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Evaluation of the effect of selective CYP 450 enzymes on NCE

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Abstract
Background: With the increasing knowledge about Cytochrome P450 enzymes, the Pharmacokinetic and toxicological profile of the drug can be studied. The determination of the cytochrome P450, on the metabolism of New Chemical Entity using an *in vitro* approach has become essential for the prospective understanding of any drug. It is an important filter in the drug screening and before checking with the animal models.
Purpose: The *in vitro* approach of incubation with the selective enzymes aims
i. To aid in preclinical screening of the drug metabolism without utilization of animals
ii. Illustrate the mechanistic pathway of the NCE via the CYP enzymes
Materials and Methods: The Test compound was incubated *in vitro,* using selective CYP enzymes like 1A2, 2B6, 2C8, 2C9, 2C19, 2D6, 3A4 in presence and absence of their CYP inhibitors and then extrapolated the observations using Microsoft excel Curves and Graphs.
Result: The influence of the selective enzymes on Test compound's metabolism is of the order CYP 3A4 > CYP 2D6 > CYP 2C9 > CYP 1A2 > CYP 2C19> CYP 2B6 > CYP 2C8
Conclusion: *In vitro* metabolism of the Test compound by the CYP is observed. CYP3A4 has shown highest activity whereas the least was shown by CYP 2C8, while CYP 2D6 & CYP 2C9 depicted an average activity. The data drawn gave an understanding about the individual activity of the commercially viable enzymes on the Test compound providing the metabolic pathway of the Test compound under the given experimental conditions.
High Lights
- The CYP enzymatic influence on the metabolism of the NCE is observed
- The selective CYP activity is measured without an authentic standard

The specific role of CYP in metabolism and the sustainability of the Test compound as a drug in the process of lead optimization are commented.

Keywords: Human liver microsomes; mixed function oxidases; CYP enzymes; high resolution mass spectrometry

Introduction
Out of the 18 families and 43 subfamilies only from families 1, 2 3 viz., CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4 and CYP3A5 were involved in the metabolism of therapeutic drugs. This Cytochrome P450 is a super family of haeme proteins that catalyze the metabolism of a large number of xenobiotics and endobiotics. The type and amount of the CYP enzymes expressed, primarily in the liver, determine the metabolic responses in that species. A majority of the CYP enzymes involved in hepatic drug metabolism has been identified and about 12 human drug-metabolizing CYP enzymes have been characterized at the molecular level. The characterization of the CYP enzymes has made it possible to "phenotype" Test compounds with respect to their relative levels and their metabolic capabilities. The various CYP enzymes differ in their substrate specificity and hence the metabolism of the probe substrate [1]. Thus screening of a Test compound using the human CYP (1A2, 2B6, 2C8, 2C9, 2C19, 2D6, 3A4) would enable the researcher understand the drug metabolic pathway. The utility of this research is demonstrated by observing the selective CYP role in the metabolism of the Test compound. This work promises to help the pre-clinical scientist in identifying the behaviour of the compound and the pathway of metabolism aiding it to move on to the next step in the drug discovery.
The *in vivo* drug interaction guidance published by the Food and Drug Administration in 1999 indicates that investigators can use *in vitro* drug interaction data to conclude that a new drug does not inhibit a specific P450 activity.

In practice, the *in vitro* evidence is usually collected from one probe reaction per enzyme, and the conclusion is extrapolated to all substrates for the same enzyme. The significant regulatory impact of this approach and potential problems associated with current practice to evaluate the appropriateness of *in vitro* methodologies commonly used to study CYP-based drug interactions, shall aid in adopting a more consistent and accurate *in vitro* approach [2].

The ultimate goal of the work up is to promote

1. Identification of the substrate specific enzymes, understand the metabolic pathway of potential drug candidate and select safer candidate at an early stage
2. Develop *in vitro* results, that provide a reliable extrapolation to *in vivo* results
3. To aid in preclinical screening of the drug metabolism and elimination routes without utilization of animals
4. Moreover the recent withdrawals of marketed drugs due to drug-drug interaction compel the need for a thorough evaluation of the new drug at an early stage for any potential drug toxicity [3].

Sample preparation and the concentrations of the drug in the presence and absence of the specific CYP help in determining the mechanistic pathway of the metabolism and the potential DDi of the Test compound.

