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IDENTIFICATION OF ALDEHYDE OXIDASE AS AN ENZYME IN METABOLISM OF ZONIPORIDE

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ABSTRACT

Aldehyde oxidases are molybdoflavoenzymes present in cytosolic compartment with broad substrate specificity, oxidizing different types of aldehydes, and heterocyclic rings has attracted increased interest in recent years. The physiological function of aldehyde oxidases is largely unknown, although the enzymes play an important role in the metabolism of numerous compounds of medicinal and toxicological interest, as they oxidize a wide range of aldehydes and heterocyclic compounds Aldehyde oxidase. This unit provides methods for identification and confirmation of AO as metabolic pathways that are AO substrate as well as the effect of different protein (Human S9 fractions) concentration on different concentrations of zoniporide. The peak at 3.29 min is Metabolized zoniporide while the peak at m/z 337 (RT = 3.22 min) suggests an addition of 16 amu to zoniporide. The maximum formation of metabolite shown up to 90 min in both protein concentrations respectively, further 90 min it decreases rapidly.

Keywords *– Aldehyde oxidase (AO), Metabolism, S9fractions, Molybdoflavoenzymes, Heterocyclic rings.*

1. INTRODUCTION

Aldehyde oxidase (AO) is the member of a group of molybdoflavo-enzyme has been found to play a role in the metabolism of many drugs and xenobiotic compounds. However, AO is also involved in the oxidation of nitrogen containing heterocyclic compounds [1]. The cytochrome P450 isoforms are responsible for phase I metabolism of approximately 80% of drugs on the market [2.] However, the contribution of this enzyme to the metabolism and clearance of drugs containing heteroaromatic rings appears to have attracted increased interest in recent years [3]. Some of the inhibitors of aldehyde oxidase have been identified in vitro, including raloxifene [4]. Drugs containing hetrocyclic rings like pyridines, pyrimidines, pyrazines, and their fused-ring analogues are all typical targets for oxidation by AO. For example, drugs such as brimonidine, carbazeran, N-[(2-diethylamino) ethyl]-acridine-4-carboximide (DACA), famciclovir, zaleplon, and zoniporide (Fig No.1) belong to a category of compounds that are primarily metabolized by AO [5].

This unit gives the identification of method for AO is one of the enzymes responsible in the metabolism of xenobiotics (See Protocol 1) (Fig. 2). Basic protocol 2 relate the effect of different protein (S9 fractions) concentration on AO as enzyme in metabolism of zoniporide.

2. MATERIALS AND METHODS

2.1 Solvents and Equipment's

Human liver S9 (Male, pool of 10 H1000.S9/Lot No.0710497,10 mL of 20mg/ml), (**Sekisui Medical Co., Ltd)**. Zoniporide hydrochloride hydrate (sigma) ,50mM potassium phosphate buffer pH 7.4, DMSO, LCMS-grade acetonitrile (J.T Backer), Milii Q water, formic acid,1.5-ml micro centrifuge tubes,37˚C shaking water bath, Vortex, Eppendorf centrifuge, LC-20AD HPLC system (Shimadzu Technologies), LC-MS/MS Triple quadruple API3200 (Applied Bio system) provided by (Advinus Therapeutics, Pune)

2.2 Reagents and Solutions

2.2.1 Water with 0.1% formic acid

Add 1 ml of formic acid to ml of HPLC-grade acetonitrile (Sigma-Aldrich) add in 1000 ml with MilliQ water, Mix thoroughly.

2.2.2 0.05M Potassium Phosphate Buffer (pH 7.4)

Weigh 0.647 g potassium phosphate, monobasic (KH_2PO_4) and 3.527g Potassium phosphate, dibasic (K_2HPO_4) bring to 500 ml with Millipore water store at 4-6ºC

2.2.3 Zoniporide Hydrochloride Hydrate

M.W. 320.35, Weigh 2.33mg mg of Zoniporide Hydrochloride in 1.5 ml tarson tube and add 727µL of DMSO to make 10 mM stock. (Stock A)

To achive 3000µM stock of zoniporide hydrochloride hydrate adds 300 from (stock A) + 700 µL of DMSO.

