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Original Article

INVESTIGATION OF *IN VITRO* METABOLITES OF ETODOLAC IN HUMAN HEPATIC CELL LINE AND CHICKEN LIVER TISSUE USING LC-MS/MS

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ABSTRACT

Objective: The main objective of this study was to investigate the *in vitro* metabolic profile of etodolac (ETD) 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 ETD, a well-known non-steroidal anti-inflammatory drug (NSAID), was investigated in normal human hepatic cell lines and chicken liver tissue employing LC-MS/MS technique. The structural details on ETD metabolites were acquired using triple quadrupole mass spectrometer (LCMS-8040, Shimadzu). The metabolites were produced by incubation of ETD with the human hepatic cell lines and chicken liver tissue, at 37 °C for 24 h. The incubated extracts were analyzed with LC-MS/MS and their production spectra were acquired, interpreted and tentative structures were proposed.

Results: Six phase I and phase II metabolites were successfully detected in the proposed study. The metabolic changes observed included-oxidation, N-acetylation, hydrogenation, decarboxylation, methylation and glucuronidation of dehydrogenated ETD. The tentative structures of the metabolites were postulated based on the chemical reactions predicted and the LC-MS/MS data obtained.

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

Keywords: Etodolac, Metabolites, Hepatic cell lines, Chicken liver tissue, LC, LC-MS/MS

INTRODUCTION

ETD (fig. 1), (*RS*)-2-(1, 8-Diethyl-4, 9-dihydro-3*H*-pyrano [3, 4-b] indol-1-yl) acetic acid is a well-known member of the pyrano-carboxylic acid group of NSAID, approved by the U. S. Food and Drug Administration (FDA). This group of drugs has anti-inflammatory, analgesic and antipyretic activities. ETD is used worldwide to relieve inflammation, swelling, stiffness and joint pain in conditions like osteoarthritis, rheumatoid arthritis, and postoperative pain, as well as for general pain relief. Their pharmacological actions are related to inhibition of prostaglandin biosynthesis at the site of inflammation [1, 2]. ETD is extensively metabolized in the liver. Renal elimination of ETD mainly as glucuronide and its other phase I and phase II metabolites is the primary route of excretion [3].

Metabolism is a pr°Cess of biotransformation where drugs are transformed into a different chemical form 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 pr °Cess. Data on metabolism are used to optimize drug candidates, namely to suggest more active compounds or support further toxicology studies. Biotransformation may °Ccur in liver, intestine, kidney, lungs, brain, nasal epithelium and skin. But, liver is by far, the most important organ for drug metabolism [4].

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 the 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 [5].

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 the 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 chromate-graphically on a high-performance liquid chromatography (HPLC) column and full scan MS and production scan MS/MS data are generated on-line. Thus, the molecular weight of drug metabolites and l°Calization of the biotransformation sites can be elucidated based on interpretation of the MS/MS data [6].

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 in order to prolong the viability to four weeks. This characteristic in combination with the prolonged regulatory pathways allows the use of this *in vitro* model in studies of up-regulation or down-regulation 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 [7, 8]. Considering these advantages, the two models were selected for the proposed study, to determine and characterize the *in vitro* metabolites of ETD.

Several techniques have been reported in the literature for the analysis of ETD and its metabolites, of which HPLC-UV [9-14] is the most common technique. Other instruments such as gas chromatography with nitrogen phosphorous detection (GC-NPD) [15], electrophoresis [16, 17] and GC-MS [18] have also been used. Until now, the mono-hydroxylated metabolites of ETD, namely, 5-hydroxy [3], 6-hydroxy, 7-hydroxy and 8-(1'-hydroxyethyl) ETD were identified and reported [19, 20]. Furthermore, the acyl glucuronides of the mono hydroxylated metabolites, as well as ETD glucuronide, were also reported [21, 22]. In addition to this, reports on the existence of 4-ureido etodolac were found in the literature [23]. However, the metabolites obtained in the present research work have not been reported in the literature as yet, to the best of our knowledge. Also, so far, no studies involving the investigation of ETD metabolites in the proposed two *in vitro* models have been conducted.

