All men by nature desire knowledge.

Aristotle

Review of Literature
Interindividual differences in drug metabolism are explained by genetic and environmental factors such as physiological, pathophysiological, pharmacological and dietary factors. The body excretes lipophilic exogenous as well as endogenous compounds due to enzymes in the liver, converting the substances to more hydrophilic and excretable substances. Genetic polymorphism (allelic variants of the same gene) is common among drug metabolizing enzymes (DMEs) and causes variability in drug metabolism between individuals. Allelic variants of genes encoding DMEs have been identified and correlated to plasma levels and adverse effects of certain drugs. Knowledge about the status of metabolic capacity in a population and dose adjustments of drugs to patients carrying certain genotypes will decrease the risk of therapeutic failure or adverse effects. Lately, the knowledge on genetic variability in drug effects has advanced due to the characterization of the human genome (Lander et al., 2001; Venter et al., 2001).

Variability in drug effects has been investigated since the 1950s (Evans and McLeod 2003). The first observations were during the Second World War when it was noticed that it was mostly black American soldiers who suffered from hemolysis when using the antimalarial drug primaquine. This could later be explained by glucose 6-phosphate dehydrogenase deficiency (Alving et al., 1956). When isoniazid was introduced as treatment for tuberculosis, it was observed that individuals either had slow or rapid acetylation of the drug (Hughes et al., 1954).
3.2 Variability in Drug Effects
The response of a drug depends on multiple factors, whose effects can be synergistic or inhibitory. A substance (drug, endogenous compound, pollutant, dietary constituent) can modify the effect of a drug by influencing its pharmacokinetics (absorption, distribution, metabolism and/or excretion) or its pharmacodynamics (effects on e.g. receptors) or both. The variability of each of these factors can be due to genetic or environmental factors, or both. Existence of profound population differences with small intraindividual variability is consistent with inheritance as a determinant of drug response. Although many environmental factors may influence the effect of a drug, there are several examples in which interindividual differences in drug response are due to variants of genes encoding DMEs, drug transporters or drug targets (Evans and Relling 1999). Most drug effects have polygenic determinants with interplay of several gene products on the pharmacokinetics and pharmacodynamics of drugs, including inherited differences in drug receptors and drug disposition (e.g. DMEs and transporters). The effect of polymorphisms (i.e. at least two allelic variants with a frequency of at least 1% of the rarest variant) in the DME will be most important when variability in the other pharmacokinetic and pharmacodynamic factors is small.

The biological response of the human body to an exogenous compound e.g. a drug is dependent on a complex network of factors, as is illustrated in figure 1. Most drugs are effective because they bind to a particular target protein like enzymes, ion channels or receptors. In general, the intensity of drug effect (pharmacodynamics) is determined by the concentration of the drug in the direct environment (biophase) of the target and the affinity of the drug for the target. The drug concentration in blood should be considered as a surrogate marker but a relationship should exist because all organs and tissues are supplied with blood. Pharmacokinetics deals with drug plasma concentration time relationships in the body.
3.3 Pharmacokinetic Aspects

3.3.1 Pharmacokinetic processes

These basic processes of absorption, distribution and elimination are responsible for the transport and distribution of drug molecules and therefore influence their overall behavior in the body. (Timbrell 1985)

Absorption is defined as the passage of a drug from its site of administration into the systemic circulation. For the oral administration route (see figure 2), drugs are absorbed from the gastrointestinal tract, for other routes other organs are involved e.g. the skin (percutaneous absorption) or the lungs (inhalation). The extent of absorption determines the fraction of a dose that gains access to the circulation (the bioavailability). In addition, for some drugs, the bioavailability may be influenced by metabolism in the gut and the liver (see figure 2) before reaching the systemic circulation (Herbert et al., 1992; Floren et al., 1997). After reaching the systemic circulation the drug is distributed into the various body compartments. The four main compartments are plasma water (~5% of body weight), interstitial fluid...
Elimination comprises all processes, which result in removal of the drug from the body. The major elimination processes are excretion and biotransformation. Excretion is the elimination of the compound in the unchanged form or as metabolites and the main routes are: the urine (renal excretion) and the feces (hepatobiliary system). The polarity of a drug is often the limiting factor in excreting the drug from the body.

Enzymatic biotransformation (metabolism) reduces the lipid solubility of a drug and thereby increases the possibilities for excretion of the drug. Biotransformation reactions are generally divided in phase I and phase II reactions. Phase I reactions consist of oxidation, reduction and hydrolysis and usually form more reactive products. Phase II reactions consist of conjugation e.g. glucuronidation, sulfation, acetylation to form highly polar and readily excretable conjugates. Phase I reactions are mainly catalyzed by a complex enzyme system known as the mixed function oxygenase system which is based on cytochrome P450 (Timbrell et al., 1985).

Besides the cytochrome P450 enzymes other non-P450 enzymes like flavin mono-oxygenase, monoamine oxidase, alcohol dehydrogenase, xanthine oxidase etc., may catalyze oxidative pathways as well. (Beedham et al., 1997)

![Diagram of drug metabolism](image)

**Figure 2** The barriers that an orally administered drug encounters before reaching the circulation to exert its biological activity (Rowland and Tozer 1994).
3.3.2 **Variation in Pharmacokinetics**

Patients are commonly given standardized treatment of a specific drug, independently of other concomitant therapies, age or relevant physiological factors. Many drugs do not exhibit the same effect, and do not obtain the same concentrations in different individuals, despite the same dosage. This effect is called interindividual variation, and compared to intraindividual variation (variation within the same subject); its importance in drug therapy is relatively large (Meyer, 2001). Genetic polymorphisms within genes involved in drug metabolism seem to be one of the major causes to the variable outcomes (Speight and Holford, 1997; Ingelman-Sundberg et al., 1999a; Phillips et al., 2001; Oscarson et al., 2002).

### 3.3.2.1 Food

For some drugs the simultaneous intake of food can change the rate and extent of absorption. Food can physically bind the drug, alter the gastric emptying, intestinal motility, and pH, and thereby influence the bioavailability of the drug. In most cases, food delays or reduces the absorption, although enhanced absorption also has been observed. For example, dietary calcium forms chelating complexes with tetracyclines and fluoroquinolones, compromising their absorption. Furthermore, certain dietary components can induce or inhibit the drug metabolizing enzymes. Flavonoids in grapefruit juice reduce the intestinal CYP3A4 activity, simultaneously inhibiting P-glycoprotein, resulting in enhanced concentrations of drugs, which are substrates of CYP3A4/P-gp. In contrast, polycyclic aromatic hydrocarbons in chargrilled meat induce the expression of CYP1A (Toothaker et al., 1980; Hathcock et al., 1985; Fontana et al., 1999; Harris et al., 2003).

### 3.3.2.2 Age

Newborns have different enzymatic capacities as compared to adults, and both phase I and phase II reactions are usually low in activity.
during the first months compared to adults, but around six months they might even have a faster clearance of certain drugs. In addition, plasma protein binding, total body fat, plasma volume, total extracellular and intracellular volumes, and rate of gastric emptying differ in infants compared to the grown-up individual. The elimination rates of indomethacin and ibuprofen have been shown to be dependent on postnatal age, and the half-life of ibuprofen was reported to be substantially longer in prematures than in adults. This is important to consider when treating premature infants for patent ductus arteriosus. In contrast, the oxidation of theophylline is much faster in children between 1 and 5 years than in adults (Rane et al., 1973; Rane, 1980; Rane, 1999; Van Overmeire et al., 2001; Strolin Benedetti et al., 2003). With increased age, several physiological functions are reduced. Renal function decreases with age, and attention should be paid to elderly patients given drugs that are eliminated mainly via urinary excretion, since there is a risk of reaching supratherapeutic levels, due to drug accumulation. Examples of drugs that are mainly excreted through the kidneys include; digoxin, sotalol, antibiotics and most ACE-inhibitors. Furthermore, increased fat and decreased water content in elderly, influence the distribution volumes of drugs, and thereby the elimination rates (Dorne et al., 2004). Liver blood flow also decreases with age, and this will mainly affect orally administered drugs with high hepatic extraction ratios.

3.3.2.3 Gender

Gender difference is another factor that can influence the pharmacokinetics of drugs, but its relative importance has been poorly evaluated (Giudicelli et al., 1977; Fletcher et al., 1994; Kashuba et al., 1998; Kaiser, 2005). Even if adjustments are made for body mass, percent body fat, renal clearance, plasma volume, and organ blood flow, unexplained differences still remain between men and women with regard to the exposure of certain drugs (Gandhi et al., 2004; Anderson, 2005). There are reports that women would have a faster
clearance of CYP3A4-substrates and also higher CYP3A4 mRNA and protein levels (Wolbold et al., 2003). The metabolic changes are usually rather moderate, and most studies have a limited number of subjects and a large interindividual variation. Thus it is difficult to draw any general conclusions about gender differences based on these studies (Harris et al., 1996; Chen et al., 2000; Chiou et al., 2001). Results from studies using human liver microsomes are incoherent, but in general data point toward similar metabolic activity, although the hepatic content of some drug metabolising enzymes can differ between men and women (Wolbold et al., 2003; Schmucker et al., 1990; Hunt et al., 1992b; George et al., 1995). During pregnancy physiological factors will change, e.g. plasma volume, body fat and body water increases, and the intestinal motility decreases. The clearance of drugs metabolised by CYP2D6, have been shown to be significantly higher during pregnancy as compared to after delivery (Hogstedt et al., 1985; Wadelius et al., 1997). Taken together, the pharmacokinetics of most drugs will be altered in pregnant women, although the final out come of these changes is difficult to predict (Fletcher et al., 1994; Kaiser, 2005; Pennell, 2003).

3.3.2.4 Disease

Infectious agents have been shown to depress cytochrome P450 dependent drug metabolism, and one of the first reports concerned the decreased elimination of theophylline in patients with upper-respiratory tract infection, caused by influenza A and adenovirus (Chang et al., 1978; Renton, 2001). Inflammatory processes and infections involve the release of cytokines, such as interferons, interleukins, and tumor necrosis factor (TNF), from macrophages and neutrophils. In animals, these inflammatory mediators have been noted to inhibit the expression of drug metabolising enzymes, resulting in a reduced clearance of drugs, which are substrates of these enzymes. During infections and inflammation, albumin concentrations are decreased, and this will alter the fraction of unbound molecules (i.e. free fraction). The final effect will
vary, depending on the initial degree of protein binding, and whether the drug is a high or low extraction compound. The clinical consequences of such alterations in protein binding are generally minor, though. Decreased hepatic blood flow and a lower oxygenation capacity can reduce the metabolic clearance in conditions like cardiac failure (Alvan, 1986; Renton, 2004).

3.3.2.5 Environment
Compounds in cigarette smoke and pollutants can generate an inflammatory response, and polycyclic aromatic hydrocarbons in smoke induce CYP1A1/1A2 expression. These enzymes are involved in the metabolism of exogenous and endogenous procarcinogens, and will thus generate substantially more toxic and harmful compounds if induced (Fuhr, 1998; Churg, 2003; Raunio et al., 2005). Exposure to heavy metals influences the cytochrome P450 levels, and lead (Pb) has been shown to decrease drug metabolism in humans. Cadmium (Cd) has been noted to induce CYP1A1 in cultured human cell lines, and increased hepatic CYP2C9 protein levels have been associated with cadmium accumulation in human liver (Moore, 2004).

3.3.2.6 Concomitant drugs
The concomitant administration of two drugs may lead to drug interactions due to altered pharmacokinetics and/or pharmacodynamics, of one or both substances. The pharmacokinetic interactions can occur at the site of absorption, as well as during distribution, metabolism and excretion. Induction is defined as an increase in the amount or activity of the enzyme.

Rifampicin is a well-known inducer of several drug metabolising enzymes, and the elimination rate of concomitantly administered drugs is usually enhanced, whereby the concentration is reduced and the therapeutic effect is attenuated. Sulphaphenazole and gemfibrozil, on
the other hand, are CYP2C9 enzyme inhibitors. Supratherapeutic concentrations of drugs, which are substrates of this enzyme, might be reached if given together with sulphaphenzole or gemfibrozil. Ketoconazole is another inhibitor, increasing the concentrations of concomitantly administered CYP3A4 substrates. Drugs can also interact with each other by forming insoluble complexes in the intestine, compromising absorption, which is the case when aluminium-containing antacids are given concomitantly with fluoroquinolones. Thus, no effect of the fluoroquinolone will be obtained. Probenecid inhibits the tubular secretion of antibiotics, and this action can be used to prolong the effect of antibiotics. Herbal remedies, such as St. Johns Wort (Hypericum perforatum), may also interact with drugs, usually by enhancing their clearance, through induction of CYP3A4 and P-glycoprotein expression (Meisel et al., 2000; Wen et al., 2001; Shapiro et al., 2002). The effects of inhibition can be rather severe, since unexpectedly high or even toxic concentrations of one or several drugs can be reached. In patients on multi-drug therapy, not only reduced or enhanced concentrations may be obtained, but synergistic or additive pharmacological effects of two or more drugs can also cause severe side effects.

