GENETIC POLYMORPHISM IN DEXTROMETHORPHAN METABOLISM BY CYP2D6 AND CYP3A4 ENZYME ISOFORMS

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Abstract

Genetic polymorphism in dextromethorphan metabolism by CYP2D6 and CYP3A4 enzyme isoforms

Most administered drugs are metabolised in the liver by Phase I enzymes and more importantly by the cytochrome P450 (CYP) system. The extent of first-pass metabolism is important in determining whether the drug will have therapeutic or adverse effects after being administered to a patient. To date the CYP family has been shown to consist of 74 families denoted as CYP1 to CYP118, and only a few families are significantly involved in drug metabolism. CYP3A4 is the most important isoenzyme followed by CYP2D6, CYP2C9, and CYP2C19 with a small contribution by CYP2E1, CYP2A6, and CYP1A4. CYP2D6 and CYP3A4 enzyme isoforms have been well established to exhibit interethnic and inter-individual variability with regard to drug metabolising capacity.

Mutation on the gene coding for a metabolising enzyme is a major cause of variation in drug metabolism. This mutation gives rise to allelic variants producing enzymes with altered metabolising activity. The presence of an allele with decreased metabolic activity in an individual gives rise to the poor metabolising (PM) phenotype. When the PM phenotype occurs at a frequency of more than 1% within a given population, then the term genetic polymorphism applies. The aberrant metabolic capacity translates into variable drug responses of more than 20-fold, leading to different susceptibility to sub-therapeutic effects or adverse drug reactions. A significant number of drugs, such as the β-adrenergic blockers, antidepressants, antipsychotic and antiarrhythmic agents, are entirely or partly metabolised by CYP2D6 and CYP3A4. Genetic polymorphism is especially important for drugs with a narrow therapeutic/toxicity window.

Phenotyping involves the use of a probe drug that is administered to the subject, followed by determination of the parent drug and its metabolites in the urine. The aim of this study was to develop and validate an HPLC method for phenotypic determination of the CYP3A4 and

CYP2D6 enzymes, followed by the application of the assay in a random heterogeneous population of males.

Dextromethorphan (DXM) was used as an in *vivo* probe for simultaneous determination of the phenotypic expression of CYP2D6 and CYP3A4. An HPLC method coupled with a fluorescence detector was developed for the phenotypic determination of CYP2D6 and CYP3A4 iso-enzymes as determined by the concentration of dextromethorphan/dextrophan (DXM/DX) and dextromethorphan/3methoxy-morphinan (DXM/3MM) metabolic ratios respectively. The compounds were separated on a phenyl column (150 x 4,6 mm, 5-μm particle size) serially connected to nitrile column (250 x 4,6 mm, 5-μm particle size) using mobile phase of 80% (1.5% glacial acetic acid and 0.1% triethyl amine in distilled water) and 20% acetonitrile. Solid phase extraction was used to extract the analytes from urine samples using silica cartridges. The suitability of the method was demonstrated in a preliminary study with sixteen healthy Caucasian males. After a single oral 30 mg DXM dose, the volunteers were required to collect all urine samples voided 8 hours post oral dose. DXM/3HM and DXM/DX metabolic ratios were determined from collected urine samples.

The method was validated for DXM and DX at a concentration range of 0.25 - 30 μg/ml, and at 0.025 - 3 μg/ml for 3MM. Calibration curves were linear with R^2 values of at-least 0.999 for all compounds of interest. Recoveries were 97%, 93%, and 65% for DX, DXM and 3MM, respectively. The method was reproducible with intra-day precision having coefficients of variation percentage (CV%) of less than 17% for all analytes. Inter-day precision had a CV% of less than 14% for all analytes. The limit of detection was 30 ng/ml for all compounds. All volunteers were classified with an extensive metaboliser (EM) phenotype. In conclusion the method described is suitable for polymorphic determination of CYP2D6 and CYP3A4 in a population study, and may have value in further studies planned at investigating the critical issue of racial genetic polymorphism in ethnic groups in South Africa.

Key words: HPLC, Cytochrome P450 2D6 (CYP2D6), Cytochrome P450 3A4 (CYP3A4), Dextromethorphan, Metabolism, Phenotyping.

Uittreksel

Genetiese polimorfisme in dekstrometorfaanmetabolisme as gevolg van CYP2D6- en CYP3A4-ensiemsisoforme

Die meeste geneesmiddels word in die lewer met behulp van Fase I ensieme, veral die sitochroom P450 (CYP) sisteem, gemetaboliseer. Die mate van eerste deurgangsmetabolisme is belangrik om vas te stel of 'n geneesmiddel terapeutiese of newe effekte gaan hê na toediening aan die pasiënt. Tot op datum bestaan die CYP-ensiemsisteem uit 74 familie groepe, aangedui as CYP1 tot CYP118, met slegs 'n paar familie wat betekenisvol betrokke is in geneesmiddelmetabolisme. CYP3A4 is die belangrikste isoensiem, gevolg deur CYP2D6, CYP2C9 en CYP2C19 met CYP2E1, CYP2A6 en CYP1A4 wat 'n kleiner bydra lewer. Inter-etniese en interindividuele variasie ten opsigte van geneesmiddelmetabolisme kapasiteit is duidelik aangetoon vir CYP2D6 en CYP3A4 isoensieme.

Geenmutasie van die metaboliserende ensiem is 'n belangrike oorsaak van variasie in geneesmiddelmetabolisme. Hierdie mutasie lei tot die vorming van alleliese variante wat ensieme produseer met veranderde metaboliese aktiwiteit. Die teenwoordigheid van 'n alleel met 'n verminderde metaboliese aktiwiteit in 'n individu, lei tot die vorming van die fenotipe wat 'n swak metaboliseerder (SM) genoem word. Indien die SM- fenotipe voorkom met 'n frekwensie van meer as 1% van die populasie, word die term genetiese polimorfisme van toepassing. Die afwyking in metaboliese kapasiteit het tot gevolg dat daar tot 'n twintig-voudige verskil in geneesmiddelrespons kan voorkom, met gepaardgaande verskille ten opsigte van die voorkoms van sub-terapeutiese effekte en newe effekte. 'n Beduidende aantal geneesmiddels, o.a. die β-adrenergiese blokkeerders, antidepressante, antipsigotika en anti-aritmiese middels, word ten volle of gedeeltelik deur CYP2D6 en CYP3A4 gemetaboliseer. Genetiese polimorfisme is veral belangrik ten opsigte van geneesmiddels met 'n nou terapeutiese- / toksisiteitsvenster.

Fenotipering behels die toediening van 'n proefmiddel aan die persoon, gevolg deur die bepaling van die oorspronklike geneesmiddel en sy metaboliete in die urien. Die doel van die huidige studie was om 'n hoëdrukvloeistofchromatografiese (HDVC) metode te ontwikkel en te valideer ten einde fenotipiese bepaling van CYP3A4- en CYP2D6-ensieme moontlik te maak. Die ontwikkeling en validering van die metode is opgevolg deur die toepassing hiervan in 'n steekproef met 'n heterogene manlike populasie.

Dekstrometorfaan is as 'n *in vivo* toetsmiddel gebruik vir die gelyktydige bepaling van die fenotipiese uitdrukking van CYP2D6 en CYP3A4. 'n HDVC-metode met fluoressensie deteksie is ontwikkel vir die fenotipiese bepaling van CYP2D6 en CYP3A4 iso-ensieme. Dit is gedoen deur die bepaling van die konsentrasie van die metaboliese verhoudings van dekstrometorfaan/ dekstrofaan (DXM/DX) en dekstrometorfaan/3metoksie-morfinaan (DXM/3MM) onderskeidelik. Die verbindings is op 'n fenielkolom (150 x 4,6 mm, 5-µm deeltjie-grootte) in lyn gekoppel aan 'n nitrielkolom (250 x 4,6 mm, 5-µm deeltjie-grootte). Die mobiele fase het bestaan uit 80% ysasynsuur (1,5%) en tri-etielamien (0,1%) in gedistilleerde water en 20% asetonitriel. Soliedefase-ekstraksie met behulp van silikonpatrone is gebruik om die geanaliseerde stowwe vanuit die urienmonsters te onttrek. Die geskiktheid van die metode is in 'n voorlopige studie met sestien gesonde manlike Kaukasiërs bevestig. Na 'n enkel orale 30 mg DXM-dosis, is die vrywilligers versoek om alle urienmonsters tot en met 8 uur na die orale dosis te versamel. Die metaboliese verhoudings van DXM/3HM en DXM/DX is uit die versamelde urienmonsters bepaal.

Die metode is gevalideer vir DXM en DX in 'n konsentrasiereeks van 0,25 – 30 µg/ml, en vir 3MM in die reeks 0,025 - 3 µg/ml. Kalibrasiekurwes was liniêr met R^2-waardes van ten minste 0.999 vir alle verbindings van belang. Herwinning was 97%, 93% en 65% vir DX, DXM en 3MM onderskeidelik. Die metode was herhaalbaar met intra-dag presisie met koëffisiënte van variasiepersentasies (CV%) van minder as 17% vir alle geanaliseerde stowwe. Inter-dag presisie het 'n CV% gehad van minder as 14% vir alle geanaliseerde stowwe. Die deteksielimiet vir alle verbindings was 30 ng/ml. Alle vrywilligers is as ekstensiewe metaboliseerder fenotipe (EM) geklassifiseer. Die gevolgtrekking kan dus gemaak word dat die metode wat beskryf is, geskik is vir polimorfiese bepaling van CYP2D6 en CYP3A4 in 'n populasiestudie en van waarde mag wees in verdere studies wat beplan word om die kritiese vraagstuk van genetiese polimorfismes wat rassensitief is, in etniese groepe in Suid Afrika te ondersoek.

Sleutelwoorde: Hoëdrukvloeistofchromatografie, Sitochroom P450 2D6 (CYP2D6), Sitochroom P450 3A4 (CYP3A4), Dekstrometorfaan, Metabolisme, Fenotipering.

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***"Kure ndokusina, kwachiri unofa wasvika"* (Shona)**

Far is where there is nothing, where there is something, you will struggle to death to reach there (Direct English translation)

Where there is a will there is a way (Closest English equivalent proverb)

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List of Abbreviations

3D QSAR:	Three dimensional quantitative structure activity relationship
3HM:	3-hydroxy-morphinan
3MM:	3-methoxy-morphinan
4HD:	4-hydroxy debrisoquine
AUC:	Area under the curve / total amount of drug
BMI:	Body mass index
Cl:	Clearance
CNS:	Central nervous system
C_{max}	Maximum serum concentration
COMT:	Catechol-*O*-methyl transferase
Conc:	Concentration
CV%:	Percentage of the coefficient of variation
CYP:	Cytochrome P450
DB:	Debrisoquine
DHS:	Dehydro-sparteine
DX:	Dextrorphan
DXM:	Dextromethorphan
HM:	Hydroxy metoprolol
HPLC:	High pressure liquid chromatography

IEM:	Intermediate extensive metaboliser
IS:	Internal standard (levallorphan in this literature)
LOD:	Limit of detection
LOQ:	Limit of quantification
M:	Metoprolol
MFO:	Mixed function oxidase
NAT:	*N*-acetyl-transferase
ND:	No data available at the given time
nm:	Nano meter
PCR-RFL:	Polymerase chain reaction restrictive fragment length
PM:	Poor metaboliser
psi:	Pascal per square inch
SULT	Sulfotransferase
SD:	Standard deviation
SEM	Slow extensive metaboliser
SSRI	Serotonin selective reuptake inhibitors
$t_{1/2}$:	Drug half time
TCA:	Tetracyclic antidepressants
UEM:	Ultra rapid extensive metaboliser
UGT:	UDP-glucuronyltransfarase

Introduction

Chapter 1

1.1 Background

"Amagazi abantu awafani" is an old Zulu saying which roughly means each individual has his unique blood type. The Inyangas (traditional herbalist) often used this idiom to explain to their patients why a recommended medicament did not produce similar results in different patients for a particular ailment. This clearly demonstrates that interindividual variation in drug response was recognised far back in African culture even before the introduction of western medicinal substances in Africa.

Interindividual variation of drug effects commonly occurs with a variety of drugs. The variation can be attributed to a number of factors among which the rate of drug metabolism is regarded as being the most important factor (Yokoi & Kamataki, 1998). Some of these variations can be explained by a genetic variation in drug transport protein (e.g. P-glycoprotein), but also of drug targets (e.g. polymorphic β_2-adrenergic receptors). The main causes of variation in the rate of drug metabolism include the following: genetic polymorphism of a metabolic enzyme; induction or inhibition of a metabolising enzyme due to concominant drug therapies or environmental factors; physiological status; and disease states. The first two are of major importance (Ingelman-Sunberg, 1999). For a number of drugs that have been studied, large interindividual variation in the rate of metabolic eliminations are under predominantly genetic rather than environmental control (Vesell, 2000). Pharmacogenetics is a discipline within pharmacology that is aimed at addressing the effects of genetic differences on a person's response to drugs.

Inter-individual variation can be accounted for by the presence of a polymorphic expressed drug metabolising enzyme within a population. Well-studied examples of genetically polymorphic metabolic enzymes are cytochrome P450 enzymes (CYPs), N-acetyl transferase (NATs), UDP-glucuronyltransferase (UGT), sulfotransferase (SULT), catechol-O-methyl transerase (COMT), and thiopurine methyl transfarse (TPMT), among others (Gaedigk, 2000). CYPs are the most extensively studied. CYPs are expressed in almost every mammalian tissue.

Mammalian cell membranes consist of a double lipophilic layer that forms the barrier between the internal and the external environment. Due to their chemical nature, lipophilic compounds

can easily cross this barrier while hydrophilic compounds require an active transport system to cross it (Correia, 1998). Applying this concept to human body design, lipophilic compounds are easily absorbed from the gastro-intestinal tract (GIT), and also quickly reabsorbed back into the system from the renal tubules. The net effect would be an indefinite life span of any lipophilic compound introduced into the body.

To overcome this, xenobiotics undergo metabolism with the aim of converting lipophilic compounds to hydrophilic compounds that are more readily excretable. Generally, metabolism is divided into phase I and phase II reactions. Virtually all pharmaceuticals are metabolised by phase I followed by phase II metabolism (Nebert, 2000). Phase I reactions include oxidation, reduction, and hydrolysis as a means of converting a hydrophobic compound to one that is hydrophilic, whereas phase II reactions achieve the same goal by conjugating the drug with endogenous hydrophilic groups. The cytochrome P450 (CYP) enzyme system catalyses a vast majority of Phase I reactions.

CYPs can act on many endogenous substrates, introducing oxidative, peroxidative, and reductive changes to small molecules of widely different chemical structures (Nerbert & Russel, 2002). Substrates identified to date include eicosanoids, sterols, steroids, bile acids, vitamin D_3 derivatives, retinoids, saturated and unsaturated fatty acids (Yamazaki & Shimada, 1999). These substrates play an important role in the human bodily functions such as homeostasis. By virtue of their involvement in the synthesis, metabolism, and activation of endogenous substances, CYPs are crucial for life systems.

The term CYPs is an umbrella term for all members, sub-members, and individual enzymes of the CYP system (Nerbert & Russel 2002). CYPs are well noted for their ability to metabolise prescribed drugs and to detoxify carcinogens. Up to date, 74 CYP families have been characterised consisting of families CYP1 to CYP118 (Yokoi & Kamataki 1998), although only a few human CYPs are significantly involved with drug metabolism. In terms of drug metabolism, CYP3A4 is the most important followed by CYP2D6, CYP2C9, and CYP2C19 with a small contribution of CYP2E1, CYP2A6, and CYP1A4 (Guengerich 1995). Hepatic CYP2D6 and CYP3A4 represent 2% and 30%, respectively, of total liver CYP enzyme content (Shimada *et al.*, 1994). Together they are capable of metabolising over 90 commonly prescribed therapeutic drugs (Gaedigk, 2000). There is a need to understand these various CYPs iso-forms so as to optimise drug treatment.

Medical science has made it possible to determine the metabolising phenotype of an individual with the use of selective analytical methods. This process, known as phenotyping, makes use of metabolic probes that are administered by various routes and the recovered metabolic probes

and their metabolites are quantified from blood plasma, saliva or urine. By applying phenotyping procedures, scientists can now tell whether an individual has an extensive, ultra-rapid or poor metabolising (PM) capability.

In healthy patients that are not taking any substances known to interfere with the enzyme metabolising capability of an enzyme, an ultra rapid or poor metabolising phenotype can be accounted for by the mutation on a gene coding for that particular enzyme (Masimirembwa & Hasler, 1997). The nature of a mutation at DNA level can be determined using a procedure known as genotyping (Meyer, 1997). Genotyping can reveal allele(s) responsible for the production of the mutated enzyme whose activity deviates from the normal enzyme.

The ability of these enzymes to metabolise drugs does not only vary from person to person, but also exhibits inter-ethnic variation. Reports of racial differences in response to drugs and other exogenous chemicals appeared very infrequently in the medical literature before the 1920s (Weber, 1999). However, in more recent times, studies have consistently shown that 5-10% of Caucasian populations fail to effectively metabolise drugs that are subjected to CYP2D6 metabolism (i.e. 5-10% of the population have CYP2D6 PM phenotype, Alvan *et al.*., 1990). Conversely studies on Orientals have consistently shown the lowest prevalence of CYP2D6 PMs of 0-1% (Johansson *et al.*, 1991; Dahl *et al.*, 1995). Few as they may be, studies on Africans show the greatest variation of PM phenotype, with a prevalence of 0.02% (Iyun 1986) to 19% (Sommers *et al.*, 1989). Interestingly, each population seems to have a common allele accounting for the prevalence of the PM phenotype that is different to another population group. Unlike CYP2D6, most of the data supporting polymorphic expression of CYP3A4 is from drug pharmacokinetic studies rather than phenotyping studies. Much of the interethnic variations are attributed to the presence of genetic polymorphism (Masimirembwa & Hasler, 1997). Genetic polymorphism CYP enzyme is well established in various ethnic groups. Comparatively, polymorphism of CYP2D6 and CYP2C19 are the best characterised, while little attention has been focused on CYP3A4 polymorphism.

A significant number of drug groups, such as the β-adrenergic blockers, antidepressants, antipsychotic and antiarrhythmic agents, are entirely or partly metabolised by CYP2D6 and CYP3A4 (Bradford & Kirlin, 1998). This is especially important because subjects presenting with a PM status will more easily develop adverse drug reactions due to failure of eliminating the administered drug dose effectively which could lead to drug accumulation. Alternatively, ultra rapid extensive metabolisers (UEM) more commonly report decreased drug efficacy due to rapid drug clearance. Depending on the nature of the interaction and the overall contribution of the drug metabolising enzyme, typical drug-drug interactions can be exaggerated to fatal conditions for subjects carrying PM phenotype. More cases of drug-drug interactions are

reported for CYP3A4 because, unlike CYP2D6, the CYP3A4 isoform can be induced or inhibited by co-administered drugs or environmental factors.

There is evidence from the literature that Black patients require lower doses of serotonin selective reuptake inhibitors than Caucasian patients to attain a similar response in the treatment of severe depression (Varner *et al.*, 1998). With reference to neuropharmacotherapeutic agents, the clinical impact of CYP2D6 and CYP3A4 polymorphism from a Caucasian aspect has been demonstrated. However studies addressing the toxicological or therapeutic implication of these agents in a Black population have not been documented.

Data from one population should be projected with caution to another population. It is for this same reason that in Japan a drug is approved based on pharmacokinetics studies performed in Japanese population. At both interindividual and interethnic level, the relevance of genetic polymorphism and its implication for drug metabolism is indisputable. It is therefore essential to understand the catalytic mechanism of CYP in order to predict adverse drug effects and drug interactions in both drug research and utilisation. This study will focus on the activities of the CYP2D6 and CYP3A4 isoforms, which collectively metabolise the largest proportion of commonly prescribed drugs.

1.2 Study objectives

The aims of this study are to:

Develop a sensitive method for identification and quantification of dextromethorphan (DXM) and its major metabolites in the urine, namely dextrorphan (DX) and 3-methoxy morphinan (3MM).

Conduct a clinical pilot study whereby the metabolic profile of CYP2D6 and CYP3A4 will be determined from human subjects by administering DXM as the probe drug.

Discuss and compare our findings to other published studies of similar nature.

1.3 Study approach

DXM is a metabolic probe of choice for both in vitro and in vivo determination of cytochrome P4503A4 and P4502D6 enzymatic catalytic activities (Wieling *et al.*, 2000). A quantitative HPLC method will be set up, and characterised for the assay of DXM and its major metabolites. Thereafter, the methodology will be evaluated in a small group of male students who will be recruited to participate in a phenotyping study. Each participant will take a pre-measured oral dose of DXM. Recovered DXM and its metabolites will be quantified from 8 hour urine samples collected post oral dose in each individual. CYP2D6 and CYP3A4 metabolic ratios will be

determined by quantifying recovered chemical species. From this, the CYP2D6 metabolic activity for each patient will be determined by using the CYP2D6 metabolic ratio parameter, introduced in later text. CYP3A4 will be determined in a similar manner.

Data will be analysed for statistical significance, and the possible applications of the method will be discussed. Data will then be compared to population norms for Caucasian, and, discussed with respect to normal distribution and possible application to population kinetics and future drug studies.

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The cytochrome P450 system

Chapter 2

2.1 Background

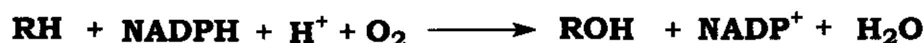
Biotransformation can take place in almost every type of living cell, although the chief organ of biotransformation is the liver (Correia, 1998). To achieve biotransformation, the liver is equipped with microsomal mixed function oxidase (MFO) enzymes. Cytochrome P450 (a super-family of haem containing oxidative enzymes) is a member of the MFO enzymes. Cytochromes are defined as haemoproteins, the principal function of which is electron and /or hydrogen transport by virtue of a reversible valency change of haem iron from the ferrous to the ferric state (Lemberg & Barret, 1973).

CYP was discovered in 1955 and described as a pigment which reacts with carbon monoxide and accompanied by cytochrome b_5 (Cooper *et al.*, 1965). Sato and co-workers suggested the name cytochrome P450 be given because of its unusual characteristic Soret and spectral peak absorption in the carbon monoxide bound state at 450 nm (Lemberg & Barret, 1973). The letter P stands for pigment. CYP-dependant drug metabolism is well distributed in a wide range of animal and plant species. In mammals, CYP expression has been reported in almost all tissues including the brain, lung, kidney, small intestines, and colon, but its highest concentration is in the liver cells (Strobel *et al.*, 1995). Not every tissue expresses the same spectrum of isoforms, but each tissue has its own profile of expression.

2.2 The catalytic cycle of CYPs

Other major components of the MFO enzymes include flavoprotein (NADPH-reductase or NADH-dependant flavoprotein), iron sulphur protein and cytochrome b_5. These components serve as electron donors for the CYP enzyme. Mitochondrial and most bacterial CYP system contain three components, a flavoprotein, an iron sulphur protein and CYP enzyme (Degtyarenko & Archakov, 1993). Eukaryotic microsomal CYP system, however, only have two components, viz flavoprotein, and CYP enzyme. The roles of these additional components become clear when one considers the catalytic cycle of CYP enzymes. A unique bacterial one-component system exist as a single polypeptide chain with two functional parts, the haem and

flavin domains that are co-joined. CYP catalysed hydroxylation reactions have the following stoichiometric reaction formula (Strobel *et al.*, 1995, Poolsup *et al.*, 2002):



$$RH + NADPH + H^+ + O_2 \longrightarrow ROH + NADP^+ + H_2O$$

Where RH represents a large variety of compounds including *N*- and *O*-alkyl drugs, polycyclic aromatic hydrocarbons, alkanes, fatty acids, pesticides, and chemical carcinogens. ROH is the hydroxylated product which may manifest more, slight, similar or no pharmacological activity. Because of the introduction of the hydroxyl group, the compound becomes polar and is much more readily excreted.

The catalytic cycle of CYPs can be summarised as follows (Segall, 1997):

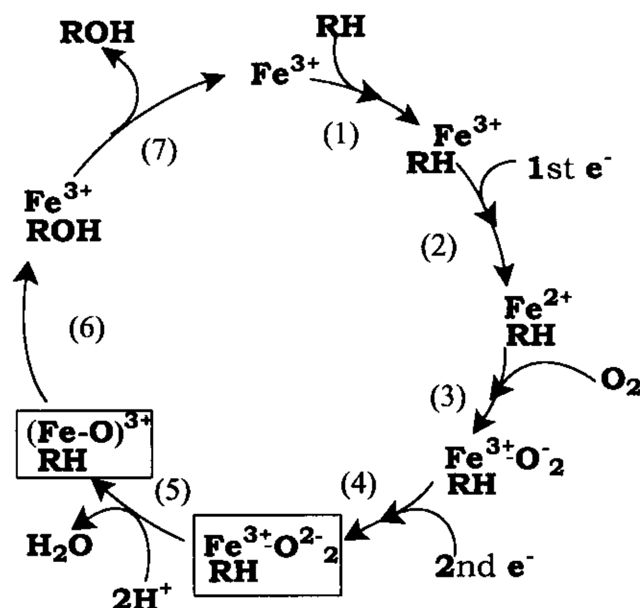


Figure 2.1. The events of the catalytic cycle of the CYPs

1) Substrate binding.

   The binding of a substrate to CYP causes a lowering of redox potential, which makes the transfer of the electron favourable for its redox partner, **NADH** or **NADPH**. It has also been suggested that substrate binding causes a conformational change in the enzyme, which triggers an interaction with the redox partner.

2) The first reduction:

   The next stage in the cycle is the reduction of the Fe^{3+} (ferric ion) into a Fe^{2+} (ferrous ion) by an electron transferred from **NAD(P)H** via an electron transfer chain.

3) Oxygen binding:
   An O_2 molecule binds rapidly to the Fe^{2+} ion forming a Fe^{2+}—O_2 complex. This complex undergoes a slow conversion into a more stable Fe^{3+}—O^{-}_2 complex.

4) Second reduction:
   Described as the rate-limiting step of the reaction. The Fe^{3+}—O^{2-}_2 (peroxoiron) complex so formed becomes the starting point for the next reaction.

5) O_2 cleavage:
   The O^{2-}_2 reacts with two protons from the surrounding solvent, breaking the O-O bond, forming water and leaving an $(Fe—O)^{3+}$ iron oxo complex.

6) Product formation:
   The Fe-ligated atom is transferred to the substrate forming a hydroxylated form of the substrate.

7) Product dissociation:
   The product is released from the active site of the enzyme, which returns to its initial state.

