International Journal of Pharmacy and Biological Sciences-IJPBS™ (2019) 9 (1): 1200-1207
Online ISSN: 2230-7605, Print ISSN: 2321-3272
Research Article | Biological Sciences | Open Access | MCI Approved
UGC Approved Journal

Preclinical Metabolite Identification in Rat Urine

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Received: 15 Oct 2018 / Accepted: 10 Nov 2018 / Published online: 1 Jan 2019
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Abstract

Drug discovery and development utilizes in *vitro* experimental models followed by *in vivo* studies, to support the investigations performed on Test molecules. Performing MET ID studies on Test molecules with the utilization of HRMS gives data with certain preliminary conclusions, thereby helps in further advancement of drug candidates. **Objective:** The main objective of this study to check the *in vivo* model for the presence of metabolites observed in *in vitro* studies. **Materials and Methods:** Sprague Dawley Rat was used to administer the Test Compound. The plasma and urine samples obtained from the experimental model were analyzed for the presence of metabolites utilizing the analytical tool LC-Q-TOF Mass spectrometry. Simultaneous extraction and separation technique in combination with high resolution accurate mass spectrometric detector was used for the parent and the metabolite identification. **Results and Conclusion**: The simultaneous acquisition and processing gives qualitative and quantitative information about the parent and its metabolites. Three oxidative metabolites were identified and are confirmed by MS/MS and Accurate mass analysis using the analytical tool LC-Q-TOF Mass spectrometry.

Keywords
Enzymes, Metabolism, UPLC, Xevo QTOF

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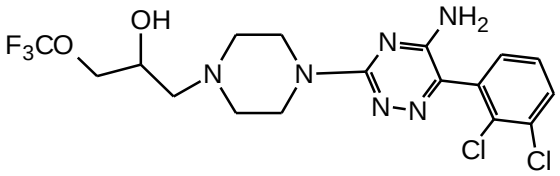
INTRODUCTION

A better understanding of drug metabolic enzymes, transporters, and differences among species and individuals would aid in understanding the drugs DMPK behaviour and its Pharmacodynamic action[1]. Following the same way, the Test Compound Fig.1 with molecular weight of 467.27 daltons is worked on. During the early stages of this research with respect to the safety aspect (USFDA, www.fda.gov / downloads / Drugs / Guidance Compliance Regulatory Information / Guidance / ucm079266.pdf) metabolite identification study was done using liver microsomes and the method is optimized. Later the molecule is dosed and checked of its *in vivo* metabolites by per oral administration in rats. This work up helped for the later studies which are more comprehensive with respect to ADME properties[2-3].

Figure: 1



Molecular Formula: $C_{17}H_{19}Cl_2F_3N_6O_2$
Formula Weight: 467.2729696
1- {4-[5-amino-6-(2, 3-dichlorophenyl)-1, 2, 4-triazin-3-yl] piperazin-1-yl}-3-(trifluoromethoxy) propan-2-ol

MATERIALS AND METHODS

Chemicals

Test compound was obtained from GPRCP, Methanol and Acetonitrile from J.T Bakers, Formic acid from Fluka.

All the chemicals used are of analytical grade.

Procedure for sample preparation and analysis

Metabolite identification for the molecules was conducted in Dawley rats administered per oral (50 mg/kg) dose of the respective drug[4]. This testing paradigm is used to understand the metabolic behaviour of the Test compound synthesized on lab scale in academia.

Sample Preparation

The Blood sample obtained after 1 hour is centrifuged at 14000 rpm for 10 minutes and the plasma is separated. The drug is extracted from the plasma using Acetonitrile with 0.1% formic acid following precipitation method. The supernatant is collected and evaporated under nitrogen water bath till dried of its solvent and reconstituted with 90:10:0.1 % Water: ACN: Formic Acid [4-5].

Whereas Urine samples obtained were prepared using simple dilution with 0.1% Formic acid in water followed by evaporation and reconstitution.