3.3.2.7 Compliance

It is common that patients forget to take their medication, or that they do not adhere to the drug prescription. This is likely to cause fluctuations in drug concentrations and could result in toxic or subtherapeutic concentrations. Lack of compliance is considered to be a great problem, but is very difficult to control (Osterberg and Blaschke, 2005).

As stated above, the large interindividual variation in drug concentration, and the resulting clinical outcome, is dependent on
several factors, one of the most important being the rate of drug metabolism.

3.3.3 Genetic Polymorphisms

3.3.3.1 Genetic polymorphism and the diversity of human genes

Genetics:
The human genome contains three billions of base pairs of nucleotides in the haploid genome of which about only 3% are genes. (Dykes et al., 1996) A gene can be defined as the basic unit of heredity that contains the information for making one RNA and, in most cases, one polypeptide (Weaver and Hedrick; 1997). The number of genes in humans is estimated at 40,000 to 100,000. (Dunham et al., 2000) Extensive genetic variation occurs in most populations at several different levels, including variants affecting color, chromosomal structure, and protein characteristics.

Polymorphism is defined as the existence of two or more genetically determined forms (alleles) in a population in substantial frequency. In practice, a polymorphic gene is one at which the frequency of the most common allele is less than 0.99 (Weaver and Hedrick; 1997). In humans polymorphism is rather common: it has been estimated that in each human individual 20% of the proteins and hence the genes exist in a form that is different from the majority of the population (Meyer 1992). Similarly, in a sample of 71 human genes it was observed that 28% was polymorphic and that the average heterozygosity was 0.067 (Weaver and Hedrick; 1997). Heterozygosity is defined as the proportion in a population of diploid genotypes in which the two alleles for a given gene are different. In a population the allelic frequencies may follow the Hardy-Weinberg Law that states: under certain conditions of stability both allelic frequencies and genotypic ratios remain constant from generation to generation in sexually reproducing populations. (Keeton et al., 1980) Conditions for this genetic equilibrium are:
The population must be large enough.
Mutations must not occur.
There must be no immigration or emigration.
Reproduction must be totally random.

The Hardy-Weinberg equilibrium is as follows:
\[ p^2 + 2pq + q^2 = 1 \]

where \( p \) = frequency of one allele and \( q \) = frequency of the other allele.

Polymorphism in drug metabolizing enzymes is caused by mutations in genes that code for the specific biotransformation enzyme. Generally, they follow the autosomal recessive trait that means that the mutation is not sex linked (autosomal) and that one mutated allele does not express the phenotype when combined with a normal, not mutated (dominant) allele. Genes can be mutated in several ways: a nucleotide can be changed by substitution (e.g. a C changes into a T), insertion or deletion of a base (Stryer 1981). If changes refer to one or a few bases, these mutations are called point mutations. Larger changes can exist also, e.g. deletion of the entire gene or duplication of the entire gene.

Figure 3 Model for control of protein synthesis by the genes. (Keeton et al., 1980)
Some point mutations are silent mutations, that is, they have no consequences at the protein level. Yet, other point mutations will affect amino acid sequence and thereby will affect the biological function of the protein. There are several mechanisms at which a point mutation can affect amino acid sequence and or expression. Mutations can be missense mutations, in which a base change alters the sense of a codon (a three base sequence that is specific for an amino acid) from one amino acid to another. For example, a missense mutation might change the proline codon (CCG) to the arginine codon (CGG). A mutation may introduce a preliminary stop codon: the cysteine codon (TGC) for example can be mutated to a stop codon (TGA). Insertion or deletion may lead to a frameshift, which means that the base sequence will be read incorrect from the insertion or deletion because the reading frame has shifted. Another frequently occurring consequence of a mutation is incorrect splicing (splicing defect). A gene consists of introns (non-coding sequences) and exons (coding sequences). The messenger RNA (mRNA; Figure 3) is read from the DNA and the primary transcription product contains both introns and exons. The primary product is further matured by removal of its introns in a process called splicing. Splicing occurs via splicing-sites: GU (first two bases of an intron) and AG (almost always last two bases). Mutations in these sequences or mutations at other sites may lead to abnormal splicing.

The most common types of polymorphisms are those called single nucleotide polymorphisms (SNP), and they occur at a frequency of about 1 per 1000 base pairs in the human genome. To be called a polymorphism the nucleotide exchange must be present in more than 1 % of the population, and the more precise definition would be “the occurrence in the same population of two or more alleles at one locus, each with a frequency of at least 1%”. A functional polymorphic gene is defined as a stable variant of the gene, with altered enzymatic activity, existing in a population with a known frequency (Tribut et al., 2002).
The first definition of genetic polymorphisms was aimed to distinguish between common and rare phenotypes (Vogel 1961; Meyer, 1991). Spontaneous mutations occur at a lower frequency and are only distinguished from polymorphisms by definition (Meyer, 1991). The frequency of a specific variant allele varies between populations, and while some variants can be rather frequent in one population, it can be totally absent in others (Solus et al., 2004).

A recent study indicates that there is a higher genetic diversity among CYP genes than among most other human genes examined. This is believed to have been an evolutionary advantage, since it created enzymes with different substrate specificities. Much of the observed diversity is however due to the occurrence of many variants with low allele frequency, and those are probably of minor importance. The clinical significance of genetic polymorphisms in genes of drug metabolising enzymes, is very much dependent on whether the metabolite is active and/or toxic, the therapeutic index of the drug, the clinical state of the patient, concomitant drug treatment, as well as dose-response relationship (Tucker, 2000). Deletions, insertions, and duplications are other examples of mutations, which can be of clinical relevance for drug treatment, when present in genes coding for drug metabolising enzymes (Gotoh, 1992; Solus et al., 2004).

The resulting phenotypes of genetic polymorphisms in drug metabolising enzymes are generally called poor and extensive metabolisers, PMs and EMs. The poor metaboliser phenotype is inherited as an autosomal recessive trait, and these subjects are usually homozygous for the defect allele, while the extensive metabolisers are homozygous for the fully functional allele. Heterozygous subjects in general have intermediate metabolic capacity. The presence of duplicated genes is defined as ultrarapid metabolisers (UM) (Kalow, 1997).
3.4 Phenotyping

3.4.1 Assessment of enzyme activity using probe drugs

The individual status of the activity of drug metabolizing enzymes can be assessed using enzyme specific probe drugs. In Phenotyping, the drug is administered to an individual and the excretion rate e.g. metabolic ratio, is measured after several hours. In selecting a probe drug the following basic elements should be considered (Pelkonen et al., 1995) kinetics/metabolite formation should be determined predominantly by metabolism and not by liver blood flow or protein binding, the probe drug should not inhibit other enzymes of interest, the probe drug should be able to detect environmental or host influences and it should be safe. Furthermore, some practical issues play a role: are the clinical procedures not too inconvenient for patients or volunteers, is the analytical methodology not too demanding and is the probe easily available (preferably a clinically used drug). Simultaneous assessment of in-vivo activities of more than one enzyme may be performed by a multi-enzyme probe approach or by the cocktail approach.

The multi-enzyme approach also called the metabolic fingerprinting approach seems to be a practical approach: only one drug has to be administered yielding information on several drug-metabolising enzymes. In the eighties and early nineties antipyrine was considered a promising multi-enzyme probe but nowadays its use as test compound has decreased because it failed to predict liver disease correctly and furthermore inhibition or induction of antipyrine metabolism cannot directly be coupled to a specific P450 enzyme (Brockmoller and Roots 1996). At least six P450 enzymes are involved in the metabolism of antipyrine and none of the routes to the major metabolites are single enzyme routes (Engel et al., 1996). Therefore, it was concluded that antipyrine is not well suited as a probe for distinct human cytochrome
P450 enzymes. Other examples of multi-enzyme probes are caffeine (Kashuba et al., 1984) (NAT2, XO and CYP1A2), warfarine (Wang et al., 1994) (CYP3A4, CYP1A2 and CYP2C19), dextromethorphan (CYP2D6 and CYP3A4) (Ducharme et al., 1996) and omeprazole (CYP2C19 and CYP3A4) (Bertilsson et al., 1997).

Table 1 An overview of multi-drug cocktails to assess P450 activity

<table>
<thead>
<tr>
<th>Compound</th>
<th>Enzymes to be assessed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dextromethorphan, mephenytoin</td>
<td>CYP2D6 and CYP2C19</td>
</tr>
<tr>
<td>Sparteine, mephenytoin</td>
<td>CYP2D6 and CYP2C19</td>
</tr>
<tr>
<td>Debrisoquine, mephenytoin</td>
<td>CYP2D6 and CYP2C19</td>
</tr>
<tr>
<td>Debrisoquine, mephenytoin and dapsone</td>
<td>CYP2D6, CYP2C19 and CYP3A4</td>
</tr>
<tr>
<td>Dextromethorphan, proguanil</td>
<td>CYP2D6, CYP2C19</td>
</tr>
<tr>
<td>Dextromethorphan, proguanil and caffeine</td>
<td>CYP2D6, CYP2C19 and NAT2</td>
</tr>
<tr>
<td>Dextromethorphan, caffeine</td>
<td>CYP2D6, NAT2, XO, CYP1A2</td>
</tr>
<tr>
<td>Pittsburgh Cocktail</td>
<td>NAT2, CYP1A2</td>
</tr>
<tr>
<td>Caffeine, chlorzoxazone, dapsone, debrisoquine, mephenytoin</td>
<td>CYP2E1, CYP3A4, CYP2D6 and CYP2C19</td>
</tr>
</tbody>
</table>

The cocktail method is a multi-drug approach in which enzyme specific probes are co-administered. (Table 1) Several combinations have been used, validated and applied successfully in both clinical and epidemiological studies. Examples of well established cocktails for simultaneous assessment of several polymorphic and non-polymorphic enzymes are given in table 1.

Some combinations, however, may lead to misinterpretations, for example the combined administration of omeprazole and caffeine showed significant induction of CYP1A2 by omeprazole (Rost et al., 1994). Furthermore, care should be taken when CYP2D6 has to be assessed in populations other than Caucasians. Dissociation has been observed in the control of metoprolol, sparteine and debrisoquine oxidation in Nigerians (Lennard et al., 1992) and of metoprolol and debrisoquine oxidation in Zambians (Simooya et al., 1993). Similar results were observed in a study to compare sparteine, debrisoquine and dextromethorphan as CYP2D6 probes in Ghanaians, Chinese and
Caucasian (Droll et al., 1988). Again, dissociation was found in the oxidation of the probes in Ghanaians whereas such dissociation did not exist in Caucasians and Chinese. It was suggested that this dissociation might be explained by allele(s), which alter substrate specificity of CYP2D6. These studies highlight the difficulties in the interpretation of data from pharmacogenetic studies in ethnic groups.

3.5 Genotyping

Genotyping is the method used for determining the allelic variants of a certain gene. The genotype refers to the genetic constitution or make-up of an individual, and is the internally coded, inheritable information. The genotype should preferentially predict the phenotype (see below), and is determined from DNA, which can be extracted from e.g., a blood sample or buccal swab. Several techniques for genotyping exist, and the most common are different types of PCR (Polymerase Chain Reaction) methods, such as RFLP (Restriction Fragment Length Polymorphism) and SSCP (Single Stranded Conformation Polymorphism). With high throughput methods such as dynamic allele-specific hybridization (DASH), matrix assisted laser desorption/ionisation time-of-flight (MALDI-TOF), and TaqMan, many samples can be analysed in one single run. In addition, different sequencing methods are also available. DNA chips and microarray methods, using the principle of hybridization of complementary nucleotide sequences, also offer the possibility of high throughput screening of polymorphisms (Bray et al., 2001; Tribut et al., 2002; Daly, 2004). The method of choice is dependent on many factors, e.g. availability, cost, number of samples to be analysed, and accuracy.

3.6 Genotyping Vs. Phenotyping

The limitations using phenotyping is that the probe drug itself can cause side effects. Usually this is a minor problem, since the doses used are relatively low, and administered only once. However,
sometimes concomitant medications must temporarily be withdrawn, in order not to influence the results by causing drug interactions. If using endogenous substrates the method will be less invasive. Since many of the factors influencing the phenotype, are not constant over time, the test usually has to be repeated. The advantage of phenotyping is the ability to provide information about the overall drug metabolising capacity at a certain timepoint, also taking hepatic and renal function, diet, and other environmental factors into account. For enzymes that do not have any polymorphisms of clinical interest, but still exhibit variation in activity, phenotyping is the method of choice for dose prediction (Caraco, 1998; Bachmann, 2002). Phenotyping tests are usually expensive and sample analysis is time consuming. When using a cocktail approach, a lot of information can be acquired simultaneously. The drawback of a cocktail is the large amount of samples required for analysis, and the potential risk of interactions between the probe drugs (Tanaka et al., 2003; Zhou et al., 2004).

