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Investigation on the in Vitro Biotrans Formation of NCE Using LC-MS/MS

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INTRODUCTION

With the dramatic increase in the number of new chemical entities (NCEs) arising from combinatorial chemistry and high-throughput processes, novel bioanalytical techniques are required for the rapid determination of the metabolites of these NCEs. Knowledge of the metabolic sites of the NCEs in early drug discovery is essential for selecting compounds with favorable pharmacokinetic. In development, elucidation of biotransformation pathways of a drug candidate it is important to understand its physiological effects. Liquid chromatography(LC) coupled with atmospheric pressure ionization (API) MS has now become the most powerful tool for the rapid detection, structure elucidation, and quantification of drug-derived material within various biological fluids [1].

Metabolism is a biochemical process by which end ogenous compounds and xenobiotics are converted to more hydrophilic (water soluble) entities, which enhance their elimination from the body. In general, metabolites are pharmacologically less active and less toxic than their corresponding parent compound. However, it is not uncommon that biotransformation reactions also lead to formation of pharmacologically activemetabolites, drug–drug interactions via inhibition or induction of drug metabolizing enzymes and/or formation of toxic metabolites Therefore, determination of an NCE's metabolicrate, biotransformation pathways in animals and humans, and pharmacological and toxicological consequences of its metabolites are very critical to pharmaceutical research and compound progression in early discovery[2-4].

Simple *in vitro* systems, such as subcellular liver fractions or hepatocytes, are used to determine the metabolic fates of NCEs. The information from these studies can assist medicinal chemists to synthesize rationally metabolically stable analogs by blocking the site of metabolism, which could result in the discovery of NCEs with superior pharmacology and safety. Metabolic pathways of drug candidates elucidated in laboratory animals provide guidance for the selection of animal species used for safety evaluation studies to ensure that the selected animal species are exposed to all major metabolites formed in humans. In the development phase, the metabolic profiles of an NCE are determined definitively in preclinical species and humans following its radiolabel administration. Subsequently, major circulatory metabolitesin humans can be synthesized for the evaluation of their pharmacological activity. Therefore, determination of an NCE's metabolic rate, biotransformation pathways in animals and humans, and pharmacological and toxicological consequences of its metabolites are very critical to pharmaceutical research and compound progression [5].

TheCytochrome 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 various CYP enzymes differ in their substrate specificity and hence the metabolism of the probe substrate in various species [6].Thus by observing the role of CYP enzymes in the metabolism of the Test compound help in identifying the safe behaviour of the compound in the body aiding it to move on to the next step in the drug discovery.

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 such substrates for that particular enzyme[7].

The work up would promote

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1. Identification of the substrate metabolic pathway of the potential drug candidate at an early stage using analytical techniques.
2. Develop in *vitro* results that provide a reliable extrapolation to *in vivo*stating a clear mechanistic way of CYP role.
3. Aid in preclinical screening of the NCE's metabolism without utilization of animals

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

This study characterizes the metabolic pathway of the new drug and the potential for the Test Compound to stand as new drug using Tandem Mass spectrometry. The prediction of the potential of the new drug in withstanding the perpetuated screening process while competing with other drugs relies on the evaluation of the new drug on the rate of a reaction that represents a specific P450 enzyme activity, and these studies are adding value and made mandatory. [8-11].

EXPERIMENTAL SECTION

Chemicals: Test Compound from GPRCP, Potassium Phosphate Mono Basic (PBS) from Sigma Aldrich, Potassium Hydroxide (KOH) and Magnesium Chloride (Mgcl2) from Merck, NADPH Regenerating Solution (NRS), Glucose - 6- Phosphate Dehydrogenase and Ammonium formate obtained from Sigma Aldrich, Formic acid from Fluka and the Microsomes from Xenotech. Methanol (MeOH), Acetonitrile of analytical grade and supplied by J.T Bakers. 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 *In Vitro* Sample Preparation for Metabolite Identification

A typical incubation (final volume 0.2 ml) consisted of 0.1 mg of microsomal protein in 100 mM potassium phosphate buffer (pH 7.4).

Stock solution of Test compound was prepared in methanol (final concentration of methanol was less than 0.3% v/v (approximately 10µM/incubation) drug. The drug, buffer, and microsomes were mixed and pre incubated at 37°C for 4 min.

Then the incubations were initiated by the addition of the NADPH, and conducted at 37°C for 60 min. For control incubations, NADPH was omitted. Reactions were terminated upon addition of 200 ml of Acetonitrile, after which 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 and10µL solution was injected onto LC Q-TOF for analysis.

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) and solvent B (0.1% Formic acid in 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 measurement was performed using Quadruple Time of Flight mass spectrometer 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.

Drug Metabolite Identification for Test Compound

The Test compound is an NCE / KSL 03 synthesized with an amino linkage in between main scaffold and a Glycol linkage found to be showing therapeutic effect and is considered for metabolic studies. Figures 1& 2.

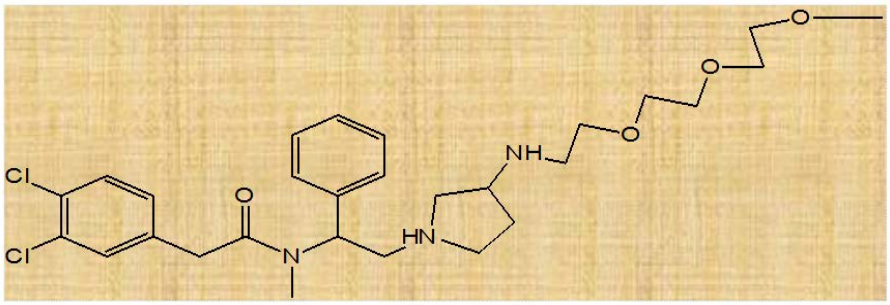


Figure 1: Test Compound
1-(2-(2-(3, 4-dichlorophenyl)-*N*-methylacetamido)-2-phenylethyl)-3-((2-(2-(2-methoxyethoxy)ethoxy)ethyl)amino)pyrrolidin-1-ium
Chemical Formula: $C_{28}H_{40}Cl_2N_3O_4^+$Exact Mass: 552.24

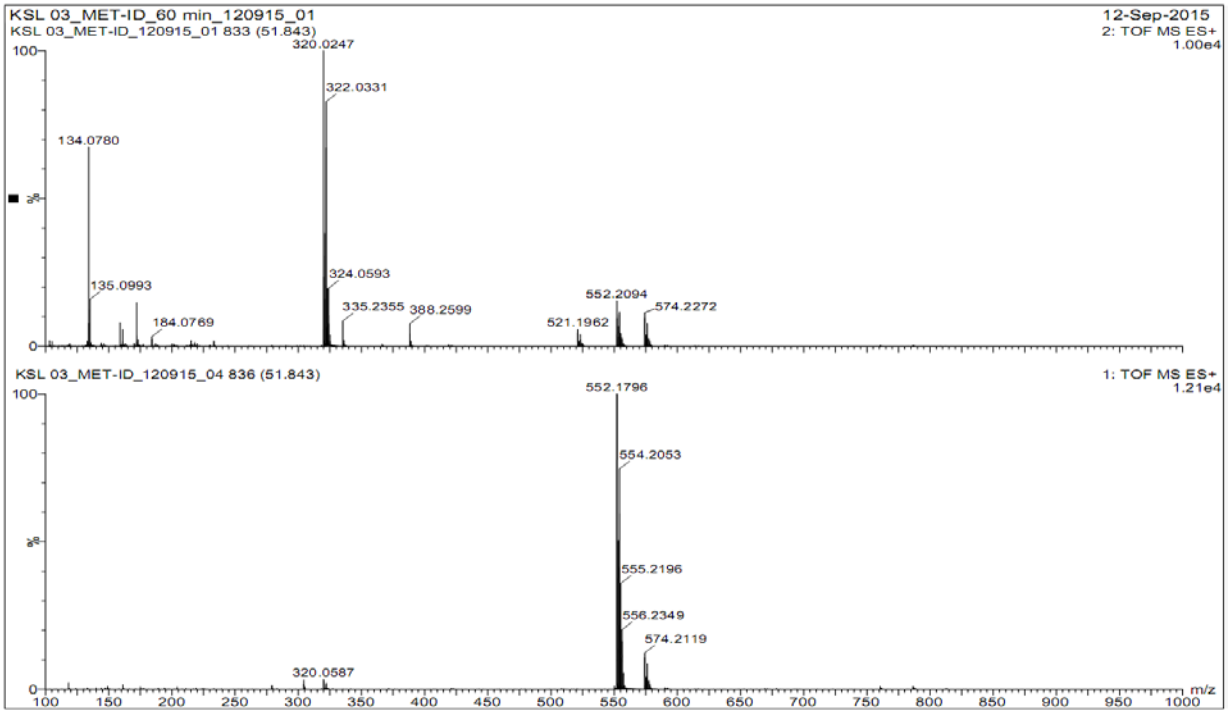


Figure 2: MS and MS/ MS spectra of Test Compound

The incubated sample were run on analytical tool LC-Q-TOF Mass spectrometryThe MS spectrum acquired after 60 minutes of incubation along the retention times for the resolved chromatographic peaks depicted the formation of various metabolites, Figures 3 & 4.

