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Research Article

A STUDY ON INFLUENCE OF REGULATING FACTORS ON FUNGAL BIOTRANSFORMATION OF LORATADINE

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ABSTRACT

Loratadine, is a non sedating anti histaminic drug used to treat chronic urticaria and allergic rhinitis. Loratadine is metabolised into an active metabolite desloratadine in mammals by CYP450 3A4 and CYP450 2D6 enzymes. Microorganisms have proficiency to imitate the biotransformation reactions that happen in mammals. In preliminary microbial screening process, out of six fungi, two *Cunnighmella* fungi and one *Aspergillus* fungus proved the ability to biotransform the loratadine. Then the study was conducted to find the effect of incubation period and substrate concentration on the capacity of fungal biotransformation of loratadine. Different concentrations of substrate i.e, 10 to 60 µg and different incubation periods 18 hrs, 36hrs, 48hrs, 56 hrs, 64 hrs and 72 hrs were selected for study, during incubation for estimation of extent of biotransformation of loratadine by selected three fungi along with their controls. Then these samples were extracted for loratadine metabolite using Diethyl ether: Dichloromethane (70:30) and analysed by HPLC to find the quantity of loratadine metabolite formed under different selected incubation periods and substrate concentrations. The percentage of metabolite formed was calculated using area of respective peaks found in the HPLC chromatograms. Percentage metabolite formed was increased with increase in the substrate concentration in case of *Cunnighmella elegans* and *Cunnighmella echinulata,* whereas, in case of *Aspergillus niger*, it was increased up to 40 µg of substrate concentraton after that a decrease in metabolite formation was found. Maximum amount of metabolite was formed at 64 hrs of incubation in case of all three fungi and further there was no change in the percentage metabolite formed with increase in the incubation time up to 72 hours. Hence, from the above results, it can be concluded that the substrate (loratadine) has shown substrate inhibition when incubated with *Aspergillus niger* during loratadine biotransformation, It was also found that the process of biotransformation was ceased after 64 hours of incubation period, might be due to exhaustion of nutrients available in the media for their growth and further biotransformation.

INTRODUCTION

Loratadine is a prototypical, competitive peripheral H1 histamine receptor antagonist. (Ahn hs and Barnett A. 1986*)* derived from azatadine and it is less basic and more polar than its parent compound (Dockhorn RJ and Schellenberger M.K. 1987; Villani FJ *et al*. 1986). Loratadine, a potent second generation anti histamine drug effectively used against allergic rhinitis and idiopathic chronic urticaria (Bousquet J 1987; Kaminszczik I. 1986). Metabolism of loratadine bring about, an active antihistamine, desloratadine, which is four times more potent and shows similarity with parent drug having clinical onset of action within one hour (Kreutner W. 1987; Batenhorst RL, *et al*. 1986). Desloratadine exists at low concentrations in the plasma as a result of metabolism to several hydroxylated metabolites (Katchen *et al*. 1985; Ramanathan *et al.* 2005; Katchen B *et al*. 1985). Human cytochrome P450 3A4 and 2D6 were considerable isoforms associated in desloratadine production. (Yumibe *et al*.1996; Barecki *et al*., 2001; Ghosal *et al*., 2003). Drug metabolism studies are essentially carried out to gain knowledge about its activity, toxicity, distribution, elimination, and residence in the body (Sepuri Asha and Maravajhala Vidyavathi 2009). CYP 450 enzymatic systems play a key role in human biotransformation of drugs. Microbial biotransformation acquired its importance in the area of research due to the comparable biotransformation capability of microbial enzymes with human CYP450 enzyme systems and it has more practical benefits than other metabolism studies. (R.K.Venisetty and V. Ciddi 2003). In the commenced study, six different species of fungi belong to two different genera were screened for their

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capability to convert loratadine. *Cunnighmella elegans*, *Cunnighmella echinulata* and *Aspergillus niger* were displayed their potential for biotransformation of loratadine to its metabolites in preliminary studies. The present paper intended to interpret the effect of incubation period and substrate concentration on the percentage of metabolite formation by three screened fungal cultures.

