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Development of analytical method for assessment of liver based Ex-vivo metabolic profile of pazopanib

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ABSTRACT

Drug biotransformation study is an integral part of preclinical screening for new drug candidates. This assessment usually involves both in vitro and in vivo models in animal species where the main pharmacokinetics, pharmacodynamics and toxicological profile are investigated. Several in vitro models utilized in studying hepatic drug biotransformation. Such models which range from whole cell system (intact perfused liver, human hepatocytes culture, hepatic and transfected cell lines)to enzymes preparations (liver Microsomes, cytosolic and S9 fraction) are now increasingly applied for quantitative and qualitative assessment in preclinical drug development, post-approval routine checks, identification of metabolic determinant factor etc. A literature survey was done to study reported metabolites and metabolic pathway of pazopanib Ex-vivo protocol for metabolic assessment was developed after collecting the appropriate enzymatic fraction. RHPLC method was developed and validated for quantitation of pazopanib in presence of metabolizing enzymes. Microsomal fraction was isolated from goat liver obtained from slaughterhouse using centrifugation technique. A sample was incubated with pazopanib and also in the presence of microsomal enzyme inducer and inhibitor and sample withdrawn at different time interval were subjected to analysis by the developed HPLC method for detection of possibly formed metabolites. The developed chromatographic method will be a handy tool for assessment of quantitative formation as such metabolites and will help in metabolic stability for pazopanib.

Keywords— *Pazopanib, Metabolites, Liquid chromatography, Liquid chromatography and mass spectroscopy, Microsomal fraction*

1. INTRODUCTION

The leading cause of death all over the world is the Renal cell carcinoma. Every year, about 14,970 people die due to this cause. The need for the drug development in anti-cancer is therefore necessary. The major issue is the safety of therapy. And so the toxicity, as well as the metabolic stability of anti-cancer, needs to be comprehensively studied. The main reason for toxicity is Metabolic Overload and the lack of specificity.

In this work, we propose to study fractions obtained from liver using simple techniques to isolate, characterize and use these for specific metabolic and toxicity studies.

Pazopanib is a multi-targeted tyrosine kinase inhibitor of vascular endothelial growth factor receptor (VEGFR)-1, -2, -3, platelet-derived growth factor receptor (PDGFR)-α and -β and stem cell factor receptor (c-kit) VEGFR-mediated signalling pathways play a critical role in renal cell carcinoma angiogenesis, tumour growth, and metastasis

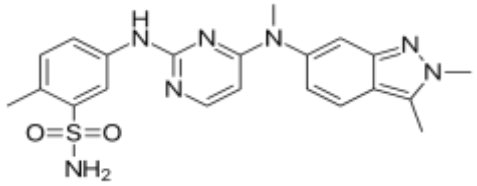


Fig. 1: Chemical structure of pazopanib

The FDA encourages drug developers to study the metabolic profile of candidate drugs in vitro, in vivo (animals) and in humans as early as feasible to avoid finding dispropthe ortionate metabolites later and potential development and marketing delays (FDA 2008). The FDA acknowledges that direct dosing of a metabolite may lead to metabolism and toxicities that were not observed with the parent drug, adding complications to the safety evaluation (FDA 2008). The European Medicine Agency revised its guidance on nonclinical safety studies (ICH guideline M3 (R2) for the conduct of human clinical trials at the end of 2009 to include short metabolite section (EMA 2009). The EMA requires nonclinical characterization for human metabolites having levels

greater than 10% of total drug-related exposure and at greater levels than maximum observed in toxicological animal studies (EMA 2009).

Drug metabolism mainly takes place in the liver, where along with metabolism of the drugs, the excretion and thus the clearance of the drugs takes place. The site of hepatic metabolism includes liver and site of extra hepatic metabolism include lungs, blood skin GIT, and kidney. As the liver contains a number of metabolizing enzymes majority of metabolism occurs in the liver. Prominent examples of metabolizing enzymes are the cytochromes P450 (CYPs) isozymes. Cytochrome P450 is a component of microsome. Human liver microsomes provide the most convenient way to study CYP450 metabolism. Microsomes are a subcellular fraction of tissue obtained by differential high-speed centrifugation. All of the CYP450 enzymes are collected in the microsomal fraction. The CYP450 enzymes retain their activity for many years in microsomes or whole liver stored at low temperature. Many forms of cytochrome P-450 have been classified into 17 families according to homologies of their amino acids sequences. [1]

