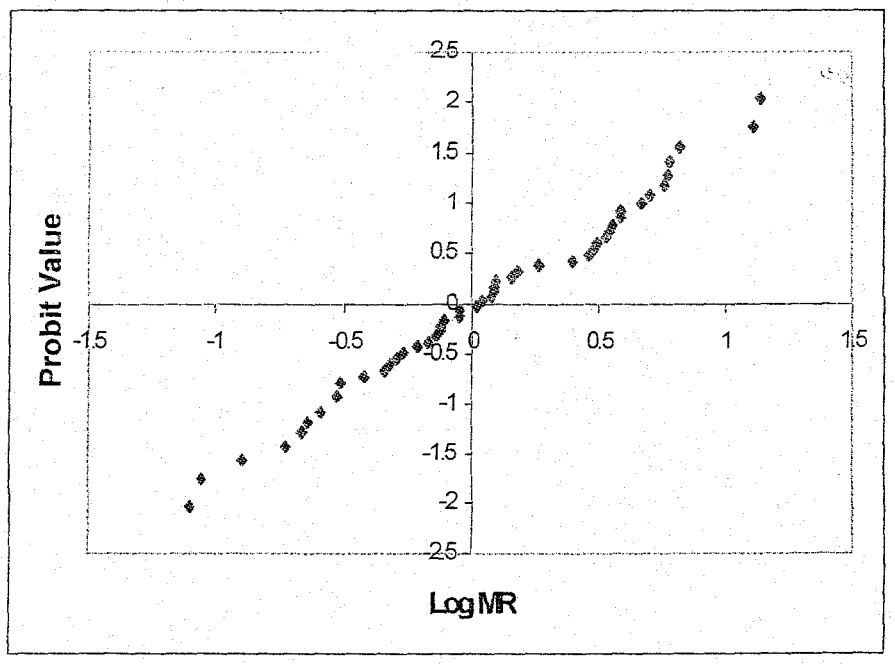


Fig. 1. Probit Analysis of log MR Values in Study Subjects

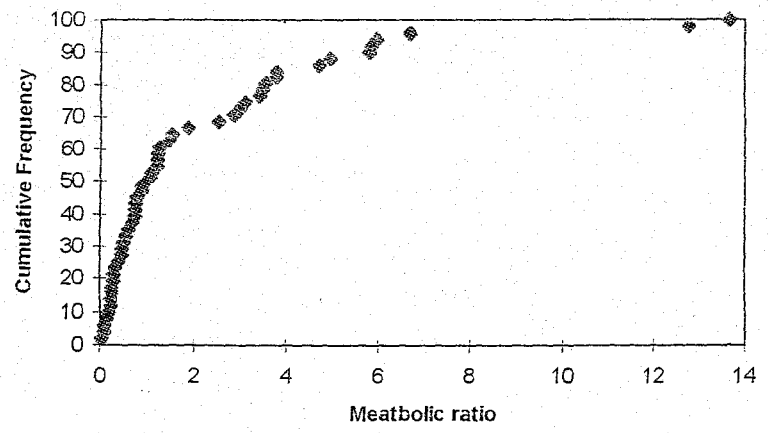


Fig. 2. Cumulative Frequency For MR in Study Subjects

The calculated MR values varied widely but there were suggestions of clustering of MR values. Although half of the subjects had values of less than 1, the frequency distribution of log MR values still revealed a skewness to the right as is shown in Fig. 3. In 34% of subjects, log MR values were between 0.2 and 1.

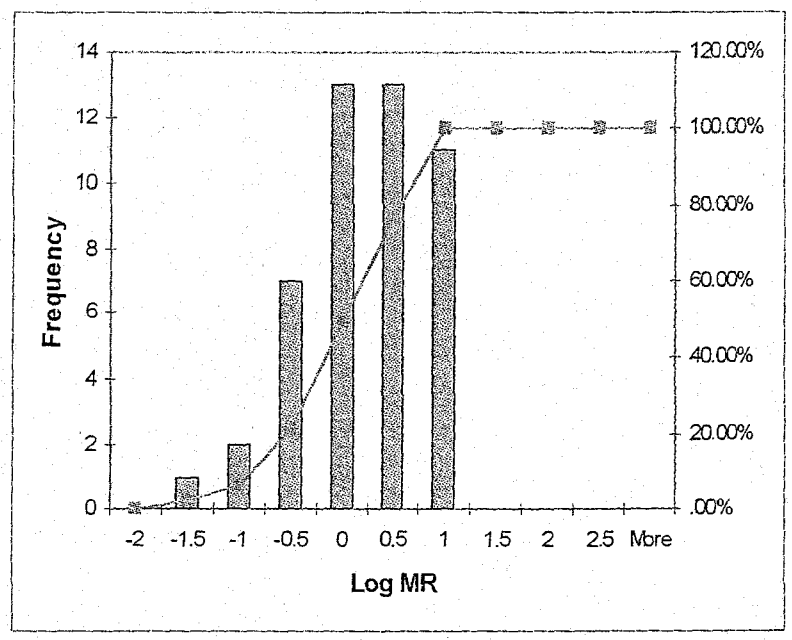


Fig. 3. Frequency Distribution of Log MR for EM Subjects.

We were only able to identify alleles in 24 of the original 51 subjects. In the other 27, the alleles could not be identified despite numerous attempts with altered conditions. Among those subjects whose alleles could be identified, the most frequent were the CYP2D6*1 and CYP2D6*1/CYP2D6*4. Two of the subjects had the CYP2D6*1/CYP2D6*3 alleles (Table 2). We did not estimate allele frequencies for this sample.

Allele	Frequency	Median MR
CYP2D6*1/*1	10	0.29
CYP2D6*1/*3	2	3.67
CYP2D6*1/*4	11	1.53
CYP2D6*4/*4	1	
Others+	27	1.20

Table 4. Frequency of CYP2D6 Genotypes and the Corresponding MR Values.

(+Alleles not identified).

Ten subjects (19.61%) were homozygous for CYP2D6*1 and 13 were heterozygous (25.49%) with CYP2D6*1/CYP2D6*3 (2) and CYP2D6*1/CYP2D6*4 (11). The MR among the former group averaged 0.31 and for the later 2.15 but the difference did not reach statistical difference (t-tests for unequal variances, p = 0.001, 95% CI for the difference -2.73, -0.93). Average MR in the 11 subjects who were heterozygous for CYP2D6*1/CYP2D6*4 was 1.87 that, although higher than the 0.3 average for the homozygous CYP2D6*1, was again not statistically different from the homozygots (t-test for unequal variances, p = 0.005, 95% CI for the difference -2.52, -0.58). Among the 2 subjects who were heterozygous with CYP2D6*1/CYP2D6*3, average MR was 3.68, statistically differently higher than the average 0.31 among the homozygots CYP2D6*1 (t-test for equal variances, p = 0.000, 95% confidence interval for the difference (-3.74, -2.75). Average MR value for the 25 subjects who exhibited PCR products that could not be identified by the current methods was, at 2.16, although higher than the average among the homozygots CYP2D6*1, was not statistically different (t-test for unequal variances, p = 0.000, 95% confidence interval for the difference -2.74, -0.96).

The DNA from the 25 subjects with unidentified PCR products yielded over 300 bp products. All the 25 subjects were phenotyped as EM's and their MR values ranged from 0.13 to 6.67. Subject # 51 who was phenotyped as PM and subject # 4 who was phenotyped EM did not show any product in the PCR of their DNA's.

The DNA from subject # 50 gave amplification for the primer 1/8, indicating the presence of a mutant CYP2D6*4 allele. Subjects # 28 and 46 whose MR's were 6.68, 2.86 respectively appeared genotypically as heterozygous EM's. The PCR product for these subjects yielded 564 bp and 300 above bp. It is possible that these individuals are heterozygous with CYP2D6 mutations that have not been identified.