Reviewing the literature from in *vitro* P450-based metabolic studies using purified cDNA-expressed enzymes, selective chemical inhibitors and inhibitory antibodies as well as studies on enzyme kinetic analyses and the rate of formation of a metabolite specifically reflects the activity of one distinct CYP enzyme. During the drug-candidate screening and development process, investigators often conduct two types of *in vitro* drug metabolism studies to assess the potential CYP based drug interactions. One type of study characterizes the metabolic pathway of the new drug and the potential for other drugs to modify the metabolism of the new drug. The other type of study evaluates the potential for the new drug to alter the metabolism of other drugs. The availability of selective chemical inhibitors against specific P450 enzymes, the unequivocal identification of the major P450 isoform responsible for the metabolism of a new drug can be used to view and establish the metabolism. However, predicting the potential for the new drug to alter the metabolism of other drugs or being perpetuated by other drugs usually relies on the evaluation of the effect of the new drug on the rate of a reaction that represents a specific P450 enzyme inhibition activity [4].

Therefore, one of the key areas to understand about new molecule is the determination of the effect of selective CYP on the metabolism of the Test compound. In the present study, the effect of 7 different CYP enzymes were checked selectively using various individual vials wherein the Test compound was incubated with the specific CYP enzyme. The rate of metabolism and the depletion of the Test compound are observed in presence and absence of specific CYP inhibitor.

Materials and method

Chemicals

Test compound obtained from GPRCP, Methanol and Acetonitrile from J.T Bakers, Phosphate Mono Basic (PBS) from Sigma Aldrich, Potassium Hydroxide (KOH) and Magnesium Chloride (Mgcl2) from Merck, NADPH Regenerating Solution (NRS) and Glucose - 6- Phosphates dehydrogenase and Ammonium formate obtained from Sigma Aldrich, Formic acid from Fluka, Human Microsomes and the CYP enzymes from Krishgen Biosystems. All the chemicals used are of analytical grade.

Probe Substrates were obtained from Sigma

Note: No specific safety considerations apply to any of these agents, although the agents shall be handled with care in a safety fume hood to avoid inhalation and contamination.

Procedure for the preparation of reagents and incubation mixtures

1. 100 mM Potassium phosphate buffer pH 7.4 at 37 °C and 400 mM $MgCl_2$ solution Store the solutions at 4°C.
2. Stock solution of 10mM Test compound was prepared in methanol.
3. A 10 mM inhibitor stocks were prepared and diluted using phosphate buffer saline to give final concentrations of 10 uM. The 10 µM inhibitor concentration is used to inhibit the enzyme activity.
4. The solutions were prepared and stored at < -70°C, brought to 4°C prior to addition, except Montelukast solution. Warm Montelukast solution to 37° C and vortex for 10 seconds prior to addition to obtain a well mix up solution just before the experimental start.
5. The respective mixtures containing various substrates were prepared in Acetonitrile, DMSO, methanol and water. The final concentration of the organic solvents did not exceed 0.1%.

Table 1: Preparation of CYP inhibitors

Cyp isoenzyme	Inhibitor	Solvent	Concentration (mM)
CYP 1A2	α Naphthoflavone	DMSO:Methanol	10
CYP 2B6	Tranylcypromine HCl	Water	10
CYP 2C8	Montelukast Sodium	Water	10
CYP 2C9	Sulfaphenazole	Acetonitrile	10
CYP 2C19	Benzylnirvanol	Acetonitrile	10
CYP 3A4	Ketoconazole	Methanol	10
CYP 2D6	Quinidine	Methanol	10

The probe reaction is allowed to proceed under initial rate conditions, for this to precede the experiment shall use optimal experimental conditions, such as substrate concentrations, incubation time and enzyme protein content. Deviation from optimal experimental conditions may result in an underestimation or overestimation of changes in enzyme activity, and thereby lead to incorrect conclusions regarding the drug interaction potential.

Prior to analysis all the samples were thawed, vortexed and centrifuged for 10 minutes at 14,000 rpm. The supernatant is collected and evaporated under nitrogen water bath till dried of its solvent and reconstituted using 50 µL of 90:10:0.1 %

Water: ACN: Formic Acid prior to analysis.

