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Study of metabolism of diclofenac-using microbial cultures

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ABSTRACT

To develop a microbial model for study of metabolism of diclofenac for further pharmacological and toxicological studies and to produce the metabolites which are difficult for chemical synthesis due to many steps and requirement of regio , stereo specificity. The metabolites of diclofenac in microbial cultures were identified and confirmed using fermentation techniques, HPLC and LCMS methods. Among different organisms screened *Pseudomonas aeruginosa* and *Cunninghmella elegans* showed extra peaks at 5, 8.6 and 8.5m. Respectively in HPLC indicating formation of metabolite by these two micro organisms. The metabolites were characterized by Liquid Chromatography Mass Spectrometry and were found as methoxy, hydroxy diclofenac formed by *Pseudomonas aeruginosa* and as dihydroxy diclofenac formed by *Cunninghmella elegans* which were similar to mammalian CYP 2C9 mediated hydroxy diclofenac and other minor mammalian metabolite methoxy hydroxy diclofenac. Thus, the above two microbes were able to metabolize diclofenac as mammalian CYP 2C9 mediated oxidation . The present study demonstrates a microbial model to produce CYP 2C9 mediated metabolites of diclofenac using simple, easy fermentation technique.

KEYWORDS

CYP 2C9;
Diclofenac;
Hydroxy diclofenac;
Methoxy diclofenac;
Microbial model;
Oxidation.

INTRODUCTION

The approval and usage of drug in human subjects require extensive studies to establish its efficacy and safety. An important factor in the evaluation of safety and efficacy of any drug is the knowledge about drug metabolism. Traditionally, drug metabolism studies were conducted on small animal models, perfused organs[1,2] *in vitro* enzyme systems and *in vitro* cell cultures. Later microbial models were developed as an alternative method to study the metabolic fate of the drug with advantage of reducing the use of animals in the early phases of drug development. Microorganisms such as bacteria and fungi have been used as *in vitro* models for the prediction of mammalian drug metabolism with successful applications[3,4,5]. Now a days microbial biotransformation systems are being used to complement mammalian drug metabolism studies[1,6].

Microbial transformation studies of drugs, mimic the corresponding metabolism in animal system, and provide technical methods for developing microbial models[7].

Emphasis was stressed on the potential for selected microorganisms to mimic the patterns of mammalian

biotransformation as, the use of microorganisms as models of mammalian metabolism has been well documented[8,9,10,11].

CYP450 plays a key role in the metabolism of xenobiotics in mammals. To enlighten the importance of CYP450 and its isoforms, significant progress has been made in the use of *in vitro* methods to characterize the pharmacokinetics of potential drugs in man. This work requires selective substrates, for each isoenzyme of the P-450 system, to allow detailed metabolism experiments[12] by using microbes.

Thus, diclofenac, an anti-inflammatory analgesic and is a substrate for CYP 2C9 isoenzyme is selected for the present metabolism experiments using microbes. Diclofenac is an *in vitro* specific CYP 2C9 substrate [13,14]. CYP 2C9 catalyses the oxidation of diclofenac to 4'-hydroxy diclofenac and the minor metabolites 3'-hydroxy diclofenac and 3'-hydroxy,4'-methoxy diclofenac[15,16,17]. These are further conjugated as glucuro conjugate and sulphate conjugate[18.19]. Therefore, the present study was aimed at developing a microbial model to use as tool for producing CYP 2C9 mediated mammalian metabolites of diclofenac and other drugs which are difficult for chemical synthesis due to many steps and requirement of regio, stereo specificity.

EXPERIMENTAL

Microorganisms

Cultures were obtained from National Chemical Laboratories, Pune, India. The cultures used in the present work were, *Proteus vulgaris* (NCIM 2027), *Pseudomonas aeruginosa* (NCIM 2053), *Nocardia hydrocarbonoxydans* (NCIM 2386), *Cunninghamella elegans* (NCIM 689) and *Saccharomyces cerevisiae* (NCIM 3090).

Chemicals

Diclofenac sodium was obtained from Sigma, Mumbai, India. Dilute hydrochloric acid, dichloro methane, glacial acetic acid were purchased from S.D. fine chem. Ltd., Mumbai, India. All the reagents used in the analysis were of HPLC grade. Acetonitrile was obtained from Spectrochem Pvt., Ltd., Mumbai, India. Culture media components were purchased from S.D. fine chem. Ltd., Mumbai, India.