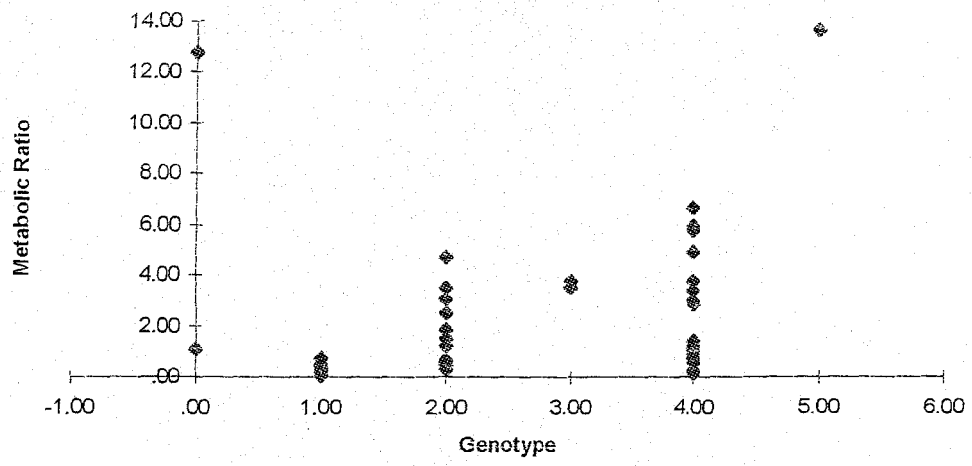


Fig. 4. Distribution of MR Values According to Investigated Genotypes

For the 97 blood donor subjects, their age ranged from 18 to 40 years (mean 24.1 ± 5.6) and 55 were males. Almost half were students but there was also a sizeable portion of technical workers (technicians and engineers, Table 3). The most common blood type found was 'O' followed by 'B' and 'A'. All had normal blood pressure with diastolic blood pressure that ranged from 70 to 90 mm Hg and systolic that ranged from 100 to 140 mm Hg.

Characteristic	Number	Characteristic	Number
Sex		Occupation	
Male	55	Army personnel	16
Female	42	Student officers	3
		Supervisor	4
		Clerk	4
		College student	43
		Engineer	2
		Forklift driver	1
		Houseboy	1
		Lecturer	1
		Technician	21
		Unspecified	2

Table 5. Characteristics of Study Population.

The quantity and quality of the DNA obtained was generally good. The average amount obtained was 88.1 mg (range 12 to 462 mg) and the average absorbance ratio obtained was 1.82 (range 1.52 to 2.09). The DNA from all but two of the subjects was successfully amplified. The DNA from the two subjects failed to amplify, despite numerous attempts. Five of the six defective alleles tested, CYP2D6*4, CYP2D6*5, CYP2D6*9, CYP2D6*10 and CYP2D6*17, were found in our population and so was gene duplication. Two subjects were homozygous for the deleted CYP2D6*5 gene giving a PM prevalence of 2.06%. Thirty-three of our Malays genotyped were heterozygous for wt-J (*1/*10), making this genotype the most common (Fig. 1). Forty-three others also carried the J-allele, 27 were homozygous. Thirteen

subjects were homozygous for the wild type gene, CYP2D6*1, and 6 carried the nonfunctional CYP2D6*4 gene, heterozygous with CYP2D6*10. One subject was heterozygous with CYP2D6*10/*17 (J-Z). CYP2D6*17, to our knowledge, has not been reported in Asians. One subject was also found homozygous for the defective C (*9) allele and two others, heterozygous, again novel among Asians.

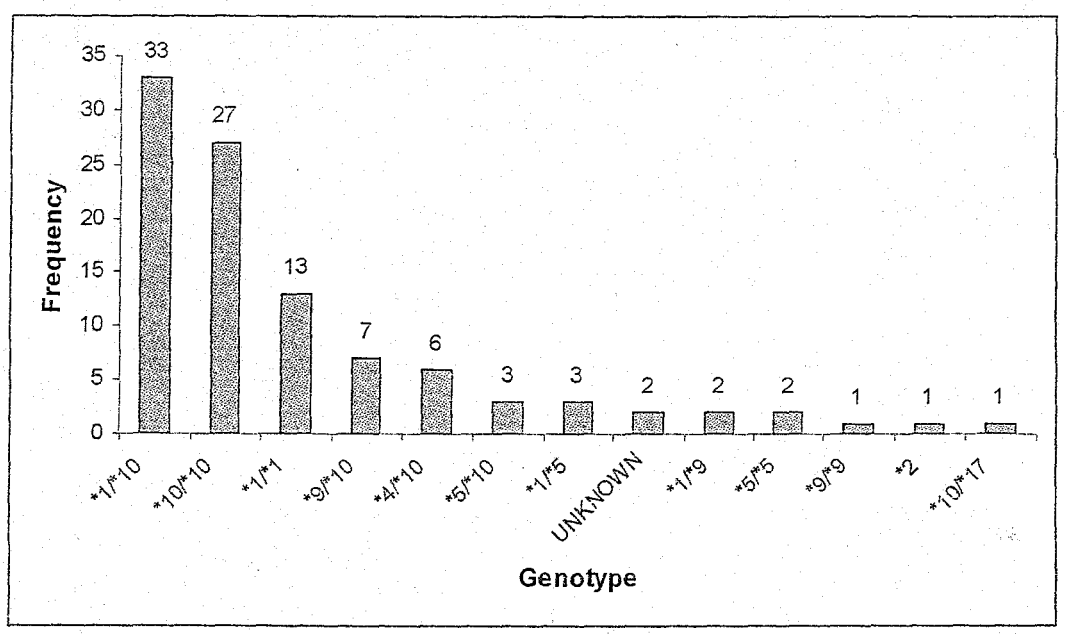


Fig. 4. CYP2D6 Genotypes Among Malay Blood Donors

Table 6 compares the prevalence of the PM phenotype among the Malays and some other races. It is apparent that, while the Malays appear to have a similarly low PM prevalence as the other Oriental races, the figure, on the average, is somewhat higher.

Race	% PM	Study Method	Number of Subjects	Source
Malays	2.06	Genotyping	97	Present study
	3.9	Phenotyping with debrisoquine	51	Present study
	2.06	Phenotyping with debrisoquine	97	Lee, Nam & Hee, 1988
Singapore Chinese	0	Phenotyping with debrisoquine	97	Lee, Nam & Hee, 1988
Han Chinese	1.4	Phenotyping with debrisoquine	140	Du & Lou,1990
Han, Mongolian, Wei and Zang Chinese	1.01	Phenotyping with debrisoquine	695	Bertilsson et al., 1992
Japanese	0	Phenotyping with debrisoquine	100	Nakamura et al., 1985
Japanese	1.02	Phenotyping with dextrometorphan	98	Tateishi et al., 1999
Chinese, Korean and Japanese	0	Phenotyping with debrisoquine	63	Dahl et al., 1995
Koreans	0	Phenotyping with debrisoquine	152	Roh et al., 1996
Thais	1.2	Phenotyping with debrisoquine	173	Wanwimolruk, Patamasucon & Lee, 1990
Khmers	2.1	Phenotyping with debrisoquine	98	Wanwimolruk, Thou & Woods, 1995
Saudi Arabs	0.98	Phenotyping with debrisoquine	102	Islam, Idle & Smith, 1980

Table 6. Prevalence of Debrisoquine PM Phenotype for Malays and Related Races

Fig. 4 gives the allelic frequencies for our study population. CYP2D6*10 (J) occurred most frequently, followed by the wild-type CYP2D6*1 gene. CYP2D6*3 (A) was not found but the two other null alleles, *4 and *5, were found.

In Table 7, allelic frequencies for the various CYP2D6 alleles tested are compared for our Malays and related ethnic groups. As for the Japanese and Chinese, the most frequently occurring allele was

CYP2D6*10 followed by the wild-type CYP2D6*1 allele. A cursory inspection also reveals that our Malays resembled Tateishi's Japanese more than they did Chinese. Their frequency for the wild-type gene was similar to Merez's Europeans and was intermediate between those for the Japanese and Chinese. The frequency for the null CYP2D6*4 allele was similar for our Malays as for the Arabs and for the South Amerindians, the later being a race that probably had its origins in Asia (Munoz et al., 1998). The frequency for the deleted gene was also similar to most of the other populations studied.

	Malays	HK Chinese	Chinese	Japanese	Amerindians	Arabs	Europeans
*1	0.3299	0.2269	0.269	0.423	0.661		0.322
*2	0.0103	0.00798	0.143	0.092	0.185	0.104	0.252
*3	0				0		0.016
*4	0.0309	0		0.02	0.036	0.035	0.158
*5	0.0515	0.0462	0.057	0.061	0.042	0.01	0.069
*9	0.0587				0		0.027
*10	0.4948	0.6471	0.878	0.408	0.018	0.03	0.014
*17	0.0052					0.03	0.001
*21				0.01			
Source	Present study	Garcia-Barcelo ct al., 2000	Johansson ct al., 1994	Tateishi et al., 1999	Munoz et al., 1998	McLellan et al., 1977	Merez et al., 1997

Table 7. CYP2D6 Allelic Frequency for Malays and Some Related Ethnic Groups

Metoprolol Study in Patients

The Patients

Sixty-one patients with varying degrees of renal impairment were enrolled. Fifty-two were Malays, and 6 were Chinese. There were 36 males and 25 females. In terms of their degree of renal impairment, 45 had moderate renal failure, 12 severe renal failure and 4 had end-stage disease. Their age averaged 62 years and their weight 60.2 kg (see also Table 8).