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

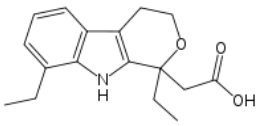


Fig. 1: Chemical structure of etodolac

MATERIALS AND METHODS

Chemicals and reagents

ETD API was procured from Hetero Chemical Lab (Hyderabad, India) with a purity of 99.4 % w/w.

HPLC grade acetonitrile (CH_3CN), formic acid (HCOOH), 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) were purchased from Merck (Mumbai, India).

Dulbecco's modified eagle's medium (DMEM) was purchased from Sigma-Aldrich (Mumbai, India), foetal calf Serum (FCS) from Biowest (Mumbai, India), Ttypsin-versene mixture from Himedia (Mumbai, India) and gentamycin from Nicholas Piramal (Mumbai, India).

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

The fresh chicken liver was collected from the l°Cal butcher shop.

Equipments

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

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

Stock preparation

To get a final concentration of 400µg/ml for cell line study and 1000µg/ml for liver tissue study, a parent stock of 10 mg/ml of ETD API was initially dissolved in minimum quantity of ethyl alcohol followed by the addition of DMEM. This was filter sterilized through 0.22 µ membrane filter. This parent stock was further used to prepare concentrations of 4000 µg/ml and 10,000 µg/ml. From these stock solutions, 10 µl was added to 90 µl of cell suspension in 96-well plate making the final concentrations of 400µg/ml and 1000µg/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, in order 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 medium supplemented with 10 % FCS. Cells with a density of $1x10^3$cells/well were seeded in 96 well plates and incubated at 37 °C in CO_2 incubator for 24 h 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 medium 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 min at 37 °C. Later, appropriate cell lysates were collected and pooled together in a micr°Centrifuge tube, centrifuged at 1000 rpm for 3 min. The pellet was discarded and the supernatant was collected in a separate sterile micr°Centrifuge 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 min. 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 the inverted microscope and are shown in fig. 2.

In vitro experiment using chicken liver tissue

Fresh chicken liver was collected from the l°Cal butcher shop and cleaned with sterile phosphate buffered saline (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 X 7 mm pieces for the experiment. The tissue samples were suspended in DMEM medium supplemented with 10 % FCS in 24 well plates and incubated at 37 °C in CO_2 incubator for 24 h. The medium was then aspirated carefully, and the cells were switched to DMEM medium 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 min at 37 °C. Later, appropriate tissue lysates were collected and pooled together in a microcentrifuge tube, centrifuged at 1000 rpm for 3 min. The pellet was discarded and the supernatant was collected in a 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 min. 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 a 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 Lab solutions software. The analysis was done using Shim-pack XR ODS column (100 mm x 2 mm, 3 µ). The mobile phase comprised of-(A) water (pH 3.0 adjusted with formic acid) (B) acetonitrile, in a gradient mode. The gradient program is given in table 1. The flow rate was maintained at 0.5 ml/min; injection volume was 1 µl and the column temperature was maintained at 40 °C. Run time for the analysis was kept 8 min. The chromatograms were monitored at 225 nm. The structure elucidation of the metabolites was done

using triple quadrupole mass spectrometer LCMS-8040 equipped with electrospray ionization (ESI) source, operated in positive mode.

Nitrogen gas was used at flow rates of 1.5 l/min for nebulization, 15 l/min for heating and as a drying gas at 15 l/min. Argon was used heating gas 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 off 0.08 seconds.