Metabolism is a process of bio-transformation where the drug is transferred into different chemical forms by an enzymatic reaction. Metabolism mainly increases drug hydrophilicity and decreases toxicity and activity of most of the drugs. The study of the metabolic fate of drug (absorption, distribution, metabolism, excretion, toxicology) is an important part of the drug development process. [2]

The goals in evaluating ex-vivo metabolism are:

1) To identify all of the major metabolic pathways that affect the test drug and its metabolites, including the specific enzymes responsible for elimination and the intermediates formed; and
2) To explore and anticipate the effects of the test drug on the metabolism of other drugs and the effects of other drugs on its metabolism. Pharmacologic effects of the test drug and its major metabolites also should be studied, if feasible. The knowledge that a particular drug is not a substrate for certain metabolic pathways is helpful.

The drug metabolized by the different reaction that is classified into phase 1 and phase 2 mostly includes phase oxidation, reduction, and hydrolysis. The function of phase 1 reaction to introduce a new functional group within the molecule to a modified existing functional group or to expose functional group that is the substrate to the phase 2 reaction. Phase 1 reaction is responsible for the enhancement of hydrophilicity of drug. Phase 2 reaction represents conjugating reactions and further increases hydrophilicity and facilitates excretion of metabolites from the body. [3]

The analysis of metabolites in complex biological matrices is a challenging task; therefore several analytical methods for qualification and quantification of drug metabolites are used. First, for the qualification and quantification of the process is carried out by High-performance liquid chromatography and then liquid chromatography coupled with mass spectroscopy (LCMS) has become a powerful tool for the screening and identification of drug metabolites in biological matrices due to its selectivity, sensitivity, and speed of analysis. The LCMS method is considered as a most appropriate method for determination of metabolites of drug and also suited for high throughput analysis. Complex metabolite mixture is resolved by chromatographically on High-performance liquid chromatography (HPLC) Column. and full scan MS and product ion scan MS data are generated online, Thus molecular weight of drug metabolites and localization of biotransformation site can be elucidated based on interpretation of MS data. [4,5]

In this study Development and validation of an HPLC method for quantization of pazopanib. Identification of biofluid fractions and other tissues i.e. Goat liver procured from the local butcher house will be cut in small pieces. Tris-buffer will be added to these pieces. Pieces will be subjected to homogenization by grinder for 10 min. Homogenate will be centrifuged for a different interval of time at different rotations per minute to get various microsomal fractions. The drug will be incubated in the collected different microsomal fractions for a specified period of time and will be analyzed by HPLC to identify and quantify the portion of drug metabolized and possible metabolite formed.

2. MATERIAL AND METHOD

2.1 Chemical and Reagents

Pazopanib API was procured from Cipla private limited Mumbai with purity 99.4% w/ w.
HPLC grade Acetonitrile (CH_2CN), Methanol, Water, Analytical grade– potassium chloride (KCl), Tris buffer.
Fresh Goat liver procured from the local butcher shop

2.2 Equipment

HPLC (High performance liquid chromatography) (jasco HPLC with 2080 plus Detector MD2010) UV visible double beam Spectrophotometry (Jasco UV-630, Japan) Analytical balance (Digital) (Shimadzu AX-200, AY200, Japan) Incubator (Nanolab India) Centrifuge (REMI-R 8C) pH meter (Digital)
(Lab Junction) Ultrasonicator (PCI enterprises, Mumbai)

2.3 Chromatographic method Development

Selection of stationary phase: Selection of chromatographic condition done on the basis of Physicochemical properties (pKa, log P, Solubility) and appropriate wavelength. The chromatographic variables such as mobile phase, stationary phase, flow rate, solvent ratio were studied. The resulting chromatograms were recorded. The chromatographic parameters like retention time capacity factor, theoretical plates, peak symmetry, tailing factor etc. were studied.

Selection of mobile phase: Various mobile phase compositions of water and acetonitrile in different ratios were tried to get desired retention time. Water: Acetonitrile (60:40) was selected to obtain good resolution of pazopanib.