Characteristics		**Number**
Race	Malay	52
	Chinese	6
	Others	4
Sex	Male	36
	Female	25

Characteristics	Mean	Std Dev	Minimum	Maximum
Age, years	62	9.1	44	85
Weight, kg	60.2	11.9	40	87

Table 8. Demography of Study patients.

Figure 5 shows the frequency distribution of daily metoprolol dose. Ninety-five percent of the patients received 200 mg or less daily of metoprolol and 2 patients received 300 mg and 1 received 400 mg daily

metoprolol. Plasma metoprolol concentrations were available in 43 of the patients. Samples for 19 were lost during storage. They ranged from 14.6 ng/ml to 789.5 ng/ml and averaged 156.3 ng/ml.

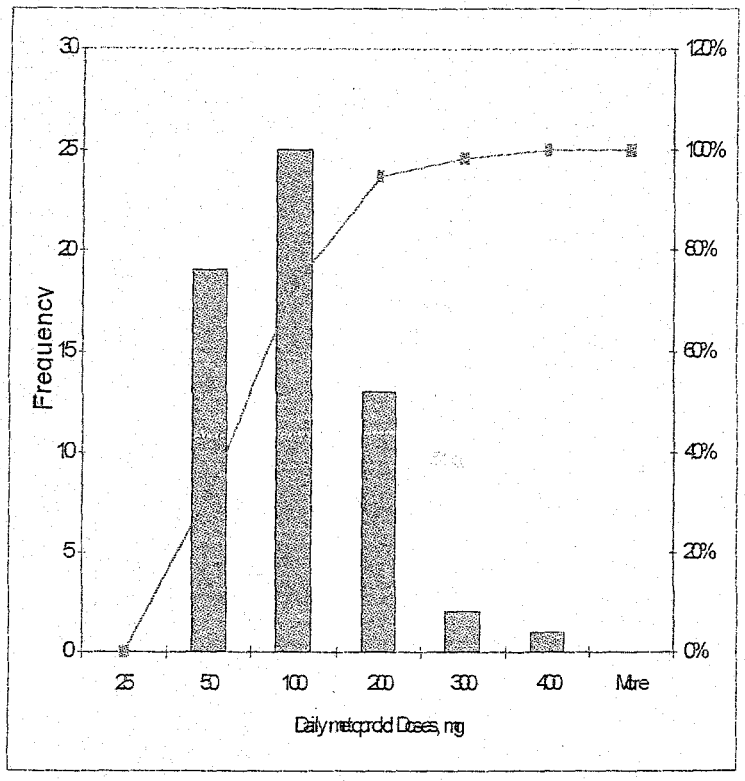
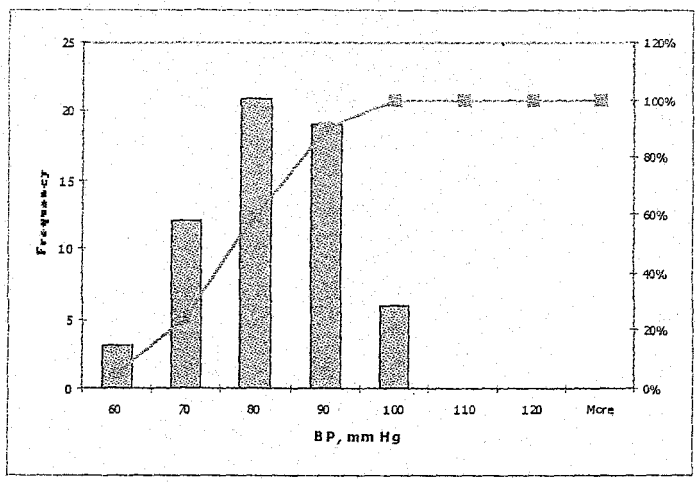


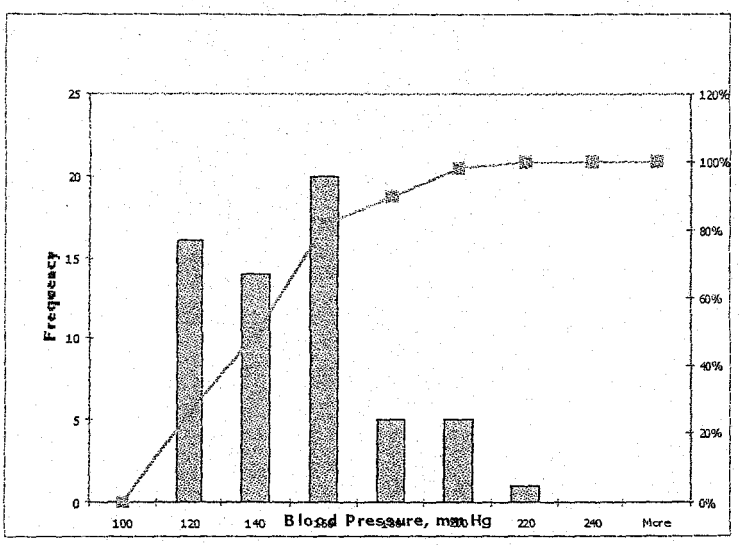
Fig. 5. Daily Metoprolol Doses.

Clinical Response

At the given metoprolol doses, pulse rates in the patients averaged 70.3 beats per minute, diastolic blood pressure, 81.7 mm Hg, systolic blood pressure 146.0 mm Hg and mean arterial pressure 103.0 mm Hg at the time of blood sampling for metoprolol concentration determinations. . All the patients had a diastolic blood pressure of 100 mm Hg or less at the time of blood sampling and 60% had a pressure of 80 mm Hg or less. For the systolic blood pressure, 26% had pressures below 120 mm Hg and 49% had pressures below 140 mm Hg.



Diastolic Blood Pressure



Systolic Blood Pressure

When pulse rate was taken as a measure of β-blockade, it is seen that daily metoprolol doses poorly predicted the rates (Fig. 6). On the other hand, as is depicted in Fig. 7, observed pulse rates paralleled plasma metoprolol concentrations categories relatively well, where category 0 corresponded to plasma metoprolol of less than 150 ng/ml, category 1, plasma metoprolol between 150 to 300 ng/ml and category 2, plasma metoprolol exceeding 300 ng/ml. Pulse rates averaged 70.6 beats per minute when plasma metoprolol was below 150 ng/ml, 67.4 when the concentrations were between 150 to 300 and 66 when concentrations exceeded 300. On the other hand, at a daily dose of 50 mg, pulse rates averaged 69.9 beats per minute, at 100 mg, 71.2 beats per minute and at 200 mg, 71.5 beats per minute. It is evident that plasma metoprolol concentrations gave a better indication of β-blockade capability compared to daily doses.

