When you know a thing, to hold that you know it; and when you do not know a thing, to allow that you do not know it - this is knowledge.

Confucius

Discussion
In the present study, a SPE extraction procedure for the preparation of human plasma samples prior to LC-tandem MS analysis was developed. Plasma samples were mixed with 2% formic acid in order to ensure that the analytes were in the unionized form prior to transferral to the SPE cartridges. Wash steps with solutions containing amounts of organic solvent greater than 50% acetonitrile or 30% methanol in water resulted in a dramatic decrease in the extraction recoveries of the metabolites. A wash step with 30% methanol in water resulted in cleaner samples and less variable recovery than wash solutions containing 50% acetonitrile, and was therefore preferred. Acetonitrile-Methanol-Formic acid (50-30-20) was used for elution of the analytes from HPLC column. A volume of 1 mL was needed in order to achieve maximum recovery for this eluent.

Separation of the compounds needed to be good in order to enable the use of MRM, which resulted in lower limits of detection and better signal: noise ratio. Due to the high signal intensity of [M-H], pravastatin and atorvastatin were normally been analyzed in negative-ion mode. Based on achieving a balance between sensitivity, separation efficiency and analysis time, a flow-rate of 0.2 ml/min was selected for the separation of atorvastatin and pravastatin (internal standard). A variety of SPE cartridges, including HLB, Bond Elute C8 and DSC-18, were investigated to find most efficient extraction method for atorvastatin. Elute C8 and DSC-18 could recover very less % of atorvastatin and therefore, HLB cartridges were used to develop the extraction method. The total time of analysis in the chromatographic system was therefore 3.5 min. The retention times of atorvastatin, metabolites and internal standard were at around 2.4 min.
Validation of the method showed linearity within the concentration range 0.2-40 ng/ml for atorvastatin and 0.25-50 ng/ml for p-hydroxyatorvastatin acid and o-hydroxyatorvastatin acid (r²>0.99, n=3 for all analytes). Relative standard deviations (RSDs) of the estimated slopes were less than 15% over the whole concentration range. The intercepts of the calibration curves were shown to be not statistically different from zero for any of the analytes. LOD was 0.06 ng/ml for atorvastatin and p-hydroxy atorvastatin, and 0.15 ng/ml for o-hydroxy atorvastatin. Recoveries for all analytes ranged between 50 and 68% on average. The specificity of the method was tested using extracted drug-free plasma from six individuals. No interfering peaks were found. Also, no interfering ion suppression or matrix effect on signal enhancement was observed. No significant concentration changes in any of the analytes were observed upon autosampler storage at 10°C for a period of up to 24 hour.

The developed method was suitable for determination of Serum concentrations of atorvastatin and its two metabolites by a liquid chromatography-tandem mass spectrometry method LC-MS/MS. The assay showed a lower limit of quantification of 0.2ng/L for all analytes. The calibration curve was linear from 0.2-40 ng/ml for atorvastatin and 0.25-50 ng/ml for p-hydroxyatorvastatin acid and o-hydroxyatorvastatin acid and the method demonstrated good precision and accuracy.

Both the preparations were generally well tolerated without any sign of clinically significant adverse effects in laboratory parameters or physical findings. Treatment-emergent adverse events (AEs) that were reported by at least 18% of the patients who received atorvastatin. The most common AEs on atorvastatin administration were digestive system related. No serious adverse events were registered in the course of the trial, eight non-serious adverse events, most commonly headache, dyspepsia and flatulence were observed in eight volunteer in
the course of the trial. All the side effects were previously reported and were expected (Hans et al., 2003). Both products were similarly well tolerated. All adverse events were primarily transient in duration, of mild to moderate intensity, and resolved without any sequelae or need for drug treatment. No subject was prematurely withdrawn from the study as a result of an adverse event. Thus the products were well tolerated.