To achive 100µM stock of zoniporide hydrochloride hydrate add 10µl (stock A) + 990 µL of DMSO

2.2.4 Basic Protocol 1: Identification of ao as enzyme in metabolism of zoniporide hydrate

Non-CYP450 aldehyde oxidase follow-up experiments are generally performed; by using S-9 fractions (fraction used 1ml at 20mg/ml vial from XenoTech) with incubate the lead compound in the absence of NADPH.

2.3 Assay Procedure

1. Thaw human liver S9 fraction by placing the tube in cool water until the contents are defrosted and then place on ice.
2. Combine the following reagents in a 1.5-ml micro centrifuge tube

   519 µl of 0.05M potassium phosphate buffer, pH 7.4

   6µl of 100µM DMSO stock of Zoniporide Hydrochloride (final 1µM)

   75µl of 20 mg/ml human liver S9 fraction (final 2.5 mg/ml)
3. Pre-incubate 2 min at 37°C.
4. Initiate reaction by adding 10µl of 100µM Zoniporide stock solution (final 1µM).
5. Incubate up to 120 min in a 37°C water bath with gentle shaking.
6. Remove the sample after time point 0min,15min,30min,60min,90min,120min
7. Terminate reaction by adding 150µl acetonitrile and vortex for 10 sec.
8. Centrifuge 5 min at 4000rpm at room temperature.
9. Transfer supernatant 200 µl of sample from incubation tube into 96-deep well plate separately.
10. Inject to LC-MS-MS for analysis

2.4 LC-MS/MS Analysis

11. Transferred 200 µl supernatant in 96-deep well plate used for LC-MS/MS Analysis.
12. Analyze 10 µl of the mixture by reversed-phase HPLC/MS/MS using mobile phase
13. A -0.1% Formic Acid and mobile phase B –Acetonitrile.
14. LC-MS/MS conditions are described in Table 3

15. Analyze data obtained in the total ion chromatogram using Analyst® 1.5.1 Software.
16. The ion detection of zoniporide hydrochloride hydrate is compare with detection of metabolite standard compound 2-oxozoniporide, mass is 237 (Dalvie et al., 2015).

2.5 Basic protocol: 2 Effect of different protein (s9 fractions) concentration on ao as enzyme in metabolism of zoniporide hydrate

This unit relate to the effect of protein (S9 Fraction) on metabolism of Zoniporide hydrochloride hydrate, by using five substrate concentrations with 1.5mg/ml and 2.5mg/ml protetin concentration.

Concentration of zoniporide hydrochloride hydrate used in reaction is 1µM, 5µM, 15µM,50µM, 100µM.

2.6 Assay Procedure

1. Take 1.5ml eppendorff tubes as a reaction tubes with naming A, B, C, D, and E for protein concentration 1.5 mg/ml and F, G, H, I, J reaction tubes for protein concentration 2.5 mg/ml. refer Table. No 1and table.No.1 and table.No.2
2. Add buffer, S9 fraction and substrate concentration as describe in table no.
3. Incubate reaction tubes in 37ºC water bath shaker for 120min.
4. At 0 min,15min,30min,90min,120min of incubation period pipette out 100 µL of sample from A, B, C, D and E tubes in both case, and transferred in to fresh 96-deep well .plate containing 100 µL of cold ACN in individual column to terminate the reaction.
5. Remove the tube stand from water bath shaker.
6. Centrifuge at 4000 rpm for 5 minutes.
7. Transfer 100µl of sample from incubation plates into second 96-deep well plates separately.
8. Inject to LC-MS/MS for analysis.
9. LC-MS/MS conditions are given in table No.3

2.7 Critical Parameters and Troubleshooting

Concentration of DMSO up to 1% to 2% is shown to be compatible with AO.at this level AO activity is not decreased. (Obach, 2004; Choughule et al., 2013). The S9 fraction used for experiment throught the same lot of vials. The rate of conversion of the substrate depends on source of S9 fraction and lot. That's why it is advisable to use a positive control that is primarily metabolized by AO (such as zoniporide) to assess and validate the rates of new chemical entities by AO.