2.4 Ex-vivo protocol for metabolic assessment

Preparation of 0.154 M KCl containing 50 m Mtris-HCl (pH 7.4): The accurately weighed quantity of 1.48 gm of KCl was transferred into 100 ml volumetric flask containing water. The accurately weighed quantity of 0.605 gm of Tris-buffer was added to it. pH was adjusted with conc. HCl to 7.4 using pH meter to make 0.154 M KCl containing 50 m Mtris-HCl (pH 7.4).

Preparation of microsomal fraction: Freshly isolated microsomal enzymes in suspension were applied for incubations from 15 m in. to 14 hrs with test substances to predict theirs in vivo metabolism. Goat liver procured from the local butcher house was cut in small pieces then tris-buffer was added. Pieces were grinded in a mixture for 10 min. to make homogenate using 0.154 M KCl containing 50 mMtris-HCl (pH 7.4) as homogenating medium. Homogenate was centrifuged firstly at 600 g/2590 rpm for 10 min, then supernatant collected and centrifuged at 5000 g/ 7477 rpm for 10 min and lastly again collected supernatant centrifuged at 10,000 gm/ 11500 rpm for 1 hr 30 min to get a microsomal fraction.

2.5 Incubation and analysis on HPLC-DAD chromatography

Positive control (Pzopanib in homogenising medium): Positive control for incubation was prepared by using 5 mg Pazopanib in 5 ml of homoginizing medium which was incubated at 37^0C for up to 14 hour

Negative control (Pellets of the microsomal fraction in homogenizing medium): Negative control for incubation was prepared by using 5 mg pellet of the microsomal fraction to the solution of 5 ml homogenizing medium, which was incubated at 37 ˚ C for up to 14 hr.

Set-I (Pazopanib): The samples of set-I control for incubation was prepared by adding 5 mg pellet of the microsomal fraction to the solution of 5 ml of homogenising medium, and 5 mg Pazopanib drug which was incubated at 37 ˚ C for up to 14 hr.

Set-II (Pazopanib with P henobarbitone as inducer): The samples for set-II control for incubation was prepared by adding 5 mg pazopanib and 1 mg Phenobarbitone as a microsomal enzymes stimulator the to5 mg pellet of a microsomal fraction of homogenising medium, which was incubated at 37 ˚ C for up to 14hr.

Set-III (Pazopanib with Ranitidine as an inhibitor): The samples for set-II, I control for incubation was prepared by adding 5 mg pazopanib and 1 mg ranitidine as a microsomal enzymes inhibitor to 5 mg pellet of a microsomal fraction of homogenising medium, which was incubated at 37 ˚ C for up to 14 hr.

3. MASS SPECTROPHOTOMETRIC ANALYSIS (LC-MS)

For the identification of metabolites present in the sample solution, LC-MS analysis was carried out. Mobile phase optimized for the chromatographic method development was employed for LC-MS work with minor modification, it helps to obtain further detailed structural information of metabolites. The instrument used for liquid chromatography analysis was Accela1200 (Thermo Fisher Scientific) and the mass spectrometer was Q exactive orbitrap (Thermo Fisher scientific. the USA). Analytical conditions were as follows: Mobile phase Water: Acetonitrile (60:40). Both solvents were LC-MS grade. Samples were injected by splitless mode. The volume injected was 1.5µl .the total running time was 20 minutes with a flow rate 350µl/min. the gradient was 90% acetonitrile throughout 10 minutes. The column used was Hypersil gold (50mm x 2.1 mm, 1.9 µm). The MS conditions were as follows; polarity was positive, the sheath gas flow rate 45 µl /min, Auxiliary gas flow rate 12 µl/min; acquisitions scan mass range of 100 – 1000 amu. Capillary voltage -4.2 kv, capillary temperature - 320ºC, drying gas temperature - 350 ºC. The software used for analysis of the data obtained from LC-MS was named" X-Caliber"

4. RESULT AND DISCUSSION

As we have mentioned earlier, the main objective of this was for the development and validation of HPLC method for quantitation of pazopanib. Identification and isolation of bio-fluid fractions. Characterisation of formed metabolite/s For characterization of the drug by UV spectroscopy, determination of the wavelength of maximum absorption (λ_{max}) was done and UV-spectrum was also recorded. The spectrum was taken in water: Acetonitrile (60:40 v/v).

It was found that the Maximum Absorption rate ((λ_{max}) was at 272 nm and Maximum Absorbance was 0.1799.

