

Fungal biotransformation of clobazam – induction, inhibition and kinetic studies

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The present study was aimed to explore the ability of six distinct fungi to biotransform the drug clobazam to its metabolites, and the nature of enzymes involved in such fungal biotransformation by performing screening, induction, inhibition and kinetic studies. Among the six organisms, a sample of *Aspergillus fumigatus* culture showed an extra peak at 3.5 min in the high performance liquid chromatography chromatogram when compared with its controls, indicating the formation of metabolite. The metabolite thus formed was confirmed by mass spectrometry and NMR spectroscopy as 4-hydroxy norclobazam. Enzyme induction and inhibition studies were conducted on the involvement of a CYP3A4-like enzyme in fungal biotransformation. Enzyme kinetic studies were conducted to determine the affinity of the enzyme to the substrate. The study revealed that *A. fumigatus* can be used as a microbial resource to analyse the complete metabolic profile of clobazam with a maximum concentration 30 µg/ml.

Keywords: *Aspergillus fumigatus*, biotransformation, induction, inhibition, kinetics.

CLOBAZAM, a 1,5-benzodiazepine derivative indicated for many 'difficult-to-treat' epilepsies, in addition to Lennox–Gastaut syndrome, is also used to treat anxiety^{1–3}. Clobazam is a comparatively better anti-anxiety drug due to less side effects than other benzodiazepines. Clobazam is abundantly transformed into its metabolites by hepatic metabolism via *N*-demethylation and hydroxylation. The metabolites of clobazam are norclobazam, an active metabolite and 4'-hydroxyclobazam. The main enzyme that mediates *N*-demethylation is CYP3A4 (ref. 4). The efficacy and safety profile of clobazam is due to its metabolite norclobazam^{5,6}. Biotransformation studies are essential measures of efficacy and safety of a drug. Bioavailability and rate of clearance of a drug highly depend on its metabolism; therefore, metabolism studies are crucial in drug design and development⁷. A microbial system can serve better to gain insight about drug metabolites under moderate conditions than those required by chemical systems and other available methods⁸. Microbial species like fungi, bacteria and yeast are efficient *in vitro*

models for mammalian biotransformation of drugs with potential applications^{9–12}.

Enzyme induction and inhibition studies are useful to predict alterations in the drug metabolism, which significantly influence drug efficacy and these are prerequisite to gain knowledge about clinically significant drug interactions¹³. Enzyme induction and inhibition studies in microbial biotransformation using a known inducer and inhibitor of mammalian enzymes are also invaluable to determine the type of enzymes of microorganisms and their comparison with mammalian enzymes. Enzyme kinetics is important in the biotransformation of drugs¹⁴. The Michaelis–Menten model assumes that the active site of an enzyme contains one binding site at which the catalytic process occurs, and the velocity of the reaction is characterized by applying Michaelis–Menten kinetic model to enzymes in a fungal environment^{15,16}. A systematic study of the kinetics of metabolite formation in microbial biotransformation is, therefore, crucial for the development of an effective control strategy to improve metabolite production in large quantities in the industry^{17–19}. Different species of fungi have different values for both substrate concentration at which half maximum velocity can be achieved (K_m) and maximum velocity (V_{max}) for the substrate because they differ in their substrate utilization capacity²⁰. The present study was aimed at screening six fungal cultures for biotransformation of clobazam by performing induction, inhibition and enzyme kinetic studies.

Materials and methods

Fungi

Aspergillus niger (NCIM-589), *Aspergillus fumigates* (NCIM-902), *Aspergillus ochraceus* (NCIM-1140) *Cunninghamella elegans* (NCIM-689), *Cunninghamella blacklesleeana* (NCIM-691) and *Cunninghamella echinulata* (NCIM-687), were obtained from National chemical laboratory (NCL), Pune, India.

Chemicals

Clobazam was a gift sample from Lake Chemicals, Bengaluru. The remaining chemicals were purchased from

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SD Fine Chemicals. HPLC grade solvents used for the analysis were obtained from Sigma Aldrich, Bengaluru.