3. RESULT AND DISSCUSION

3.1 Results for Basic protocol: 1

When zoniporide was incubated using Basic Protocol I, the total ion chromatogram of the incubation mixture showed two peaks at 3.22 and 3.29 min with molecular ions *m/z* 337 and 321, respectively (Fig16). The peak at 3.29 min is Metabolized zoniporide Hydrcholoride hydrate while the peak at *m/z* 337 (RT = 3.22 min) suggests an addition of 16 amu to Zoniporide. Zoniporide decreases gradually with respective time points

Shown in table No.4 and Fig.No.3

The position of hydroxylation is identified by comparing mass spectra of the two molecular ions, which indicates addition of the oxygen atom on the quinolone ring and their interpretation (Fig No.6 and 7). Since the metaboliteis formed in the absence of NADPH, a cofactor required for P450-mediated oxidation, the role of AO in metabolism of this compound is speculated.

3.2 Results for Basic protocol: 2

The method described in Basic Protocol 2 the selection of protein concentration requires for the metabolic stability assay, the role of protein concentration used for assay development. the test concentration of Zoniporide used in protocol-2 is 1µM, 5µM, 15µM, 50µM, 100µM.Fig.no 15 and fig.no 16 shown the S9 fraction (protein concentration) taken 1.5mg/ml and 2.5mg/ml respectively. The maximum formation of metabolite shown up to 90 min in both protein concentrations respectively, further 90 min it decreases rapidly shown in Fig.No.8 and 9.

Table 1: Reaction condition for Protein concentration 1.5 mg/ml

Reaction Tube.	Substrate concentration(µM)	Sodium phosphate Buffer pH-7.4(µl)	S9 Fractions (20mg/ml) in(µl)	Volume of substrate (µL)	Final reaction volume (µL)
A	**1**	549	45	6 µL from 100 from 100µM DMSO stock	600
B	**5**	545	45	10µL from 300µM DMSO stock	600
C	**15**	546	45	9µL from 1000µM DMSO stock	600
D	**50**	545	45	10µL from 3000µM DMSO stock	600
E	**100**	549	45	6µL from 10000µM DMSO stock	600

Table 2: Reaction condition for Protein concentration 2.5 mg/ml

Reaction Tube	Substrate concentra-tion (µM)	Sodium phosphate Buffer pH-7.4(µl)	S9 Fractions (20mg/ml) in(µl)	Volume of substrate (µL)	Final reaction volume (µL)
F	**1**	519	75	6 µL from 100 from 100µM DMSO stock	600
G	**5**	515	75	10µL from 300µM DMSO stock	600
H	**15**	516	75	9µL from 1000µM DMSO stock	600
I	**50**	515	75	10µL from 3000µM DMSO stock	600
J	**100**	519	75	6µL from 10000µM DMSO stock	600

Table 3: LC-MS/MS Settings Used in Conducting the Experiment for Zoniporide

Instrumentation		
Sr.no	**Conditions**	**Information**
1.	Model	SIL-HTC
2.	Column	HPLC-Agilent-Zorbax-Eclipse-XDB-C18-4.6x50mm
3.	Pump	LC-10ADvP Binary gradient pump
4.	Auto injector	LC-10ADvP Auto injector
5.	Shimadzu LC system Injection volume	10µl.
6.	Pressure Range	0-4999 psi
7.	Sampling Speed	5.0µL/sec.
8.	Purge Time	25min
9.	Total Flow	0.600 ml/min
10.	*Mobile phase* Solvent A Solvent B	 0.1% Formic acid in water Acetonitrile
11.	*Gradient* Time (min) 0.50-3.00 3.00-3.50 3.50-3.60 3.60-5.00 5.00- Stop	 Solvent A Solvent B 95 5 5 95 95 5 5 95
12.	Detection	SCIEX-API 3200 LC-MS/MS mass spectrometer
13.	Zoniporide	(M+H)321
14.	Oxidative metabolite	(M+H)337
15.	Mass spectrometer	API 3200 LC-MS/MS
16.	Source	Turbo spray
17.	Polarity	Positive
18.	Acqisition Duration	5min1sec
19.	Number of scans	208
20.	Scan Type MR transitions	MRM
21.	Collision gas (CAD)	6.00
22.	Temperature (TEM)	450.00
23.	Ion source Gas1 (GS1)	40.00
24.	Ion source Gas1 (GS2)	70.00
25.	Ion Spray Voltage (IS)	5000.00
26.	DE clustering Potential (V)	40.00
27.	Entrance Potential (EP)	10.00
28.	Collision cell exit potential (CXP)	10.00
29.	Software version	Analyst 1.5
30.	Multiple reaction monitoring transitions Zoniporide	Q1(m/z) Q2(m/z) 321 337