Various mobile phase compositions of water and Acetonitrile in different ratios were tried to get desired retention time. Water: Acetonitrile (60:40) was selected to obtain good resolution of Pazopanib. It was found that the retention time for the mobile phase of Water: Acetonitrile (60:40) was 6.5 minutes.

For Quantitation of pazopanib and its metabolite/s formed by extracted enzymes using the developed HPCL method following conditions were created:

Positive control: 5mg drug + 5 ml homogenising medium.
Negative control: 5mg pellet + 5ml homogenising medium
Set I (Drug sample): 5 mg drug + 5mg pellet + 5 ml homogenising medium.
Set II (Drug + Inducer): 5 mg drug + 1 mg inducer + 5 mg pellet + 5 ml homogenising medium
Set III (Drug + inhibitor): 5 mg drug + 1 mg inhibitor + 5 mg pellet + 5 ml homogenising medium

4.1 Sampling

All Samples from of Set-I, Set-II, and Set-III, and negative control were withdrawn at 15 min, 30 min, 1 hr, 2 hr, 4 hr,6 hr, 8 hr.10 hr,12 hr, 14 hr. and these samples were analyzed on HPLC chromatography using following chromatographic conditions.

Table 1: Chromatoghrapic condition

Chromatographic mode	Chromatographic Conditions
Stationary phase	Hypersil BDS C18(150mm x4.4mm)5um
Mobile phase	Water: Acetonitrile (60:40)
Detection Wavelength	272 nm
Flow rate	1 ml/min
Sample size	20 µl
Runtime	10 min.

From the HPLC analysis, I sample showing significance concentrations of metabolites was selected for LC-MS analysis.LC-MS analysis of samples was carried out to determine the probable structure of metabolites. All the LC-MS chromatograms and fragmentation patterns are given below.

4.2 LC-MS analysis of pazopanib drug sample Set I (12-hour incubation)

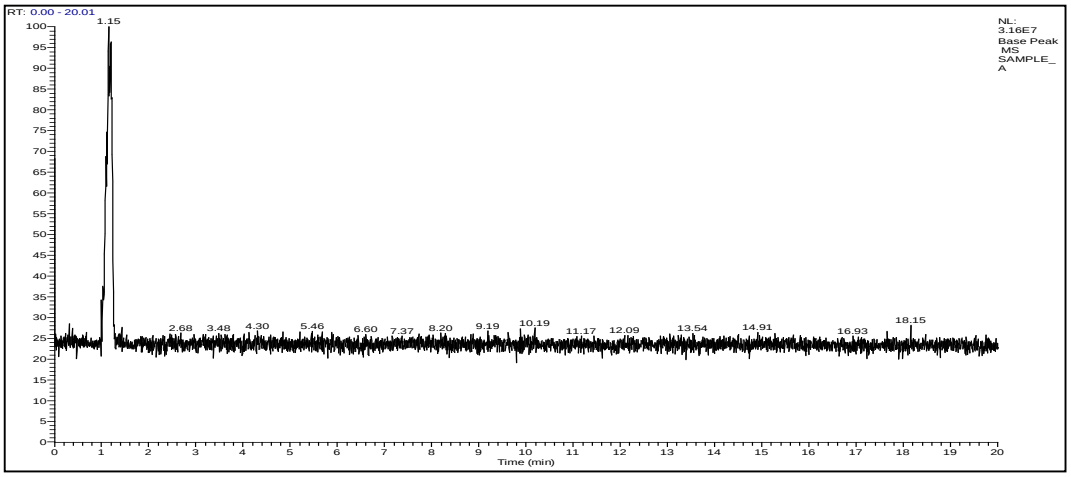


Fig. 2: LCMS of Pazopanib Drug sample (Set 1) after incubation of 12 hours at 1.20 min.

