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Research Article

STUDY OF THE IN-VITRO METABOLIC PROFILE OF AMLODIPINE IN HUMAN HEPATIC CELL LINE AND CHICKEN LIVER TISSUE USING LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY/MASS SPECTROMETRY

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ABSTRACT

Objective: The main objective of this study was to investigate the *in-vitro* metabolic profile of Amlodipine (AMD) using normal human hepatic cell lines and chicken liver tissue and to characterize the metabolites obtained using liquid chromatography-tandem mass spectrometry (LC-MS/MS).

Methods: In the present study, the metabolic profile of AMD, a well-known calcium channel blocker, was investigated in normal human hepatic cell lines and chicken liver tissue employing LC-MS/MS technique. The structural details on AMD metabolites were acquired using triple quadrupole mass spectrometer (LCMS-8040, Shimadzu). The metabolites were produced by incubation of AMD with the human hepatic cell lines and chicken liver tissue at 37 °C for 24 h. The incubated extracts were analyzed on LC-MS/MS and their product ion spectra were acquired, interpreted and tentative structures were proposed.

Results: Twelve Phase I and Phase II metabolites were successfully detected in the proposed study. The main metabolic changes observed were oxidative deamination, N-acetylation, de-esterification, hydrogenation, de-methylation, aliphatic hydroxylation, and glucuronidation of dehydrogenated AMD. Based on this information, the tentative structures of the metabolites were postulated.

Conclusion: The *in-vitro* metabolites of AMD were successfully investigated and characterized in human hepatic cell lines and chicken liver tissue. Furthermore, both models were found to be equally effective for carrying out the *in-vitro* metabolic study of AMD.

Keywords: Amlodipine, Metabolites, Hepatic cell lines, Chicken liver tissue, Liquid chromatography, Liquid chromatography-mass spectrometry/ mass spectrometry.

INTRODUCTION

Amlodipine (AMD) (Fig. 1), R, S-2-[(2-aminoethoxy) methyl]-4-(2-chlorophenyl)-3-ethoxycarbonyl-5-methoxycarbonyl-6-methyl-1, 4-dihydropyridine, is a potent, long-acting calcium channel blocker of the dihydropyridine group. It blocks the calcium entry into the smooth muscle cells, causes the blood vessel to relax, and then lowers the blood pressure. It is widely used in the treatment of hypertension and angina [1-3]. It has the capacity to inhibit binding of the oxidized low-density lipoprotein (LDL) lipids as a result of its high lipophilicity and positively charged amino group. *In-vitro* studies have suggested that the dihydropyridine calcium antagonists act as antioxidants by directly quenching several radical species and thereby reduces leukocyte-induced oxidation of LDLs. AMD is extensively metabolized in the liver, and biotransformation primarily involves oxidation to the pyridine derivative [4].

Metabolism is a process of biotransformation where drugs are transformed into different chemical forms by enzymatic reactions. Metabolism mainly increases drug hydrophilicity and decreases the toxicity and activity of most drugs. The study of the metabolic fate of drugs (absorption, distribution, metabolism, excretion, and toxicology) is an essential and important part of the drug discovery development process. Data on metabolism are used to optimize drug candidates, namely to suggest more active compounds or support further toxicology studies. Biotransformation may occur in liver, intestine, kidney, lungs, brain, nasal epithelium, and skin. However, liver is by far, the most important organ for drug metabolism [5].

Drugs are metabolized by different reactions that are classified into two groups: Phase I and Phase II. Phase I reactions mostly include oxidation, reduction, and hydrolysis. The function of Phase I reactions is to introduce a new functional group within a molecule, to modify an existing functional group, or to expose a functional group that is a substrate for Phase II reactions. Phase I reactions are responsible for enhancement of drugs' hydrophilicity. Phase II reactions represent conjugating reactions and mainly further increase the hydrophilicity and facilitate the excretion of metabolites from the body [6].

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. Liquid chromatography coupled with mass spectrometry (LC-MS) has become the most powerful analytical tool for screening and identification of drug metabolites in biological matrices due to its selectivity, sensitivity, and speed of analysis. The LC-MS/MS methods are considered as most appropriate for determination of drugs and their metabolites and are also best suited for high throughput analysis. Crude extracts of *in-vitro* incubation and *in-vivo* samples can be subjected to metabolite profiling and identification by LC-MS/MS. Complex metabolite mixtures are resolved chromatographically on a high-performance liquid chromatography (HPLC) column, and full scan MS and product ion scan MS/MS data are generated on-line. Thus, the molecular weight of drug metabolites and localization of the biotransformation sites can be elucidated based on interpretation of the MS/MS data [7,8].

