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Significance of vitamins, substrate concentration and agitation on biotransformation of anti-inflamatory meloxicam by *Cunninghamella blakesleeana*

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ABSTRACT

Influence of vitamins, substrate concentration and shaking speed on biotransformation of meloxicam was studied by employing *Cunninghamella blakesleeana* NCIM 687 with an aim to achieve maximum transformation of meloxicam and in search of new metabolites. Based on HPLC, LC-MS/MS data and previous reports the metabolites were predicted as 5-hydroxymethyl meloxicam, 5-carboxy meloxicam and a novel metabolite. The quantification of metabolites was performed using HPLC peak areas. The results obtained indicate that folic acid as vitamin source, 0.01% concentration of meloxicam and 120rpm were found to be optimum for maximum transformation of meloxicam. The study suggests the significance of these factors in biotransformation of meloxicam using microbial cultures.
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KEYWORDS

5-hydroxymethyl meloxicam;
5-carboxymeloxicam;
LC-MS/MS;
HPLC;
Biotransformation;
Cunninghamella blakesleeana.

INTRODUCTION

Meloxicam is a non steroidal anti-inflammatory drug (NSAID) derived from enolic acid. Its mechanism of action may be attributed to inhibition of prostaglandin synthetase (cyclooxygenase). This leads to anti-inflammatory, analgesic, and antipyretic effects[1]. Meloxicam is more selective for the cyclooxygenase-2 (COX-2) isoform of prostaglandin synthetase than the COX-1 form. Its selectivity for COX-2 is dose dependent and is reduced at higher doses. Therefore meloxicam has been labeled a "preferential" inhibitor instead of a "selective" inhibitor of COX-2[2]. Meloxicam is indicated for the relief of signs and symptoms of osteoarthritis in adults. It has also been used to treat signs and symptoms of rheumatoid arthritis, ankylosing spondylitis[2], acute low back pain, and acute sciatica[3]. Gastrointestinal adverse effects are most commonly associated with meloxicam therapy (26.6%). These include abdominal pain (2.6%), constipation (1.2%), diarrhea (2.7%), dyspepsia (7.4%), flatulence (0.4%), nausea (4.7%), and vomiting (0.8%). Other adverse effects that occurred in more than 2% of patients included headache (8.3%), infection (4.7%), anemia (4.1%), dizziness (3.8%), edema (4.5%), rash (3.0%), coughing (2.4%), insomnia (3.6%), and pruritis (2.4%)[1]. Some of the rare adverse effects included leukopenia and gastrointestinal ulcers[3]. Meloxicam is practically insoluble in water. The poor solubility and wettability of meloxicam leads to poor dissolution and thereby variation in

bioavailability. Thus increasing the aqueous solubility and dissolution of meloxicam is of paramount therapeutic importance[4].

Microbial transformation is a process involving the use of biological agents as catalysts to perform transformations of chemical compounds and is useful technique for producing medicinal and agricultural chemicals from both active and inactive products. Microorganisms are able to perform a large variety of reactions, including those inaccessible by chemical processes. Biotransformation process involves enzymatic or microbial biocatalysts, when compared to their chemical counterparts, offer the advantages of high regioselectivity, stereo specificity and mild operating conditions.

In our previous work we reported the transformation of meloxicam by the filamentous fungus *Cunninghamella blakesleeana* NCIM 687 into three metabolites viz., 5-hydroxymethyl meloxicam, 5-carboxy meloxicam and a novel metabolite[5].

In the present study, effect of vitamins, shaking speed and concentration of meloxicam was studied for maximum transformation of meloxicam and to achieve novel metabolites.

MATERIALS AND METHODS

Chemicals and microorganism

Meloxicam was gifted by Unichem Laboratories Mumbai, India. Methanol and acetonitrile were of HPLC grade obtained from Ranbaxy, New Delhi, India. Peptone, yeast extract, potato dextrose agar, glucose and all other chemicals of highest available purity were obtained from Himedia, Mumbai, India. The culture *Cunninghamella blakesleeana* NCIM 687 was procured from National Collection of Industrial Microorganisms (NCIM), Pune, India. Stock cultures were maintained on potato dextrose agar slants at 4 °C and subcultured for every 3 months.