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

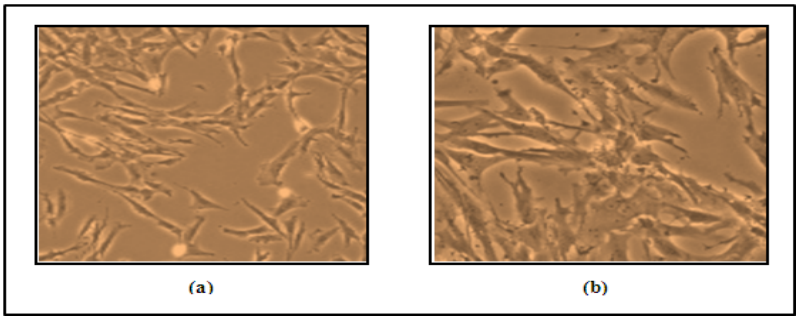


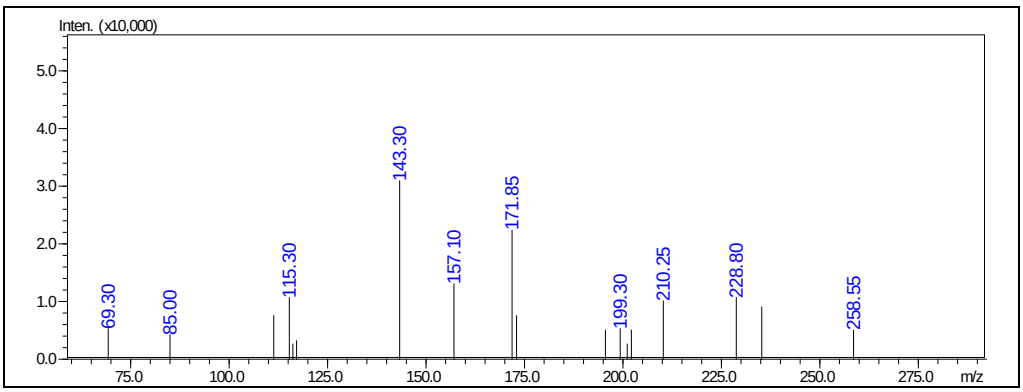
Fig. 2: Images of the cells taken from inverted microscope (a) cells of the blank sample (b) cells spiked with drug, showing fine granulation in the nuclear region and expected stressed conditions as compared to the control

RESULTS AND DISCUSSION

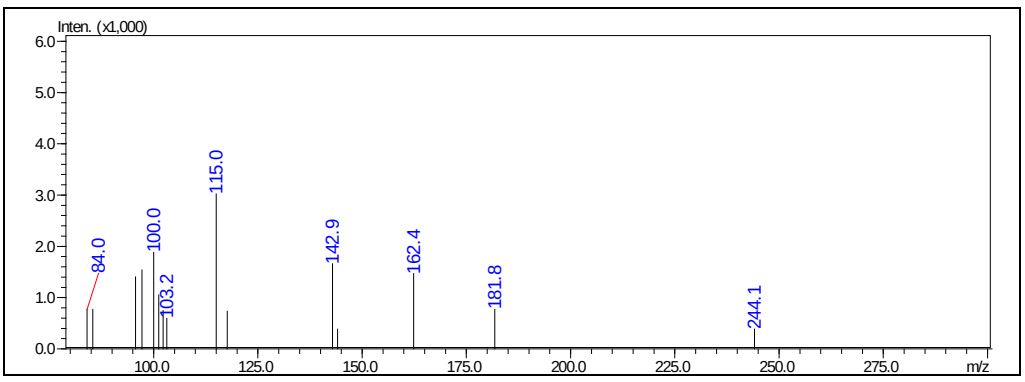
As already mentioned, the main aim of the present study was to investigate the *in vitro* metabolic profile of ETD employing LC-MS/MS technique. For this, two *in vitro* models were used, namely, normal human hepatic cell lines and chicken liver tissue.

Though the metabolites of ETD have been studied in different types of samples including blood, urine, plasma etc. [19, 20] 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.