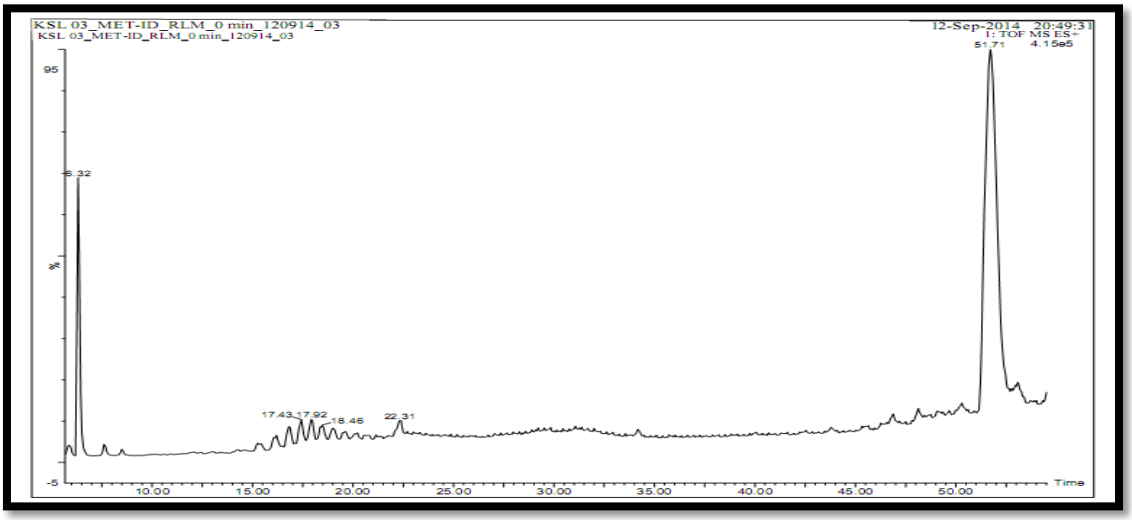


Figure 3: Chromatograph of Blank Sample of Rat Liver Microsomes (RLM)

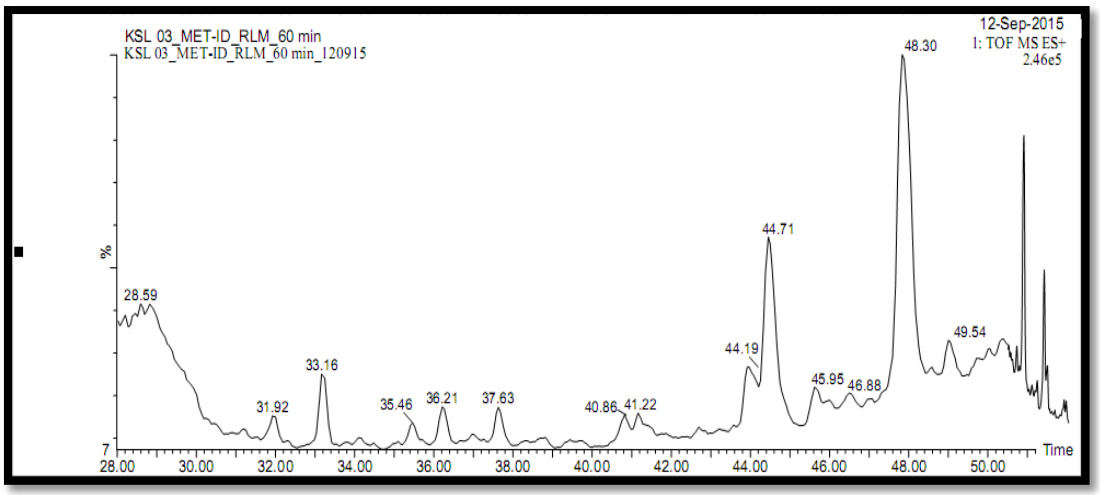


Figure 4: Chromatograph of KSL 03 upon 60 min incubation in RLM

The hypothetical proposed metabolic changes which are likely are the

1. oxidative cleavages and carboxylate metabolites formation on one side and
2. Aromatic Hydroxylation along with the oxidative cleavages and carboxylate metabolites formation on other side.

These two types of oxidative metabolites observedwere confirmed by taking the MS/MS and Accurate mass analysis data.

RESULTS AND DISCUSSION

The MS spectrum acquired after 60 minutes of incubation along the retention times for the resolved chromatographic peaks depicted the formation of various metabolites; the metabolite numbering was given based on their retention times starting from the retention time of Test compound, Table 1.

Oxidation Cleavage by the CYP Enzymes

The MS spectrum acquired after 60 minutes of incubation, is observed all along the retention times for the resolved chromatographic peaks .The peaks depicted the formation of various metabolites, and the approach for drug metabolite identification with HR-MS, is accomplished via post-acquisition data mining where the chromatograph obtained were checked of their full-scan HR-MS and MS/MS data sets over the entire retention window .Once metabolite ions were found, multistage product ion scans (MSn) were carried out to obtain more detailed fragmentation pathways for structure elucidation. HR-MS instrument is utilized for the determination of MS/MS and empirical formulae of metabolites. The mass-based identification was observed as the main annotation technique for, all the resolved chromatographic peaks.

The full-scan HR-MS and MS/MS data sets acquired and processed were with an intensity-dependent method. This process help in finding expected metabolites by following predicted molecular masses based on the similarity of metabolites to that of the parent drug. To state, more clearly - the fragmentation of the parent would give m/z 320 which can be cross checked in the MS/MS of the metabolites formed during the biotransformation process.

Fragment ion which is likely to be observed during fragmentation of parent is m/z 320

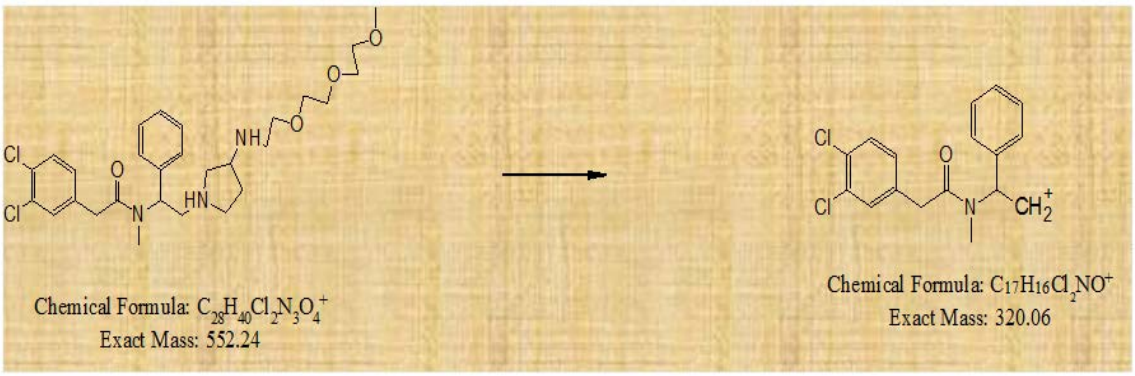


Figure 5.0: MS/ MS Fragment of Parent

So metabolites formed from parent shall have m/z 320 as fragment at most of the times, as they are formed from parent, this is taken as an important annotation technique for identification of metabolites formed all along the Chromatogram.

Hence the use of MS/MS of the Test compound and the metabolites is important in detecting the metabolites, while the elemental compositions and their respective accurate masses with a mass difference of less than 35 mDa within a PPM error of less than 20 would give, a high assurance of the data generated thus aiding in confirmation of the structures proposed.

The MS/ MS Spectra of Oxidative Metabolites were shown from Figure 5.1 to 5.7respectively.