Stock culture maintenance

All cultures were retained on the respective agar slants at 4°C and sub-cultured every six months to maintain viability²¹. Potato dextrose broth was used as the medium for growth of fungal cultures²². The components of potato dextrose broth were potato chips (200 g/1000 ml, boiled for 30 min), dextrose 20 g and yeast extract 0.1 g. The prepared medium was sterilized using autoclave at 121°C for 30 min before the study²³.

Fungal screening studies

Screening was implemented in 250 ml Erlenmeyer flasks containing 50 ml of broth media designated as drug control, culture control and sample using six different fungal strains. Drug control flask had only drug in broth medium without culture, while the culture control flask contained broth medium inoculated with a loopful of the respective fungi. Sample flask consisted of both drug and culture. All the flasks were kept in a shaker incubator (CIS 24Remi instruments, Mumbai, India) for 72 h under identical conditions to achieve prominent growth of fungi for biotransformation of the drug²⁴. Then the drug and metabolites were extracted for further analysis by HPLC.

Inhibition and induction studies

In mammals, enzyme CYP3A4 is involved in the metabolism of clobazam²⁵. Soon after confirmation of metabolites formed in fungal biotransformation, as a part of the present work, enzyme inhibition and induction studies were conducted using CYP3A4 inducer and inhibitor to determine the nature of the enzyme responsible for fungal production of clobazam metabolite with a selected fungus. For the selected culture, six flasks, one sample and five controls were specified. These include blank I (drug control), blank II (culture control) consisting of the selected fungus, blank III (inhibitor/inducer control), blank IV (inhibitor/inducer and substrate control), and blank V (culture + inhibitor/inducer control) to explore the interference of substrate and inhibitor/inducer in association with the media. Sample for induction and inhibition studies contained drug, inducer/inhibitor and culture. Stock solutions of clobazam, CYP3A4 inducer (carbamazepine) and inhibitor (fluoxetine) were prepared by dissolving 10 mg of each in 10 ml of methanol separately, for further use. From the prepared stock solutions, 0.5 ml of drug solution, inhibitor solution and inducer solution and cultures was aseptically transferred to the assigned flasks consisting of 50 ml potato dextrose broth. All the

flasks were incubated, extracted and analysed similar to screening studies. Then the percentage metabolite formed was calculated with the help of peak areas found in the HPLC chromatograms.

Enzyme kinetic studies

These studies were conducted in continuation to the induction and inhibition studies, to evaluate the affinity of the enzyme to the given substrate involved in fungal biotransformation. The selected fungus was inoculated in prefixed sample flasks containing different substrate (clobazam) concentrations, viz. 10, 20, 30, 40, 50 and 60 µg/ml, and samples were aseptically collected from each flask at different incubation time intervals, viz. 24, 36, 48, 60 and 72 h to extract the metabolite and calculate the percentage of metabolite formed at each concentration of substrate and each time interval²⁶.

Extraction method

Samples collected after the predetermined incubation period in the above studies were heated in a water bath at 50°C for 30 min to stop further growth of the fungi. Then, contents of all flasks were centrifuged at 3000 rpm

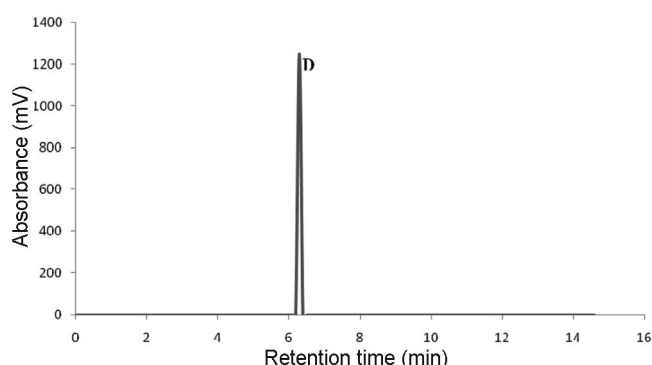


Figure 1. HPLC chromatogram of clobazam.