MATERIALS AND METHODS

Fungal Cultures

Aspergillus niger (NCIM-589), *Cunnighamella elegans* (NCIM-689), *Cunnighamella echinulata*(NCIM-687), were purchased from National chemical laboratory(NCL) Pune, India.

Chemicals

Loratadine was a gift sample obtained Shasun pharmaceuticals, Guindy, Chennai, Tamil Nadu, India. Remaining chemicals were acquired from S.D fine chemicals. Analytical grade HPLC solvents were used.

Fungal conversion studies

The fermentation was executed in 250ml erlenmeyer flasks accommodated with 50ml of potato dextrose broth as medium, which is common in all flasks. For each culture three flasks were specified and labelled as Blank-I, Blank-II and sample. Blank-I comprises only drug and incubated without fungus, Blank-II composed of respective fungi without drug and sample flask incorporated with both substrate (loratadine) and fungus. Incubation of three flasks for each culture was completed in an orbital shaker under identical conditions of 120 rpm and 32 º C temperature.(Moffat, A.C.1986).

Extraction

The incubated flasks were shifted to water bath to kill grown microbes after completion of selected incubation period. Flasks were heated at 50°C for 30 minutes to assassinate grown microbes. Further step proceeded with centrifugation of the contents of the flasks in centrifuge tubes at 3000 rpm for 10 min to collect the supernatant. (Laboratory Centrifuge C-854/8, Remi instruments, Mumbai, India). The collected supernatant was transferred to separate boiling tubes and added with similar proportion of dichloromethane and diethyl ether (30:70) mixture and mixed in cyclomixer. The organic layer was departed to screw cap bottles and kept for drying at room temperature. HPLC analysis was conducted after reconstitution of dried extract with mobile phase (acetonitrile:water: methanol (1:2:1) mixture) (Nagwa, A.S. *et al*., 2014).

Study of effect of Incubation period on metabolite formation

Selected fungi were used to discriminate the effect of incubation period on percentage metabolite formation. During incubation period, samples were aseptically withdrawn from flasks after 18,36,48,56,64, and 72 hrs. All samples were heated, centrifuged, extracted, dried and analysed in a similar manner, as discussed above.

Study of effect of substrate concentration on metabolite formation

The selected fungi were inoculated in sample flasks containing different substrate concentrations like 10µg, 20µg, 30 µg, 40 µg, 50 µg and 60 µg ofloratadine. After incubation of 72 hrs, flasks were heated, contents were centrifuged. Clear supernatant was used for extraction and dried at room temperature, dried extracts were reconstituted to perform HPLC analysis.

High performance liquid chromatography analysis:

Loratadine and formed metabolites were identified using a HPLC system (Shimadzu, Kyoto, Japan) consisted with LC 20 AD pump and its sensitivity was 0.0001aufs. Separation of drug and metabolites was done by phenomenex luna 5µ C18 (2) 100A 250 X 4.60mm (Phenomenex, USA). Mobile phase used was Acetonitrile: Water: Methanol (1:2:1) at a flow rate of 1ml/min (Gajjela Ramulu 2011), UV detection wavelength was set at 254 nm and run time was 20 min (Rajeev Soni and Gali Vidya Sagar 2013). The formation of metabolites was noticed by correlating the HPLC chromatograms attained with extracts collected from all control and sample flasks. Then, the quantity of the metabolite formed was calculated from the area of respective peaks found in HPLC chromatogram.

Calculation of percentage of metabolite

Percentage of metabolite formed for different incubation periods at different substrate concentrations was calculated from the area of metabolite peak in HPLC chromatogram in corresponding to the area of drug peak.

RESULTS

Preliminary screening studies of biotransformation of loratadine by different fungi revealed that, the metabolites of loratadine were found by three fungi, *Cunnighmella elegans, Cunnighmella echinulata and Aspergillus niger.* HPLC chromatograms of samples from the above three culture extracts shown an extra peak when compared with their respective controls as represented in figures 1-3.