Table 4: Metabolic stability of Zoniporide

Time (min)	% R at 120'	LN % R
120	34	3.52
90	48	3.87
60	63	4.14
30	83	4.42
15	86	4.46
0	100	4.61

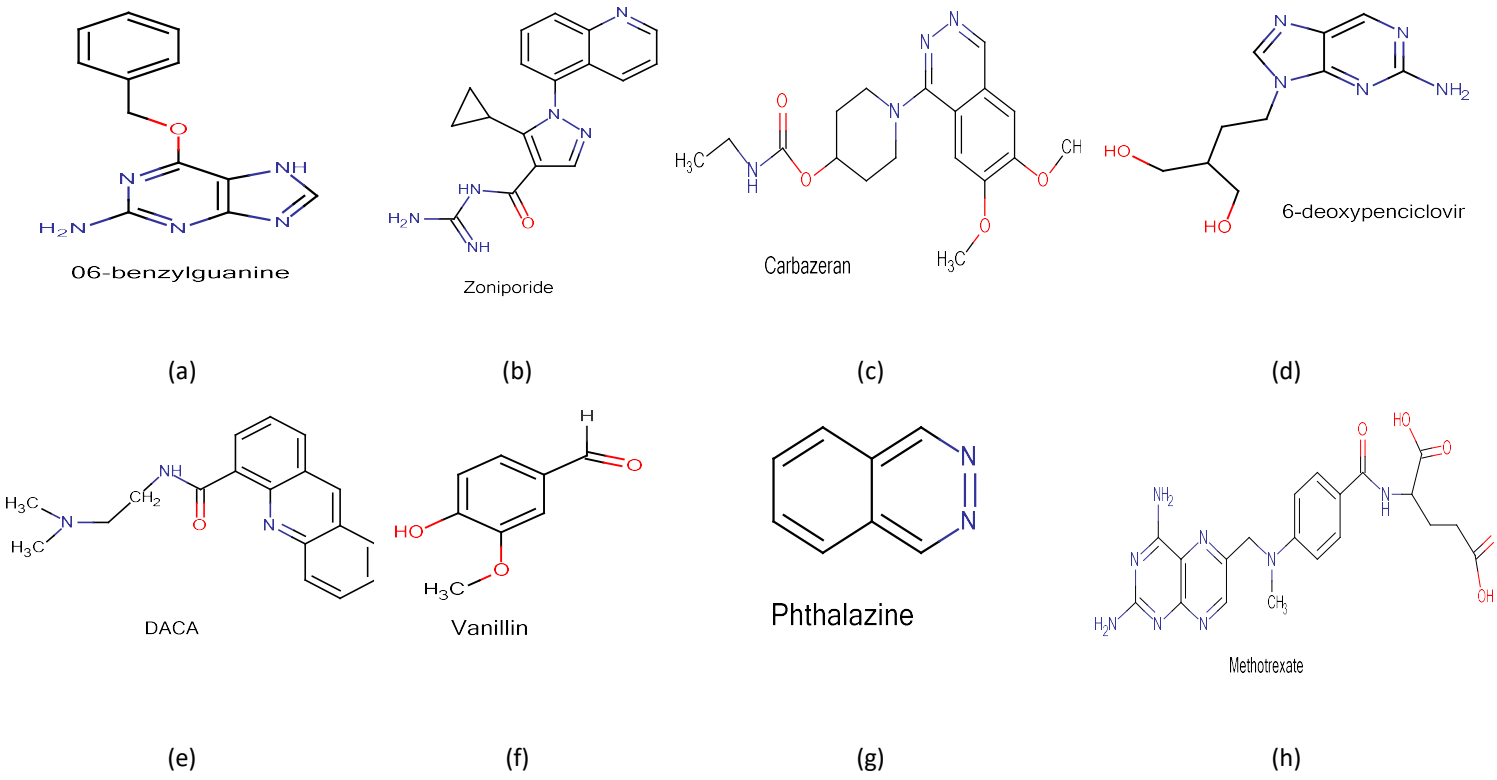

Fig 1: Structures of some drugs that are substrates for aldehyde oxidase (AO).