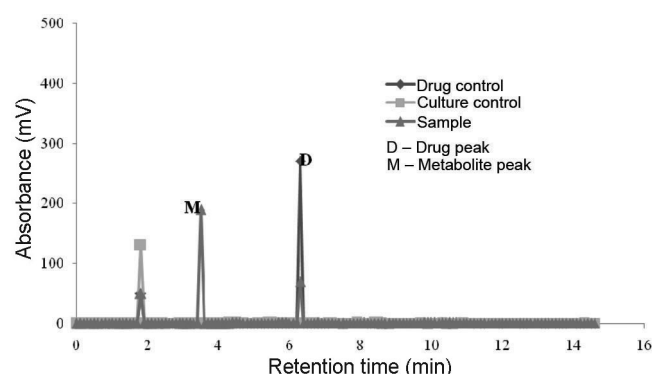
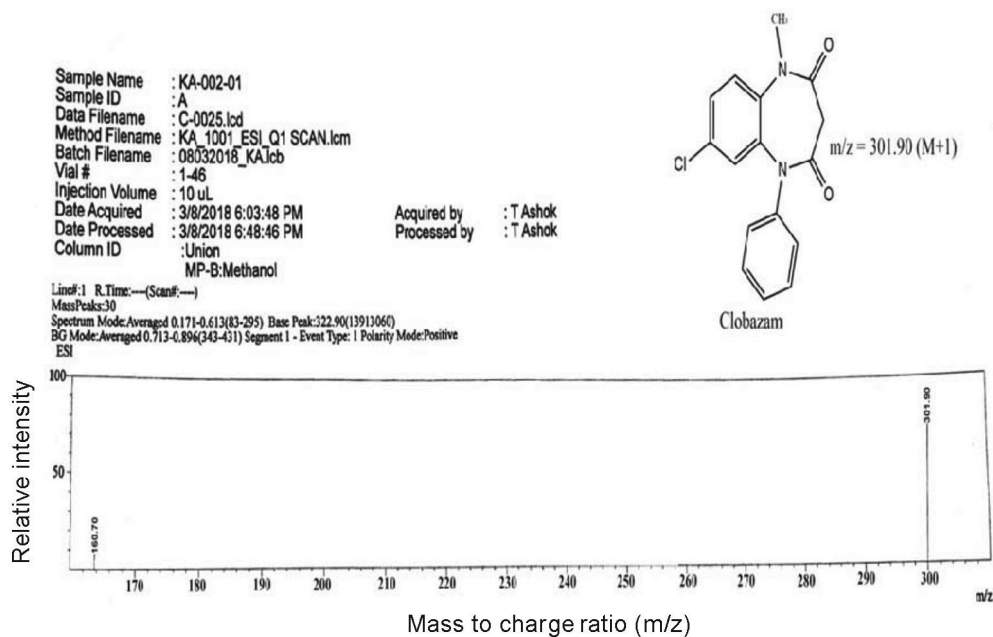
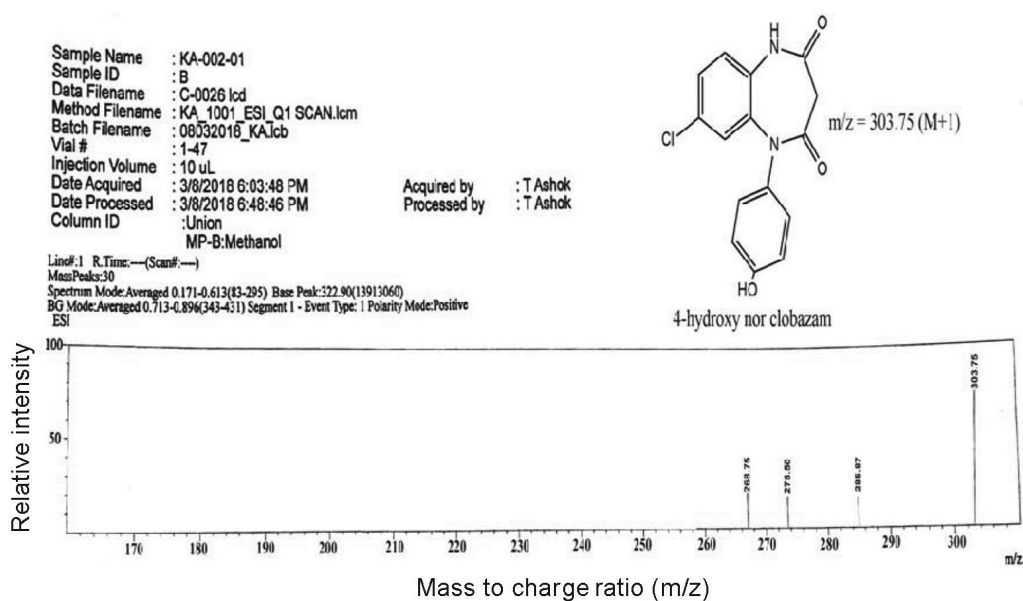