The samples prepared are injected to HRMS with the following conditions.

UPLC and MS Method

Acquity UPLC

Solvents	Mobile Phase A: 0.1% Formic Acid in H_2O
	Mobile Phase B: 0.1% Formic Acid in ACN
Column	Polaris 5C18-A column, 2.1×250 mm
Injection Volume (µl)	10.00
UPLC Flow Rate (ml/min)	0.400
Column Temperature (ºC)	Ambient (Room Temperature)

Gradient Time table

Time	A%	B%
Initial	99	1
40.00	75	25
51.00	63	37
60.00	55	45
70.00	45	55
75.00	40	60
75.01	99	1

Xevo QTOF Parameters

Source (ES+)

Retention window (mins)	:	0.0 to 60.0
Sampling cone Voltage (kV)	:	35.0
Extraction cone	:	4.0
Capillary Voltage (kV)	:	0.2
Source Temperature (°C)	:	100
De solvation temperature (°C)	:	20
Cone Gas Flow (L/Hr)	:	5.0

Purge Gas Flow (L/Hr)	:	600.0
Instrument		
Collision Energy (eV)	:	6-32
Scan	:	MS^e & MS / MS
Detector Voltage	:	2050
LM Resolution	:	4.7
HM Resolution	:	15.0
Aperture 1	:	0.0
Pre-filer	:	10.0
Ion Energy	:	1.0

RESULTS AND DISCUSSIONS

The samples drawn from the experiment were processed and analysed for the presence of the probable metabolites as per the theoretical propositions shown in Fig.2. There observed no metabolites and the experiment is repeated. After repeating twice, it is confirmed that the metabolites were not present in the plasma samples Fig.3. Then the urine collected during the study is analyzed for the presence of any metabolites Fig 4 [6-7].

The evident data observed after running the HRMS is shown in the tabular form of Table.1

Table 1: Metabolites of Test Compound observed in Rat Urine

Name	m/z Observed Mass	Retention Time (min)	Mass Peak Areas		
			0-4 hr	4-8 hr	8-24 hr
Parent	467.0806	72.4	2.15E+05	1.12E+04	1.23E+03
M1	399.1052	49.35	1.30E+04	1.87E+04	5.02E+03
M2	325.0716	45.23	2.20E+04	3.37E+04	1.37E+04
M3	397.0816	48.13	2.77E+07	1.90E+04	3.40E+03

In Fig- 3 & 4, full-scan HR-MS and MS/MS data sets were acquired with a generic intensity-dependent method and then processed. This process helped in finding expected metabolites by following predicted molecular masses based on the similarity of metabolites to those of the parent drug. The parallel use of the fragmentation pathway of the parent drug and its metabolites helped in detecting the oxidative metabolites.

The approach for drug metabolite identification with HR-MS is accomplished via post-acquisition data mining[8-9].