Fermentation procedure

The experiments were carried out using respective growth media for different microorganisms consisting of the following composition:For bacteria:Peptone 1g, sodium chloride 0.5g, beef extract 1 g, distilled water 100 ml and pH adjusted to 7.0-7.2.

For fungi

Potato extract, dextrose 2 g, yeast extract 0.3 g, peptone 0.5 g, distilled water 100 ml. For yeasts : Malt extract 0.3 g, glucose 1 g, yeast extract 0.3 g, peptone 0.5 g, distilled water 100 ml. pH adjusted to 6.4-6.8. Stock cultures were stored at 4°C on agar slants, and transferred for every 2 months to maintain viability. The media were sterilized in an autoclave for 20 m. at 121°C and 15 lb/sq.in. Microbial metabolism studies were carried out by shake flask cultures in an incubator shaker, operating at 120 rpm at 32°C.

The experiments were carried out in culture flasks (250 ml) each containing 50ml. growth medium. Fermentations were carried out according to standard protocol[21]. In brief, the substrate (diclofenac sodium) was prepared as a 1% (w/v) solution in sterile water and added to the culture medium of selected organisms at a concentration of 10 µg/ml of medium in samples and incubated on shaker. The study also maintained the substrate control to which diclofenac sodium was added and incubated without microorganisms and culture controls consisted of fermentation blanks in which the microorganisms were grown under identical conditions without the substrate. The incubation was continued for 24-48 h.

Extraction procedure

The pre incubated medium was heated on water bath at 50°C for 30 min. and centrifuged at 3000 rpm for 10 m. at 37°C (Remi instruments Pvt. Ltd., Mumbai, India).

A clear supernatant liquid was collected and 4ml. of supernatant with 4ml. of dilute hydrochloric acid was vortexed for 5m.. Then, diclofenac and its metabolites were extracted by vortexing with 6ml. of dichloromethane for 5m. The organic layer was separated and dried in vacuum. Later it was reconstituted with 0.5 ml methanol (HPLC grade, Ranbaxy Fine Chemical Ltd., Delhi, India) and centrifuged at 13000 rpm/ 8 min. at 37°C in

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Biofuge fresco centrifuge (Hercaus, Germany). 20 µl portions were injected into HPLC for analysis. Controls (substrate and culture controls) were also prepared similarly to provide suitable blanks.

TABLE 1 :HPLC data for diclofenac and its metabolite from microbial culture extracts

Organism	Retention time in minutes			
	(Blank I) Drug control	(Blank II) Culture control	(Control) Pure diclofenac	Sample
	2.3	2.3	2.3	2.3
Proteus vulgaris	3.5	3.5	-	3.5
	14.6	-	14.5	14.6
	2.3	2.3	2.3	3.3
Pseudomonas aeruginosa	-	-	-	5*(MI)
	-	-	-	8.6*(MIII)
	14.5	-	14.5	14.5
Nocardia hydrocarbonoxydans	2.3	2.3	2.3	2.3
	14.5	-	14.5	14.5
Cunninghamella elegans	2.3	2.3	2.3	2.3
	-	-	-	8.5*(MII)
	14.5	-	14.5	14.5
Saccharomyces cerevisiae	2.1	2.1	2.3	2.1
	2.6	2.6	-	2.6
	14.5	-	14.5	14.5