Methods

The step wise procedure followed is as follows

Cyp Activity Studies

i. To 398µL of PBS in 2.0 ml centrifuge tube add ~2µl of Test compound (10,000µM conc.) and vortex for 5 sec. To make the final analyte conc. 20µM in sample.
ii. Add 100 µL of thawed Microsomes with CYP (25 – 62.5 pm CYP/mL) to the above centrifuge tube and vortex for 10 sec and pre incubated at 37°C for 4 min
iii. Add 500 µL of Activated NADPH Regenerating Solution to 500µL microsomal samples (Microsomes+ analyte), Vortex for 10 seconds and incubate in water bath at 37°C.
iv. For 75 mins, collect the microsomal sample at the time intervals of 5, 15 30, 45, 60, 75 min of incubation. Add Chilled Acetonitrile for quenching and vortex for 10 sec, then centrifuge at 14000 rpm for 10 mins. Collect the supernatant and evaporate under nitrogen water bath till dried and reconstitute the sample (10:90 ACN: water 0.1%FA).
v. Inject 5µl of sample on to LC- MS/MS

Cyp Inhibitory Studies

i. To 398µL of PBS in 2.0 ml centrifuge tube added 2µL of Inhibitory molecule and vortex for 5 sec. To make the final analyte conc. 20µM in sample
ii. Add 100 µL of thawed Microsomes with CYP (25 – 62.5 pm CYP/mL) to the above centrifuge tube and vortex for 10 sec and pre incubated at 37°C for 4 min
iii. Add 250 µL of Activated NADPH Regenerating Solution to 500µL microsomal samples Vortex for 10 seconds and incubate in water bath at 37°C for 30 min.
iv. After 30 min add ~2µl of Test compound (10,000µM conc.) and 250 µL of Activated NADPH Regenerating Solution
v. For 75 mins, collect the microsomal sample at the time intervals of 5, 15 30, 45, 60, 75 min of incubation. Add Chilled Acetonitrile for quenching and vortex for 10 sec, then centrifuge at 14000 rpm for 10 mins. Collect the supernatant and evaporate under nitrogen water bath till dried and reconstitute the sample (10:90 ACN: water 0.1%FA).
vi. Inject 5µl of sample on to LC- MS/MS
vii. This testing paradigm is customized in order to understand the CYP metabolic potential on the molecules synthesized on lab scale in academia.

Experiment: Test compound was incubated in pooled inactivated HLM plus selective enzymes in one set and along with their inhibitors in aseparate experimentation. For 75 minutes the experiment continued and the reactions in the collected samples were terminated with the addition of double the quantity of Acetonitrile. The samples were vortexed and centrifuged for 15 minutes at 14,000 rpm. The subsequent supernatants were evaporated and reconstituted using 50µL of 90: 10 water and Acetonitrile with 0.1% Formic acid and transferred for analysis to HR-MS connected to UPLC.

UPLC: Analysis of the Test compound was achieved using the Waters Acquity UPLC system. The Waters Acquity system consisted of a binary UPLC Pump, column oven, a sample manager, and a dual UV detector. Separation was carried out on an analytical column of X Bridge C18 (250 × 4.6 mm, 3 µ 5 mm particle size) in a column oven maintained at 25°C. The mobile phase used consisted of solvent A (10 mM Ammonium formate in water with 0.1% Formic acid) and solvent B (100% Acetonitrile). Initial mobile phase conditions (100% solvent A) at a rate of 0.2 ml/min were held for 5 min, followed by a step gradient to 30% solvent B in 45 min, followed by a second step gradient to 95% solvent B in 10 min, the final conditions were held for 5 min, then returned to the original starting conditions.

Mass Spectrometry: High-resolution mass spectrometric (HR-MS) measurement was performed using Quadruple Time of Flight (QTOF) mass spectrometer (Waters) with dual orthogonal Z Spray ESI Source. The Q TOF was operated under V-Mode and calibrated with polyethylene glycol, 50 pg/µl Leucine Enkephalin was used as lock spray at a flow rate of 3 µL/min, Electron Spray Ionization under positive ion mode with collision energy ramp of 20 to 40 eV under MSe scan were used all along the retention window of 0 – 60 mins. The capillary and tube lens voltages were 32 and 60 V respectively. Nitrogen was used as a drying gas at a sheath pressure of 78 psi with auxiliary flow.

Results

The potential influence of the CYP on the assessment of *in vitro* drug metabolism has a significant impact on the drug development process.

The study of enzyme activity on the Test compound gives sufficient in-site about the selective role of individual enzymes on the metabolism of investigative molecule. The data drawn give an understanding about the individual activity of the commercially viable enzymes on the Test compound providing the metabolic pathway of the Test compound under the given experimental conditions.

CYP role in metabolism and inhibition studies involve incubation of Test compound with selective CYP enzymes, where the rate of disappearance of Test compound is measured with respect to time, Fig.1 & 2 and Table 2 & 3 respectively.