Since the genotype is constant over time, and is not affected by other interacting factors, such as concomitant drug treatment, dietary habits, hormonal levels, and diseases, genotyping for a specific gene variant does not have to be repeated. In addition, genotyping results are obtained relatively fast, and the test can be performed without withdrawal of any ongoing drug therapy. Only small amount of sample is required, and the problem with interindividual variation does not exist (Kirchheiner and Brockmoller, 2005). Irrespective of which method is being used, it has to be thoroughly validated. Usually, genotyping methods have proven to be highly accurate (high sensitivity and specificity). As can be concluded from above, the method of choice is dependent on many factors, but despite the many advantages with genotyping, its use in clinical practice has so far been limited (Evans et al., 2004). This might be due to the lack of conclusive results proving that the therapeutic outcome would benefit from performing genetic
The term pharmacogenomics is sometimes used interchangeably with pharmacogenetics, and there seems to be some confusion about the exact interpretation of the two words. Pharmacogenetics is usually defined as the inherited genetic components of variability in individual responses to drugs. Pharmacogenomics, on the other hand, deals with the development of drugs, based on the knowledge of the human genome, and can be defined as the correlation between the genotype of an individual and the response to drug treatment. The field of pharmacogenetics was already recognised by Pythagoras, when he observed that some people, but not others, were at danger when eating fava beans. This was later associated to glucose-6-phosphate dehydrogenase deficiency, resulting in anaemia. The first thorough description of the field was introduced by Vogel in the beginning of the 1960s (Vogel, 1961; Kalow, 1965; Kalow, 1967; Kalow, 2002).

When pharmacogenetic studies started, very little was known about the genes responsible for either intraindividual or interethnic difference. In fact nobody knew for sure whether or not most of the interethnic difference had a genetic or a cultural basis; within a population, family or twin, studies can easily show that a trait is genetic.

In short, differences in drug response between human populations first drew attention 4-5 decades ago. However, because of initial methodological shortcoming, science of interethnic pharmacogenetics is in a strict sense only 20-30 yr old. Its true that majority of genes in different human populations are identical. Nevertheless it would be wrong and inappropriate to take this overall similarity as an
opportunity to disavow the medical importance of interethnic divisions of the human species.

A revolutionary book by Masstoshi Nei entitled Molecular evolutionary genetics divided populations by their genetic diversities, more recently this work has been greatly extended, mostly confirmed but with some changes by Cavalli-Sforza et al., in a book entitled the history and geography of human genes. These authors tested 120 alleles frequency in 42 human populations. They calculated genetic distances between populations and estimated the time of their separation. They summarized and constructed 9 clusters. Greatest difference was between African and all non African population in support of the theory that all humans derived from a wave of emigrants who left Africa 100,000 years ago.

An interesting point in this figure below is indication that the separation between SE and NE Asians (~50,000 yrs) is older than that between NE Asians and Europeans (~40,000 yrs). Most Chinese are NE Asians and Japanese and Koreans are SE Asians, hence drug tests comparing Japanese and Koreans tend to be more similar than equivalent comparison between Japanese and Chinese.
It's largely a matter of economic and cultural factor that we know more about pharmacogenetic difference between Caucasian and Asian population than between these and African populations.

Data obtained with African–American must be interpreted as having a Caucasian admixture of approximated 30%; African populations are not a uniform group and differ substantially from one another.
3.7.1 Individualized Drug Therapy (Personalised Medicine)

The recommended standard doses of a certain drug are not appropriate for all patients. Due to interindividual variations such as genetic factors, diagnosis, drug interactions, as well as the actual disease and physiological status of the patient, the initial dose must be adjusted in order to achieve optimal effect, without causing any side-effects (Meyer, 2001; Tsai et al., 2002; Kalow, 2002). Pharmacogenetics is thought to be one of several useful tools for optimising drug treatment and developing new therapies. The aim of pharmacogenetic research is to identify genetic differences, which could be responsible for unpleasant or harmful reactions, so called adverse drug reactions (ADRs), or in the opposite case, lack of therapeutic response (Oscarson and Ingelman-Sundberg, 2002; Pirmohamed and Park, 2003).

Since approximately 56 % of all known drugs are completely or partly cleared via cytochrome P450 enzymes, and 40 % of those are polymorphic, it is of importance to understand the over all influence of polymorphisms on drug metabolism, in order to avoid unwanted effects. The CYP2D6 gene, having about 50 genetic variants, demonstrates the complexity of the issue (Ingelman-Sundberg et al., 2000). Results have been presented, stating that 59 % of the 27 drugs occurring most frequently in ADR reports, were metabolised by at least one enzyme having a variant allele known to cause decreased enzymatic activity. This figure can be compared with 7-22 % of randomly selected drugs that were metabolised by polymorphic enzymes (Phillips et al., 2001; Pirmohamed et al., 2001; Pirmohamed and Park, 2003). Although no exact figure regarding the involvement of polymorphic drug metabolising enzymes in adverse effects can be provided, pharmacogenetics should be recognised as an important
research field that can help optimise drug treatment and reduce patient suffering, and hopefully also health care costs (Bates et al., 1997; Lazarou et al., 1998; Anderson, 2005). Traditionally, studies have been performed on single genes, trying to determine their association to a given outcome, but lately polymorphic patterns for several different genes have been established. Data derived from the human genome project (HUGO) has been able to associate polymorphisms in drug receptors, transporters, drug metabolising enzymes and disease genes to drug responses. The are a lot of examples demonstrating the potential benefits of pharmacogenetic research, but still few conclusive results from prospective, randomized, clinical trials have been presented, which are able to prove the advantages, i.e. improved efficacy or reduced number of adverse events, of knowing the genotype of patients before initiating a drug treatment (Evans and Relling, 2004).

The lack of substantial benefit of knowing the genotype of certain genes before initiating drug therapy is dependent on several factors. First, the genotype does not always reflect the actual phenotype, due to factors such as phenocopy, i.e. different factors giving rise to the same trait or phenotype. Other common problems are unknown affecting environmental factors, the physiological changes caused by the actual disease, drug interactions, and last but not least the fact that most drugs are metabolised by several enzymes. The high frequency of single nucleotide polymorphisms also limits the possibility to establish a phenotype-genotype association. This is in contrast to the general belief that the identification of SNPs will help us predict the phenotype, and thereby the ultimate dose of a specific drug to a certain patient. Further more, the expression of genes can be affected by the drug treatment itself, as well as the disease. The aim to find a specific genotype, which will affect the phenotype, has thus been more complex than first anticipated, and it is rather complicated to predict the optimal drug therapy and dose for each patient. Anyway, efforts to establish dose recommendation charts for some drugs, metabolised by polymorphic
enzymes, have been done (Meisel et al., 2000; Kirchheiner et al., 2001). In the future there are however hopes that a CYP genotype chart might be available for each patient, helping the physician choosing the right drug and the right starting dose. It must however be remembered that if the frequency of the variant allele is low, a large number of people would have to be genotyped in order to identify one exceptional case at risk of adverse effects, and this would mean substantial additional costs.

Despite the complexity of pharmacogenetic research described above, there are examples proving that genotyping can be a helpful tool in order to optimise drug therapy for the individual patient, and save money, before initiating certain therapies. Studies have shown that poor metabolisers of CYP2C19 have better effect of proton-pump inhibitor treatment than extensive metabolisers, given the recommended doses. In a study, based on patients with duodenal ulcers and H. pylori infection, the cost-effectiveness analysis supported the use of CYP2C19 genotyping before initiating drug treatment (Lehmann et al., 2003). It has also been implicated that side effects in patients treated with amitriptyline would be reduced if the CYP2D6 and CYP2C19 genotypes were known beforehand (Steimer et al., 2005).

### 3.7.2 Characteristics of Cytochrome P450 Enzymes

#### 3.7.2.1 Nomenclature

The name P450 was originally a description of a red pigment found in liver microsomes with an absorbance maximum at 450 nm in its reduced carbon-monoxide-bound form (Omura and Sato, 1964). As the number of P450 genes identified and cloned increased, a system for classification of the genes was adopted (Daly et al., 1996; Nelson et al., 1996). It is based on protein sequence identity using a global alignment of the P450 sequences. The enzymes are denoted CYP (Cytochrome
Cytochrome P450 genes can be polymorphic, i.e. exist in at least two allelic variants with a frequency of at least 1% of the most rare variant. Such variant alleles can contain one or several SNPs. To be assigned as a unique allele it should contain SNPs or larger genetic rearrangements that have been shown to cause at least one amino acid exchange or to affect transcription, splicing or translation. The wild-type allele was previously used to describe a fully functional allele that occurred at a high frequency in the studied population. Lately, the wild-type definition has been used for the first allele sequenced and refers to a consensus (reference) allele and is designated as *1 (or *1A and *1B in case of slightly variant sequences) (http://www.imm.ki.se/CYPalleles) (Ingelman-Sundberg et al., 2000). In many instances, the first genetic material sequenced has been from a Caucasian population, and therefore it has often been the most frequent allele among Caucasians that is named as wild type.

3.7.2.2 Evolutionary Perspective
As the concentration of atmospheric oxygen started to increase about 2.5 million years ago, living organisms were required to protect themselves from oxidative stress, and this was partly achieved by ancestral forms of the CYP enzymes. Later, further development of these protective enzyme systems occurred in animals, when they had to detoxify the harmful substances that were present in plants, which
they used as an energy source. Throughout evolution, plants and animals successively adapted, and new gene variants emerged, and an association between dietary composition and overall cytochrome P450 activity has been implied. Comparative sequence analyses have revealed a frequent replacement of amino acids within the substrate recognition sites of cytochrome P450 enzymes, showing a successive adaptation to the environment. Accordingly, enzymes with divergent substrate specificities seem to be an evolutionary advantage. In man, 57 functional cytochrome P450 genes have been identified at present (Nebert and Gonzalez, 1987; Gotoh, 1992; Nebert et al., 2000; Pirmohamed et al., 2003; Salisbury et al., 2003).

3.7.2.3 Historical Perspectives

The leading early workers in the field of Drug Metabolism were chemists. Woehler was the pioneer person who identified potential chemical transformations occurring in the body and conducted experiments in dogs. He speculated the conversion of benzoic acid to hippuric acid, but his initial experiments in dogs led to the conclusion that only benzoic acid was excreted in the urine. This was due to the fact that hippuric acid was not fully characterized until 1829 by Liebig. Alexander performed the first human metabolism study in 1841, he observed the conversion of benzoic acid to hippuric acid and proposed the use of benzoic acid for the treatment of gout. W. Keller, in Woehler's laboratory provided the confirmation of Ure's experiments. Thus, not only had Keller and Woehler delineated the first biochemical study they unveiled a ready source for new compounds. Since then a lot many novel and innovative works have been conducted in the field of Drug metabolism.

Drug metabolizing enzymes are responsible for degradation of drugs and environmental pollutants to aid their excretion and are important
determinants of drug action (Lu et al., 1998). Williams (1959) found out that drug metabolism is carried out in two phases: Phase I and Phase II. In the first phase the hydrophobic moieties are converted into more hydrophilic ones by oxidation, reduction, hydrolysis, hydroxylation or deamination reactions, and then the conjugation/synthesis/addition starts in which there can be sulphation, glucuronidation, methylation, acetylation (to name the few) to eliminate the hydrophilic toxic material outside the body.

Demographic evolutionary factors (i.e. drift and migration/gene flow) affect all loci similarly, in contrast to loci under selection pressure (i.e. dietary or environmental stress) in the event of shaping genetic variability. Frequency of alleles can be influenced by adaptive selection through dietary stress alternatively through random genetic drift (Tishkoff and Verrelli 2003). In two Amerindian populations where the effect of selection through diet on human CYP2D6 evolution was studied, no disagreements between genotypic and phenotypic data were detected (Jorge et al., 1999). The two tribes diverged 8000 to 10,000 years ago and have low degree of genetic admixture (Torroni et al., 1994). It was therefore concluded that human CYP2D6 among these Amerindians was preferentially affected by random genetic drift and not by adaptive selection. However, local aggregation of subjects with duplicated and multiduplicated CYP2D6 genes was suggested to be the result of dietary pressure by plant alkaloids (Ingelman-Sundberg et al., 1999b). Moreover, the role of population history and natural selection in shaping genetic diversity in CYP1A2 was studied (Wooding et al., 2002). It was shown that haplotypes found outside Africa were mostly a subset of those found within Africa and that African populations had the lowest level of linkage disequilibrium of the populations studied.
3.7.2.4 Cytochrome P450-Mediated Reactions

\[
\text{NADPH + O}_2 + \text{SH} + \text{H}^+ \rightarrow \text{NADP}^+ + \text{SOH} + \text{H}_2\text{O}
\]

S = substrate

Figure 4 The equation for cytochrome P450-dependent reactions

Cytochrome P450s used to be called mixed function oxidases since many reactions lead to incorporation of an oxygen atom, but P450s can also catalyse reduction reactions. The P450s use electrons from NADPH to catalyse activation of molecular oxygen, leading to regio and stereospecific oxidation of a large number of substrates. In mammalians, P450s and NADPH-P450 reductases are anchored on the cytosolic side of the endoplasmic reticulum (Werck-Reichhart and Feyereisen 2000). There are also P450s in the inner membrane of the mitochondrias that catalyse steroid synthesis and fatty acid metabolism. These P450s are well conserved between species and have high affinity for their substrates.