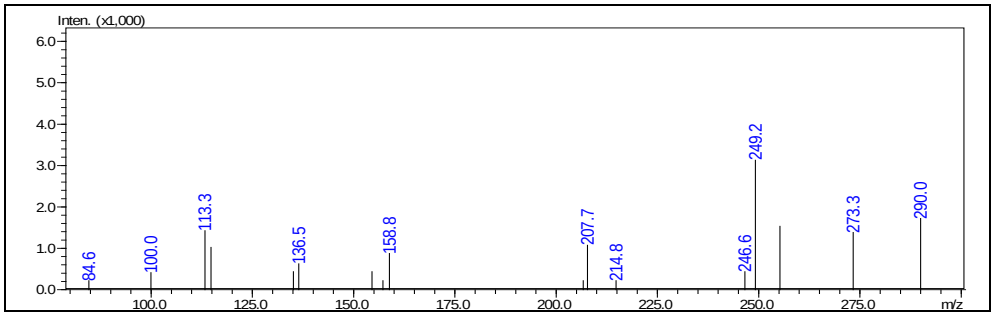
Finally, the metabolites were produced by incubating the desired amount of drug with the liver cell lines and chicken liver tissue at optimized test conditions. The incubated extracts were analyzed on LC-MS/MS and their product ion scan spectra were acquired, interpreted, and tentative structures were proposed. The production scans of the metabolites are given in fig. 3, while the product ion scan obtained, are mentioned in table 2.