The proposed study was carried out using normal human hepatic cell lines and chicken liver tissue. Liver cell lines are easy to culture and have stable enzyme concentration. These can be cultured in a monolayer to prolong the viability to 4 weeks. This characteristic in combination with the prolonged regulatory pathways allows the use of this *in-vitro*

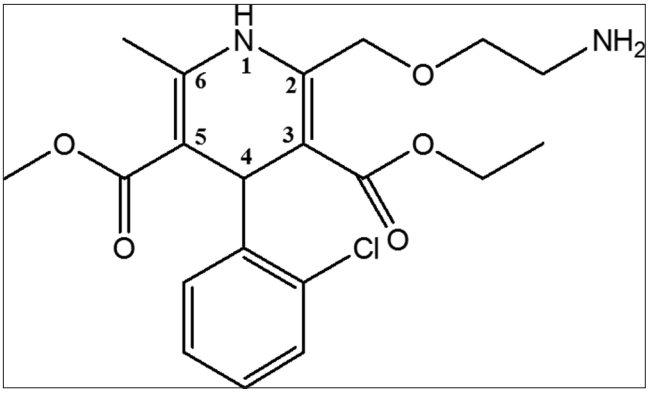

Fig. 1: Chemical structure of amlodipine

model in studies of upregulation or downregulation of metabolites. The major sources of cell lines are primary tumors of liver parenchyma. On the other hand, isolated liver also gives an excellent representation of the *in-vivo* situation. The additional advantages of this *in-vitro* model are three-dimensional architecture and presence of hepatic as well as non-hepatic cell types [9-11]. Considering these advantages, the two models were selected for the proposed study to determine and characterize the *in-vitro* metabolites of AMD.

Several different analytical techniques have been reported in the literature for the estimation of AMD in biological samples [12-18], LC-MS/MS being the most common one. Metabolism of AMD has been studied using human liver microsomes to determine the role of cytochrome enzymes in its metabolism [19]. It has been proved that the biotransformation of AMD primarily involves oxidation to the pyridine derivative with species differences in humans, rats, and dogs [20-23]. Furthermore, a stereoselective study of AMD biotransformation in rat hepatocyte has also been reported, where eight metabolites of AMD were characterized [24]. In addition to this, *in-vitro* metabolic profile of AMD in rat has been studied, and 21 Phase I and Phase II metabolites were determined [25]. However, so far, no studies involving the investigation of AMD metabolites in the proposed two *in-vitro* models have been conducted, to the best of our knowledge. In addition, three new Phase I metabolites were obtained in the present research work which were not reported in the literature as yet.

Thus, the main aim of the present study was to investigate the *in-vitro* metabolic profile of AMD in normal human hepatic cell lines and chicken liver tissue using LC-MS/MS technique and to compare the metabolic profile of AMD in the two *in-vitro* models. Based on the MS and MS/MS data, the chemical structures of the metabolites were proposed and three new *in-vitro* metabolites of AMD which were not published as yet were presented.

METHODS

Chemicals and reagents

AMD besylate API was procured from Hetero Chemical Lab, Hyderabad, India, with a purity of 99.4% w/w.

HPLC grade - acetonitrile (CH_3CN), water; analytical grade - ethyl alcohol (C_2H_5OH), potassium chloride (KCl), potassium diphosphate (KH_2PO_4), sodium chloride (NaCl), disodium phosphate (Na_2HPO_4), sodium hydroxide (NaOH), sodium lauryl sulfate (SLS), and LCMS grade - ammoniumacetate (NH_3COOH) were purchased from Merck (Mumbai, Maharashtra, India).

Dulbecco's modified eagle's medium (DMEM) was purchased from Sigma Aldrich (Mumbai, India), fetal calf serum (FCS) from Biowest (Mumbai, Maharashtra, India), Trypsin-versene mixture from Himedia (Mumbai, Maharashtra, India) and Gentamycin from Nicholas Piramal (Mumbai, Maharashtra, India).

THLE-3 (ATCC – CRL-11233) Normal human liver cell line was procured from ATCC.

Fresh chicken liver was collected from the local butcher shop.

Equipment

Centrifuge (EMTEK), CO_2 Incubator (Thermo Forma Series II), Epi-Fluorescent Inverted Microscope (AXIOVERT S-100).

From the literature, a few similar research works on AMD were referred to carry out the present study [19,24,25].

Stock preparation

To get a final concentration of 400 µg/ml for cell line study and 1000 µg/ml for liver tissue study, a parent stock of 10 mg/ml of AMD API was initially dissolved in minimum quantity of phosphate buffered saline (PBS) followed by the addition of DMEM. This was filter sterilized through 0.22 µ membrane filter. This stock was further used to prepare concentrations of 4000 µg/ml and 10,000 µg/ml. From these two concentrations, 10 µl from the stock was added to 90 µl of cell suspension in 96-well plate making the final concentration of 400 µg/ml and 1000 µg/ml for cell lines and chicken liver tissue, respectively.