The P450s are heme-thiolate proteins. They have a conserved structural fold, but only a few completely conserved amino acids. The highest structural conservation is found in the core of the protein around the heme and reflects a common mechanism of electron and proton transfer and oxygen activation. The most variable regions are associated with amino-terminal anchoring and substrate binding (Graham and Peterson 1999; Werck-Reichhart and Feyereisen 2000). The latter regions are located near the substrate access channel and catalytic site and are often referred to as substrate recognition sites (Gotoh et al., 1992).

In mammalian cells, the P450s are membrane bound and have therefore proven difficult to crystallise. The bacterial P450s are non-
membrane bound and are possible to solubilise and further crystallise, i.e. P450cam or CYP101 (Poulos et al., 1987). Since, six additional P450 structures (bacterial and fungal) have been described. A molecular model of CYP2D6 was constructed from the bacterial form CYP102 via homology alignment between the CYP2D subfamily and the CYP102 protein sequence (Modi et al., 1996; Lewis et al., 1997).

3.7.3 Characteristics of Human CYP3A

3.7.3.1 Tissue Localization
A considerable amount of information exists on the characteristics of the members in the CYP3A subfamily of enzymes in both animals and humans (Maurel 1996). At least three functional proteins exist in humans. CYP3A4 is universally found in the liver, where it constitutes the major isoform—on average about 30% of total CYP protein (Shimada et al., 1994). Relatively high CYP3A4 levels about 50% of hepatic levels and 70% of total CYP protein are also present in small intestinal epithelium, particularly in the apical region of mature enterocytes at the tip of the microvillus (Kolars et al., 1994; McKinnon et al., 1995). The amount of isoform progressively falls along the remainder of the gastrointestinal tract.
In the kidney, however, CYP3A4 is present in only about 30% of renal tissue samples, mainly in the collecting ducts (Schuetz et al., 1992, Haehner et al., 1996); the mechanism for such polymorphic expression is not currently understood. CYP3A3 is a very closely related isoform to CYP3A4 (>98% cDNA sequence similarity), but it is not known whether this reflects a separate gene product or an allelic variant. Therefore, the term CYP3A4 is generally taken to indicate a collective contribution of the two isoforms. By contrast, CYP3A5 is structurally distinct from CYP3A4. It is also found in the liver but only in about 10–30% of hepatic samples and then at levels 10–30% of CYP3A4 (Wrighton et al., 1989; Wrighton et al., 1990). A point mutation resulting in the synthesis of an unstable protein may account for such polymorphism (Jouna'ydi 1996). CYP3A5 is the predominant CYP3A isoform that is universally expressed in the kidney (Schuetz et al., 1992; Haehner et al., 1996). It is also heterogeneously expressed throughout the gastrointestinal tract but, generally, in lower amounts than CYP3A4,
except in parietal cells of the stomach (Kolars et al., 1994; McKinnon et al., 1995) and possibly the colon (Gervot et al., 1996). CYP3A5 also differs from CYP3A4 in that its expression does not appear to be up regulated by agents that are well established inducers of CYP3A4 (Wrighton et al., 1989, Schuetz et al., 1993). The third functional CYP3A isoform is CYP3A7, which was originally found in fetal liver; however, it may also be selectively expressed in adult livers at lower levels than CYP3A4 and CYP3A5 (Schuetz et al., 1994).

3.7.3.2 CYP3A Substrate Specificity
The substrate specificity of the CYP3A enzymes is very broad; accordingly, an extremely large number of structurally divergent chemicals are metabolized by a variety of different pathways, often in a regio- and stereoselective fashion (Maurel 1996, Guengerich et al., 1995). Estimates, based primarily on in vitro studies, suggest that the metabolism of perhaps 40–50% of drugs used in humans involves CYP3A mediate oxidation to some extent. Whether this reflects the importance of such metabolism in drug elimination in vivo is, however, not so apparent. Nevertheless, it is clear that CYP3A is of major importance in the metabolism of drugs by humans. CYP3A4 is the most thoroughly investigated isoform, and it is generally assumed that the other isoforms have essentially similar characteristics. However, limited studies suggest the likelihood of possibly important differences in the isoforms' substrate specificities. For example, neither quinidine nor erythromycin appeared to be metabolized by CYP3A5, although they are good CYP3A4 substrates (Wrighton et al., 1990, Aoyama et al., 1989), and only one of three primary metabolites of cyclosporine A formed by CYP3A4 was produced by CYP3A5 (Aoyama et al., 1989).

In other studies, the level of CYP3A5-mediated metabolism, when it was present, was less than that of CYP3A4, although in one instance, namely, the 10-hydroxylation of midazolam, CYP3A5 exhibited greater catalytic activity (Gorski et al., 1994). A difficulty with such
comparative studies is that the optimal in vitro conditions may not be the same for the two isoforms (see below). An important characteristic of CYP3A is the large interindividual variability in activity, which reflects a genetic effect combined with modulation by environmental factors. For example, hepatic microsomal activity often differs by up to 40-fold (Shimada 1994, Guengerich 1995), and large variability also has been noted with intestinal (Lown et al., 1994) and renal microsomes (Haehner et al., 1996).

Human in vivo studies have also indicated considerable interindividual variability, but generally, this has been smaller (fivefold), although, of course, the range can be significantly increased by deliberate modulation, i.e. inhibition and induction. The reason for such a discrepancy is not clear, but it has been observed with other CYP isoforms and indicates that care must be taken in quantitatively extrapolating in vitro studies to the in vivo situation. Because of the difficulty in distinguishing between the isoforms' catalytic activities, the fact that in many instances several isoforms may be present in a single organ, and in vivo the relative contribution of a specific organ to overall metabolism cannot be readily determined, the term CYP3A is usually understood to reflect the collective activity of all of the isoforms. However, it primarily reflects CYP3A4.

3.7.3.3 Interindividual Variability
A major characteristic of CYP3A is the large range of interindividual variability in the level of its activity in various populations (see above), and this is particularly the case following oral drug administration. Moreover, variability in bioavailability increases both in theory (Wilkinson 1987) and in practice (Hellriegel et al., 1996) as the extent of first-pass metabolism increases. While these considerations apply to the enzyme's basal level, it has also become apparent that considerable interindividual variability is present with respect to the extent of CYP3A-mediated drug interactions, especially those involving
inhibition. For example, terfenadine’s plasma levels, which are usually below the assay detection limit of 5 ng/ml, were measurable in some but not all subjects pretreated with erythromycin (Honig et al., 1992; Wilkinson et al., 1997), and the interaction with ketoconazole resulted in 16- to 73-fold reduction in the antihistamines’ oral clearance (Honig 1993).

Pronounced variability was also found in the interaction of grapefruit juice with various CYP3A substrates (Wilkinson et al., 1997). For example, the increase in relative oral bioavailability ranged from 0 to 200% for cyclosporine (Yee et al., 1995), 5 to 470% for felodopine (Bailey et al., 1991; Edgar et al., 1992; Bailey et al., 1996), 26 to 100% for midazolam (Kupferschmidt et al., 1995), and 21 to 485% for terfenadine (Benton et al., 1996; Rau et al., 1997; Clifford et al., 1997). Several factors probably contribute to such variability. One is possibly the basal level of CYP3A, since the relative change in midazolam’s oral clearance produced by erythromycin and ketoconazole (Olkkola et al., 1994) tended to be related to the magnitude of the pretreatment clearance value. By contrast, no such relationship was observed with itraconazole (Olkkola et al., 1994). The observed heterogeneity in the intestinal expression of CYP3A activity in biopsied tissue (Lown et al., 1994; Paine et al., 1996) and the finding that certain subjects have essentially no intestinal midazolam 10-hydroxylating ability (Thummel et al., 1996) also indicate that the extent of inhibition of metabolism is likely dependent on the amount of available enzyme. As a result, interindividual variability is reduced when an inhibitory drug interaction is present. Another likely contributing factor is the individual variability in the inhibitor’s pharmacokinetics that results in different intestinal and hepatic tissue time profiles between subjects. Such wide interindividual variability in the extent of inhibited metabolism possibly accounts for observations indicative of the lack of an interaction when other studies have clearly demonstrated such an effect (Vanakoski et al., 1996, Jones et al., 1996).
3.7.3.4 In Vivo Probes of CYP3A Activity
Since numerous drugs are metabolized by CYP3A, any of these could be used to determine the enzyme's overall catalytic activity in the body and its modulation. In fact, this is the approach used to establish whether a significant interaction occurs between a specific CYP3A substrate and a known inhibitor/inducer. On the other hand, defining the determinants of CYP3A activity—e.g., race, age, sex, and others—and possible alteration by, for example, interaction with a new drug candidate require the use of an appropriate in vivo probe. Considerable effort has been extended to identifying a compound for this purpose; however, because of several characteristics of CYP3A, an ideal and universally applicable drug has yet to be defined (Watkins et al., 1994). Accordingly, the choice of an in vivo probe largely depends on the purpose of the study. Of particular importance is whether the selected phenotypic trait is to be used as a qualitative indicator of changes in CYP3A function or as a quantitative and predictive measure of the enzyme's activity.

3.7.3.4.1 Cortisol
For many years, the 6β-hydroxylation of endogenous cortisol, provided by the ratio of 6β-hydroxycortisol to free cortisol in urine, has been used as a measure of changes in overall CYP activity (Park et al., 1981; Ohnhaus et al., 1989). The finding that the 6β-hydroxylation step was predominantly CYP3A mediated (Waxman et al., 1988) suggested that it might serve as a more selective trait measure for this particular isoform(s). Several studies, for example, showed that the urinary cortisol ratio increased following treatment with inducers known to be selective for CYP3A, e.g., rifampin (Horsmans et al., 1992, Roots et al., 1979) and anticonvulsant agents (Roots et al., 1979; Saenger et al., 1981; Saenger et al., 1983). However, an analogous study with the CYP3A-selective, mechanism-based inhibitor troleandomycin noted no
consistent change in cortisol 6β-hydroxylation, despite the fact that the erythromycin breath test (see below) was markedly affected (Watkins 1992).

In addition, other studies were unable to find a correlation between the urinary excretion of 6β-hydroxy cortisol and CYP3A activity measured by this breath test (Lown et al., 1992, Hunt 1992a), consistent with the lack of a significant relationship between the urinary cortisol ratio and the uninduced level of CYP3A activity in a liver biopsy. Collectively, these data seriously question whether cortisol 6β-hydroxylation, as measured by the urinary cortisol ratio, is, in fact, a valid and useful in vivo probe for measuring CYP3A activity. The reason(s) for the discrepancies is unknown but could reflect the importance of CYP3A-mediated cortisol metabolism in the kidney.

3.7.3.4.2 Erythromycin
CYP3A selectively N-demethylates erythromycin and, if (14C)-,N-methyl drug is used, the cleaved carbon of the methyl group is eventually expired as 14CO2. Measurement of this radioactivity over a 1-h period, following an intravenous dose, forms the basis of the erythromycin breath test. Considerable validation and application of this simple and relatively rapid approach as a measure of in vivo CYP3A activity have been reported (Watkins, 1994), and the test provides a measure of CYP3A activity under certain circumstances and for some types of investigation. A limiting factor of the erythromycin breath test, however, is that it only appears to measure CYP3A4-mediated metabolism and not that mediated by CYP3A5 (Wrighton et al., 1990; Gorski 1994). Thus, overall CYP3A activity is underestimated, especially in the 25–30% of individuals with significant hepatic levels of this isoform. More importantly, however, is the fact that the erythromycin breath test predominantly reflects CYP3A4 activity in the liver (Gorski et al., 1994,
Watkins et al., 1992; Lown et al., 1992), and therefore, metabolism within the intestinal epithelium is not measured (Gorski et al., 1994).