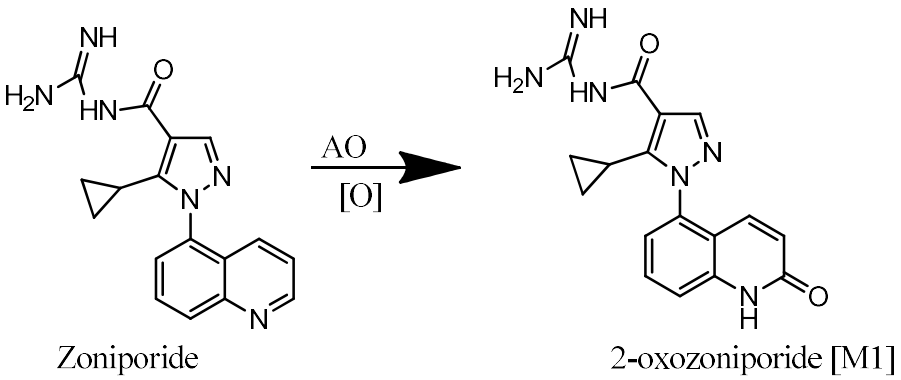

Fig 2: Conversion of zoniporide to its oxidative metabolite, 2-oxozoniporide (M1)

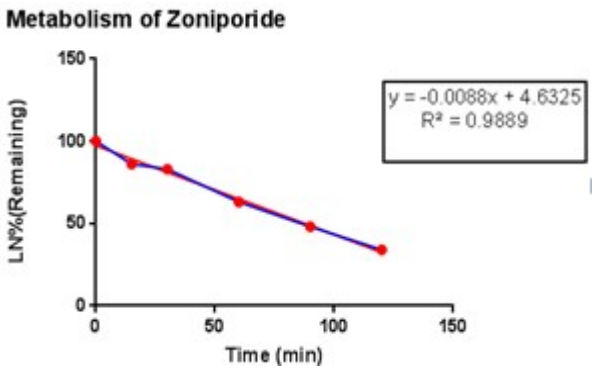

Figure 3: Plot showing disappearance of Zoniporide hydrochloride Hydrate upon incubation with pooled liver S9 fraction.