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 is 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). The absolute bioavailability of atorvastatin (parent drug) is approximately 14% and the systemic availability of HMG CoA reductase inhibitory activity is approximately 30%. The low systemic availability is attributed to presystemic clearance in gastrointestinal mucosa and/or hepatic first-pass metabolism. Although food decreases the rate and extent of drug, LDL-C reduction is similar whether atorvastatin is given with or without food. Atorvastatin is extensively metabolized to ortho- and para-hydroxylated derivatives (Jacobsen et al., 2000). In vitro inhibition of HMG CoA reductase by ortho- and para-hydroxylated metabolites is equivalent to that of atorvastatin. 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. Moreover, as the metabolite is also active a steady state pharmacokinetic study was done to assess the bioequivalence of atorvastatin for test formulation with the reference formulation in healthy human volunteers.
Methods for testing the bioequivalence of drug formulations have been evolving over the past quarter century. Plasma equivalent concentrations of atorvastatin have been quantified using a validated radioimmunoassay. In this assay, the plasma concentrations of atorvastatin were determined by displacement of 125I-atorvastatin from antibody by unlabelled drug (Posvar et al., 1996; Radulovic et al., 1995). Atorvastatin has also been measured by a Gas Chromatography-Mass Spectrometry (GC-MS) method with a limit of quantification (LOQ) of 0.1μg/L (Gibson et al., 1997).

There has been a growing recognition of the problems that are likely to arise in studies investigating the bioequivalence of two drug formulations when there is high within-subject variability among the test population. Recent practice for the assessment of bioequivalence was based on the fundamental bioequivalence assumption that when two formulations of the same drug are equivalent with regard to the rate and extent of drug absorption they will reach the same therapeutic effect (Chen et al., 2000). The method presently being used is referred to as average bioequivalence, or the bootstrap-type method, in which the 90% confidence interval must fit entirely within fixed bioequivalence limits of 0.8 to 1.25 on the log scale for the two formulations to be in bioequivalence (Schuirmann, 1987).

In our study, the equivalence study was performed only in healthy male subjects. As in case of women intrasubject variability is higher than among men as a result of factors such as differences in menstrual cycle, hormonal makeup, increase in body water, and so on (Schwartz, 2003).

The formulation was administered in fasting conditions to eliminate the influence of food and dosing time on drug absorption (Fleisher et al., 1999; Whitfield et al., 2000). A dose of 40 mg was selected expecting
taking into consideration the sensitivity of analytical method and the Maximum Concentration (Mendoza et al., 2006; Koytchev et al., 2004).

In a previous study for the bioequivalence of atorvastatin formulations, Koytchev et al., 2004, used a dose of 10 mg and they found that the dose was too low for detection of the drug and its metabolites in plasma, and thus we selected the dose of 40 mg which was intermediate dose for atorvastatin.

In the case of new drug development, clinical trials are generally performed with "experimental" formulations before efficacy is established. After efficacy has been established, final formulations undergo rigorous bioequivalence testing to meet the requirements of regulatory authorities. Thus the innovator manufacturer frequently uses bioavailability and pharmacokinetic testing to confirm the bioequivalence of the final marketed formulation to the "experimental" formulation that was originally used to establish efficacy in clinical trials. After patent expiration, other manufacturers use the same standards of bioavailability and pharmacokinetics used by innovator companies to establish the bioequivalence of their products to the originator product. 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. However, drugs used in the prophylaxis or treatment of chronic disease like hypercholesterolemia (atorvastatin), schizophrenia (aripiprazole) (Xiao et al., 2006), and Alzheimer's disease (rivastigmine) (Joshua et al., 2006), and recurrent infections like cystic fibrosis (levofloxacin) (Manjunath et al., 2006) are almost always administered on a long-term basis. Therefore we have studied the bioequivalence of atorvastatin, which is used on a long-term basis with a multiple-dose experimental model that more closely represents therapeutic use. Levy (1972) recommended comparative bioavailability assessments based on steady-state plasma concentrations that offer several advantages over
single-dose studies. We have obtained results of a multiple-dose "chronic" study of 2 formulations of atorvastatin tablets in contrast to the single dose Bioequivalence studies (Mendoza et al., 2006; Koytchev et al., 2004).

As the metabolites significantly contribute to the net activity of an active substance, 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). We measured the concentrations of ortho-and para-hydroxy atrovastatin and evaluated them separately for bioequivalence.

It has been suggested that: (i) bioequivalence should be based upon predominant active moiety; (ii) if the parent compound and metabolite are equipotent and present in equivalent concentrations, if the parent compound fails the CI criteria then the metabolite should be used; and (iii) if the parent compound is active and the metabolite inactive, then only the parent compound should be used for bioequivalence (Turnheim et al., 1999).