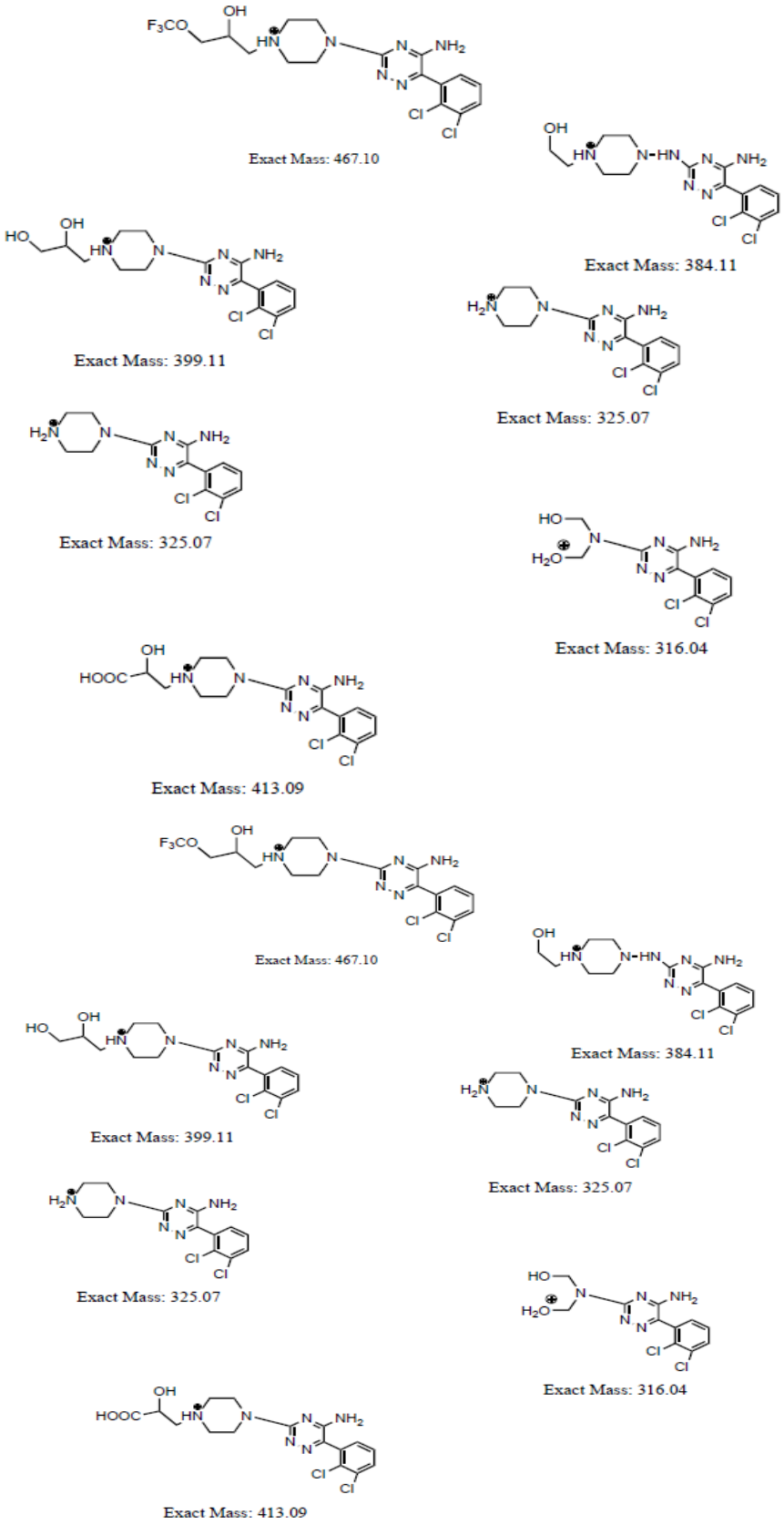
Preliminary data about the metabolism in rat urine suggested the formation of oxidative metabolites such as M1, M2, M3 (the numbering was given based on metabolic profile identified)

The MS spectra and the accurate mass analysis were used in conforming the parent and metabolites structures Fig 6 to 10 & Table 2.

Table 2: Accurate Mass Analysis

Name	Observed Mass m/z	Theoretical mass	mDa	PPM	Formula
P	467.0977	467.0938	-3.9	-8.3	$C_{17}H_{20}Cl_2F_3N_6O_2$
M1	325.0688	325.0735	-4.7	17.1	$C_{13}H_{15}Cl_2N_6$
M2	399.1103	399.1107	0.4	1.0	$C_{16}H_{21}Cl_2N_6O_2$
M3	397.0947	397.1015	6.8	17.1	$C_{16}H_{19}Cl_2N_6O_2$

Figure: 2 *In silico* Proposed metabolites based on Theoritical oxidative metabolism