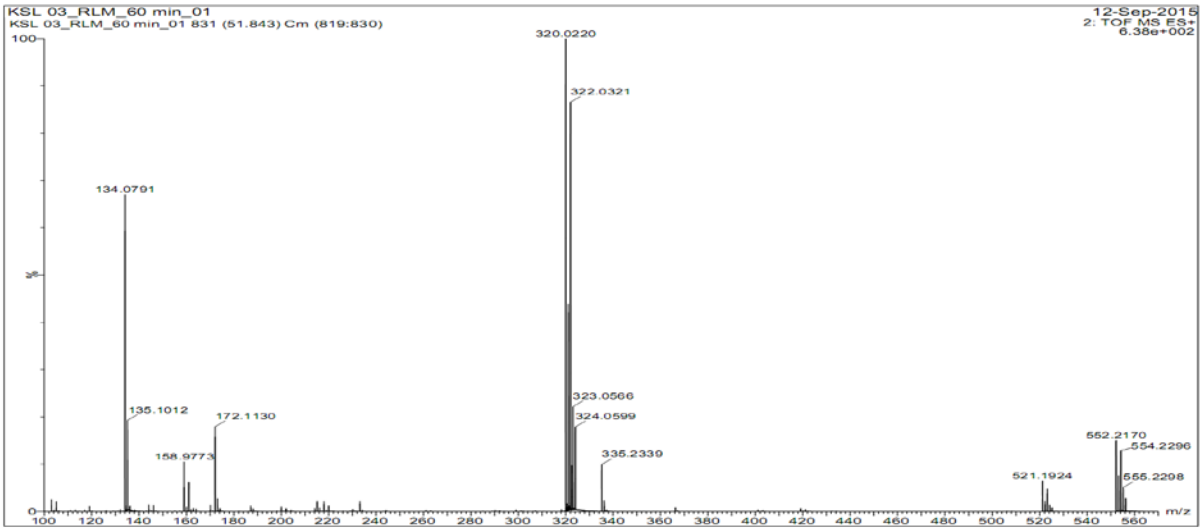


Figure 5.1: MS/ MS spectra of Parent m/z 552.24

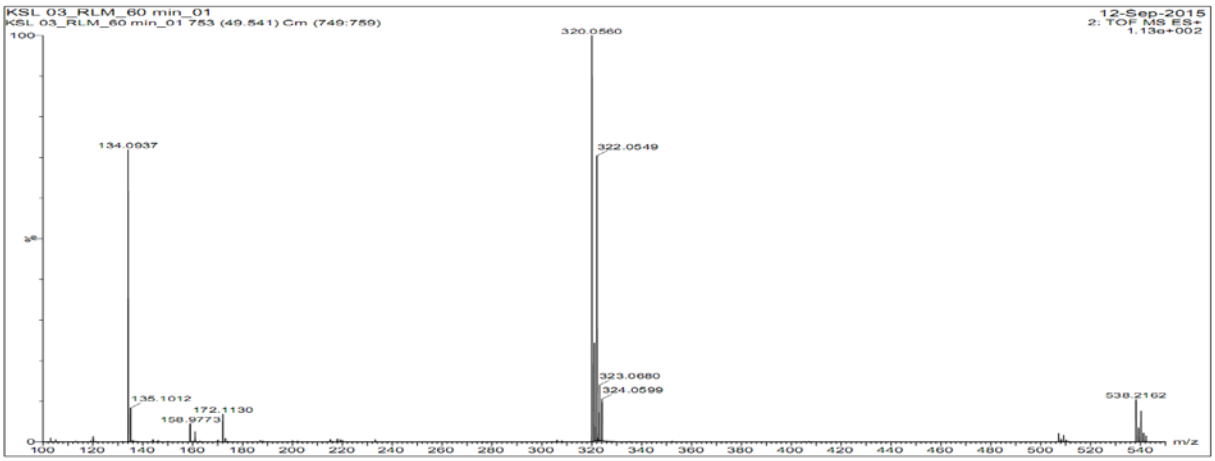


Figure 5.2: MS/ MS spectra of m/z 538.22 M1

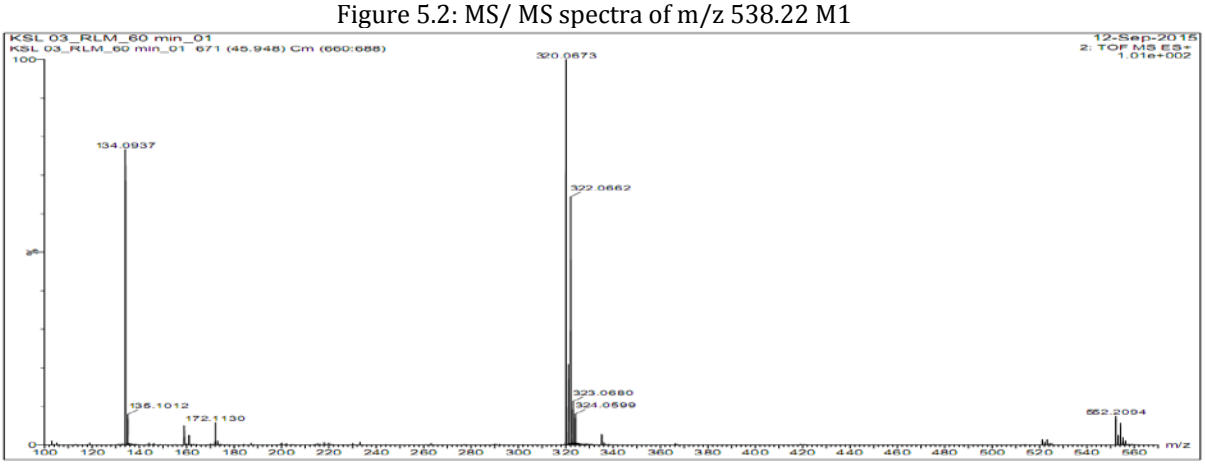


Figure 5.3: MS/ MS spectra of m/z 552.20 M2

Note: The carboxylate metabolite of the parent with m/z 552 in Figure 5.3 observed can be differentiated from parent by its retention time and the elemental composition.

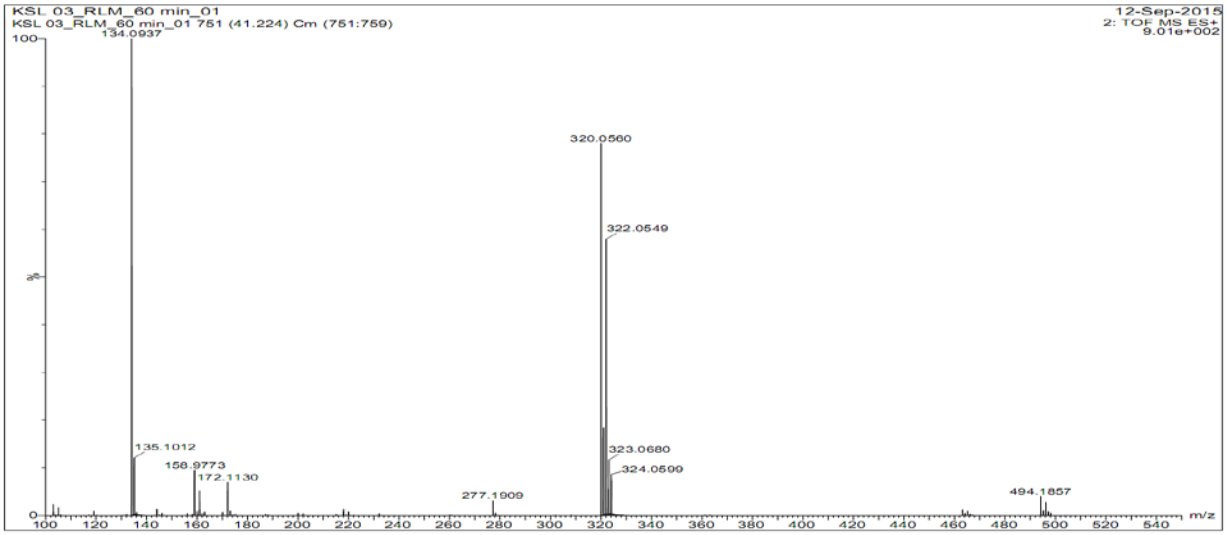


Figure 5.4: MS/ MS spectra of m/z 494.20 M3

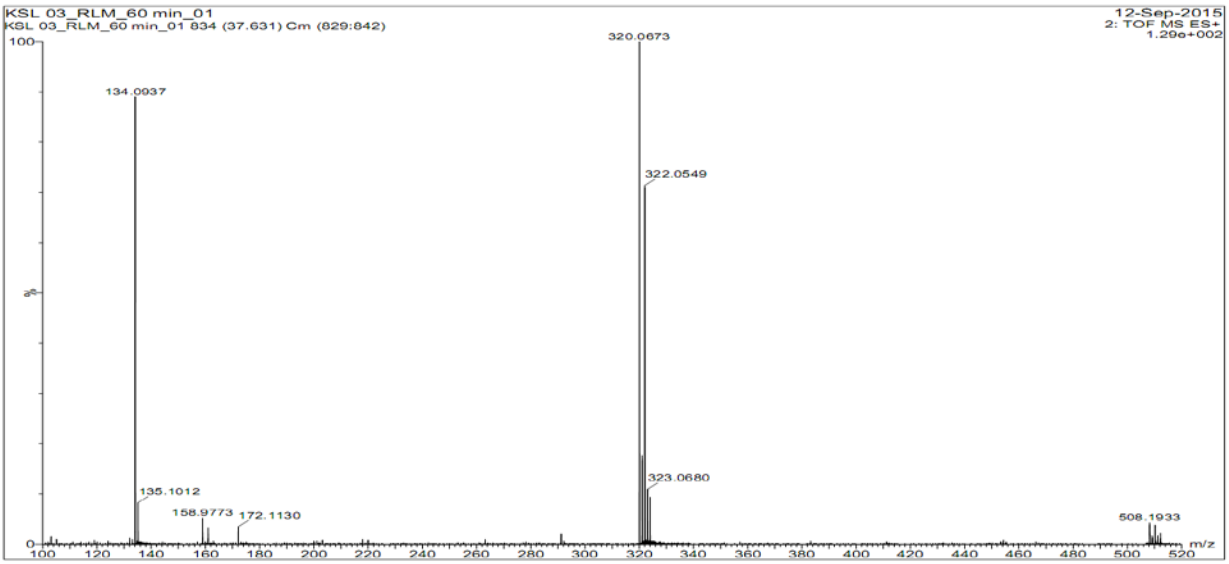


Figure 5.5: MS/ MS spectra of m/z 508.18 M4

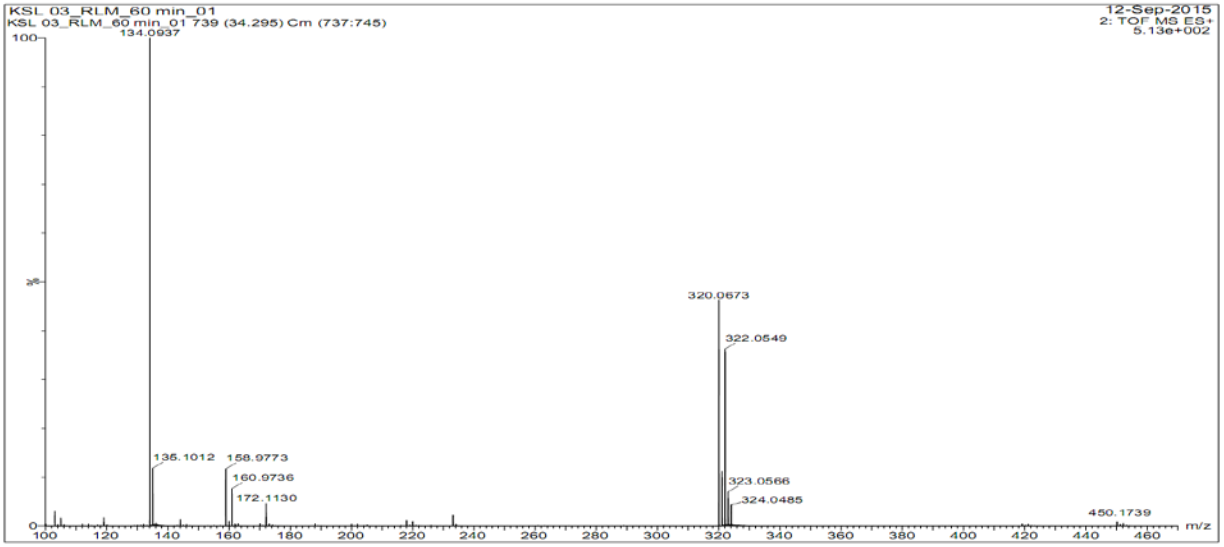


Figure 5.6: MS/ MS spectra of m/z 450.17 M5

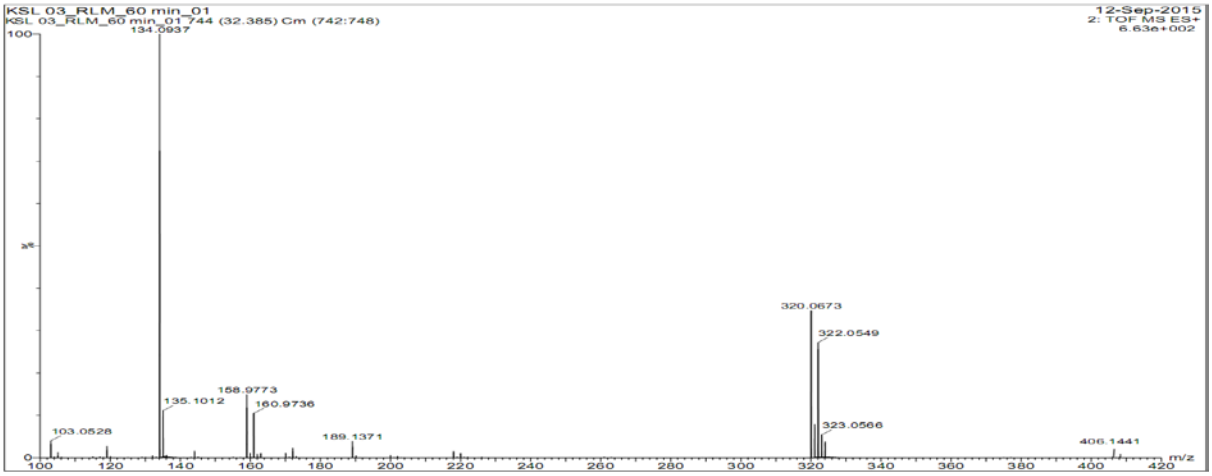


Figure 5.7: MS/ MS spectra of m/z 406.14 M6

MS/ MS Spectra of Aromatic Oxidative Metabolites

The Test compound is having two aromatic rings which make it prone to aromatic hydroxylation by the microsomal enzymes. Hence the fragmentation of the parent with aromatic hydroxylation can be observed after microsomal incubation.