The two boxed transitional complexes from figure 2.1 have not been monitored spectroscopicaly and are hypothesised on the basis of analogy with other haemoproteins.

2.3 Families of CYP and their naming

An isoform in this context is a CYP enzyme variant that derives from one particular gene. The total number of CYP enzymes is approximately 750 and at least 30 different human P450 genes have been purified, sequenced and characterised (Guengerich, 1995).

2.3.1 Naming CYPs

During the late 80's scientist realised the need to adopt a universal CYP nomenclature. Nebert and co-workers subsequently proposed and developed the naming system over a period of 6 years (Nebert *et al.*, 1987; Nerbet & Nelson, 1991). When naming CYP subtypes, the capitalised CYP root is prefixed (denoting cytochrome P450) followed by an Arabic numeric denoting the individual family, followed by a letter indicating a subfamily (if subfamilies are known to exist within that family; Nerbet & Nelson, 1991). Naming the gene coding for the enzyme applies the same criteria, but the writing is italised, e.g. CYP2D6 is the protein product of *CYP2D6* gene. In depth details on naming CYP genes is provided on the world wide web (Oscarson, 2002).

2.3.2 Classification criteria

The CYP isoforms are classified according to the similarities of their amino-acid sequences. The system is based on the evolution of CYPs, a classification that allows division of CYP isoforms into the following:

- Families
  Families contain genes that have at least a 40% sequence homology. There are 74 gene families described so far, of which 14 families exist in all mammals (Nelson *et al.*, 1996). From 74 families only about seventeen have been described in man.

- Subfamilies
  Members of a subfamily must have at least a 55% identity. About thirty subfamilies are well characterised in man

- individual genes - Human genome project identified 57 human CYP genes.

With regard to families, a few cases of exception to the 40% rule have been made; these changes were made on the basis of the observation of the phylogenic tree of CYPs. These involve one subfamily in the *CYP4, CYP11, CYP105,* and *CYP2* gene families. An example is the CYP2D subfamily that can easily stand as a separate family with clusters of more than 40% identity to other members of the CYP2 family. The CYP2D is however grouped together because of close clusters it forms with its submembers (Nebert & Nelson, 1991).

Clearly it can be seen that this naming system has various shortcomings relating to consistency of application. It is for this reason that Degtyarenko and Archakov (1993) suggested that in the future, a classification criterion employing both evolutionary and sequence-clustering parameters should be adopted. Such a system is however yet to be described.

CYPs are widely distributed in various life forms, and have been characterised in Animalia, Fungi, Plantae, and Bacteria kingdoms, with a range of CYP1 to CYP132. Nelson and co-workers (1996) compiled a list of 481 CYP genes and 22 pseudogenes, which have been described in 85 eukaryote and 20 eukaryote species.

CYP2D6 and CYP3A4 are microsomal enzymes, which account for approximately 4 % and 28 % of total liver content, respectively (Correia, 1998).

2.4 Chemistry of the CYP2D6 and CYP3A4

CYP enzymes are made up of between 400 to 500 amino acids, all containing a haem moiety in the centre of the protein (Poulos, 1991, Segall, 1997). The haem moiety is responsible for the

oxidation reactions of the enzyme (see fig. 2.1;Goves & Han 1995). Joining the haem moiety to the protein is a cystein residue (see figure 2.2). Due to the membrane bound nature of mammalian CYPs, information regarding their crystal structure is lacking (Nerbet & Nelson, Ekins, 1999). However, recent developments have allowed elucidation of the crystal structures of CYP2C9 and CYP2C5 (Williams *et al.*, 2000; Williams *et al.*, 2003). More often scientist resort to sequence alignment and computational methods to infer the structure, and particularly the active sites, of these enzymes.

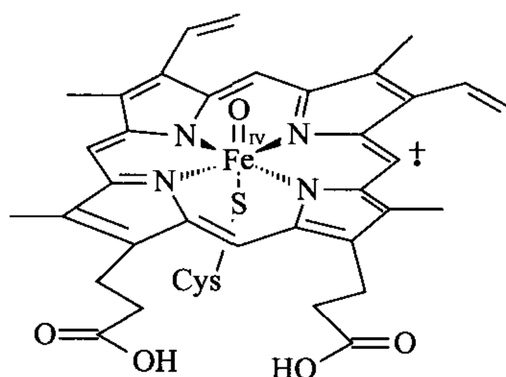


Figure 2.2. The oxoiron (IV) porphyrin cation radical (Groves & Han 1995)

Pharmacophore modelling, homology modelling, and 3 dimensional quantitative structure activity relationship (3D QSAR) techniques have been applied to predict the enzyme structures of CYP2D6 (Strobel *et al.*, 1993; Groot *et al.*, 1999). Up to now only pharmacore modelling has been applied to CYP3A4 (Ekins *et al.*, 1999a,b). Pharmacore modelling studies involve the use of well known substrates of the enzyme and relate their chemical properties to those of the enzyme (de Groot and Ekins, 2002). Homology modelling is the means of deciphering an unknown protein structure with reference to a known protein structure, such as $P450_{cam}$ (Poulos, 1991). 3D-QSAR techniques employ the following: selecting a group of molecules, each possesing a measured response from a given biological system; aligning molecules according to some predetermined orientation rules; calculate a set of spatially dependant parameters for each molecule determined in the receptor space surrounding the aligned series; derive a function that relates each molecule's spatial parameters to their respective biological property; establish self-consistency and predictability of the derived function (Green and Marshal, 1995).

Instead of focusing on one approach, Groot and co workers (1999a), combined different computational methods to produce a protein model of CYP2D6 (see fig. 2.3). The resulting model was further refined to a full protein with primary and secondary protein structures (i.e. the

beta sheet alpha helices), and, most impressively, it could account for the metabolism of numerous CYP2D6 substrates (Groot *et al.*, 1999b).

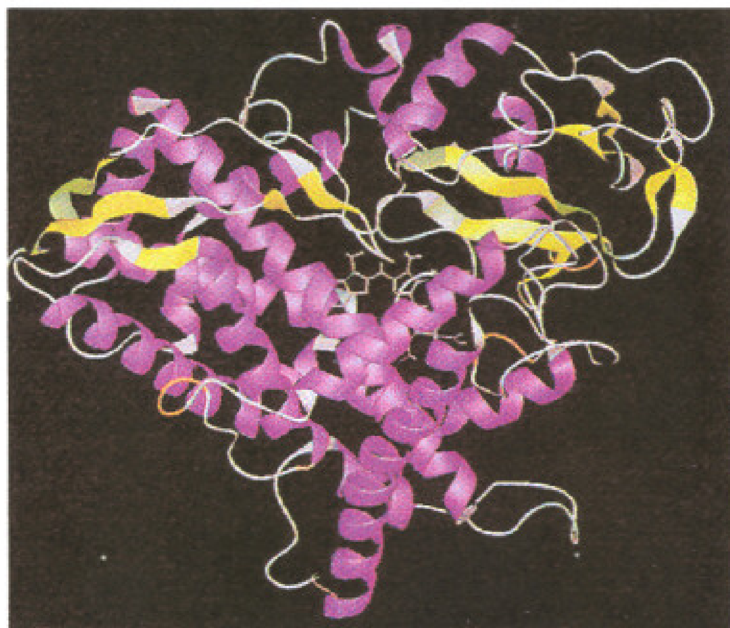


Figure. 2.3 CYP 2D6 protein model. (Groot *et al.*, 1998). Take note of the haem moiety at the centre and α helices in purple and β sheets in yellow.

Enzyme specificity is controlled by the surface loops and those groups that directly interact with the substrate (Poulos, 1991; Kaminsky, 1991). All CYP2D6 substrates have in common at least one basic nitrogen atom at 5 or 7 Å. (10 Å also possible) from the site of oxidation, a flat hydrophobic region (e.g. an aromatic ring) that has a negative electrostatic potential (Strobel *et al.*, 1995; Groot, 1999a).

CYP2D6 hydroxylation and *O*-dealkylation protein occurs between the Asp^{301} and basic nitrogen in the substrate, while the interaction between Phe^{481} and the aromatic ring and/or side chain in the substrate provides an additional stabilizing interaction. CYP2D6 catalysed *N*-dealkylations, the interaction of Phe^{481} with the aromatic ring, is the major stabilising interaction, strengthened by binding interaction with Leu^{121} and Leu^{213} (see fig. 2.4. Groot *et al.*, 1999a,b). Common features for CYP3A4 substrates consist of four structural features: two hydrogen bond acceptors 7.7 Å apart, one hydrogen donor and a hydrophobic region (Ekins *et al.*, 1999b).

Structures provided by these computational methods helps us understand the nature of interaction between the enzyme and its substrates, and is of great significance in drug research and development (Groot & Ekins, 2002). Enzyme structure has an important role in its specificity

and efficiency in drug metabolism. A minor change in protein configuration might lead to significantly altered catalytic properties.

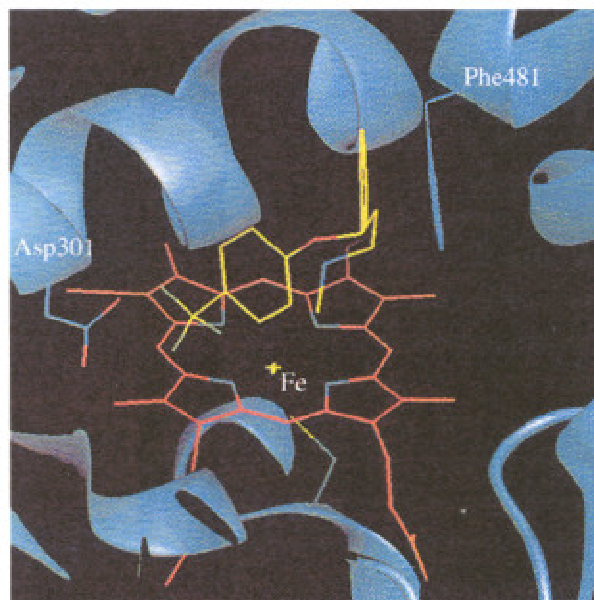


Figure.2.4. Orientation of fluoxetine (in yellow), leading to *N*-demethylation in the CYP2D6 model. An interaction between the aromatic ring of the substrate and aromatic side chain of Phe^{481} stabilises this orientation, in the absence of the interaction with Asp^{301}. In blue, is the amino acid helices of the protein structure. Red depicts the porphyrin ring with the iron at the centre of the protein (Groot *et al.*, 1999a).

2.5 Physiological role of CYPs

CYPs have three main metabolic functions in the body and these include steroid biosynthesis (steroidogenesis) and metabolism, fatty acid metabolism, and xenobiotic metabolism.

2.5.1 Steroidogenesis

CYPs involved in steroid metabolism include: CYP2G1, CYP7, CYP8B1, CYP11, CYP17, CYP19, CYP21, CYP27A1, CYP46, and CYP51. The initial and rate-limiting step in all steroidogenic pathways is the conversion of cholesterol to pregnenolone (Kagawa and Waterman, 1995). This reaction can take place inside the mitochondria of any type of steroidogenic cells, such as the adipose tissue, retina, stomach etc. (Kagawa & Waterman, 1995). The reaction is catalysed by the CYP11A1 formerly known as the cholesterol side chain cleavage (CYPscc) enzyme (Simpsons, 1979). Individuals who have a deficiency of CYP11A1 suffer from congenital adrenal hyperplasia (Anaesthetic, 2002). The remaining biosynthetic

pathways take place inside steroidogenic cells of either the adrenal cortex or the gonads (i.e. testes or ovaries).

2.5.1.1 Adrenal cortex steroidogenesis

The following steroidogenic pathways take place in the adrenal cortex where the largest number of steroidogenic CYPs are found, including CYP21, CYP11B1, and CYP11B2, formerly known as P450c21, P45011β and P450aldo, respectively (Anaesthetic, 2002). Cellular constituents of steroidogenic cells of the adrenal cortex can generally be divided into those that contain CYP17 and CYP11B1 and that synthesise glucocorticoids (fasciculata/reticularis), and those that CYP17 is absent but contain CYP11B2 which synthesise mineralocorticoids (glomerulosa). CYP21 is contained in both fasciculata/reticularis and glomerulosa and it catalyses two reactions, namely the formation of 11-deoxycortisol from 17α-hydroxyprogesterone which is the intermediate in cortisol biosynthesis, and the formation of deoxycorticosterone which is an intermediate in aldosterone biosynthesis (Anaesthetic, 2002).

2.5.1.2 Gonadal steroidogenesis

Testis steroidogenic cells contain CYP11A1 and CYPC17 that participate in the biosynthesis of the male hormone testosterone and dihydrotestosterone from cholesterol. Theca cells in the ovarian follicle contain CYP11A1 and CYPC17 while the granulosa steroidogenic cells contain CYP11A1 and CYP19 (former P450arom). Oestrogen production occurs in the granulosa cells, while oestrogen's immediate precursor, androsteridione, is synthesised in the theca cells (Anaesthetic, 2002).

2.5.1.3 Vitamin D_3 biosynthesis and metabolism

Vitamin D is classified as a steroid because of its structural chemistry and with CYP27A1, CYP27B1, and CYP8B participating in its metabolism. Mitochondrial enzyme CYP27A1, with a possible contribution of CYP2D6 and CYP3A4, catalyse the 25-hydroxylation of cholecalciferol (a vitamin D_3 precursor) in the liver to form 25-hydroxyvitamin D_3 (Nebert and Russell, 2002). In the kidneys, the CYP27B1 enzyme catalyses the 1α-hydroxylation of 25-hydroxy vitamin D_3 to form 1α,25-hydroxy vitamin D_3, the active ligand of the vitamin D_3 receptor. The 1α,25-hydroxy vitamin D_3 ligand, and the vitamin D_3 receptor system is responsible for the mobilisation of calcium ions from the bone and increased re-absorption of calcium from the intestines. Dietary vitamin D deficiency or hereditary diseases cause rickets. Hereditary rickets is classified into vitamin D dependant class I, class II, and X-linked hypophosphatemic vitamin D-resistant rickets. Mutation of the CYP27B1 is an underlying cause of vitamin D-dependant rickets type I (Kato, 1999).

Vitamin D_3 and its intermediates undergo 24-hydroxylation catalysed by CYP24A1 enzyme, 25-hydroxylation prevents subsequent ligand binding to the vitamin D_3 receptor and represents the major initial step in the metabolic inactivation of vitamin D_3.

2.5.2 Fatty acid metabolism

CYPs that metabolise fatty acids (especially arachidonic acid metabolites) are CYP2J2, CYP4, CYP5, and CYP8A1.

2.5.2.1 Metabolism of arachidonic acid its and metabolites

Arachidonic acid metabolism through the CYP system proceeds in three ways: epoxidation resulting in the formation of epoxyeicosatrienoic acids (EET); allylic oxidation resulting in the formation of hydroxyeicosatetraenoic acids (HETEs); and ω or ω-1 hydroxylation reaction resulting in the formation of HETEs and carboxy arachidonic acid (Rahman *et al.*, 1997).

The ω/ω-1 hydroxylation reaction is the major pathway for arachidonic acid metabolism and is catalysed by the CYP4A family (Rahman *et al.*, 1997: Capdevila *et al.*, 1995). Animal studies show that various fatty acids, such as prostaglandins and leukotrienes are metabolised by the members of the CYP4As, although prostaglandins and leukotrienes are poor substrates of CYP4As, with the exception of CYP4A4 (Kikuta *et al.*, 2002).

To date only CYP4F2, CYP4F3, CYP4F8, CYP4F11, CYP4F12 and CYP4F22 have been shown to be expressed in humans. This CYP4F diversity contrasts with the CYP4A subfamily, where only CYP4A11 is expressed in humans (Kikuta *et al.*, 2002). The exclusively myeloid cell expressed CYP4F3 is capable of ω-hydroxylation of arachidonate, leukotriene B_4, lipoxin A_4, lipoxin B_4, 5-, and 12-HETE. CYP4F2 is expressed in liver and other tissues but, unlike CYP4F3, it is not expressed in polymorphonuclear leukocytes. CYP4F2 catalyses ω-hydroxylation of arachidonate, leukotriene B_4, lipoxin A_4, 8- and 12-HETE.

CYP2J2 largely catalyses the epoxidation of arachidonic acid, with the formation of 5-, 6-, 8-, 9-, 11-, 12-, 14-, 15-EET and 19 HETE (Wu *et al.*, 1996). Arachidonic acid, and its coversion to prostaglandins and leukotrienes, play an important role in pain and inflammatory reactions.

CYP5A1, also known as the thromboxane A_2 synthase, catalyses the conversion of prostaglandin H_2 (PGH_2) to thromboxane A_2 (TXA_2), which is a potent mediator of platelet aggregation, vasoconstriction and bronchoconstriction. CYP8A1, also known as the prostacyclin synthase, catalyses the conversion of PGH_2 to prostaglandin I_2 (PGI_2) also known as prostacyclin, the principle action of which is to inhibit platelet aggregation. PGI_2 is also a strong vasodilator and inhibits the growth of vascular smooth muscle cells (Chevalier *et al.*, 2001;

Nebert and Russell, 2002). As it can be deduced from the above statement, the two enzymes have opposing physiological roles with regard to blood clotting. Indeed, mutation of CYP5A1 and CYP8A1 genes is linked to clotting and inflammatory disorders, including coronary artery disease and pulmonary hypertension (Hennan *et al.*, 2001; Tuder *et al.*, 2001). A balance of the production of TXA_2 and PGI_2 is a very important factor in maintaining vessel integrity.

2.5.2.2 Cholesterol metabolism and bile acid biosynthesis

Members of the *CYP3, CYP46, CYP7, CYP8, CYP27, CYP39,* and *CYP51* families take part in the metabolism of cholesterol and the formation of bile acids. Cholesterol biosynthesis is achieved by the removal of two methyl groups via oxidative reaction from lanosterol. Conversion of lanosterol to cholesterol is a six step reaction whose initial step is catalysed by CYP51A1. Cholesterol is metabolised by the CYPs into bile acids or into oxysterols and then into bile acids, such as cholic acid, chenodeoxycholic acid and at least another ten derivatives.

Cholesterol to bile acid metabolism is catalysed by the CYP3A4, CYP7A1, CYP8B1, CYP27A1, and possibly CYP2D6 (Nebert & Russell, 2002). CYP3A4, CYP27A1, CYP46A1, and again the possible contribution of CYP2D6, catalyse the formation of oxysterols from cholesterol. Oxysterols are metabolised into bile acids by CYP7B1, CYP8B1, and CYP39A1. Tissues, such as the lung and the brain, secrete measurable amounts of oxysterols into the circulation, which are then transported to the liver and converted into bile acids. The CYP7A1, CYP7B1, and CYP39A1 initiate bile acids synthesis from cholesterol and oxysterol substrates by the introduction of a hydroxy group in the α-configuration at carbon number 7 of the B-ring. CYP8B1 is a sterol 12α-hydroxylase that is essential for the synthesis of the primary bile acid, cholate. CYP27A1 is a sterol 27-/26-hydroxylase with a role in the synthesis of oxysterols and oxidation of the sterol side chain.

Biosynthesis of bile acids requires the metabolism of cholesterol, thus the two are interrelated. CYP46A1 is almost exclusively expressed in the neurons of CNS where its primary role appears be ridding the brain of excessive cholesterol. CYP46A1 catalyses the conversion of cholesterol to oxysterol and 24*S*-hydroxycholesterol, which, unlike cholesterol, is freely permeable to the blood brain barrier (Bogdanovic *et al.*, 2001). Both metabolites are excreted into the circulation where they will be metabolised further into bile acids.

2.5.3 Xenobiotic metabolism

A xenobiotic (alias foreign chemical) is any chemical constituent that is introduced to body which is alien to body chemistry. Suitable examples of xenobiotics include drugs, plant derived secondary metabolites consumed with food, agrochemicals, and are to a large extent environmental pollutants (Nebert and Russell, 2002). All CYPs except CYP2D6 are inducible.

The inducers are often substrates of the induced enzyme, the induction of which enhances detoxification, particularly when low to moderate concentrations of the substrate are present (Whitlock and Denison, 1995). From the above statement it can be seen that this induction phenomena is designed to rid the body of foreign chemicals. Unfortunately the above statement is not always true as, in some instances, induction leads to increased metabolism of another compound or may enhance the chemical toxicity of the xenobiotic. CYPs that mainly degrade xenobiotics include CYP1, CYP2A, CYP2E, and CYP3 (Anaesthetic, 2002).

Polycyclic aromatic hydrocarbons, such as those found in industrial incineration products, cigarette smoke, and charcoal grilled food, induce and are subjected to metabolism by the CYP1 enzyme family (Anaesthetic, 2002). The enzyme induction of this family is believed to be mediated via the aryl hydrocarbon receptor binding of the substrate, leading to an immediate (i.e. in minutes) and direct (i.e. require no protein synthesis) induction (Whitlock and Denison, 1995). CYP1A1 and CYP1B1 are the most efficient in metabolising aromatic hydrocarbons, whereas CYP1A2 preferentially metabolises arylamines and *N*-heterocyclics (Nebert and Russell, 2002).

Consequences of enzyme induction lead, for example, to the following: enhanced inactivation of prostaglandin G_2 by the CYP1A1; CYP1A2 and CYP1B1 hydroxylation of oestrogen at carbon number 2 and carbon number 4 respectively; CYP1A1 oxidation of uroporphyrinogen and melatonin; CYP1A2 metabolism of various co administered drugs (Anaesthetic, 2002). Gene members of the CYP2 and CYP3 families seems to be concentrated on the metabolism of various prescription drugs (Nebert and Russell, 2002).

2.5.4 Regulation of CYP expression

Liver CYP enzyme expression is regulated by hormones such as the growth hormone (GH), thyroid hormone (TH), and gonadal hormones (i.e. testosterone and oestrogen) (Waxman and Chang, 1995). GH and the TH play a much more significant role as compared to the gonadotropins. GH regulates the sex specific expression of liver CYPs and their associated roles in steroid hydroxylation and xenobiotic metabolism through transcriptional mechanisms. TH acts directly to influence the expression of individual CYP enzymes as well as indirectly via effects on the pituitary GH secretion and NADPH-cytochrome reductase gene expression (Waxman and Chang, 1995).

2.5.5 Summary of the physiological roles

The cytochromes play a very important role in the digestion and biosynthesis of endogenous substrates. This makes CYPs important in the control of vascular tone, pain and inflammatory

reactions and mineral and water balance. Because of their role in xenobiotic metabolism, they can be described as the second immune system.

2.6 Relationship of CYPs to P-glycoprotein

There is an interesting association between some CYPs and the important transmembrane pump protein, P-glycoprotein (the product of the MDR1 gene). Generally, if P-glycoprotein is present, then CYP3A4 is also present. This seems to be part of a concerted strategy by the body to eliminate xenobiotics since P-glycoprotein pumps out what it can, and CYP3A metabolises the rest (Thummel & Wilkinson, 1998). This association makes for even more interesting drug interactions. For example, calcium-channel blockers interact with the membrane pump and also the CYP. The same holds for drugs as diverse as azole antifungals, immunosuppressants and macrolides.

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Genetics: a practical overview

Chapter 3

3.1 Introduction

The nature of this study emphasises genetic polymorphism. This chapter discusses the concepts and terms relating to genetics, the nature of the genetic material, how it mutates and how it affects the direct structure and function of proteins.

3.2 Chromosomes

Flemming (1879) described darkly stained threads inside stained nuclei that had a peculiar dance-like behaviour during cell division. These threads were later named chromosomes (Greek chroma, "colour" soma, "body"), and their role in cell division was recognised.

All organisms have cells, and every somatic cell (non-reproductive cells) contains chromosomes that are presented in pairs. Chromosomes are located in the nucleus of every living cell. Each member of the chromosome pair carries the same genes, and is referred to as the homologues. The chromosome comprises of a very long molecule of deoxyribonucleic acid (DNA). The DNA molecule is coiled, compacted and held in place by specialised molecules called histones. Histones are a group of five highly basic proteins known as H1, H2A, H2B, H3, and H4. Collectively these proteins serve a common purpose of organising the DNA strand in a chromosome, giving the primary structure of all eukaryotic chromosomes (see fig 3.1).

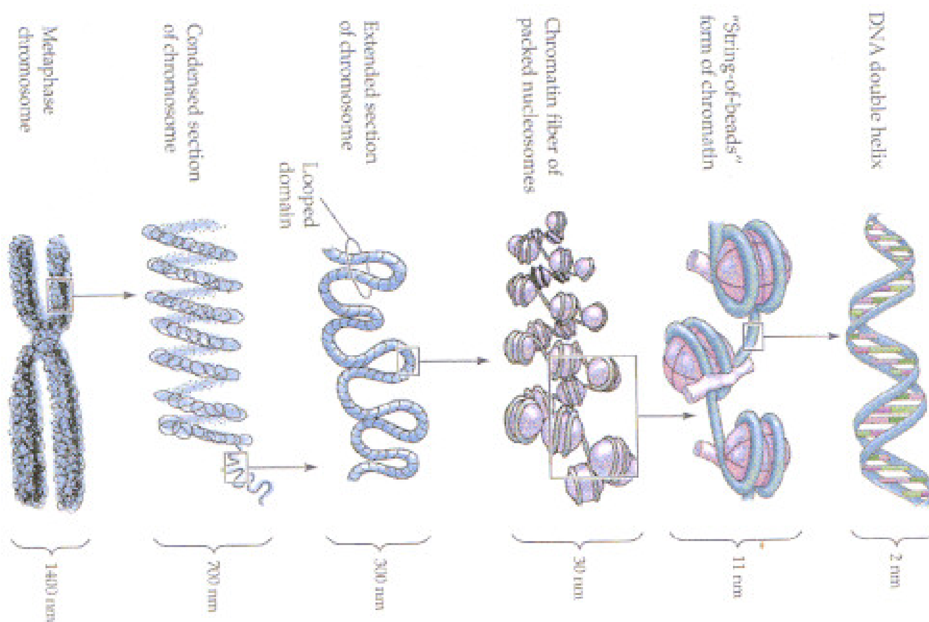

Figure 3.1. Organisation of the DNA on the chromosomes.

Chromosomes have a distinguishable "pinched" centre region called the centromere region. The centromere region divides the chromosome into divisions of unequal lengths referred to as arms, hence the short and long arm. Humans have 23 pairs of chromosomes. Each pair is assigned a number from 1 to 22 according to their length, with chromosome number 1 being the longest. An exception to this numbering applies to the sex chromosomes, this pair is not assigned a number but is referred to as either X or Y chromosomes.