Maintenance of cell line

THLE-3 (ATCC – CRL-11233) normal human liver cell line was procured from ATCC. Cells were maintained at 37°C in humid conditions (5% CO_2/95% air) of CO_2 incubator; suspended in DMEM supplemented with 10% FCS. Cells were sub-cultured on every 4th day using 0.25% trypsin-versene mixture for harvesting the cells, to confirm the normal growth pattern of the cells before using it for the experiment.

In-vitro experiment using liver cell line

Exponentially growing cells in the flasks were harvested by trypsinization with 0.25% trypsin-versene and suspended in DMEM supplemented with 10% FCS. Cells with a density of 1×10^3 cells/well were seeded in 96 well plates and incubated at 37°C in CO_2 incubator for 24 hrs to allow them to adhere to the surface and form a monolayer. The medium was then aspirated carefully and the cells were switched to DMEM consisting of required concentration of API without disturbing the adhered cells. The supernatant was aspirated and the cells were lysed using 100 µl of lysis buffer (1N NaOH solution with 0.5% SLS) in each well. Lysis of the cells was facilitated by incubating the plates for 30 minutes at 37°C. Later, appropriate cell lysates were collected and pooled together in a microcentrifuge tube, centrifuged at 1000 rpm for 3 minutes. The pellet was discarded and the supernatant was collected in separate sterile microcentrifuge tube. To this acetonitrile was added in order to precipitate the proteins present. The extract was vertex and then centrifuged at 8000 rpm for 10 minutes. The pellet was discarded and the supernatant was filtered through 0.22 µ millipore membrane filter and used for further analysis on LC-MS/MS.

Blank was prepared in a similar fashion without spiking of the drug.

Images of the cells of the blank as well as the test samples, during the incubation time, were taken from inverted microscope and are shown in Fig. 2.

In-vitro experiment using chicken liver tissue

Fresh chicken liver was collected from the local butcher shop and cleaned with sterile PBS to remove traces of blood and dust particles. The intact tissue was surface sterilized with absolute ethyl alcohol for 30 seconds and washed with sterile PBS to remove the traces of alcohol. The tissue was then aseptically sliced to approximately 7 mm×7 mm pieces for the experiment. The tissue samples were suspended in DMEM supplemented with 10% FCS in 24 well plates and incubated at 37°C in CO_2 incubator for 24 hrs. The medium was then aspirated carefully and the cells were switched to DMEM consisting of required concentration of API without disturbing the tissue. The tissue was incubated further for 24 h. The supernatant was aspirated and the tissue was lysed using

250 µl of lysis buffer in each well. Lysis of the cells was facilitated by incubating the plates for 30 minutes at 37°C. Later, appropriate tissue lysates were collected and pooled together in a microcentrifuge tube, centrifuged at 1000 rpm for 3 minutes. The pellet was discarded and the supernatant was collected in separate sterile microcentrifuge tube. To this acetonitrile was added in order to precipitate the proteins present. The extract was vertex and then centrifuged at 8000 rpm for 10 minutes. The pellet was discarded and the supernatant was filtered through 0.22 µ millipore membrane filter and used for further analysis on LC-MS/MS.

Blank was prepared in a similar fashion without spiking of the drug.

LC-MS/MS analysis

HPLC system (Shimadzu Prominence Binary Gradient System, Shimadzu Corporation, Japan) equipped with binary pump (LC-30AD), autosampler (SIL-30ACMP), a temperature controlled column compartment (CTO-30A) and photodiode array detector (SPD-M20A) was used. Chromatographic data was acquired using Labsolutions software. Analysis was done using Shim-pack XR ODS column (100 mm×2 mm, 3 µ). The mobile phase comprised of - (A) 30 mM ammonium acetate buffer (B) acetonitrile, in a gradient mode. The gradient program is given in Table 1. The flow rate was maintained at 0.5 ml/minutes, injection volume was 1 µl and the column temperature was maintained at 40°C. Run time for the analysis was kept 8 minutes. The chromatograms were monitored at 238 nm. The structure elucidation of the metabolites was done using triple quadrupole mass spectrometer LCMS-8040 equipped with electrospray ionization source, operated in positive mode. Nitrogen gas was used at flow rates of 2 l/minute for nebulization, 15 l/minute for heating and as a drying gas at 15 l/minute. Argon was used as a collision gas. The collision energy was optimized and set to −30.0 V. Mass spectra were acquired over m/z range of 80-700. Event time was of 0.08 seconds.