There is a varied scientific opinion related to role of metabolites in equivalence studies according to Health Protection Branch, Canada. In 1992, it established the following criteria for immediate-release and modified-release formulations. The determination of bioequivalence is based upon measurement of the active ingredient, or its metabolite, or both, as a function of time. Normally, the parent compound is sufficient but in some cases the metabolite could be required. When a prodrug is administered, the active metabolite should be measured.

However, the European Agency for Evaluation of Medicinal Products' guidance paper (Committee for Proprietary Medicinal Products, 2004) states that the applicant must measure the parent compound.
Metabolites are required in the following cases: (i) if the concentration of parent compound is too low; and (ii) if the parent compound is unstable or half-life is too short. If bioequivalence is to be based upon the metabolite, it must be justified in each case. In particular, if metabolites significantly contribute to the net activity of an active substance and the pharmacodynamics are nonlinear, it is necessary to measure both the parent compound and the active metabolite and evaluate them separately.

US FDA is of the opinion that the parent compound is measured only when a metabolite is formed as a result of gut wall or other presystemic metabolism and the metabolite contributes to safety and efficacy is the metabolite measured to provide supportive evidence. In all other instances only the parent compound is measured for bioequivalence (Food and Drug Administration, 2000).

In our study relative bioavailability of atorvastatin for Test vs. Reference was 98.9%, 79.1% and 84.7% for Css min, Cssmax, and AUCss respectively. The ANOVA test for Css min, Cssmax, and AUCss of Test vs Reference showed that forCss min, there was no significant difference among formulations (p>0.05) but forCss max and AUCss there was significant difference among formulations (p<0.05).

For ortho-atorvastatin Test vs. Reference, the relative bioavailability of Test compared with Reference is 96.5%, 77.9% and 86.0% forCss min, Cssmax, and AUCss respectively. Similar to atorvastatin, The ANOVA for Css min, Cssmax, and AUCss of Test vs Reference showed that for Css min, there was no significant difference among formulations since p>0.05 but for Css max and AUCss there was significant difference among formulations (p<0.05).

In case of para-atorvastatin relative bioavailability of Test compared with Reference is 88.6%, 82.1% and 89.6% forCss min, Cssmax, and
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AUCss respectively. ANOVA for AUCss, there is no significant difference among formulations since, p>0.05 but for Css min and Cssmax there is significant difference among formulations since, p<0.05.

Thus, we can conclude that the test formulation was not bioequivalent for atorvastatin and orthohydroxy-atorvastatin to reference formulation with respect to all the pharmacokinetic [Css min,Cssmax and AUCss] parameters. For all pharmacokinetic parameters it cannot conclude equivalence. The test formulation was also not bioequivalent for parahydroxy-atorvastatin to reference formulation with respect to Cssmin and Cssmax but was bioequivalent with respect to AUCss.

Overall, the test formulation was not bioequivalent to the reference formulation and thus hypothesis was accepted at the 5% level of significance i.e., 90% confidence interval for μA/μB does not lies within (0.8, 1.25).

It is estimated that the developing field of pharmacogenetics may have an impact on the use of metabolites in bioequivalence studies in those cases where there are clear genotype and phenotype correlations for a drug(s) metabolized in the gut wall. In these cases, the area of drug release and the rate and amount of metabolite formed during transport through the mucosal cells in the lumen may be critical to the accurate determination of bioequivalence. Thus in our case both the drug and metabolites were estimated for the bioequivalence (Food and Drug Administration, 2000).

In general, oral bioavailability of a drug is affected by factors such as dissolution rate, stability issues, transit time, food, age, gender, disease, environment, concomitant drugs, compliance, and the first pass effect (Kwan et al., 1997). In case of bioequivalence study all the factors are kept constant so that there is no variation.
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Based on the results of the present study it can be stated that the two-atorvastatin formulations i.e test and reference tablets were not bioequivalent. The possible reason could be due to the formulation effect or genetic variation in the metabolising enzyme CYP 3A4. The regulation of CYP enzymes is influenced by a multiplicity of genetic, endocrine, environmental, pharmacologic, and dietary factors, resulting in substantial inter-subject and intra-subject variability in the expression and activity of individual CYPs. This variation is, in turn, responsible, at least in part, for wide differences in the rate and extent of metabolism of drugs between individuals and intersubject variation in drug response. Multiple studies that have compared the activity of individual enzymes have often been performed in small samples of subjects and have produced inconclusive and conflicting data on the contribution of age or sex to the variation in metabolizing capacity (Harris et al., 1995).