Figure 2. HPLC chromatogram of clobazam and its metabolite from culture extracts of *Aspergillus fumigatus*.

Table 1. HPLC analysis of clobazam incubated with six fungi

Organism	Retention time (min)			
	Drug control	Culture control	Standard	Sample
<i>Cunninghamella elegans</i> (NCIM-689)	1.8, 6.3	1.8	6.3	1.8, 6.3
<i>Cunninghamella echinulata</i> (NCIM-687)	1.8, 6.3	1.8	6.3	1.8, 6.3
<i>Cunninghamella blackesleeana</i> (NCIM-691)	1.8, 6.3	1.8	6.3	1.8, 6.3
<i>Aspergillus niger</i> (NCIM-589)	1.9, 6.3	1.9	6.3	1.9, 6.3
<i>Aspergillus fumigatus</i> (NCIM-902)	1.9, 6.3	1.9	6.3	1.9, 3.5*, 6.3
<i>Aspergillus ochreus</i> (NCIM-1140)	1.8, 6.3	1.8	6.3	1.8, 6.3

**Figure 3.** Mass spectrum of clobazam.**Figure 4.** Mass spectrum of clobazam metabolite formed by *A. fumigatus*.

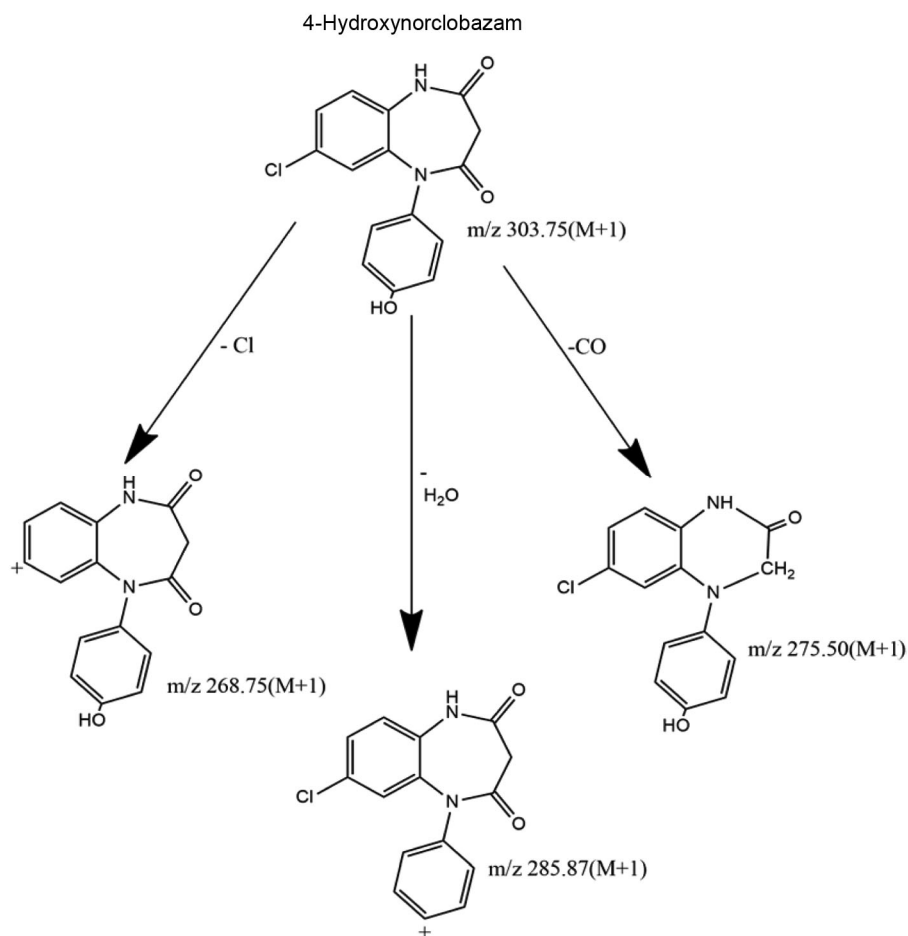


Figure 5. Mass fragmentation pattern of clobazam metabolite formed by *A. fumigatus*.

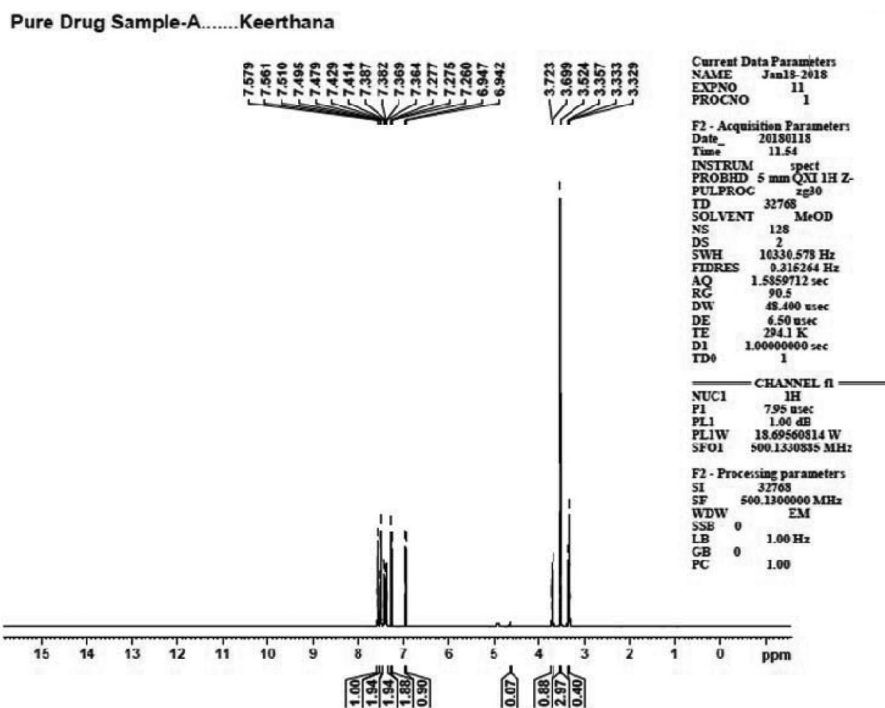
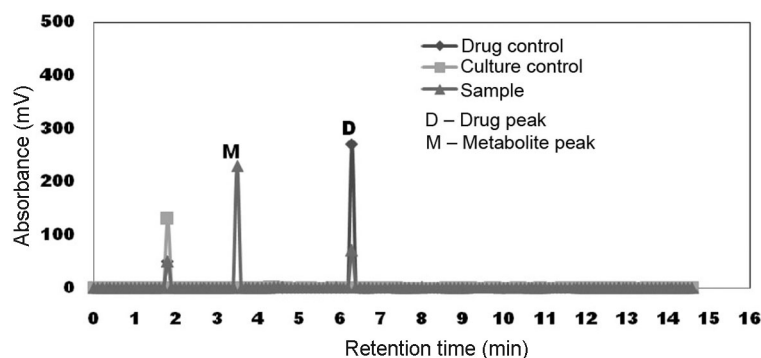
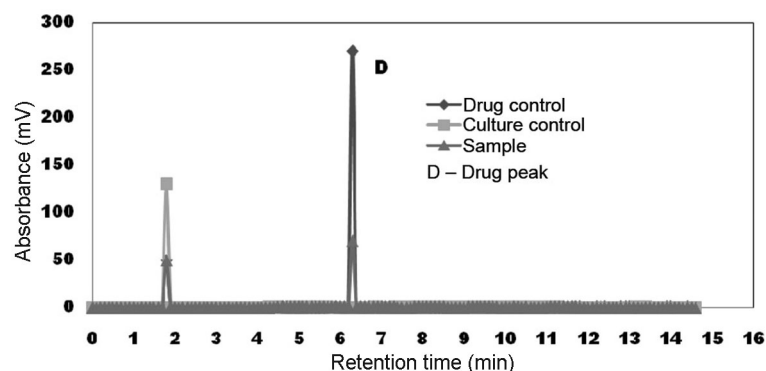