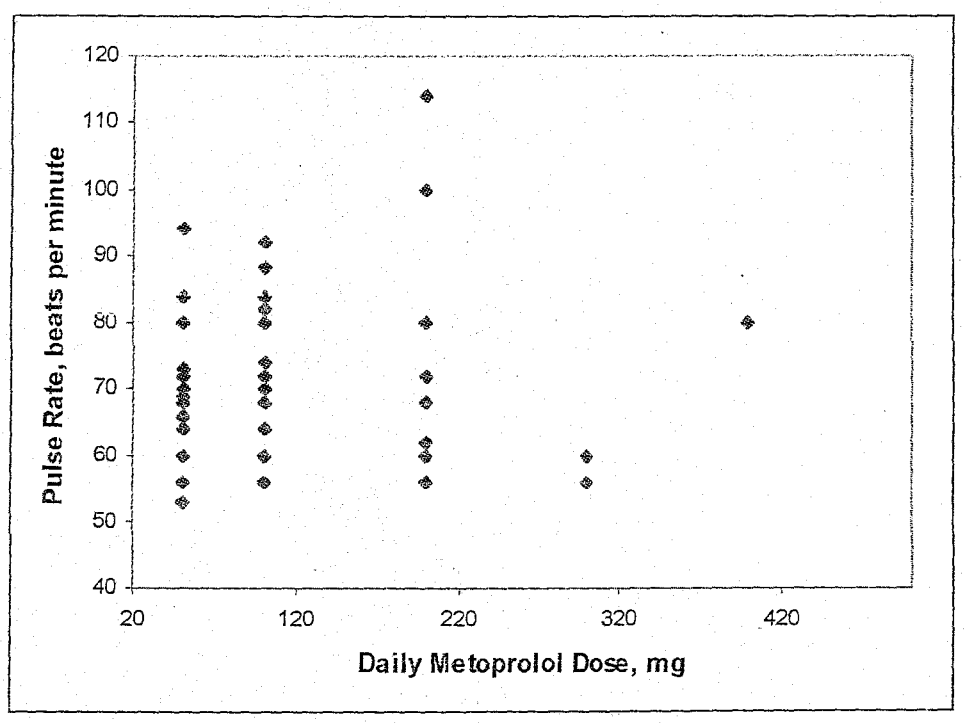


Fig. 6. Pulse Rates as a Function of Daily Metoprolol Doses

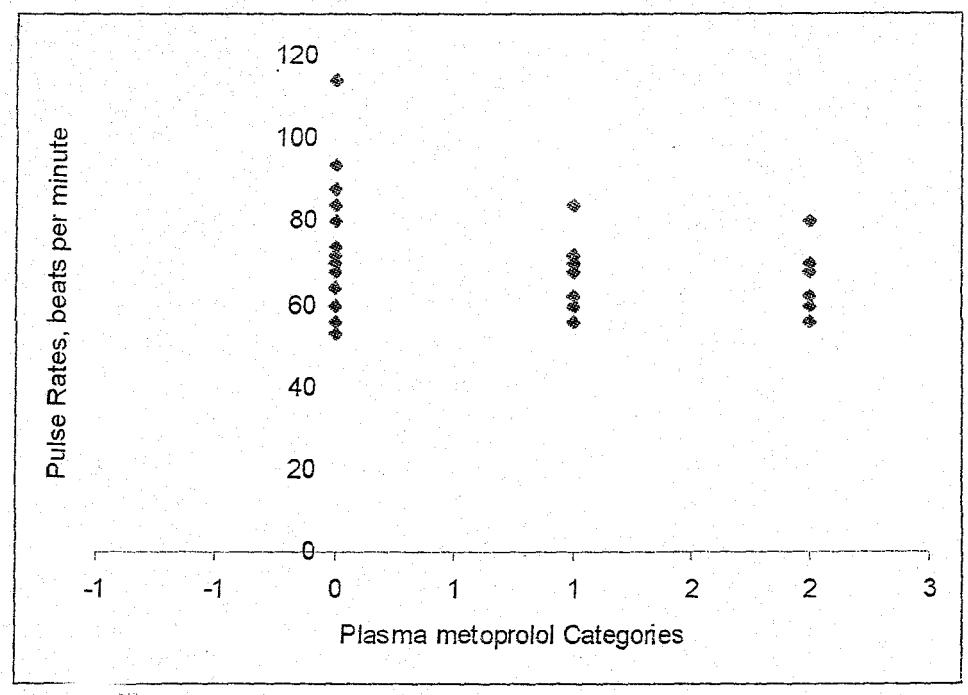


Fig. 7. Pulse Rates as a Function of Plasma Metoprolol Concentration categories1.

[1] 0 = plasma metoprolol less than 150 ng,ml
1 = plasma metoprolol between 150 - 300 ng/ml
2 = plasma metoprolol more than 300 ng/ml

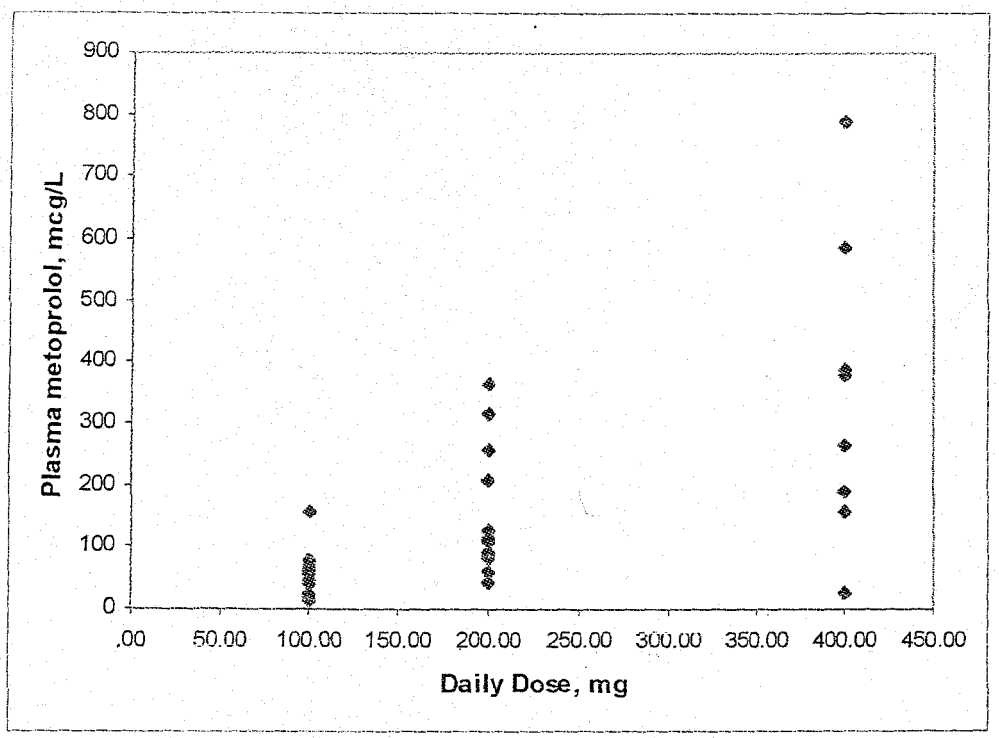


Fig. 8. Daily Dose and Plasma Metoprolol Concentration

Metoprolol undergoes hydroxylation by the CYP2D6 enzymes of the liver where 4-hydroxymetoprolol is formed. In an attempt to find a better predictor of plasma metoprolol concentrations, we determined the 'metabolic ratios', MR for metoprolol where MR was defined as the ratio of plasma metoprolol concentration at 4 hours post-dose over the simultaneously obtained plasma hydroxy-metoprolol concentration. The relationship between plasma metoprolol concentrations at 4 hours and the calculated metabolic ratio is depicted in Fig. 9. It is evident that there is a good correlation between calculated metabolic ratio and measured plasma metoprolol ($R^2 = 0.6$).

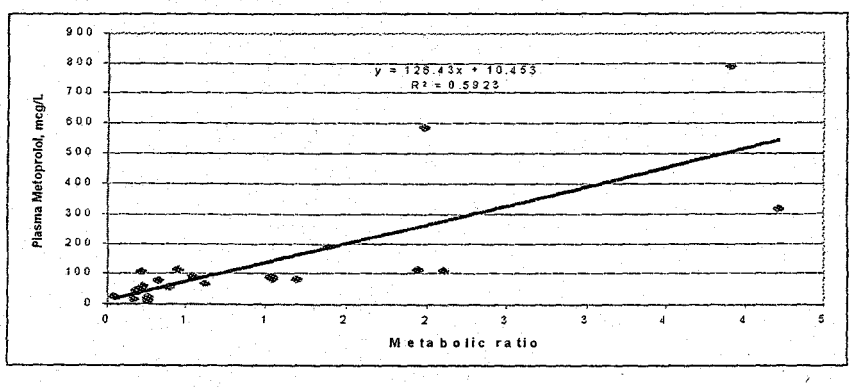


Fig. 9. Relationship Between Plasma Metoprolol Concentrations at 4 hours Versus metoprolol 'Metabolic ratio"

We also developed a probit analysis of the frequency distribution of metoprolol MR. As is shown in Fig. 10, the frequency distribution of metoprolol MR is not unimodal and there is a clear demarcation between

MR values among 'average' patients and that of patients who evidently was a slow metabolizer of metoprolol.

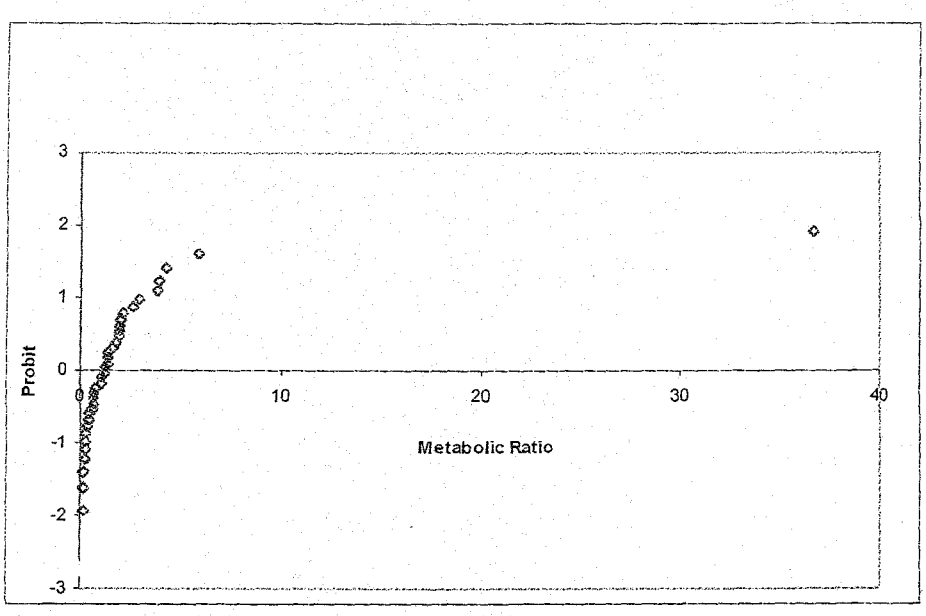


Fig. 10. Probit Plot of Metoprolol Metabolic Ratio to Show Polymorphism.