3.2.1 Chromosome bands

In the 1970s, newer chromosome staining techniques using dyes were developed. These dyes produced light and dark cross bands of varying widths on chromosomes. These bands formed patterns that were unique for each chromosome (see fig. 3.2). The patterns reflected the differences in: the finity of DNA replication during synthesis period; the relative content of the different base pair content, and finally, the relative high length of the repeated gene sequence they contain.

The band patterns not only make it possible to distinguish each chromosome from the other, but also make it possible to map a specific region of a chromosome. When describing a particular region on a chromosome, the first consideration is the chromosome number, followed by the arm (p designates the short arm and q the long arm), and finally the specific band within that region (see fig 3.2). For example 1p13 refers to chromosome number 1, short arm, region 1, band 3 (a narrow light band).

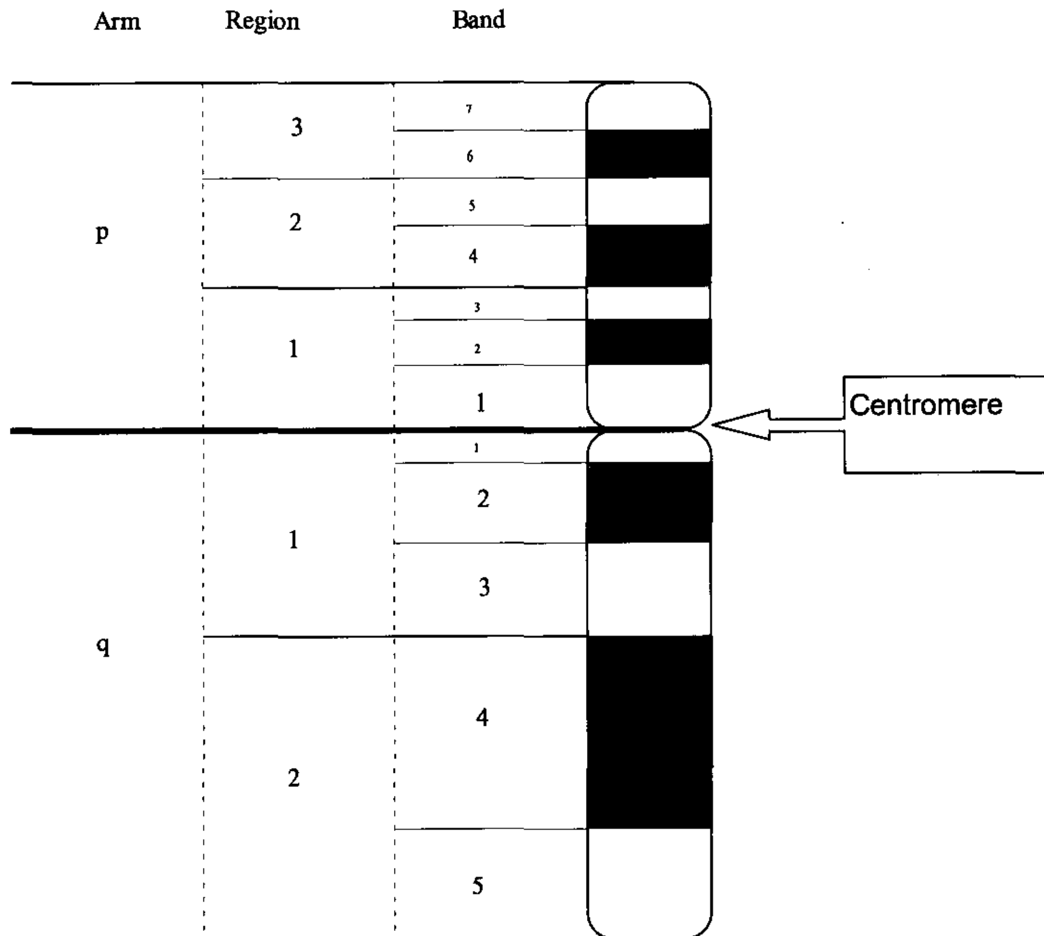

Figure. 3.2. An ideogram of a chromosome. The regions are according to a band pattern of a chromosome.

A much more sophisticated and advanced mapping method has since been developed, and is well described by Griffiths and colleagues (1999).

3.3 DNA

In 1953, Watson and Crick proposed that the DNA chemical structure is in the form of a double helix. Indeed, the double helix can be visualised as a twisted ladder with a vertical support consisting of a sugar-phosphate backbone. Projecting inward from each sugar forming horizontal step are base pairs. Four base pairs occur in DNA, that is to say, two purines, adenine (A) and guanine (G), and two pyrimidines thymine (T) and cytosine (D). Each pyridine pairs with a purine in a double helix such that either A pairs with T or G pairs with C (see fig 3.3).

A nucleotide is the basic building block of a DNA molecule. It consist of a deoxy sugar, a phosphate group attached to one end of the sugar carbon (called the 5' carbon), and a base attached to the middle carbon of the sugar (called 1" carbon). The 3" carbon attaches the next nucleotide (see fig 3.4). Clearly, it can be noted that the DNA nucleotides differ form one to

another only in the base they carry, hence the terms nucleotide pairing and base pairing express the same meaning.

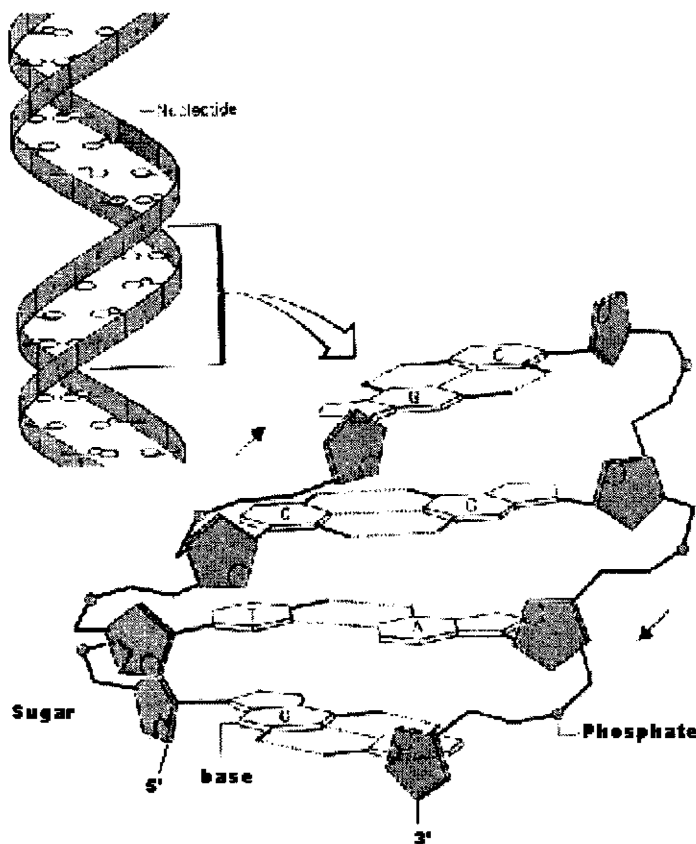

Figure. 3.3. The structure of the DNA helix. Note that as represented by the arrows, the strand run in opposite directions.

3.4 Gene expression

A gene is a segment of the DNA wherein a genetic code is encrypted, and can be transcribed to form proteins. The genetic code comprises of base pairs. Successive three bases (triplets) code for a single amino acid, although two different triplets can code for a single protein. The genetic code follows four laws (Lewin, 1990), namely:

1. It is expressed as a triplet.

2. The triplets do not overlap.

3. It is commaless (i.e. it does not have any breaks).

4. Successive triplets are read from a fixed starting point.

Transcription and translation of a gene, resulting in the synthesis of a protein is called gene expression. Expression follows the order DNA ➔ ribonucleic acid (RNA) ➔ protein. Gene expression takes place in a living cells and involves the transcription and translation stages:

3.4.1 Transcription

This process takes place in the nucleus of the cell. Usually each gene contains a special sequence of bases called the promoter. The first step of transcription is the attachment of the RNA polymerase (an enzyme catalysing RNA formation by linking together nucleotides) to the promoter region. The RNA molecule is similar to the DNA molecular arrangement but differs in that it is single stranded (as opposed to the double helix), contains the sugar ribose (rather than deoxy-ribose) and the base uracil in place of thymine.

Transcription begins at a spot after the promoter region. The new ribonucleotides are introduced at the 3' end of the growing RNA. The growing chain continues, until the RNA polymerase reaches the transcription termination sequence on the DNA molecule.

Regulatory proteins called transcription factors, bind to DNA promoter sequences and have control over which genes are turned on or off according to the requirement of the cell. In this way the cells have control over which proteins are synthesised at which times. The end product is a transcribed RNA, which then leaves the nucleus and enters the cytoplasm.

3.4.1.1 Types of RNA

Three types of RNA exist: messenger RNA (mRNA), transfer RNA (tRNA), and ribosomal RNA (rRNA). These are transcribed in a similar manner as described above, but from different specific regions of the DNA. Each type of RNA plays its role in the translation step as described below.

3.4.1.1.1 mRNA

mRNA plays the key role of information transfer from the DNA to the actual protein to be synthesised. This is the only type of RNA that is actually translated. The genetic information representing one of the 20 amino acid is expressed by a successive group of three nucleotides in mRNA, called the codon. Each codon codes for only one of the 20 human amino acids.

3.4.1.1.2 tRNA

tRNA is transcribed but it is not actually translated (i.e. has no protein product). It plays an important role in the incorporation of amino acids into a synthesised protein during translation. The transcribed tRNA is long, coiled and folded into a three dimensional structure. This structure enables the tRNA to have dual specificity, with one end of the molecule attaching itself

to a specific protein and the other end containing specific three bases called anticodons. The actual function of tRNA will be expanded upon later.

3.4.1.1.3 rRNA

Similarly to the tRNA, the rRNA has no protein product. It is also arranged in a complex three-dimensional structure. As the name suggests, rRNA exerts its function on the ribosome during translation, which occurs on the ribosomes. The ribosome is made of two normally separated subunits, which clamp on the mRNA. The rRNA helps form these ribosomes by becoming a part of them. All the RNA's converge at the ribosomes where the translation takes place.

3.4.2 Translation

The function of a ribosome can be conceptualised as that of a factory, all the components (all the RNA's) are brought together and assembled to yield a final product (proteins).

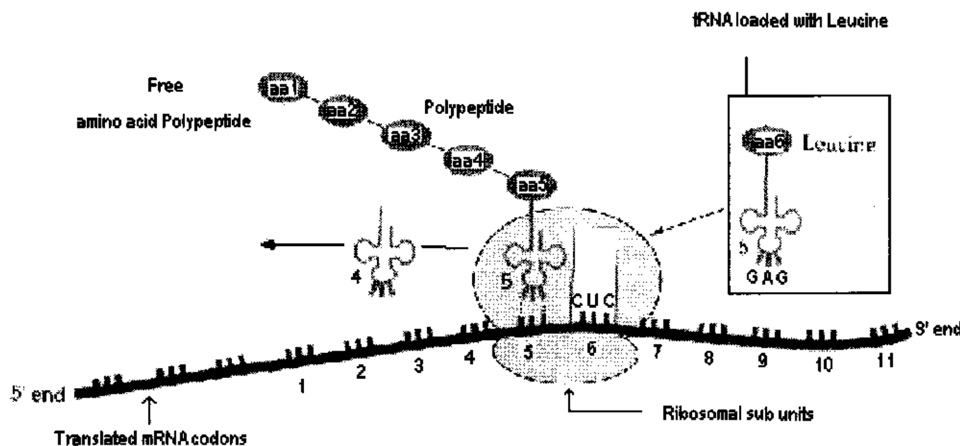

Figure 3.4. Translation of the mRNA on the ribosome. The ribosome has two units that are fused at this stage. aa = amino acid.

The larger of the two ribosomal subunits has two pockets for positioning tRNA attached with an amino acid on one side and the anticodon on the other. A pocket will fit only the tRNA whose anticodon complements the codon of the mRNA that forms the lower boundary of the pocket. The next pocket will also only fit the complementary tRNA loaded with its specific protein, as governed by the base pairing rules. The introduction of the second tRNA causes the adjacent amino acids to fuse, thus adding an amino acid into a growing polypeptide. Once a single amino acid is added, the mRNA moves one codon towards 3' direction, allowing the next pocket to be free to accept the next tRNA. Thereafter the leading 5' tRNA is freed from the amino acid already joined into a polypeptide chain.

Not all codons from the mRNA code for an amino acid, certain 5' (head) and 3' (tail) codons are translated into start and stop codons, respectively. When the process is completed the resulting protein is freed from the ribosome, allowing it to perform its biological function.

3.4.3 mRNA processing

A DNA molecule contains thousands of genes, coded on even more base pairs, although not all base pairs carry a genetic code. Within a gene sequence, there are regions that are expressed (exons) and regions that have no corresponding mRNA product (introns). Researchers have revealed that genes of vertebrates have at least one intron segment that may be longer than the actual gene. A well-studied example of this phenomenon is that of haemoglobin (see fig. 3.5).

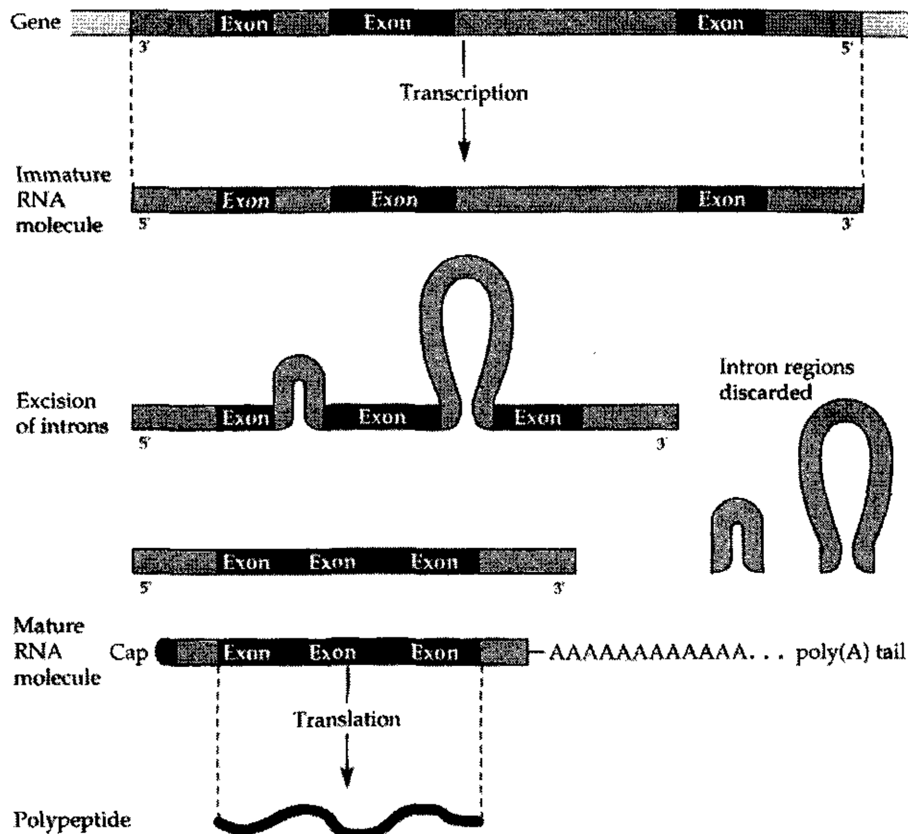

Figure 3.5 Protein synthesis. Messenger RNA from immature RNA is modified, such that the regions corresponding to introns are cut out and the exons are joined together into an uninterrupted sequence. Final modifications include a special sequence on the 5' end, and a string of adenine at the 3' end. Portions designated as exons are the only read portions having corresponding amino acid.

Haemoglobin protein has two α and two β protein units. A different gene encodes each chain, and each gene has three exons separated by two units (see fig 3.5). The gene that codes for the β polypeptide chain contains 1.606 bases. The first mRNA transcript of β polypeptide contains the same number of nucleotides, an exact compliment of the entire gene (see fig 3.4).

The RNA' s 130 complimentary bases of intro1 and 850 of intron 2, are cut out of the molecule before it leaves the nucleus. The splicing together of the successive exons is called RNA splicing. This process must occur with high precision to preserve the codons of the joined ends. A specific sequence at the two ends of an intron mark the region to be cut out, mutation of these cause inaccurate splicing, leading to a genetic disease. The changes occurring at converting an original transcript to a mature mRNA is called mRNA processing.

In addition to mRNA triplets coding for amino acids, there are special sequences that signal the beginning and the end of the translation. The AUG codon (initiator codon) signals the commencing of translation while the UAA, UAG, and UGA signal the end of translation (Smith-keary, 1991).

3.5 Nature of mutations

Mutation describes any change in the sequence of genomic DNA, and is classified into four types, namely germinal, somatic, spontaneous, and induced mutation. Germinal mutation occurs in the germ line (cells that are destined to become eggs or sperm). This mutation doesn't have any physical manifestation in the person in whom it occurs, but can be transmitted to and affect the offspring. Somatic mutations, however occur on the somatic cells, and although it affects the carrier of such a mutation, it is not transmitted to, or affects, the offspring.

Spontaneous mutation seems to occur irrespective of known environmental conditions. It is believed that it results from random thermal motions of the atoms and molecules in and near the DNA, this motion is characteristic of all matter. On the other hand, agents that cause gene alteration (mutagens), cause induced mutation. Mutagens include pesticides, radioactive materials, drugs and the like.

3.5.1 Mutation at the molecular level

The nature of a mutation can be generally categorised into point mutation and rearrangement mutation. Point mutation affects the change of a single base pair in a gene, the common example being the single base substitution. Rearrangement affects a larger region of a gene, common examples being insertion of additional material or deletion of a stretch of nucleotides. These mutations can occur in various ways, and will now be discussed.

3.5.1.1 Missense mutation

As alluded to earlier, a codon has three base pairs coding for a single amino acid. For example, CAC codes for the amino acid valine. Mutation can cause the substitution of the first C in the CAC triplet to a T. This substitution causes a methionine instead of valine incorporation in the

amino acid sequence. The resulting protein will have an altered activity due to dissimilarities in amino acid sequences. Such single point substitution leading to one amino acid being replaced by another is referred to as missense mutation.

A popular example of a disease caused by missense mutation is that of sickle cell anaemia, which affects thousands of the Black population group. This disease is caused by single point mutation on the DNA triplet coding for the sixth amino acid of the β -chain subunit of haemoglobin. It is characterised by a CTC to CAC substitution causing a glutamic acid to valine amino acid substitution. The resulting protein product after translation is a deformed "sickle" shaped haemoglobin that clogs blood vessels causing excruciating pain in the affected subject.

3.5.1.2 Chain termination mutation

Another type of missense mutation occurs when the triplet specifying for an amino acid changes to a triplet of a stop codon. Although sometimes referred to as a missense mutation, a better name is chain termination mutation. The consequence of this mutation is the premature stopping of the translation, producing short non-functional protein products.

3.5.1.3 Silent mutation

Silent mutation occurs when a single point mutation leads to a base triplet synonymous to the original base triplet information. An example is the changed DNA encoded AAA triplet, to AAG. Both AAA and AAG code for the same phenylalanine amino acid. Consequently the protein product is not altered and the carrier of such a mutation does not have any phenotypic effect.

3.5.1.4 Frame shift mutation

The afore mentioned types of mutation take place on the exon regions of a DNA. However, mutation is not restricted only to exon regions, but also to sequences surrounding the exons and even within intronic regions. An example is the mutation of the promoter region of the gene, thereby markedly reducing the efficiency of transcription. Although the protein products are normal, very few are synthesised leading to a deficiency disorder.

The genetic code is read in non-overlapping triplets. For a base sequence of ACGACGACGACG the sequence can be read as ACG **or** CGA **or** GAC depending on the starting point. These are called reading frames of a sequence. Frame shift mutation occurs when there is an insertion or deletion of one or more bases. This causes changes in the entire DNA triplet frame. Single base changes converting the whole frame into a meaningless sequence. Obviously this mutation leads to the addition of the wrong amino acids making an incorrect sequence, producing non-functional proteins.

3.5.1.5 Splicing defect

As explained in section 3.4.3, RNA splicing is a normal aspect of the transcription pathway. However, inaccurate splicing can occur leading to a gain or loss of one or more bases. The consequences of a splicing defect can be equated to those of frame shift mutations.

3.5.1.6 Unequal cross over

This is similar to germinal mutation, but involves deletions, duplications, insertion and fusion of large blocks of DNA, larger than those discussed above. These duplications and repetitions range from a few nucleotides, to a loss or gain of a whole gene. It happens when the homologous chromosomes undergo very close pairing called synapsis during the first meiotic division. During synapsis, two double helices that have a similar nucleotide sequences lie along each other. After synapsis, the strands undergo cross over, which can be explained as a breakage of two different DNA strands at exactly the same point and the reunion in opposite positions. However when unequal cross over occurs, the chromosomes are miss-aligned such that the DNA break off and reattach themselves at incorrect positions. This results in one DNA losing genetic material and the other strand gaining genetic material.

3.5.1.7 Trinucleotide repeats

This mutation is characterised by the abnormal repetition of similar base triplets. Such a repetition on a gene causes an amino acid repeat in the encoded polypeptide. An example of a disease caused by trinucleotide repeat is Huntington's disease. Patients suffering from this disease have an abnormally long glutamine amino acid chain in the huntingtin protein.

3.5.1.8 Insertion mutations

Chromosomes have transposable elements, that have the ability of removing themselves from one part of the chromosome and inserting themselves elsewhere on the chromosomes (Shapiro 1983). Insertion mutations occur when the transposable units insert themselves on a DNA segment that they don't belong to.

Insertion mutations interrupt the gene function such that at times, the protein product of the affected gene is absent. Indeed this is true for all haemophilic A patients, who have an absent clotting factor VIII, caused by this mutation.

3.6 Mutation nomenclature

Different authors have used different notations to describe mutations, and there is therefore a need to have a uniform nomenclature for all mutations. Den Dunnen and Antonarakis (2000) have propsed a system of nomenclature for mutations, which has been adopted and used by

the majority of researchers in the human and clinical genetics field. In this section, this system of nomenclature will only be briefly discussed, since elements of this nomenclature are still under development.

In this chapter, the concept that genes are mutable units was introduced. It was also stated that mutation could occur at DNA, RNA and protein levels, that is to say at DNA, RNA processing, and at translation level. This method of nomenclature is useful in understanding later chapters of this dissertation.

3.6.1 DNA level

Sequence variations are best described at a DNA level. The small case letter "g" or "c" proceeds the description of a mutation in a genomic or reference cDNA from a database. For example, g.76>T for a genomic sequence and c.76>T for a cDNA sequence. The difference between the two is that a cDNA sequence is derived from mature mRNA and thus has no introns, while genomic DNA includes introns in its sequence.

3.6.1.1 Nucleotide numbering

Nucleotide numbering assigns the nucleotide +1 as being A of the ATG-translation initiation codon, the nucleotide 5' to +1 is numbered -1 and there is no nucleotide 0. Therefore, the non-coding regions 5' to the initiation codon are preceded by a minus sign. The nucleotide 3' of the termination codon is *1. For intronic nucleotides, the number of the last nucleotide preceding an exon, a plus or minus sign (depending on whether its at the beginning or end of the intron) and the position in the intron, is assigned, e.g. 77+1G. Alternatively, if the exon number is known, the notation becomes IVS1+1G (the "IVS" designates an intronic change). Nucleotide changes start with the nucleotide number and the change follows this number in the format; [nucleotide interval][sequence changed nucleotide][type of change][sequence new nucleotide].

3.6.1.2 Substitutions

Substitution mutations are designated by a ">" character. For an example 76A>C denotes that at nucleotide 76 an A is changed to C. The g.88+1G>1 or IVS2+1g>T denotes the G to T substitution at nucleotide +1 of intron 2, relative to nucleotide 88 of genomic DNA.

3.6.1.3 Deletions

Deletion are designated by "del" after the nucleotide(s) flanking the deletion site. For example, deletion of the TG in the ACTTTG**TG**CC (A is nucleotide 76) is designated as 82_83del or 82_83delGT. *IVS2_IVS5* or *88+?_923+?* designates a deletion (including exonic deletion)

starting at an unknown position in intron 2 after nucleotide 88 and ending at an unknown position in intron 5 after nucleotide 923.

3.6.1.4 Insertions

Insertions are designated by the "ins" after the nucleotide flanking the insertion site and followed by the inserted nucleotides. An insertion of TG in a sequence ACTTTGTG**TG**CC (A is nucleotide 76), is denoted as 83_84insTG.

3.6.2 RNA level

Sequence changes at the RNA level are basically described as those at the DNA level with a few changes. The small case "r" is used to denote changes on RNA level. Nucleotides are designated as bases in lower case, such as a (adenine), c (cytosine), g (guanine) and u (uracil). For example, r.54a>u designates a substitution at nucleotide 54 from A to U.

3.6.3 Protein level

It is important to note that protein mutations are a consequence of mutations either at DNA or RNA levels, hence it is important to include the nature of nucleotide changes when describing a mutation at a protein level. Changes at this level are designated by a small case letter "p". The codon for initiator Methionine is numbered as +1. Each amino acid is designated with a single letter as opposed to a previous three-letter format, with "X" designated as the stop codon. A complete list of amino acids and their corresponding shorthand letter abbreviation are tabulated in table 3.1. Amino acid changes are described in a format: [code of the first amino acid changed][amino acid interval][code of new amino acid or type of change].

3.6.3.1 Substitutions

A missense mutation leading to W26C denotes that the amino acid Tryptophan is changed to Cysteine at amino acid position 26 of a protein (also refer to table 3.1. for the abbreviation of amino acids). W26X denotes that an amino acid 26 (tryptophan) is changed to a stop codon.

3.6.3.2 Deletions

Deletions are designated by "del" after the nucleotide flanking the deletion site. An amino acid deletion of an amino acid 29 from a sequence C**K**MGHQQQCC (C being amino acid 28) to CMGHQQQCC is described as K29del. A deletion of three amino acids, from 28 to 30 from a sequence R**CKM**GHQCC (R being amino acid 27) is described as C28_M30del.

3.6.3.3 Insertions

Insertions are designated by "ins" after the nucleotides flanking the insertion sites, followed by the nucleotides inserted. An insertion of the QSK between Lysine at amino acid number 29 and Methionine at number 30, changing the sequence from CKMGHQQQCC (C is 28) to CK<u>QSK</u>MGHQQQCC is designated as K29_M30insQSK. Insertion of Q at a position between amino acid 35 and 36 to form a sequence CKMGHQQQ<u>Q</u>CC (C is 28) is designated as Q35_C36. This can also be assigned as Q35dup, because of the duplication insertion of Glutamine amino acid.

Table. 3.1. Amino acids. Twenty amino acids and their abbreviations.