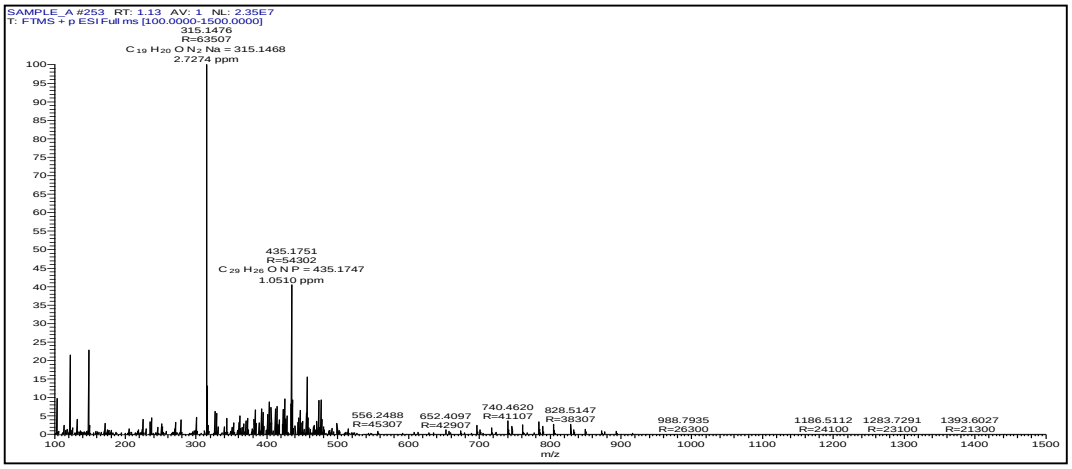


Fig. 3: Fragmentation pattern of I of Pazopanib drug sample (Set I) after of incubation of 12 hours at 1.20 min retention time

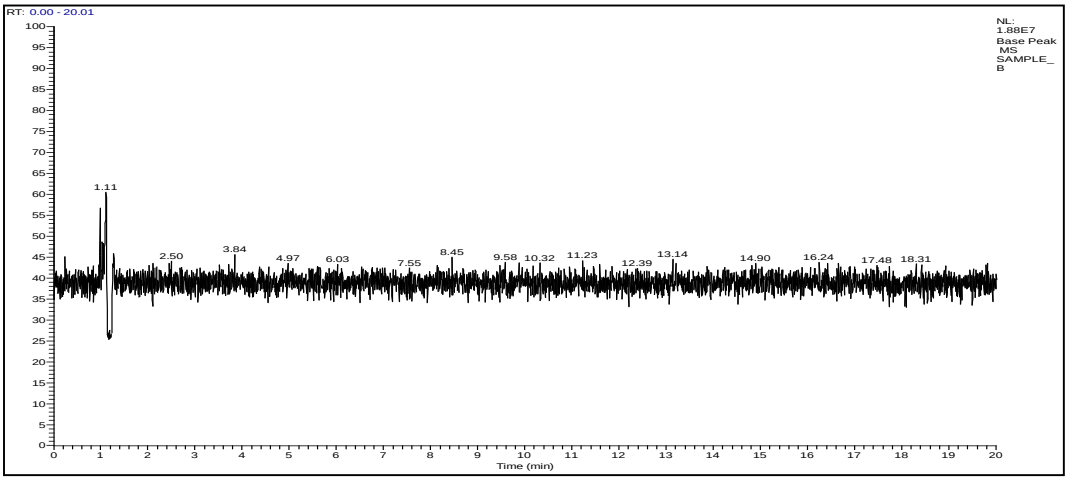


Fig. 4: LCMS of Pazopanib Drug sample (Set 3) after incubation of 14 hours at 1.11 min.