***Metabolite peak, MI-Metabolite I, MII-Metabolite II, MIII-Metabolite III**

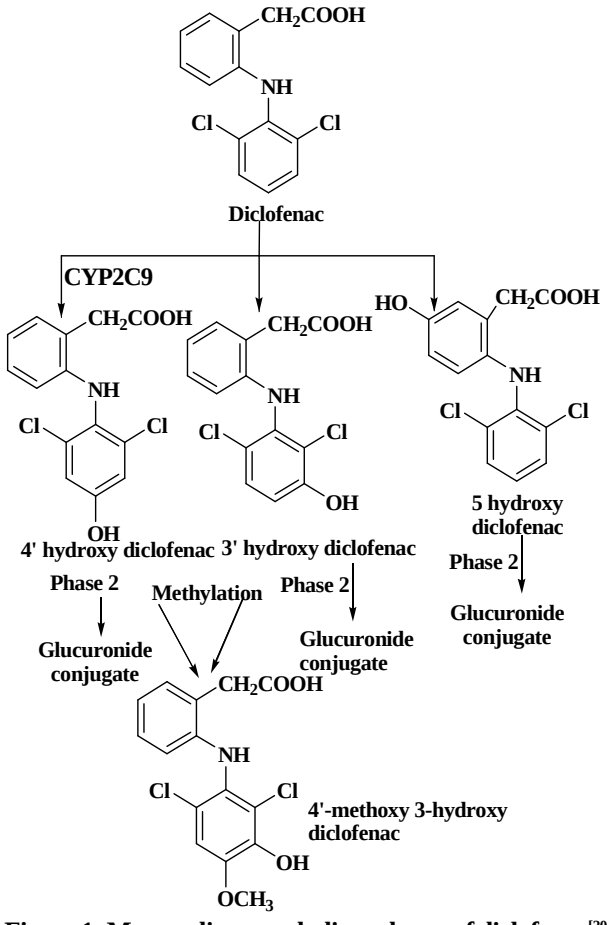

Figure 1: Mammalian metabolic pathway of diclofenac[20]

Analytical techniques

Chromatographic procedure

High performance liquid chromatography (HPLC) analysis was carried out using a HPLC system (Shimadzu, Kyoto, Japan) consisted of LC-10A solvent delivery module and SPD-10AVP UV-visible spectrophotometer detector and a Wakosil II5C-18RS-100a. 5UM, 4.6 250 MM SS column (SGE Japan). Sensitivity was set at 0.001 aufs. Mobile phase consisted of acetonitrile and 0.5% v/v glacial acetic acid at ratio of 65:35 with a flow rate of 1 ml/m.[22]. Elution was monitored using a UV/Vis detector set at 280 nm.

Mass spectrometry

Mass spectral data were obtained using Liquid Chromatography Mass Spectrometry (Agilent technologies, Germany). Model is LC/MSD-trap SL-1100 series, LC coupled to a mass spectrometer operating in the electron spray ionization (ESI) mode. Ionization was carried out in positive ion mode using Ion trap detector (5.5 KV, 300°C, 40 psi).

RESULTS

The results of HPLC analysis of diclofenac and its metabolite in different extracts are given in TABLE 1. Two to three peaks were seen in all extracts with retention times of 2.3m., 2.6m., 3.3m. and 14.5m.. It was found that the peaks of first three represent broth constituents where as the peak at 14.5m. corresponds to diclofenac. The additional peaks at 5.m.(M I), 8.6m.(M III) and 8.5m.(M II) were observed in samples from *Pseudomonas aeruginosa* and *Cunninghmella*

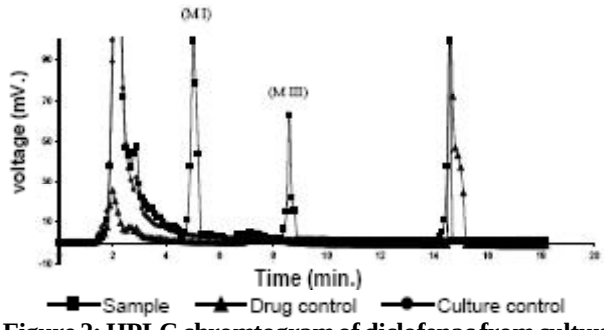

Figure 2: HPLC chromtogram of diclofenac from culture extracts of *Pseudomonas aeruginosa*

elegans cultures respectively (TABLE 1, figures 2 and 3). It indicates diclofenac has been metabolized by two organisms. The structures of observed metabolites were confirmed by LCMS as shown in figures 4, 5 and 6.

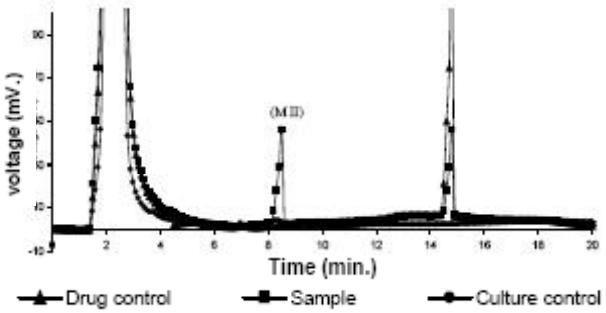

Figure 3: HPLC chromtogram of diclofenac from culture extracts of *Cunninghamella elegans*

The mass spectrum of diclofenac showed a molecular ion peak at m/z 295 at retention time of 14.3 with the fragment ion peaks at m/z 240, 220 and 100 (figure 4).