EXPERIMENTAL

Effect of shake speed on biotransformation

Biotransformation was performed using a two stage fermentation protocol. In the first stage, fermentation was initiated by inoculating a 250ml culture flask consists of 50ml of liquid broth. The liquid broth used contain (per litre) glucose (20g), peptone (5g), yeast extract (5g), K_2HPO_4 (5g) and sodium chloride (5g). The pH of the broth was adjusted to 6.0 with 0.1M HCl or 0.1M NaOH. The media was autoclaved and inoculated with a loopful of culture obtained from freshly grown potato dextrose agar slants. The flasks were incubated at 120rpm and 28°C for 48 hours. Second stage cultures were initiated in the same media using an inoculum of 1ml of first stage culture per 20ml of medium in 100ml culture flask. The second stage cultures were incubated for 24 hours at 28°C and the substrate albendazole in dimethyl formamide was added to get a final concentration of 100mg/L. The flasks were incubated under similar conditions for 5 days. The shake speed for the biotransformation was studied from 60rpm to 160rpm. Culture controls consisted of fermentation blank in which the microorganism was grown under identical conditions and no substrate was added. Substrate controls comprised of albendazole added to the sterile medium and incubated under similar conditions. Each culture was studied in triplicate.

The initial reaction rate was increased up to a maximum of 120rpm, which was then used for all subsequent work.

Biotransformation in presence of various vitamins

The effect of various vitamins was studied using the procedure similar to that described under 'Effect of shake speed on biotransformation. The biotransformation of meloxicam by *C. blakesleeana* NCIM 687 was performed by adding different vitamins viz. riboflavin, niacin, choline, folic acid, inositol, pantothenic acid, thiamine, pyridoxine and ascorbic acid in to the medium before inoculation of the organism to observe their influence on the extent of biotransformation. Each vitamin was studied at a concentration of 10μg per culture flask (20ml media).

Influence of substrate concentration

The effect of substrate concentration was studied using the procedure similar to that described under 'Effect of shake speed on biotransformation'. The biotransformation of meloxicam by *C. blakesleeana* NCIM 687 was performed at substrate concentrations, 0.01,

0.02, 0.03, 0.04 and 0.05 %w/v while maintaining the other parameters constant.

Extraction and analysis of transformed products

The cultures were extracted with three volumes of ethyl acetate, the combined organic extracts were evaporated using a rotary vacuum evaporator and dried over a bed of sodium sulfate. The resultant residues were analyzed by HPLC and LC- MS-MS for identification of metabolites.

HPLC analysis was performed according to the method described by Elbary *et al.*[6] with a slight modification. The samples were analysed using an LC-10AT system (Shimadzu, Japan) by injecting 20µl of sample into the syringe-loading sample injector (Model 7725i, Rheodyne, USA). The column used was Wakosil II, C18, 250×4.6 mm and 5µm (SGE, Australia). The mobile phase consisted of a mixture of methanol and water (pH adjusted to 3.0 with orthophosphoric acid) in the ratio of 60:40. The analysis was performed isocratically at a flow rate of 1ml/min and the analytes were detected at 360nm using a photodiode array detector (Model SPD M10Avp, Shimadzu, Japan). LC-MS-MS analysis was carried out using a Waters system, column X Terra C18, 25×0.46 cm, 5 µm and a mobile phase consisting of methanol and water (pH adjusted to 3.0 with formic acid) in 60:40 ratios. The ESI detection was set to positive mode. A temperature of 300°C and scan range of 50-500 was set for the analysis. The transformed compounds were identified from the masses of the fragmentation products obtained.

RESULTS AND DISCUSSION

In our previous work[5] we reported that the fungus *Cunninghamella blakesleeana* was found to transform meloxicam into three metabolites: 5′-hydroxy meloxicam (M_2), 5′-carboxy meloxicam (M_1) and a new metabolite (M_3). In the present investigation the fungus was found to produce similar metabolites in presence of all the parameters studied.

Identification of metabolites

The metabolites formed were identified basing on observation of new peaks in HPLC (Figure 1) and characterized with the help of the mass values of fragmentation ions obtained in LC-MS-MS analysis (Figure 2).