	Abbreviation			Abbreviation	
Amino acid	**3-letter**	**1-letter**	**Amino acid**	**3-letter**	**1-letter**
Alanine	Ala	A	Leucine	Leu	L
Arginine	Arg	R	Lysine	Lys	K
Asparagine	Asn	N	Methionine	Met	M
Aspartic acid	Asp	D	Phenylalanine	Phe	F
Cysteine	Cys	C	Proline	Pro	P
Glutamic acid	Glu	E	Serine	Ser	S
Glutamine	Gln	Q	Theorine	Thr	T
Glycine	Gly	G	Tryptophan	Typ	W
Histidine	His	H	Tyrosine	Tyr	Y
Isoleucine	Ile	I	Valine	Val	V

3.7 Conclusion

While the information contained in this chapter may appear excessive, it has relevance in better understanding later chapters where mutations of the CYP system and their clinical relevance, are reviewed, especially in light of the results presented in chapter 6.

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Cytochrome P450 and genetic polymorphism

Chapter 4

4.1 What is genetic polymorphism?

Mutation on the gene coding for metabolising enzymes can give rise to variants (alleles) with higher, lower, no activity, or altered substrate specificity. If the mutant allele occurs with a frequency of at least 1-2 % in the normal population, the term polymorphism applies. Among numerous CYP isoforms identified, eight of these are polymorphic, including CYP1A1, CYP1A2, CYP2A6, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4 (Weber, 1999: Topic *et al.*, 2001).

Genetic polymorphism was discovered when it was noticed that some patients experienced adverse drug reactions even when the drug was given at doses lower than the standard recommended dose, e.g. orthostatic hypotention after administering an antihypertensive drug (Eichelbaum & Gross, 1990). Adverse reactions associated with the antihypertensive agent debrisoquine, and the anti-arrhythmic and oxytocic agent sparteine, eventually led to the discovery of polymorphic CYP2D6 enzyme (Meyer *et al.*, 1997).

Genetic polymorphism is not restricted to only phase I enzymes. Phase II enzymes, such as *N*-acetyltransferases (NATs), UDP-glucuronyltransferases (UGTs), sulfotransferases (SULTs), catechol-O-methyltransferase (COMT), and thiopurine methyltransferase (TPMT) have also been characterised as being polymorphic (Gaedigk, 2000). NATs is a classical example of the polymorphic phase II reaction enzyme. The mutated allele of this enzyme occurs at a population frequency of 20 % or less in Japanese and Canadian Eskimos (Weber, 1999).

It has not been conclusively determined how polymorphic alleles originate and to what extent the interethnic differences in the distribution of these alleles are dependent on the environment. A possible explanation offered by Ingelman-Sundberg (1999) is that the influence originates from plants that have continuously evolved biosynthetic pathways. Since the animals began consuming plants, the plants responded by evolving gene to synthesise toxic metabolites as a measure of self-defence. Humans then adapted by evolving new metabolising genes to detoxify

these new plant derived toxins. Another possible explanation, based on the existence of physiological functions of CYPs, suggests that *CYP* gene products in vertebrates probably first evolved for important life functions, before developing plant metabolite metabolism and drug metabolic capabilities (Nebert & Dieter, 2000). In the modern world of science and technology, toxins from both pollutants and diet sources introduced into our bodies it can therefore be expected that new alleles will develop as the body attempts to adapt to the increasing presence of the foreign substances.

4.2 Phenotyping and genotyping

The form taken by some characteristic (or group of characteristics) in a specific organism that forms the observable trait or attributes of that organism, is referred to as the phenotype. The specific genetic composition for a certain gene or a set of genes that manifests a given phenotype in an organism is referred to as the genotype. In simple terms, the genotype are the genes that an organism possesses, and the observable outward manifestations of those genes is the phenotype.

4.2.1 Phenotyping

The influence brought about by an individual's genotype produces that individual's phenotype. Phenotype can be measured using analytical methods, using a procedure known as phenotyping. In the current context of drug metabolism, this procedure determines the metabolic activity of a particular drug metabolising enzyme by introducing an enzyme-specific substrate and monitoring its metabolic conversion over time.

4.2.1.1 Metabolic probe drugs

As alluded to earlier, phenotyping determinations employ the use of drugs that are substrates (probe drugs) of a specific enzyme, and that will reflect that enzyme's activity *in vivo*. *In vitro* determinations are also possible, but factors such as the difficulty with which to obtain samples and patient records make this an arduous route. For the purpose of the current study, only probe drugs for CYP2D6 and CYP3A4 will be presented.

4.2.1.1.1 CYP2D6 probes

Probe drugs used to measure CYP2D6 activity include debrisoquine, sparteine, metoprolol and dextromethorphan (DXM) (see Figure 4.1), although debrisoquine is the more frequently used. This is partly due to the fact that polymorphism was originally discovered through the use of debrisoquine as an antihypertensive agent (Eichelbaum & Gross, 1990). 4-Hydroxy debrisoquine formation, exclusively catalysed by the CYP2D6 enzyme, forms the basis of this procedure. Recent studies, however, make use of DXM because it is more accessible and

better accepted by the patient, a feature which it owes to its lower side effect profile. Schmid and co-workers (1985) were the first to demonstrate that DXM is largely metabolised by the same enzyme as debrisoquine, and suggested its use as an alternative to debrisoquine for probing CYP2D6.

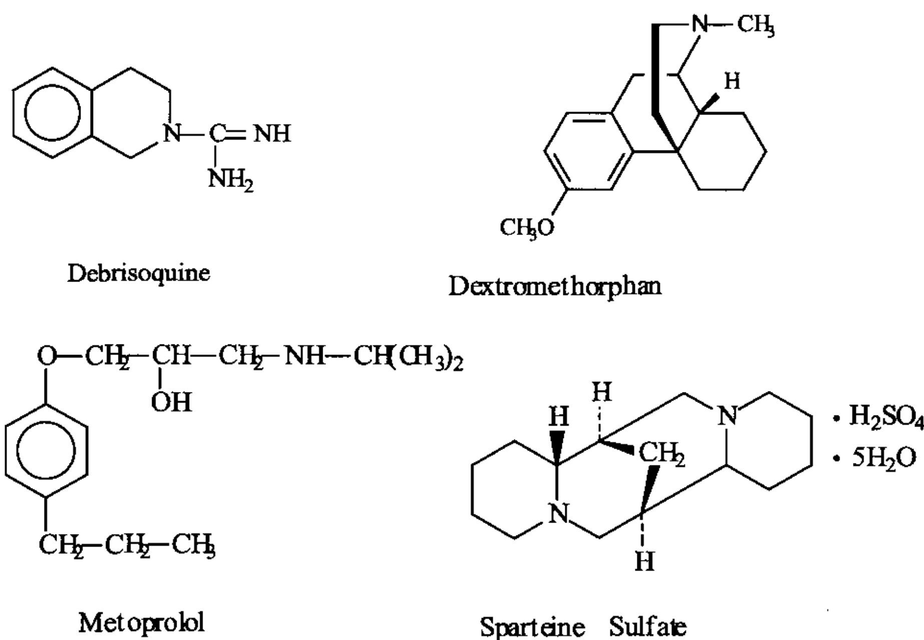


Figure 4.1. Chemical structures of CYP2D6 probe drugs (Katzung *et al.,* 1998; Heller, 1972; Batrolleti *et al.,* 1995)

4.2.1.1.2 CYP3A4 probes

The erythromycin breath test formed the procedure of choice for CYP3A phenotyping. This rapid procedure was based on the selective *N*-demethyltion of erythromycin by CYP3A in the lungs. When radio-labelled [^{14}C]-*N*-methyl erythromycin is used, the cleaved carbon of the methyl group is expired as CO_2 (Thummel and Wilkinson, 1998). Following an intravenous dose of erythromycin, the radioactive $^{14}CO_2$ can be measured to determine enzymatic activity. This method, however, has shortcomings. For one, it only measures CYP3A4 activity and not CYP3A5 activity. Secondly, the intravenous route of administration neglects the important contribution by CYP3A4 activity expressed in the GIT. On the contrary, the use of orally administered DXM as a metabolic probe considers CYP3A5 activity and also accounts for GIT expressed CYP3A4 activity. Moreover DXM has been described as meeting all the necessary requirements for CYP3A4 phenotyping (Thummel and Wilkinson, 1998). As an alternative, midazolam is also suitable as in vivo probe for CYP3A activity.

4.2.1.1.3 Dextromethorphan as a dual phenotypic marker

DXM can therefore probe both CYP2D6 and CYP3A4 metabolic activities. A brief overview of DXM metabolism will clarify its unique ability to act as a dual phenotypic marker. Eighteen different metabolites of dextromethorphan have been identified in urine (Koppel *et al.,* 1987). For the purpose of this study, however, only two major metabolites will be discussed, notably the metabolites of *N*- and *O*-demethylation, which are the major metabolic pathways of DXM (see fig 4.2).

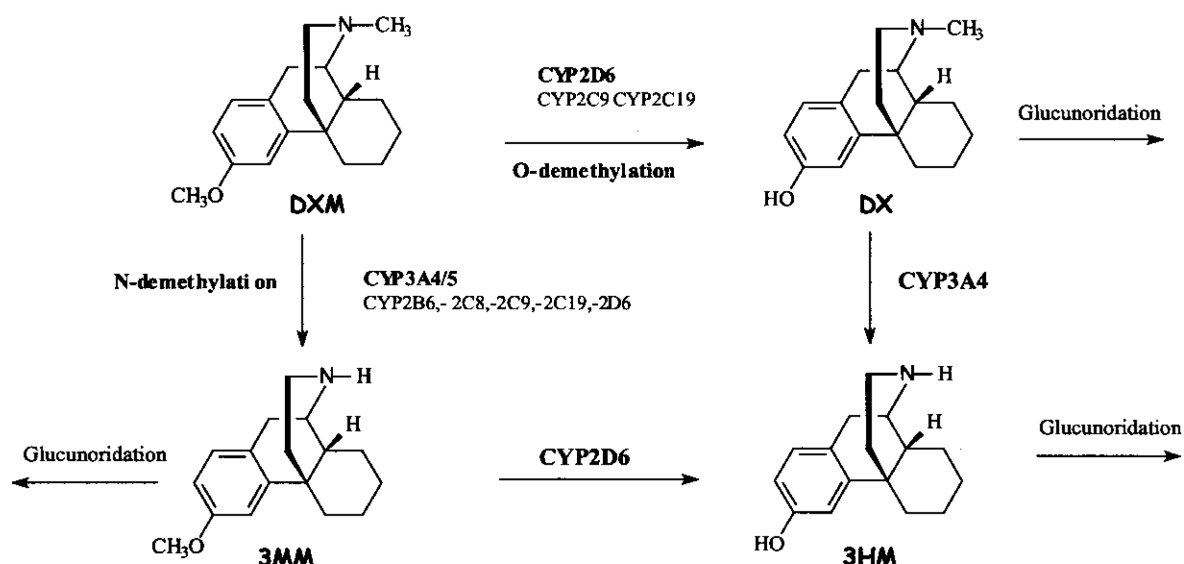


Figure. 4.2. Dextromethorphan metabolism. Enzymes that mainly characterise the reaction are printed in bold letters. DXM = dextromethorphan, DX = dextrophan, 3MM = 3-methoxy-morphinan, 3HM = 3-hydroxy-morphinan.

DXM is metabolised to either dextrophan (DX) or 3-methoxy-morphinan (3MM). DX is either conjugated and excreted by the kidneys or further metabolised to 3-hydroxy-morphinan (3HM). The 3HM metabolite is also formed from the metabolism of 3-methoxy-morphinan (3MM) (see figure 4.2). Alternatively, the 3MM metabolite is conjugated and excreted in the urine. Studies conducted by Von Motlke and co workers (1998) on human liver microsomes showed that DXM O-demethylation to DX is largely mediated by CYP2D6 followed by a minor contribution from CYP2C9, and CYP2C19 isoforms. DXM *N*-demethylation is predominantly carried out by CYP3A4 although other CYPs play a role in this reaction. The remainder of 3MM formation is mediated by CYP 2B6, -2C8, -2C9, -2C18, -2C19, -2D6 and 2E1 activity (Schmider *et al.*, 1997; Von Moltke *et al.*, 1998; Wang and Unadkat, 1999). Using CYP2B6 antibodies, Wang and co workers demonstrated that CYP2B6 is the major contributor to residual 3MM formation.

Although it seems that 3MM formation has various contributors, DMX is a suitable probe for CYP3A4 phenotyping in clinical applications due to low activity of other isoenzymes (Wieling *et al.*, 2000).

4.2.1.2 Metabolic ratios

During phenotyping, the probe drug is administered to a patient under well-controlled conditions. Recovered probe drug and its metabolite(s) are quantified from urine, saliva or blood samples collected over a period of time post drug intake. Quantification of the various chemical species makes use of chromatographic methods fitted with an appropriate detector. A high performance liquid chromatographic (HPLC) method, coupled with fluorescence detection, has gained popularity for its high sensitivity. After quantifying the chemical species of interest, the results are then used to calculate the metabolic ratios, which in turn are used to determine the phenotype.

The metabolic ratio of a probe drug is expressed as the concentration of the recovered probe drug divided by the concentration of its metabolite(s). Metabolic ratios for assessing CYP2D6 activity are as follows:

Debrisoquine : $\dfrac{[debrisoquine]}{[4\text{ - }hydroxy\ debrisoquine]}$ or simply [DB] / [4HD]

Sparteine : $\dfrac{[sparteine]}{[2\text{ - }dehydro\ and\ 5\text{ - }dehydro\text{ - }sparteine]}$ or simply [SP]/[2&5DHS]

Metoprolol : $\dfrac{[metoprolol]}{[\alpha\text{ - }hydroxy\ metoprolol]}$ or simply [M]/[HM]

Dextromethorphan: (This ratio determines CYP2D6 activity) $\dfrac{[dextromethorphan]}{[dextrophan]}$ or simply [DXM]/[DX]

Dextromethorphan: (This ratio determines CYP3A4 activity) $\dfrac{[\mathrm{dextromethorphan}]}{[\mathrm{3\text{ - }methoxy\text{ - }morphinan}]}$ or simply [DXM]/[3MM]

From the above, it can be seen that the higher the quotient, the lower the metabolising activity because it will imply that the numerator (the intact drug) is higher than the denominator (the metabolised form).

4.2.1.3 The antimode

Once the metabolic ratios have been determined, their frequency of metabolic ratios is then plotted on a graph. For most cases, the graph displays a bimodal distribution, splitting the population into two metabolising phenotypes, namely poor metabolisers (PMs) and extensive metabolisers (EMs). The value of the antimode is then determined, and used to classify individuals into their respective metabolising status.

A suitable example is the antimode of debrisoquine which is 12.6 based on results obtained in Caucasians (see figure 4.3.). Using on this criteria, individuals with a metabolic ratio above 12.6 are classified as PMs and the opposite for EMs. For the other antimodes of the remaining three probes see Figure 4.4. Bimodal distribution is not always evident. Sometimes the population can have only one mode, which implies there are neither PMs nor EMs. Sometimes the population is divided into three or four modes, in this case the EMs phenotype is further divided into slow extensive metabolisers (SEMs), intermediate extensive metabolises (IEMs), and ultra rapid extensive metabolises (UEMs) (Sommers *et al.*, 1988). The above feature of multi-modal distribution can only be shown in few populations but for the sake of convenience, only the criteria for classification of Caucasians will be discussed.

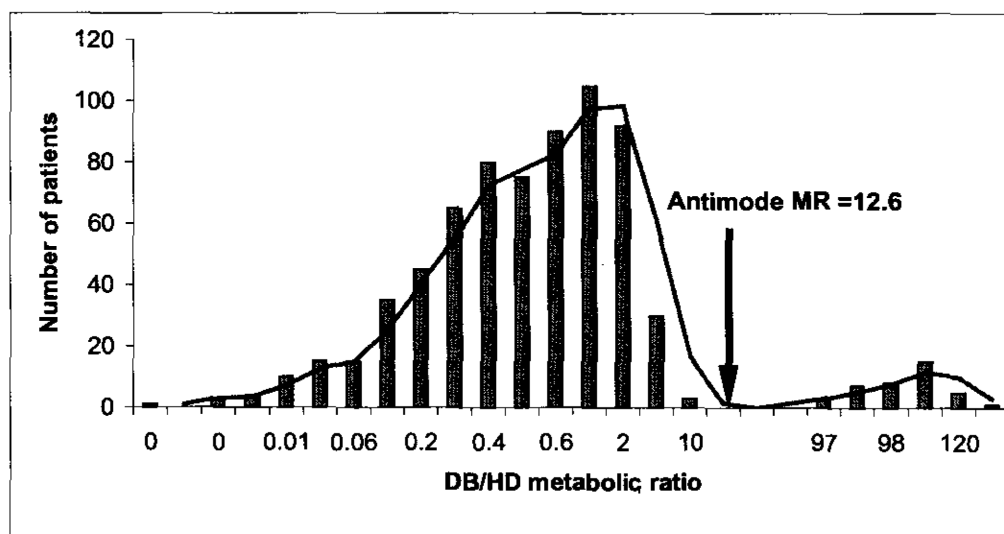
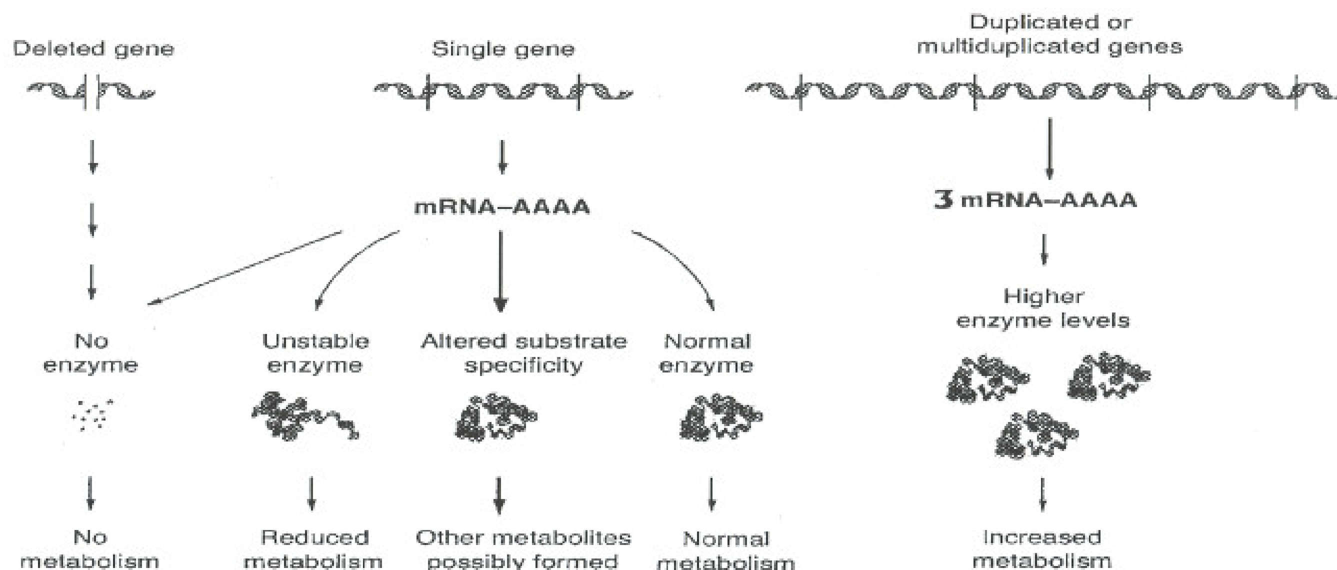


Figure 4.3. Example of bimodal distribution. The antimode value of 12.6 is indicated by the arrow. Note that there are no patients presenting with 12.6 metabolic ratio. MR = metabolic ratio, DB= debrisoquin, HD = 4-hydroxy-debrisoquine.



Phenotyping

	PM	EM		UEM
Derisoquine	PM D/DH >12.6	EM D/DH< 12.6		UEM D/DH<0.2
Metoprolol	PM M/HM > 0.5	EM M/HM< 0.5		
Sparteine	PM SP/2&5DHS>20	EM SP/2&5DHS<20		
dextromethorphan	PM DXM/DX>0.3	EM DXM/DX<0.3		

Genotyping

	PM	SEM	IEM	UEM
	PM *CYP2D6 * 3* *CYP2D6 *4* *CYP2D6*5*	SEM *CYP2D6*5* *CYP2D6*10* *CYP2D6*17* *CYP2D6*9*	IEM *CYP2D6*1* $(CYP2D6\ ^*2)_{N=1}$	UEM $(CYP2D6^*2)_{N>1}$

Figure 4.4. Illustration of possible enzymes that can be formed from different genes after replication. The symbol mRNA-AAA represents the messenger RNA and its chain. The intensity of the shading increases with the increase in metabolising activity. Metabolising status can be characterised into 4 groups at a genotype level, to date metabolic ratios have been unable to achieve this, but there is a general acceptance that a metabolic ratio of debrisoquine less than.0.2 belongs to a UEM. Poor metabolisers may arise from combination of *CYP2D6*1* and a mutant allele, those indicated here are responsible for that phenotype as homozygous. Taken from references (Ingelman-Sundberg, *et al.*, 1999; Laforest *et al.*, 2000)

4.2.2 Genotyping

Phenotyping is very reliable in healthy subjects, but poses a problem when a patient is suffering from a disease he is not aware of e.g. undiagnosed renal impairment. Although some phenotyping methods are able to distinguish subgroups within the EMs phenotype, genotyping is capable of dividing the various groups which are within the EMs phenotype, namely, slow extensive metabolisers (SEMs), intermediate extensive metabolisers (IEMs), and ultra rapid extensive metabolisers (UEMs). Alleles responsible for various phenotypes are summarised in the last frame of Figure. 4.4.

Two alleles (one from the father the other from the mother) carried at a given gene locus are referred to as genotype. With the help of ever-advancing technology, the genotype can be determined at a DNA level. Up to date over 70 CYP2D6 alleles have been found and are continuously updated on the internet (Oscarion, 2001). Genotype determining methods operate by identifying specific mutations on a specific locus. Genotyping techniques include polymerase chain reaction restriction fragment length polymorphism (PCR-RFL). This technique has gained the widest popularity and use.

4.2.3 The human *CYP2D6* gene

From the *CYP2D* gene family CYP2D6 is the only enzyme expressed in humans (Guengerich, 1995). The human CYP2D6 gene is located on chromosome 22 (Eichelbaum *et al.*, 1987). The major variants of CYP2D6 that account for most phenotypes in different populations are *CYP2D6*1, CYP2D6*2, CYP2D6*3, CYP2D6*4, CYP2D6*5* and *CYP2D6*17* (Dandara *et al.* 2001). *CYP2D6*1,* also known as the wild type is considered the normal allele.

4.2.3.1 Mechanism of mutation of the *CYP2D6* alleles and their functionality

The *CYP2D6*2* allele on its own produces an enzyme with slightly reduced activity but has a tendency to appear as a duplicate and, in some cases, may express up to 13 copies. The affected individuals have a very high metabolic rate because a larger number of enzymes will be produced from a single stimulation (see figure 4.4). Genetic mechanisms for duplication and multiplication have been described as an unequal cross-over at a specific break point for individuals with 1-5 copies. Extrachromosomal replication accounts for individuals with 13 copies (Lundqvist *et al.*, 1999). It is important to note that this allele is very rare in most populations.

The *CYP2D6*4* polymorphic variant has a 1934G>A splice site defect at the intronic 3-exon 4 junction (Topic *et al.* 2000). The *CYP2D6*4* allele is associated with the PMs phenotype, owing

to the inability to metabolize its substrate as efficient as the wild type. The resulting protein from this mutation has a P34S, L91M, H94R and S486T amino acid substitution.

*CYP2D6*5* allele mutation represents the entire gene deletion. This allele therefore produces no enzyme and thus has zero functionality.

*CYP2D6*10* allele has a single nucleotide polymorphism (SNP) of 188C>T causing a P34S substitution (Bertillson, 2002). The protein product (i.e. CYP2D6.10) has diminished activity because of an impaired folding capacity resulting in an unstable protein and it also has lower expression levels than the normal CYP2D6.1 (Fukuda *et al.*, 2000).

*CYP2D6*17* bears a nucleotide exchange of 1111C>T, 2938C>T, and 4268G>C associated with amino acid exchange T107I, R296C and S486T, respectively (Babiro *et al.*, 2002). Babiro and co-workers (2002) showed that the CYP2D6.17 allele has a diminished ability to clear common CYP2D6 probe drugs *in vitro*.

4.2.4 The human *CYP3A4* gene

Discriminating between the CYP3A5 and CYP3A4 is not simple. The two enzymes display an overlapping role in metabolism and, in some instances, show similar metabolising capacity (Gorski *et al.*, 1994). Moreover they have an amino acid sequence homology of more than 85% (Dresser *et al.*, 2000). Available technology has also failed to convincingly distinguish between the two with respect to their metabolising capacities (Lamba *et al.*, 2002). For the purpose of this study, we are not separating the two, but treat them as a single entity which will be designated by the CYP3A4 symbol.

CYP3As metabolise the greatest proportion of drugs when compared to other CYPs (Hardman *et al.*, 1996). The CYP3A subfamily include CYP3A3, CYP3A4, CYP3A5 and CYP3A7 (Dresser *et al.*, 2000). CYP3A5 is the predominant form in the stomach and lungs, but its effect on drug metabolism is not clear. CYP3A4 is the most predominant isoform of the CYP3As and has the highest content in the liver. This enzyme is expressed not only in the liver, but also in the stomach. CYP3As have received little attention as compared to CYP2D6 and some studies suggest that this enzyme might not be polymorphic since it displays unimodal distribution.

The human CYP3A4 gene is located on chromosome 7 (http://www.ebi.ac.uk /proteome /HUMAN /chromosomes/ 7.html). The most common alleles are *CYP3A4**1B. and *CYP3A5*3A*. Unlike CYP2D6, there is no evidence for the null allele for *CYP3A4* (Lamba *et al.*, 2002).

4.2.4.1 Mechanism of mutation of the *CYP3A4* alleles and their functionality

CYP3A4*1B (alias nifedine response element) has a SNP in the 5' flanking region. This variant is in the promoter region of the *CYP3A4* gene located at -287 to -296 base pairs from the start of the start site of the *CYP3A4* gene (Ball *et al.*, 1999). The basis of this mutation is a -392A>G nucleotide substitution (Sata *et al.*, 2000). CYP3A4*1B has been shown by Sata and colleagues (2000) to have an inability to clear nifedine effectively. CYP3A4*2 allele has a exon 7 base change causing a S222P amino acid substitution (Sata *et al.*, 2000).