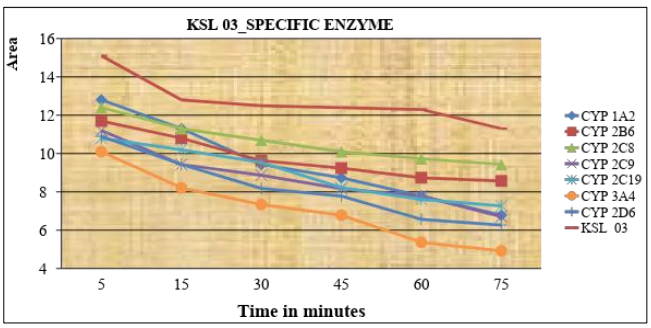

Fig 1: Graphical representation of the Test compound under the influence of the individual enzymes vs time.

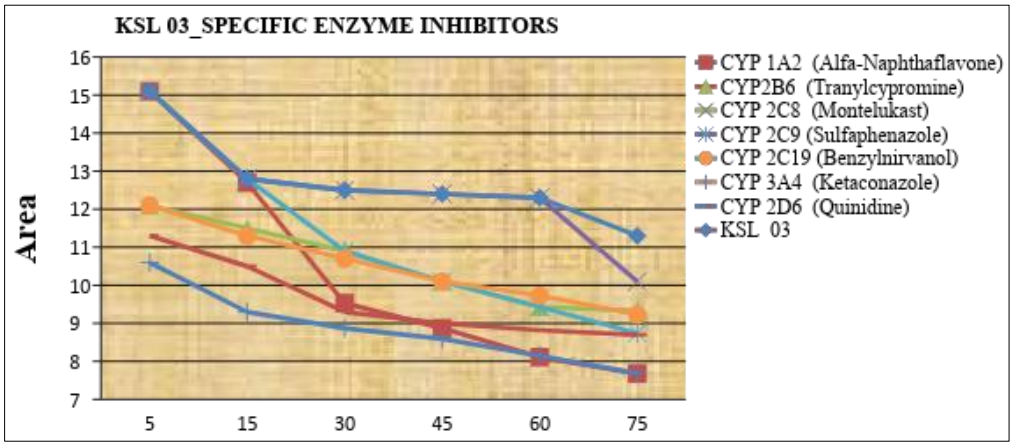

Fig 2: Graphical representation of the metabolism of the Test compound with specific enzyme inhibitor vs time.

Table 2: Test compound's response under the influence of CYP enzymes

Substrate - KSL 03_ RT_ 2.26 m/z = 552.3/320.4							
Time Minutes	**CYP 1A2**	**CYP 2B6**	**CYP 2C8**	**CYP 2C9**	**CYP 2C19**	**CYP 3A4**	**CYP 2D6**
5	1280000	1170000	1240000	1120000	1080000	1010000	1090000
15	1130000	1080000	1130000	943000	1020000	821000	943000
30	943000	963000	1070000	887000	953000	734000	818000
45	874000	924000	1010000	816000	822000	678000	778000
60	778000	874000	973000	778000	761000	537000	657000
75	678000	857000	943000	671000	727000	492000	627000

Table 3: Test compound's response under specific enzyme inhibitor effect

Inhibitors - KSL 03_ RT_ 2.26 m/z = 552.3/320.4							
Time Minutes	**CYP 1A2 (Alfa-Naphthaflavone)**	**CYP2B6 (Tranylcy promine)**	**CYP 2C8 (Montelukast)**	**CYP 2C9 (Sulfaphenazole)**	**CYP 2C19 (Benzylnirvanol)**	**CYP 3A4 (Ketaconazole)**	**CYP 2D6 (Quinidine)**
5	1510000	1210000	1510000	1510000	1210000	1060000	1130000
15	1270000	1150000	1280000	1280000	1130000	930000	1050000
30	953000	1090000	1250000	1090000	1070000	887000	930000
45	887000	1010000	1240000	1010000	1010000	859000	900000
60	811000	943000	1230000	943000	973000	815000	882000
75	768000	933000	1010000	874000	923000	768000	870000

The influence of the selective CYP on Test compound till 75 minutes starting from 5 minutes can be demonstrated by the individual CYP Vs inhibitor through an overlap of either curves as observed from the data obtained. The cumulative effect with respect to the individual capability of the CYP enzymes can be observed graphically in Fig.3 to 10 for CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, and CYP3A4 respectively.