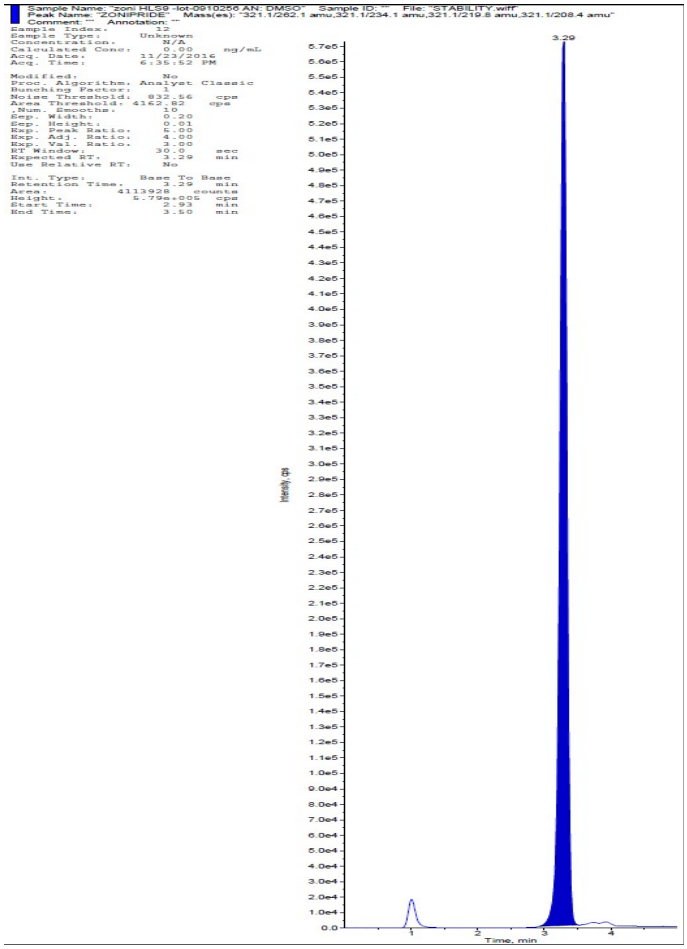

Figure 4: Chromatogram of Zoniporide Hydrochloride Hydrate (RT 3.29)

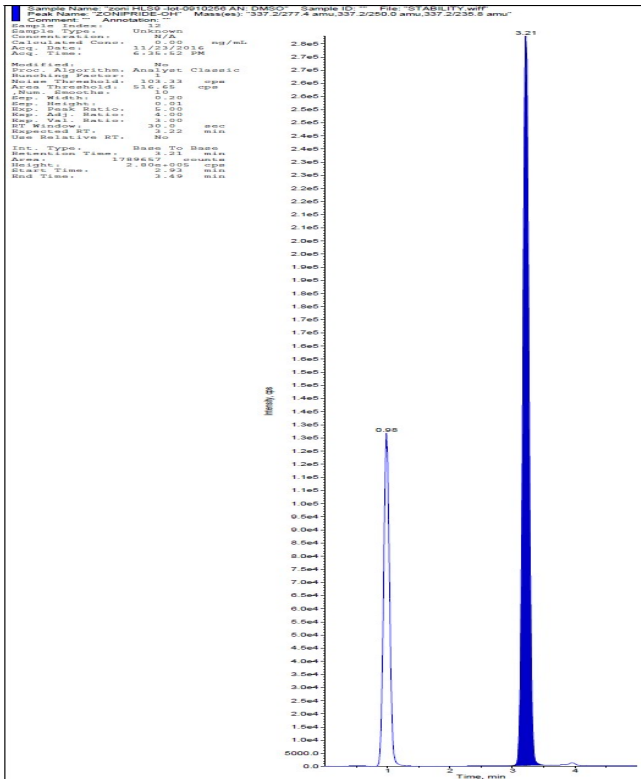

Fig. 5: Chromatogram of 2-oxozoniporide (RT 3.21)