RESULTS AND DISCUSSION

As already mentioned, the main aim of the present study was to investigate the *in-vitro* metabolic profile of AMD employing LC-MS/MS technique. For this, two *in-vitro* models were used, namely, normal human hepatic cell lines and chicken liver tissue. Though the metabolites of AMD have been studied in different types of biological samples, but these two *in-vitro* models were not used in the past. The desired drug concentration to be used and incubation time to be set for the experiment were initially optimized. Finally, the metabolites were produced by incubating the desired amount of drug with the liver cell lines and chicken liver tissue at optimized test conditions. The incubated extracts were analyzed on LC-MS/MS and their product ion spectra were acquired, interpreted and tentative structures were proposed. The product ion scans of AMD and its metabolites are given in Fig. 3 while the product ions are mentioned in Table 2.

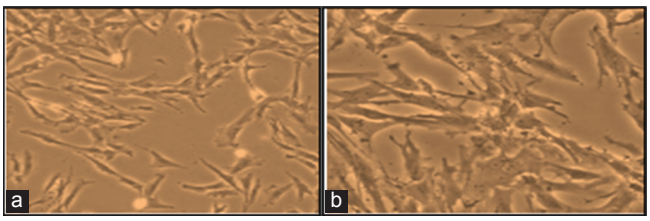

Fig. 2: Images of the cells taken from inverted microscope (a) cells of the blank sample (b) cells spiked with drug, showing fine granulation in the nuclear region and expected stressed conditions as compared to the blank

Table 1: Gradient program used for the analysis

Time	A (%)	B (%)
0.01	60	40
1.00	60	40
3.00	0	100
5.00	0	100
5.50	60	40
8.00	60	40

Twelve Phase I and Phase II metabolites were identified in the proposed study. The masses ($[M+H]^+$) of the obtained metabolites and the possible chemical biotransformation reactions are mentioned in Table 3. The masses (m/z) of the metabolites obtained were found to be 349, 351, 381, 391, 411, 425, 433, 435, 437, 449, 467, and 584.

The possible biotransformations were predicted based on the mass difference of the metabolite with respect to the parent drug and the acquired product ion scan. There was a mass difference of 60 units for the metabolite-M1 with mass 349 ($C_{18}H_{22}ClN_2O_3$). The nearest possible reaction therefore suggested was de-esterification (loss of –$COOCH_3$ moiety). M1 produced the following ions with mass (m/z) as 220 ($C_{13}H_{15}ClN$), 196 ($C_{10}H_{14}NO_3$), 187 ($C_9H_{17}NO_3$), 167 ($C_8H_9NO_3$), 151 ($C_8H_9NO_2$), 144 ($C_8H_{17}NO$). From these, fragment m/z 220 was the base peak and same as one of the fragments of AMD while fragments m/z 167, m/z 151, and m/z 144 were found to be present in other metabolites too. Likewise, other chemical changes were also predicted; a mass loss of 58 units for M2 (m/z 351 - $C_{17}H_{18}ClNO_5$) was described to be a result of deamination followed by demethylation and de-alkylation. M2 fragmented into ions having mass (m/z): 180 ($C_{10}H_{13}NO_2$), 150 ($C_9H_{11}NO$), 144 ($C_8H_{17}NO$). The fragment m/z 150 showed a close similarity with one of the AMD fragment; while the remaining two fragments were also found in other metabolites. A mass loss of 28 units for M3 (m/z 381 - $C_{18}H_{21}ClN_2O_5$) was explained as de-alkylation, loss of CH_2=CH_2 group. The main fragments of M3 consisted of m/z 364, m/z 350, m/z 239, m/z 125. Fragment m/z 239 was similar with the fragment m/z 238 present in the parent drug while fragment m/z 125 was also present in M6. A loss of water molecule (18 units) resulted in formation of M4 (m/z 391 - $C_{20}H_{23}ClN_2O_4$). M4 on losing the chlorophenyl ring and modification of the side chains at the 2nd and 3rd position provided fragments with mass: m/z 261 ($C_{12}H_{24}N_2O_4$), m/z 167($C_8H_9NO_3$), m/z 149 ($C_9H_{11}NO$), and m/z 113 ($C_7H_{15}N$). From these, fragment m/z 149 was same as that of AMD and fragment m/z 261 was

Table 2: Precursor ions and their respective product ions obtained from liquid chromatography-tandem mass spectrometry study

Metabolites	Precursor ions (m/z)	Collision energy used	Product ions obtained (m/z)
M1	349	−30 V	116, 144, 151, 167, 187, 196, 220, 248, 266
M2	351		106, 128, 144, 150, 163, 180, 218
M3	381		110, 125, 239, 277, 298, 350, 364
M4	391		113, 149, 167, 200, 261, 279
M5	411		139, 142, 151, 167, 170, 208, 222, 240
M6	425		114, 125, 143, 165, 186, 195, 233, 259, 281, 331
M7	433		108, 119, 178, 319, 398
M8	435		123, 153, 186, 207, 233, 251, 286, 318, 360
M9	437		139, 185, 211, 244, 357, 392, 402, 420
M10	449		112, 125, 149, 184, 210, 245, 285, 316, 332, 422
M11	467		113, 238
M12	584		124, 133, 185, 212, 231, 456