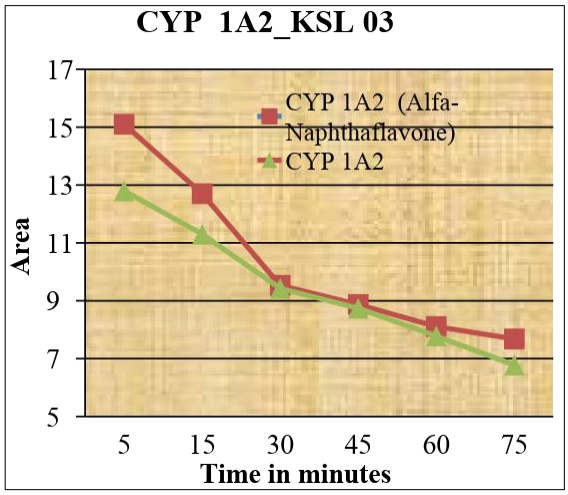

Fig 3: Graphical illustration of CYP1A2. In presence and absence of Alfa-Naphthaflavone

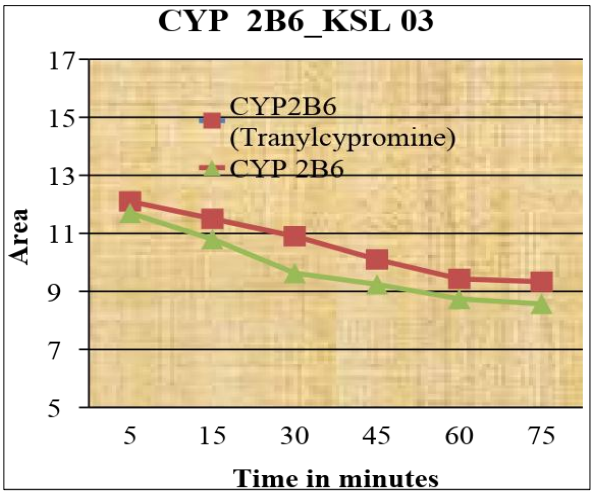

Fig 4: Graphical illustration of CYP2B6. In presence and absence of Tranylcypromine.

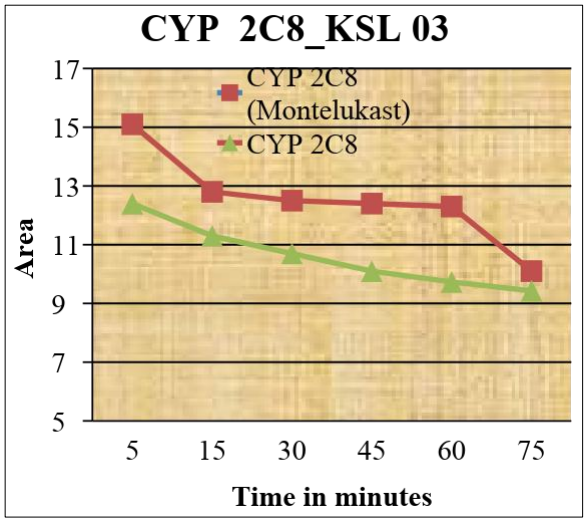

Fig 5: Graphical illustration of CYP2C8. In presence and absence of Montelukast.

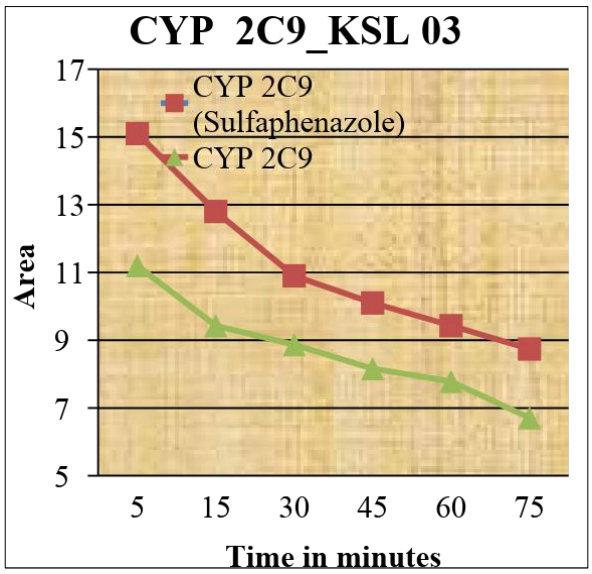

Fig 6: Graphical illustration of CYP2C9. In presence and absence of Sulfaphenazole.