Just like the CYP3A4*1B allele, the CYP3A4*2 allele shows a reduced ability to metabolise nifepidine. From the above same study, the CYP3A4*3 allele was discovered to have a nucleotide exchange of T1334G causing a M445T amino acid substitution. This allele was shown to have no activity on testosterone (a well known substrate of CYP3A4 next to nifepidine).

CYP3A5*3A has intronic mutations with a nucleotide change at 6986+1A>G and 31611-2C>T which also presents with a cryptic splice defect (Lamba *et al.*, 2002).

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Materials and Methods

Chapter 5

5.1 Study objectives

As earlier discussed in chapter 3, phenotypic determinations can be achieved with the use of various analytical techniques. For the purpose of this study, the CYP2D6 and CYP3A4 phenotypes will be determined by employing DXM as a metabolic probe for the activity of CYP2D6 and CYP3A4 enzymes. DXM is a metabolic probe of choice for determining the catalytic activities of the CYP2D6 and CYP3A4 enzymes (Wieling *et al.*, 2000). The immediate objectives are the following:

- Setting up and validation of a sensitive HPLC method for identification and quantification of DXM, and its major metabolites, DX and 3MM in the urine. This is necessary for the determination CYP2D6 and CYP3A4 enzymatic activities, as dictated by the CYP2D6 and CYP3A4 metabolic ratios.

- Application of the validated HPLC method in a clinical study, whereby the metabolic profile of CYP2D6 and CYP3A4 will be determined in human subjects. This step ascertains the accuracy and reproducibility of the HPLC method to phenotype a given population.

5.2 HPLC

The HPLC method of analysis of DXM and its metabolites essentially follows that of Bendriss, and co-workers (2001), but validated for own laboratory conditions. Metabolic ratios and antimode determination followed that of norms for this procedure.

5.2.1 Chemicals and reagents

Analytical grade 3-methoxymorphinan hydrochloride ([±]-*cis*-1,34,9,10,10a-hexahydro-6-methoxy-2H-10,4a-iminoethanophenanthrene hydrochloride), (+)-3-hydroxymorphinan hydrobromide (9α,13α,14α-morphinan-3-ol), dextrophan D-tartrate ([+]-3-hydroxy-*N*-methylmorphinan D-tatrate) and the internal standard (IS) levallorphan tartrate (17-[2-propenyl]

morphinan-3-ol tartate), were purchased from Sigma® RBI®chemical Co, Saint Louis, Missouri, USA. Dextromethorphan, β-glucoronidase type H-5 from Helix pomatia 600 000 units/g solid, Supelclean LC-Si, 1 ml cartridges, glacial acetic acid, and acetonitrile, were purchased from Sigma-Aldrich, Kempton-Park, South Africa. All chemicals and their solutions were stored under dark conditions at 4°C.

Double distilled water was used throughout the study. Sodium acetate buffer 1M (pH 5.5) was prepared in a same fashion as described by Walpole (1914). Sodium carbonate-bicarbonate buffer 1M (pH 9.2) was prepared as prescribed by Delory and King (1945). Fine adjustment of pH for both buffers was achieved with the use of either 0.1M NaOH or 0.1*M* HCl solution. All other reagents were analytical grade and from commercial sources.

5.2.2 Chromatographic conditioning

The chromatographic system consisted of a Waters M45 pump, fitted with a Sphere Clone 150 x 4.6 mm, 5μ phenyl column serially connected to a Luna 250 x 4.6 mm 5μ nitrile column and a security guard column (Luna, phenomex, Torrance, CA, USA). The pump was operated at a flow rate of 1 ml/minute and had a 50 μl loop for sample injection. Fluorescence detection was achieved with the use of Shimadzu RF551 fluorescence detector at an excitation wavelength of 280 nm and emission at 310 nm wavelength. A Hewlett Packard 3395 integrator was used for plotting and for calculation of the peak areas. Mobile phase was made up of 80% glacial acetic acid (1.5 %), 0.1% triethyl amine, and 20% acetonitrile.

5.2.3 Standard Solution preparation

Four stock solutions of DMX, 3HM, 3MM, and DX were prepared in double distilled water at concentration of 50 μg/ml free base and stored at 5°C. The stock solution of internal standard, levallorphan, was prepared by dissolving 5 mg in 2.5 ml of water. Test solutions were prepared in the concentration range of 0.05 μg/ml, 0.1 μg/ml; 0.25 μg/ml; 0.5 μg/ml; 1 μg/ml; 2 μg/ml; 3 μg/ml; 10 μg/ml; and 30 μg/ml free base. Volumetric flasks were completely wrapped in aluminium foil and stored at 4°C for all solutions.

5.2.4 Sample preparation

Normally, biological fluids contain a large number of chemical constituents and proteins. Sample pre-treatment allows the exclusion of interfering components, while chemical constituents of interest are retained in the sample for analysis.

The DXM metabolic pathway is discussed in detail in chapter 4, and also illustrated by figure 4.2. Clearly from figure 4.2, it can be seen that 3MM and DX can undergo conjugation prior to excretion. There is accordingly a need to convert both 3MM and DX conjugated forms into glucuronide-free, de-conjugated forms. For this reason, the first step of sample treatment is de-conjugation with the enzyme β-glucoronidase. This enzyme functions optimally at pH 5.5 and temperature of 37 ^{0}C. The complete hydrolysis of the conjugated metabolites takes 18 hours.

As discussed earlier, solid phase extraction operates on chromatographic principles. The packing material of the extracting columns is a silica material, which is composed of Si-OH groups. This material is suitable for separating polar organic compounds. The samples are made alkaline by the addition of a buffer, to ensure that the compounds of interest are non-polar. This means that all non-polar compounds will be retained on the packing material while the remainder will elute. To remove the retained chemical species from the packing material, dichloromethane (non-polar solvent) is used to wash all the previously retained chemicals into a clean vial. The next step is to evaporate the samples to dryness, and then reconstitute them with the mobile phase such that the final volume equals the initial volume from untreated urine sample (i.e. 250 μl).

From the patient sample, an aliquot of 250 μL human urine was added to 2,5 ml centrifuge tubes, to which 250 μL sodium bicarbonate buffer solution pH 5.5 and 250 μL β-glucoronidase (1000 units/ml in bicarbonate buffer [pH 5.5]) was added followed by vortex mixing for 10 seconds. The tubes were sealed, transferred to a warm water bath, and incubated at 37°C for a period of 18 hours to ensure complete hydrolysis of the conjugated metabolites.

After 18 hours, the tubes were removed and allowed to cool at room temperature. To each tube, 25 μl IS and 250 μl of sodium bicarbonate buffer (pH 9.2) were added to alkalinise the sample. The sample volume was adjusted to 1 ml using distilled water and vortexed for 10 seconds. The tubes were subjected to centrifuge at 22000 *g*.

One ml silica cartridges (Supelco, Bellefonte, USA) were conditioned by sequential addition of 2 X 1 ml methanol followed by another 2 X 1 ml distilled water. The samples from the centrifuge tubes were then applied to the cartridges. Care was taken not to allow the packing material to dry during the conditioning and sample application steps.

The compounds of interest were retained in the cartridges while the urine was allowed to elute. Vacuum was applied to ensure complete removal of urine from the sample. Under slight vacuum (5 mm Hg), the chemicals of interest were eluted into clean 10 ml test tubes using 2 X 4 ml dichloromethane/ hexane solution (95:05 v/v). The eluent was evaporated to dryness under

gentle stream nitrogen. The resulting extract was reconstituted with 250 μl of the mobile phase (80% [glacial acetic acid (1.5 %), 0.1% triethyl amine] 20% acetonitrile) and mixed with a vortex mixer for 5 seconds. This solution (250 μl) was then injected into the HPLC.

5.3 Patient study

Thirty apparently healthy male volunteers, with the mean age 31 (range 18 and 55 years) were recruited and gave written informed consent before entering the study as approved by the Ethics committee for Human Research of the Potchefstroom University for CHE (3m04). The clinical study was perfomed at the department of Pharmacology, PU for CHE and was carried out under the supervision of a qualified medical officer. The following exclusion criteria was applied :

- History of allergic reactions to probe drugs to be used
- Alcohol use
- Smoking history
- Renal impairment or any cardiovascular disease
- Subjects taking medication known to alter the CYP2D6 or CYP3A4 enzyme activity

After an obligatory overnight fast, each subject was phenotyped for CYP2D6 and CYP3A4 using dextromethorphan as a probe. For this purpose, commercially available Benylin cough syrup containing dextromethorphan (Parke Davis, South Africa). An initial screening procedure will include a medical history, blood pressure, heart rate and body mass index determination.

Patients were requested to report to the research facility at 7h:30 am, and were required to empty their bladder, the voided urine serving as a blank. Fluids and food were allowed two hours after the ingestion of metabolic probe. Prescription drugs or food containing substances known to alter the test enzyme's activity were not allowed one week prior to and for 8 hours post-dose.

A pre-measured dose equivalent to 30 mg of dextromethorphan was administered orally to each patient. Samples from urine voided over 8 hours post-dose were then collected in Rotalibo plastic bottles laced with two to three drops of sodium azide (0.6 M). The volume of the sample was measured and a 20 ml aliquot stored at -20°C until assayed.

5.4 Data analysis

The goal of this study is to validate our HPLC procedure is able to consistently and reliably quantify the chemicals of interest, first from water spiked samples, then urine spiked samples, and later to apply this to a clinical trial situation. Data were analysed with respect to precision, accuracy and linearity. The recovery, limit of detection (LOD), and limit of quantification (LOQ) will also be used for data analysis.

5.4.1 Precision

Precision expresses the degree of scatter between a series of measurements obtained from multiple sampling. The percentage coefficient of variation (CV%) will be used as a measure of precision. The CV% is given by the formula: $\frac{standard\ deviation(SD)}{mean\left(\bar{x}\right)} \times 100\%$

From published analytical methods of similar nature (e.g. Ducharme *et al* 1996; Jones *et al* 1996; Hoskins *et al*, 1997), the CV% ranges from 5 to 25%. For the purpose of this study, a CV% of less than 15% was considered as acceptable. Intra- and inter-day precision will be evaluated by spiking urine samples with the known amount of the analytes and extracting them using the method described in section 5.2.4.

5.4.2 Specificity

Specificity is the ability to determine unequivocally the analylate of interest in the presence of compounds that may be expected in a standard urine sample. In this study, the absence of interfering peaks will be the confirmation of specificity.

5.4.3 Linearity

Linearity is the ability of an analytical procedure, within a given range, to obtain test results that are directly proportional to the concentration of analylate in the sample. The R^2 value is the measure of linearity. The r value is given by the formula:

$$r = \frac{n\sum xy - (\sum x)(\sum y)}{\sqrt{(n\sum x^2 - (\sum x)^2)}\sqrt{(n\sum y^2 - (\sum y)^2)}}$$

the R^2 value has the corresponding meaning.

5.4.4 Range

Range describes the interval from the lowest concentration to the highest concentration that can be determined using a given analytical method.

5.4.5 Recovery

Absolute recovery is achieved if the response from urine sample equals the response from water samples. The recovery of each compound will be measured at five concentrations, each done in triplicate by comparing the quantity of an extracted urine sample per quantity of unextracted water sample at a given concentration point. Using the linear curves of water standards, the concentration will be calculated using the equation for a linear curve,

$$y = mx + c$$

where y= the response (in this case the peak area ratio of a compound) and

x = the concentration of the compound.

By making x (concentration of the extracted quantity) the subject of the formula, the equation becomes

$$x = \frac{y - c}{m}$$

The resulting equations for each compound will be used to calculate the urine sample concentrations at each point. Recovery will be expressed as the % of $\frac{conc.found}{spiked\ conc.} \times 100\%$

5.4.6 Accuracy

Accuracy expresses the degree of agreement between the value considered to be true (spiked concentration) and the value found (calculated concentration). The accuracy of the method will be determined as a percentage of the concentration found, divided by the true concentration (i.e. spiked concentration).

5.4.7 LOD and LOQ

LOD represents the lowest amount of an anaylate in a sample which can be detected but not necessary quantified. The LOD value will be the concentration at which the peak height is three times above baseline. LOQ represents the lowest amount of an anaylate in a sample which can be quantified with suitable precision and accuracy. The criteria for LOQ was set as the lowest point of the concentration range for that compound.

5.5 References

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Results

Chapter 6

6.1 Background

This study consist of two experimental procedures, one being the development and validation of an HLPC method for analysis of DXM, DX and 3MM in urine, followed by its application in a group of human subjects.

6.2 HPLC analytical method validation

The method adopted was that originally described by Bendriss and colleagues (2001), although various methods from Batroletti and colleagues (1996); Jones and colleagues (1996); Ducharme and colleagues (1996); and Hoskins and colleagues (1997) were consulted.

6.2.1 HPLC optimisation

The HPLC system, consisting of the pump, sample injection system, detector, integrator, mobile and stationery phase all represent variable components. These components need to be optimised to best suit the planned trial procedure. Thereafter, the analysis will be validated with respect to linearity, precision, method of extraction, accuracy, recovery and limits of quantification and detection.

6.2.1.1 Pump speed and mobile phase

The primary function of the pump is to deliver the mobile phase through the system as reproducibly as possible (Robards *et al.,* 2001). The above mentioned published methods used a pump speed of 1 ml/min which, according to our findings, confirmed it as the ideal speed for this analytical procedure and analytical column diameter. Pump speeds lower than 1ml/min delayed the retention times of the eluting peaks, resulting in peak broadening and difficulty to integrate consistently. Flow rate higher than 1.5 ml/min produced excessive pressure (greater than 4000 p.s.i), as expected when coupling two columns in series.

The mobile phase employed in the publications varied from one to the next. In common, using varying means, the mobile phase pH for all methods was adjusted to a highly acidic environment of pH equal to 3 or less, although the mobile phase differed. The variations in

mobile phase selection reflect the various extractions, separation and modes of detection for analytes. A solution containing 1.5% glacial acetic acid, 0.1% triethylamine and acetonitrile (75:25 v/v) was used by Bendriss and co-workers (2001). The findings of this study showed that this solution eluted the compounds a little too fast for the required proper integration (see fig 6.1). Decreasing the organic phase to 20% produced the required result (see fig 6.2). Increasing the organic phase concentration above 25% caused the species of interest to elute too fast and interfered with peak separation between 3HM and DX (results similar to fig 6.1).

The sharp peaks are perfect for integration purposes, but when urine extracted samples were introduced, the peaks formed clusters with the solvent front peaks and could not be separated clearly. These peaks also posed a problem when concentrations of higher than 3 μg/ml were employed, since they overlaped significantly rather than to separate at the base line. Running the system at lower organic phase concentration produced similar results as when the pump speed was reduced below than 1 ml/min (see fig 6.2). A pump speed of 1 ml/min. and mobile phase containing solution containing 1.5% glacial acetic acid, 0.1% triethylamine, and acetonitrile (80:20 v/v) was used henceforth throughout the study (see fig 6.3)

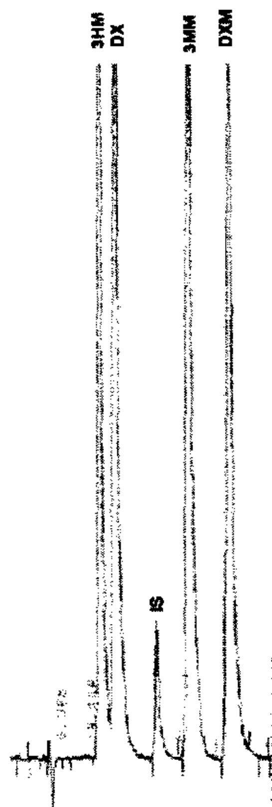


Figure 6.1. Chromatogram of all four compounds at high organic phase conditions. Note that the 3-hydroxy morphinan (3HM) and the dextrophan peak are fused at the base.

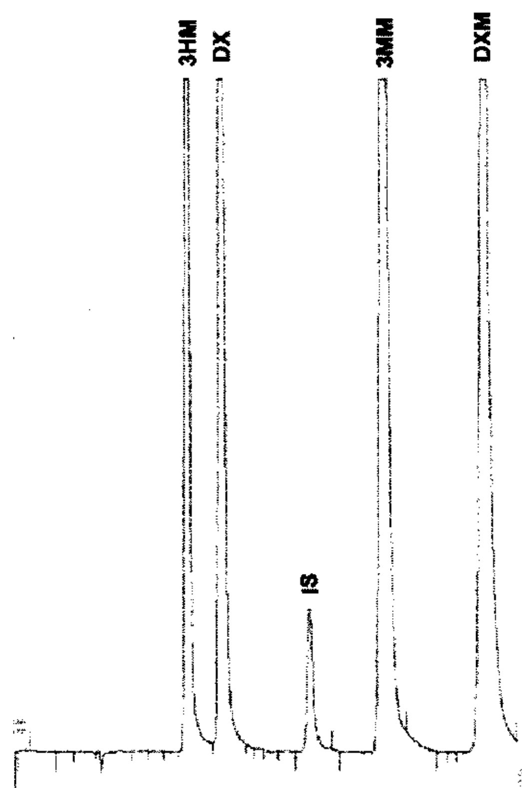


Figure 6.2. Chromatogram of slow eluting compounds caused by low pump speed or low organic phase concentration in the mobile phase.

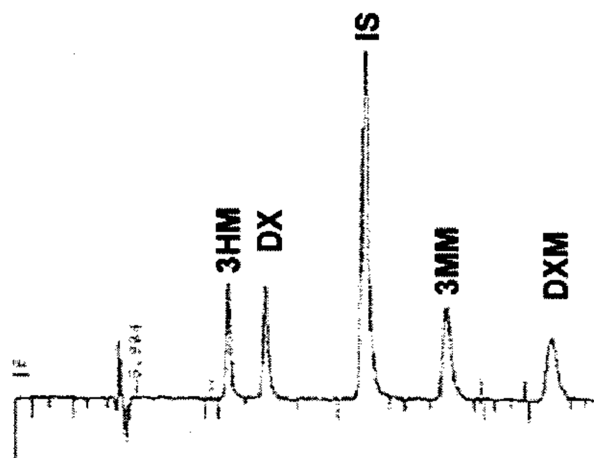


Figure 6.3. Optimised chromatogram of all four compounds at 20:80 mobile phase ratio.

6.2.1.2 Loop size

Loop size regulates the final volume of the sample that is injected into the HPLC system. The previously mentioned methods have used varying loop sizes ranging from 5 to 120 μl. Modern HPLC system have a variable loop injection volume without manually removing the loop. The

100 μl loop was tested and found to produce good peaks with high reproducibility, although it introduced the largest amount of impurities resulting in frequent changes of the guard column cartridges. This expensive practice came to an end when we realised that it was time consuming. The 50 μl loop was subsequently used throughout the study, more than doubling guard cartridge lifetime.

6.2.1.3 Detector

A Shimadzu fluorescence (RF551) detector was used for detection at wave length 280 nm and 310 nm for excitation and emission, respectively. This detector required one and a half-hour waiting period to allow the lamp to stabilise and to avoid an unsteady and wavy baseline.

6.2.2 Water standards

After optimisation of the operating conditions for the HPLC, the next step was to construct a standard curve for each compound using aqueous samples. Samples of all four compounds of interest at various concentration ranges, together with an internal standard, were injected onto the HPLC column. Peak area ratio for each compound (area of a compound per peak-area of internal standard) was plotted against its concentration. Each analysis was tested for linearity, precision, limit of quantification and limit of detection.

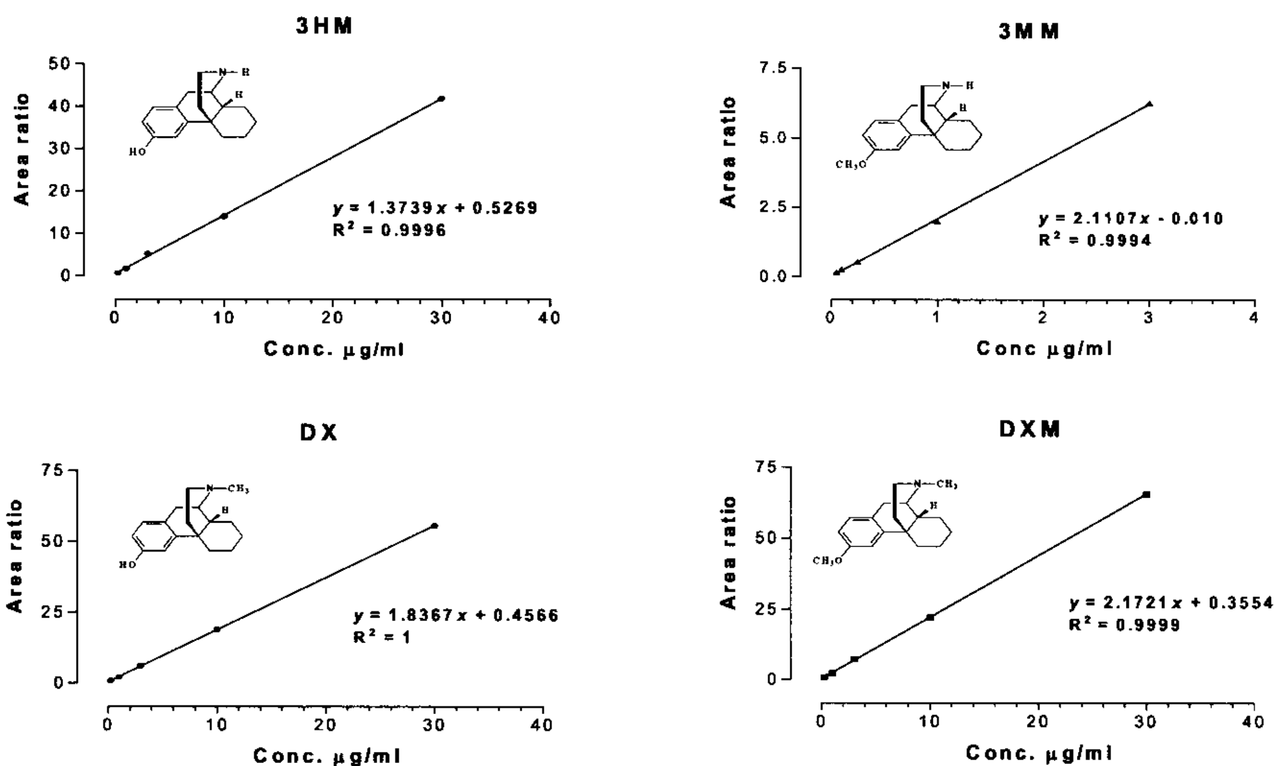


Figure 6.4. Linear curves from 3HM, DX, 3MM, and DXM from quality control water standards

6.2.2.1 Linearity

The R^2 value is the measure of linearity. All four compounds were found to be linear over their respective concentration ranges (see fig 6.4). R^2 values were 1.000, 0.9994, and 0,9999 for 3HM, DX, 3MM and DXM respectively (see fig. 6.4).

6.2.2.2 Precision

Precision expresses the degree of scatter between a series of measurements obtained from multiple sampling. Coefficient of variation (CV) was used as a measure of precision. Results are presented in tables 6.1 to 6.4. The coefficient of variation for all compounds was less than 10%. Generally the C.V. increased with a decrease in concentration; this was most evident with DX and DXM.

Table 6.1. Precision for 3HM water standards.

Conc.	3HM			Ave (SD)	C.V. %
	Area $Ratio_1$	Area $Ratio_2$	Area $Ratio_3$		
30 µg/ml	45.074	37.857	42.908	41.780 (3.549)	8.37
10 µg/ml	13.401	13.938	14.699	14.015 (0.652)	3.13
3 µg/ml	5.442	5.079	5.077	5.199 (0.210)	4.04
1 µg/ml	1.754	1.653	1.653	1.762 (0.114)	6.46
0.25 µg/ml	0.683	0.698	0.646	0.676 (0.027)	3.96

Ave = average, SD = Standard deviation, Conc. = concentration.

Table 6.2. Precision for DX water standards

Conc.	DX			Ave (SD)	C.V. %
	Area Ratio$_1$	Area Ratio$_2$	Area Ratio$_3$		
30 μg/ml	59.870	50.484	56.225	55.526 (4.732)	8.53
10 μg/ml	18.132	18.798	19.717	18.882 (0796)	4.22
3 μg/ml	6.423	6.062	5.994	6.160 (0.23)	3.74
1 μg/ml	2.091	1.966	2.222	2.093 (0.125)	6.11
0.25 μg/ml	0.995	0.830	0.866	0.897 (0.087)	9.67

Ave = average, SD = Standard deviation, Conc. = concentration.

Table 6.3. Precision for 3MM water standards.

Conc.	3MM			Ave (SD)	C.V. %
	Area Ratio$_1$	Area Ratio$_2$	Area Ratio$_3$		
3 μg/ml	6.658	6.062	5.994	6.160 (0.231)	3.17
1 μg/ml	1.982	1.863	2.130	1.992 (0.134)	6.73
0.25 μg/ml	0.512	0.534	0.507	0.855 (0.014)	2.77
0.1 μg/ml	0.252	0.266	0.243	0.254 (0.126)	4.58
0.05 μg/ml	0.119	0.199	0.115	0.118 (0.002)	1.62

Ave = average, SD = Standard deviation, Conc. = concentration.

Table 6.4. Precision for DXM water standards.

Conc.	DXM			Ave (SD)	C.V. %
	Area Ratio$_1$	Area Ratio$_2$	Area Ratio$_3$		
30 μg/ml	70.705	60.008	65.830	65.514 (5.35)	8.33
10 μg/ml	22.169	21.263	22.615	22.016 (0.69)	4.32
3 μg/ml	7.533	7.069	7.027	7.210 (0.280)	3.89
1 μg/ml	2.350	2.119	2.428	2.299 (0.161)	7.00
0.25 μg/ml	0.849	0.927	0.790	0.855 (0.07)	8.05

Ave = average, SD = Standard deviation, Conc. = concentration.

6.3 Solid phase vs. liquid-liquid phase extraction

The next step was to successfully extract DXM and its major metabolites from urine samples for the purpose of clinical quantification in patients using HPLC. Either solid phase or liquid-liquid extraction methods can be used. The principal differences between these extraction techniques are as follow:

Liquid-liquid extraction involves the use of a mixture composed of solvents that are either electron donors or acceptors, having the ability to form intermolecular hydrogen bridges (i.e. hydrophilic or polar solvents), and solvents that lack these abilities (i.e. lipophilic or non-polar solvents) (Heftmann 1963). By manipulating the pH of the extracting solution, selective partition takes place whereby the chemical species from a sample separate into either aqueous or organic phase.