This is an obvious limitation with respect to an orally administered drug and may account, in part, for the relatively poor correlation between the breath test value and the oral clearance of several CYP3A substrates (Hunt et al., 1992a; Kinirons et al., 1993; Lown et al., 1995; Krivoruk et al., 1994). Unfortunately, application of an oral erythromycin breath test to overcome this problem has not been successful (PB Watkins, personal communication). Additionally, it has not been possible to quantitatively interpret either the basal level or any change in the erythromycin breath test value with regard to CYP3A activity, i.e. it is essentially a relative measure whose relationship to erythromycin’s clearance is unknown. Nevertheless, the breath test has considerable potential for monitoring drug interactions involving hepatic CYP3A4. For example, treatment with troleandomycin markedly reduced the breath test value, whereas CYP3A-inducing agents such as dexamethasone and rifampin resulted in large increases. Furthermore, several published reports and ongoing research with new drugs under development indicate the utility of serial erythromycin breath testing to determine whether hepatic CYP3A induction or inhibition occurs. In this fashion, the chronic administration of delavirdine was found to result in rapid and substantial reduction in CYP3A activity that was dose related and led to the yet untested prediction that delavirdine would likely cause drug interactions when coadministered with other CYP3A substrates (Cheng et al., 1997).

Similarly, the time course of ketoconazole-associated inhibition of CYP3A activity, after a single oral dose, was followed for 30–36 h with repeated erythromycin breath tests (Jamis-Dow et al., 1997). Also, this study showed that paclitaxel’s metabolism was not affected despite CYP3A activity being inhibited, indicating the ability of the test to identify situations where a drug interaction does not occur. Such data,
therefore, strengthen the conclusions that omeprazole did not alter CYP3A activity (Tateishi et al., 1995) and that interferon only causes a small degree of inhibition (Craig et al., 1993).

3.7.3.4.3 Midazolam

In humans, midazolam is almost completely and rapidly metabolized to its primary 10-hydroxy metabolite and, to a much lesser extent, to 4-hydroxymidazolam. Importantly, both of these metabolic pathways are selectively mediated by CYP3A (Gorski et al., 1994). Accordingly, midazolam has been investigated as an in vivo probe for CYP3A activity and found to meet most, if not all, of the necessary criteria suggested for such an application (Watkins et al., 1994). For example, microsomes from both the liver (Thummel et al., 1996; Thummel et al., 1994) and small intestine (Thummel et al., 1996) were found to have high midazolam 10-hydroxylating activity, which in the case of the liver was significantly correlated with the drug's systemic clearance (Thummel et al., 1994). Liver dysfunction markedly impaired midazolam's elimination (MacGilchrist et al., 1986; Pentikainen et al., 1989), and plasma levels following intravenous administration during the anhepatic phase of liver transplantation were elevated (Paine et al., 1996). In addition, known inhibitors and inducers of CYP3A produced consistent alterations in midazolam's clearance (Olkkola et al., 1993; Kharasch et al., 1997a; Olkkola et al., 1994; Backman et al., 1996). It was also possible to scale-up results from in vitro measures of midazolam's 10-hydroxylation by liver and intestinal microsomes to predict the in vivo extraction ratios of these organs (Thummel et al., 1997). Importantly, close agreement was obtained between such estimates and their values measured in vivo (Thummel et al., 1994; Backman et al., 1996).

Midazolam has substantial advantages over other putative probes: in particular, the fact that the drug may be given safely by both intravenous and oral routes of administration. Thus, a measure of
CYP3A activity relative to firstpass metabolism is provided following an oral dose, whereas, after intravenous administration, CYP3A-mediated hepatic metabolism is primarily obtained. Importantly, using clearance as the phenotypic value allows quantification of CYP3A function in a way that can be related to drug dosage regimens and possible changes resulting from a drug interaction. This does not, of course, apply to the use of an alternative trait measure, namely, the plasma level ratio of 10-hydroxy midazolam to midazolam at a single time point after drug administration (Thummel et al., 1994). However, this latter phenotypic trait value now appears to be less valid and useful than originally suggested (Thummel et al., 1996). Importantly, it is also apparent that CYP3A-mediated metabolism of midazolam, especially as measured by the drug's oral clearance, is very sensitive to modulation of the enzyme's level of activity. For example, rifampin pretreatment reduced the area under the drug's plasma concentration-time curve by 96% (Backman et al., 1996), whereas ketoconazole increased this parameter by 15-fold (Olkkola et al., 1994), and itraconazole also resulted in a large effect (Olkkola et al., 1994; Ahonen et al., 1995). More modest changes (two- to fourfold) were found following a single dose of grapefruit juice (Kupferschmidt et al., 1995) and a period of erythromycin (Olkkola et al., 1993), clarithromycin, roxithromycin (Backman et al., 1994a), fluconazole (Ahonen et al., 1997), or diltiazem (Backman et al., 1994b) administration. By contrast, several days pretreatment with azithromycin (Backman et al., 1995; Zimmermann et al., 1996; Mattila et al., 1994) and terbinafine (Ahonen et al., 1995) had no effect on midazolam's metabolism. Thus, at the present time, measurement of changes in midazolam's clearance and the fractional clearance associated with the 10-hydroxy pathway of metabolism after oral drug administration probably provides the best means by which quantitative alterations in intestinal and/or hepatic CYP3A resulting from a drug interaction activity can be determined in vivo.
Moreover, the additional determination of any change in midazolam systemic clearance after intravenous administration (ideally using a differentially labeled form of the drug) permits estimation of the separate changes in CYP3A activity at these two anatomical sites (Thummel et al., 1996). *In vitro* studies using human liver microsomes invariably indicate a high degree of correlation between the rates of CYP3A-mediated catalysis of a variety of substrates. However, an as yet unexplained situation apparently exists *in vivo* whereby only relatively weak correlations have been found between various phenotypic trait measurements of CYP3A activity (Lown et al., 1992; Hunt et al., 1992a; Kinirons et al., 1993), and the ability of any such test to accurately predict the clearance of another CYP3A substrate has generally been poor (Kinirons et al., 1993, Watkins et al., 1990; Turgeon et al., 1992; Turgeon et al., 1994; Stein et al., 1996; Yeates et al., 1996).

In certain instances, this may be because some *in vivo* probes are not as valid as previously considered, e.g. the urinary cortisol ratio (see above) and the urinary dapsone hydroxylation index, which probably measure CYP2E1 and CYP2C9 activities in addition to that of CYP3A (Mitra et al., 1995; Gill et al., 1995). Furthermore, others, such as the erythromycin breath test, although possibly reflective of CYP3A activity, do not necessarily measure the enzyme's catalytic level in as quantitative a fashion as, for example, a clearance value.

Differences in the route of administration of the test drugs may also be contributory, although poor relationships have been observed following the intravenous administration of both putative probes, e.g. the erythromycin breath test value relative to fentanyl's clearance by N-dealkylation (Krivoruk et al., 1994) and midazolam clearance (Kinirons et al., 1994). Additional studies are required in this area, especially those based on clearance determinations rather than other more indirect types of trait measures. Other factors possibly contribute to
differences in the catalytic activities and levels of CYP3A4 and CYP3A5 and, also, their differential localization in various extrahepatic tissues. However, the interaction between CYP3A and its substrates may be more complex than previously thought. For example, if the allosteric effects observed in vitro also occur in vivo, CYP3A’s catalytic activity is likely to be relatively evanescent and dependent on active site interactions with substrates and effectors at any given time.
3.7.4 Population Characteristics

3.7.4.1 Indian Populations

The population in India as on 1st March 2001 stood at 1,027,015,247 persons. With this, India became only the second country in the world after China to cross the one billion mark. India's population rose by 21.34% between 1991 - 2001. The first human species, Homo habilis, originated in Africa about 2.5 million years ago. Later, about 100,000 to 200,000 years ago, it evolved into the modern human species Homo sapiens, which spread to other parts of the world (Cavalli-Sforza et al., 1994; Tishkoff and Verrelli, 2003). The dating of the occurrence of Homo sapiens is however controversial (Brooks et al., 1995; Tishkoff and Verrelli, 2003).

Human populations can be classified by different approaches, such as by genetic, linguistic, and cultural characteristics. Populations have been classified by means of measuring genetic distances based on the counts of numerous gene frequencies (Cavalli-Sforza et al., 1994). Significant correspondence between linguistic superfamilies and major genetic clusters has been reported (Chen et al., 1995). Linguistic classifications may be useful as an evolutionary tool but only when combined with genetic information. India is a country that is genetically and linguistically diverse. Indian populations typically have higher levels of genetic diversity and a more complex population substructure compared to non-Indian populations. While 22 major languages are recognized as "Official Languages" by the Constitution of India and these can be divided into four major families the Caucasio Aryans, Caucasio Dravidians, and Mongoloids and perfect mixture of Caucasio Aryans and dravidians. Linguistic barriers strengthen genetic isolation (minimising genetic mix-up) between groups speaking different
languages and hence the four major groups are believed to be genetically different.

Pharmacogenetic data are relatively scarce in India compared to European and other Asian populations. Ethnicity is a multidimensional classification that may encompass shared origins, social background, culture and environment (Xie et al., 2001). The use of ethnic classification in medicine is debated as it risks to undervalue the great diversity within groups, however, it may be useful for generating and exploring hypotheses about environmental and genetic risk factors in complex diseases (Burchard et al., 2003; Phimister 2003).

3.7.4.2 Gujaratis

The Gujarati people sometimes, also referred to as Gujarati are Gujarati language speaking people. They are mostly of Indo-Aryan Origin. Three modern populations from Gujarat are Garasia, Bhils and Rajputs, studies are used to estimate biological distance. In an earlier odontometric analysis (Lukacs and Hemphill, 1993), the Garasia were morphologically intermediate between Bhils and Rajputs, but clustered more closely to the tribal Bhils. Because the Garasia were most similar to groups from proximal geographic regions, Hemphill and Lukacs (1993) concluded that the biological data supported the admixture and Hinduization described in historical and ethnographic sources (Unnithan-Kumar, 1997). The close association between the Garasia and Bhils may indicate that the division between high and low status groups is more significant than that between caste and tribe. But Linguistically, there are regional differences in Bhil dialects, which reflect the cultural matrix, but few non-Aryan elements have been recorded for any of the Bhil groups (Naik, 1956). In Gujarat, these non-Aryan elements represent about 6% of the Bhili language, which appears most, influenced by Rajasthani, Gujarati, Khandeshi, and
elements of Garasia dialects are interspersed. Thus it's a perfect mixture of the Aryans and Dravidians.

3.7.5 CLINICAL RELEVANCE OF CYTOCHROME P450 DRUG METABOLISM

3.7.5.1 Effects of Variability in Drug Metabolism

Cytochrome P450-catalysed metabolic reactions show marked interindividual variability leading to large differences in plasma concentrations; e.g. a 30-fold variability in steady-state plasma concentration of nortriptyline was seen in patients with the same dose (Hammer and Sjöqvist 1967). It was also shown that subjects with three functional CYP2D6 genes needed three times the normal dose of nortriptylin, i.e. 300-500 mg daily (Bertilsson et al., 1985; Bertilsson et al., 1993). Schizophrenic patients phenotyped as PMs of CYP2D6 were shown to be at risk of adverse drug reactions upon treatment with haloperidol (Brockmoller et al., 2002). In contrast, patients with extra CYP2D6 gene copies were at risk of low therapeutic efficacy when treated with haloperidol (Brockmoller et al., 2002). Also, the CYP2D6 substrate codeine, which is a pro-drug of morphine, has a lower analgesic effect in PMs (Eichelbaum and Evert 1996; Poulsen et al., 1996), whereas it was reported that an ultrarapid metaboliser got severe abdominal pain after codeine intake (Dalen et al., 1997).

Polymorphisms in DMEs may have clinical importance when 1) a substantial amount of the drug is metabolized by the enzyme with no or minor alternative pathways available, 2) the resulting variability in elimination rate has clinical impact (i.e. drugs with narrow therapeutic ranges), 3) the drug is a prodrug, 4) there is low variability in other pharmacokinetic processes, and 5) there is low pharmacodynamic variability.
In PMs, the potential consequences of polymorphic drug metabolism are extended pharmacological effect, adverse drug reactions from the substrate, lack of prodrug activation, metabolism by alternative, deleterious pathways or drug-drug interactions. UMs may have an increased effective dose and may have adverse drug reactions from formed metabolites. Adverse effects are a serious clinical issue and it has been estimated that about 100,000 American patients die each year and 2.2 million are injured by adverse drug reactions to prescribed drugs (Lazarou et al., 1998). This data have been criticised for overestimation of figures though (Fremont-Smith 1998). In Sweden, 10 to 15% of patients acutely admitted to a clinic of internal medicine were admitted due to an adverse drug reaction (Sarlöv et al., 2001; Mjröndal et al., 2002). Known risk factors for adverse drug reactions include polytherapy, old age, liver and renal disease. Moreover, it has been estimated that more than half of the drugs in reports on adverse drug reactions are metabolised by polymorphic enzymes (Phillips et al., 2001). Even though drug-drug interactions may cause adverse drug reactions, it has been difficult to improve pharmacogenetic awareness in the clinic. It is believed that to do so, prospective studies may have to be performed, where measurable differences in clinical outcome that patients and physicians care about are considered (Meisel et al., 2000; Meisel et al., 2003).