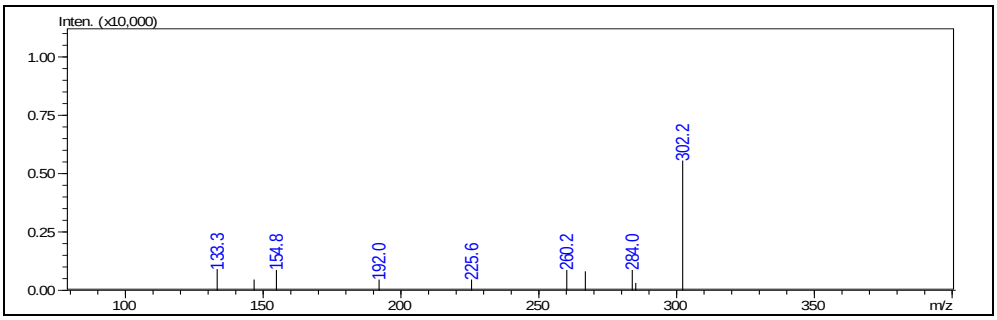
(a) etodolac



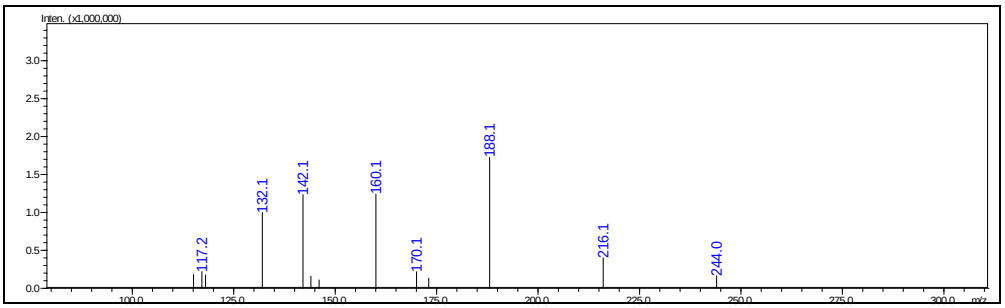
(b) m/z 244



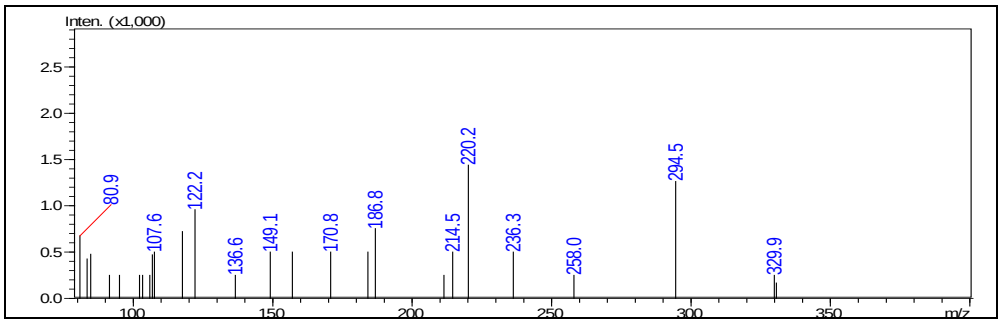
(c) m/z 290



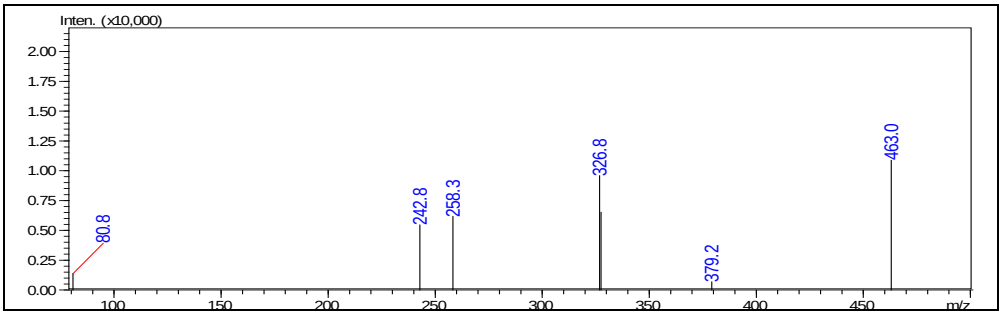
(d) m/z 302



(e) m/z 304



(f) m/z 330



(g) m/z 463

Fig. 3: (a–g): Product ion scans of etodolac and its metabolites

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

Metabolites	Precursor ions (m/z)	Collision energy used	product ion scan obtained (m/z)
M1	244	-30 V	84, 100, 103, 115, 143, 162, 182
M2	290		85,100, 113, 137,159, 208, 247, 249, 273
M3	302		133, 155, 192, 226, 260, 284
M4	304		132, 142, 160, 170, 188, 216, 244
M5	330		81, 108, 122, 137,149, 171,187, 215,220, 258, 295
M6	463		81, 243, 258, 327

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

Masses of the metabolites (m/z)	Mass difference w. r. t. t drug molecule (Δ m)	Possible reactions
244	-44	Decarboxylation (loss of CO_2)
290	+2	Hydrogenation (addition of two H)
302	+14	Methylation (loss of H, addition of CH_3)
304	+16	Oxidation (addition of O)
330	+42	N-acetylation (loss of H, addition of CH_3CO)
463	+175	Glucuronide conjugation (loss of H and addition of glucoronic acid)

m/z: mass/charge value; Δ m: mass difference; mass of Etodolac = m/z 288

Six 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 were found to be 244, 290, 302, 304, 330 and 463.

The possible biotransformations were predicted based on the mass difference of the metabolites with respect to the parent drug and the acquired product ion scan scan. There was a mass difference of 44 units for the metabolite-M1 with mass 244 ($C_{16}H_{21}NO$). The nearest possible reaction therefore suggested was decarboxylation (loss of CO_2 moiety). M1 produced the following ions with mass (m/z) as: 182 ($C_{13}H_{12}N^{\cdot}$), 162 ($C_{11}H_{15}N$), 143 ($C_{10}H_9N^{\cdot\cdot}$), 115 ($C_8H_5N^{\cdot\cdot}$), 103 ($C_6H_{14}O$), 100 ($C_6H_{12}O^{\cdot\cdot}$), 84 (C_5H_8O). From these, fragments with mass (m/z) 84,115 and 143 were same to that of ETD. Likewise, other chemical changes were also predicted; a mass gain of 2 unit for M2 (m/z 290-$C_{17}H_{23}NO_3$) was described to be hydrogenation. M2 gave the following fragments with mass (m/z) as: 273 ($C_{17}H_{22}NO_2^{\cdot}$), 249 ($C_{15}H_{23}NO_2$), 247 ($C_{15}H_{21}NO_2$), 208 ($C_{13}H_{21}NO$), 159 ($C_{11}H_{13}N$), 137 ($C_9H_{15}N$), 113 ($C_7H_{15}N$), 100 ($C_6H_{12}O^{\cdot\cdot}$), 85 (C_5H_8O). From these, fragment with mass (m/z) 85 was same as that of the parent drug, while fragments with mass 100 and 137 were same as that of M1 and M5 respectively. Also, fragment m/z 159 could be said to be similar to the fragment m/z 157 present in ETD on the basis of 2 unit mass gain of M2 (hydrogenation), the same being reflected in the fragment m/z 159. A mass gain of 14 units for M3 (m/z 302-$C_{18}H_{23}NO_3$) was explained as methylation. M3 fragmented into ions with mass (m/z): 284 ($C_{18}H_{21}NO_2$), 260 ($C_{16}H_{21}NO_2$), 226 ($C_{15}H_{15}N$), 192 ($C_{12}H_{17}NO$), 155 ($C_{10}H_{19}N$), 133 ($C_9H_{11}N$). From these, fragments with mass (m/z) 155 and 260 bear a close similarity with fragments of the parent drug; while fragment m/z 133 was same as that of M4. A mass gain of 16 unit for M4 (m/z 304-$C_{17}H_{21}NO_4$) was proposed to be oxidation. M4 produced the following ions with mass (m/z): 244 ($C_{15}H_{18}NO_2^{\cdot}$), 216 ($C_{13}H_{14}NO_2^{\cdot}$), 188 ($C_{11}H_{10}NO_2^{\cdot}$), 170 ($C_{11}H_8NO^{\cdot}$), 160 ($C_{10}H_9NO$), 142 ($C_{10}H_8N^{\cdot}$), 132 (C_9H_9N). From these, fragments with mass (m/z) 244, 216 and 188 showed the corresponding16 unit mass gain when compared with the fragments of the parent drug.