Solid phase extraction, on the other end, employs chromatographic principles. A sample is introduced into a packing material that will either retain or elute the chemical species of interest while the unwanted portion of the sample elutes or is retained.

Liquid-liquid extraction is a less expensive option, since the required chemicals are readily available and affordable. It was for the latter reason alone that this procedure was opted for the study.

6.3.1 Liquid extraction

Liquid extraction, as described by Jones and colleagues (1996) and Hoskins and colleagues (1997), were applied in an attempt at finding a fast, reproducible, and cost-effective method. The method described by Hoskins was slightly modified by using levallorphan instead of pholcodine as an IS, and an injection of 50 µl instead of 5 µl. In brief, a 500 µl urine sample was alkalinised with a saturated sodium carbonate solution, thereafter the analytes were extracted with 4 ml of diethyl ether chloroform propan-2ol, (20:9:1 v/v/v). The aqueous phase is discarded and the organic phase acidified with 100 µl of 0.1M HCl. Fifty µl of the resulting aqueous phase was injected onto the HPLC column. In this method, the initial untreated urine volume was 500µl, and in the final volume, 100 µl, of which only 50 µl was injected. A concentrating factor is therefore evident, since the chemical entities remain constant while the volume decreases, and is accounted for in the final calculations.

The resulting peaks from this method were of appreciable size because of a concentrating factor of 2.5 and the solvent front separated clearly from the 3HM peak. The extracted samples were linear with the R^2 values of 0.9995, 0.9997, 0.9972, 0.9982 for 3HM, DX, 3MM, and DXM respectively (also see Table. 6.5). The co-efficient of variance were less than 10% for all compounds and thus highly reproducible. Although recoveries were generally low (28% for DXM), it was consistent as seen in the low C.V. and therefore acceptable as a reproducible and accurate method. However, the search for a method yielding appreciable DXM recovery continued.

Table 6.5. Recoveries based on a method described by Hoskins and colleagues (1997).

	3HM	DX	3MM	DXM
Ave Recovery %	64.5	56.3	34.5	27.7
SD $_{n=6}$	2.6	3.9	3.3	2.58
C.V. %	3.4	6.9	9.7	9.3
R^2	0.9995	0.9997	0.997	0.998

Average recoveries taken from 6 points each in triplicate over their respective concentration range. R^2 taken from linear regression plots of 6 concentration points.

The method described by Jones (1996) employs an extensive extraction procedure with high recoveries claimed for DXM, DX and 3MM. In this method, levallorphan was used as an IS, instead of either codeine or ethylmorphine, while a better suited carbonate-bicarbonate buffer instead of NaOH-glycine buffer was used (this buffer has a good capacity at pH of 11.3 and

contains inorganic residues as opposed to the NaOH-gylcine buffer). Finally a fixed 50 µl injection as opposed to the variable 5-120 µl injection was used.

This extraction procedure, which proved to be long, involved the alkalinisation of a 500 µl urine sample, followed by a two step extraction with hexane-ethylacetate. The resulting aqueous phase was discarded. The two extracted portions were combined and acidified with 1 ml 0.1 M HCl. The organic phase was discarded and the remainder alkalinised with a carbonate-bicarbonate buffer (pH 11.3). The resulting solution was extracted again with hexane-ethylacetate, and the organic phase evaporated to dryness before reconstituting it with 250 µl of mobile phase (similar to that described in section 6.2.1.1). This solution was injected into the HPLC system.

The method described by Jones and colleagues (1996) yielded results with much improved recoveries of 62.7 %, 78.8%, 53.8%, 52.5% for 3HM, DX, 3MM, and DXM respectively (see table 6.6) while maintaining acceptable variability. All four compounds were linear with R^2 values well above 0.994. Coefficients of variation for all compounds was less than 20% and this method provided additional sensitivity of all compounds due to the concentrating factor.

Table 6.6. Recoveries based on a method described by Hoskins and colleagues (1996).

	3HM	DX	3MM	DXM
Recovery Ave%	62.7	78.8	53.8	52.5
$SD_{n=5}$	9.6	9.2	8.9	10.3
C.V. %	13.2	13.2	19.1	15.9
R^2	0.999	0.999	0.996	0.994

Average recoveries taken from 5 points each in triplicate over their respective concentration range. R^2 taken from linear regression plots of 5 concentration points

The amount of impurities, however, was large, causing the guard column to clog constantly and the recoveries were not all above the predetermined satisfactory 60% (based on similar studies). In an attempt to minimise this, half (i.e. 250 µl) the recommended urine sample was subjected to the same method as described above, but with no changes in other constituents. The results are presented in table 6.7.

Table 6.7. Recoveries based on a modified method described by Jones and colleagues (1996).

	3HM	DX	3MM	DXM
Recovery Ave%	92.7	83.4	79.3	64.4
$SD_{n=5}$	12	8.2	15.2	15.6
C.V. %	13.9	9.8	19.1	18.1
R^2	0.9998	1	0.9995	0.992

The modifications yielded generally good linearity and recoveries. Although reasonable recoveries were achieved, the reproducibility was worse but still acceptable at lower concentrations. Our major concern was the recovery and reproducibility of DXM, which had the lowest recovery, a high CV percentage and the poorest linearity (see Table 6.7.). Considering that consistent determination of DXM is crucial for the success of the method, we opted for a suitable solid phase extraction method.

6.3.2 Solid phase extraction

Methods that were considered for the procedure for solid phase extraction were those published by Bartoletti and colleagues (1996) and Bendriss and colleagues (2001). The results of the solid phase extraction described by Bendriss and colleagues (2001) are described in the next section. In brief the results are presented in table 6.8.

Table 6.8 Recoveries based on a modified method described by Bendriss and colleagues (2001).

	3HM	DX	3MM	DXM
Recovery Ave%	62.2	98.2	67.6	96.6
$SD_{n=5}$	21.44	8.3	6.5	7.5
C.V. %	36.7	14.9	9.1	8.3
R^2	0.9989	0.9985	0.9989	0.9998

3HM had the lowest average recovery and a high SD value of 21%. The CV % of 3HM was also unacceptably high, with a value of 36.7%. Using this method, the best recoveries were achieved for DX and DXM. Impressive recovery for 3MM was also achieved with the exception of the modified method from table 6.7. Except for 3HM, all compounds had CV% of less than 15%.

6.3.3 Comparison between solid and liquid phase extraction

Fig 6.5 and 6.6 illustrate typical chromatograms from liquid-liquid and solid phase extraction respectively, obtained under the current laboratory conditions. The two chromatograms are representative of the same urine sample concentration and attenuation. Comparing chromatograms between solid and liquid phase extraction revealed the following:

The peaks from liquid extraction are much bigger than that from solid phase extraction. The reason is the concentrating factor used in the liquid-liquid extractions as compared to solid phase extraction, wherein no concentrating factor was present. Nevertheless, peaks form solid phase extractions remained more that adequate for our procedure.

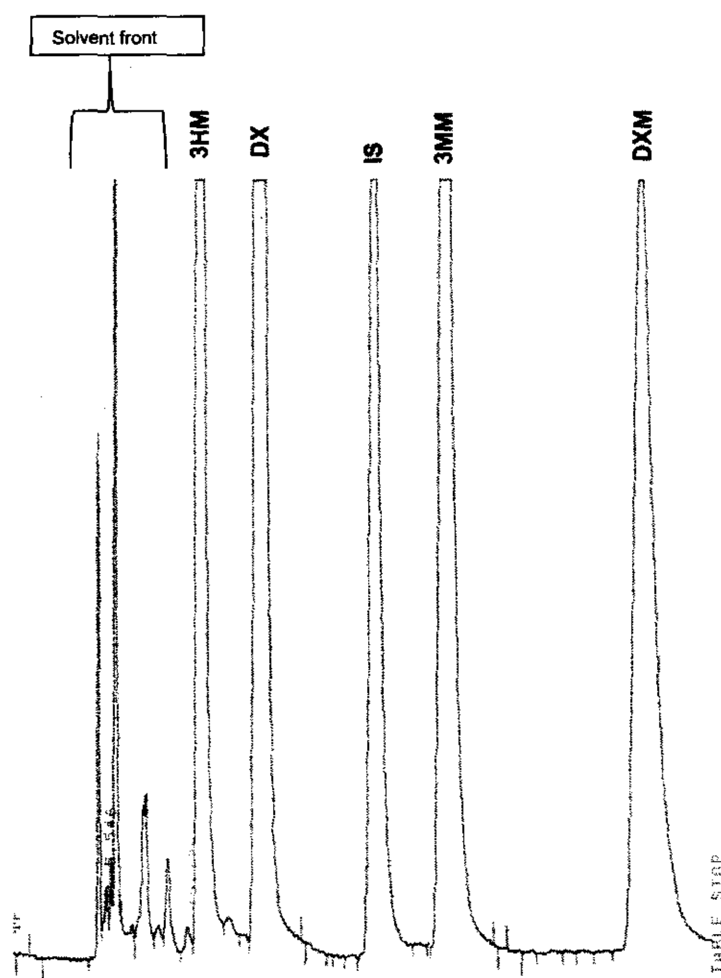


Figure 6.5. Liquid-liquid extraction chromatogram taken at 1 µg/ml urine sample concentration. Attenuation = 4, chart speed = 0.3 cm/minute

The solvent front from liquid extraction had fewer fluorescent chemical constituents as opposed to solid extraction; the fewer peaks at the beginning of the chromatograms illustrate this (Fig 6.5 and 6.6). The most interesting finding was that the recovery and reproducibility of 3HM was generally superior with liquid extraction compared to solid phase extraction. This was most evident at concentrations higher than 1 µg/ml. However the quantitation of 3HM is not necessary for the determination of CYP2D6 and CYP3A4 activity. Treatment of sample using solid phase extraction was quicker as compared to liquid-liquid extraction which at times took as long as 8 hours for a 15 sample treatment. Although the liquid-liquid extraction proved to be less expensive, Bendriss's solid phase method was adopted for this study.

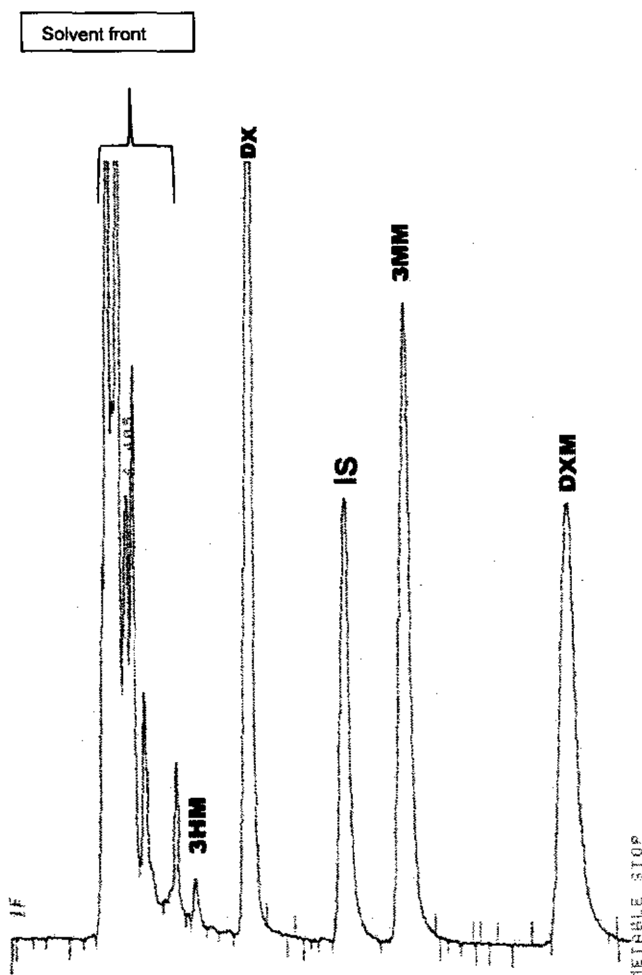


Figure 6.6. Solid phase extraction chromatogram taken at 1µg/ml urine sample concentration. Attenuation = 4, chart speed = 0.3 cm/minute

6.4 Adopted method

The solid phase extraction method described by Bendriss and co-workers (2001) was therefore adopted throughout the remainder of the study. Modifications of this method included the use of a 50 μl loop instead of a 100 μl injection, while the mobile phase was filtered though a better suited 0.22 μm Millipore membrane filter instead of 0.45 μm. Furthermore, the mobile phase was not sonicated prior to use. Finally, sample evaporation to dryness was achieved by blowing a nitrogen gas onto the samples in a warm water bath at 40° C rather than using a speed-vac. The method was subsequently validated with respect to range, specificity, linearity, accuracy and precision.

6.4.1 Range

Based on a preliminary test, we were able to make a reasonable estimation of the anticipated concentration ranges at which the DMX and its metabolites are excreted in the urine over a period of 12 hours. These concentration ranges were chosen to cover all possible expected concentrations that would be obtained from a typical population. These data were based on a preliminary study prior the clinical trial.

6.4.2 Specificity

After extraction, the retention times of all the compounds was found not to interfere with any compound normally excreted in urine. The intake of caffeine also did not interfere with any peaks and produced the same chromatogram as in fig 6.6. The method was therefore found to be sufficiently specific for the determination of all compounds of interest.

6.4.3 Linearity

Linearity was determined at various concentration levels for each of the three compounds. Five points, each in triplicate, were used to determine linearity for all the compounds. Fig 6.7 illustrates all four plots that were constructed using area ratio of the compound of interest relative to the internal standard. All four compounds had a R^2 value greater than 0.99 indicating acceptable linearity.

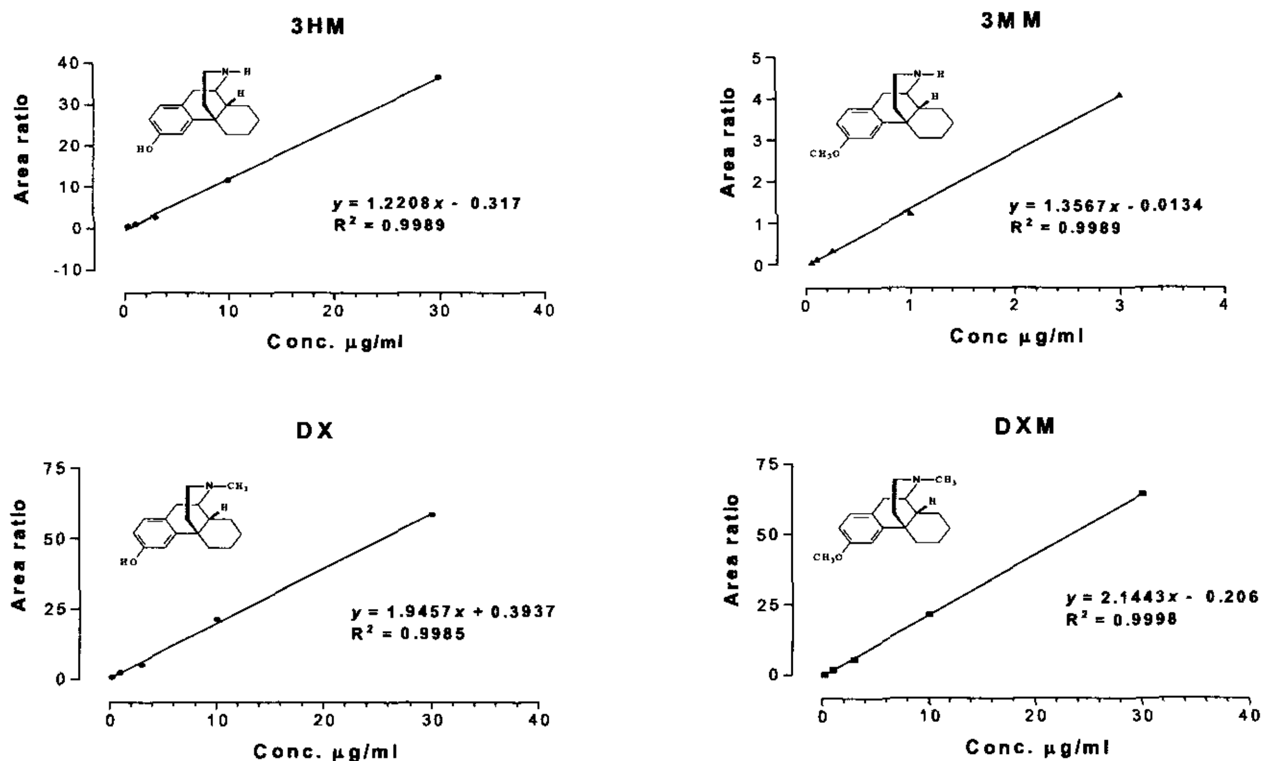


Figure 6.7. Plots of area vs. concentration from urine samples of 3HM, 3MM, DX, DXM in urine extracts constructed from 5 points.

6.4.4 Accuracy and Recovery

Accuracy of each compound was determined by comparing the response from water standards and response from urine extracts, the degree thereof was expressed as a percentage. From nine determinations, the recovery of the IS was 96.9% with a CV% of 4.4%. Accuracy was determined at five concentration points for each compound. For this method the accuracy and the recovery of the compound are determined in the same manner and thus can be discussed together.

Recovery measures the extraction efficiency of the method. The recovery of each compound was measured at five points each in triplicate at a given concentration point. Using the linear curves of water standards, the concentrations were calculated using this formula: $x = \frac{y-c}{m}$ and were expressed as

$x = \frac{y - 0.5269}{1.3739}$ $x = \frac{y - 0.4566}{1.8367}$, $x = \frac{y + 0.0100}{2.1107}$, and $x = \frac{y - 0.1355}{2.1767}$,

for 3MM, DX, 3MM, and DXM respectively.

Recovery of each compound is graphically presented Fig 6.8. The average recovery of 3MM, DX, and DXM were all satisfactory, with recoveries of 64, 97, and 94% respectively (see table 6.9). The recovery of 3HM was satisfactory but problems were encountered with its accuracy, which is indicated by a C.V. of 35.25 % (see table 6.9). For this reason, and the fact that its determination is not critical in phenotyping the CYP2D6 or CYP3A4 (refer to figure 3.2), this compound was not measured in later analyses.

The variability in recovery of 3HM appears to reflect non-specific protein binding at low concentration (see table 6.9).

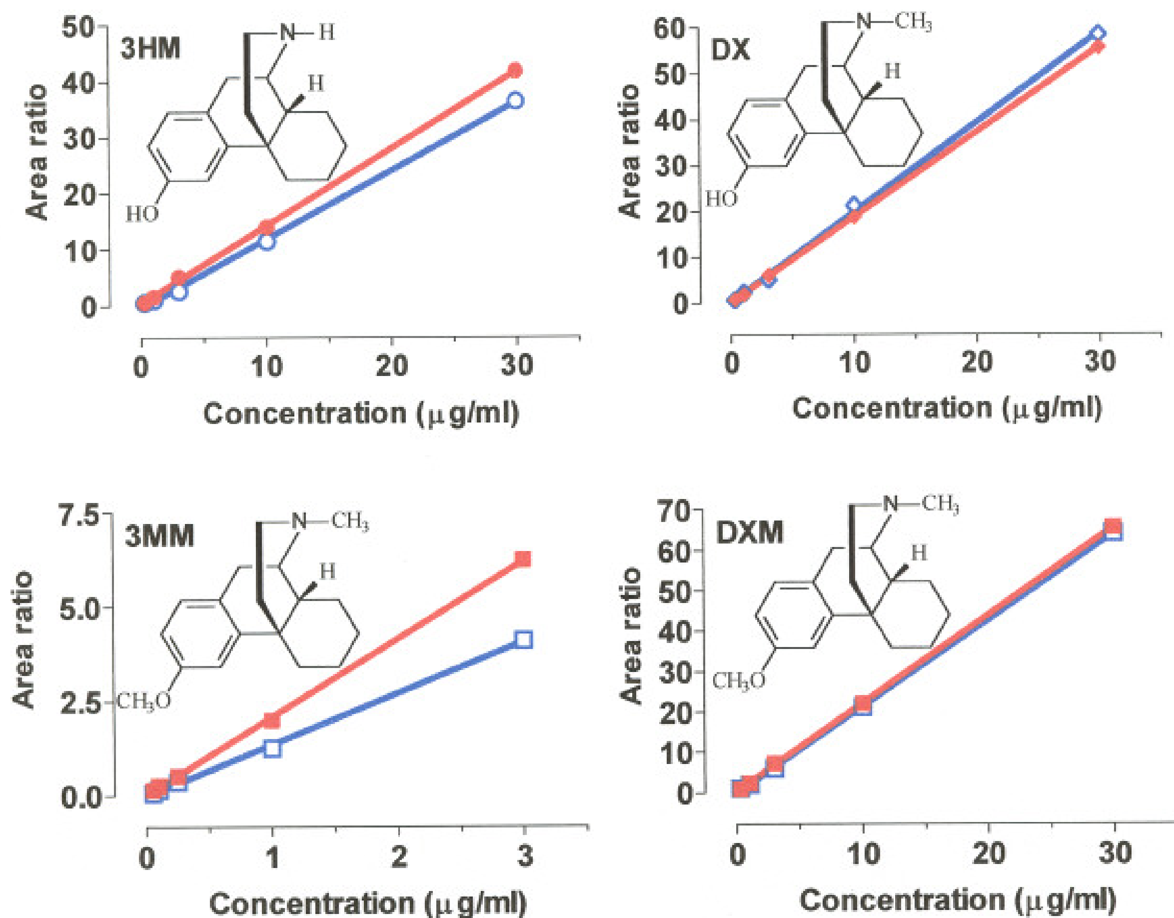


Figure 6.8. Plots illustrating the recovery of 3HM, DX, 3MM and DXM from urine samples. The solid points represent points from water standards and the unfilled legends represent urine extracts. 3HM (● ○), 3MM (▲ Δ), DX (◆ ◇), DXM (■ □). -Blue- line = water samples. -red- line = urine samples. Take note that the scale of the 3MM plot is ten times less than that of the other plots, thus making it look as if it displays poor recovery.

Table 6.9. Recovery and precision of all compounds from urine samples.

Nominal Conc. (µg/ml)	% recovered 3HM	% recovered DX	% recovered DXM	Nominal Conc. (µg/ml)	% recovered 3MM
30	87.21	105.09	98.00	**3**	64.67
10	80.38	113.88	97.59	**1**	59.55
3	51.49	86.32	83.91	**0.1**	71.01
1	44.08	105.15	87.73	**0.25**	70.47
0.25	40.99	76.81	101.45	**0.05**	56.56
Ave recovery	60.83	97.45	93.73	**Ave recovery**	64.45
CV%	35.25	15.71	8.00	**CV%**	9.98

6.4.5 Precision

Three determinations were made for each point, one at a low concentration level (0.25 µg/ml), one intermediate concentration level (3 µg/ml), and one at a high concentration level (30 µg/ml). 3MM concentrations were 3 µg/ml, 0.25, and 0.05 µg/ml for lowest, in-between, and highest concentration level respectively. The following parameters of precision were determined:

6.4.5.1 Intra day precision (repeatability)

Intra day precision measures the precision of an analytical method over a short space of time. It is also sometimes referred to as in-between run precision. The results are presented in a table format on table 6.10. Intra day precision for DX and DXM were impressive at all concentration levels. The C.V. % of 3MM was at an average of 14%.

Table 6.10. Intra day precision

	DX			DXM			3MM		
run#	30µg/ml	3µg/ml	0.25µg/ml	30µg/ml	3µg/ml	0.25µg/ml	3µg/ml	0.25µg/ml	0.05µg/ml
1	32.41	2.40	0.21	28.96	2.41	0.30	1.58	0.19	0.04
2	31.21	2.62	0.17	28.35	2.64	0.25	2.05	0.16	0.03
3	30.96	2.71	0.21	30.89	2.50	0.26	2.19	0.19	0.03
mean	31.53	2.58	0.20	29.40	2.52	0.27	1.94	0.18	0.04
CV %	2.46	6.34	12.92	4.51	4.37	11.14	16.44	10.00	15.25

6.4.5.2 Inter day precision

The interday precision was used as a measure of intermediate precision. This measure quantifies the degree of variation as given by day to day variations. Spiked urine samples were

extracted at three different days and the variation of the three concentrations was measured. The results of DX, DXM, and 3MM are tabulated in table 6.11, 6.12 and 6.13, respectively. Inter day precision was excellent for all compounds at all concentration levels. The C.Vs % were all less than 10, with the exception of DX at 0.25 ug/ml concentration level.

Table 6.11. Inter day precision of DX

Conc.			**DX**	
		day1	*day2*	*day3*
30 μg/ml		28.57578	31.65924	31.03217
	Mean	30.42		
	STD	1.63		
	C.V. %	5.36	**Accuracy %**	101.4
3 μg/ml		2.558278	2.825908	2.777621
	Mean	2.72		
	STD	0.14		
	C.V. %	5.24	**Accuracy %**	90.7
0.25 μg/ml		0.161314	0.207837	0.19135
	Mean	0.1868		
	STD	0.0236		
	C.V. %	12.6252	**Accuracy %**	74.8

Table 6.12 Inter day precision of DXM

Conc.		DXM		
		day1	*day2*	*day3*
30 µg/ml		28.65045	30.69062	30.95957
	Mean	30.10		
	STD	1.26		
	C.V. %	4.20	**Accuracy %**	100.3
3 µg/ml		2.417829	2.635447	2.498352
	Mean	2.52		
	STD	0.11		
	C.V. %	4.37	**Accuracy %**	84.0
0.25 µg/ml		0.227605	0.254519	0.231868
	Mean	0.24		
	STD	0.01		
	C.V. %	6.08	**Accuracy %**	96.0

Table 6.13 Inter day precision of 3MM

Conc.		3MM		
		day1	*day2*	*day3*
3 µg/ml		2.06536	2.03562	2.19011
	Mean	2.10		
	STD	0.08		
	C.V. %	3.91	**Accuracy %**	70.0
0.25 µg/ml		0.15137	0.15722	0.16945
	Mean	0.16		
	STD	0.01		
	C.V. %	5.79	**Accuracy %**	64.0
0.05 µg/ml		0.04305	0.03675	0.03707
	Mean	0.04		
	STD	0.00		
	C.V. %	9.10	**Accuracy %**	80.0

6.4.6 Limit of detection (LOD)

On the basis that the LOD represents the concentration where the peak height is three times above the baseline, this criteria was evident in the LOD for urine samples (fig. 6.9) which was found to be 30 ng/ml for all compounds.