To explore the polymorphism we observed in the metabolism of metoprolol, we also determined the CYP2D6 genotypes among our study patients. Of the 61 patients, 22 (36.1%) had the wild type CYP2D6 gene, and 21 (34.4%) had one mutation. The remainder of the patients had multiple mutations. Results of the genotype determinations are shown.

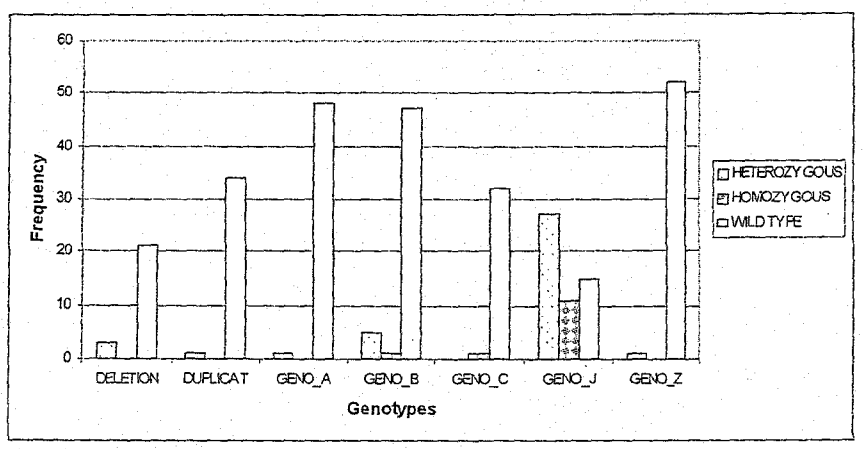


Fig. 11. Frequency of CYP2D6 Alleleic Variants in the Study Patients

Metoprolol MR values tended to be higher when there occurred mutations in the CYP2D6 gene in the study patients (Fig. 12). It averaged 0.999 when no mutation was present and 20.434 when 3 mutations occurred. One-way ANOVA performed on the means showed that there existed statistically significant

differences between the groups ($p<0.05$). Furthermore, the patient who was identified as a poor metaboliser, with a metoprolol MR of 36.65, was genotyped homozygous for the CYP2D6B allele, a null allele.

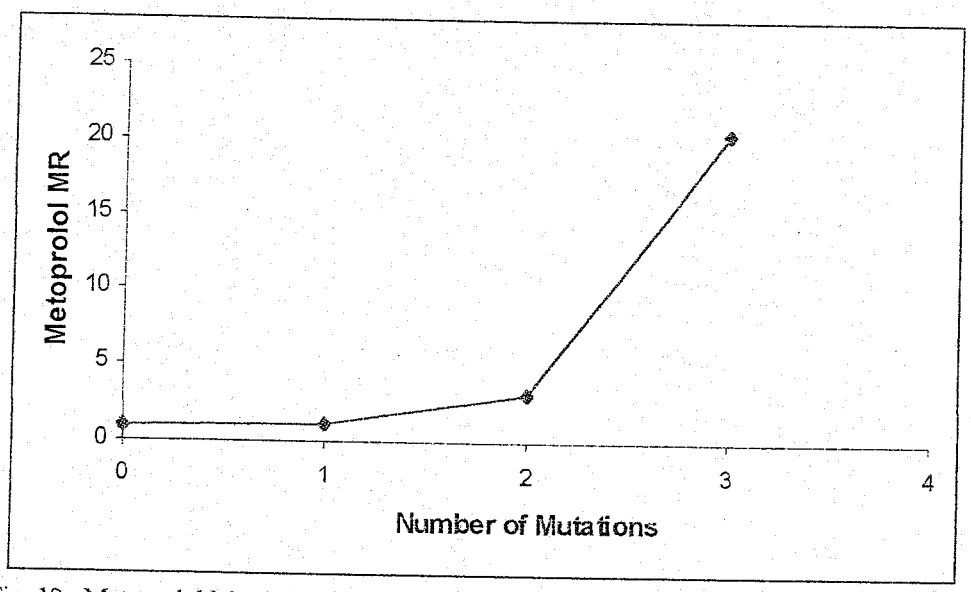


Fig. 12. Metoprolol Metabolic ratio as a Function of Number of Mutations.

DISCUSSION

Ancient Malays originated from Yunnan in China. Their expansion from the coasts of Borneo to the Malay Peninsula and much of South East Asia, and their evolution into Modern Malays, both resulted from their trading and seafaring ways. They traded with and married their early trading partners, the Arabs, Indians, Chinese and Thais. This could have lead to the mixing of genetic materials.

We phenotyped 51 Malay subjects by administering 10 mg of DEB and collecting their urine over an eight-hour period. Median urinary concentrations of DEB of 1.8 and 4OHDEB of 1.4 μg/ml were well within our linear calibration curves and were at concentrations where our assay precision was good. Indeed, one problem one may face in performing phenotyping study is to obtain an assay of sufficient sensitivity and precision at the concentrations most commonly obtained. With our HPLC methods, this problem was overcome even when using small aliquots of 1 ml of urine samples.

We found 2 (3.9%) PMs from of our 51 Malays and we found one who was probably an UM. Our diagnosis of ultra-rapid metaboliser status in that one subject may however need to be reconfirmed because it was based on a measured urinary DEB concentration of 0.5 μg/ml, the limit of detection. The % CV for the determination at this concentration was estimated to be high, the standard deviation for the determination for DEB being described as = -0.0074x2 + 0.253x - 1.64. The measured urinary 4OHDEB in this subject was 15.3 μg/ml and the corresponding assay CV would similarly be again high. The combined error that can be expected from the MR determination in this subject would therefore be relatively large.

It should also be noted that the identification of the DEB metabolic status in the present study is based on the criteria of the MR derived from Caucasian populations (MR > 12.6 for PM). Nevertheless, our PM rate of 3.9% was comparable to the 2.1% found in Singapore (Lee et al., 1988), using an MR cut-off point of 10 for PM diagnosis. Zahurin also found One PM in her 21 subjects (4.8%) phenotyped with dextrometorphan (Zahurin- personal communication). PM prevalences among Chinese were low and ranged from 0% to 1.4%. None was found among the 97 Singaporean Chinese (Lee, Nam & Hee, 1988) but 2 (1.4%) were found among 140 Han Chinese (Du & Lou, 1990). In a study that compared Chinese and Swedish populations (Bertilsson et al., 1992) the prevalence was 1.01%, significantly lower than the 6.82% for the white Swedish subjects. Lower prevalences have also generally been reported for other Oriental races. Thus, no PM was found from 100 Japanese phenotyped with debrisoquine (Nakamura et al., 1985) and 1 (1.02%), genotyped as homozygous for CYP2D6*5, was found from another 98 (Tateishi et al., 1999). There was also none among the 63 Chinese, Korean and Japanese phenotyped with debrisoquine (Dahl et al., 1995) and a similar observation was made in a study of 152 Koreans (Roh et al., 1996). Due to the small sample size, Lee abstained from describing conclusions as regards difference between Chinese and Malays (Lee et al., 1988). Our results, although again using a small sample size, taken together with Lee's and Zahurin's may be seen as suggesting that Malays may indeed differ from Chinese and other ethnic Asians in relation to CYP2D6 polymorphism, PM frequencies among ethnic Asians being generally less than 1% (Garcia-Barcelo et al., 2000, Roh et al., 1996).

Among Arabs, few PMs were also found. There was 1 from among 102 of one study (Islam, Idle & Smith, 1980) and none (based on genotypes) from 101 of another (McLellan et al., 1977). It is however interesting to note that higher prevalences have been reported among Indian subjects. In one South Indian study, 3.2% (Mamidi et al., 1999) were PMs and in another, 4.8% (Abraham et al., 200). The figure for North Indians was similarly high at 3% (Lamba et al., 1998). Before they, together with the Arabs, brought Islam to Malaysia, Indians came as Hindus and during both the periods, they intermarried the Malays, thus probably bringing with them the PM phenotype genes. Similar to Malays, 2% of Thais (Wanwimolruk, Patamasucon & Lee, 1990) and 2.1% of Khmers (Wanwimolruk, Thou & Woods, 1995)

were PMs. Thus it appears that the Malays on the average, have a greater probability of being a CYP2D6 PM compared to the other Oriental races. Together with Indians, Thais and Khmers, they probably represent a group that is intermediate between Caucasians and Asians in terms of debrisoquine PM prevalences. It would appear that Malays could have inherited genes responsible for the PM phenotype from their Indian trading partners. It thus interesting to note that PM prevalence among Khmers, a race originating from China but with a lot of Indian intermarriages, was also relatively high at 2.1% (Wanwimolruk et al., 1995).

