The aim of science is not to open the door to infinite wisdom, but to set a limit to infinite error

Introduction
INTRODUCTION:

Atorvastatin is a selective, competitive inhibitor of HMG-CoA reductase, the rate-limiting enzyme that converts 3-hydroxy-3-methylglutaryl-coenzyme A to mevalonate, a precursor of sterols, including cholesterol. Thus it's used in the treatment of hypercholesterolemia. (Superko and Krauss et al 1994; Levine et al., 1995; Gotto 1995). Atorvastatin is rapidly absorbed after oral administration; maximum plasma concentrations occur within 1 to 2 hours. Extent of absorption increases in proportion to atorvastatin dose (Amidon et al., 1995). As there is a difference in the rate of absorption but not in the extent of absorption of atorvastatin and there is excessive variability in bioavailability from subject to subject (Hans et al., 2003). Bioequivalence of atorvastatin formulations was done by average bioequivalence, or the bootstrap-type method, (Koytchev et al., 2004). The trial was performed according to an open, crossover design with a wash-out period of 7 days in one study center. They have used 10mg dose of atorvastatin and measured the concentration of atorvastatin over a period of time after single drug administration.

The interchangeability of a multi-source pharmaceutical product should result from a). pharmaceutical equivalence; b). bioequivalence; and c). therapeutic equivalence. The bioequivalence of 2 formulations of the same product is generally established by evaluation of the bioavailability and pharmacokinetics after a single dose of each is administered. In general, single-dose studies would suffice for the purpose of assessing the relative bioavailability and bioequivalence of two formulations.(USFDA 2002; EMEA 2001) According to the FDA, (2002) single-dose studies are considered to be more sensitive for addressing the primary question of bioequivalence (i.e., release of drug substance from the drug product into the systemic circulation) and
thus multiple-dose studies are generally not recommended. However, drugs used in the prophylaxis or treatment of chronic disease like hypercholesterolemia (atorvastatin), schizophrenia (aripiprazole) and Alzheimer's disease (rivastigmine) and recurrent infections like cystic fibrosis (levofloxacin) are almost always administered on a long-term basis a steady state study is required. (Xiao et al., 2006; Joshua et al., 2006; Manjunath et al., 2006)

Thus the bioequivalence of atorvastatin, which is used on a long-term basis should be with a multiple-dose experimental model that more closely represents therapeutic use.

Further Levy (1972) recommended comparative bioavailability assessments based on steady-state plasma concentrations that offer several advantages over single-dose studies. In vivo atorvastatin is metabolised by cytochrome P450 (CYP) 3A4 to two active metabolites, 2-hydroxy-atorvastatin acid and 4-hydroxy-atorvastatin acid. 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). In case of atorvastatin, the primary metabolites formed are active and might substantially exceed the parent compound in terms of concentration and/or activity. In these cases, it is imperative to elucidate the pharmacokinetics of the metabolite in order to understand and predict expected changes in active metabolite concentration and/or activity. This is especially important when the parent compound is administered on a long-term basis. During repeated administration, parent compound concentrations might reach steady state but the metabolite may continue to accumulate. Therefore, it is important to assess the accumulation index of both the parent compound and the active metabolite. Moreover the currently valid CPMP Note for guidance on the investigation of Bioavailability and Bioequivalence recommends to measure both parent drug and active
metabolite plasma concentrations and evaluate them separately (CPMP/ICH 2004).

Thus in the above light the aim of the present work was to perform bioequivalence study of two formulations i.e Test and Reference by steady state model and measure bioequivalence with both of its metabolites.

Despite that the fact that HMG-CoA inhibitors seem to be structurally similar, analytical methods for their quantitative determination in blood have been developed individually for each drug because of their different solubility, stability and optic characteristics. Generally, hyperlipidemic patients are treated with multiple-drug regime and some patients may benefit from combination treatment of antihyperlipidemic agents. Since statins are not used with other statins simultaneously, there is no need for simultaneous analysis of statins.