Figure 6. NMR spectrum of clobazam.

Table 3. ^1H NMR proton assignment of clobazam and its metabolite

Compound	^1H NMR proton assignment
Clobazam ($\text{C}_{16}\text{H}_{13}\text{ClN}_2\text{O}_2$)	3.329–3.357 (t, 2H, CH_2) J -value = 12 Hz, 3.524 (s, 3H, CH_3), 6.942 (s, H, CH), 7.260 (s, H, CH), 7.275 (s, H, CH), 7.414–7.429 (s, 2H, CH), 7.479 (m, 3H, CH) J -value = 8 Hz.
Metabolite ($\text{C}_{15}\text{H}_{11}\text{ClN}_2\text{O}_3$)	3.187–3.190 (d, 2H, CH_2) J -value = 3 Hz, 4.917 (s, 1H, OH), 6.949–6.954 (d, 2H, CH) J -value = 2.5 Hz, 7.266–7.599 (m, 4H, CH) J -value = 3.5 Hz, 7.930 (s, H, CH), 8.187 (s, H, NH).

**Figure 8.** HPLC chromatogram of clobazam and its metabolite in the presence of CYP 3A4 inducer (carbamazepine) from culture extracts of *A. fumigatus*.**Figure 9.** HPLC chromatogram of clobazam and its metabolite in the presence of CYP 3A4 inhibitor (fluoxetine) from culture extracts of *A. fumigatus*.

for 10 min using centrifuge tubes (Laboratory Centrifuge C-854/8, Remi instruments, Mumbai, India). Supernatant was collected from all the tubes and stored in a refrigerator. The supernatant was treated with diethyl ether to extract clobazam and the metabolites formed²⁷. Then organic layer was collected in screw-cap bottles and kept for air-drying. The dried samples were analysed by HPLC after reconstitution with mobile phase. Besides, pure drug clobazam was also analysed by HPLC to set it as a standard.

Analytical methods

High performance liquid chromatography^{4,28}

Extracted drug and metabolites were separated using a Phenomenex Luna 5 μ C18(2) 100A 250 \times 4.60 mm

(Phenomenex, USA) column and the solvent system consisted of acetonitrile : water (0.6 : 1) delivered at a flow rate of 1 ml/min in the HPLC system (Shimadzu, Kyoto, Japan) with LC 20 AD pump solvent delivery module and SPD 20AV UV detector. Sensitivity was set at 0.0001 a.u. The UV detector wavelength was set at 230 nm. The mobile phase was filtered through a 0.45 μm filter, degassed using a sonicator for 15 min and runtime was set for 20 min.

Mass spectrometry

The metabolite identified in one of the fungi samples screened during HPLC analysis were collected and further analysed using mass spectrometry to confirm their molar mass. The mass spectrometer (Agilent

Table 4. Percentage metabolite formed during induction, inhibition and kinetic studies by *A. fumigatus*

Type of study	Drug	Percentage metabolite formed				
Biotransformation	Clobazam	7.05				
Induction	Carbamazepine (inducer)	10.8				
Inhibition	Fluoxetine (inhibitor)	0.10				
Kinetic	Incubation time (h)	24	36	48	60	72
Clobazam concentration ($\mu\text{g/ml}$)	10	0.9	1.57	2.13	7.73	8.14
	20	0.93	1.63	3.18	10.1	10.7
	30	1.01	1.69	3.20	11.4	12.1
	40	1.04	1.7	3.21	11.5	12.17
	50	1.06	1.71	3.26	11.8	12.2
	60	1.08	1.72	3.26	11.8	12.4

Technologies, Germany; model API 3000MS was operated in the electron spray ionization (ESI) mode. Ionization was carried out in positron mode using ion trap detector (3.5 kV, 325°C, 210 psi).