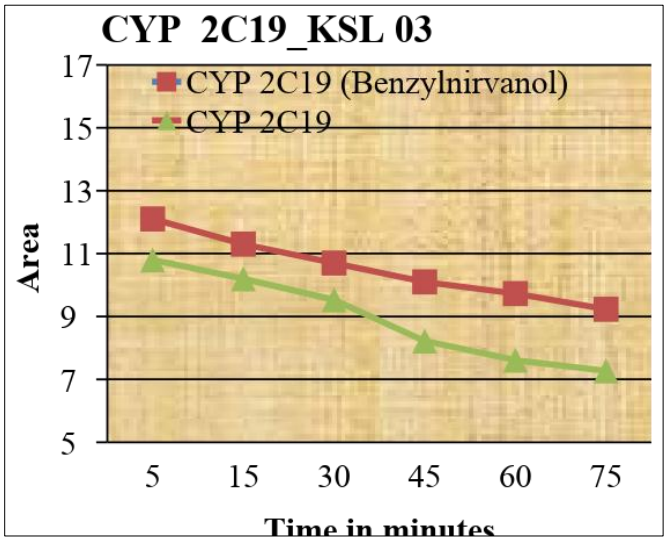

Fig 7: Graphical illustration of CYP2C19. In presence and absence of Benzylnirvanol