**Reference structures were drawn using the chembio software*

Figure 3: UPLC-MS/MS Chromatograph for the 1 Hr Rat plasma sample

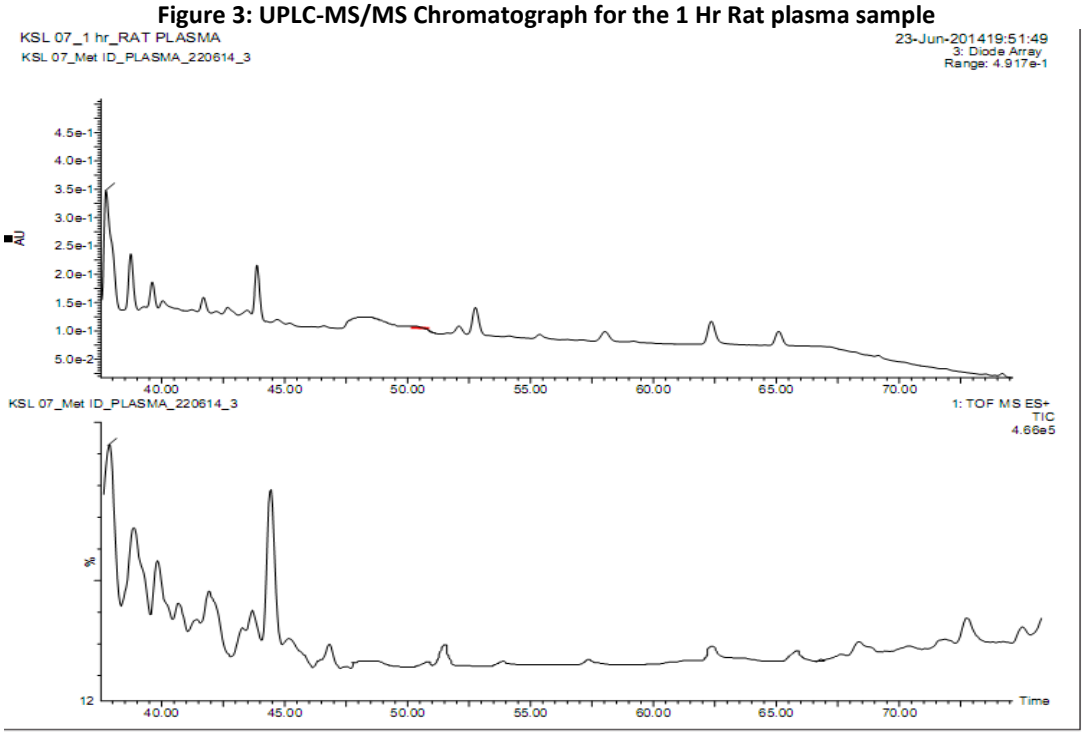