Genetic polymorphism in CYP3A4 have been observed (Dai et al 2001, Sata et al 2000; Eiselt et al 2001), but the importance of this in explaining the interindividual variation in vivo remains to be investigated. Moreover genetic polymorphism in CYP3A, which is expressed in about 30% of Caucasians and 60% of African Americans (Kuehl et al 2001), may contribute to the interindividual variability in biotransformation of CYP3A4 substrates (Ball et al 1999). A prior phenotyping-genotyping screening of volunteers could identify PMs (Poor Metabolisers) who should not be used in the trial. However, this approach, albeit mandatory for steady state studies, is time consuming (Marzo 1999) Specific drug probes should be used to phenotype PMs and EM (Extensive Metabolisers) of xenobiotics. It's now days recommended by regulatory authorities to perform the phenotyping of the study subjects before enrolling subjects for bioavailability bioequivalence studies.
Thus, the genetic polymorphism of human Cytochrome P450 is an important factor for the bioavailability and bioequivalence studies. A prior phenotyping of the study subjects is critical and very important for the bioequivalence studies.

CYP3A4 is the dominant CYP3A enzyme in the liver and small intestine. CYP3As expression is clearly polymorphic, with individuals exhibiting a relatively high or low level of protein (Wrighton et al., 1990; Paine et al., 1997). It is responsible for metabolism of more than 50% of the drugs.

Genetic polymorphism is one of the factors resulting in variation of CYP450 activity and gives rise to distinct subgroups in the population that differ in their ability to perform certain drug biotransformation reactions. With respect to the possible polymorphism of CYP3A activity, findings in a number of studies have been inconsistent. Kleinbloesem et al., 1984, found that the frequency distribution of the area under the plasma concentration-time curve (AUC) of nifedipine, a CYP3A substrate, was bimodal in a population of 53 healthy subjects. However, in another two studies with 172 and 130 healthy subjects, respectively, bimodality was not observed (Schellens et al., 1988). In the study in which midazolam was administered intravenously to 168 Caucasian patients, polymorphism in the oxidative metabolism of midazolam was not evident (Kassai et al., 1988). The metabolism of midazolam reflects hepatic CYP3A activity when administered intravenously, but reflects both intestinal and hepatic CYP3A activity after oral administration (Wandel et al., 1998).

In our study we used atorvastatin as a probe drug as vitro studies have indicated that after incubation of atorvastatin with human microsomes, ortho-hydroxy atorvastatin and para-hydroxy atorvastatin are formed (Michniewicz et al., 1994; Christians et al., 1998). As shown by using specific CYP inhibitors and isolated CYP enzymes, CYP3A4 is the major
enzyme involved in formation of the two metabolites. In our study, we measured ortho atorvastatin as it is formed by the metabolism of atorvastatin with CYP3A. 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, due to marked inhibitory effects on CYP3A4 substrates (Kantola et al., 1998; Siedlik et al., 1999). Rifampicin, which is known potent inducers of cytochrome P450 3A4; it markedly decreases the plasma concentrations of atorvastatin and its metabolites (Janne et al., 2005). 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.

Statins have been accepted sensitive probe drug for CYP3A4 activity by the U.S. Food and Drug Administration (FDA, 1999). There are data suggesting that simvastatin is proportionally metabolized by other metabolic pathways in addition to CYP3A. Approximately 20% of the metabolism of simvastatin can be attributed to the polymorphic enzyme CYP2C8 (Ellen et al., 2006). Therefore, simvastatin may not be considered as an ideal CYP3A probe. Atorvastatin was considered as a suitable probe drug for this study because its mainly metabolized by CYP3A4 only and it also fulfilled major requirement of probe validation proposed by Watkins, 1994, and modified by Zaiglerm 2000. Moreover, it is well tolerated (Connie et al., 2003) and free from radioactivity and phenotyping is technically easy. Based on the above reports we attempted to use atorvastatin as a probe for Phenotyping of CYP3A enzyme.