There are various methods available for the detection of atorvastatin in plasma like in an attempt to study the effect of food on bioavailability of atorvastatin plasma equivalent atorvastatin concentrations were quantitated using a validated, unpublished radioimmunoassay. They employed solid-phase extraction. Determination of recovery of atorvastatin from the solid-phase extraction cartridges was not found necessary, as calibration standards and quality controls were treated similarly. (Louis et al., 1995) 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 concentration was therefore expressed in terms of atorvastatin equivalents. In this method atorvastatin was isolated from 0.25 mL of human plasma by protein precipitation using acetonitrile. (Donald et al., 1996)
Moreover metabolic properties of statins in human hepatic microsomes was studied in which simvastatin, cerivastatin, atorvastatin, and their metabolites were analyzed using HPLC methods (Prueksaritanont et al., 1999). 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.

In order 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). Protein precipitation was done by addition of 0.4 ml of ice-cold acetonitrile containing the corresponding internal standard to the reaction mixtures (Arthur et al., 2000). In various methods mentioned above some purification steps have been used before the samples are injected to chromatographic system as liquid–liquid extraction, solid-phase extraction, etc. Moreover, some derivatization procedures have been used to decrease sensitivity in some of the statin assay. Internal standards leading to more correct results in analytical methods have not been used in some of the assays. However, in most of the assays one of the statins has been chosen as internal standard owing to the similar structures of the substances. Thus aim of present study was to Develop and validate a method for the detection of atorvastatin and its metabolites in plasma.

Variability in drug effects has been investigated since the 1950s (Evans and McLeod 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 (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. 7 to 22% of randomly selected drugs were metabolised by polymorphic enzymes (Phillips et al., 2001; Pirmohamed et al., 2001; Pirmohamed and Park, 2003).

During the Second World War it was noticed that it was mostly black American soldiers who suffered from hemolysis when using the antimalarial drug primaquine. This was later explained to be due to 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). 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). Thus 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). 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). Thus 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).
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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). 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 (Tishkoff and Verrelli, 2003). It has been suggested that genetic variability account for approximately 90% of the inter-individual differences in hepatic CYP3A activity, based on an analysis of inter- and intra-subject variability in the elimination of CYP3A-selective probes (Ozdemir, et al., 2000). 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).

It is seen that there is a significant correspondence between linguistic superfamilies and major genetic clusters have 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.

A "phenotype" is the sum of genetic, physiological, and environmental factors, determination of phenotype is assumed to be a better predictor
of a patient's abilities to metabolize certain agents than genotype alone. Drugs, which are primarily metabolized by CYPs, may be first choice compounds for a CYP-phenotyping test. The phenotyping procedure is straightforward by giving the patient a "probe"-drug, (which is ideally a simple, safe, cheap and easily administered and detectable compound), and which is metabolized by the enzyme in question. The cytochrome P450 (CYP) superfamily comprises a large number of enzymes that are often critical for the oxidative metabolism of endogenous and exogenous compounds (Nelson et al., 1996). 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). It has been suggested that genetic variability accounts for approximately 90% of the inter-individual differences in hepatic CYP3A activity, based on an analysis of inter- and intra-subject variability in the elimination of CYP3A-selective probes (Ozdemir et al 2000). The CYP3A enzymes are of major importance in India, since they metabolize 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. This emphasizes the importance of phenotyping CYP3A4 in Indian population. 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. Pharmacogenetic data are relatively scarce in India compared to European and other Asian populations. It is hence important to know the effects of differences in drug metabolism to make informed choices on drug dosages.
Traditionally used probes have several disadvantages. For example, erythromycin breath test is used as a tool for detection of CYP3A4, but there is discordance between Erythromycin breath test and the disposition of other CYP3A substrates in vivo has also been reported (Lown et al., 1994; Kinirons et al., 1993; Kinirons et al., 1999). Moreover, it must be administered intravenously and involves nontherapeutic administration of radioactive substances. Midazolam is used as another probe for CYP3A enzyme but despite the low doses of midazolam used for assessment of CYP3A activity, transient sedation, muscle relaxation, ataxia, and amnesia are commonly observed (Streetman et al., 2000). Cisapride is an orally administered prokinetic agent used for the Phenotyping of CYP3A4 but it’s associated with the production of serious cardiac arrhythmias (Michaelets et al., 2000; Dresser et al., 2000). However, in case of 6-β 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). However, there was no correlation between the urinary excretion of 6-β hydroxycortisol and CYP3A activity when measured by breath test (Lown et al., 1992, Hunt, 1992a). Thus the aim of the present study was to phenotype Gujarati (western Indian) population using novel CYP3A4 probe.