Within the EM phenotype, the distribution of debrisoquine MR among Chinese is shifted to the right, indicating lower enzyme activity (Bertilsson et al., 1992; Lee & Jeyaseelan, 1994; Garcia-Barcelo et al., 2000). Thus while most Swedes were found to have debrisoquine MRs of less than 1, the opposite was found for Chinese (Bertilsson et al., 1992). Half our subjects had a debrisoquine MRs of 1 or less, again suggesting some distinctions between Malays and Chinese. MR values for Asians are generally shifted to the right, a feature that has been attributed to the high frequency of occurrence of a defective CYP2D6 gene among Chinese and Japanese (Lee & Jeyaseelan, 1994). This shift to the right was also observed among Indians (Abraham et al., 2000) but was not seen in Arabs (Islam et al., 1980). The latter did not seem to have a high frequency figure for the defective 'Asian' allele (McLellan et al., 1977). Lee and Jeyaseelan (Lee & Jeyaseelan, 1994) further reported an enrichment of the log distribution of MR at log MR between 0.2 and 1.0 among Singaporean Chinese where they reported that 44% of their subjects had a log MR in this range. Only 34% of our Malay subjects on the other hand had a log MR in this range that can again be taken as suggesting their difference from Chinese.

To further study the apparent differences, we used allele-specific PCR to characterise CYP2D6 alleles in Malays. Using the allele-specific PCR method described to detect CYP2D6*1, *3 AND *4, we were however only able to identify alleles in 24 of the 51 subjects phenotyped. In 2 no PCR product were amplified and in the other 25, the PCR products did not correspond to the specific primers used. Among those subjects whose alleles could be identified, the most frequent were homozygous CYP2D6*1 (10 subjects) and heterozygous CYP2D6*1/CYP2D6*4 (11 subjects). Two subjects were heterozygous with CYP2D6*1/CYP2D6*3 allele and one was homozygous with CYP2D6*4 allele. Alleles (homozygous CYP2D6*1, heterozygous CYP2D6*1/CYP2D6*3 and CYP2D6*1/CYP2D6*4) were therefore detectable in about half of the 48 EM's and in one of the 2 PM's (CYP2D6*4). The method did not therefore seem to be similarly sensitive in detecting CYP2D6 alleles among our Malay subjects.

Several null CYP2D6 alleles have been characterized and in addition, 2 variant CYP2D6 alleles have also been discovered that have altered catalytic activity (Gonzalez & Idle, 1994). They include the CYP2D6*4 which is the most common null allele found in Caucasians, CYP2D6*6, CYP2D6*3, CYP2D6*7, CYP2D6*11, CYP2D6*9 and CYP2D6*10. The major defective alleles associated with the PM phenotype among Caucasians, CYP2D6*3 and CYP2D6*4 are rarely found among Chinese in accordance with the low incidence of PM in this population (Dahl et al., 1995). Both the alleles were however found among our Malay subjects. One of our two PM carried the -B allele and 11 of our EM were heterozygous with the -B allele while 2 were heterozygous with the -A allele. Thus it would appear that the presence of the -B allele at least have accorded a higher incidence of the PM status among our Malay subjects in comparison to their Chinese cousins. No homozygous CYP2D6*3 allele was however found among the small number of the Malay subjects studied but its presence should not at all be surprising if large numbers of subjects are studied.

Some of our subjects showed amplification at 300 bp above suggesting the presence of CYP2D6*9 allele (Eleni Aklillu et al. 1996). The allele carrying this mutation has been classified as a variant allele on the basis of in vitro enzymatic criteria in human liver and its presence may cause reduced activity (Broly & Meyer, 1993). The possibility therefore exists that our EM subjects may also carry this CYP2d6*9.

Among Caucasians, single PCR assay for CYP2DB alone had a sensitivity of 92% in detecting homozygous PM and 64% in detecting heterozygous PM. Double PCR for CYP2D6*3 and CYP2D6*4 on the other hand had a sensitivity of 98% for homozygous PM and 70% for heterozygous PM, CYP2D6*4

being the most common null allele among the population (Gonzalez & Idle, 1994). Coupled with RFLP for CYP2D6*6 and CYP2D6*7, the sensitivity for heterozygous PM also reached 98%. The putative alleles were also able to be identified in almost 100% of the EM's among the Caucasians, using the combined RFLP and double PCR methods (Heim & Meyer, 1990). From our results, it appears that the method as employed here was not sufficiently sensitive in picking up CYP2D6 variants among our Malay subjects. Other primers may need to be employed in order to elicit the presence of other null alleles and/or gene variants and RFLP may also need to be employed for yet unidentified genes.

Genotypes have been shown to closely correlate with phenotypes for DEB metabolism except in a few individuals (Gonzalez & Idle, 1994). This appears to hold with our present study with Malay subjects. Thus our homozygous EM's (CYP2D6*1) on the average appeared to have the lowest MR values and our homozygous CYP2D6*4 PM the highest MR value. Heterozygous CYP2D6*1/CYP2D6*3 and CYP2D6*1/CYP2D6*4 appeared to have intermediate MR values. Firm correlation could not however be obtained because of our relatively small sample size and the large number of individuals who were not adequately genotyped. One subject who was phenotypically EM failed to yield identifiable PCR product. This raises the possibility that this individual may have a variant allele with reduced activity. The presence of the relatively commonly occurring CYP2D6*10 allele for instance, may accord this individual an EM phenotype but with a higher MR value compared to the individuals with wild type alleles (Gonzalez and Idle JR. Pharmacogenetic Phenotyping and Genotyping. Present Status and Future Potential. Clin Pharmacokinet 26 (1): 59-70, 1994). However it is also conceivable that this represents a misclassification of the phenotype arising from assay error and or (less likely), an error occurred while doing the PCR.

The MR among the ten subjects who were homozygous for CYP2D6*1 averaged 0.31 and for the subjects who were heterozygous either with CYP2D6*1/CYP2D6*3 or CYP2D6*1/CYP2D6*4 averaged 2.15 but the difference did not reach statistical difference. As alluded firm conclusions could not be drawn from this due to our small sample size. It would however not be surprising to find that the heterozygotes had higher MR values corresponding to lower rates of DEB metabolism as a consequence of gene dose effect (Broly and Meyer A. Debrisoquine Oxidation Polymorphism: Phenotypic Consequences of a 3-Base-pair Deletion in Exon 5 of the CYP2D6 gene. Pharmacogenetics 3: 123-130, 1993). Such a difference would be expected with individuals who were homozygous with either CYP2D6*1/CYP2D6*3 or CYP2D6*1/CYP2D6*4 notwithstanding.

Average MR in the 11 subjects who were heterozygous for CYP2D6*1/CYP2D6*4 was 1.87 and that for the 2 subjects who were heterozygous with CYP2D6*1/CYP2D6*3 was 3.68. Indeed the difference in the MR values between the homozygous CYP2D6*1 and the heterozygous CYP2D6*1/CYP2D6*3 reached a statistical significance. Similarly, average MR value for the 25 subjects who exhibited PCR products that could not be identified by the current methods was, at 2.16, higher than that among the homozygous CYP2D6*1 even though the difference was not statistically significant. Among these individuals, the presence of the CYP2D6*10 allele (see supra) or the CYP2D6*9 allele would accord them the EM phenotype status but with reduced activity (Broly and Meyer A. Debrisoquine Oxidation Polymorphism: Phenotypic Consequences of a 3-Base-pair Deletion in Exon 5 of the CYP2D6 gene. Pharmacogenetics 3: 123-130, 1993).

Subjects 28 and 46 whose MR were 6.68, 2.86 respectively appeared genotypically as heterozygous EMs. The PCR for these subjects yielded products at 564 bp and 300 above bp. Conceivably these individuals are heterozygous with CYP2D6 mutations that have not been identified. This may also be the case for the one subject in the PM group and one of the EM's who failed to amplify by PCR analysis. They may be homozygotes/heterozygotes with different fragments. One of the PM subjects produced a band with primer 1/8 indicating the presence of CYP2D6*4 allele. The MR value for this subject was 12.78.