The Test compound upon aromatic Hydroxylation would be with m/z 568, so when the metabolite, m/z 568 undergoes fragmentation it would give m/z 326ion as fragmentby the MS.Implies all the metabolites formed after aromatic hydroxylation from metabolitem/z 568 shall have m/z 326 as the fragment.

Fragment ion to be observed for Aromatic Hydroxylated Test compound is m/z 326.

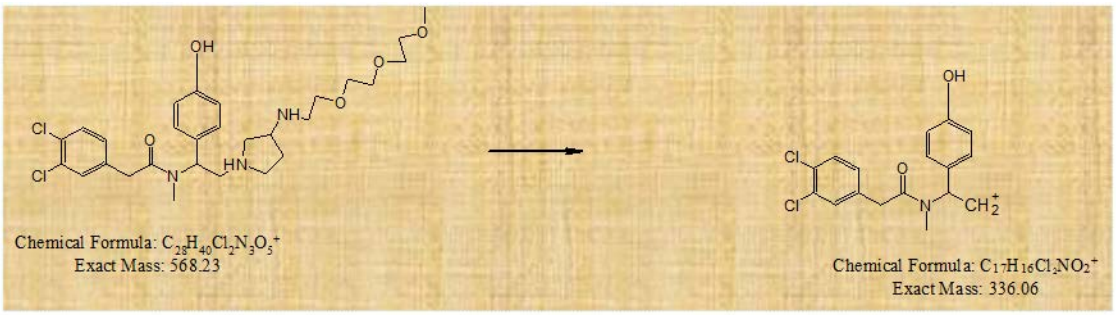


Figure 6.0: MS/ MS Fragment of Parent with Aromatic Hydroxylation

Hence, the metabolites with a MS/MS of m/z 326 were screened all along the chromatograph followed by cross verification with their elemental composition.

The MS/ MS Spectra of Aromatic hydroxylated Metabolites were shown from Figure 6.1 to6.8respectively.

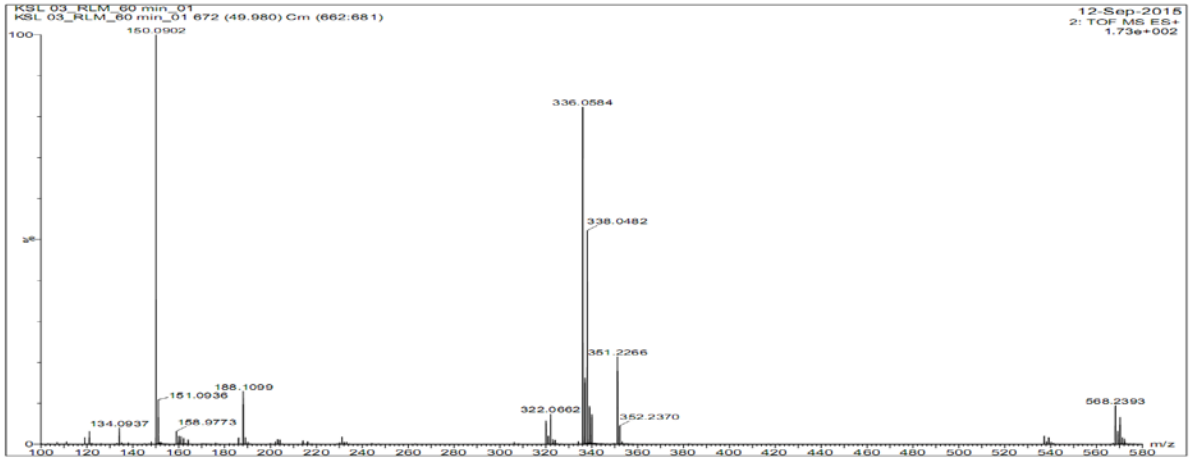


Figure 6.1: MS/ MS spectra of m/z 568.23 M7

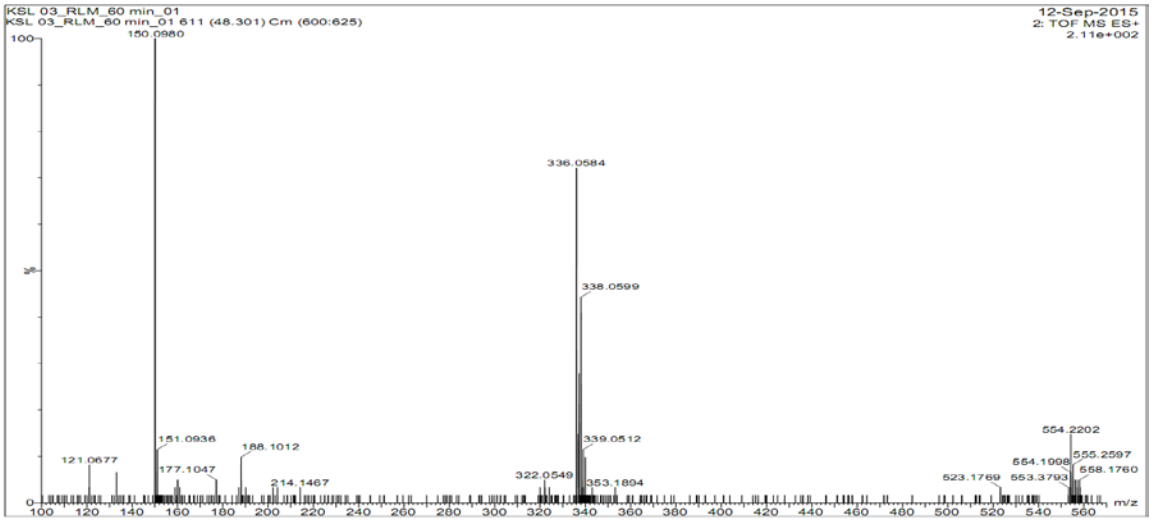


Figure 6.2: MS/ MS spectra of m/z 554.22 M8

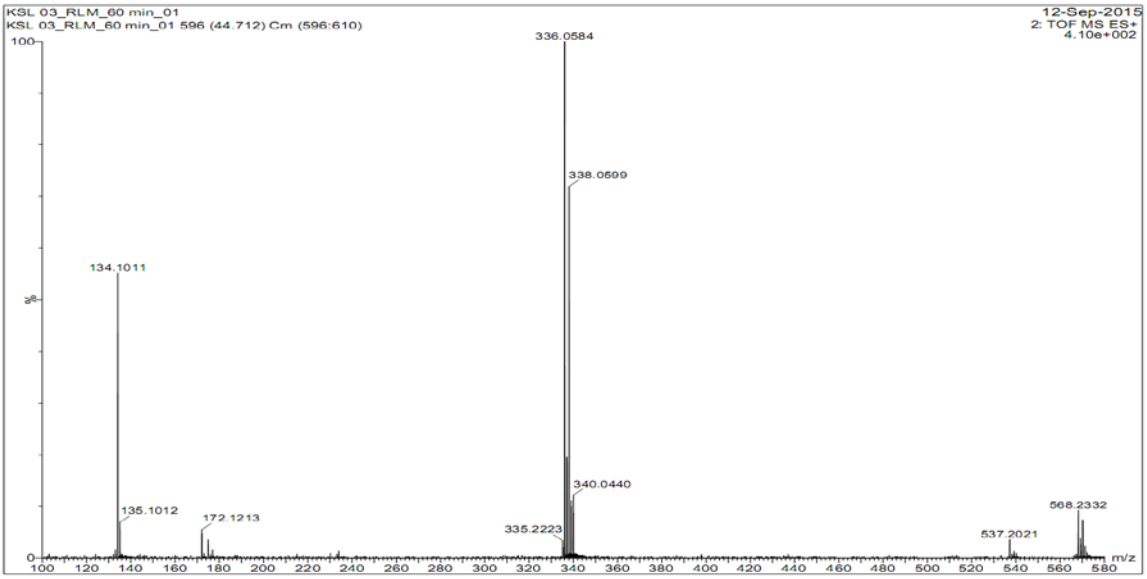


Figure 6.3: MS/ MS spectra of m/z 568.23 M9

Note: Similar metabolites with m/z 568 were observed in Figure 6.3 and Figure6.1. The aromatic hydroxylated metabolite is differentiated from its carboxylate metabolite by its retention time and the elemental composition