A mass gain of 42 unit for M5 (m/z 330-$C_{19}H_{23}NO_4$) was explained as N-acetylation. The fragments (m/z) obtained for M5 were: 295 ($C_{19}H_{21}NO_2$), 258 ($C_{16}H_{19}NO_2$), 220 ($C_{14}H_{21}NO$), 215 ($C_{14}H_{17}NO$), 187 ($C_{12}H_{13}NO$), 171 ($C_{12}H_{13}N$), 149 ($C_{10}H_5N$), 137 ($C_9H_{15}N$), 122 ($C_8H_{11}N$), 108 (C_8H_{12}), 81 (C_6H_8). The fragment with mass m/z 258 was same as that of ETD while fragments with mass (m/z) 81, 187, 171 and 187 were found to be similar with the other metabolites. M6 with a mass gain of 175 units was explicated as the glucuronide of dehydrogenated ETD (M6-m/z 463). M6 ($C_{23}H_{29}NO_9$) fragmented into ions with mass (m/z) as: 327($C_{19}H_{21}NO_4$), 258 ($C_{15}H_{15}NO_3$), 243 ($C_{15}H_{17}NO_2$), 81 (C_6H_8). The fragment with m/z 258 was same as that of ETD.

These metabolites were so far not reported in the literature. The previous studies were only successful in finding the mono-hydroxylated metabolites of the drug and their acyl-glucuronides [3, 19-23]. These were assumed to be ETD metabolites on comparison with the blank (incubation of the cell lines and the chicken liver tissue without ETD) and also on the basis of the justification given above for the fragmentation pattern of the metabolites.

It was also found that two of the metabolites, with masses-m/z 244 and m/z 304 were reported as the degradation products of ETD in one of the study [26].

Based on all the above information, the tentative structures of the investigated metabolites were postulated (fig. 4) and a tentative scheme of biotransformation of ETD in the two studied *in vitro* models was presented in fig. 5.

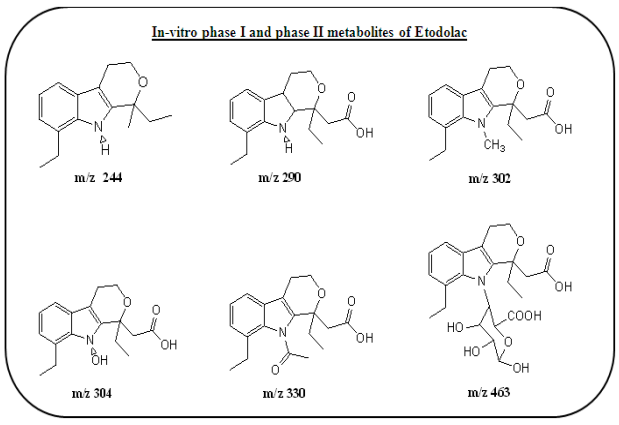


Fig. 4: Tentative postulated structures of the *in vitro* phase I and phase II metabolites of etodolac

Besides characterizing the metabolites of ETD, 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 the same metabolites were formed in both the *in vitro* test models, it could be indicated that the drug underwent a similar kind of 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 ETD.