Figure 4: UPLC-MS/MS Chromatograph for the Rat Urine sample

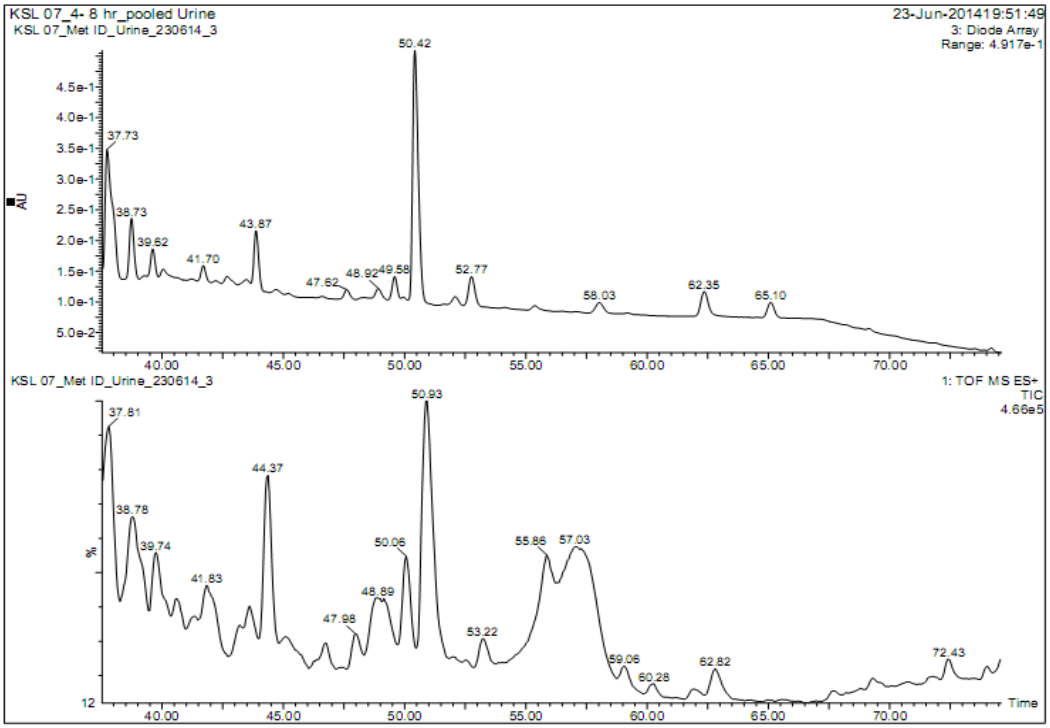
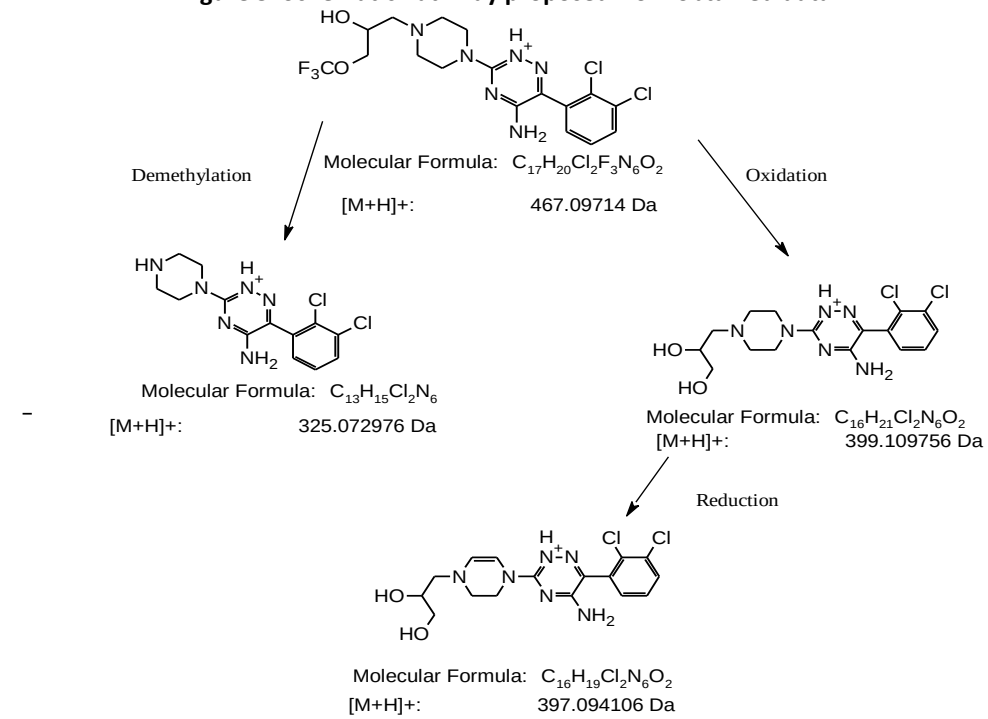