In our study data of 125 Gujarati (West Indian) subjects showed a bimodal distribution with respect to metabolism of atorvastatin. The
sample size was calculated assuming a 5% prevalence of PM with 95% confidence interval at 5% significance. The prevalence for sample size calculation was taken as 5% based on the data of 1% and 5% prevalence in Caucasians and Orientals. Interindividual differences of drug metabolism expressed as log Metabolic Ratio (MR) were plotted in the form of a frequency distribution histogram with the log MR of atorvastatin hydroxylation (ortho) on the x-axis and the number of subjects on the y-axis. Therefore, our findings are in contrast to the results of Kassai et al., 1988.

Several published reports have described 25 different alleles in CYP3A4 and 12 different alleles in CYP3A5 (Sarah, 2006). Genetic polymorphism is an important determinant of individual CYP3A activity. In our study, a 7-fold variation of CYP3A activity was observed, which may be due to a combination of genetic, environmental, pathological, hormonal, and dietary factors. Such a remarkable variability of CYP3A activity results in potential difficulties in determining a drug dosage regimen in individual patients whose metabolizing ability is unknown a priori (Wilkinson, 1996). Now pharmaceutical companies are screening new chemical entities during the drug development process to determine whether CYP3A enzymes metabolize new chemical entity.

Recent advances in the characterization of specific isozymes involved in drug metabolism now allow for the preliminary identification of enzyme systems that are affected by gender. The significance of these gender differences will be most important in the administration of drugs that have a narrow therapeutic range (Harris et al., 1995). N-demethylation of erythromycin is mediated exclusively by CYP3A4. Erythromycin is metabolized 25% more rapidly by female than male human liver microsomes (Hunt et al., 1992b). Krecic-Shepard et al., 2000, reported that the clearance of nifedipine was significantly lower in men compared with women. Midazolam was found to be cleared 20-40%
faster by women than by men, although this did not reach statistical significance (Harris et al., 1995). By comparison, there are other reports that have not found gender differences in CYP3A4-mediated drug metabolism. Lobo et al., found that gender did not appear to influence the clearance of nifedipine (Lobo et al., 1986). Contradictory results have been observed in alfentanil metabolism in relation to gender (Lemmens et al., 1990; Sitar et al., 1989). Some studies failed to detect a significant gender difference in CYP3A4 concentrations in human liver microsomes and thus do not support the notion of a gender difference in CYP3A4 activity (Harris et al., 1995).

In our study of the 28 women phenotyped, one women subject was poor metabolizer. Thus the frequencies of occurrence of extensive and poor metabolizers of atorvastatin in women were 96.43% and 3.57%, respectively. Of the 97 male volunteers, two were poor metabolizers of atorvastatin, demonstrating that the frequency of occurrence of extensive metabolizers and poor metabolizers of atorvastatin in men was 97.94% and 2.06%, respectively. Thus using the metabolism of atorvastatin as an index, we demonstrated that CYP3A activity was significantly lower in women than in men in the Gujarat population (P < 0.05). Therefore, women may eliminate many drugs metabolized by CYP3A slower. CYP3A plays an important role in the metabolism of several sex steroid hormones including oestrogen, progesterone and testosterone (Harris et al., 1995; Mäenpää et al., 1998; Waxman et al., 1988).

The mechanism of gender-related difference of CYP3A activity may be due to the fact that steroid hormones may regulate CYP3A activity at the level of gene expression. Hashimoto et al (1993) has identified several consensus sequences for transcription factors including three ERE (oestradiol responsive element) and one progesterone/glucocorticoid responsive element (PRE/GRE) in the
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The promoter region of CYP3A4, CYP3A5 and CYP3A7 (Jounaidi et al., 1994).

The menstrual cycle is a potential source of variability in drug metabolism and disposition in young women. This variation has been a specific focus of FDA guidelines, specifically with regard to new drug development (Merkatz et al., 1993). A previous investigation found no difference in the plasma elimination and systemic clearance of the CYP3A4 probe alfentanil between days 2, 13 and 21 of the menstrual cycle, suggesting no menstrual cycle difference in CYP3A4 activity (Kharasch et al., 1997b). Kharasch et al., (1999), examined CYP3A4 activity during the menstrual cycle using midazolam clearance as the metabolic probe. Midazolam (1 mg i.v.) was administered to 11 female volunteers with normal menstrual cycles on three separate occasions during the same cycles. The results revealed no difference in hepatic CYP3A4 activity on menstrual cycle days 2, 13, and 21. Hormonal changes occurring during the menstrual cycle, especially the varying concentrations of progesterone may contribute to differences of CYP3A activity.