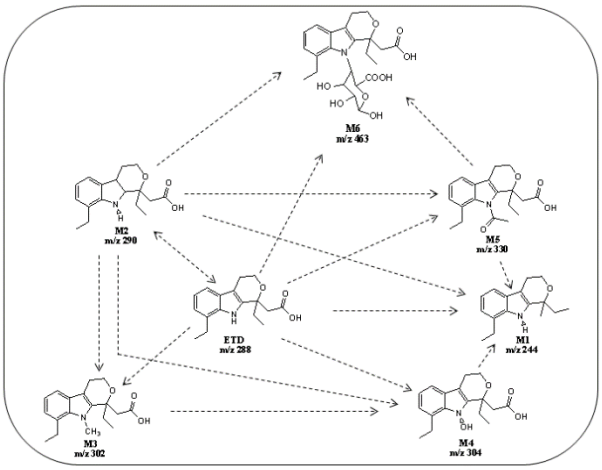


Fig. 5: Tentative scheme of biotransformation of etodolac

CONCLUSION

Thus, in the proposed study, six phase I and phase II metabolites of ETD 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 in producing the metabolites and produced the same metabolites. The masses ($[M+H]^+$) of these metabolites were found to be-m/z 244, m/z 290, m/z 302, m/z 304, m/z 330 and m/z 463. The main chemical changes observed in the drug molecule during the pr°Cess of metabolism included oxidation, N-acetylation, hydrogenation, decarboxylation, methylation and glucuronide conjugation. Production scans were acquired and interpreted for the detected metabolites. Based on the interpretations and the remaining information, the tentative structures of the investigated metabolites and a biotrans-formation scheme of ETD in the two studied *in vitro* models were postulated.