Figure 5: Schematic Pathway proposed from obtained data



Reference structures were drawn using the chembio software

Figure 6: MS Spectra of Parent

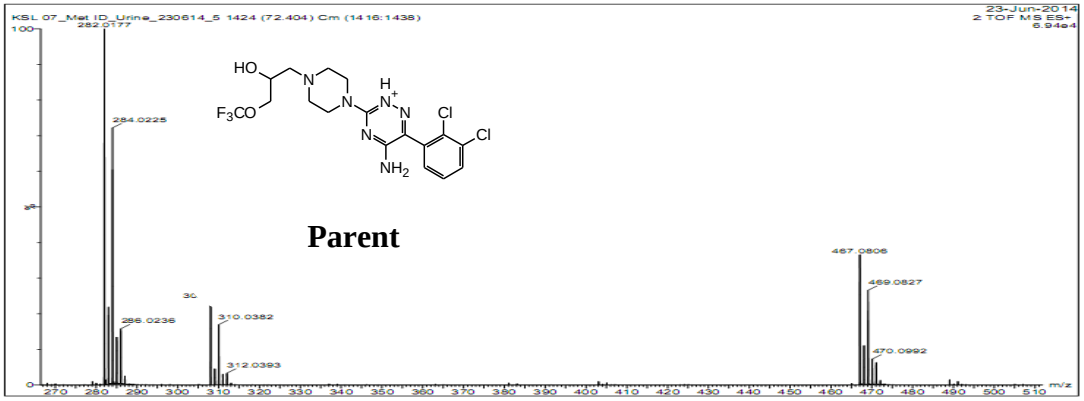


Figure 7: MS Spectra of Metabolite M1

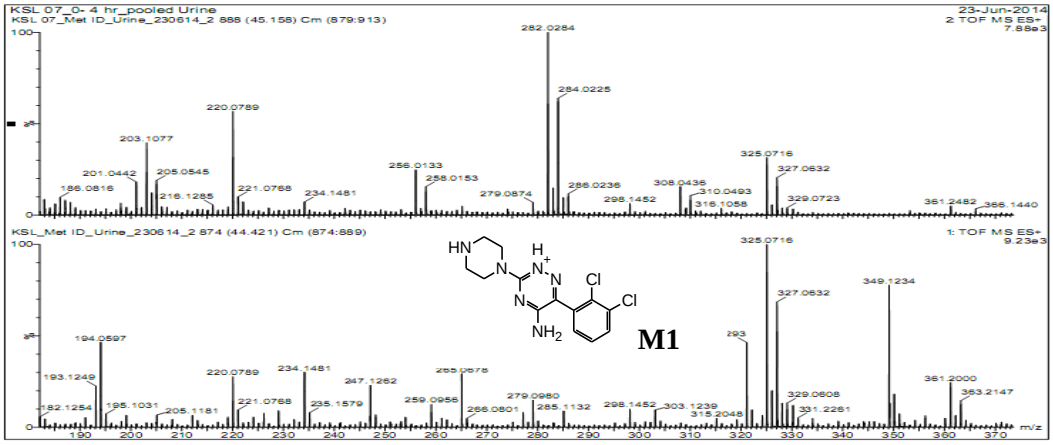


Figure 8: MS Spectra of Metabolite M2

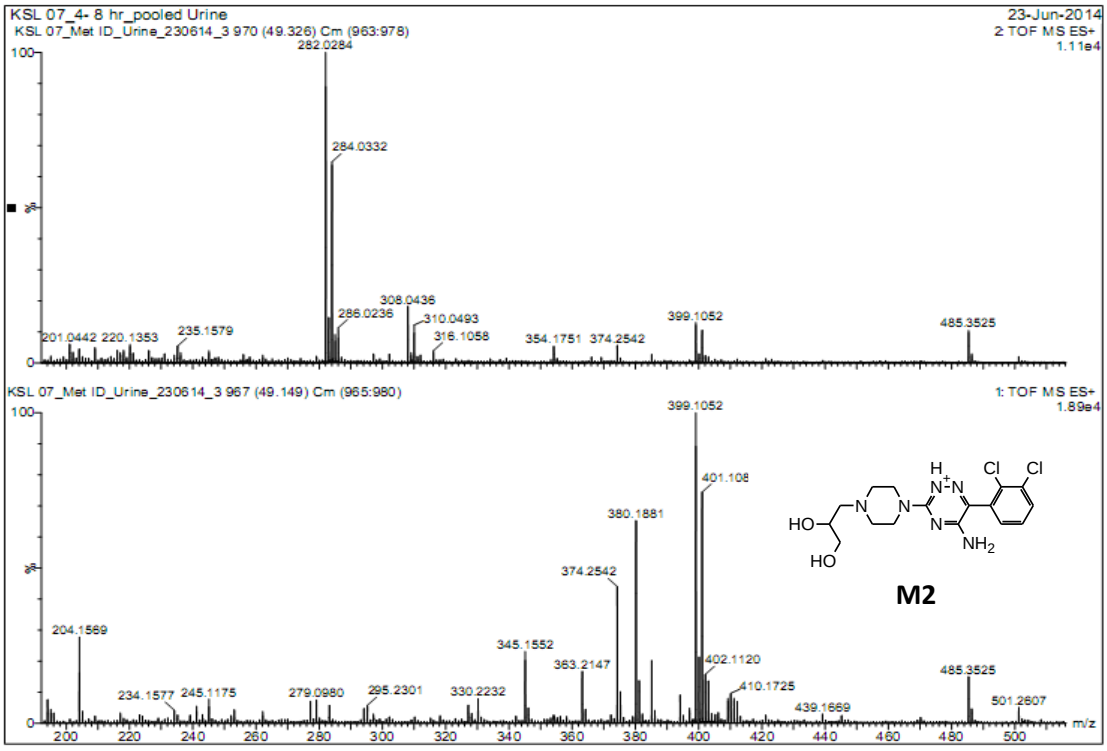


Figure 9: MS Spectra of Metabolite M3

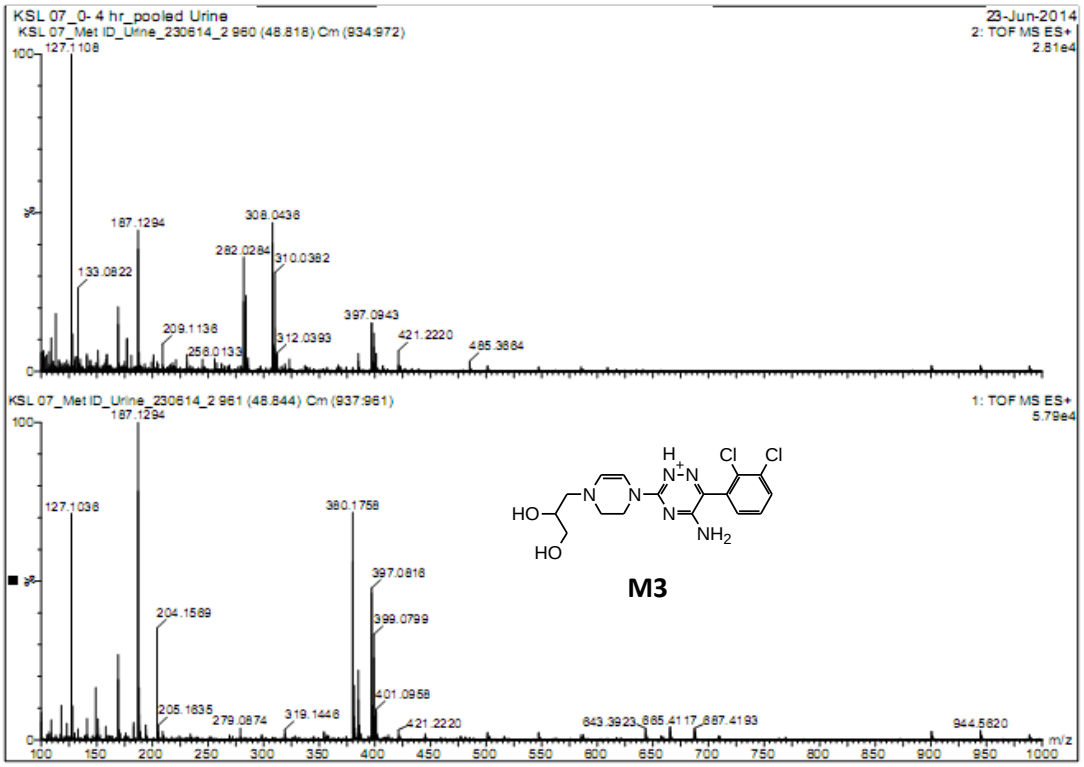
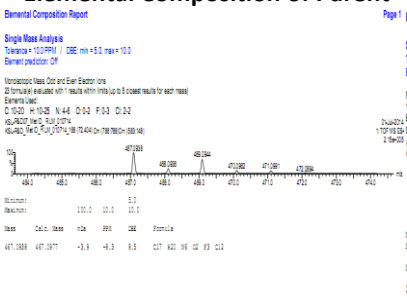
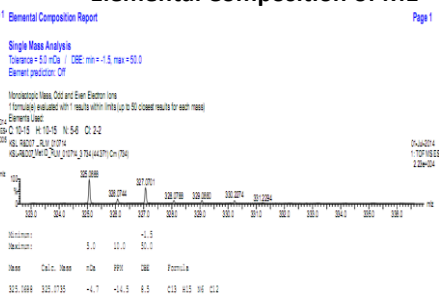


Figure 10: Elemental Composition of Parent and metabolites