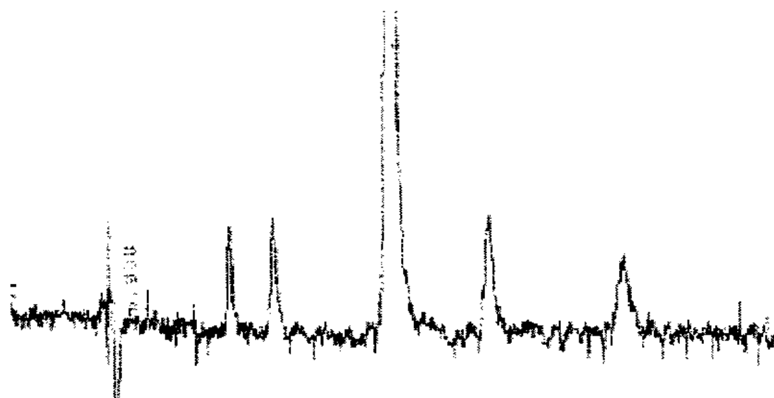


Figure 6.9. Chromatogram of all compounds showing the minimum of detection at 30 ng/ml

6.4.7 Limit of quantification (LOQ)

Following the description that the LOQ represents the lowest concentration of the standard curve, it was found that the LOQ was 50 ng/ml for 3MM, and 250 ng/ml for 3DX and DXM.

6.4.8 Conclusion

The sequence of procedures has therefore allowed for successful validation of the method and that it may be used effectively for the identification and quantification of DX, DXM, and 3MM in urine samples. The next phase was the application of the developed method on a group of human subjects.

6.5 Patient study

The Ethics Committee for human studies of the Potchefstroom University for CHE approved the clinical trial under registration number 03MO4. A total of 16 student and staff Caucasian male volunteers were recruited from the Potchefstroom University for CHE. All participants provided written informed consent, were to be non-smokers, and were Caucasian by declaration. Each volunteer was randomly assigned a number from #1 to #16, and were all confirmed to have adhered to rules set out in section 5.3, i.e. overnight fast, no breakfast and abstinence of and consumption of alcohol containing beverages or medicaments.

6.5.1 Medication history

Volunteer #1 reported in with minor symptoms of common coryza and had taken ibuprofen and acetyl-cysteine at 19:h00 a day prior. Volunteers #3; #5; and #9 had taken 150 mg aspirin during the trial and #6 was taking vitamin C (ascorbic acid) and vitamin B complex daily as a vitamin supplement. The rest of the volunteers had not taken any medication over the past three months.

6.5.2 Health status

Volunteers ranged from 19 to 55 with a mean of 31 years of age. The initial health screening performed by a registered nurse showed that all volunteers had a normal blood pressure and heart rate. The BMI for all volunteers was found to fall within the 15% of the normal BMI.

6.5.3 Concentrations of compounds and urine volume

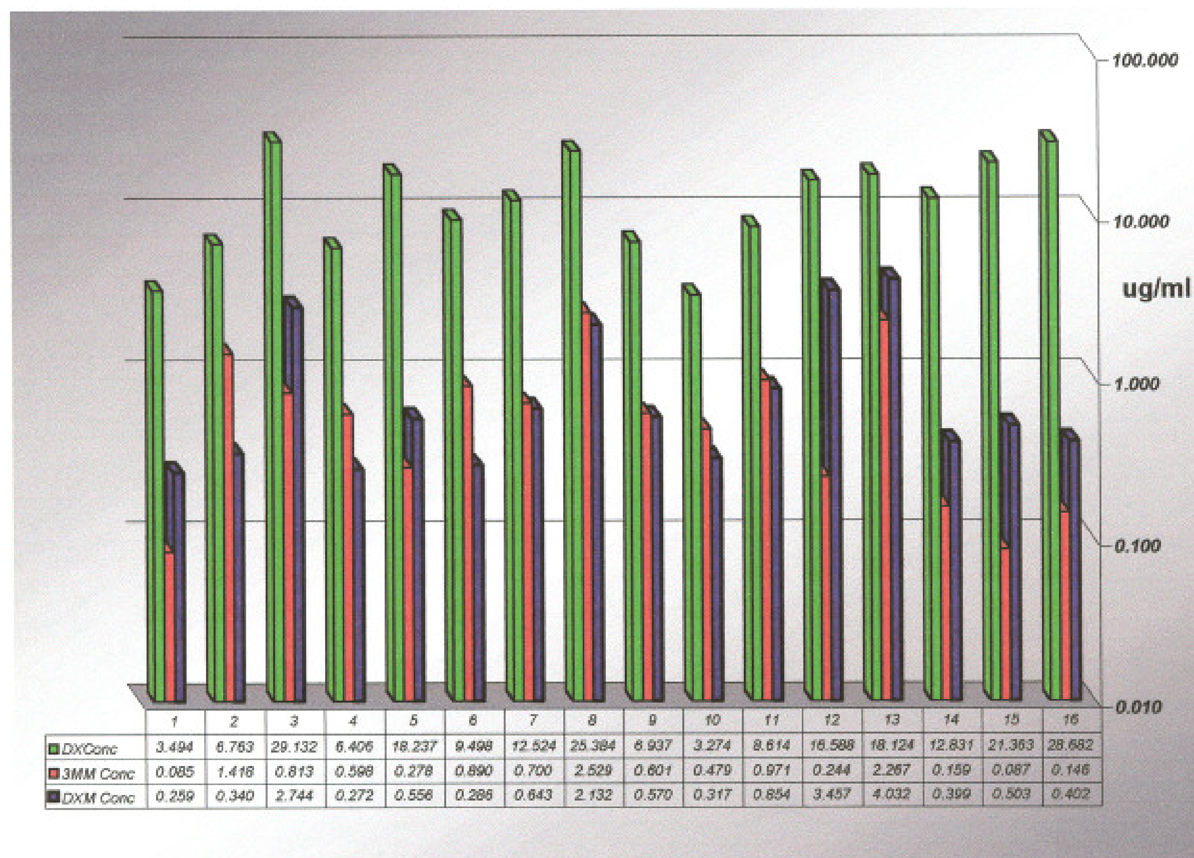
The concentration of all compounds were found within the expected range of the calibration curves (see table 6.14 for all concentrations found). Correcting the concentrations for the volume will give the amount of the analytes in a given sample. However, in this scenario it is unnecessary to do so, since the metabolic ratio can be determined using the concentrations directly. Moreover the concentrations also indicate whether the determination is within or outside the standard calibration curves.

The concentration of DX ranged from 3.27 to 29.1 μg/ml with the mean of 14 μg/ml. The concentrations of 3MM ranged from 0.085 to 2.53 μg/ml, with a mean of 0.77 μg/ml. For DXM, concentrations ranging from 0.25 μg/ml to 30 μg/ml were anticipated. However, DXM concentrations were found to range between 0.26 μg/ml to 4.03 μg/ml with a mean concentration of 1.11 μg/ml (Table 6.14).

Table 6.14.DXM and metabolites. Concentrations found and their respective metabolic ratios

Analyte concentration				Metabolic ratio	
Volunteer#	**DX (µg/ml)**	**3MM (µg/ml)**	**DXM (µg/ml)**	**CYP2D6**	**CYP3A4**
#1	3.494	0.085	0.259	0.074	3.064
#2	6.763	1.418	0.340	0.050	0.240
#3	29.132	0.813	2.744	0.094	3.377
#4	6.406	0.598	0.272	0.043	0.455
#5	18.237	0.278	0.556	0.031	2.003
#6	9.498	0.890	0.286	0.030	0.321
#7	12.524	0.700	0.643	0.051	0.918
#8	25.384	2.529	2.132	0.084	0.843
#9	6.937	0.601	0.570	0.082	0.948
#10	3.274	0.479	0.317	0.097	0.662
#11	8.614	0.971	0.854	0.099	0.880
#12	16.588	0.244	3.457	0.208	14.16
#13	18.124	2.267	4.032	0.222	1.779
#14	12.831	0.159	0.399	0.031	2.503
#15	21.363	0.087	0.503	0.024	5.791
#16	28.682	0.146	0.402	0.014	2.749

The results confirmed that the major metabolite of DXM is DX and in agreement with that illustrated in figure 6.10. Referring to fig. 6.10, the concentration of DX (in blue) dominated in each sample. The volume of urine sample collected ranged from 150 ml to 900 ml. A relationship between the total volume of the collected sample and the concentration of the compounds was established. As may be expected, it was noted that for the samples that had higher concentrations of all compounds also had lower volume of the total sample collected, and vice versa for low concentration. For example, volunteer #3 had highest concentrations of all compounds and the total volume of his urine was 150 ml (see fig. 6.10). Volunteer #1, on the other hand, had the lowest concentrations of all the compounds and his total urine sample amounted to 900 ml. However both #1 and #3 had similar metabolic ratios. These findings can be explained by the fact that, since each volunteer had the same amount of the probe drug, the concentration of the drug and its metabolites will depend on the total urine volume, since the quantity of the metabolite excreted is the same.



	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
DXConc	3.494	6.763	29.132	6.406	18.237	9.498	12.524	25.384	6.937	3.274	8.614	16.588	18.124	12.831	21.363	28.682
3MM Conc	0.085	1.416	0.813	0.598	0.278	0.890	0.700	2.529	0.601	0.479	0.971	0.244	2.267	0.159	0.087	0.146
DXM Conc	0.259	0.340	2.744	0.272	0.556	0.286	0.643	2.132	0.570	0.317	0.854	3.457	4.032	0.399	0.503	0.402

Figure 6.10. Concentrations of analytes obtained from the urine from healthy volunteers. Concentration of dextrorphan (in green), 3methoxy-morphinan (in red), and dextromethorphan (in purple) obtained from each volunteer. Please note that the scale is in multiples of ten.

6.5.4 Metabolic ratios

Metabolic ratios are tabulated on table 6.14. The metabolic ratios were smaller for CYP2D6 activity as compared to that of CYP3A4 activity. Metabolic ratios dictating the activity of CYP3A4 ranged from 0.2399 to 14.16, and 0.014 to 0.22 for CYP2D6 activity. The mean metabolic ratio for CYP3A4 and CYP2D6 activity was 2.54 and 0.08, respectively. From figure 6.11 volunteer #12 had a CYP3A4 metabolic ratio that seems to have characteristic of an outliner based on visual inspection.

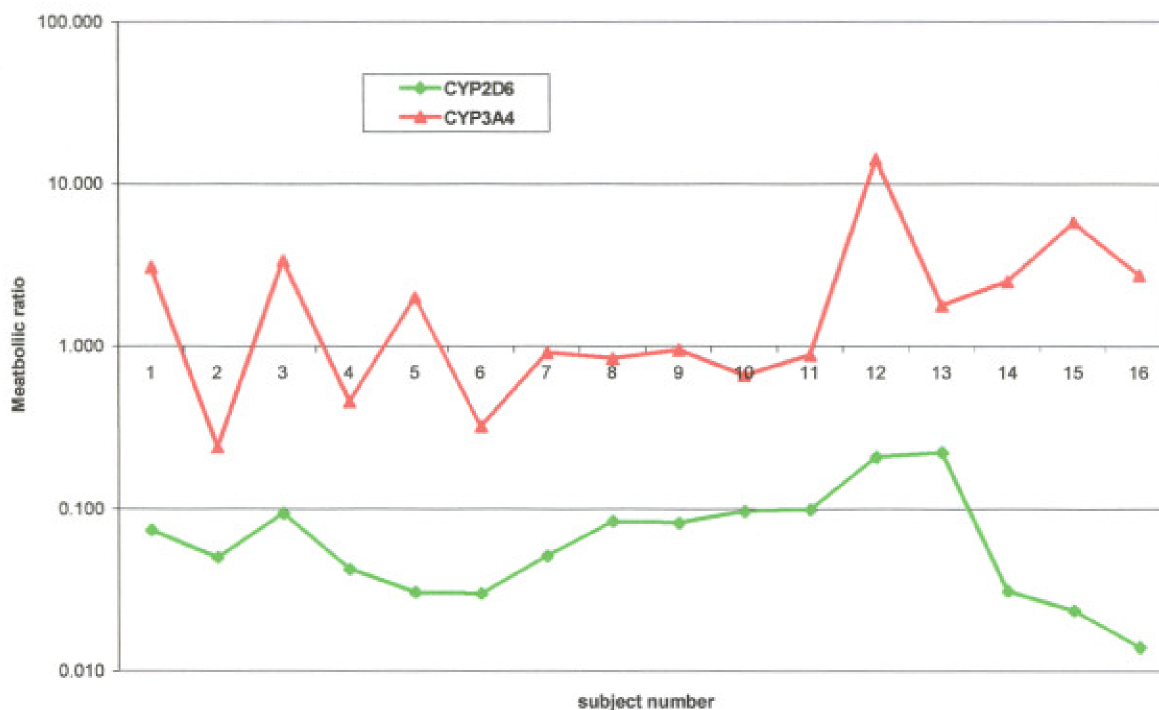


Figure 6.11. Metabolic ratios of CYP2D6 and CYP3A4 activity.

6.5.5 CYP2D6 and CYP3A4 metabolic activity

The classification of CYP2D6 activity was based on the criteria for Caucasian (i.e. CYP2D6 metabolic ratio greater than 0.3 equates to PM phenotype). Based on this criteria, none of the recruited 16 volunteers were classified with the PM phenotype. Since the number of volunteers was too small and the fact that a clear bimodal distribution of the CYP3A4 activity could not been established, it was not possible to discriminate between the PM and EMs for CYP3A4 activity.

An example of the chromatogram of CYP2D6 EM from the current study is presented in figure 6.12. This chromatogram illustrates even further, the fact that DX is the major metabolite, illustrated by the relatively large DX peak as compared to 3HM, 3MM, and DXM. For some chromatograms there were additional peaks like the one in figure 6.13 (the peak between 3MM and DXM). However none of these peaks interfered with either retention times or proper integration of the peaks of interest.

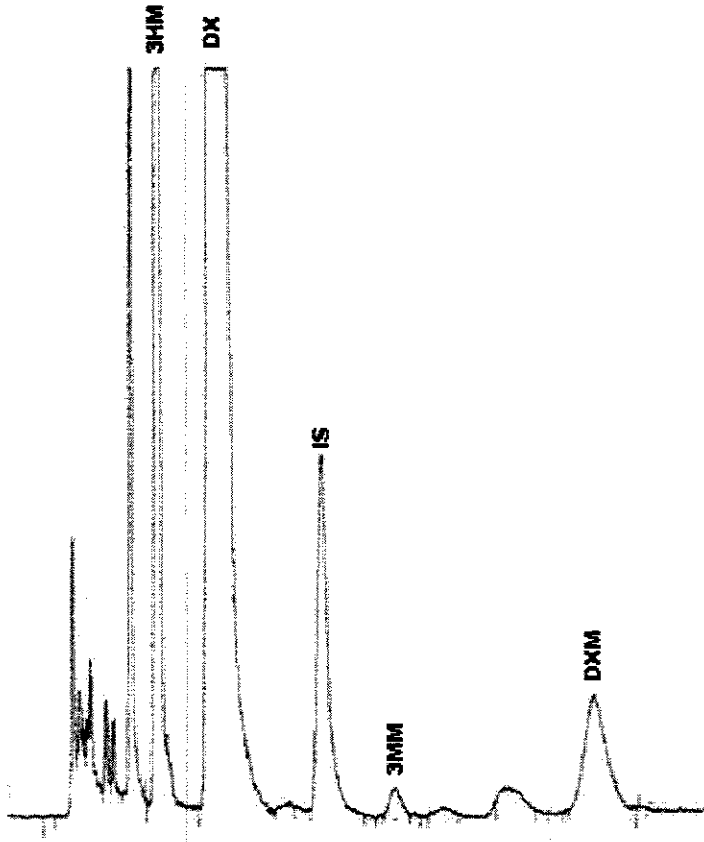


Figure 6.12. Chromatogram of CYP2D6 EM in the current study. Taken from volunteer #12.

Figure 6.13 to 6.15 represent CYP2D6 EM chromatograms from published methods that have been applied in the clinical screening of metabolic phenotypes. These methods relate to the current study in that they were performed on urine samples using fluorescent detection and solid-phase extractions.

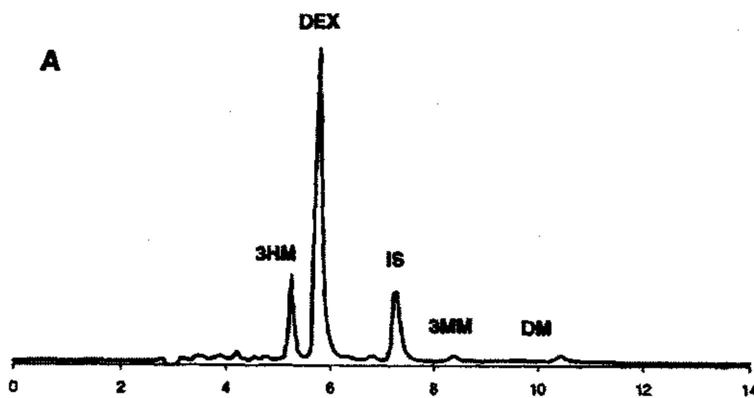


Figure 6.13. CYP2D6 EM chromatogram (Ducharme *et al.*, 1996). Excitation 228 and no cut off filter for emission. IS= levallorphan, DEX= dextrorphan, DM= dextromethorphan.

Figure 6.13 illustrates a chromatogram of an EM in a study involving 65 subjects. As in all above mentioned chromatograms (including the current study), this chromatogram shows a major DX peak and a minor DXM peak. Striking similarities of chromatograms in this regard emphasise the suitability of the current method in later application in future pharmacogenetic studies.

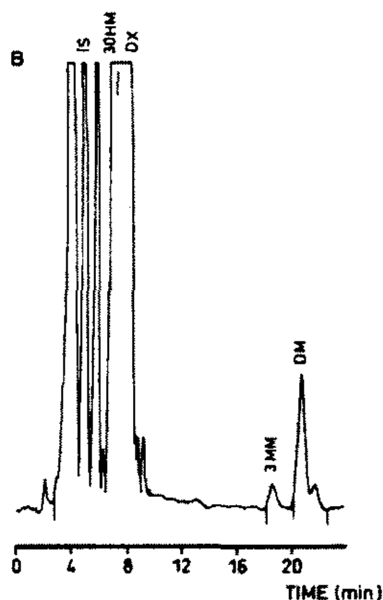


Figure 6.14. CYP2D6 EM chromatogram (Batroletti *et al.*, 1996). Excitation 200, emission 310. IS= pholcodine, 3OHM= 3 hydroxy-morphinan, DM= dextromethorphan.

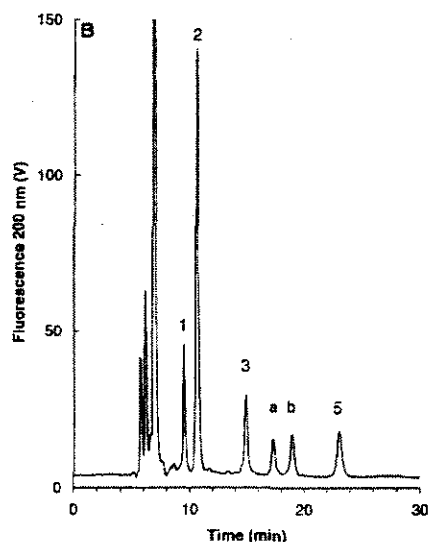


Figure 6.15. CYP2D6 EM chromatogram (Bendriss *et al.*, 1996). Excitation 200 and no cut off filter for emission. 1= 3-hydroxy-morphinan, 2= dextrorphan, 3= levallorphan, a/b= unidentified peaks, 5= dextromethorphan.

6.6 References

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Discussion

Chapter 7

7.1 Discussion

This study has established and validated an analytical method for determining the activities of CYP2D6 and CYP3A4 and, thereafter, undertook a small pilot study to establish the suitability of the developed HPLC method to determine the polymorphic expression of these two enzymes in the given population. The study also investigated DXM as a phenotypic probe.

7.1.1 HPLC method

The determination of individual oxidative phenotype has been performed by various methods, including GC, HPLC and even TLC methods (Guttendorf *et al.,* 1988). In the HPLC used in this study, and unlike most published methods, the buffer was not included in the mobile phase. This is advantageous especially in the event of a power failure that can lead to the clogging of the HPLC system due to the precipitation of the buffer in the system. The chosen HPLC method yielded excellent calibration curves for all compounds at their respective concentration ranges (fig. 6.4).

Using a sample volume of between 0.5 ml to 5 ml urine, most published methods have reported an LOQ of 2 ng/ml, 0.1 μg/ml 0.02 μg/ml for DXM, 3MM and DX respectively. In this study, a LOQ of 0.25 μg/ml was achieved for DX and DXM and 25ng/ml for 3MM, from a 250 μl urine sample using a 50 μl sample injection. The limit of detection was 30 ng/ml for all compounds. The Method separated DXM and its metabolites were well separated with good peaks over a reasonable run time of 25 minutes, as shown by the chromatogram of an extracted urine sample in fig. 6.6.

7.1.1.1 Adopted extraction method

Extraction of the compounds of interest from urine was necessary for the assay of these compounds. Comparing liquid-liquid extraction to solid phase extraction, it was demonstrated that solid phase extraction was superior. Generally, liquid-liquid extraction yielded low

recoveries of DX, 3MM, and DXM (Table 6.5-6.7). Inter-day precision of liquid-liquid extractions (C.V.% of Table 6.5-6.6) were also generally higher than those of solid phase extraction. This could be explained by the tedious techniques involved with the separation of large organic and aqueous phases in liquid extractions, as compared to solid phase extraction.

The literature reports recoveries of between 57 to 90% for DXM and DX, and 60 to 80% for 3MM (Park *et al.*, 1984; Chen *et al.*, 1990; East *et al.*, 1985; Jacqz-Aigrain *et al.*, 1989). In this study we report appreciable recoveries of 97%, 64%, 93% for DX, 3MM and DXM respectively (see Table 6.8), which compares favourably with the earlier data. Excellent recovery of levallorphan as the IS was achieved.

An unknown peak in figure 6.12. was noted in chromatograms of volunteers, after an oral DXM dose. This peak did not appear in all chromatograms from volunteers, was of variable size within volunteers, but was absent in spiked urine standards (fig. 6.5-6.6) and blanks. Bendriss and colleagues also noted this peak, although instead of one peak, two were observed (see fig. 6.16). Bendriss and colleagues suggested that this peak could be one of the 15 metabolites formed from DXM metabolism (Koppel *et al.,* 1986). The fact that Bendriss used fluorescence detection at an excitation of 200 nm and no cut off filter for emission, while in this method the excitation of 280 nm and emission of 310 nm was used, could explain why only one instead of two peaks were noted in this study. This anomaly does not appear to influence the accuracy and validity of the data.

7.1.2 Clinical study

7.1.2.1 DXM as a dual probe

DB has been extensively used in the past for CYP2D6 phenotyping. However, the use of this agent is becoming restricted due to its potential side effects. In addition, this agent is not available in South Africa for human use. DXM, a codeine analogue is a centrally acting antitussive agent chosen as a metabolic probe for this study. This study, like earlier studies, has demonstrated its usefulness in performing CYP2D6 and CYP3A4 phenotyping. The single 30 mg oral dose was well tolerated by all subjects. None of the volunteers reported any side effects associated with this single dose, although it may be argued that this is because none of the volunteers presented with the PM phenotype. Nevertheless, none of the studies of a similar nature reported PM presenting with any life threatening side effects from the single 30 mg dose (Di Marco *et al.*, 2002).

7.1.2.2 Metabolite concentrations

A schematic presentation of DXM metabolism is presented in fig. 4.2. In this study it was found that DXM *O*-demethylation to DX is the major metabolic pathway of DXM metabolism. This is illustrated by the high DX concentrations in all deconjugated urine samples from volunteers (fig. 6.10.). The findings also suggest that DXM *N*-demethylation to 3MM proceeds at a slower rate as demonstrated by the low 3MM concentrations in all collected urine samples. These findings concur with previously published studies on DXM pharmacokinetics in man (East & Dye, 1985; Park *et al.*, 1984; Chen *et al.*, 1990; Jones *et al.*, 1996a).

Interestingly, the DXM concentration range was unexpectedly lower than that suggested by a preliminary study on three volunteers, which suggested that the concentration of DXM would be found at a minimal concentration of 0.25 μg/ml and a maximum 25 μg/ml. In the final clinical trial a concentration range for DXM averaged 1.11 μg/ml, which strongly suggests that a better suited concentration range for DXM would be 0.1 μg/ml to 10 μg/ml for a 30 mg dose followed by an 8 hour sample collection. Higher DXM concentrations may be expected from both CYP2D6 and CYP3A4 PM. Based on the latter pilot study, it is not believed that these concentrations would exceed 10 μg/ml.

The absence of the PM phenotype in this small study precluded the possibility of observing the metabolite profiles characteristic of PM. A study conducted by Jones and colleagues (1996b) demonstrated that urine samples from PMs have relatively higher DXM concentrations, indicating the inability of the subject to effectively metabolise DXM. Since all volunteers in the current study were phenotyped with the EM phenotype, this was not evident in any of the samples.

7.1.2.3 Metabolic ratios

The literature reports an average metabolic ratio from CYP2D6 EM over a wide range (see table 7.1). The range of metabolic ratios for all CYP2D6 and CYP3A4 phenotypes is even wider, ranging from 0.0002 to 196.81. For the purpose of this discussion, metabolic ratios of only the EM phenotype will be discussed.

Table 7.1. Average metabolic ratios of CYP2D6 EM from earlier studies compared to that of the current study.

Author	number of subjects	Average metabolic ratio	
		CYP2D6	CYP3A4
Jones *et al.* 1996a	*n*=8	0.03	7.0
Jones *et al.* 1996b	*n*=135	0.008	7.32
Min *et al.* 1999‡	*n*=11	0.03	5.40
Ducharme *et al.* 1996	*n*=61	0.10	0.82
Hägg *et al.* 2001	*n*=285	0.08	ND
Schmid *et al.* 1985	*n*=127	0.013	ND
Chou *et al.* 2003	*n*=236	0.004	ND
Tamminga *et al.* 1999	*n*=3029	0.014	ND
Markowitz, *et al.* 2000	*n*=7	0.006	#
Mgwabi 2003 (this study)	*n*=16	0.08	1.36

ND = no data was available, # a different probe was used to assay the CYP3A4 activity, ‡ = Based on a 24hour urine collection, n = number of subjects.

7.1.2.3.1 CYP2D6 metabolic ratios

From table 7.1 it can be seen that CYP2D6 metabolic ratios range from 0.004 to 0.1. It can also be noted that generally, with the exception of Markowitz and colleagues (2000), studies with fewer subjects (***n*** <20) had higher metabolic ratios. The same applies to the current study where the average PM metabolic ratio was found to be 0.08 (see table 7.1). Studies with a large number of subjects (i.e. ***n*** >120) had low metabolic ratios. This could be attributed to a better population representation from larger subject numbers.