### 3.7.5.2 Clinical Relevance in Indian (Gujarat) Populations

Parasitic and infectious diseases are major threats to the health of most populations in India. It is recognised that pronounced differences in drug metabolism exist between as well as within ethnic groups (Bertilsson et al., 1995; Masimirembwa and Hasler 1997). New drugs have seldom been studied for use in developing countries where unique factors influence the effect of a given dose, e.g. polymorphic DMEs, nutritional status, interfering diseases. It is now accepted in principle that differences in genetic inheritance and environment preclude
extrapolation of results of drug studies from non-Indians to Indians. Therefore, it is important to do specific molecular genetic and clinical studies in Indian populations to understand the mechanisms behind differences that may require population-specific dosage schedule of important drugs. Besides, combination therapy is particularly common in tropical medicine. It was first used for treatment of tuberculosis (TB) and leprosy, but with time this strategy has also been used to treat HIV and malaria. Due to the limited number of locally available antiparasitic drugs it has become necessary to prolong the efficacy of these drugs (Bloland and Ettling 1999). This has lead to the use of combination therapy that may help to avoid resistance development (White et al., 1999). The high incidence of parasite diseases as well as HIV and its associated infections means increased exposure to multiple drugs, thus increasing the risk of drug-drug interactions. Drugs in a combination therapy can interact in several different ways, either by competing for the same DME or by inhibiting the enzyme metabolising one of the other drugs. The DMEs involved may also exist in allelic variants affecting the drugs differently. It is important to study whether such polymorphisms influence suitable dosage of these drugs in Indian populations. The CYP3A enzymes are of major importance in India, since they metabolise so many drugs including certain protease inhibitors used in HIV treatment (Barry et al., 1997). Some protease inhibitors are also inhibitors of the CYP3A4 enzyme (van Heeswijk et al., 2001) thus requiring studies of their inhibitory potential in Indian populations, which might differ from that observed in Caucasians due to differences in genetic variants between the populations.

Knowledge of the CYP3A enzymes is also important for future treatment of TB, since one of the drugs in TB treatment, rifampicin, is a potent inducer of CYP3A4 (Hebert et al., 1992). In a study in healthy volunteers, dosing of rifampicin (600 mg per day) for 7 days increased the oral clearance of midazolam 22-fold (Gorski et al., 2003). Rifampicin being a potent P-gp inducer complicates the issue further
(Greiner et al., 1999). The complexity of handling combination treatments for HIV patients with active or latent tuberculosis is recognized. In future, anti-HIV and TB treatments will become more readily available to risk groups of patients also in India. It is hence important to know the effects of differences in drug metabolism to make informed choices on drug dosages. This emphasises the importance of phenotyping methods that are possible to use in larger population studies.

Quinoline drugs (e.g. chloroquine and quinine) have been among the most widely used drugs for treatment of Plasmodium falciparum. However, resistance development has decreased their utility. It was shown that chloroquine resistance could be reversed in vitro by verapamil, an inhibitor of multi-drug resistance in cancer cell lines (Martin et al., 1987; Martiney et al., 1995). Chloroquine resistance has been associated to a P-glycoprotein homologue present in the parasite (Foote et al., 1989), and resistance of the malaria parasite may therefore in some aspects be compared to P-gp-mediated multidrug resistance to chemotherapy in patients. A chloroquine resistance reversing agent, chlorpheniramine, together with chloroquine was successfully used in the treatment of malaria in children in an area where chloroquine-resistance reaches 35% (Sowunmi et al., 2000). Some malarial drugs (e.g. mefloquine, quinine and halofantrine) show cross-resistance in their malaria activity, which also indicates a multidrug resistance mechanism (Price et al., 1999). The World Trade Organisation (WTO) (August 2003) decided that developing countries would be allowed to import cheaper copies of drugs protected by patents for the treatment of HIV and other serious infections. This is a continuation of the decision taken at a WTO meeting in 2001, where it was decided that developing countries had the right to produce generics of certain drugs protected by patents. A condition for the agreement was that the drugs would only be used for treatment of
3.7.5.3 Population-Specific Drug Dosages

Oriental populations have been shown to require lower doses of antipsychotics compared to Caucasians (Poolsup et al., 2000). Also, slower metabolism in Asians due to the CYP2D6*10 allele has resulted in lower dosing recommendations (Lou 1990; Lin et al., 1991) and Japanese federal requirements that pharmacokinetic studies should be performed in Japanese subjects (Shah 1993). Moreover, major steps towards subpopulation (extensive, intermediate and poor metabolisers) specific drug dosages relevant for Caucasian populations have been taken for psychiatric drugs (Kirchheiner et al., 2001). In these, dose reductions around 50% were generally recommended for tricyclic antidepressants in PMs of substrates of CYP2D6 or CYP2C19, whereas differences were smaller for the selective serotonin re-uptake inhibitors.

It is important to study whether genetic diversity in Africans translates into clinically important variations in responses to drugs. It was reported that African-Americans showed significantly higher plasma concentrations of nortriptyline than Caucasians after administration of the same drug dose (Ziegler and Biggs 1977). It has also been shown that depressed Tanzanian patients required lower doses of clomipramine compared to those recommended for Caucasians (Kilonzo et al., 1994). Population-specific drug dosages may also be important in the light of drug interactions caused by polymorphic P450 enzymes. Several clinically used drugs are known inhibitors of these enzymes, which affect possible drug combinations as well as drug dosages. In the CPMP/ICH guideline "Note for Guidance on Ethnic Factors in the Acceptability of Foreign Clinical Data" (CPMP/ICH 1998), issued by the European Agency for the Evaluation of Medicinal Products, recommendations are given for evaluation of the impact of ethnic factors. They divide drugs into those that are less likely to be sensitive
to ethnic factors and those that are more likely to be sensitive to ethnic factors. Factors rendering a drug sensitive to ethnic factors are, e.g. steep pharmacodynamic curve for efficacy and safety, extensively metabolised, metabolism by polymorphic enzymes, low bioavailability (more susceptible to dietary absorption effects) and high likelihood of use in a setting of multiple co-medications.
3.8 HMG-CoA Reductase Inhibitors (Statin)

A major breakthrough in the pharmacological treatment of hypercholesterolaemia has been the introduction of inhibitors of 3-hydroxy-3-methyl-glutaryl coenzyme A (HMG-CoA) reductase (statins). The progression of atherosclerosis can be efficiently delayed by treatment with statins with or without concomitant therapy with resins, fibrates or nicotinic acid derivatives. (Superko and Krauss., 1994; Levine et al., 1995; Gotto., 1995) Indeed, an actual regression of atherosclerosis was observed in a substantial number of subjects. Furthermore, there was a reduction in complications of atherosclerosis and this was even significant in occasional studies inspite of limited sample sizes and follow-up periods. (Brown et al., 1993) Consequently, correction of hypercholesterolaemia with statins leads not only to reversal of the atherosclerotic process itself but also to appreciable reductions in clinical complications.

A number of studies of the clinical use of statins have been described. In general, they significantly reduced cardiovascular-related morbidity and mortality in patients with and without CHD. (Pedersen et al., 1994, Shepherd et al., 1995, Sacks et al., 1996; Downs et al., 1998) Large intervention trials have reported a positive outcome from statin therapy in primary as well as secondary prevention. Subsequently, statins have been recommended as first-line therapy for hypercholesterolaemia. (Pedersen et al., 1994; Shepherd et al., 1995) Results from a secondary prevention trial showed that a reduction in LDL-cholesterol of 28% and in major CHD events of 24% was achieved on pravastatin 40mg daily, which indicated that benefit can be obtained in subjects with rather normal cholesterol levels. (Sacks et al., 1996) Statins have been shown to slow the progression or even promote regression of coronary atherosclerosis. This results in a reduction of new lesions and total artery occlusions when compared with untreated hypercholesterolaemic patients. (Vaughan et al., 1999; Christians et
al., 1998) This has been suggested to be a consequence of a reduced lipid core of the atherosclerotic plaque, which avoids plaque rupture that would otherwise trigger intramural haemorrhage and intraluminal thrombosis. Statins also show other beneficial cardiovascular effects independently of their lipid-modifying properties. (Corsini et al., 1999) These pleiotropic effects may be explained by inhibition of synthesis of non-sterol isoprenoid compounds, which also are formed from mevalonic acid, (Christians et al., 1998) and include improvement of endothelial cell function, modification of inflammatory responses, and reduction of smooth muscle cell proliferation and cholesterol accumulation. (Corsini et al., 1999) Recently, it has been reported from animal studies that pravastatin inhibits vascular inflammation and atherosclerosis by actions independent of lipid-lowering effects, possibly involving increased endothelial NO synthase activity and inhibition of Rho activity. (Ni et al., 2001)

Treatment of hypercholesterolemia is life-long, and the drugs will be used in patients who will never encounter a CHD endpoint in spite of an increased statistical risk. Therefore, useful drugs must be effective, safe and well tolerated. The clinical benefit and safety of lipid-lowering drugs are best documented for the statins, and they are also better tolerated than any other drugs used for treating dyslipidemia. However, safety issues arise in these patients due to drug-drug interactions, and this is a crucial issue for future safe use of these drugs as patients with hypercholesterolaemia often have co-existing disease and are exposed to multiple drug therapy. For atorvastatin and other statins, the liver is the primary site of action, and accordingly the oral dose administered, rather than plasma exposure, will predict the pharmacological response. However, increased systemic exposure of active drug or metabolites may increase the risk for clinically important adverse events. Therefore, there is a need for better characterization of the pharmacokinetics of the statins, particularly the mechanisms behind
the drug interactions that may cause serious adverse effects, such as myopathy or rhabdomyolysis.

3.8.1 Pharmacodynamics

Statins represent the most efficient drugs for treatment of hypercholesterolaemia and have significantly affected the management of lipid disorders. They reduce plasma lipid concentrations and are generally considered to be safe. Plasma levels of LDL-cholesterol are lowered by inhibition of HMG CoA reductase, which affects endogenous cholesterol synthesis and subsequent increases the expression of the LDL receptor. This results in an upregulated catabolic rate for LDL-cholesterol. (Lennernet al., 1997) The effect of statins is not closely related to the plasma concentrations of the drug and its active metabolites. Instead, several reports have shown that the pharmacological response was better correlated with the daily dosage of statins. (Cilla et al., 1996a; Lennernet al., 1997) This observation is not surprising, when considering that the liver is the main site of HMG-CoA reductase action. As a consequence, liver concentrations of statins and active metabolite(s) (i.e. total active inhibitors) might better correlate with pharmacological effects on plasma LDL-cholesterol.

Unfortunately, such data are not available and they are difficult to model because of the complex interplay among processes such as portal drug concentrations, plasma protein and liver tissue binding, hepatic uptake, intracellular metabolism and secretion into bile. Even if the plasma concentration of atorvastatin is a poor predictor of the effect, steady-state dosages and relevant potency are used as a robust comparator between different statins in human trials. (Cilla et al., 1996b; Lennernet al., 1997; Malhotra and Goa, 2001) It has been reported that statins produce an immediate change in the biochemical profile of patients.
In a study with healthy subjects (a double-blind parallel-group design), atorvastatin was given once or twice daily for 14 days, with rising single and multiple doses of 2.5–80 mg/day (Cilia et al., 1996b). Atorvastatin was well tolerated up to 80 mg/day and had significant cholesterol-lowering effects. The most common adverse events reported were headache and nausea, which occurred as frequently as after placebo administration. Reductions in mean total cholesterol and LDL-cholesterol ranged from 13–22% to 45–58% after 2.5 and 80 mg/day, respectively. (Cilia et al., 1996b) The efficacy of atorvastatin was better predicted by drug dose rather than by peak concentration (Cmax) and AUC, despite these parameters being based on plasma equivalent concentrations of atorvastatin (total inhibitors). *In vivo* atorvastatin is metabolised by cytochrome P450 (CYP) 3A4 to two active metabolites, 2-hydroxy-atorvastatin acid and 4-hydroxy-atorvastatin acid, both of which are in equilibrium with their inactive lactone forms. (Kantola et al., 1998) It has also been reported that about 70% of the circulating inhibitory activity for HMG-CoA reductase is attributable to these active metabolites (Kantola et al., 1998).