It may be that high concentrations of steroid hormones could potentially inhibit CYP3A activity. In an in-vitro study, it was found that CYP3A activity could be inhibited by 30% with 50 mM progesterone. The activity of CYP3A4 and other isozymes could be inactivated by Oral Contraceptives (Ocs) containing steroid hormones (Guengerich et al., 1990). In the study of Lobo et al., in which the gender did not appear to influence the clearance of nifedipine, due to one-third of the women who in the study were taking OCs (Lobo et al., 1986). In our study, none of the women used OCs.

The mechanism of the effect of menstrual cycle on CYP3A activity still needs to be clarified. Thus it can be said that CYP3A activity is bimodally distributed and considerable interindividual variability exists.
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Gender differences in CYP3A activity may influence individual susceptibility to adverse effects of particular drugs. Further investigations are required to clarify the mechanisms underlying the gender differences of CYP3A activity and the effect of menstrual cycle on CYP3A activity.

Interethnic differences in CYP3A4-mediated drug metabolism have been studied in vitro and in different populations (Shimada et al., 1994). Caucasian liver microsomes had higher nifedipine oxidase activity and significantly higher testosterone 6-β hydroxylase activity than Japanese samples (n = 30 each). Hepatic P-450 3A (principally P-450 3A4) content correlated well with nifedipine oxidation (r = 0.79) and testosterone 6 β -hydroxylation (r = 0.81) activities. Also, CYP3A4-mediated metabolic activation of aflatoxin B1 and sterigmatocystin correlated well with microsomal P-450 3A content (r = 0.78 and 0.83, respectively) and was significantly higher in Caucasian than in Japanese samples (Shimada et al., 1994). Apparent oral clearance of alprazolam was found to be similar in native and American-born Asians, but to be significantly higher in Caucasians than in Asians (Lin et al., 1988). Similarly, the area under the plasma concentration–time curve of nifedipine was significantly higher in Asians than Caucasians (Yu et al., 2000a; Ahsan et al., 1993; Ahsan et al., 1991; Rashid et al., 1995). The ability to metabolize oral nifedipine was similar in Asian Indians and Malaysians who resided in the same geographic area (Mohamed et al., 1998) and codeine N-demethylation, mediated by CYP3A4 (Ladona et al., 1991) was more extensive in Caucasian than Chinese subjects (Yue et al., 1989). These data suggest that CYP3A4 activity may be higher in Caucasians than other populations. However, such an ethnic variation may be substrate dependent, since erythromycin N-demethylation did not show a good correlation with the content of hepatic P-450 3A (r = 0.28), and no difference was present between Asians and Caucasians when erythromycin (Yu et al., 2000a), triazolam (Kinirons et al., 1996) or
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Cerivastatin (Muck et al., 1998) were used as metabolic markers of CYP3A4 activity.

CYP3A4 is involved in the metabolism of many statins (Han, 2003). Thus, a genetic polymorphism in CYP3A4 can be associated with the pharmacokinetic, pharmacodynamic and toxic effects of many statins. There have been no reports of pharmacokinetic changes in statins produced by SNPs in CYP3A4 (Yoshihisa and Yuichi, 2006). However, there are some reports of a changed pharmacological effect associated with SNPs in CYP3A4 (Kajinami et al., 2004; Wang et al., 2005). Wang et al. reported stimulation of the pharmacological effect of simvastatin by the genetic variation of CYP3A4*4(I118V) in CYP3A4, which resulted in reduced metabolism (Wang et al., 2005). Reduced metabolism (CLmet) results in an increase in the plasma concentration and the AUC of statins and enhancement of the pharmacological effect.

Kajinami et al., 2004, examined the effects of a variation in the promoter region, A-290G, and amino acid substitution, M455T, on the pharmacological effect of atorvastatin. They found a significant reduction in the pharmacological effect only in patients with A-290G (i.e, CYP3A4*1B). The variation A-290G might alter the transcription of CYP3A4. However, they did not measure the enzyme activity. A reduction in the metabolic rate may be associated with enhancement of the pharmacological effect.