The average metabolic ratio of 0.08 is comparable to the findings of Hägg and co-workers (2001), where an average of 0.08 was found based on 285 subjects phenotyped as EMs. A metabolic ratio of 0.08 is also within the published metabolic ratio range published by various authors (see table 7.1). The current study, however, suggests a right shift of the distribution of the metabolic ratios, since most metabolic ratios from literature are far less than 0.08. This right shift is towards the PMs metabolic ratios, taking into account that the antimode is 0.3. In essence, this suggest that although all the volunteers were found to be EMs, this group of volunteers would metabolise CYP2D6 substrates less effectively than EMs reported in other studies.

Low CYP2D6 metabolic ratios as compared to those of CYP3A4 (table 7.1), confirms that DX is the predominant metabolite from DXM metabolism and that the 2D6 is the major isoform involved in its metabolism. The possibility of one metabolic pathway being increased so as to compensate for another ineffective metabolic pathway does exist. In this case, the possibility of induction of the CYP3A4 DXM metabolism to compensate for the ineffective CYP2D6 pathway in PM is questioned. It is not known whether CYP3A4 metabolic ratios could be much lower in PMs. This is a subject of future interest, although findings from Ducharme (1996) dismiss this theory.

7.1.2.3.2 CYP3A4 metabolic ratios

Clearly, from the studies indicated in table 7.1, published phenotyping data for CYP3A4 activity is limited. However from the available studies, the average metabolic ratio ranges from 0.8 to 7.32. A definite DXM metabolic ratio antimode has not yet been established for the CYP3A4 activity classification. It is for this reason that the CYP3A4 phenotype evident in this study can not be stated. Here the average metabolic ratio was found to be 1.36, well within the range of metabolic ratios from previously published studies (table 7.1).

Upon visual inspection, volunteer #12 presented with a CYP3A4 ratio (14.2) characteristics of PM outlier (see figure 6.12). An earlier study found that out of eleven participants three had metabolic ratios of 16.3, 12.1 and 11.1 (Jones *et al.,* 1996a). In addition, another study had one subject presenting with a metabolic ratio of 196.18, distinct characteristic of an outlier (Ducharme *et al.,* 1996). This information suggests that the metabolic ratio of volunteer #12 may not necessarily be an outlier.

7.1.2.4 Allele associated with EM

All 16 of the volunteers in this study were phenotyped as EM. These results from such a small number of subjects are not unusual considering the reported 5% PM frequency. More than 20 inactivating mutations in the CYP2D6 genes have been reported. In Caucasians, the most common inactivating allele are the *CYP2D6*4* and *CYP2D6*5*. Earlier it has been mentioned (chapter 5) that the CYP2D6 EM phenotype can be brought about by individuals with homozygous wild type (i.e. *CYP2D6*1 / CYP2D6*1*) or heterozygous wild type with a mutated allele (i.e. *CYP2D6*1 / mutated*).

In a genotype-phenotype study, EM metabolic ratios ranging from 0.0009 to 0.001 were found to be associated with individuals having a homozygous *CYP2D6*1* allele (Schadel *et al.,* 1995). In the same study, one subject was genotyped as having a heterozygous *CYP2D6*1 / CYP2D6*4*, with a metabolic ratio of 0.003.

Another study involving 3 959 volunteers showed that the individuals who were homozygous with the wild type allele had metabolic ratios ranging from 0.0009 to 0.2 (Tamminga *et al.,* 2001). Metabolic ratios ranging from 0.002 to 0.25 were associated with a heterozygous *CYP2D6*1 / CYP2D6*4,* while individuals with the heterozygous *CYP2D6*1 / CYP2D6*3* had metabolic ratios ranging from 0.003 to 0.06. As for the heterozygous *CYP2D6*1 / CYP2D6*6* genotype, metabolic ratios ranged from 0.007 to 0.012. One subject was genotyped as *CYP2D6*1 / CYP2D6*7,* and had the metabolic ratio of 0.003.

In the above mentioned study (Tamminga *et al.,* 2001), *CYP2D6*2, CYP2D6*8, CYP2D6*9, CYP2D6*10, CYP2D6*11, CYP2D6*12, CYP2D6*13, CYP2D6*14, CYP2D6*15* and *CYP2D6*16* allele screening was excluded, whereas the study conducted by McElroy and colleagues (2000), these alleles were screened. In the latter study involving 481 EM, it was found that 29% were genotyped as *CYP2D6*1 / CYP2D6*2,* with metabolic ratios from 0.0003 to 0.1. Another 16% of the population were homozygous *CYP2D6*1 / CYP2D6*1* with metabolic ratios of 0.0003 to 0.1. *CYP2D6*1 / CYP2D6*4* was found with metabolic ratio of from 0.001 to 0.11, while *CYP2D6*2 / CYP2D6*4* was found with metabolic ratios of 0.0011 to 0.3.

Clearly, a genotype-phenotype relationship with at-least DXM seems to be poor. For this reason, only a genotyping study can state with absolute certainty the genotype of the EMs in the current study. It can, however, be noted that the metabolic ratio of 0.08 was not found in individuals with the homozygous wild type allele. Also, individuals homozygous with an inactive allele had a metabolic ratio greater than 0.3, hence PMs. Therefore, it is a reasonable hypotheses that, in the current study, all the individuals were heterozygous *CYP2D6*1 /* mutated allele.

The mutated allele could be any of the inactive alleles in the bottom panel of fig 4.3. It has been mentioned that *CYP2D6*4* and *CYP2D6*5* occur at a high frequency in the Caucasian population (Jurima-Romet *et al.*, 1997). The *CYP2D6*4* was found at the highest frequency in a genotyping study on Dutch Caucasians (Tamminga *et al.,* 2001). Although the protocol of the current study did not exclusively identify Dutch Caucasians per se, considering the two above mentioned statements and the fact that this current study was conducted on South African Caucasians who may be descendants of the Dutch Caucasians, it is plausible that all the volunteers from the current study had a *CYP2D6*1 / CYP2D6*4* genotype.

Unfortunately, very little data (both in number of studies and number of subjects used) are available for an extensive discussion on the CYP3A4 genotype. In addition, there is conflicting evidence supporting bimodal distribution of this enzyme (Haehmer *et al.,* 1996; Thummel & Wilkinson, 1998).

7.2 Future application

The current pilot study is a foundation for much larger genetic polymorphism studies in the South African population. The following brief discussion will illustrate the relevance of this ideal, showing that genetic polymorphism studies not only is relevant, but also extremely important in the South Africa and African context.

A race, ethnic group, or population can be defined in terms of geographic distribution, anthropological features, linguistics or more precisely, in terms of common gene distribution (Gaedigk, 2000). For the purpose of this discussion, the word "Black" will refer to dark skinned race of African origin. This term will exclude mixed races, which may come close to this definition. Further, ethnic and racial will be used interchangeably.

Racial genetic polymorphism dates as far back as the 1920's when Marshall and colleagues (1918) observed that the black population group, which was then referred to as the Negroid race, was less susceptible to blistering from an exposure to mustard gas as compared to the Caucasian counterpart (Marshall *et al.,* 1918). Increased susceptibility of Black soldiers to primaquine haemolysis was also observed during Second World War, with deficient glucose-6-phosphate dehydrogenase found to be the culprit.

Polymorphism in drug metabolising enzymes has been demonstrated in many other populations, with differences between Caucasians and Orientals having been the most extensively studied.

What makes polymorphism of metabolising enzymes important, is that the frequency of occurrence of mutated alleles differs from one race to another. This implies that different races will exhibit different profiles of PMs and EMs. Table 7.2 tabulates some of studies that have been conducted on different ethnic group and the subsequent distribution of PMs.

Table 7.2 Population distribution of PMs. Frequency of distribution of PMs as compared between African populations and Non African population groups using different probe drugs.

Population	Prevalence in % of PMs (reference)			
	Probe drugs			
	Debrisoquine	**Metoprolol**	**Sparteine**	**Dextromethorphan**
African				
Black Americans	N.D.	N.D.	N.D.	6.1 (Marinac 1995)
Ethiopian	1.7 (Aklillul, 1996)	ND	ND	ND
Gabonese	N.D.	N.D.	ND	1.9 (Panserat, 1999)
Ghananians (2 studies)				
a)	7.1 (Woolhouse, 1984)	N.D.	0.0 (Woolhouse, 1984)	
b)	5 (Woolhouse, 1979)	N.D.	ND	ND
Nigerians (2 studies)				
a)	0.0 (Iyun, 1986)	0.0 (Iyun, 1986)	ND	ND
b)	8.1 (Mbanefo, 1980)	N.D.	ND	ND
San-Bushmen (SA)	18.8 (Sommers, 1988)	4.1 (Sommers, 1989)	ND	ND
Tanzanians	N.D.	N.D.	0.5 (Bathum, 1999)	N.D.
Tunisians				
a)Berbers	3.1 (Attilah, 2000)	N.D.	N.D.	ND
b)Numids	2.3 (Attilah, 2000)	N.D.	ND	ND
Venda (SA)	4 (Sommers, 1989)	7.4 (Sommers, 1989)	ND.	ND
Zimbabwean	1.9 (Masimirembwa, 1996)	5.3 (Masimirembwa, 1996)		
Non African				
Arabs				
a)	2.4 (Attilah, 2000)	ND	ND	ND
b)	1.0 (Islam, 1980)	ND	ND	ND
Caucasians	5-10	5-10	5-10	5-10
Orientals	1-5	1-5	1-5	1-5

ND means data was not available at a time of printing. Black Americans have been grouped as Africans, but their genetic make up is questionable since it may carry ancestral Caucasian gene through inter breeding. References for Orientals and Caucasians are too numerous to be included here. SA refers to groups found in South Africa.

7.2.1 Populations and genetic polymorphism

Alleles differ from the normal (wild type) by mutation such as exchange of amino acids, or gene duplication or even a complete gene deletion (see fig 3.3). However, few studies have been conducted on Africans and even fewer in Southern Africa. From Table 7.2 it can be seen that there is a large variation in frequency of PMs which ranges from 0 – 18.8 %, with the Nigerians having the lowest (0.02%) and the San presenting with the highest frequency (19%).

Observations from the San study showed a wide range of DB metabolic ratios ranging from 0 to 4000, which divided this population into four metabolising groups, nine out of 96 were classified as UEMs. Sommers and colleagues (1988) interpreted this as an indication of the presence of three or more alleles in the San population. Tri-modal DB distribution was also shown in Ghanaians (Woolhouse *et al.*, 1997). In contrast to these findings, a study conducted on the Nigerians showed a single mode of distribution, using S/R spartein ratio; although with metoprolol M/HM ratio, the distribution was bimodal (Lennard *et al.*, 1989).

7.2.2 Population distribution of *CYP2D6* allelic variants.

Caucasians have *CYP2D6*4* and *CYP2D6*3* alleles which accounts for up to 75% and 3% of their PM phenotype, respectively (Jurima-Romet *et al.*, 1997). The *CYP2D6*4* allele is almost absent in Orientals (Bertilsson *et al.*, 2002), however a *CYP2D6*10* variant is predominantly found in Orientals and accounts for up to 50 % of their PM phenotype (Dandara *et al.*, 2001). The *CYP2D6*10* allele has not been found anywhere else with such a high prevalence. When it was initially discovered it was dubbed the Chinese allele. Masimirembwa was the first to identify the *CYP2D6*17* which is found almost exclusively in the Black population and accounts for up to 34% of PM phenotype (Masimirembwa *et al.*, 1996).

7.2.3 Population distribution of *CYP3A4* allelic variants.

An American study on the polymorphic *CYP3A4*1B* (alias nifedipine specific element) across five population groups has been documented (Ball *et al.*, 1999). The population distribution of this allele was as follows: Black Americans had the highest frequency of this allele that accounted for 54.6% of the test population. Hispanic and Caucasians Americans had the lower prevalence of 3.6% and 9.3%, respectively. The most interesting finding was the absence of this allele in both Japanese and Chinese Americans, both populations presenting with a frequency of 0.0 %.

Sata and colleagues (2000), confirmed these findings across Caucasians, Black and Chinese subjects. Black subjects had a *CYP3A4*1B* prevalence of 66.7%, while Caucasians had a 4.2% prevalence. This allele was absent in Chinese subjects. In the same study, the *CYP3A4*2* was

found at a frequency of 2.7% in the Caucasian subjects while neither Black nor Chinese subjects presented with this variant.

7.2.4 African specific alleles

It is uncommon to find a population with a frequency of 3% or higher for UEMs. However this is the case with Ethiopians (see Table. 7.2). Genotyping has revealed that this is due to a high prevalence of duplicated *CYP2D6*2* allele (Aklillu *et al.*, 1996). PMs phenotype can be brought about by any of the combinations of defective genes including combinations with the wild type (CYP2D6*1). With the few studies on Africans, it seems that the *CYP2D6*17* accounts for PMs phenotype in Africans (Woolhouse *et al.*, 1984). This means that for the PMs phenotype, in most Africans this will be due to either the homozygous *CYP2D6*17* allele or heterozygous *CYP2D6*17* and *CYP2D6*1* allele.

With the above in mind, conclusive statements can not be made. However, the varying prevalence of allele distribution from one population to another is noted. It is also apparent that Black populations present with a genotype that is unique. Clearly, more genotyping studies in all parts of Africa, including South Africa are urgently needed.

7.3 Clinical relevance

Currently there is no documentation on the therapeutic or toxicological response of Africans to CNS drugs, including antidepressants, anti-epileptic, anti-psychotic, but also various cardiovascular drugs, that are substrates of CYP2D6 and/ or CYP3A4 enzymes. It is speculative at best to state that observations of therapeutic or toxicological responses in one population will equally apply to a Black population. Indeed, a safe assumption can be made that a given genotype or phenotype status will simulate analogous pharmacokinetic observations.

The term "recommended dose" is used with the assumption that the drug will produce the same effects in all patients. Indeed, it is generally known that this is not always the case. The clinical relevance of polymorphism of a drug metabolising enzyme has distinct implications and depends on a number of factors including:

- Whether the parent drug, metabolite(s) or both are metabolised by the enzyme
- Whether the parent compound, metabolite(s) or both are pharmacologically active
- The potency of the active species
- The therapeutic index of the drug.

- The overall contribution of the enzyme in the clearance drug.

Moreover, the overall pathway for total drug elimination also needs serious consideration bearing in mind that some substrates have alternative pathways should the enzyme reach some point of saturation.

Drug $t_{1/2}$ is prolonged in PMs. This implies that the PMs will require only a fraction of the "recommended dose" to achieve the same steady state level as the EMs. The consequences of drug accumulation in PMs may range from harmless adverse effects, such as headaches, to serious fatal reactions.

UEMs will not attain any therapeutic effect because the drug is metabolised at a very fast rate, so that it is unable to reach the minimum therapeutic plasma level. These individuals require very high, and sometimes impractical and costly doses, to attain the same therapeutic plasma levels as "normal individuals".

Drugs dependant on CYP and also with high toxicity or narrow therapeutic index, such as the tricyclic antidepressants (TCAs), selective serotonin reuptake inhibitors (SSRIs) and antipsychotics (APs), have the highest risk of adverse effects and are the group that require urgent attention.

7.3.1 Antidepressant and antipsychotic metabolism by CYP3A4 and/or CYP2D6

Pharmacokinetic studies have been conducted with a number of antidepressants and antipsychotics with regard to their metabolism and disposition within different metabolic phenotypes. These studies, in general, show clinically significant differences in the maximum serum concentration (C_{max}), total amount of drug (AUC), clearance (Cl) and halt-life ($t_{1/2}$) of these drugs (Masimirembwa & Hasler, 1997).

Table 7.3 reviews data on antidepressants and neuroleptic agents, with regard to their metabolism by CYP2D6 and CYP3A4 enzymes. Implications for few-selected agent within relevant phenotypes will also be covered.

Table 7.3. Antidepressant and antipsychotic drugs subjected to polymorphic CYP3A4 and/or CYP2D6 metabolism

	CYP2D6	**CYP3A4**
TCA		
Amitriptyline	YES	YES
Notriptyline	YES	
Imipramine	YES	YES
Desipramine	YES	
Clomipramine	YES	YES
Maprolitine	YES	
Mianserin	YES	
Nefazodone		YES
SSRI		
Fluoxetine	YES (*N*-demethylation)[a]	
Norfluoxetine		YES
Citalopram		YES (*N*-demethylation)[a]
Desmethylcitaprolam	YES (*N*-demethylation)[a]	
Fluvoxamine	YES (*O*-demethylation)[a]	
Paroxetine	YES (oxidative cleavage)[a]	
Sertaline		YES (*N*-demethylation)[a]
Venlafaxine	YES	YES
Antipsychotic		
Clozapine	YES[b]	YES[b]
Haloperidol	YES(*N*-dealkylation)[b]	YES (oxidation of a reduced metabolite)[c]
Perphenazine	YES (dealkylation)[f]	YES (dealkylation)[f]
Risperidone	YES[e]	YES[e]
Thioridazine	YES[b]	
Zuclopenthixol	YES[e]	

Adapted from Steimer and colleagues (2001). a= (Hiemke & Härtter 2000). c= (Fang *et al.*, 2001). d= (Koymans *et al.*, 1992). e (Fang *et al.*, 1999). f= (Olesen, & Linnet, 2000)

7.3.1.1 Tricyclic Antidepressants (TCA)

Most clinically employed antidepressants are substrates of the polymorphic CYP2D6 and CYP3A4 enzymes (see Table 4.2). TCAs have a narrow therapeutic index, with adverse effects such as tachycardia, dry mouth, constipation, cardio-toxicity and fatigue.

7.3.1.2 Amitriptyline

Amitriptyline undergoes N-demethylation and E&Z-10 hydroxylation as its major metabolic pathways. Nortriptyline is the product of amitriptyline N-demethylation which is pharmacologically active (Mellström *et al.*, 1981; Coutts *et al.*, 1996). Using varying techniques studies show that N-demethylation is catalysed by CYP2B6, CYP2C8, CYP2C9, CYP1A2, CYP 2D6 with an important contribution of CYP2C19 and CYP3A4 (Ghahramani *et al.*, 1997; Venkatakrishnan *et al.*, 1998; Venkatakrishnan *et al.*, 2001). E- and Z- hydroxy-amitriptyline formation is believed to be mediated CYP2D6, CYP2B6 and CYP3A4 with minor contribution of other CYP isoenzymes (Masubuchi *et al.*, 1996, Venkatakrishnan *et al.* 2001).

Shimoda and colleagues (2002) carried out a study on Japanese psychiatric patients with the aim of investigating the *CYP2D6* and *CYP2C19* genotype effects on steady state concentration of amitriptyline and its metabolites. Patients carrying two mutated *CYP2D6* alleles showed significantly higher nortriptyline/E-nortriptyline ratios as compared to patients with a homozygous *CYP2D6*1* genotype. Meanwhile, two mutated *CYP2C19* alleles were associated with higher amitriptyline/E&Z-hydroxy-triptyline ratios than with homozygous *CYP2C19* genotype. The above mentioned study was based on therapeutic concentrations of amitriptyline, and there is a possibility of enzyme saturation whereby enzymes like CYP3A4 and CYP2D6 may play an important role at toxic plasma concentrations (Venkatakrishnan *et al.*, 2001).

7.3.1.3 Nortriptyline

Nortriptyline is demethylated product of imipramine. As early as in 1960s, nortriptyline was shown to have variation in steady state plasma concentration levels as high as 30 fold. Nortriptyline is metabolised by CYP2D6 into E and Z-10-hydroxy nortriptyline, whereby the E isomer is half as potent as nortriptyline, and characterised by fewer side effects (Mellström *et al.*, 1981). A pharmacokinetic study of nortriptyline conducted on Chinese subjects showed that patients with homozygous *CYP2D6*10* genotype demonstrated a doubling in $t_{1/2}$, AUC and half plasma Cl as compared to homozygous *CYP2D6*1* (Yue *et al.*, 1998). Subjects heterozygous with *CYP2D6*10* and *CYP2D6*1* genotype had shorter $t_{1/2}$, higher oral Cl as compared to homozygous *CYP2D6*10* subjects. In a relatively recent study on nortriptyline by Dalén and colleagues (1998), patients with homozygous *CYP2D6*4* (has zero functionality) had the highest AUC, while subjects with 13 duplicated *CYP2D6*2* copies had the lowest AUC. The

opposite was true for AUC of 10-hydroxy-nortriptyline. Clearly the *CYP2D6*10* and *CYP2D6*4* allele are associated with slow metabolism of nortriptyline. It is likely that other alleles associated with a PM phenotype might elicit analogous drug pharmacokinetics.

7.3.1.4 Serotonin selective reuptake inhibitors (SSRI)

With regard to therapeutic efficacy, SSRI are regarded as equipotent to TCA, but because of their selective inhibition they are almost devoid of life threatening side effects (Potter & Hollister, 1996b). As indicated in table 4.2. SSRI and some of their metabolites are metabolised by the polymorphic CYP3A4 and CYP2D6 enzymes.

7.3.1.5 Fluoxetine

Fluoxetine was the first SSRI to reach general world-wide clinical use (Potter & Hollister, 1996). It exists as a racemic mixture of the S-enatiomer and the R-enatiomer, whereby the S-enatiomer is approximately 1.5 times more potent an inhibitor of serotonin reuptake than the R-enatiomer (Gram, 1994). Norfluoxetine is the pharmacological active metabolite of fluoxetine whose S-enatiomer has approximately 20 times more activity than the R-enatiomer in blocking serotonin reuptake (Fuller *et al.*, 1992). Normally, the steady state concentration of the racemic norfluoxetine exceeds the concentration of racemic fluoxetine (Hiemke & Hartter, 2000).

Fluoxetine undergoes *N*-demethylation to form norfluoxetine. The *N*-demethylation is catalysed by the CYP2D6 and CYP2C9. The CYP2D6 and CYP2C9 catalyse the formation of R-norfluoxetine from R-fluoxetine, while S-norfluoxetine formation from S-fluoxetine is catalysed by CYP2D6 (Ring *et al.*, 2001). Although fluoxetine is claimed to have fewer side effects, extrapyramidal symptoms have been reported. A plausible hypothesis forwarded by Hiemke and & Hartter, 2000) suggests that drug accumulation and subsequent loss of specificity as the cause of adverse effect with patients carrying a CYP2D6 PM phenotype.

Other clinically important drugs to be considered are the cardiovascular agents such as the ß-adrenoceptor antagonist and antiarrhythmic agent e.g. metoprolol and flecainamide, respectively.

7.3.2 ß-adrenoceptor antagonists

ß-adrenoceptor antagonists used in cardiovascular medicine invariably have a high affinity for β receptors. However, this selectivity is lost when present in high concentrations, as will be the case to PMs. This results in adverse reactions such as broncho-constriction. Examples of ß-adrenoceptor antagonists metabolised by the polymorphic CYP2D6 and or CYP3A4 iso-enzymes are propranolol, timolol, metoprolol, bufuralol, alprenolol, and pindolol.

While ß- adrenoceptor antagonists require receptor specific actions, their receptor specificity is also governed by their stereo selectivity. Metoprolol for example, is administered in a racemate form. Elimination of R-metoprolol appears to be rapid in the EMs, while a faster clearance of the S-enantiomer is evident in PMs (Lennard *et al.,* 1989). Consequently, there are significant differences in enantiomer disposition between different phenotypes. This translates into different therapeutic effects between PMs and EMs.

7.3.3 Antiarrhythmic agents

A relationship exists between suppression of arrhythmia and antiarrhythmic drug serum concentration, such that the drug dosage should be such that it achieves and maintains the dose of the steady state concentration with suppression of arryhthmias (Eichelbaum & Gross, 1990). However, antiarrhythmic agents elicit proarrhythmic effects when plasma concentration are high. Antiarrythmic agents such as propafenone, encainide, flecainide, perhexiline, spartein, *N*-propylamajmaline and quinidine are all subject to CYP2D6 metabolism (Poolsup *et al.*, 2000)

The Class 1C antiarrhythmic agent flecainide has been shown to achieve almost 50% less C_{max} concentration with PMs as compared to EMs (Eichelbaum & Gross, 1990). Class 1 antiarrhythmic agent also achieved higher steady concentrations in PMs that coincided with CNS side effects in 67% of PMs as opposed to 14% of EMs (Siddoway *et al.* 1987).

7.3.4 Pro drugs

Drugs that require metabolism to their active form prior to exerting their pharmacological effect (pro drugs) also need to be seriously considered in the PMs. Codeine for example, will fail to produce analgesic effect in CYP2D6 PM because there will be a lack of contribution from the morphine metabolite. Conversely, UEMs will be highly prone to the respiratory, psychomotor and pupillary adverse effects of codeine. Interestingly, this could also imply that UEM genotype may be a factor that predisposes certain individuals to codeine addiction, the reason being that giving codeine to UEMs produce many of the physiological effects of morphine

7.3.5 Polymorphism and disease predisposition

It has been proposed that the two most important determinants of the risk of developing an illness is exposure and genetic constitution (Nerbert and Roe, 2001). The polymorphic CYP enzymes have been implicated as a predisposing factor in various non-infective diseases. Lung, liver and gastric cancers, as well Parkinson's disease, are more likely to occur to patients carrying a polymorphic *CYP2D6* gene (Ayesh *et al.*, 1984; Dykes 1996; Watanabe 1998).

Breast, head and neck cancer has been associated with the *CYP2D6*2* allele (Topić *et al.*, 2000). Ankylosing spondoylitis has been associated with *CYP2D6*4* allele (Brown *et al.*, 2000). Although an association between *CYP2D6*2* allele and tardive dyskinesia has been suggested, Ohmori and colleagues have not found an association between *CYP2D6*2* and the occurrence of tardive dyskinesia (Ohmori *et al.*, 1999).

A possible mechanism for the association of disease with the alleles is the production of reactive metabolites by the defective enzymes which have lost their substrate specificity (see Figure 4.3). The metabolites so produced might be carcinogenic or toxic to cells thus causing cell death.

7.3.6 In summary

The prevalence of allele distribution varies from one population to another. It is also apparent that the Black population presents with a genotype that is unique. The clinical relevance of this is wide and clinically significant as briefly described in 7.3. Genotyping studies from all parts of Africa, including South Africa, must therefore be viewed as important and are to be encouraged.

The planned future study therefore is planned at addressing the existence and extent of genetic polymorphism in a South African population. The initial phenotyping will be performed using the HPLC method described in this study. It is with great expectancy that the studies will realise the possibility of genotyping selected subsequent phenotypes observed in a population, and then go further to reveal the underlying genotypes.

7.4 References

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