Thus, apart from the previous allele-specific PCR methods we used previously, we also adopted other PCR techniques to identify some of the commoner null and defective CYP2D6 alleles in our 97 donor subjects. In the 97 blood donors, we again found the null CYP2D6*4 allele from the subjects and in total, 5 of the 6

defective alleles tested were found. The finding of CYP2D6*9 and *17 was novel and we are not aware of any similar reports from other Asian populations. The latter, commonly seen in Zimbabweans, occurred at a frequency of 3% in Saudi Arabs (McLellan et al., 1977), who may have passed it to the Malays. Although we did not find CYP2D6*3 in our subjects, CYP2D6*4 occurred with a frequency of 3.1%. The defective CYP2D6*3 and *4 alleles are responsible for the PM phenotype among Caucasians. Their rarity among Chinese (Roh et al., 1996; Garcia-Barcelo et al., 2000) contributed to their low PM prevalence. CYP2D6*4 frequency is 0.8% in Taiwanese Chinese (Wang et al., 1993) but was not found among Hong Kong Chinese (Garcia-Barcelo et al., 2000). A low 0.5% occurrence has also been reported in Japanese (Tateishi et al., 1999) and none was found in 152 Koreans (Roh et al., 1996). Its frequency is slightly higher at 3.5% in Saudis (McLellan et al., 1977). It is therefore conceivable that the higher frequency for Malays may have come from the Arabs and the Indians who seem to have a relatively higher PM prevalence, given the relative constancy of CYP2D6*5 allele frequencies across populations (McLellan et al., 1977), another allele that commonly causes the PM phenotype.

Within the EM phenotype, the distribution of debrisoquine MR among Chinese is shifted to the right, indicating lower enzyme activity. This has been attributed to the high prevalence of the CYP2D6*10 allele and its variants (Bertilsson et al., 1992; Lee & Jeyaseelan, 1994; Garcia-Barcelo et al., 2000), rare among Caucasians. This allele was also the most prevalent in our Malays. At 50%, it was however lower than the 62% reported in Singapore Chinese (Lee & Jeyaseelan, 1994), 70% in Taiwanese Chinese (Wang et al., 1993), and 65% in Hong Kong Chinese but comparable to the 48% and 51% occurrences among Mainland Chinese and Chinese living in Sweden (Garcia-Barcelo et al., 2000). This probably explains why we did not see such a marked shift to the right of our MR frequency distribution curve in our earlier study. This corresponds to a lower average MR value for Malays compared to their other Asian counterparts. The combined effect of lower CYP2D6*4, CYP2D6*2 and CYP2D6*1 prevalences compared to Caucasians with presence of CYP2D6*9, CYP2D6*10 and CYP2D6*17 however, would probably tend to cause Malays to have a higher average MR value than Caucasians.

CONCLUSIONS

We conclude that we have successfully developed an HPLC assay for the determination of DEB and 4OHDEB which can easily be applied for routine use to phenotype individuals for the genetic polymorphism of DEB metabolism. Careful attention needs to however be paid to specific error patterns at DEB and 4OHDEB urinary concentrations found in the subject so as to add credibility to the data generated. We also conclude that the PM phenotype for DEB metabolism is relatively common among our Malay subjects and this was probably associated with the relatively common occurrence of the CYP2D6*3 and CYP2D6*4 alleles among them, on top of the presence of other less active variants. We further conclude that a trend exists that suggests association/correlation between genotype and phenotype for DEB metabolism among our Malay subjects as shown with our results with DEB and metoprolol and this may be useful for developing strategies in designing dosage regimens for drugs which are co-substrates for DEB metabolism.

End of Project Report

A.	**Project number:** **06-02-05-6079**
	Project title: **DRUG METABOLISM AND ITS IMPACT ON DRUG DOSING: TOWARDS A MORE COST-EFFECTIVE DRUG THERAPY**
	Project leader: RUSLI ISMAIL, IC NO 4453670
	Tel: **09 7602624** **Fax:** **09 765 3370**

B Summary for the MPKSN Report (***For publication in the annual MPKSN Report, please summarise the project objectives, significant results achieved, reasearch approach and team structure***)

The polymorphic cytochrome P450 CYP2D6 (debrisoquine metabolism) is involved in the metabolism of various drugs of wide therapeutic use and is a presumed susceptibility factor for certain environmentally-induced diseases. The polymorphism shows pronounced interethnic variations . Even though the polymorphism has been relatively extensively studied in the West, data for Malays are still scarce. The only study was done in Singapore and reported the occurrence of PM in 2.1% of the Malays studied. The authors not attest much importance to the figure but this figure was higher than most Chinese and Asian figures. Ancient Malays originated from Yunnan in China. Their expansion from the coasts of Borneo to the Malay Peninsula and much of South East Asia, and their evolution into Modern Malays, both resulted from their trading and seafaring ways. They traded with and married their early trading partners, the Arabs, Indians, Chinese and Thais. This could make them differ from Chinese in their genetic make up and such a difference would be expected in the genetic polymorphism of CYP2D6 that is highly polymorphic. It was our objective therefore to study the genetic polymorphism of debrisoquine in Malays, to characterise it and to assess its impact on drug dosing in our Malay patients.

The study of the genetic polymorphism of debrisoquine (DEB) metabolism usually involves the investigation of the phenotypes that is subsequently followed by a genotyping confirmation We phenotyped 51 Malay subjects by administering 10 mg of DEB and collecting their urine over an eight-hour period. We found 2 (3.9%) PMs from of our 51 Malays, a figure higher than found in most Asian populations, and we found one who was probably an UM. Half our subjects had a debrisoquine MRs of 1 or less, again suggesting some distinctions wth Malays MR values for Asians are generally shifted to the right, a feature that has been attributed to the high frequency of occurrence of a defective CYP2D6 gene among Chinese and Japanese.

Using allele-specific PCR methods, we were able to identify alleles in 24 of the 51 subjects studied. The most frequent were homozygous 29wt (10 subjects) and heterozygous 29wt/B (11 subjects). Two subjects were heterozygous with 29wt/A allele and one was homozygous with 29B allele. Alleles (homozygous 29wt, heterozygous 29wt/A and 29wt/B) were therefore detectable in about half of the 48 EM's and in one of the 2 PM's (29B). The method does not therefore seem to be similarly sensitive in detecting CYP2D6 alleles among our Malay subjects. Genotypes have been shown to closely correlate with phenotypes for DEB metabolism. This appears true with our study. Thus homozygots EM's (29wt) on the average had the lowest MR and homozygot 29B PM the highest MR value. Heterozygous 29wt/A and 29wt/B had intermediate MRs. Firm correlation could not however be obtained because of our relatively small sample size and the large number of individuals who were not adequately genotyped. We therefore increased our sample size by taking subjects from among blood donors. We used other specific primers to better characterise the alleles but unfortunately the DNA from our initial 51 subjects could still not be amplified.

Our study with 97 Malay donors found them as relatively heterogeneous in terms of their CYP2D6 genotypes. We again found the null CYP2D6*4 (B) allele from the subjects. Thus there were 2PM from our 97 subjects. Five of the 6 defective alleles tested were found. The finding of CYP2D6*9 and *17 was novel and we are not aware of any similar reports from other Asian populations. The latter, commonly seen in Zimbabweans, occurred at a frequency of 3% in Saudi Arabs, who may have passed it to the Malays. Although we did not find CYP2D6*3 in our subjects, CYP2D6*4 occurred with a frequency of 3.1%. The defective CYP2D6*3 and *4 alleles are responsible for the PM phenotype among Caucasians. Their rarity among Chinese contributed to their low PM prevalence. CYP2D6*4 frequency is 0.8% in Taiwanese Chinese but was not found among Hong Kong Chinese. A low 0.5% occurrence has also been reported in Japanese and none was found in 152 Koreans. Its frequency is slightly higher at 3.5% in Saudis. It is therefore conceivable that the higher frequency for Malays may have come from the Arabs and the Indians who seem to have a relatively higher PM prevalence, given the relative constancy of CYP2D6*5 allele frequencies across populations, another allele that commonly causes the PM phenotype.

Within the EM phenotype, the distribution of debrisoquine MR among Chinese is shifted to the right, indicating lower enzyme activity. This has been attributed to the high prevalence of the CYP2D6*10 allele and its variants, rare among Caucasians. This allele was also the most prevalent in our Malays. At 50%, it was however lower than the 62% reported in Singapore Chinese, 70% in Taiwanese Chinese, and 65% in Hong Kong Chinese but comparable to the 48% and 51% occurrences among Mainland Chinese and Chinese living in Sweden. This probably explains why we did not see such a marked shift to the right of our MR frequency distribution curve in our earlier study. This corresponds to a lower average MR value for Malays compared to their other Asian counterparts. The combined effect of lower CYP2D6*4, CYP2D6*2 and CYP2D6*1 prevalences compared to Caucasians with presence of CYP2D6*9, CYP2D6*10 and CYP2D6*17 however, would probably tend to cause Malays to have a higher average MR value than Caucasians.