From the frequency distribution histogram and probit plots of our study there were three individuals were found to demonstrate the PM phenotype. Thus CYP3A polymorphism occurs with a frequency of 2.4% in Gujaratis (Western Indians). The observed frequency in Gujaratis is found to be within the range demonstrated for other ethnic groups (<1% in Orientals, 5% in Caucasians).

Nearly 40 variants of CYP3A4 are known (Oscarson et al., 2006). The first common variant is CYP3A4*1B (Chowbey et al., 2003, Ball et al.,
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1999; Sata et al., 2000; Wandel et al., 2000), present in about 4% of Caucasians and not found in East Asians (Ball et al., 1999; Sata et al., 2000). It does not appear to alter significantly the metabolism of CYP3A4 substrates (Ball et al., 1999; Wandel et al., 2000; García-Martín et al., 2002). CYP3A4*2 showed substrate-selective altered kinetic parameters compared to the reference (Sata et al., 2000). The frequency of this variant is below 3% in Caucasians and is not found in Chinese (Eiselt et al., 2001; García-Martín et al., 2002; Sata et al., 2000). CYP3A4*3 frequency in Caucasians is 1.1% (van Schaik et al., 2001). In contrast to other CYPs such as CYP2D6 and CYP2C, CYP3A4 is likely to have relatively few null variants.

Bimodal distribution of the human subjects was shown by the inflection point in probit plot. From the inflection point in the probit plot it can be inferred that 3 of 125 subjects are poor metabolizers. Antimode value from probit plot were computed with the multiple regression method and was found to be 1.7 for log DM. Individuals demonstrating log DM values between 0.4 to 1.7 were therefore categorized as extensive metabolizers, whereas individuals demonstrating values higher than 1.7 were categorized as poor metabolizers. We have attempted to report the antimode value from the O-hydroxyatorvastatin metabolized in blood. The results of our study showed that the frequency of the occurrence of the poor metabolizers of atorvastatin in the Gujarati subjects was 2.4%.

Our results suggest that Gujarati population is ethinically quite close to Caucasians.

The human CYP3A subfamily, CYP3A4, CYP3A5, CYP3A7 and CYP3A43 are one of the most versatile of the biotransformation systems that facilitate the elimination of drugs, other xenobiotic compounds, and endogenous molecules from the body. Although there has been no systematic analysis of the extent of its contribution, it is generally
accepted that CYP3A enzymes play a dominant role in the metabolic elimination of more drugs than any other biotransformation enzyme. CYP3A metabolic versatility may be due to a large active site that permits the binding of structurally diverse molecules (Guengerich, 1999). Moreover, CYP3A enzymes are known to accommodate multiple ligands (different molecules or two or more of the same molecule) in the active site. This phenomenon can result in enhanced product formation (activation), or reduced product formation (non-competitive inhibition), depending on the concentration of substrate and nature of the second or third ligand (Korzekwa et al., 1998).

The recognition that CYP expression is altered in disease (Elbekai et al., 2004; Gharavi & El-Kadi, 2004; Korashy et al., 2004; Liu et al., 2004) and that genetic differences contribute to interindividual differences in enzyme activity seen within a patient population have resulted in an explosion of research on the role of these enzymes in diseases.

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). Emerging studies have documented the role of these endogenous CYP metabolites in the maintenance of cardiovascular health (Roman, 2002), thus it comes at no surprise that dysregulation of their production may be associated with the onset and progression of the various cardiopathies.

Human endothelial cells express CYP3A4 as identified by immunohistochemistry of the endocardium and coronary vessels (Minamiyama et al., 1999). Interestingly, CYP3A4, CYP3A5, and CYP3A7 mRNA transcripts were not found in the heart tissue of patients with dilated cardiomyopathy (Thum & Borlak, 2000).
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). Fromm et al. (2005) also genotyped young Caucasian males but did not see a difference in diastolic blood pressure between individuals with the CYP3A5*1/*3 genotype and those with the CYP3A5*3/*3 genotype. However, systolic blood pressure was significantly higher in individuals with low CYP3A5 genotype (*3/*3). Ho et al. (2005) found ethnic differences in the association of CYP3A5 alleles with hypertension. A greater proportion of African-American participants with poor blood pressure control had the CYP3A5*1/*1 allele but systolic blood pressure was higher in African-Americans with the *3/*3 allele than with the *1/*3 genotype. The same study however did not find a difference in allele frequency between hypertensive and nonhypertensive Caucasians.