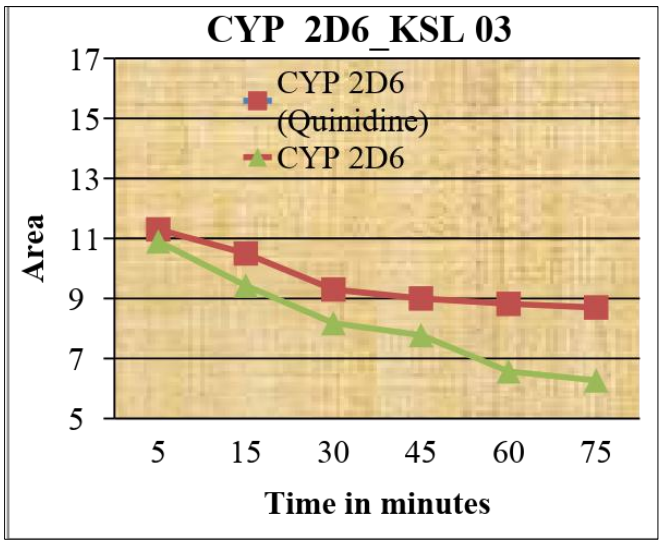

Fig 8: Graphical illustration of CYP2D6. In presence and absence of Quinidine

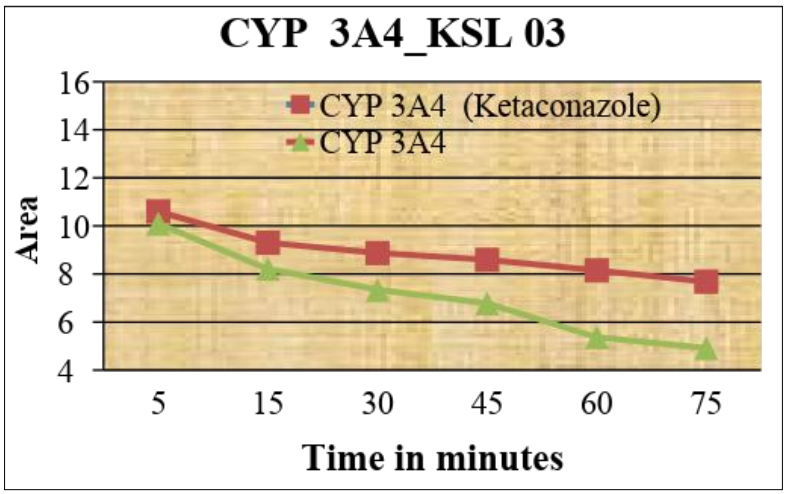

Fig 9: Graphical illustration of CYP3A4. In presence and absence of Ketaconazole

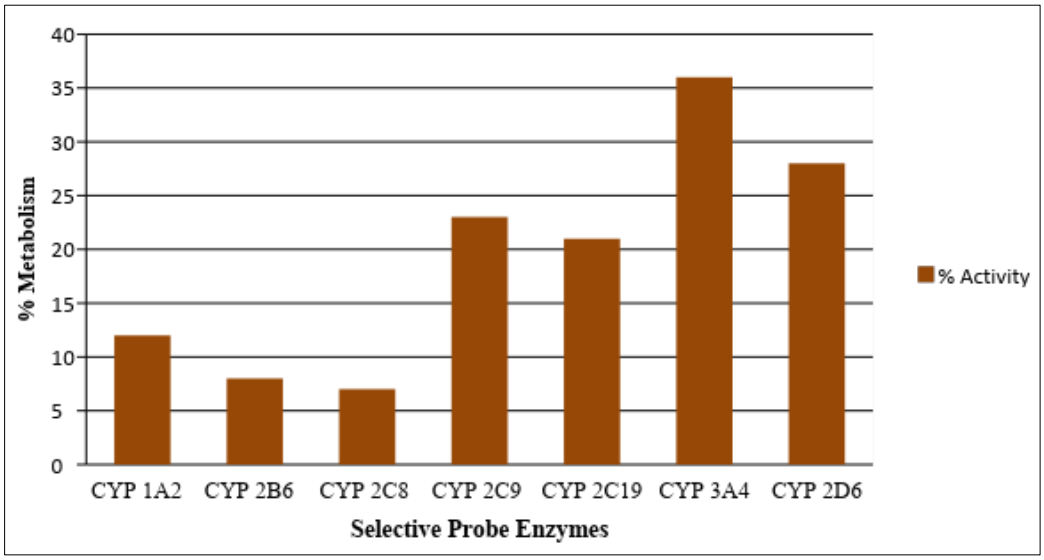

Fig 10: Effect of Selective Enzymic Activity on Test Compound

Discussion

For the evaluation although it's not a known procedure to use for investigating a Test compound in *in vitro* studies, this exercise drafted the selective and specific involvement of the individual metabolic enzymes.

Reviewing the literature information about *in vitro* CYP-based metabolism studies using purified enzymes, cDNA-expressed enzymes, and selective chemical inhibitors as well as studies on enzyme kinetic analyses, the rate of formation of a metabolite specifically reflects the activity of distinct CYP enzymes. When the NCE undergo metabolism and the reaction is selective, the formation of a metabolite being carried out by a single enzyme, the practical criteria which have to be checked are the commercial availability of the assayed molecular species, in order to quantitate the amount of metabolism using a standard, and the availability of an assay that is sensitive, rapid, simple and reasonable for in *vitro* experimental conditions. In addition one shall be cautious enough to exercise the difficulties encountered while extrapolating the *in vitro* information to *in vivo* use, in consideration to the appropriateness of probe substrates and experimental conditions while conducting the *in vitro* studies [5 - 6].

For this in the current research work the influence of the metabolic pathway was inferred by the selective inhibition of the specific enzymes. This enzyme inhibitory study specifically gives the inference about the metabolic activity of distinct CYP enzymes used in *vitro* which may later be extrapolated to *in vivo*. This approach clearly demonstrates the individual CYP capability of metabolism on the Test compound.

All the inferences obtained were comparing the data found in presence and upon inhibition of the selective enzymes. Calculations were drawn from the percent area difference, taking in the responses of the test compound in presence and absence of the selective CYP.

CYP1A2

Human liver microsomes contain relatively high constitutive levels of CYP1A2 i.e., 10–15% of the total P450 content of human liver under induced conditions. Environmental factors usually effect the CYP1A2 expression levels, complicating the *in vitro-to-in vivo* extrapolation. However in the current study of incubation with the CYP1A2 and the other with its inhibitor Alfa-Naphthaflavone there is a selective decrease in the area of the Test compound to an extent of 12 % at the end of 75 minutes time period.

CYP2B6

CYP2B6 is an isozyme which is expressed at low levels and found only in humans. It's observed that few industry investigators characterize the activity of this enzyme only in, *in vitro* studies.

The percent decrease in the concentration of the Test compound in this experiment can be accounted upto 8% in comparison to incubations without the enzyme.

The result of the current study infers that the Test compound is not substrate of CYP2B6 activity.

CYP2C8

CYP2C8 is one of the principal enzymes responsible for attacking various long-chain polyunsaturated fatty acids.

Upon incubation with the CYP2C8 there observed a selective decrease in the area of the Test compound to an extent of 7 % at the end of 75 minutes time period as observed from the comparison data obtained in presence and absence of Montelukast.

CYP2C9

CYP2C9 is the primary enzyme responsible for metabolizing nonsteroidal antiinflammatory drugs, oral antidiabetic agents, and angiotensin II receptor blockers. CYP2C9 also is the major enzyme involved in the disposition of warfarin.

Probing into its activity on the Test compound, there is a selective decrease in the area of the Test compound to an extent of 23 % at the end of 75 minutes time period.