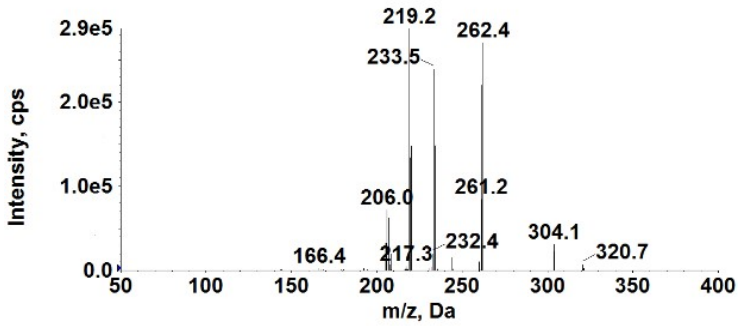

Fig. 6: LC-MS/MS spectra of Zoniporide Hydrochloride hydrate

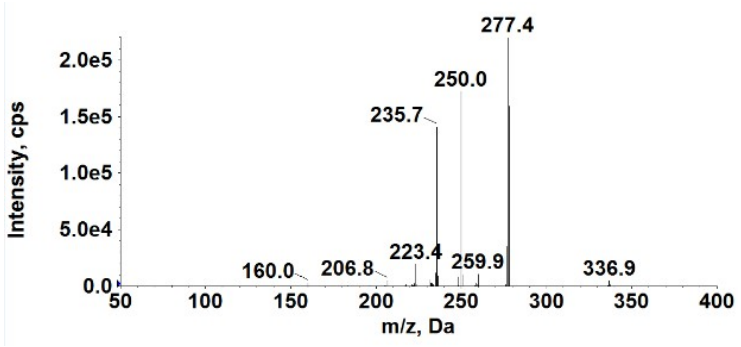

Fig. 7: LC-MS/MS spectra of metabolite 2-Oxozoniporide

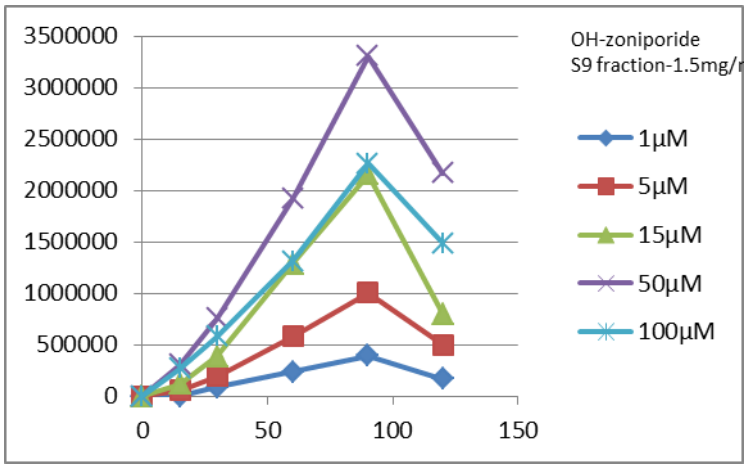

Fig 8: Zoniporide hydrochloride with at protein concentration 1.5 mg/ml

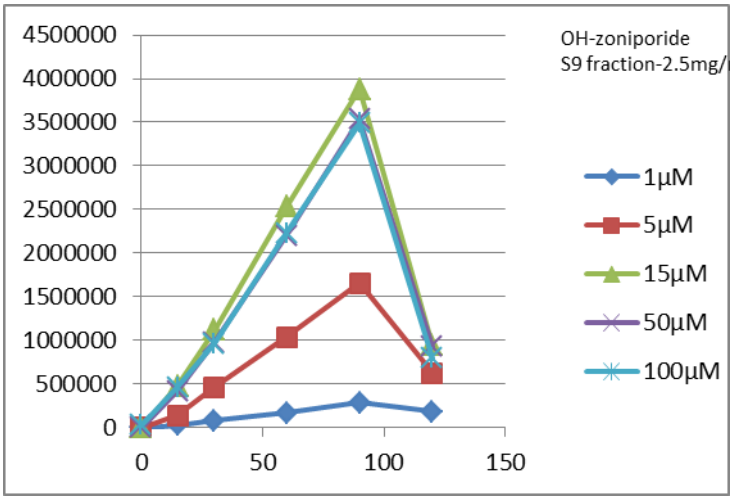

Fig 9: Zoniporide hydrochloride with at protein concentration 2.5 mg/ml

4. CONCLUSION

It can be concluded that the nitrogen containing heterocyclic compound are metabolized by aldehyde oxidase. As per our experimental study it can be concluded that the Zoniporide hydrochloride hydrate shows metabolism by aldehyde oxidase. The effective incubation time developed for zoniporide hydrochloride hydrate is 90min, which can be used for metabolic stability and inhibition assay for *in vitro* study of new chemical entities.