Table 3: The masses and possible chemical reactions of the identified metabolites of amlodipine

Metabolites	Masses of the metabolites (m/z)	Mass difference w.r.t drug molecule (Δm)	Possible reaction
M1	349	−60	De-esterification (loss of CH_3COO-)
M2	351	−58	Deamination (loss of NH_2), demethylation (loss of methyl group), de-alkylation (loss of –CH=CH- group)
M3	381	−28	De-alkylation (loss of CH_2=CH_2 group)
M4	391	−18	Loss of a water molecule (H_2O)
M5	411	+2	Hydrogenation (gain of 2 H)
M6	425	+16	Hydroxylation (addition of –OH)
M7	433	+24	Addition of carbonyl (–CO) group, loss of 4 H atoms
M8	435	+26	Addition of carbonyl (–CO) group, loss of 2 H atoms
M9	437	+28	Addition of carbonyl (–CO) group
M10	449	+40	N-acetylation (loss of H and addition of $COCH_3$) and dehydrogenation (loss of 2 H)
M11	467	+58	N-acetylation (Loss of H and addition of $COCH_3$) and Oxidation (addition of O)
M12	584	+175	Glucuronide conjugation (loss of H and addition of a glucuronic acid)

m/z: Mass/charge value, Δm: Mass difference, mass of protonated amlodipine=m/z 409

also similar to the parent drug; while the remaining two fragments were found in other metabolites too. A mass gain of 2 unit for M5 (m/z 411 - $C_{20}H_{27}ClN_2O_5$) was explained as hydrogenation. M5 gave the following fragments of mass (m/z): 240 ($C_{12}H_{17}NO_4$), 222 ($C_{11}H_{11}NO_4$), 208 ($C_{10}H_{11}NO_4$), 180 ($C_9H_9NO_3$), 170 ($C_8H_{11}NO_3$), 167 ($C_8H_9NO_3$), 151 ($C_8H_9NO_2$), 142 ($C_7H_{11}NO_2$), 139 ($C_7H_9NO_2$). From these, Fragments with mass (m/z) 208,170 and 142 were exactly same as that of AMD while fragments 222, 180 and 167 could be said to be same as that of the parent drug on the basis of the 2 unit mass gain of the metabolite w.r.t AMD. The remaining two fragments: 151 and 139 were also found in other metabolites. Addition of a hydroxyl group (16 units) resulted in the formation of M6 (m/z 425 - $C_{20}H_{27}ClN_2O_6$). M6 formed product ions with masses (m/z) - 287 ($C_{14}H_{25}NO_5$), 259 ($C_{12}H_{21}NO_5$), 233 ($C_{10}H_{19}NO_5$), 195 ($C_9H_9NO_4$), 186 ($C_9H_{15}NO_3$), 165 ($C_9H_{11}NO_2$), 143 ($C_8H_{17}NO$), 125 ($C_8H_{15}N$) and 114 ($C_7H_{15}N$). The fragment m/z 165 was exactly same as AMD, while fragments with mass (m/z): 287, 259, 233, and 143 were also similar to AMD. Fragments m/z 186 and m/z 195 showed the corresponding 16 unit mass gain of the metabolite w.r.t the parent drug. The remaining two fragments m/z 125 and m/z 114 were common in other metabolites too.