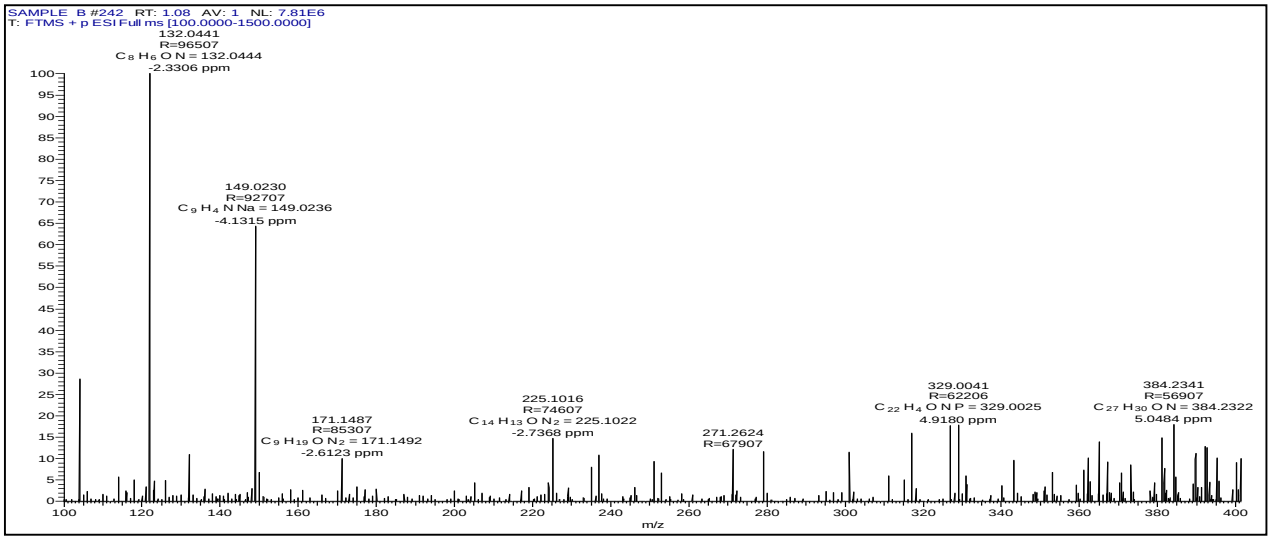


Fig. 5: Fragmentation pattern of Pazopanib Drug sample (Set 3) after incubation of 14 hours at 1.11 min.

Five phase 1 and phase 2 metabolites were identified in the proposed study. The masses of the obtained metabolites and possible chemical reaction are mentioned in table 2. The masses (m/z) of metabolites obtained were found to be 329.01, 315.14, 435.17,132.04,149.023.

Table 2: The masses and the possible chemical reaction of the identified metabolites of pazopanib

Metabolites	Mass of metabolites [m/z]	Mass differences w.r.t Drug molecule [Δm]	Possible reaction
M1	329.01	-108.507	Hydroxylation
M2	315.14	-122.377	Hydroxylation
M3	435.17	-2.347	N-Demethylation
M4	132.04	-305.477	Glucuronidation
M5	149.023	-288.287	Hydroxylation, glucuronidation

m/z: mass/ charge value, Δm: Mass difference , mass of protonated pazopanib = m/z 437

4.3 Structure of Pazopanib metabolites

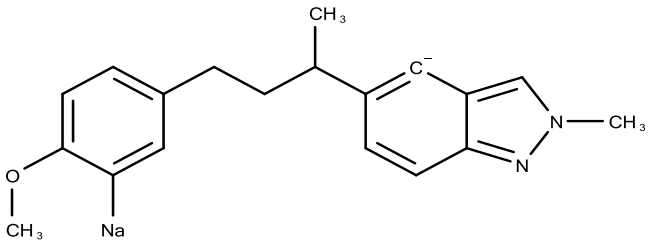
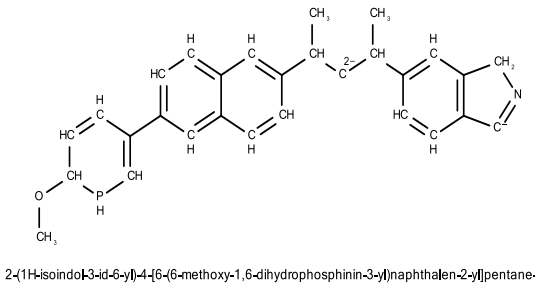


Fig. 6: Structure of Pazopanib metabolites (M1) (Mol.Wt. 329.01 g/mol)



2-(1H-isoindol-3-id-6-yl)-4-[6-(6-methoxy-1,6-dihydrophosphinin-3-yl)naphthalen-2-yl]pentane-3,3-diide

Fig. 7: Structure of Pazopanib metabolites (M2) (Mol.Wt. 315.14 g/ mol)