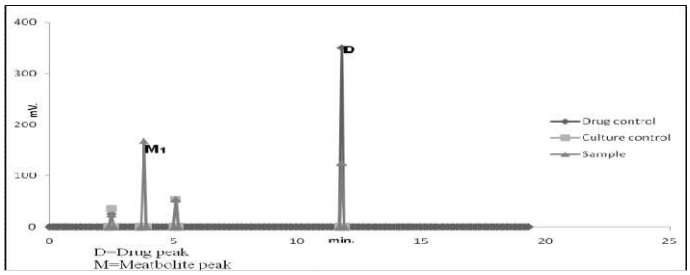

Figure 1 HPLC chromatogram representing the metabolite production by *Cunnighmella elegans*

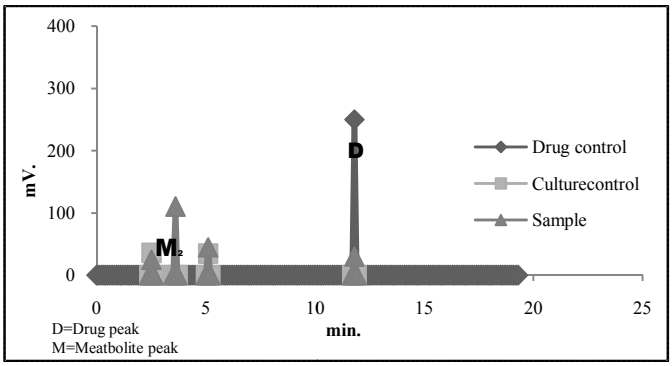

Figure 2 HPLC chromatogram representing the metabolite production by *Cunnighmella echinulata*

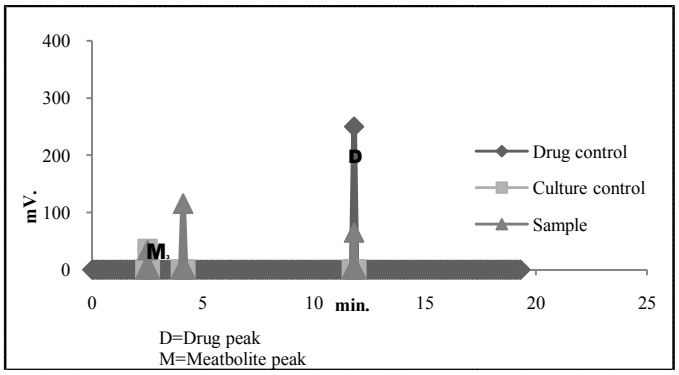

Figure 3 HPLC chromatogram representing the metabolite production by *Aspergillus niger*

In order to find out the influence of incubation time on metabolite production by three fungi, during incubation period of 72 hours, the samples were withdrawn aseptically at specified time intervals calculated the percentage metabolite formed as and results are shown in the table 1. Formation of metabolite was increased with increase in the incubation period up to 64 hours, further on incubation no increase in the metabolite formation was observed. Maximum metabolite production was observed at 64 hours of incubation and initiation of metabolite formation was observed only after 36 hours of incubation by all the three cultures.

Table 1 Percentage of loratadine metabolites formed at different incubation periods by three fungi.

Incubation time (hours)	Percentage of metabolite formed		
	Cunnighmella elegans M_1	*Cunnighmella echinulata* M_2	*Aspergillus niger* M_3
0	0	0	0
18	0	0	0
36	1.32	1.28	1.2
48	2.22	2.16	2.11
56	4.13	4.12	4
64	8.10	7.97	8.2
72	8.10	7.9	8.2

To gain the familiarity about effect of substrate concentration on production of metabolite, substrate (loratadine) concentration was varied from 10 to 60 µg/ml in this study. Area of metabolite peaks observed in HPLC chromatograms of different cultures at different substrate concentrations after incubation of 72 hours was represented in table 2 and figure 4

Table 2 Percentage of loratadine metabolite formed at different substrate concentrations by three fungi

Substrate concentration (µg/ml)	Percentage of metabolite formed		
	Cunnighmella elegans M_1	*Cunnighmella echinulata* M_2	*Aspergillus niger* M_3
10	7.8	7.44	7.45
20	9.73	9.58	10.5
30	10.9	10.4	11.5
40	13.5	12.8	17.7
50	15.6	14.4	16.7
60	16.8	15	14.5

In case of *Cunnighmella elegans* and *Cunnighmella echinulata* as the substrate concentration was increased from 10 µg/ml to 60 µg/ml metabolite production was also increased from 7.8 % to 16.8 % and 7.44% to 15%.