5. ACKNOWLEDGEMENT

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6. REFERENCES

1. Brandänge S and Lindblom L (1979) The enzyme "aldehyde oxidase" is an iminium oxidase. Reaction with nicotine delta 1'(5') iminium ion. Biochem Biophys Res Commun 91:991-996.
2 Barr JT and Jones JP (2011) Inhibition of human liver aldehyde oxidase: implications for Potential drug-drug interactions. Drug Metab Dispos 39:2381–2386 Pryde DC, Dalvie D, Hu Q, Jones P, Obach RS, and Tran TD (2010) Aldehyde oxidase: an enzyme of emerging importance in drug discovery. J Med Chem 53:8441– 8460
3. Hutzler J, Obach R, Dalvie D, and Zientek M(2013) Strategies for a comprehensive understanding of metabolism by aldehyde oxidase. *Expert Opin Drug Metab Toxicol* **9:**153-168.
4. Obach RS (2004) Potent inhibition of human liver aldehyde oxidase by raloxifene. Drug Metab Dispos 32:89 –97
5. Dalvie, D. and Zientek, M. 2015. Metabolism of Xenobiotics by Aldehyde Oxidase. Curr. Protoc. Toxicol. 63:4.41.1-4.41.13.
6. Houston, J.B. and Galetin, A. 2008. Methods for predicting in vivo pharmacokinetics using data from in vitro assays. Curr. Drug Metab. 9:940- 951.
7. Hutzler, J.M., Obach, R.S., Dalvie, D., and Zientek, M.A. 2013. Strategies for a comprehensive understanding of metabolism by aldehyde oxidase. Expert Opin. Drug Metab. Toxicol. 9:153-168.
8. Hutzler, J.M., Yang, Y.S., Brown, C., Heyward, S., and Moeller, T. 2014. Aldehyde oxidase activity in donor-matched fresh and cryopreserved human hepatocytes and assessment of variability in 75 donors. Drug Metab. Dispos. 42:1090- 1097.
9. Hutzler, J.M., Yang, Y.S., Albaugh, D., Fullenwider, C.L., Schmenk, J., and Fisher, M.B. 2012. Characterization of aldehyde oxidase enzyme activity in cryopreserved human hepatocytes. Drug Metab. Dispos. 40:267-275.
10. Johnson, C., Stubley-Beedham, C., and Stell, J.G. 1985. Hydralazine: A potent inhibitor of aldehyde oxidase activity in vitro and in vivo. Biochem. Pharmacol. 34:4251-4256.
11. Kitamura, S., Sugihara, K., and Ohta, S. 2006. Drug-metabolizing ability of molybdenum hydroxylases. Metab. Pharmacokinet. 21:83-98.
12. Obach, R.S. 1999. Prediction of human clearance of twenty-nine drugs from hepatic microsomal intrinsic clearance data: An examination of in vitro half-life approach and nonspecific binding to microsomes. Drug Metab. Dispos. 27:1350- 1359.
13. Obach, R.S. 2004. Potent inhibition of human liver aldehyde oxidase by raloxifene. Drug Metab. Dispos. 32:89-97.
14. Obach, R.S., Huynh, P., Allen, M.C., and Beedham, C. 2004. Human liver aldehyde oxidase: Inhibition by 239 drugs. Br. J. Clin Pharmacol. 44:7-19.
15. Pryde, D.C., Dalvie, D., Hu, Q., Jones, P., Obach, R.S., and Tran, T.D. 2010. Aldehyde oxidase: An enzyme of emerging importance in drug discovery. J. Med. Chem. 53:8441-8460.

16. Strelevitz, T.J., Orozco, C.C., and Obach, R.S. 2012. Hydralazine as a selective probe inactivator of aldehyde oxidase in human hepatocytes: Estimation of the contribution of aldehyde oxidase to metabolic clearance. Drug Metab. Dispos. 40:1441-1448.
17. Zientek, M., Jiang, Y., Youdim, K., and Obach, R.S. 2010. In vitro-in vivo correlation for intrinsic clearance for drugs metabolized by human aldehyde oxidase. Drug Metab. Dispos. 38:1322- 1327