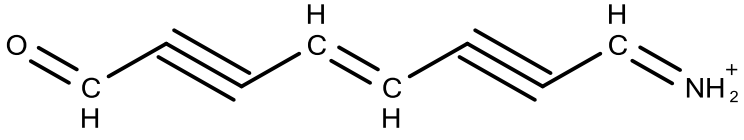


Fig. 8: Structure of Pazopanib metabolites (M3) (Mol.Wt. 435.17 g/mol

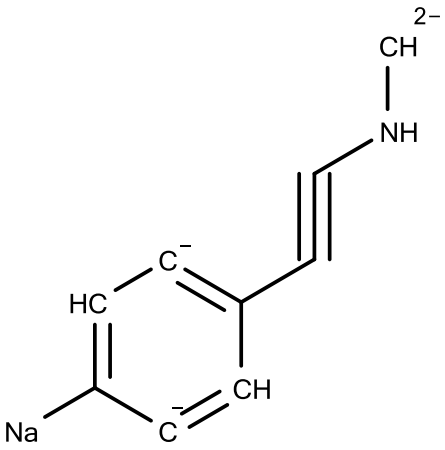


Fig. 9: Structure of Pazopanib metabolites (M4) (Mol.Wt. 132.04 g/mol)

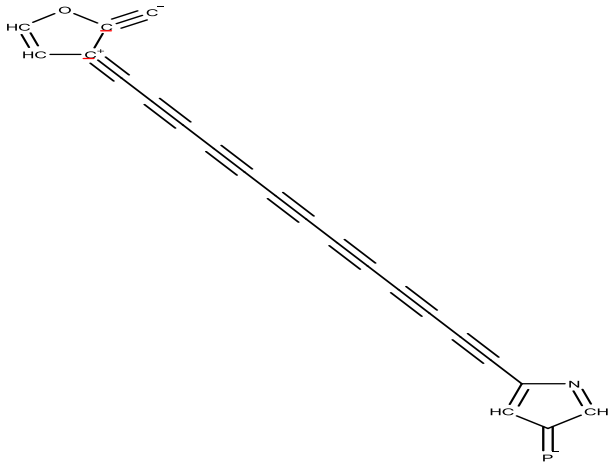


Fig. 10: Structure of Pazopanib metabolites (M5) (Mol.Wt. 149.023 g/mol)

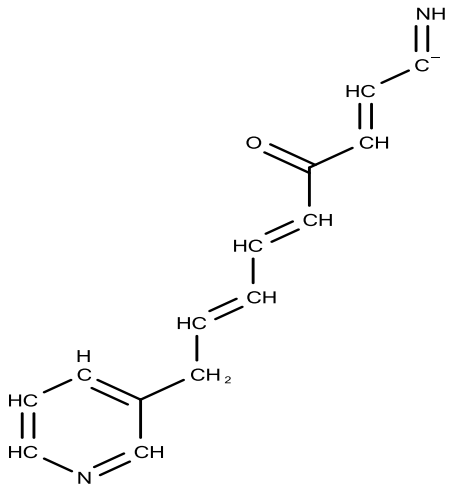


Fig. 11: Structure of Pazopanib metabolites (M6) (Mol.Wt. 225.02 g/mol)

Table 3: Probable fragments and their molecular weights

Probable phase I metabolites	Molecular weights
M_1	329.01
M_2	315.14
M_3	435.17
M_4	132.04
M_5	149.023

The results of LC-MS shows the presence and formation of the metabolite. It can be concluded that isolated microsomal enzyme fraction is valuable tools to study human liver drug metabolism. The system remains viable up to 14 h and exhibit drug metabolism at appreciable rates. The results obtained provide an insight into the metabolic properties of pazopanib.

5. CONCLUSION

Thus, in the proposed study 5 phase I metabolites of pazopanib were successfully detected and identifying employing HPLC followed by LCMS techniques by using goat liver tissue.

From this study, we conclude that the developed liquid chromatographic methods in this project will be a handy tool for assessment of the quantitative formation of such metabolites and help in metabolic stability and related toxicity estimations.
The method proved to be simple, accurate, precise, specific and selective, hence can be used for industrial application in analysis of the Pazopanib and metabolic products of Pazopanib. The study highlights the benefit of the use of metabolic profile in the establishment of structural information by LC-MS analysis for Pazopanib. The development of sensitive and specific analytical methods for quantization of a drug and its metabolites is critical to the study of drug metabolism. Development of such analytical methods has long been a high priority for drug development programs. These techniques once developed will be readily available for assessing drug metabolism. The results also emphasize that metabolic studies are suitable tools for safety pharmacological profiling with a time- and cost-effective method and more complex mechanistic investigation, than is possible with the existing methods. This will help in the determination of the drug development process and have the potential to complement, reduce, and possibly, in the future, replace some existing animal-based assays.

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