Proton nuclear magnetic resonance spectroscopy

The structure of metabolite isolated from HPLC was confirmed by ^1H NMR spectroscopy (BRUKER AVANCE 500 MHz). Deuterated methanol was used as the solvent to analyse clobazam and its metabolite.

Quantification of metabolite formed

The peak area of metabolites obtained by HPLC from samples collected during induction, inhibition and kinetic studies was used to quantify the metabolites formed in terms of percentage in relation to peak area of the drug.

Kinetic studies²⁹

The data of percentage metabolites formed during kinetic studies were fitted in MS EXCEL by plotting a graph between time and percentage metabolites formed at different concentrations to obtain the velocity of reaction from the slope of the graph. Then the velocity obtained for each concentration by the above method was fitted in the Michaelis–Menten equation using nonlinear regression in Graph Pad Prism 5.0 (ref. 30), and best-fit values for K_m and V_{max} were obtained in terms of correlation coefficients to find the extent of affinity of fungal enzymes with clobazam.

Results and discussion

Screening of fungi and identification of metabolite

In this study, six different fungi were screened for biotransformation of clobazam using fermentation protocol

and HPLC analysis. Figure 1 shows HPLC chromatogram of pure drug. Blank I (drug control) chromatogram showed only drug peak at retention time of 6.3 min and no drug peak was observed in blank II (culture control) chromatogram (Table 1). HPLC chromatogram of clobazam incubated with *A. fumigatus* showed an extra peak at 3.5 min when compared with its two controls (Figure 2 and Table 1). The peaks at 1.8 and 1.9 min represent the media components. Since *A. fumigatus* biotransformed clobazam, elute of the extra peak was collected to evaluate the formed metabolite structure by mass and NMR analysis.

Mass spectrum of clobazam showed a molecular ion peak at m/z 301.90 ($M + 1$) which is equal to the molecular weight of clobazam (Figure 3). Metabolite formed by *A. fumigatus* showed a molecular ion peak at m/z 303.75 ($M + 1$) which is equal to the molar mass of 4-hydroxy norclobazam (Figure 4). This structure was also supported by fragments at m/z 268.75, m/z 275.50 and m/z 285.87 (Figure 5). Clobazam metabolite fragment ions formed by *A. fumigates* and their significance are shown in Table 2. The structure of metabolite was further affirmed by the comparison of NMR spectra of drug with the metabolite (Figures 6 and 7 respectively) formed from culture extracts of *A. fumigates*. As per NMR spectrum of metabolite shown in Figure 7, presence of a peak at 4.91 δ indicated the addition of hydroxyl group and absence of peak at 3.52 δ indicated the removal of methyl group.

^1H NMR proton assignment data of the drug and its metabolite (Table 3) also suggested the structure of the metabolite. J -value for doublet and triplet in ^1H NMR spectrum of the metabolite ranged from 2.5 Hz to 3.5 Hz. It indicated the formation of 4-hydroxy norclobazam through an active metabolite, norclobazam. Thus, 4-hydroxy derivative of norclobazam formed by *A. fumigatus* was confirmed using HPLC, mass, and NMR analysis, and was also supported by reports on *in vitro* liver microsomes⁴, human and other mammals³¹.

CYP 3A4 enzyme is responsible for biotransformation of clobazam in humans and other mammals; hence enzyme induction and inhibition studies were conducted using CYP3A4 inhibitor (fluoxetine) and inducer

(carbamazepine) in order to determine the type of enzyme responsible for fungal biotransformation of clobazam. Figures 8 and 9 show HPLC chromatograms of extracts obtained during induction and inhibition studies with *A. fumigatus* respectively. The areas of HPLC peaks of clobazam and the formed metabolite when incubated with *A. fumigatus* in the presence of inducer and inhibitor were compared with their controls (Table 4). The percentage metabolite formed had increased from 7.05 to 10.8 in the presence of inducer (carbamazepine; Figure 8 and Table 4), and the metabolite peak disappeared with inhibitor (fluoxetine; Figure 9 and Table 4), indicating that the formation of metabolite was inhibited. Hence, CYP3A4-like enzyme might be involved in the metabolism of clobazam by *A. fumigatus*.