Cholesterol, an important component of cellular membranes, is also essential for biological activity as it serves as a precursor for the biosynthesis of many biologically active compounds that are involved in multiple physiological processes. Although cholesterol has no direct role in cardiovascular physiological processes, the regulation of cholesterol homeostasis is essential in maintaining cardiovascular health. The accumulation of cholesterol in the body increases the risk of CVD as excess cholesterol may lead to atherosclerosis. A large number of the CYP superfamily members play important roles in both the synthesis and the metabolism of cholesterol.

Many members of the CYP have been shown to alter both the pathogenesis of atherosclerosis and its contribution to the risk of MI and stroke. As mentioned previously, major participants in the formation of atheromatous plaques are oxidized LDL. It has been hypothesized that since CYP enzymes are known to be major contributors to the production of cellular oxidative stress and LDL are
oxidized by reactive oxygen species (ROS), it may be plausible to assume that CYP inhibition may decrease the production of oxidized LDL (Hunter et al., 2004).

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).

In our study the patients of CAD who are on chronic treatment with atorvastatin had an increase in the metabolic ratio of atorvastatin, which can be seen from the graph. As compared to normal patients there is a significant increase in the drug metabolite ratio, which is representative to the decrease in the CYP3A4 activity in CAD patients.

The increase in the drug metabolite ratio is proportional to the number of coronary arteries blocked, which suggest that, as the Hypercholesterolemia blocks the artery there is a proportional decrease in the CYP3A4 activity.

Endothelial nitric oxide synthase (eNOS) produced in the vascular endothelium has a role in the physiological cellular regulation of this tissue. Nitric oxide is generated in vascular endothelial cells by eNOS and is responsible for endothelial dependent vasorelaxation, inhibition of smooth muscle cell proliferation, and decreased synthesis of extracellular matrix proteins (Wilcox et al., 1997). In the presence of oxidised low density lipoprotein, eNOS is downregulated by destabilizing eNOS mRNA (Lubrano et al., 2003).
Discussion

Feron and colleagues (Feron et al., 1999) have previously shown that eNOS concentrations are decreased with hypercholesterolemia. Myers and colleagues (1998) have further shown that decreased nitric oxide concentrations are associated with an increase in collagen matrix production within the vasculature. These experiments in the vasculature are important studies supporting the hypothesis tested in this current study.

NO plays important bioregulatory roles in physiological processes such as regulation of vascular tone, platelet aggregation, neurotransmission and cytotoxicity of activated macrophages (Moncada et al., 1991; Nathan, 1992). These physiological actions are based on a modulation of enzyme activity through binding of NO to proteins with heme or non-heme iron cofactors. NO production increases in many cell types in response to immunological stimuli such as bacterial lipopolysaccharide (LPS) and cytokines (Geller et al., 1993; Nussler et al., 1992). It has been shown that NO inhibits CYP activities and the expression of CYP mRNA (Carlson and Billings, 1996; Hara et al., 2000; Minamiyama et al., 1997). NO may inhibit CYP activities by binding to the heme groups, but the mechanism by which NO suppresses CYP gene expression remains unknown.

Given that NO interacts with hemoproteins, such as CYP to throttle enzyme activity, and EDHF-mediated responses are only unequivocally detectable when NO synthase is inhibited, it was proposed that a decrease in the bioavailability of NO (eg, in endothelial dysfunction) would be associated with an increase in CYP activity as well as EDHF-mediated responses (Bauersachs et al., 1996).

Atorvastatin treatment increased the eNOS production (Cameron et al., 2006). The increased NOS was responsible for the NO production which
Discussion

further inhibited the CYP and thus produced the increased drug metabolite ratio in patients suffering from CAD.

Atorvastatin was found to be effective in treatment of CAD and the patients chronically on atorvastatin showed increased metabolic ratio.

There was a very good correlation between the metabolic ratio in single vessel, double vessel and multiple vessel disease patients when compared against either hypertension or diabetes, which proves that diabetes and hypertension are one of the risk factors for Coronary artery disease.