Earlier investigations suggested that statins exert beneficial effects beyond their favourable lipid-lowering effects. Modification of thrombus formation and degradation, alteration in inflammatory response, plaque stabilisation and improved endothelial function are thought to be responsible for additional reduction of morbidity and mortality due to cardiovascular events. However, it is still unclear whether these effects are mediated by all statins and their active metabolites. A randomised, double blind clinical study investigated the effects of three statins (atorvastatin, simvastatin and pravastatin at equipotent cholesterol-lowering dosages) on haemostatic and inflammatory markers in 99 hypercholesterolaemic patients over 3 months of treatment. (Joukhadar et al., 2001) The short-term effects of atorvastatin, simvastatin and pravastatin on selected haemostatic and inflammatory parameters were similar (von Willebrand factor antigen, fibrinogen, d-dimer,
prothrombin fragment 1 + 2 and C-reactive protein). Thus, chemical and pharmacological differences between statins appear to exert no major influence on these parameters. (Joukhadar et al., 2001)

3.8.2 Pharmacokinetics
In general, oral bioavailability of a drug is affected by factors such as dissolution rate, stability issues in the lumen, transit time, intestinal permeability, and the first-pass effect (metabolism, hepatic uptake and biliary secretion) in the gut and liver. (Amidon et al., 1995) The solubility of atorvastatin acid is high at pH 6.0, which is the physiologically relevant intestinal pH. (Kearney et al., 1993) Consequently, after doses of between 10 and 80mg, the estimated maximal concentrations in the gut lumen of atorvastatin acid will be 70–550 μmol/L. (Amidon et al., 1995; Wu et al., 2000)

3.8.2.1 Plasma Protein Binding and Distribution
Volume of distribution for atorvastatin acid was 381L, determined following administration of 5mg as an intravenous infusion, with the plasma concentrations measured with a specific GC-MS method. (Gibson et al., 1997) The plasma protein binding has been reported to be >95%, >98% or 80–90%. (Christians et al., 1998; Gibson et al., 1997, Malhotra and Goa, 2001) Vd exceeds total body water, which clearly indicates that peripheral tissue binding is extensive. Consequently, the mass transport of atorvastatin acid into tissue is high, which is in accordance with its high membrane permeability, due to the nonpolar nature of the drug, and a plasma protein binding not exceeding 99%. (Ishigami et al., 2001) There is no data available for the plasma protein binding and distribution of the two active metabolites. There is no risk for displacement interactions plasma protein for any of the statins since they are only given by oral administration.
3.8.2.2 Metabolism and Elimination

Atorvastatin acid probably undergoes complete metabolism, and the major organ for elimination seems to be the liver, even if gut wall metabolism makes a significant contribution to the first-pass effect. After a Single-dose study of (14C) atorvastatin in patients with a T-tube, it was found that the biliary route was the major route of elimination for the drug and its metabolites. (Le Couteur, et al., 1996)

The renal route is a minor route for the elimination of atorvastatin and its metabolites in humans, as only about 1% of orally administered radioactive atorvastatin was excreted in urine in a mass balance study. (Stern et al., 1997)

This clearly demonstrates that the elimination of atorvastatin acid and its metabolites predominantly occurs in the liver. Plasma half-life of active HMG-CoA reductase inhibitors was 13–16 hours (Kantola et al., 1998; Lilja et al., 1999). The half-life in plasma of total radioactivity has been reported to be approximately 60 hours, indicating the presence of long-lived inactive metabolites (Christians et al., 1998). The clearance of atorvastatin acid is about 625 mL/min, which corresponds to an EH of 0.4. This classifies atorvastatin acid as a drug with an intermediate liver extraction. Two active metabolites have been detected in plasma, 2-hydroxy-atorvastatin acid and 4-hydroxy-atorvastatin acid, both of which are in equilibrium with their inactive lactone forms (Kantola et al., 1998; Jacobsen et al., 2000) 2-Hydroxy-atorvastatin acid and its lactone form are the dominant metabolites detected in plasma (Kantola et al., 1998, Lilja et al., 1999).

3.8.2.3 Effect of Age and Sex

The influence of age and sex on the pharmacokinetics of atorvastatin after a single oral dose of 20mg was investigated in 16 young and 16 elderly volunteers (8 men and 8 women in each age group) (Gibson et al., 1996). Plasma equivalent concentrations of atorvastatin were measured by enzyme inhibition bioassay. The Cmax of atorvastatin
equivalents was 42.5% higher in elderly (age 66–92 years) than in young (age 19–35 years) participants, and 17.6% higher in women than in men. In addition, AUC and elimination half-life were 27.3% greater and 36.2% longer, respectively, in elderly adults than in young adults, and 11.3% lower and 19.9% shorter, respectively, in women than in men. (Gibson et al., 1996) Because of the use of an unspecific assay it is unclear what mechanism(s) explain these age- and sex-related differences in pharmacokinetics, although it is known that drugs are frequently metabolised faster in women than in men (Cummins et al., 2002). This has been related to enzyme activity.

Nineteen subjects aged 18–65 years with calculated creatinine clearances ranging from 13 to 143 mL/min were administered atorvastatin 10 mg orally daily for 2 weeks. It was clear from this study that renal impairment had no significant effect on the pharmacokinetics and pharmacodynamics of atorvastatin (Stern et al., 1997).

3.8.2.4 Diurnal variation
The diurnal variation of cholesterol synthesis leads to the recommendation that statins when administered only once a day should be taken at night (Cilia et al., 1996a). In a study by Cilia et al., the pharmacodynamics and pharmacokinetics of atorvastatin equivalents were investigated in 16 normolipidaemic subjects after administration of 40mg daily for 15 days in the morning or evening (Cilia et al., 1996a). The effects on lipid and apolipoprotein values were similar after morning and evening administration of atorvastatin. Cmax and tmax of atorvastatin equivalents were 95.0 versus 65.9 μg-equiv/L and 1.9 and 2.9 hours after morning and evening administration, respectively. The plasma AUC for atorvastatin equivalents was also higher after the morning than the evening dose, 649 versus 461 μg-equiv h/L, respectively, but the values of elimination half-life were
similar (Cilla et al., 1996a). The authors claimed that morning and evening administration of atorvastatin 40mg are equally effective in decreasing total and LDL-cholesterol levels, as the pharmacokinetic differences did not correlate with effects on serum lipids (Cilla et al., 1996a).

3.8.3 Novel Probe of CYP3A4:

Atorvastatin is used in the treatment of hypercholesterolemia. Atorvastatin, a HMG CoA reductase inhibitors reduce blood cholesterol concentrations by competitive inhibition of HMG-CoA reductase, a rate-limiting factor in cholesterol biosynthesis. In vitro studies have indicated that after incubation of atorvastatin acid with human microsomes, para-hydroxy and ortho-hydroxy atorvastatin are formed (Michniewicz et al., 1994; Christians et al., 1998). As shown by using specific cytochrome P450 (CYP) inhibitors and isolated CYP enzymes, CYP3A4 is the major enzyme involved in formation of the two metabolites. In the case of atorvastatin, CYP3A4 plays a central role in its metabolism (Bolego et al., 2002) Grapefruit juice, which is known to inhibit the activity of intestinal CYP3A4 significantly increased plasma concentrations of atorvastatin (Lilja et al., 1999). A significant increase in the plasma concentration of atorvastatin has also been reported after the concomitant use of itraconazole or erythromycin, both of which had marked inhibitory effects on CYP3A4 substrates (Kantola et al., 1998; Siedlik et al., 1999) Therefore, it is plausible that variation in the CYP3A4 gene may lead to variation in CYP3A4 activity and, in turn, to the differences in drug metabolism. This is further confirmed by Rifampin which is a known potent inducers of cytochrome P450 3A4, it markedly decreases the plasma concentrations of atorvastatin and its metabolites (Janne et al., 2005).

Accordingly, statins have been accepted sensitive probe drug for CYP3A4 activity by the U.S.Food and Drug Administration (FDA
There are data suggesting that simvastatin is proportionally metabolized by other metabolic pathways in addition to CYP3A, and therefore simvastatin may not be an ideal CYP3A probe.

Approximately 20% of the metabolism of simvastatin can be attributed to the polymorphic enzyme CYP2C8 (Ellen et al., 2006). Atorvastatin was considered as a suitable probe drug for this study also because this fulfilled major requirement of probe validation proposed by Watkins (1994) and modified by Zaigler (2000). Moreover it is very well tolerated (Connie et al., 2003) and free from radioactivity and phenotyping is technically easy.
3.9 Analytical Methods for estimation of atorvastatin:

3.9.1 Invitro studies (Thomayant et al., 2002)
Metabolic properties of statins in human hepatic microsomes was studied in which simvastatin (SVA), cerivastatin (CVA), atorvastatin (AVA), and their metabolites were analyzed using HPLC methods (Pruksaritanont et al., 1999). In brief, samples held in an autosampler set at 5°C were chromatographed on a C18 Zorbax column (150 X 4.6 mm, 5 µm; Waters, Inc., Milford, MA) preceded by a C18 guard column, with a linear gradient of ACN and 25 mM ammonium acetate, pH 4.5. The eluate was monitored by UV absorption at 240 nm (SVA and AVA) or 280 nm (CVA). Due to the unavailability of authentic standards for glucuronide conjugates of statins, quantitation of these metabolites in the in vitro incubation mixtures was accomplished using standard curves for their respective parent statins, assuming identical extraction recoveries and extinction coefficients between the parent drug and its corresponding glucuronide conjugate. For the three statins, standard curves showed satisfactory linearity and precision (15% coefficient of variation). The limits of assay detection were 5 pmol (on column) for all three statins.

Identification of the statin metabolites was accomplished by using LC-MS techniques (HP-1050 gradient system; Hewlett Packard, San Fernando, CA; Finnigan MAT LCQ ion trap mass spectrometer; Thermo Finnigan MAT, San Jose, CA). Separation of the metabolites was carried out on a Betasil C18 column (2 _ 150 mm, 5 _m), with a linear gradient of ACN and 0.1% formic acid (30% ACN to 80% ACN in 20 min) delivered at a constant flow rate of 0.2 ml/min. Mass spectral analyses were performed using electrospray ionization in the negative ion mode (for SVA and AVA glucuronide conjugates) or positive ion mode (for statin lactones and CVA glucuronide). The electrospray ionization voltage was set at 4 kV, with the heated capillary
temperature held at 230°C. For estimation of atorvastatin and its metabolites, NMR was used as a detector.

For NMR studies, the dried extract from in vitro incubates containing the metabolite were reconstituted in approximately 250 µl of 30% ACN/70% water in 1 mM ammonium acetate, pH 4.5 (SVA), or 10% ACN/90% water/0.1% CF3COOD (AVA and CVA) before injection. All NMR spectra were acquired under stopped-flow conditions. Once the apex of the metabolite peak was detected, the HPLC pump was stopped after a precalibrated delay time, at the end of which the metabolite was located in the NMR flow cell. The HPLC conditions were optimized such that the LC peak volume matched that of the NMR flow cell volume (60 µl) to maximize the signal-to-noise ratio of the NMR spectrum. Deuterated mobile phase was used for all LC-NMR runs, and no solvent suppression techniques were applied. The following HPLC conditions were used for LC-NMR studies: Symmetry C18, 5-µm, 3.9 X150-mm column (SVA) or Phenomenex phenylhexyl, 5µm, 2 X150-mm column (AVA and CVA); flow rate, 1.0 ml/min (SVA) or 0.3 ml/min (CVA and AVA); and UV detection at 239 nm (SVA), 244 nm (AVA), or 283 nm (CVA). Separation of glucuronide metabolites from parent statins was achieved using the gradient conditions. The parent LC-NMR spectra were obtained by injecting 25 µg of the corresponding statin under the same LC conditions as used for the metabolite. 1H chemical shifts (in parts per million) are referenced relative to residual CD2HCN at 1.99 ppm. NMR spectra were obtained using an Inova (11.7 T/500 MHz) 51-mm, narrow-bore spectrometer (Varian, Inc., Palo Alto, CA) equipped with a 60µl flow.

In an in vitro study, to compare the cytochrome P450 (CYP)-dependent metabolism and drug interactions of the acid and lactone forms of the 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase atorvastatin analysis was done after protein precipitation by addition of 0.4 ml of ice-cold acetonitrile containing the corresponding internal standard to the
reaction mixtures (vide supra), samples were mixed on a vortex for 10 s and centrifuged at 4°C and 10,900g for 10 min. Supernatants were transferred into 1.8-ml HPLC brown vials (Hewlett-Packard, Palo Alto, CA) and were kept in the temperature-controlled autosampler at 14°C until analysis. Atorvastatin, atorvastatin lactone, and their metabolites were quantified by HPLC/electrospray-MS in combination with an on-line column switching extraction step using a Hewlett-Packard series HP1100 liquid chromatograph consisting of a G1313A autosampler in combination with a G1330A thermostat, a G1312A binary pump, a G1322A degasser, a G1316A column thermostat, and a G1946A mass selective detector (all components Hewlett-Packard, Palo Alto, CA). Solvent for the on-line extraction column was delivered by an additional quaternary G1311A HPLC pump. The HPLC/MS system was controlled, and data were processed using ChemStation software revision C.06.02 (Hewlett-Packard). The column switching LC/LC-MS system was set up as described in detail by Christians et al., 2000. In brief, 15 ml of the extracted sample was injected into the LC/LC-MS system. Samples were loaded onto the 20 3 4 mm extraction column (Hewlett-Packard) filled with Hypersil MOS of 5 mm particle size (Shandon, Chadwick, UK) and washed using a mobile phase consisting of 2 mM ammonium acetate. The flow was 6 ml/min. After 1 min, the switching valve (model 7240, Rheodyne, Cotati, CA) was activated, and the analytes were eluted in the backflush mode from the extraction column onto a 100 3 2.1 mm analytical column filled with Hypersil ODS of 5 mm particle size (Hewlett-Packard). After 4 min, the switching valve was activated again, and the extraction column was cleaned with acetonitrile/2 mM ammonium acetate (9:1, v/v) (2 ml/min, 2 min) and re-equilibrated to the starting conditions.