The affinity of CYP 450 3A4-like enzyme of the fungus to the substrate (clobazam) was determined by performing enzyme kinetic studies. Using nonlinear regression, Michaelis–Menten curves were independently generated. Best-fit values for K_m were $7.064 \pm 0.936 \mu\text{g/ml}$ and for V_{\max} $0.2165 \pm 0.005 \mu\text{g/ml/min}$. Correlation coefficient value was 0.9957. K_m value denotes the substrate concentration at which half maximum velocity of the reaction is seen. The Michaelis–Menten plot shows metabolism at different concentrations of clobazam by *A. fumigatus* shown in Figure 10.

A substantial increase in percentage metabolite formation was observed by increasing the substrate concentration up to $60 \mu\text{g/ml}$ (Table 4, Figure 10), but saturation of the process was noticed after $30 \mu\text{g/ml}$ concentration because no significant increase in the metabolite quantity was observed though it is increased up to $60 \mu\text{g/ml}$. The Michaelis–Menten kinetics can be best fitted to the present fungal biotransformation of clobazam because the saturation profile was a hyperbolic curve and kinetic parameters were estimated by calculating percentage metabolite formed at different time intervals of various concentrations of the substrate. K_m depends on the type of substrate, and incubation conditions like pH, temperature

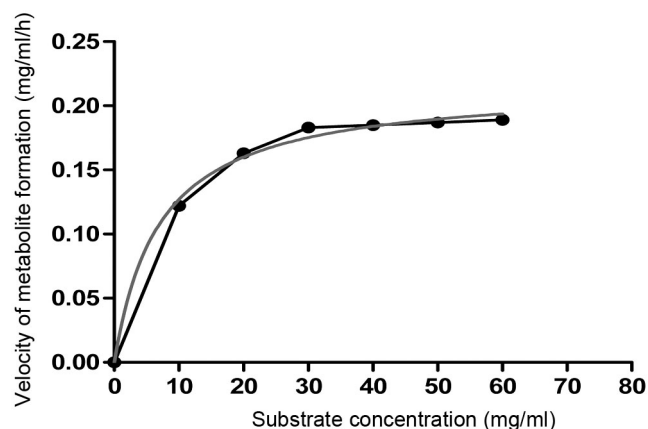


Figure 10. Michaelis–Menten plot representing clobazam metabolism by *A. fumigatus*.

and presence of other substrates or inhibitors. So, K_m gives an account of the substrate concentration essential to initiate significant catalysis³². From the above facts and the present results, it is evident that the CYP3A4-like enzyme present in *A. fumigatus* has less affinity towards clobazam in fungal biotransformation, as the K_m value derived from best-fit values was more than 10^{-1} .

Conclusion

This study proves the ability of *A. fumigatus* to biotransform the drug clobazam to its metabolite 4-hydroxy norclobazam, similar to humans and other mammals. The above findings were confirmed by HPLC, mass and NMR analysis. The enzyme induction and inhibition studies confirmed that the nature of enzyme involved in the metabolism of clobazam by *A. fumigatus* was identical to the mammalian CYP3A4 enzyme. The fungal biotransformation of clobazam followed Michaelis–Menten kinetics up to $60 \mu\text{g/ml}$ of substrate concentration. The enzyme involved in the fungal biotransformation showed less affinity towards clobazam, which was confirmed by higher K_m values found in the kinetic studies. Hence, *A. fumigatus* can be used as an *in vitro* model to study biotransformation of clobazam similar to mammalian metabolism with a substrate concentration of $60 \mu\text{g/ml}$, and also to produce an active metabolite of clobazam for further applications.

Conflict of interest: The authors declare no conflict of interest.

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