It is concluded that there is some difference in the genetic polymorphism of CYP2D6 among Malays as compared to other Asian counterparts. Their CYP2D6 alleles, probably due to the nature of their ancestry, were more heterogeneous. There seemed to be an increased occurrence of the null and defective alleles among them as compared to Chinese, Japanese and Koreans. They may also have inherited the CYP2D6*17 allele from the Arabs. Comparative studies of related ethnic groups in relation to CYP2D6 genotypes and prevalences of diseases allegedly linked to the polymorphism may provide another angle in delineating cause-effect relationships. Further work is also proposed to help define what is the effect of heterogeneous genotypes on the pharmacokinetic of CYP2D6 drugs in Malays.

C. **Objectives achievement**

Original project objectives (*Please state the specific project objectives as described in Section II of the Application Form*)

DEVELOPMENT OF HPLC ASSAYS
RAISING MONOCLONAL ANTIBODIES USING HYBRIDOMA TECHNOLOGY
DEVELOPMENT OF GENOTYPING TECHNIQUE USING PCR
PHENOTYPING STUDY
CLINICAL STUDY
TO GENOTYPE PHENOTYPE CORRELATE

- **Objectives Achieved** (*Please state the extent to which the project objectives were achieved*)

  Assays have been developed for DEB, 4OHDEB, metoprolol, propranolol, amitryptylline, dextrophan and dextrometorphan and a few other tricyclics
  PCR techniques were successfully employed to detect CYP2D6*1, *2, *3, *4, *5, *9, *10, and *17
  51 Malays successfully phenotyped
  Clinical study is partially completed and is on-going
  Genotype phenotype correlate was obtaine.

- **Objectives not achieved** (*Please identify objectives that were not achieved and give reasons*)

  Monoclonals not raised because money was not approved for equipment. Objective was altered to developing PCR techniques

D. **Technology Transfer/Commercialisation Approach** (*Please describe the approach planned to transfer/commercialise the results of the project*)

1. Research publications
2. Making genotype test available to drug houses involved with the marketing of CYP2D6 drugs

E. **Benefits of the Project** (*Please identify the actual benefits arising from the project as defined in Section III of the Application Form. For examples of outputs, organisational outcomes and sectoral/national impact, please refer to Section III of the Guidelines for the Application of R&D Funding under IRPA*)

- **Output of the project and potential beneficiaries** (*Please describe as specifically as possible the outputs achieved and provide an assessment of their significance to users*)

  Genotyping and phenotyping techniques established - can serve as a test-house for pharmaceutical houses.
  Population characterestic of CYP2D6 polymorphism characterised in Malays.
  Useful for planning therapeutic strategies.

- **Organisational Outcomes** (*Please describe as specifically as possible the organisational benefits arising from the project and provide an assessment of their significance*)
  Genotyping and phenotyping techniques established on-site and can be offerred for outside use.
  One MSc and 1 MMed have graduated.
  Three PhD's and one MSc on-going

- **National Impact** (*If known at this point in time, please describe as specifically as possible the potential sectoral/national benefits arising from the project and provide an assessment of their significance*)
  The only centre locally that can do Genotyping CYP2D6

F. **Assessment of project structure**

- **Project Team** (*Please provide an assessment of how the project team performed and highlight any significant departures from plan in either structure or man-days utilised*)

  Generally there was no major departures from plan excepting the failure to proceed with the objective of developing monoclonals and thus the essentially non-participating of the particular project member.

- **Collaboration** (Please describe the nature of collaborations with other research organisations and/or industry).

  Prof. Inger Johansson of Karolinska Institutet, Sweden has provided invaluable advice in PCR techniques.
  Dr Jennie Wong of the National University of Singapore provided positive controls for CYP2D6* 5 and *10
  Prof. Inger Johansson of Karolinska Institutet, Sweden provided positive controls for the duplicated gene of and
  Dr. rer. nat. Ulrich Griese of the Dr. Margarete Fischer-Bosch - Institut fuer Klinische Pharmakologie Auerbachstr provided positive controls for CYP2D6*3, and *4.

G. **Assessment of Research Approach** (*Please highlight the main steps actually performed and indicate any major departures from the planned approach or any major difficulty encountered*)

Plan to develop monoclonals and assays based on it had to be abandoned due to inavailability of equipment which was not approved for.
There was also some difficulty in enrolling psychiatric patients to study genotype pharmacokinetic correlation to develop dosing normograms.

H. **Assessment of the Project Schedule** (*Please make any relevant comment regarding the actual duration of the project and highlight any significant variation from plan)*

Genotyping and phenotyping proceeded smoothly because they involved volunteers only. Patient studies however took longer than plannned due to difficiculties in enrolling patients.

I.	**Assessment of the Project Cost** (*Please comment on the appropriateness of the original budget and highlight any major departures from the planned budget*) Approved budget was lower than what was needed but this was overcome by the approval of a new grant that was essentially a continuation of this.
J.	**Additional Project Funding Obtained** (***In case of involvement of other funding sources, please indicate the sources and total additional funding provided***) NONE
K.	**Other Remarks** (***Please include any other comment which you may feel is relevant for the evaluation of this project***) **The major finding was perhaps that Malays are quite distinct from other East Asians in relation to CYP2D6 polymorhism and this may neccesitate us to rethink about some of our assumptions about susceptibility to certain diseases and therapies.**
	Date: 6/7/2000 **Signature:**

Technology Transfer/Commercialisation Approach (*Please describe the approach planned to transfer/commercialise the results of the project*)

Patent (Please state full title of the patent number or application number)

NONE

Publication pertaining to the research finding

Report/Conference Paper

1. Rusli Ismail, Ahmad Hussein, LK Teh. "Heterogeneity of CYP2D6 Polymorphism in Malays" IXth SEA Drug Metabolism Workshop, Kuala Lumpur, 13-15th Apr 1998
2. Rusli Ismail, Ahmad Hussein. "HPLC Assay Error" IXth SEA Drug Metabolism Workshop, Kuala Lumpur, 13-15th Apr 1998
3. Rusli Ismail, Ahmad Hussein. "Genetic Polymorphism of Debrisoquine Metabolism in Malays" Xth SEA Drug Metabolism Workshop, Indonesia 1996

Journal Publication (*Use only the standard accepted abbreviations for journal titles*)

1. Rusli Ismail, Ahmad Hussein, LK Teh, M Nizam Isa. CYP2D6 PHENOTYPES AMONG MALAYS IN MALAYSIA. J Clin Pharm Ther - submitted for publication
2. Rusli Ismail, LK Teh, Rohana Yusoff, Ahmad Hussein, M Nizam Isa, A.R.A Rahman. HETEROGENEITY OF CYP2D6 GENE AMONG MALAYS IN MALAYSIA. Pharmacogenetics - submitted for publication
3. Rasool AH, Rahman AR, Ismail R, Hatim S, Abdullah AR, Singh R, Haron R. Ethnic differences in response to non-selective beta-blockade among racial groups in Malaysia. Int J Clin Pharmacol Ther. 2000 May;38(5):260-9;
4. Rasool AH, Ismail R, Rahman AR. Simple and Sensitive HPLC Method For the Detection of Plasma Propranolol. Mal J Med Sci 4(2): 23-27, 1997;

Others:

Post Graduates (Who graduated or who are still participating the project)

	Student Name & Year of Registration?Nationality	Thesis Title	PhD/MSc	Year of Completion
	Ahmad Hussein, 1996, Bangladesh	Probing the Genetic Polymorphism of Debrisoquine Metabolism in Malays	MSc	1998
	Norita Ahmad, 1999, Malaysia	CYP2D6 Genotype and Metoprolol	Mmed	2000
	Teh Lay Kek, 1997, Malaysia	Population Genetics of CYP2D6 and Relevance in Hypertension	PhD	Ongoing, part-time
	Gan SH, 1999, Malaysia	Pharmacogenetics of Tramadol in Post-Op Patients	PhD	Ongoing,
	Noraini Ariffin, 1999, Malaysia	CYP2D6 Polymorphism and Drug Abuse	PhD	Ongoing, part-time

No. of Research Assistants or Officers funded by the project:

Research Officers: Research Assistants:: 2

- **Collaboration** (Please describe the nature of collaborations with other research organisations and/or industry).

(a) Local Institutions:

Departments of Pharmacy and Biochemistry, Universiti Malaya where part of the study was done

(b) International Institution:

Karolinska Institutet, Sweden has provided invaluable advice in PCR techniques.
National University of Singapore provided positive controls for CYP2D6* 5 and *10
Dr. Margarete Fischer-Bosch - Institut fuer Klinische Pharmakologie Auerbachstr provided positive controls for CYP2D6*3, and *4.

Objective achievement

Original project objectives (*Please state the specific project objectives as described in Section II of the Application Form*)