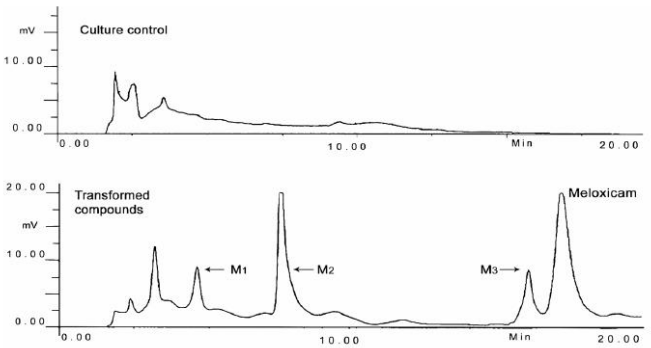

Figure 1 : HPLC chromatogram showing culture control and transformed compounds obtained in culture broth of *C. blakesleeana*

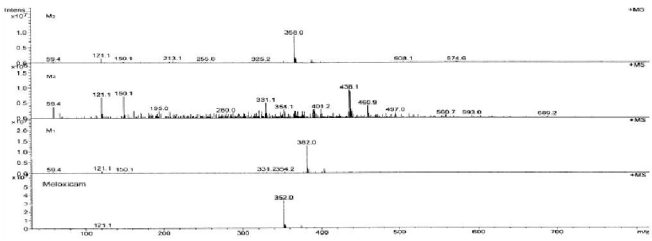

Figure 2 : LC-MS-MS spectra of metabolites detected in meloxicam fed culture broth of *C. blakesleeana*

Mass spectrometric analysis of the metabolite M_2 showed a molecular ion at m/z 367 (an increase of 16 units) indicating addition of single oxygen atom which results in formation of 5-hydroxymethyl meloxicam. Another molecular ion was found at m/z 381 (an increase of 14 units) indicating further addition of an oxygen atom and removal of two hydrogens to M_2 which results in the formation of 5-carboxy meloxicam (M_1). A third metabolite was observed with m/z of 437 indicating the addition of 86 units to meloxicam. This might be arising from oxamic acid analogue of meloxicam. These analyses indicated that the metabolites were 5-hydroxymethyl meloxicam (M_2, eluting at 7.0 min), 5-carboxy meloxicam (M_1, eluting at 5.0 min) and a new metabolite (M_3, eluting at 17 min), where the substrate meloxicam was eluted at 19.0 min. The proposed pathway of the metabolite formation is shown in Figure 3. The metabolites were quantified based on the peak areas obtained in HPLC analysis taking the drug and metabolites' peak areas together as 100%.

Influence of shaking on biotransformation

Influence of shaking on biotransformation of meloxicam was performed for maximum transformation and results are presented in TABLE 1.

From TABLE 1 it us clear that shaking was found

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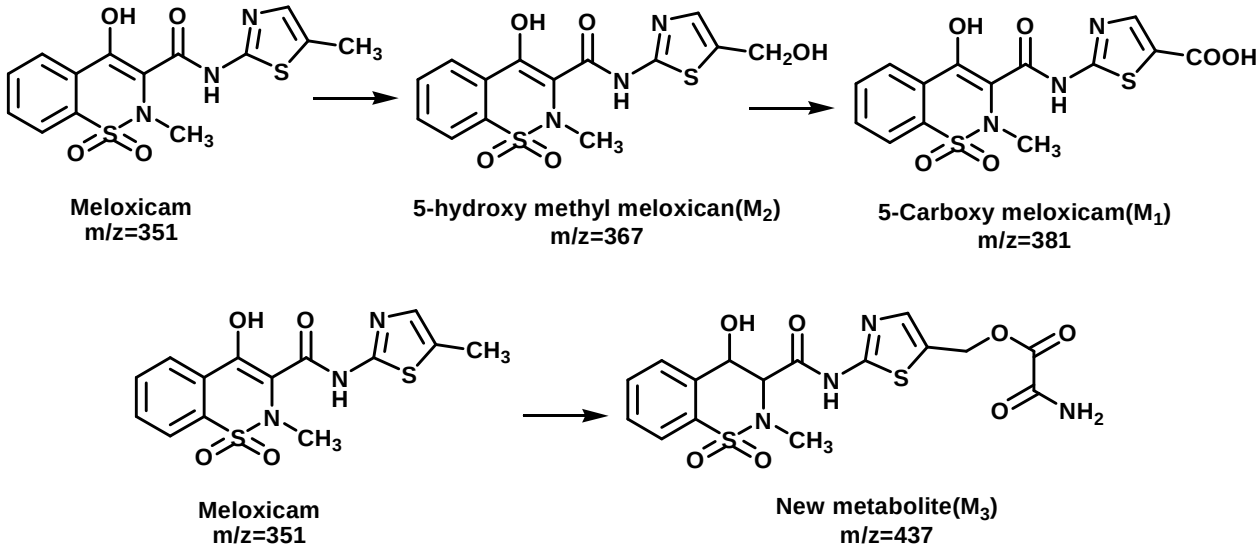