M7 (m/z 433 - $C_{21}H_{21}ClN_2O_6$) was thought to be produced by addition of a carbonyl group (-CO) and loss of four hydrogen atoms. M7 fragmented into ions with mass (m/z): 319 ($C_{15}H_{10}ClNO_5$), 178 ($C_9H_7NO_3$), 119 (C_8H_9N). The fragment m/z 178 was same as that of AMD while fragment m/z 319 was also found in M8. M8 (m/z 435 - $C_{21}H_{23}ClN_2O_6$) and M9 (m/z 437 - $C_{21}H_{25}ClN_2O_6$) were thought to be formed in a similar manner as that of M7. M8 gave fragments with masses (m/z): 318 ($C_{15}H_{10}ClNO_5$), 286 ($C_{13}H_{22}N_2O_5$), 251 ($C_{12}H_{13}NO_5$), 233 ($C_{10}H_{19}NO_5$), 207 ($C_8H_{17}NO_5$), 186 ($C_8H_{11}NO_4$), 167 ($C_8H_9NO_3$). The fragments m/z 233 and m/z 207 were similar to the fragments of AMD; fragment m/z 286 was reported in the literature [25] for the same metabolite; while the fragments m/z 318, m/z 186 and m/z 167 were also present in other detected metabolites. M9 fragmented into the following ions with mass (m/z): 420 ($C_{21}H_{25}ClN_2O_5$), 402 ($C_{20}H_{19}ClN_2O_5$), 392 ($C_{19}H_{21}ClN_2O_5$), m/z 357 ($C_{19}H_{17}ClNO_4^-$), 244 ($C_{11}H_{17}NO_5$), 211 ($C_9H_9NO_5$), 185 ($C_8H_{11}NO_4$), 139 ($C_8H_{13}NO$). The fragments m/z 420 and m/z 392 were similar to the fragments reported in the literature [24] for the aforementioned metabolite; while fragments m/z 244, m/z 211, m/z 185 and m/z 139 were similar to the ones present in the other detected metabolites of the proposed study. A mass gain of 40 units for M10 (m/z 449 - $C_{22}H_{25}ClN_2O_6$) was described as N-acetylation and loss of two hydrogen atoms. M10 gave the following fragments with mass (m/z): 316 ($C_{17}H_{14}ClNO_3$), 285 ($C_{13}H_{20}N_2O_5$), 245 ($C_{11}H_{19}NO_5$), 210 ($C_9H_{11}NO$), 184 ($C_8H_9NO_4$), 125 ($C_8H_{15}N$) and 112 ($C_7H_{13}N$). The fragment m/z 149 was same as the parent drug, also fragment m/z 210 showed the corresponding 40 unit mass gain of the metabolite w.r.t the drug; fragments m/z 285 and m/z 316 were reported in one of the study [25] for the mentioned metabolite, remaining fragments m/z 245, m/z 184, m/z 125 and m/z 112 were also found in the other metabolites. A mass gain of 58 units for M11 (m/z 467 - $C_{22}H_{27}ClN_2O_7$) was proposed to be a result of N-acetylation and oxidation. This on loss of the chlorophenyl ring and the side chain at 2nd position gave fragments with mass m/z 238 ($C_{12}H_{16}NO_4^-$) and m/z 113 ($C_7H_{15}N$). Fragment m/z 238 was the base peak of AMD; while m/z 113 was also present in M4. M12 (m/z 584 - $C_{26}H_{30}ClNO_{12}$) was explained to be the glucuronide of dehydrogenated AMD with a mass gain of 175 units. It fragmented into m/z 231, m/z 212, m/z 185, m/z 133 and m/z 124. Out of these, fragments m/z 212, m/z 185 and m/z 124 were similar to the fragments obtained for other metabolites.

All the above mentioned products (M1-M12) were assumed to be AMD metabolites on comparison with the blank (incubation of the cell lines and the chicken liver tissue without AMD), due to the presence of molecular ions accompanied by characteristic chlorine isotopic peaks with 2 unit higher m/z ratios in the mass spectra and also on the basis of the justification given above for the fragmentation pattern of the metabolites. However, after a careful scrutiny, it was found that metabolites M2, M3 and M12 do not have a direct justification of being a metabolite of AMD as they don't have any common fragments when compared with the parent drug; still the possibility of their being the metabolite of AMD could not be totally ruled out because they possess a similarity in the fragmentation pattern w.r.t other metabolites and also these masses were not obtained in the blank.

Few studies have been done in the past which have proved that the primary route of metabolism for AMD is oxidation to its pyridine analogues [20-23]. These studies were carried out in human volunteers, rats, mice, and dogs. There were two studies found in literature [24,25] which have shown formation of similar metabolites as that of the present work. However, these two studies were performed using rat hepatocytes; while the *in-vitro* models used in the present study were human hepatic cell lines and chicken liver tissue, which are not used before for a similar study on AMD. Also, three new Phase I metabolites were detected in the proposed research work namely m/z 351, m/z 381 and m/z 411. These three metabolites are not reported in the literature as yet. Three metabolites with masses - m/z 349, m/z 351, and m/z 381 were also found to be reported as the degradation products of AMD in one of the study [1].

Based on the above information, the tentative structures of the investigated metabolites were postulated (Fig. 4) in the two studied *in-vitro* models.