CYP2C19

CYP2C19 is a genetically polymorphic enzyme and its metabolism represents 2.5 to 5% of Caucasian populations, 19% of African populations, and up to 30% of Asian populations. The contribution of this enzyme depends on the ratio of its expression levels in the body and here it's observed there is a selective decrease to an extent of 21 % at the end of 75 minutes incubation time period.

CYP2D6

CYP2D6 is a polymorphically expressed P450 enzyme. About

5 to 10% of Caucasians are poor metabolizer's of CYP2D6 substrates. As with polymorphically expressed CYP2C19, the assessment of CYP2D6-based drug interaction is meaningful only in the extensive metabolizer's. Although CYP2D6 only constitutes about 2% of total CYP enzymes in the liver, it is responsible for metabolizing drugs, next to CYP 3A4 in a variety of therapeutic classes. In the current study its impact on metabolism is noticeable showing a selective decrease of 28% in the area of Test compound.

CYP3A4

CYP3A4 is the most abundant P450 enzyme in humans, accounting for an average of 30 to 40% of total CYP P450 protein in the liver. Current literature data clearly indicate that CYP3A4 is the most important CYP member with regard to clinically significant drug metabolism. The substrate used here demonstrate that in the current study the influence of CYP3A4 enzyme is greater than all other enzymes that are been probed.

It has shown a selective decrease in the area of the Test compound to an extent of 36%, which is the highest effect found, when compared with other enzymes.

The action of the specific inhibitor's on CYP enzymes would lead to increase in the half life of the Test compound, demonstrating the molecule's metabolism by the microsomal enzymes using subtraction method. Thus the Test compound being metabolized by a group of enzymes at different levels during its metabolism are obtained.

The effect of selective enzymes and the metabolic pattern of Test compound under the influence of the inhibitor are demonstrated as percentage – given as the graphs under results Figures 1 - 10.

Conclusions

The objective of the study to use the Human Liver Microsomes with the selective enzymes gave sufficient insight about the individual effect of major CYP enzymes on the metabolism.

The relative role of a particular enzyme on the Test compound is obtained from the area of the probe molecule in presence and absence of the selective enzymes, where the clear inferences about the action of selective CYP enzyme under the given conditions were obtained by comparing the changes in the areas of the Test compound in presence and absence of the selective enzymes.

The order of activity of the representative enzymes on Test compound were CYP 3A4> CYP 2D6> CYP 2C9> CYP 2C19> CYP 1A2 > CYP 2B6 > CYP 2C8.

The inference drawn by comparing the data obtained in presence and upon absence of the selective enzyme and calculations obtained are from the percent area difference of the test compound in presence and absence of the selective CYP is shown in Table 4.

Table 4: The relative percentage of metabolism upon incubation with the CYP enzymes.

Enzyme	% Activity
CYP 1A2	12
CYP 2B6	8
CYP 2C8	7
CYP 2C9	23
CYP 2C19	21
CYP 3A4	36
CYP 2D6	28

The role of CYP 2B6 & CYP 2C8 on the metabolism of the NCE are less when compared with other 5 CYP enzymes. The contribution of CYP 3A4, CYP 2D6 & CYP 2C9 are more than 20% and comparable to each other. CYP3A4 has shown highest activity of 38% whereas the least was shown by CYP 2C8. The data obtained ascertains that the Test compound is a promising entity from safety perspective as is metabolized by the enzymes CYP 3A4 & CYP 2D6 whose availability is more. However the real activities have to be confirmed from the *in vivo* studies where the involvement of the respective enzymes would vary as there real expressions are significantly different in various species under live conditions. Irrespective of the above it can be concluded that these enzymes are capable of showing clear metabolic action on the Test compound.

The action of the specific inhibitor's on CYP enzymes would lead to increase in the half life of the Test compound, demonstrating the molecule's metabolism by the microsomal enzymes. The Test compound is been metabolized by a group of enzymes at different levels during its metabolism.

This assay demonstrates that the Test compound offers a good substrate for most of the popular enzymes available, evident from the work up representing the particular enzyme activity under specific experimental conditions. While the sustained level of concentration for the test compound is probably due to its structural chemistry, known from the drug design and the objective of its construction shall be taken into consideration during the interpretation of the results obtained. The inferences drawn act as a valuable tool in obtaining the necessary SAR to re-design the molecule. This customized approach represents a significant efficiency and selectivity improvement over traditional methods, and can be used to understand the drug's Pharmacokinetic and Pharmacodynamic nature.

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