Figure 3 : Proposed pathway of meloxicam in culture broth of *C. blakesleeana*

TABLE 1 : Effect of shaking speed on biotransformation of meloxicam by *C.blakesleeana*

Agitation speed (RPM)	metabolites in %				Final pH		Dry weight mg/ml	
	M1	M2	M3	M	C	T	C	T
60	0.00	14.81	0.00	85.19	6.10	6.37	10.12	9.23
80	0.00	14.96	0.00	85.04	6.15	6.22	9.11	9.41
100	2.98	73.86	1.10	22.06	6.30	6.21	9.55	9.41
120	3.21	78.23	1.12	17.44	6.21	6.18	9.32	9.54
140	3.15	68.32	0.00	28.53	6.12	6.32	9.14	9.50
160	1.30	39.19	0.00	59.51	6.29	6.22	6.04	6.91

M1=5-carboxy meloxicam, M2=5-hydroxymethyl meloxicam, M3=New metabolite, M=Meloxicam
C=culture control, T=meloxicam fed culture

to have profound effect on biotransformation of meloxicam. Similarly, many researchers Liras and Umbreit[7], Fernandes *et al.*[8], Bastida *et al.*[9], Zhao *et al.*[10] reported influence of agitation on biotransformation reactions. At 60 and 80rpm the transformation of meloxicam was very less and there was no formation of metabolite M_3 and M_1. . This may be due to non availability of toxic levels meloxicam to the fungus. At 120rpm maximum transformation of meloxicam was observed which may be due to proper mixing and availability of nutrients, meloxicam and proper supply of oxygen to the fungus. As rpm increased beyond 120, the transformation was decreased. This may be due to non availability of the nutrients and the substrate meloxicam to the fungus to perform the transformation.

There was negligible influence of shaking speed on mycelial growth of the fungus.

TABLE 2 : Effect of vitamin source on transformation of meloxicam by *C.blakesleeana*

Vitamins	metabolites in %				Final pH		Dry weight mg/ml	
	M1	M2	M3	M	C	T	C	T
Ascorbic acid	0.00	83.96	0.00	16.03	6.10	6.47	10.17	9.26
Cholin	2.78	93.17	0.00	4.04	6.25	6.24	9.13	9.61
Folic acid	3.98	93.64	0.00	2.36	6.20	6.27	9.75	9.71
Inositol	3.21	81.22	0.11	15.44	6.21	6.15	9.52	9.84
Niacin	3.35	87.32	0.00	9.21	6.12	6.32	9.14	9.70
Pantothenic acid	2.30	84.19	0.00	13.49	6.29	6.22	6.07	9.99
Pyridoxine	0.00	73.61	0.00	26.38	6.20	6.39	6.56	9.31
Riboflavin	1.92	94.57	0.00	3.49	6.19	6.29	8.40	7.18
Thiamin	1.57	79.40	0.00	19.02	6.10	6.13	8.80	6.28

M1=5-carboxy meloxicam, M2=5-hydroxymethyl meloxicam, M3=New metabolite, M=Meloxicam
C=culture control, T=meloxicam fed culture

Influence of vitamins

Vitamins are organic compounds which are necessary for the growth of some organisms. They function as co-enzymes or constituent parts of co-enzymes which catalyze special reactions. Vitamins are necessary for proper utilization of carbohydrates, fats and proteins. The capacity of different organisms to synthesize the vitamins varies. Fungi with regard to their vitamin requirement occupy a position in between the totally independent green plants and completely dependent animals[11]. In the present investigation, the effect of vitamins on the biotransformation of meloxicam by *C. blakesleeana* was studied and the results obtained are presented in TABLE 2.

From TABLE 2 it is clear that addition of vitamins stimulated the transformation of meloxicam. Major amount of meloxicam was transformed in the medium containing riboflavin, choline, folic acid, pantothenic acid and inositol. Pyridoxine and ascorbic acid favoured the formation of 5′-OH methyl meloxicam only. Inositol induced the formation of 5′-OH methyl meloxicam (M_2), 5-carboxy meloxicam (M_1) and the new metabolite (M_3). The new metabolite (M_3) could not be detected in medium containing riboflavin, niacin, choline, folic acid, pantothenic acid, thiamine, pyridoxine and ascorbic acid.