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CONFLICT OF INTERESTS

Declare none

REFERENCES

1. Patel A, Shah B. RP-HPLC method development and validation using the factorial design for simultaneous estimation of thi°Colchicoside and etodolac with forced degradation studies. J Pharma Sci Biosci Res 2014;4:374-82.
2. Pandey R, Patil PO, Bari SB, Dhumal DM. Simultaneous estimation of etodolac and thi°Colchicoside in bulk and in tablet formulation by UV spectrophotometry. Chem Ind Chem Eng Q 2014;20:9-17.
3. Dirk BS, Gottfried B. Isolation of an unknown metabolite of the non-steroidal anti-inflammatory drug Etodolac and its identification as 5-hydroxy Etodolac. J Pharm Biomed Anal 2001;25:977-84.
4. Devarakonda RK, Ulrich K. Extrahepatic metabolism of drugs in humans. Clin Pharmacokinet 1994;26:144-60.
5. Schonborn LJ. The role of the liver in drug metabolism. Anaesthesia Tutorail Week 2010;179:1-6.
6. Prakash C, Shaffer CL, Nedderman A. Analytical strategies for identifying drug metabolites. Mass Spectrom Rev 2007;26:340-69.
7. Brandon EF, Raap CD, Meijeram I, Benjnen JH, Schellens JH. An update on *in vitro* test methods in human hepatic drug biotransformation research: pros and cons. Toxicol Appl Pharmacol 2003;189:233-46.
8. Fasinu P, Bouic PJ, Rosenkranz B. Liver based *in vitro* technologies for drug biotransformation studies-a review. Curr Drug Metab 2012;13:215-24.
9. Becker SU, Blaschke G. Evaluation of the stereoselective metabolism of the chiral analgesic drug Etodolac by high-performance liquid chromatography. J Chromatogr 1993;621:199-207.
10. Jamali F, Mehvar R, Lemko C, Eradiri O. Application of a stereospecific high-performance liquid chromatography assay to a pharmacokinetic study of Etodolac enantiomers in humans. J Pharm Sci 1988;77:693-6.
11. Wright MR, Jamali F. Limited extent of stere°Chemical conversion of chiral non-steroidal anti-inflammatory drugs induced by derivatization methods employing ethyl chloroformate. J Chromatogr 1993;616:59-65.
12. Turcant A, Premel-Cabic A, Cailleux A, Allian P. Toxicological screening of drugs by microbore high-performance liquid chromatography with photodiode-array detection and ultraviolet spectral library searches. Clin Chem 1991;37:1210-5.
13. Lapicque F, Netter P, Bannwarth B, Trechot P, Gillet P, Lambert H, *et al.* Identification and simultaneous determination of non-steroidal anti-inflammatory drugs using high-performance liquid chromatography. J Chromatogr Biomed Appl 1989;496:301-20.
14. Pirkle WH, Murray PG. The separation of the enantiomers of a variety of non-steroidal anti-inflammatory drugs (NSAIDs) as their anilide derivatives using a chiral stationary phase. J Liq Chromatogr 1990;13:2123-34.
15. Singh NN, Jamali F, Pasutto AS, Coutts RT, Russel AS. Stereoselective gas chromatographic analysis of Etodolac enantiomers in human plasma and urine. J Chromatogr 1988;382:331-7.
16. Schmutz A, Thomann W. Assessment of the impact of physic°Chemical drug properties on monitoring drug levels by micellar electrokinetic capillary chromatography with direct serum injection. Electrophoresis 1994;15:1295-303.
17. Mayer S, Schurg V. Enantiomeric separation by electro chromatography in open tubular columns coated with Chiral-DEX. J Liq Chromatogr 1993;16:915-31.
18. Giachetti C, Assandri A, Zanolo G, Brembila E. Gas chromatography-mass spectrometry determination of Etodolac in human plasma following single epicutaneous administration. J Chromatogr Biomed Appl 1994;8:180-3.
19. Dirk BS, Chankvetadze B, Blaschke G, Claudia D, Salvatore F. Separation and identification of etodolac and its urinary phase I metabolites using capillary electro chromatography and on-line capillary electro chromatography–electrospray ionization mass spectrometry coupling. J Chromatogr A 2000;887:393-407.
20. Mohammed R, Abyazani K, Esaw B. Etodolac in equine urine and serum: Determination by high-performance liquid chromatography with ultraviolet detection, confirmation, and metabolite identification by atmospheric pressure ionization mass spectrometry. J Anal Toxicol 1999;23:200-9.
21. Ferdinandi ES, Sehgal SN, Demerson CA, Dubuc J, Zilbert J, Dvornik D, *et al.* Disposition and biotransformation of 14C-etodolac in man. Xenobiotica 1986;16:153-66.
22. Berendes U, Blaschke G. Simultaneous determination of the phase II metabolites of the non-steroidal anti-inflammatory drug Etodolac in human urine. Enantiomer 1996;1:415-22.
23. Ferdinandi ES, C°Chran D, Gedamke R. Identification of the Eodolac metabolite, 4-ureidoetodolac, in mouse, rat, dog, and man. Drug Metab Dispos 1987;15:921-4.

24. Suchanova B, Kostiainen R, Ketola R. Characterization of the *in vitro* metabolic profile of amlodipine in the rat using liquid chromatography-mass spectrometry. J Pharm Sci 2008;33:91-9.
25. Suchanova B, Sispera L, Wsol V. Liquid chromatography-tandem mass spectrometry in the chiral study of Amlodipine biotransformation in rat hepat°Cytes. Anal Chim Acta 2006;573:273-83.
26. Saxena D, Damale S, Datar A, Bhandarkar D, Raju S, Rane S, *et al.* Characterization of products formed by forced degradation of Etodolac by LC-MS/MS. Presented ASMS 2015;15:1-7.