The mass spectrum of diclofenac metabolite formed by *Pseudomonas aeruginosa* revealed a molecular ion peak at m/z 314 (M I) between retention time of 4.5 to 4.9 and at m/z 340 (M III) between retention time of 8.2 to 8.8 with fragment ion peaks at m/z 329,240 (figure 5). The mass spectrum of diclofenac metabolite

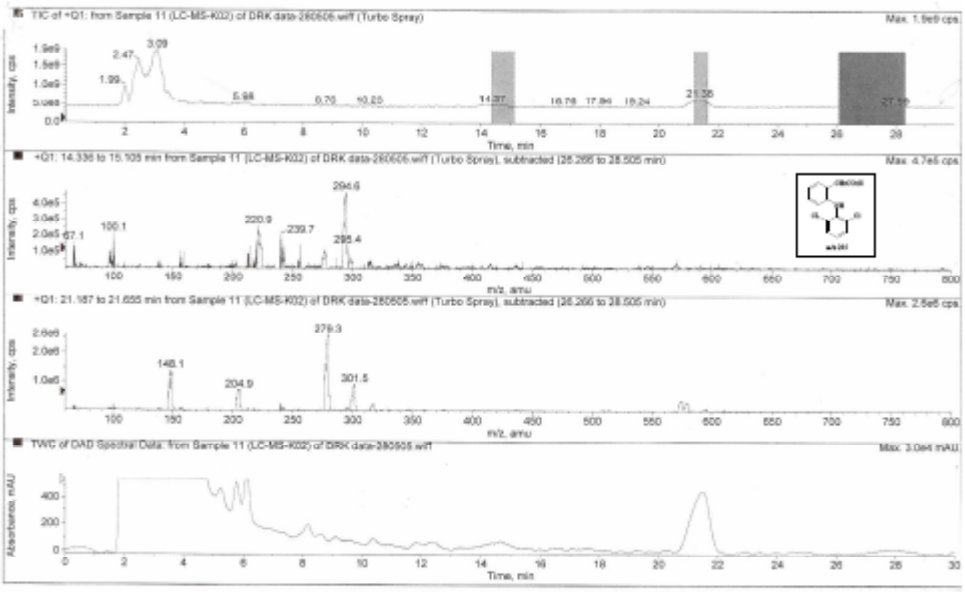

Figure 4: Mass spectrum of diclofenac

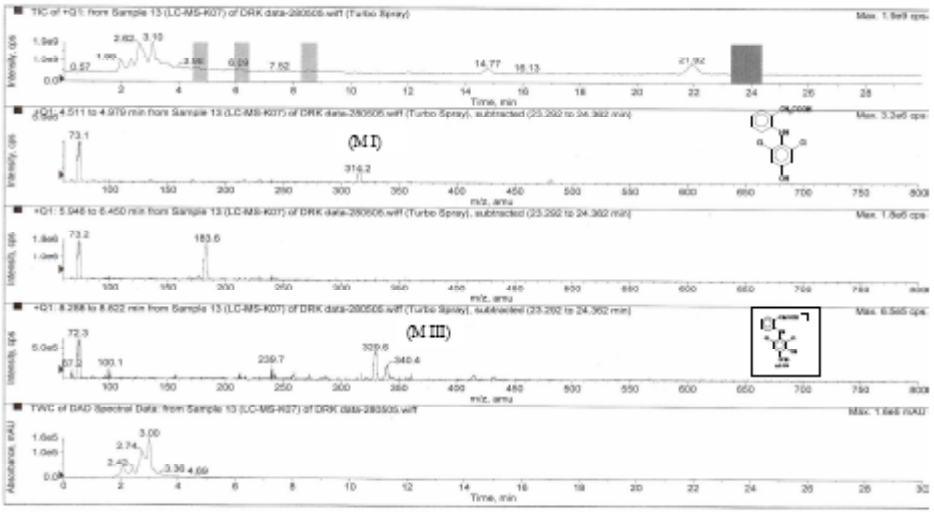

Figure 5: Mass spectrum of diclofenac metabolite produced by *Pseudomona aeruginosa*