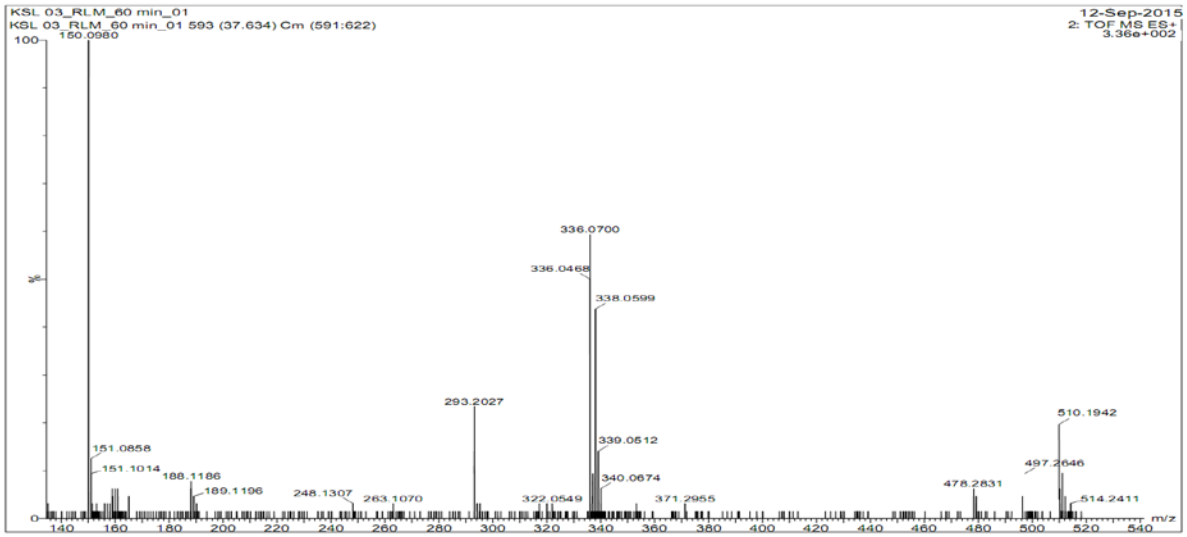


Figure 6.4: MS/ MS spectra of m/z 510.11 M10

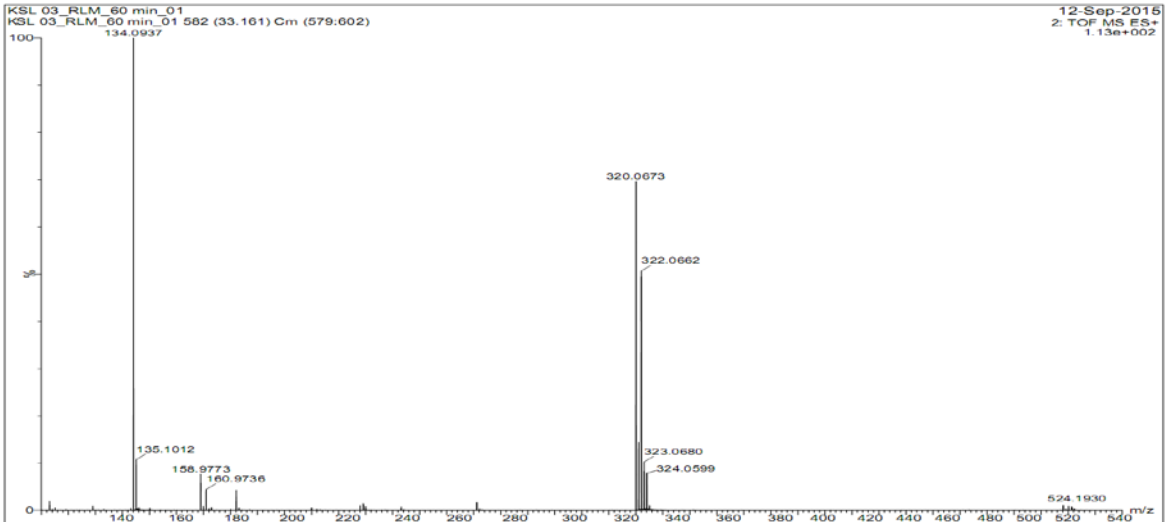


Figure 6.5: MS/ MS spectra of m/z 524.19 M11

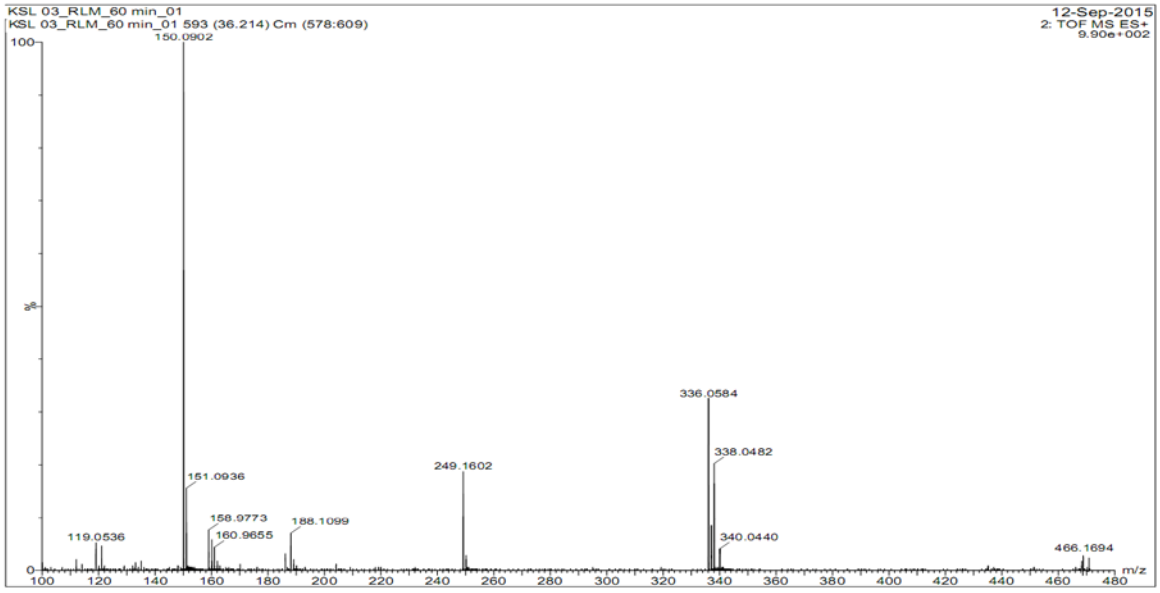


Figure 6.6: MS/ MS spectra of m/z 466.17 M12

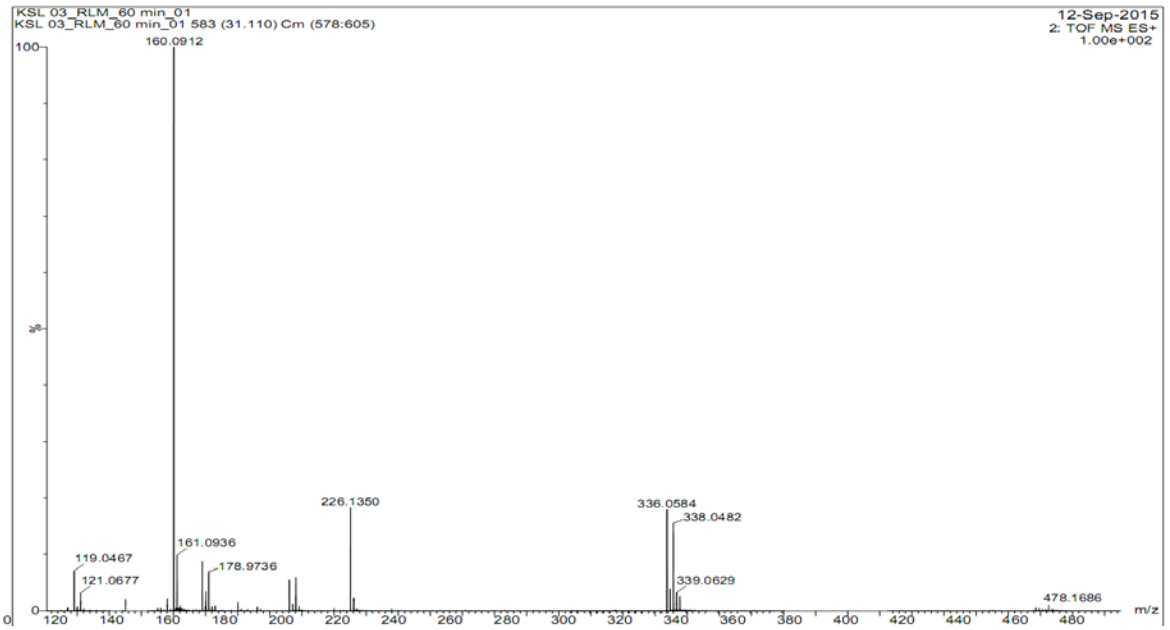


Figure 6.7: MS/ MS spectra of m/z 478.17 M13

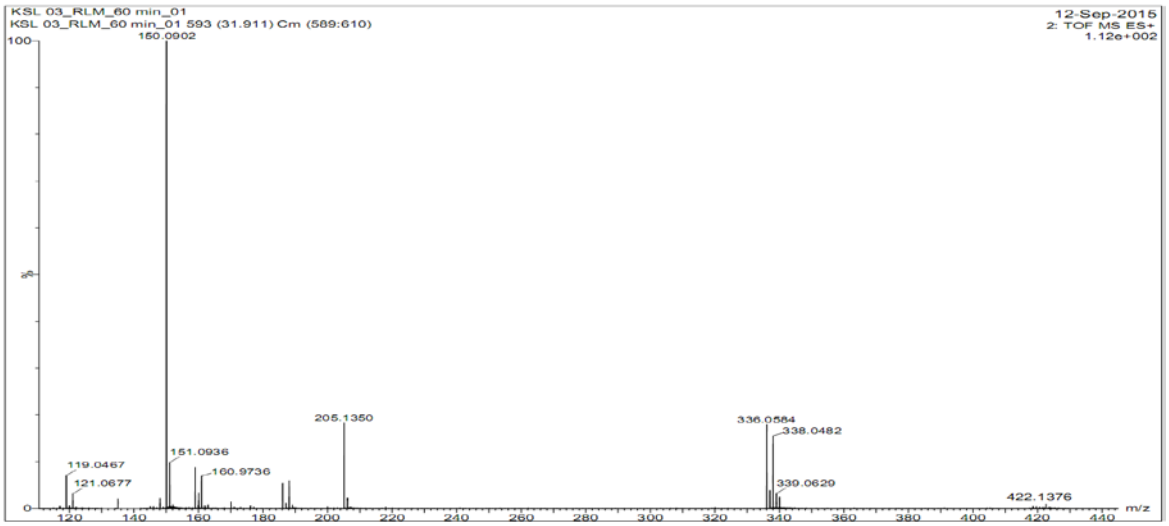


Figure 6.8: MS/ MS spectra of m/z 422.14 M14

Elemental Composition of the Metabolites Confirmed for Oxidation

The use of MS/MS data for the Test compound and the metabolites is important in detecting the metabolites, while the elemental compositions and their respective accurate masses with a mass difference of less than 35 mDa within a PPM error of less than 20 would give, a high assurance of the data generated thus aiding in confirmation of the structures proposed.

The co injection of analyte and the lock mass compound directly into ion source give authenticated mass measurements all through the MS and MS/MS scans which is an instrument driven check for the reliability of results.