The mobile phase of the analytical column consisted of 0.01% formic acid and acetonitrile. The following gradient was run: 0 min, 50% acetonitrile; 1 min, 50% acetonitrile; 8 min, 70% acetonitrile. For the following 1.9 min, the column was washed with 95% acetonitrile and
was re-equilibrated to the starting conditions for 2 min. The flow was 0.5 ml/min, and the column temperature was 40°C. The mass selective detector was adjusted to the following parameters (nomenclature according to the ChemStation software): nebulizer gas: nitrogen of grade 5.0 purity (Bay Airgas, Hayward, CA), 30 p.s.i. (5207 kPa); drying gas nitrogen, 5.0; flow, 7 l/min; 300°C; capillary voltage (Vcap), 26000 V; ion energy (octopole), 15V; quadrupole temperature, 100°C; capillary exit (fragmentor) voltage, 1100 V for atorvastatin, atorvastatin lactone, and their metabolites and 130 V for the internal standards, mevastatin and mevastatin acid. Positive ions (M+H)\(_1\) of atorvastatin, atorvastatin lactone, and their metabolites as well for the internal standards gave the best signal-to-noise ratio and were recorded in the single ion mode: m/z 5 391 for the internal standard mevastatin (retention time: mevastatin, 4.5 min; mevastatin lactone, 6.5 min); m/z 5 559.2 for atorvastatin retention time, 4.6 min); m/z 5 575.2 for both para- and ortho-hydroxy atorvastatin (retention time, 3.0/4.4 min); m/z 5 541.2 for atorvastatin lactone (retention time, 6.3 min); and m/z 5 557.2 for both para- and ortho-hydroxy atorvastatin lactone (retention time, 4.0/5.8 min) (Fig. 2). The dwell time for each ion was 116 ms. The assay was validated according to the guidelines of good laboratory practice and had the following specifications: lower limit of quantitation, 0.5 mg/l (atorvastatin and atorvastatin lactone) or 1 mg/l (their metabolites); linearity, 0.5 (1.0) to 500 mg/l (r\(^2\) = 0.998); interassay variability (3 days, three concentrations, each n 5 9), #9% for para-hydroxy atorvastatin, #5.4% for ortho-hydroxy atorvastatin, #11.1% for atorvastatin, #10.9% for para-hydroxy atorvastatin lactone, #10% for ortho-hydroxy atorvastatin lactone, and #9.8% for atorvastatin lactone. The recovery of atorvastatin, atorvastatin lactone, and their metabolites was .90%. Within-batch stability of extracted samples was established for at least 24 h at 14°C, and all samples were analyzed within this time period (Wolfgang et al., 2000).
3.9.2 Estimation of Atorvastatin in *In Vivo* studies in Plasma and Aqueous Samples

3.9.2.1 Radioimmunoassay (*Louis et al., 1995*)

In an attempt to study the effect of food on bioavailability of atorvastatin plasma equivalent atorvastatin concentrations were quantitated using a validated, unpublished radio immunoassay procedure employing solid-phase extraction. Determination of recovery of atorvastatin from the solid-phase extraction cartridges was unnecessary, as calibration standards and quality controls were treated similarly. In brief, 600 ml of standard, unknown, or quality control sample plasma were mixed with an equal volume of potassium phosphate buffer, pH 4.0. One milliliter of diluted plasma was then applied to a 3-mL Bond Elut C-18 solid-phase extraction cartridge (Varian, Harbor City, CA) previously conditioned with methanol and water. Atorvastatin was eluted from the cartridge with acetonitrile. Eluent was evaporated to dryness and the residue reconstituted in phosphate buffer, pH 7.4, containing 0.1% pigskin gelatin. Atorvastatin plasma equivalent concentrations were determined by displacement of (125I)-atorvastatin from the antibody by unlabeled drug. The minimum quantification limit was 0.35 ng/mL; concentrations below this limit were reported as zero. Assay precision, expressed as percent relative standard deviation, ranged from 6.0% to 32.9% for calibration standards (0.350, 0.700, 1.40, 2.80, and 5.60 ng/mL) and from 5.6% to 18.1% for quality control samples (0.600, 1.50, and 30.0 ng/mL). Accuracy, expressed as percent relative error, ranged from -5.5% to 5.0% for quality control samples. Plasma atorvastatin concentrations were linear over the range of 0.350 to 5.60 ng/mL (logit-log transformed data). To date, at least 3 unknown radioactive peaks have been observed in human plasma after oral (14C)-atorvastatin. However, the extremely low levels of radioactivity precluded structural identification of these metabolites.
3.9.2.2 Enzyme Inhibition Bioassay (*Donald et al.*, 1996)

In another study to see the effect of age and gender on pharmacokinetics of atorvastatin the plasma samples were analyzed for equivalent concentrations of atorvastatin by means of a validated enzyme inhibition bioassay procedure. Atorvastatin was isolated from 0.25 mL of human plasma by protein precipitation using acetonitrile: acetone (95:5). The supernatant (700 L) was evaporated to dryness under nitrogen and the residue was reconstituted with distilled water (50 tL). The reconstituted mixture was incubated with a buffer solution containing (14C) HMG-CoA, cofactors, and cloned human HMG-CoA reductase. The (14C)mevalonate produced was lactonized under acidic pH to yield (14C)- mevalonolactone and isolated on an AG 1-X8 anion exchange column. (14C) Mevalonolactone was quantitated by liquid scintillation spectrometry. The relationship between HMG-CoA reductase inhibition and atorvastatin concentration was used to construct a calibration curve. Atorvastatin metabolites and/or endogenous materials in plasma capable of inhibiting HMG-CoA reductase were quantified. Atorvastatin concentration is therefore expressed in terms of atorvastatin equivalents. The lower limit of quantification was 0.36 ng eq/mL; concentrations below this limit were assigned a value of zero. Assay precision, expressed as relative standard deviation (%RSD) of calibration standards (0.36, 0.50, 1.0, 2.0, 3.0, 6.0, 12.0, and 16.0 ng eq/mL) and quality control samples (0.65, 3.1, and 9.1 ng eq/mL) during study sample analysis, ranged from 1.03% to 9.65%. Experimentally determined concentrations of atorvastatin in quality control samples ranged from 88.5% to 112% of nominal values. (14C)atorvastatin recovery from human plasma at concentrations of 8.6 and 36 ng eq/mL was 94.7%. There were least three radioactive peaks had been observed in human plasma after administration of (14C)atorvastatin. Structural identification of atorvastatin metabolites was not determined because of low concentrations in human plasma.
These methods were used when estimation of atorvastatin was not possible as there were no specific methods available and availability of sophisticated instrument was very poor. This was an indirect method to estimate the concentration of atorvastatin in plasma.

As Analytical Chemistry progressed and new-sophisticated instruments like HPLC were available, the estimation method of atorvastatin also changed.

It was now possible to estimate drug to a very lower concentration.

With a aim to Determination of cholesterol-lowering statin drugs in aqueous samples using liquid chromatography-electrospray ionization tandem mass spectrometry Analytes were separated using a Waters 2695 liquid chromatograph (Waters, Milford, MA, USA) with a Genesis C column (2.1350 mm, 3 mm) 18 (Jones Chromatography, Hengoed, UK). The two mobile phase solvents, A and B, were acetonitrile and water, respectively, containing 2 mM methylamine with 0.1% acetate acid. The mobile phase gradient was started at 60% of A, which was increased linearly to 100% within 3 min and held for 2 min. The flow-rate of the mobile phase was 0.2 ml/min and the injection volume was 20 ml.

SPE cartridges with different packing materials, HLB, Bond Elute C8 and DSC-18 were tested for the best recovery of the analytes. The cartridges were installed on a vacuum manifold and sequentially preconditioned with 6 ml acetone, 6 ml methanol and 6 ml HPLC grade water (pH 4.5). Thereafter, the aqueous samples (500 ml STP influent, 1000 ml STP effluent and 2000 ml surface water) were allowed to pass slowly through the cartridges at a rate of approximately 10 ml/min. After passage of the samples, each sample bottle was rinsed with 10 ml of pH 4.5 HPLC grade water, and the rinse was allowed to flow through the cartridge. The cartridges were eluted using three successive 3-ml
aliquots of methanol. Each aliquot of methanol was eluted through the column for a minimum of 10 min. The eluates were collected in a 10-ml collection tube and concentrated to almost dryness with a vacuum centrifuge. Then the samples were reconstituted to 1.0 ml with acetonitrile: aqueous methylammonium acetate (3:2, 2 mM methylammonium acetate, pH 4.5).

An official method for atorvastatin determination has not yet been described in any pharmacopoeia. In the one study, a new, simple, and selective HPLC method was applied to the determination of atorvastatin in bulk drug, tablets, and spiked human plasma. The HPLC system consisted of a membrane degasser, binary solvent delivery system, a Rheodyne injector equipped with a 20mL sample loop, and UV/VIS detector (1100 Series, Agilent Technologies, USA). The detection wavelength was at 240 nm, and the peak areas were integrated automatically with Windows NT based LC ChemStation Software. The chromatographic analysis was performed at ambient temperature onto a Supelcosil C18 column (150 X 4.6mm.i.d, 5μm particle size) and a mobile phase composed of acetonitrile: methanol: water (45: 45: 10 v/v/v). The flow rate was maintained at 1.0mL/min.

Standard solutions (1.0 mg/mL) of atorvastatin and ibuprofen were prepared daily by dissolving appropriate amounts of these substances in methanol. Stored at 5°C in the dark, these solutions were shown to be stable during the period of study. Working standard solutions containing 0.5- 86.0 mg/mL of atorvastatin and 40.0 mg/mL of ibuprofen (I.S.) were prepared in methanol. A volume of 20 mL of each sample was injected into the column. All measurements were repeated five times for each concentration. The calibration curve was constructed by plotting the peak area ratios of analyte to I.S. vs. the corresponding drug concentration.
In case of human plasma Trichloroacetic acid, perchloric acid, sulphuric acid, ethanol, and acetonitrile were tested in order to precipitate human plasma proteins. Acetonitrile was found to be the best precipitant, because when this substance was used in small volumes the precipitation was successfully completed. Acetonitrile (1.0 mL) was added to human plasma (0.5 mL) containing standard solutions of atorvastatin and I.S. Addition of acetonitrile prevents atorvastatin binding to proteins and coagulate plasma proteins. The mixtures were then vortexed for 10 min. After deproteinization and centrifugation of samples for 15 min at 6000 rpm, supernatant (0.5 mL) was taken carefully and analyzed as described above. No anticoagulant was used in these proposed methods.

Validations of the procedures for the quantitative assay of the drug were examined via evaluation of the limit of detection (LOD), limit of quantitation (LOQ), repeatability, recovery, specificity, and robustness. The LOD and LOQ were calculated from the calibration curves as kSD/b, where k = 3 for LOD and 10 for LOQ, SD is the standard deviation of the intercept and b is the slope of the calibration curve. The values of LOD and LOQ were 0.0084 and 0.0179 mg/mL, respectively (Altuntas et al., 2004).

In an attempt to check whether Itraconazole alters the pharmacokinetics of atorvastatin the analysis of drug was done where serum concentrations of atorvastatin, atorvastatin lactone, and 2-hydroxy atorvastatin were determined by liquid chromatography-tandem mass spectrometry (LC/MS/MS). Serum samples (0.25 mL) were precipitated with acidified acetonitrile containing an internal standard (the M1 metabolite of cerivastatin). The clear supernatant was analyzed by HPLC with a YMC J' sphere column (Waters Corporation, Milford, Mass) (4 μm, 2.0 X150 mm) with ammonium acetate (pH 3.0)/acetonitrile as the mobile phase. Column effluent was delivered to an API 3000 mass spectrometer (PE Sciex., Foster City, Calif) by use of
a turboion spray in the negative mode. The ion transitions monitored were m/z 557.0 to 396.9 for atorvastatin, m/z 521.0 to 396.9 for atorvastatin lactone, m/z 573.1 to 133.6 for 2-hydroxy atorvastatin, and m/z 444.2 to 309.9 for internal standard. For all of the analytes, the standard curves ranged from 0.5 ng/mL to 50 ng/mL. For all analytes intra-day and inter-day precision ranged within 0.54% to 11.02%, and accuracy ranged within 80.2% to 114.3% (Arthur et al., 2000).