Besides characterizing the metabolites of AMD, another objective of the proposed research work was also to perform a comparative study of the metabolic profile of the drug obtained in the two selected *in-vitro* models. Since same metabolites were formed in both the *in-vitro* models, it could be indicated that the drug underwent a similar kind of

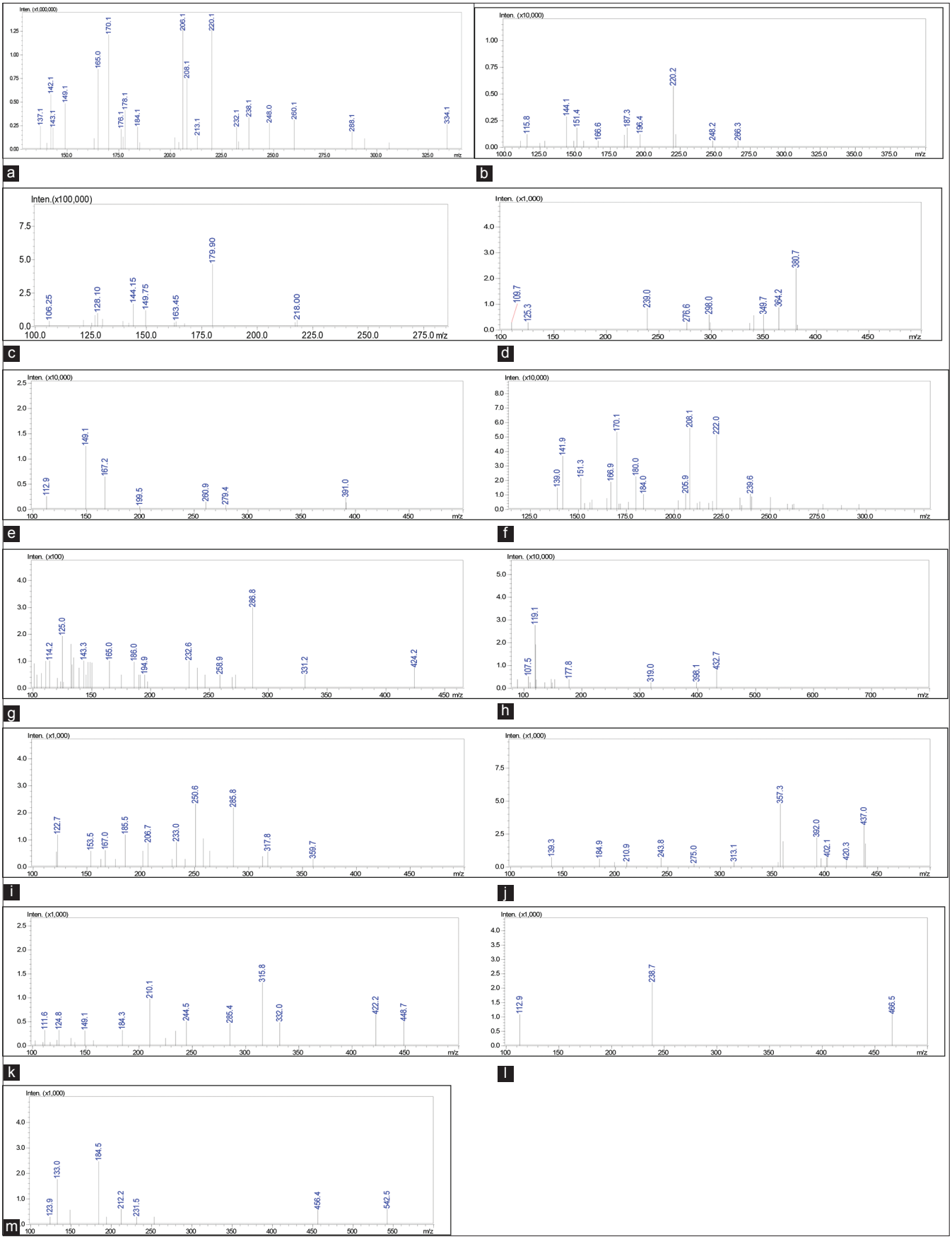

Fig. 3: Product ion scans of amlodipine and its metabolites (a) Amlodipine, (b) m/z 349, (c) m/z 351, (d) m/z 381, (e) m/z 391, (f) /z 411, (g) m/z 425, (h) m/z 433, (i) m/z 435, (j) m/z 437, (k) m/z 449, (l) m/z 467, (m) m/z 584