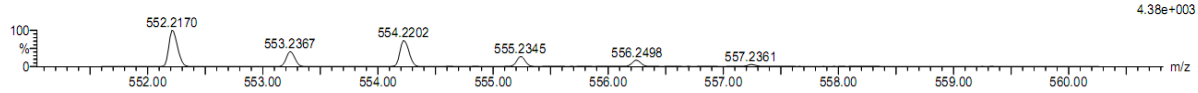
The Elemental Composition of all the metabolites observed were shown from Figure 7.1 to 7.15 respectively

Elemental Composition Report Page 1

Single Mass Analysis
Tolerance = 55.0 PPM / DBE: min = 0.0, max = 9.5
Element prediction: Off

Monoisotopic Mass, Odd and Even Electron Ions
57 formula(e) evaluated with 2 results within limits (all results (up to 1000) for each mass)
Elements Used:
C: 0-30 H: 0-40 N: 0-3 O: 0-6 Cl: 1-2

KSL 03_MET-ID_RLM_60 min_120915
KSL 03_MET-ID_RLM_60 min_120915 830 (51.843) Cm (819:830)
12-Sep-2015
1: TOF MS ES+
4.38e+003



Minimum:				0.0	
Maximum:		30.0	55.0	9.5	
Mass	Calc. Mass	mDa	PPM	DBE	Formula
552.2170	552.2284	-19.0	-34.4	9.5	C29 H40 N O5 Cl2
	552.2472	-30.2	-54.7	9.5	C28 H40 N3 O4 Cl2

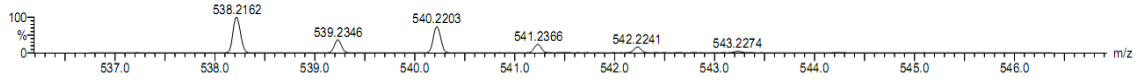
Figure 7.1: Elemental Composition of Parent

Elemental Composition Report Page 1

Single Mass Analysis
Tolerance = 12.0 PPM / DBE: min = 0.0, max = 10.5
Element prediction: Off

Monoisotopic Mass, Even Electron Ions
66 formula(e) evaluated with 1 results within limits (all results (up to 1000) for each mass)
Elements Used:
C: 0-30 H: 0-40 N: 0-3 O: 0-6 Cl: 1-2

KSL 03_MET-ID_RLM_60 min_120915
KSL 03_MET-ID_RLM_60 min_120915 752 (49.541) Cm (750:758)
12-Sep-2015
1: TOF MS ES+
1.50e+003



Minimum:				0.0	
Maximum:		30.0	12.0	10.5	
Mass	Calc. Mass	mDa	PPM	DBE	Formula
538.2162	538.2198	-3.6	-6.7	9.5	C27 H38 N3 O4 Cl2

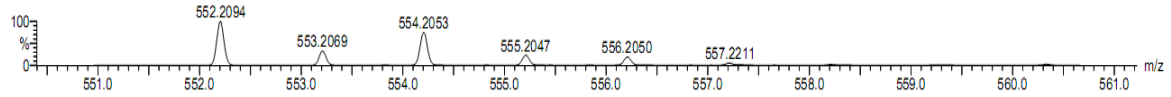
Figure 7.2: Elemental Composition of M1

Elemental Composition Report Page 1

Single Mass Analysis
Tolerance = 25.0 PPM / DBE: min = 0.0, max = 11.0
Element prediction: Off

Monoisotopic Mass, Even Electron Ions
57 formula(e) evaluated with 1 results within limits (all results (up to 1000) for each mass)
Elements Used:
C: 0-30 H: 0-40 N: 0-3 O: 0-6 Cl: 1-2

KSL 03_MET-ID_RLM_60 min_120915
KSL 03_MET-ID_RLM_60 min_120915 672 (45.948) Cm (661:683)
12-Sep-2015
1: TOF MS ES+
2.55e+003



Minimum:				0.0	
Maximum:		30.0	25.0	11.0	
Mass	Calc. Mass	mDa	PPM	DBE	Formula
552.2094	552.2032	6.2	11.2	10.5	C27 H36 N3 O5 C12

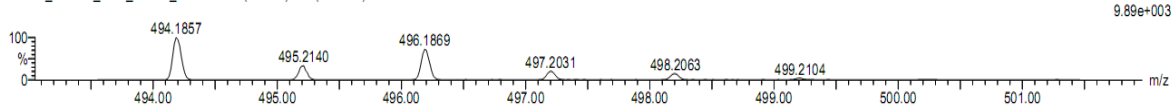
Figure 7.3: Elemental Composition of M2

Elemental Composition Report Page 1

Single Mass Analysis
Tolerance = 25.0 PPM / DBE: min = 0.0, max = 10.5
Element prediction: Off

Monoisotopic Mass, Odd and Even Electron Ions
126 formula(e) evaluated with 5 results within limits (all results (up to 1000) for each mass)
Elements Used:
C: 0-30 H: 0-40 N: 0-3 O: 0-6 Cl: 1-2

KSL 03_MET-ID_RLM_60 min_120915
KSL 03_MET-ID_RLM_60 min_120915 751 (41.224) Cm (751:759)
12-Sep-2015
1: TOF MS ES+
9.89e+003



Minimum:				0.0	
Maximum:		30.0	25.0	10.5	
Mass	Calc. Mass	mDa	PPM	DBE	Formula
494.1857	494.1865	-0.8	-1.6	9.5	C26 H34 N O4 C12
	494.1825	3.2	6.5	5.5	C21 H34 N3 O6 C12
	494.1950	-9.3	-18.8	5.0	C22 H36 N2 O6 C12
	494.1739	11.8	23.9	10.0	C25 H32 N2 O4 C12
	494.1977	-12.0	-24.3	9.5	C25 H34 N3 O3 C12

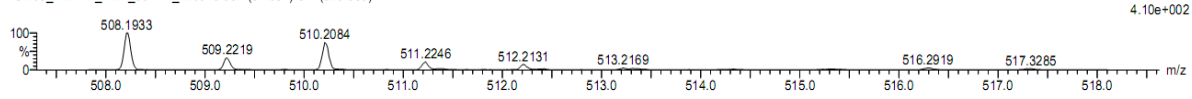
Figure 7.4: Elemental Composition of M3

Elemental Composition Report Page 1

Single Mass Analysis
Tolerance = 60.7 PPM / DBE: min = 0.0, max = 11.0
Element prediction: Off

Monoisotopic Mass, Odd and Even Electron Ions
105 formula(e) evaluated with 14 results within limits (all results (up to 1000) for each mass)
Elements Used:
C: 0-30 H: 0-40 N: 0-3 O: 0-6 Cl: 1-2

KSL 03_MET-ID_RLM_60 min_120915
KSL 03_MET-ID_RLM_60 min_120915 834 (37.631) Cm (829:839)
12-Sep-2015
1: TOF MS ES+
4.10e+002



Minimum:				0.0	
Maximum:		30.0	60.7	11.0	
Mass	Calc. Mass	mDa	PPM	DBE	Formula
508.1933	508.2107	-3.0	-5.9	5.0	C23 H38 N2 O6 C12
	508.2021	5.6	11.0	9.5	C27 H36 N O4 C12
	508.2134	-5.7	-11.2	9.5	C26 H36 N3 O3 C12
	508.2147	-7.0	-13.8	9.0	C28 H38 O4 C12
	508.1981	9.6	18.9	5.5	C22 H36 N3 O6 C12
	508.2214	-13.7	-27.0	9.5	C25 H35 N3 O6 C1
	508.2233	-15.6	-30.7	4.5	C24 H40 N O6 C12
	508.1896	18.1	35.6	10.0	C26 H34 N2 O4 C12
	508.2259	-18.2	-35.8	9.0	C27 H38 N2 O3 C12
	508.2340	-26.3	-51.8	9.0	C26 H37 N2 O6 C1
	508.2345	-26.8	-52.7	4.5	C23 H40 N3 O5 C12
	508.1783	29.4	57.9	10.0	C27 H34 O5 C12
	508.1626	30.7	60.4	10.5	C25 H32 N3 O4 C12

Figure 7.5: Elemental Composition of M4

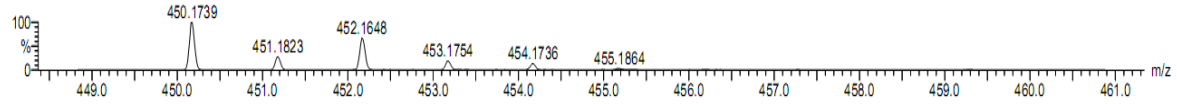
Elemental Composition Report Page 1

Single Mass Analysis
Tolerance = 6.0 PPM / DBE: min = 0.0, max = 10.5
Element prediction: Off

Monoisotopic Mass, Even Electron Ions
178 formula(e) evaluated with 1 results within limits (all results (up to 1000) for each mass)
Elements Used:
C: 0-30 H: 0-40 N: 0-3 O: 0-6 Cl: 1-2

KSL 03_MET-ID_RLM_60 min_120915
KSL 03_MET-ID_RLM_60 min_120915 739 (34.295) Cm (739:747)

12-Sep-2015
1: TOF MS ES+
5.70e+003



Minimum:				0.0	
Maximum:		30.0	6.0	10.5	
Mass	Calc. Mass	mDa	PPM	DBE	Formula
450.1739	450.1715	2.4	5.3	9.5	C23 H30 N3 O2 Cl2

Figure 7.6: Elemental Composition of M5

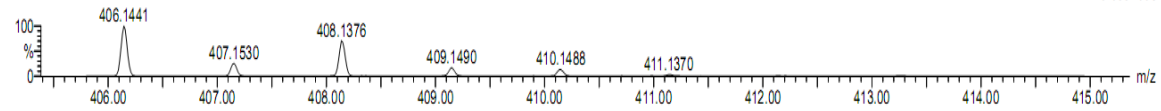
Elemental Composition Report Page 1

Single Mass Analysis
Tolerance = 3.0 PPM / DBE: min = 0.0, max = 10.5
Element prediction: Off

Monoisotopic Mass, Even Electron Ions
193 formula(e) evaluated with 1 results within limits (all results (up to 1000) for each mass)
Elements Used:
C: 0-30 H: 0-40 N: 0-3 O: 0-6 Cl: 1-2

KSL 03_MET-ID_RLM_60 min_120915
KSL 03_MET-ID_RLM_60 min_120915 744 (32.385) Cm (744:745)

12-Sep-2015
1: TOF MS ES+
6.68e+003



Minimum:				0.0	
Maximum:		30.0	3.0	10.5	
Mass	Calc. Mass	mDa	PPM	DBE	Formula
406.1441	406.1453	-1.2	-3.0	9.5	C21 H26 N3 O Cl2

Figure 7.7: Elemental Composition of M6

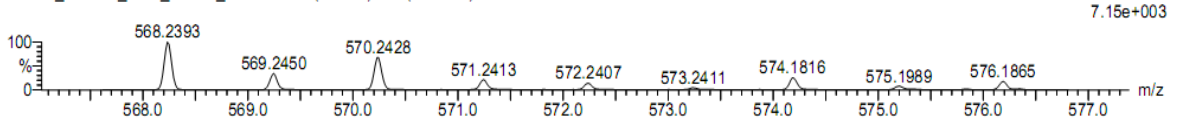
Elemental Composition Report Page 1

Single Mass Analysis
Tolerance = 10.0 PPM / DBE: min = 0.0, max = 11.0
Element prediction: Off

Monoisotopic Mass, Even Electron Ions
51 formula(e) evaluated with 1 results within limits (all results (up to 1000) for each mass)
Elements Used:
C: 0-30 H: 0-40 N: 0-3 O: 0-6 Cl: 1-2

KSL 03_MET-ID_RLM_60 min_120915
KSL 03_MET-ID_RLM_60 min_120915 672 (49.980) Cm (662:679)