It has been reported that CYP expression is altered in disease (Elbekai et al., 2004; Gharavi & El-Kadi, 2004; Korashy et al., 2004; Liu et al., 2004). 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). Pathophysiological states that arise from acute and chronic disease can result in CYP modifying levels of various cytokines (Abdel-Razzak et al., 1993). The CYPs genes, implicated in phase I metabolism, convert several antineoplastic agents into intermediate reactive metabolites, some of which can damage DNA
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(Friche et al., 1992). There is an initial report on the genetic variations in the activity of these enzymes with the risk of developing secondary cancers (Felix et al., 1998). Chang et al (2001), reported in abstract form of a higher frequency of CYP3AP1 T-369G heterozygotes among patients with hereditary prostate cancer (HPC), compared to unaffected controls.

CYP reaction products, or metabolites, have been detected in cardiovascular tissue (Roman, 2002; Gottlieb, 2003; Spiecker & Liao, 2005) and recently, specific isoforms of the enzyme superfamily have been detected (Thum & Borlak, 2000; Thum & Borlak, 2002). Presence of CYP in the vascular system where they may play a crucial role in tone regulation through the transformation of arachidonic acid (AA) to epoxygenase (epoxytrienoic acids, EET's) and N-hydroxylation (20-hydroxyeicosatetraenoic acid, 20-HETE) products (Fleming, 2001; Zeldin, 2001; Capdevila et al., 2002). Various studies have documented the role of these endogenous CYP metabolites in the maintenance of cardiovascular health (Roman, 2002), Xenobiotics that could be drugs or procarcinogens whose transformation via CYPs in coronary arteries may adversely affect the structure and function of coronary vessels. Thus it comes at no surprise that dysregulation of CYP production may be associated with the onset and progression of the various cardiopathies. Study found that the CYP3A5 6986A/G single nucleotide polymorphism (SNP) (CYP3A5*1/*3 alleles) was higher in patients with hypertension when compared to their nonhypertensive controls (Kivisto et al., 2005).

Animal studies also suggest a role for CYP3A in hypertension. Renal CYP3A activity is markedly increased in SHR compared with Wistar-Kyoto (WKY) rats and troleandomycin, a selective inhibitor of CYP3A, decreases blood pressure and in SHR (Basu et al., 1994). There is evidence suggesting that CYP expression (Yu et al., 2000b; Frisbee et al., 2000; Kroetz et al., 1997) and EET generation are increased in
hypertension (Omata et al., 1992) during salt loading, (Holla et al., 1999) and in hypercholesterolemia (Pfister et al., 1991). Thus the objectives of the present study were to study the effect of atorvastatin in patients of coronary artery disease.

Thus the objectives of the present investigation are

1. To develop and validate a method for the detection of atorvastatin and its metabolites in plasma;
2. To perform bioequivalence study of two formulations i.e., Test and Reference by steady state model and measure bioequivalence with both of its metabolites;
3. To study genetic polymorphism of CYP 3A4 in Gujarati population (western Indian) using novel CYP3A4 probe; and
4. To study genetic polymorphism of CYP 3A4 in patients with coronary artery disease.