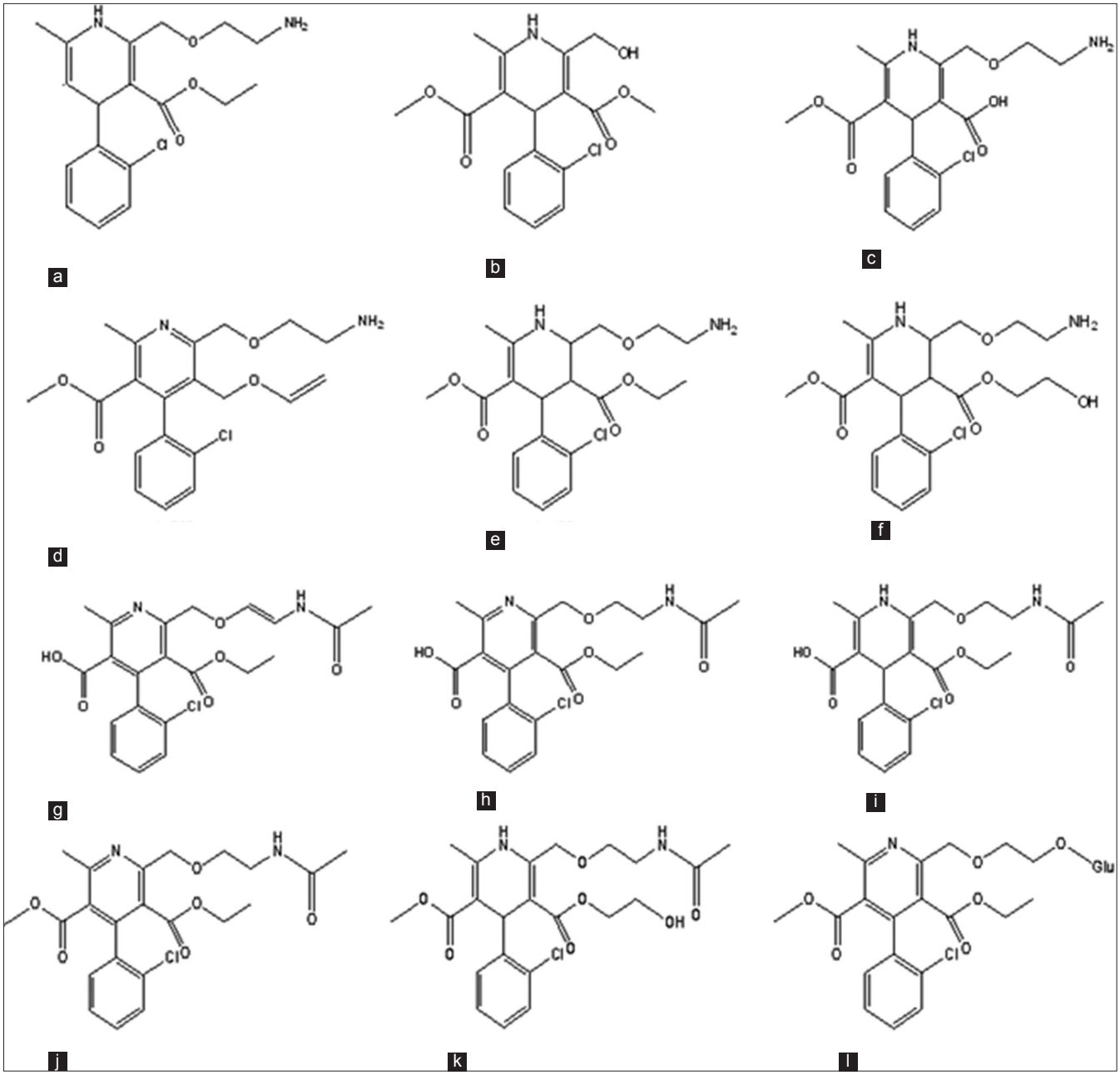

Fig. 4: Tentative postulated structures of the in-vitro metabolites of amlodipine. (a) m/z 349, (b) m/z 351, (c) m/z 381, (d) m/z 391, (e) m/z 411, (f) m/z 425, (g) m/z 433, (h) m/z 435, (i) m/z 437, (j) m/z 449, (k) m/z 467, (l) m/z 584

biotransformation in both the cases. Hence, it could be stated that both, human hepatic cell lines and chicken liver tissue are equally effective *in-vitro* models for studying the *in-vitro* metabolic profile of AMD.

CONCLUSION

Thus, in the proposed study, 12 Phase I and Phase II metabolites of AMD were successfully detected and identified employing LC-MS/MS technique, in two *in-vitro* models, namely, human hepatic cell lines and chicken liver tissue. Both the *in-vitro* models were found to be equally effective as same metabolites were formed in both the case. The masses ($[M+H]^+$) of the metabolites obtained were found to be: m/z 349, m/z 351, m/z 381, m/z 391, m/z 411, m/z 425, m/z 433, m/z 435, m/z 437, m/z 449, m/z 467 and m/z 584. The main chemical changes observed in the drug molecule during the process of metabolism included oxidative deamination, N-acetylation, de-esterification, hydrogenation, de-methylation, aliphatic hydroxylation and glucuronide conjugation. Product ion scans were acquired and interpreted for the detected metabolites. Based on the interpretations and the remaining information, the tentative structures of the investigated metabolites of AMD in the two studied *in-vitro* models were postulated.

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