Ascorbic acid and pyridoxine favoured the formation of 5-hydroxy methyl meloxicam only; this may be due to inhibition of enzyme system responsible for synthesis of other metabolites. Hence these two vitamins can be employed in production of selected metabolite in large quantities. Where as inositol induced the formation of all the three metabolites 5-OH methyl meloxicam (M_1), 5-carboxy meloxicam (M_2) and a new metabolite(M_3). Based on this result, inositol can be employed in drug metabolism studies by employing microorganisms. How ever further investigations are needed to find out the exact mechanism of action and influence of vitamins on biotransformation reactions.

Different vitamins induced varying amount of mycelial growth. Ascorbic acid was responsible for maximum stimulation of growth of *C. blakesleeana*. Pyridoxin and pantothenic acid were not much useful in production of mycelial growth of *C.blakesleeana*. The final pH recorded in different media was near neutral.

Influence of meloxicam concentration

Concentration of substrate was found to have profound influence on its biotransformation. Chatterjee and Bhattacharya[12] reported the influence of limonene concentration on its biotransformation by employing *Pseudomonas putida*. Similarly, Huang *et al.*[13] during their studies on biotransformation of tolbutamide to 4′-hydroxytolbutamide by the fungus *Cunninghamella blakesleana* found the influence of concentration of tolbutamide on its biotransformation. Sedarati *et al.*[14] also reported the effect of concentration of chlorophenols on its transformation by *Trametes versicolor*. Above reports prompted us to study the influence of concentration of meloxicam on its transformation by employing *C.blakesleeana* and the results are presented in TABLE 3.

TABLE 3 : Effect of concentration of meloxicam on its transformation by *C.blakesleeana*

Conc. of meloxicam (In %)	Metabolites in %				Final pH		Dry weight mg/20ml	
	M1	M2	M3	M	C	T	C	T
0.01	0.09	87.19	9.43	3.29	7.13	7.21	7.69	10.38
0.02	0.00	58.35	3.88	37.75	7.01	7.89	9.55	9.63
0.03	0.03	46.64	0.60	52.71	6.89	7.30	8.57	10.55
0.04	0.00	62.14	2.83	35.02	6.99	7.61	9.00	8.86
0.05	1.05	53.57	3.62	41.73	6.55	6.60	9.00	10.41
0.06	0.20	46.70	0.35	52.73	7.07	6.34	10.35	6.69

M1=5-carboxy meloxicam, M2=5-hydroxymethyl meloxicam, M3=New metabolite, M=Meloxicam
C=culture control, T=meloxicam fed culture

TABLE 3 reveals that transformation of meloxicam was maximum at 0.01% concentration and decreased with the increase of concentration. In contrast to our observation, Mountfield and Hopper[15] reported that 0.03% concentration of 1-methylnaphthalene was optimum for its transformation by *C. blakesleeana*. At concentrations of 0.02 and 0.04%, the compound 5′carboxy meloxicam (M_1) formation could not be observed. Similarly at 0.03% and 0.06% concentration the formation of the new metabolite (M_3) was minimum. 5'OH methyl meloxicam (M_2) formation was maximum at 0.01% concentration followed by 0.04% concentration. Rest of the concentrations of meloxicam (0.02%, 0.03, 0.05 and 0.06%) could show only marginal formation of 5′-OH methyl meloxicam. Such inconsistency in degree of transformation of meloxicam with substrate concentration needs to be investigated more intensively.

No consistency in biomass production by *C. blakesleeana* in relation to concentration of meloxicam was observed. With a few exceptions biomass production was more in meloxicam containing medium when compared to culture control. The pH was shifted towards alkaline side with the addition of meloxicam and the final pH recorded was near neutral.

CONCLUSION

The studies indicate that meloxicam could be transformed to 5-hydroxy methyl meloxicam, 5-carboxy meloxicam and a new metabolite using *C. blakesleeana*

in an ecofriendly way. The present investigation reveals the importance of agitation, vitamins and meloxicam concentration for optimum biotransformation of meloxicam. Among the factors studied, the use of suitable vitamins, agitation and meloxicam concentration were found to be critical for the development of a biotransformation system. Further investigations are needed to produce meloxicam metabolites in large quantities by optimizing the fermentation conditions.

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