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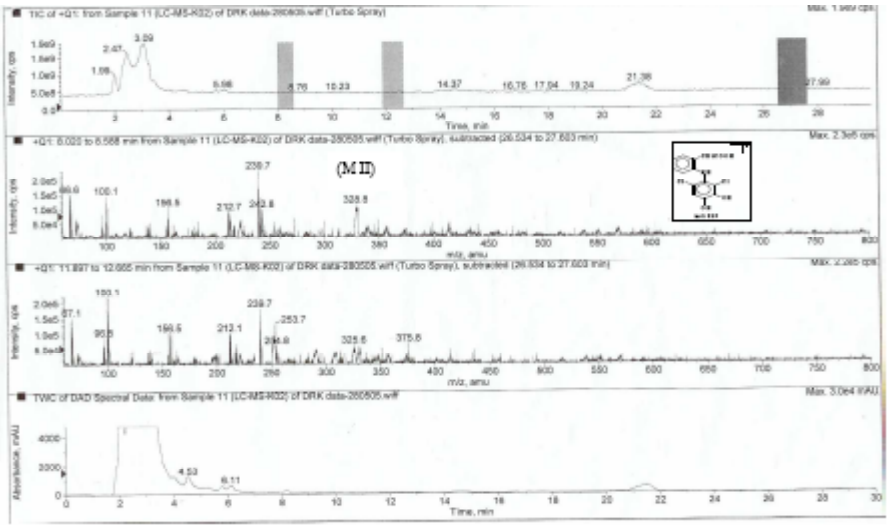

Figure 6:Mass spectrum of diclofenac metabolite produced by *Cunninghamella elegans*

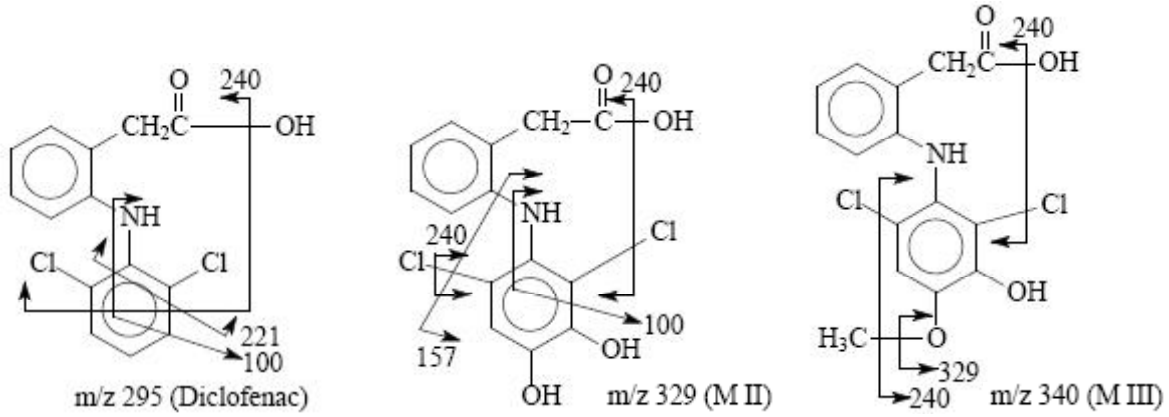

Figure 7: Mass fragmentation patterns

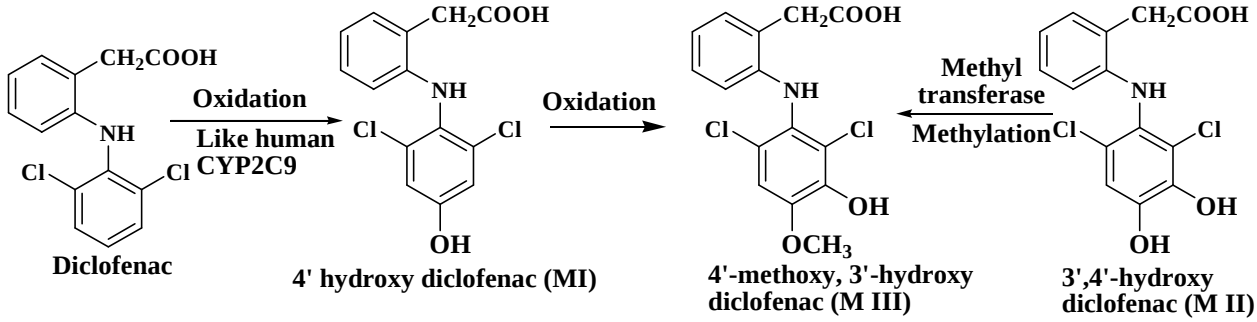

Figure 8: Proposed metabolic pathway of diclofenac by *Pseudomonas aeruginosa*

formed by *Cunninghmella elegans* exhibited a molecular ion peak at m/z 329 (M II) at retention time of 8 to 8.5m. with fragment ion peaks at m/z 240,100 (figure 6). The fragmentation pattern of diclofenac and its microbial metabolites were showed in figure 7. The metabolic pathways of diclofenac in above two microbes are proposed in figures 8 and 9.