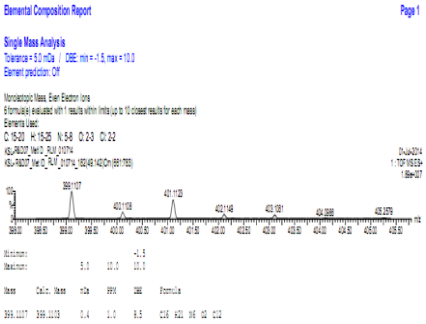
Elemental Composition of Parent



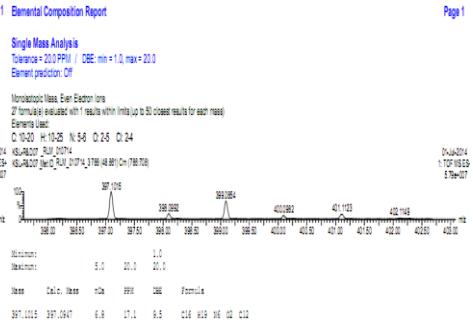
Elemental Composition of M1



Elemental Composition of M2



Elemental Composition of M3



DISCUSSIONS AND CONCLUSION

The mass-based identification observed as the main annotation technique for all the resolved chromatographic peaks. The MS spectrum acquired along the retention times for the resolved chromatographic peaks depicted the formation of 3 oxidative metabolites with very low traces of the parent compound.

The Semi-Quantitation based on Mass Spec data for the metabolites and parent clearly showed the complete metabolism of the Test compound. Thus the *in vivo* screening in the rat depicts the compound to be having a very fast metabolic pathway via Oxidation, and the main route of elimination being Urine.

ACKNOWLEDGEMENT

The authors express sincere thanks to management and staff of G. Pulla Reddy College of Pharmacy (GPRCP), Mehdipatnam, Hyderabad, Telangana, for giving all encouragement and valuable support to carry out this work.

CONFLICTS OF INTEREST: Nil

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