12-Sep-2015
1: TOF MS ES+
7.15e+003



Minimum:				0.0	
Maximum:		30.0	10.0	11.0	
Mass	Calc. Mass	mDa	PPM	DBE	Formula
568.2393	568.2406	-1.3	-2.3	9.5	C28 H40 N3 O5 Cl2

Figure 7.8: Elemental Composition of M7

Elemental Composition Report Page 1

Single Mass Analysis
Tolerance = 5.0 PPM / DBE: min = 0.0, max = 11.0
Element prediction: Off

Monoisotopic Mass, Even Electron Ions
56 formula(e) evaluated with 1 results within limits (all results (up to 1000) for each mass)
Elements Used:
C: 0-30 H: 0-40 N: 0-3 O: 0-6 Cl: 1-2

KSL 3_MET-ID_RLM_60 min_120915
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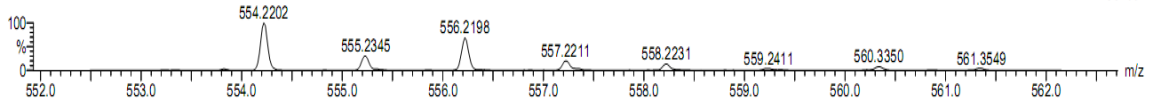
12-Sep-2015
1: TOF MS ES+
2.23e+004



Minimum:				0.0	
Maximum:		30.0	5.0	11.0	
Mass	Calc. Mass	mDa	PPM	DBE	Formula
554.2202	554.2189	1.3	2.3	9.5	C27 H38 N3 O5 Cl2

Figure 7.9: Elemental Composition of M8

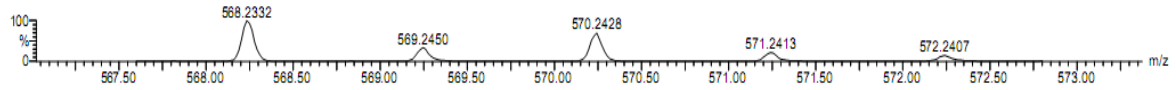
Elemental Composition Report Page 1

Single Mass Analysis
Tolerance = 100.0 PPM / DBE: min = 0.0, max = 11.0
Element prediction: Off

Monoisotopic Mass, Odd and Even Electron Ions
51 formula(e) evaluated with 4 results within limits (all results (up to 1000) for each mass)
Elements Used:
C: 0-30 H: 0-40 N: 0-3 O: 0-6 Cl: 1-2

KSL 03_MET-ID_RLM_60 min_120915
KSL 03_MET-ID_RLM_60 min_120915 596 (44.712) Cm (596:605)

12-Sep-2015
1: TOF MS ES+
8.20e+003



Minimum:				0.0	
Maximum:		30.0	100.0	11.0	
Mass	Calc. Mass	mDa	PPM	DBE	Formula
568.2332	568.2345	-1.3	-2.3	9.5	C28 H40 N3 O5 Cl2
	568.2233	9.9	17.4	9.5	C29 H40 N O6 Cl2
	568.2107	22.5	39.6	10.0	C28 H38 N2 O6 Cl2
	568.1981	35.1	61.8	10.5	C27 H36 N3 O6 Cl2

Figure 7.10: Elemental Composition of M9

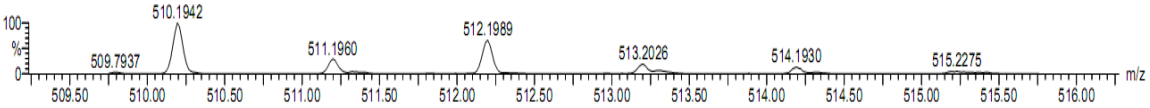
Elemental Composition Report Page 1

Single Mass Analysis
Tolerance = 3.2 PPM / DBE: min = 0.0, max = 11.0
Element prediction: Off

Monoisotopic Mass, Even Electron Ions
102 formula(e) evaluated with 1 results within limits (all results (up to 1000) for each mass)
Elements Used:
C: 0-30 H: 0-40 N: 0-3 O: 0-6 Cl: 1-2

KSL 03_MET-ID_RLM_60 min_120915
KSL 03_MET-ID_RLM_60 min_120915 592 (37.634) Cm (591:602)

12-Sep-2015
1: TOF MS ES+
9.49e+003



Minimum:				0.0	
Maximum:		30.0	3.2	11.0	
Mass	Calc. Mass	mDa	PPM	DBE	Formula
510.1942	510.1926	1.6	3.1	9.5	C25 H34 N3 O4 Cl2

Figure 7.11: Elemental Composition of M10

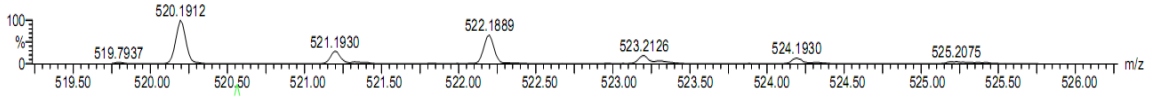
Elemental Composition Report Page 1

Single Mass Analysis
Tolerance = 3.2 PPM / DBE: min = 0.0, max = 11.0
Element prediction: Off

Monoisotopic Mass, Even Electron Ions
102 formula(e) evaluated with 1 results within limits (all results (up to 1000) for each mass)
Elements Used:
C: 0-30 H: 0-40 N: 0-3 O: 0-6 Cl: 1-2

KSL 03_MET-ID_RLM_60 min_120915
KSL 03_MET-ID_RLM_60 min_120915 582 (33.161) Cm (579:592)

12-Sep-2015
1: TOF MS ES+
5.94e+002



Minimum:				0.0	
Maximum:		30.0	3.2	11.0	
Mass	Calc. Mass	mDa	PPM	DBE	Formula
524.1930	524.1914	1.6	3.5	10.0	C25 H32 N3 O5 Cl2

Figure 7.12: Elemental Composition of M11

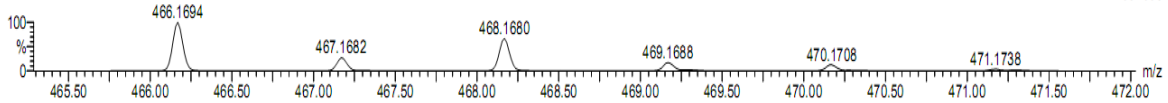
Elemental Composition Report Page 1

Single Mass Analysis
Tolerance = 10.0 PPM / DBE: min = 0.0, max = 11.0
Element prediction: Off

Monoisotopic Mass, Even Electron Ions
161 formula(e) evaluated with 1 results within limits (all results (up to 1000) for each mass)
Elements Used:
C: 0-30 H: 0-40 N: 0-3 O: 0-6 Cl: 1-2

KSL 03_MET-ID_RLM_60 min_120915
KSL 03_MET-ID_RLM_60 min_120915 593 (36.214) Cm (578:609)

12-Sep-2015
1: TOF MS ES+
1.10e+003



Minimum:				0.0	
Maximum:		30.0	10.0	11.0	
Mass	Calc. Mass	mDa	PPM	DBE	Formula
466.1694	466.1664	3.0	6.4	9.5	C23 H30 N3 O3 Cl2

Figure 7.13: Elemental Composition of M12

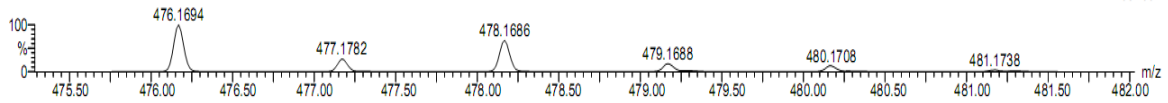
Elemental Composition Report Page 1

Single Mass Analysis
Tolerance = 10.0 PPM / DBE: min = 0.0, max = 11.0
Element prediction: Off

Monoisotopic Mass, Even Electron Ions
161 formula(e) evaluated with 1 results within limits (all results (up to 1000) for each mass)
Elements Used:
C: 0-30 H: 0-40 N: 0-3 O: 0-6 Cl: 1-2

KSL 03_MET-ID_RLM_60 min_120915
KSL 03_MET-ID_RLM_60 min_120915 583 (31.110) Cm (578:599)

12-Sep-2015
1: TOF MS ES+
4.10e+002



Minimum:				0.0	
Maximum:		30.0	10.0	11.0	
Mass	Calc. Mass	mDa	PPM	DBE	Formula
478.1686	478.1646	4.0	7.5	10.5	C23 H26 N3 O4 Cl2

Figure 7.14: Elemental Composition of M13

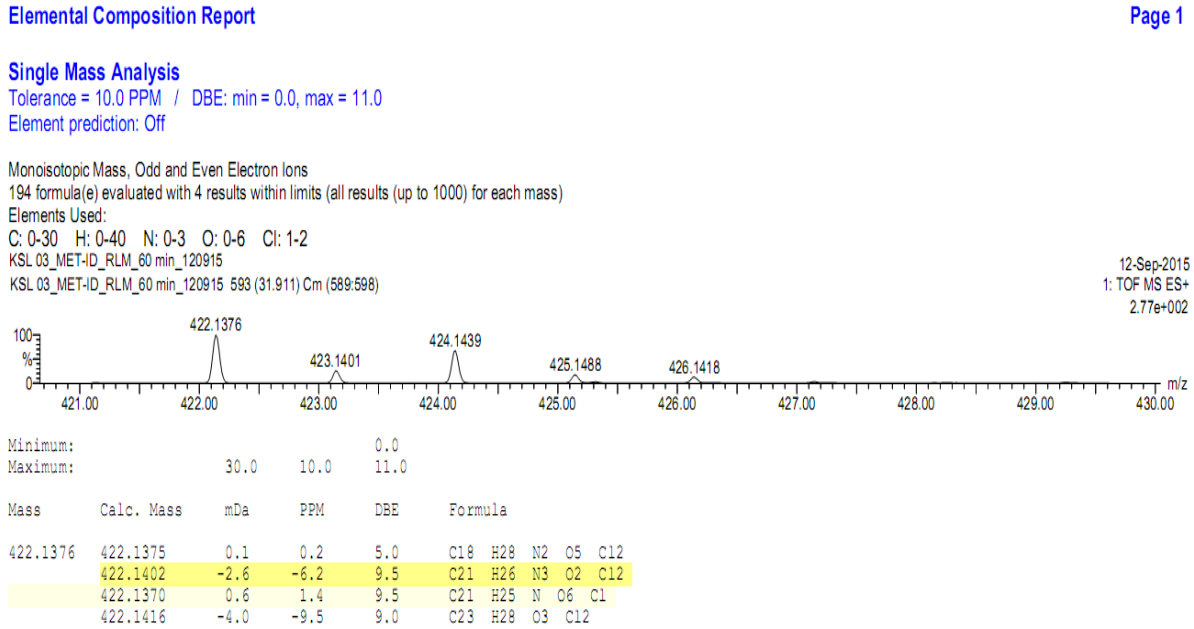


Figure 7.15: Elemental Composition of M14

The Accurate mass analysis of Test Compound and its metabolitesarelisted under Table 1.