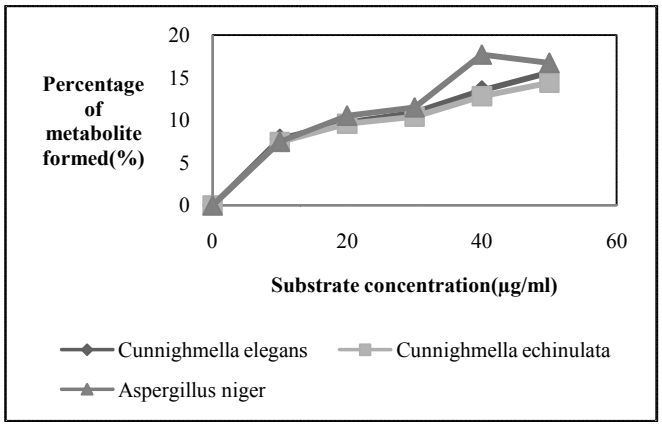

Figure no 4 Effect of substrate concentration on metabolite production by three fungi

Whereas in case of *Aspergillus niger* as the substrate concentration was increased from 10 µg/ml to 40 µg/ml, the metabolite production was also increased from 7.45% to 17.7 %, but further increase in the substrate concentration from 40 µg/ml to 60 µg/ml, there was a decrease in the metabolite production from 17.7% to 14.5% was observed (fig no-5 and table no-2).

DISUCSSION

Production of metabolites can be attained solely after a specific incubation time, during which culture was allowed to grow at a steady state (Pandey *et al*, 2000). The rate of metabolite production and quantity of metabolite produced by the process of fungal biotransformation were influenced by the incubation period and substrate concentration. Metabolite production of each strain depends on the specific growth rate of the culture under optimised conditions and amount of the substrate used in the study (Miyazawa *et al*., 1995). The present study was concentrated to trace out the optimum incubation period for biotransformation of loratadine and effect of varying substrate concentration on metabolite production using three cultures, which were potentially capable of converting the drug loratadine to its metabolites. After summarising results of influence of incubation time on the biotransformation of a loratadine, it was found that, by increasing the incubation time, metabolite production was increased up to certain time i.e., 64 hrs and on further incubation up to 72 hrs there was no change in production of metabolite quantity indicated the complete utilisation of nutrients and are insufficient after 64 hrs for further their growth and catalytic activity. This was also proved in case of (L)-citronellal biotransformation by *Rhodotorula minuta*. (Harshad and Mohan, 2003). Different substrate concentrations used in the study were also found to influence the percentage of metabolite formation. The increased substrate concentration has shown increased metabolite formation by *Cunnighmella* species, might be due to availability of increased quantities of precursor for metabolite, which was consistent with the results of (R)-(+)-limonene biotransformation by *Penicillium digitatum* DSM 62840 (Prieto S *et al.,* 2011). In contrast the increased substrate concentration after 40 µg/ml has shown decreased metabolite formation in bio transformation by *Aspergillus niger* , might be due to substrate inhibition capacity of loratadine on enzymes of *Aspergillus niger*. The conditions, which were optimised in the present study, can be prefixed in industrial

level to obtain the product in large quantities which will be the important effort to produce the product commercially in maximum yields.

CONCLUSION

The present study concluded that incubation time and substrate concentrations showed their impact in the process of biotransformation of loratadine using all the three cultures. High substrate concentrations produce high quantity of metabolites in case of *Cunnihmella elegans* and *Cunnighmella echinulata,* while in case of *Aspergillus niger,* high substrate concentrations after a specific concentrations lead to inhibition of the product formation might be due to substrate inhibition. Varying the incubation period, change in the metabolite production was observed by the three cultures and after 64 hours of incubation, no change in the percentage metabolite formed was observed, this might be due to exhaustion of media components after 64 hours. The present exploration, revealed that *Cunninghamella elegans, Cunnighmella echinulata* and *Aspergillus niger* can be used as an *in vitro* models for production of metabolites of loratadine in larger amounts adopting the optimized conditions.

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