DISCUSSION

HPLC chromatograms of samples of *Ps. aerugenosa* and *Cunn.elegans* showed additional peaks when compared with controls which represented diclofenac was metabolized to the metabolites M I, M II and M III (TABLE 1, figures 2 and 3). In case of *Noc.hydrocarbo*

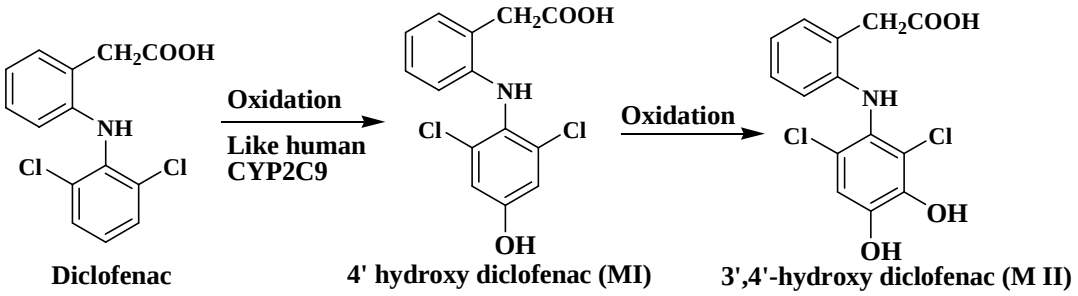

Figure 9 : Proposed metabolic pathway of diclofenac by *Cunninghamella elegans*

noxydans, Pr.vulgaris and *Sacc. cerevisiae* the peaks obtained with samples were identical with those of respective controls. The structures of M I, M II and M III were confirmed by LCMS.

The mass spectrum of diclofenac exhibited a molecular ion peak at m/z 295 at retention time of 14-15m.(Figure 4) which was supported by fragment ion peaks at m/z 240, 220 and 100 (Figure 7). The molecular ion at m/z 314 (M I) at retention time of 4.5-4.9m. and a molecular ion at m/z 340 (M III) at retention time of 8-8.8m.were observed in mass spectrum of diclofenac metabolite formed by *Ps. aerugenosa*. (Figure 5) .M I indicates 4'hydroxy diclofenac similar to major mammalian metabolite and M III represents formation of methoxy, hydroxy diclofenac as other minor mammalian metabolite[20] which is evident from fragment ion peaks at m/z 329 and 240 (Figure 7).

These fragment ions indicate dihydroxylation at two positions on aromatic ring of diclofenac similar to mammals as reported by Bort et. al.1999[15]. It was found that the conversion of diclofenac into a major metabolite 4'hydroxy diclofenac is mediated by hepatic CYP 450 2C9 and diclofenac is further metabolized to minor metabolites 3'hydroxy, 4'methoxy diclofenac in mammals[15].

The mass spectrum of diclofenac metabolite produced by *Cunninghamella elegans* showed a molecular ion peak at m/z 329 M II at retention time of 8-8.5m. (Figure 6) indicates the formation of dihydroxy diclofenac as CYP 2C9 catalysed mammalian metabolism of diclofenac[15]. It was supported by fragment ion peaks at m/z 240 and 100 (Figure 7). The fragment ion at m/z 240 represents formation of lactam of diclofenac which was also observed in diclofenac spectrum and in mass spectrum of M III metabolite formed by *Ps. aerugenosa* (Figure 7).

Based on above discussion the metabolic pathways of diclofenac in *Pseudomonas aeruginosa* and *Cunninghamella elegans* are proposed in figures 8 and 9. Thus, it is evident from our investigation that the metabolism of diclofenac by *Pseudomonas aeruginosa* is through oxidation to hydroxy diclofenac M I and to dihydroxy metabolite M II similar to CYP 2C9 mediated mammalian metabolism and further methylation of one of the hydroxyl groups by methyl transferase like in mammals. Where as *Cunninghamella elegans* metabolises diclofenac by oxidation to dihydroxy metabolite as CYP 2C9 mediated metabolism in mammals. It is suggestive of the presence of CYP 2C9 isoenzyme in these two organisms as both are metabolizing diclofenac similar to mammals[15] and other species like *Aspergillus*[23].

It is concluded that *Pseudomonas aeruginosa* and *Cunninghamella elegans* can be used as models for oxidative metabolism involving CYP 2C9 isoenzyme to simulate human metabolism.

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