Table 1: The Accurate mass analysis of Test Compound and its metabolites detected along with their elemental composition

Name	Observed Mass (m/z)	CalcMass (m/z)	mDa	PPM	Molecular Formula
Parent	552.2170	552.2472	-30.2	-54.7	C28H40Cl2N3O4
M1	538.2162	538.2198	-3.6	-6.7	C27H38Cl2N3O4
M2	552.2094	552.2032	6.2	11.2	C27H36Cl2N3O5
M3	494.1857	494.1977	-12.0	-24.3	C25H34Cl2N3O3
M4	508.1933	508.1626	30.7	60.4	C25H32Cl2N3O4
M5	450.1739	450.1715	2.4	5.3	C23H30Cl2N3O2
M6	406.1441	406.1453	-1.2	-3.0	C21H26Cl2N3O
M7	568.2393	568.2406	-1.3	-2.3	C28H40Cl2N3O5
M8	554.2202	554.2189	1.3	2.3	C27H38Cl2N3O5
M9	568.2332	568.1981	35.1	61.8	C27H36Cl2N3O6
M10	510.1942	510.1926	1.6	3.1	C25H34Cl2N3O4
M11	524.1930	524.1914	1.6	3.5	C25H32Cl2N3O5
M12	466.1694	466.1664	3.0	6.4	C23H30Cl2N3O3
M13	478.1694	478.1664	4.0	7.5	C23H26Cl2N3O4
M14	422.1376	422.1402	-2.6	-6.2	C21H26Cl2N3O2

Metabolite Profiles of KSL 03 in Human, Rat, Mouse, Monkey Rat, and Dog Microsomes

Met ID at early stage of drug discovery was considered as a good practice for drug development and regulatory filing. The i*n-vitro* metabolite profiling guide to select i*n-vivo* animal model to conduct PK and TK studies. Today extensive metabolic studies of NCEs on animals lead to high cost and significant challenges in drug development when performed *in vivo*. Hence *in vitro* MET-ID studies are encouraged to successfully guide for the lead optimization by studying the metabolic profiles in different species at early stages.

The Test compound was incubated in hepatic microsomes of various species likeHuman (HLM), Rat (RLM), Mouse (mLM), Dog (DLM) and Monkey liver microsomes (MoLM) for 60 minutes and the metabolic pathway was observed.

The percentage of metabolites found after 60minute incubation, along with their retention times were given in Table 2

Table 2: The percentage of metabolites found after 60minute incubation, along with their retention times

Name	m/z	Metabolic Pathway	RT	HLM	RLM	mLM	MoLM	DLM
Parent	552.2170	**Side chain cleavage and carboxylation**	51.843	5.80	5.46	25.69	7.62	14.22
M1	538.2162		49.541	42.21	1.87	22.09	34.29	24.64
M2	552.2094		45.948	3.59	3.18	2.43	6.64	5.11
M3	494.1857		41.244	13.93	12.33	16.90	21.54	17.01
M4	508.1933		37.631	6.32	0.51	4.84	1.91	11.20
M5	450.1739		34.295	8.13	7.11	14.80	17.30	11.42
M6	406.1441		32.385	9.40	8.33	8.13	7.74	13.38
M7	568.2393	**Aromatic Hydroxylation + Side chain cleavage and carboxylation**	49.980	4.72	8.91	0.72	0.96	0.71
M8	554.2202		48.301	4.97	27.80	3.34	0.46	1.10
M9	568.2332		44.712	0.06	10.22	0.04	0.13	0.09
M10	510.1942		37.634	0.61	11.83	0.91	1.24	0.90
M11	524.1930		33.161	0.05	0.74	0.91	1.24	0.09
M12	466.1694		36.214	0.11	1.37	0.91	0.13	0.11
M13	478.1686		31.110	0.10	0.51	0.00	0.15	0.12
M14	422.1376		31.911	0.05	0.35	0.02	0.05	0.11

The comprehensive approach of single extraction using a single analytical platform was worked up for the Test compound.

The metabolites identified, based on the theoretical interpretation in support of MS/MS and Elemental composition. Preliminary data obtained after microsomal incubation suggested the formation of metabolites M1 to M14 and a proposed metabolic pathway for the NCE is shown in Figure 8.

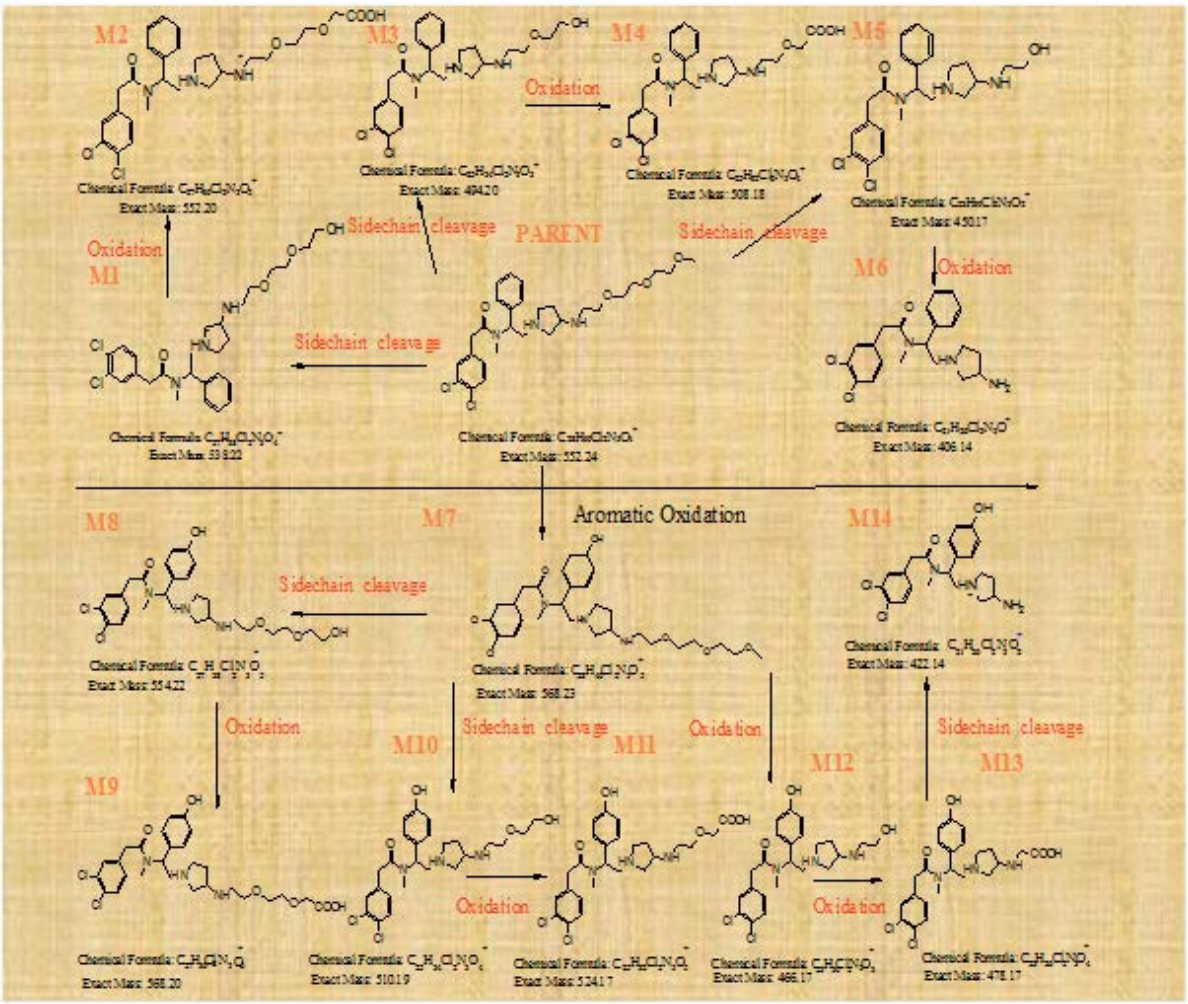


Figure 8: The Proposed metabolic pathway of Test Compound by Simple &Aromatic oxidation

Metabolite profiling being one of the main drives in the drug discovery process to optimize pharmacokinetic properties and to increase the success rate of drugs. The work up has built an *in vitro* methodology for identification of the probable metabolites, and the metabolitesobserved were through the oxidative cleavage of side chain and Aromatic hydroxylation. The main annotation for identification of the metabolites is through the MS/MS fragmentation and Elemental composition apart from the isotopic pattern observed due to presence of Chlorine atom.

The metabolites observed uponincubation in hepatic microsomes of various species likeHuman (HLM), Rat (RLM), Mouse (mLM), Dog (DLM) and Monkey (MoLM) for 60 minutes gave the information such as -the metabolism of parent to M1 is high in Human, Monkey and Rat, Aromatic metabolites formed are less in comparison to the normal ones and the compound is showing sustained levels of the parent in mice, however the data have to be confirmed by *in vivo* experimentation.

Note: The inferences were based on Mass Spec as the UV signal was weak for the compound, and the mass spec response might be quite different for different metabolites and parent compound.

CONCLUSIONS

The metabolite Identification study performed in various hepatic liver microsomes hasclearly shown that the metabolic pathway for the Test compound is through microsomal oxidation.

This *in vitro*work up using Test compound demonstrates that the compound undergoes direct and aromatic oxidativemetabolism under *in vitro* conditions. It offers a good substrate for the hepaticmicrosomal enzymes, under specific experimental conditions. While the observed sustained level of concentration for the Test compound is probably due to its structural chemistry involved in the drug

design .This built in construction shall be taken into consideration during the interpretation of the results obtained. Thus the inferences drawn in this manuscript act as a valuable tool in obtaining the necessary SAR and 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 Biotransformation under the influence of microsomal enzymes.

CONFLICTS OF INTEREST

The authors report no conflict of Interest and want to convey that this experiment doesn't restrict the scope of further evaluation, and the inferences drawn were purely based on the test procedure adopted under academia.

ACKNOWLEDGEMENTS

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