

## Predicting drug interaction of diltiazem on microbial metabolism of midazolam

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**ABSTRACT:** The present investigation was aimed at studying the biotransformation of midazolam, a marker substrate for CYP3A4 metabolic reactions using nine microbial cultures. The study was performed using a two stage fermentation protocol and the results indicate that the microbial cultures produce hydroxylated metabolites of midazolam, based on HPLC-DAD and LC-MS-MS analysis. The metabolism of midazolam was also studied in presence of diltiazem, a CYP3A4 specific inhibitor in order to elucidate the nature of enzymes involved in the biotransformation. The results of the inhibition study reveal that diltiazem produced a concentration dependent inhibition of midazolam metabolism in four cultures indicating that the enzymes participating in the metabolism in these cultures were similar to that of CYP3A4. The results indicate that microbial cultures possess enzyme systems similar to mammals and they can be used to predict drug interactions in mammalian systems. The study will be helpful in developing models for simulation of mammalian drug metabolism and drug interactions. This will greatly reduce the number of animals used in early stages of drug development.

**KEYWORDS:** Biotransformation, HPLC, LC-MS-MS, metabolites, microorganisms, midazolam, diltiazem, drug interaction

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### I. Introduction

Metabolic studies play important role in the drug discovery and development. Metabolism not only helps in the detoxification of xenobiotics, but is also important from the activity/toxicity perspective since the metabolites produced may be active/toxic in nature and produce significant effects in the body. Once the metabolic routes of a drug are characterized, the *in vivo* prediction and correlation requires elucidation of drug metabolizing enzymes. This is usually achieved by *in vitro* biotransformation studies of the drug followed by the use of substrates and inhibitors selective or specific for the respective cytochromeP450's (CYPs).

Several *in vitro* methods using human liver microsomes, purified enzymes, cDNA expressed enzymes etc., were reported for identifying the CYP(s) involved in the metabolism of a new chemical entity where different probe reactions are used. The use of selective and specific inhibitors will help to confirm the nature of enzymes involved in the metabolism. Among the various CYPs identified in mammals, CYP3A4 is involved in majority of clinically significant drug interactions with as many of the drugs available clinically, which are either the substrates and/or inhibitors of this isozyme. Testosterone, midazolam, nifedipine, erythromycin, triazolam and alprazolam are used in various probe reactions to elucidate the activity of CYP3A4.

Midazolam is a short-acting benzodiazepine used as sedative and anesthetic. It is a selective substrate for CYP3A4 mediated metabolic reactions in mammals *in vitro* and *in vivo* where it is converted by hydroxylation to form 1'-hydroxy midazolam (Figure 1). Diltiazem is a calcium ion cellular influx inhibitor (channel blocker) used in the treatment of hypertension and angina pectoris. It is a specific and selective inhibitor of CYP3A4 isozyme and responsible for drug interactions involving CYP3A4. Two metabolites of diltiazem, N-desmethyl diltiazem and N, N-didesmethyl diltiazem were found to be 11 and 200 fold more potent than diltiazem as inhibitors of CYP3A4 in humans [1] and rat liver microsomes [2]. Studies showed that diltiazem increased the AUC of midazolam by three to four-fold and the  $C_{max}$  by two-fold, compared to placebo. The elimination half life of midazolam also increased (1.5-2.5 fold) during coadministration with diltiazem. These pharmacokinetic effects seen during diltiazem coadministration can result in increased clinical effects (e.g., prolonged sedation) of midazolam. Increased AUC and prolonged elimination half-life ( $t_{1/2}$ ) of midazolam when coadministration with diltiazem suggested that the dose of midazolam should be reduced during diltiazem treatment [3]. Several reports suggest that CYP3A4 mediated midazolam  $\alpha$ -hydroxylation was inhibited by diltiazem in human liver microsomes, in a concentration dependent manner [4, 5].

Microorganisms particularly fungi possess enzyme systems that are similar to humans and can be used to predict mammalian drug metabolism [6]. Our previous reports [7, 8] reveal that microorganisms can be used to simulate the mammalian metabolism of celecoxib and valdecoxib. The metabolic pathways in mammals were effectively simulated using microorganisms, and thus it paved the way to study the effect of clopidogrel, an

enzyme inhibitor, on the microbial metabolism of diclofenac, a specific substrate for CYP2C9 metabolic reactions [9]. Clopidogrel inhibited the metabolism of diclofenac in a dose dependent manner.

Basing on the previous reports, midazolam and diltiazem were considered to be the suitable substrate and inhibitor to study the CYP3A4 mediated drug interaction using microbial cultures. The present investigation deals with the microbial metabolism of midazolam and its inhibition by diltiazem using eight fungal and one yeast cultures, viz., *Absidia coerulea* MTCC 1335, *Cunninghamella blakesleana* NCIM 687, *Cunninghamella blakesleana* NCIM 688, *Cunninghamella echinulata* NCIM 691, *Cunninghamella echinulata* NCIM 693, *Cunninghamella elegans* NCIM 690, *Cunninghamella* sp. NCIM 1184, *Rhizopus arrhizus* NCIM 997 and *Streptomyces rimosus* NCIM 2213. The cultures were selected based on the production of hydroxylated metabolite of valdecoxib, a substrate of CYP3A4, in our previous study [7, 8].

## **II. Material And Methods**

### **2.1 CHEMICALS**

Midazolam hydrochloride was obtained from Neon Laboratories Ltd., Thane (M.S.), India. Diltiazem hydrochloride was kindly gifted by Dr. Reddy's Laboratories, Hyderabad. All other chemicals used were of highest available purity. Microbial cultures were purchased from Microbial Type Culture Collection and Gene Bank (MTCC), Chandigarh, India or National Collection of Industrial Microorganisms (NCIM), Pune, India.

### **2.2 CULTURE PROCEDURE**

A two stage fermentation protocol was used in the present study. The fungal cultures were grown in liquid medium containing (per liter) dextrose 20 g, yeast extract 5 g, peptone 5 g, sodium chloride 5 g and  $\text{KH}_2\text{PO}_4$  5 g supplemented with 0.02% Triton X 100. The actinomycetes culture was grown in liquid medium containing (per liter) malt extract 3 g, glucose 10 g, yeast extract 3 g and peptone 5 g and pH adjusted to 6.8. Fifty ml conical flasks containing 10 ml of sterile liquid medium were inoculated with 50  $\mu\text{l}$  of culture obtained from a freshly grown agar slant, and orbital shaken at 180 rpm and 30 °C in a refrigerated shaker incubator. The cultures were allowed to grow for 48 h to obtain second stage cultures.

### **2.3 BIOTRANSFORMATION**

The second stage cultures (10 ml culture in 50 ml capacity culture flask) were added with 2 mg each of midazolam (in 100  $\mu\text{l}$  water) to obtain a final drug concentration of 0.2 g  $\text{l}^{-1}$ . Each culture was studied in quadruplicate while running suitable controls. Culture controls consisted of culture blanks in which the organisms were grown under identical conditions but without adding the substrate. The culture controls were added with 100  $\mu\text{l}$  sterile distilled water, the solvent used to dissolve the drug. Drug controls were composed of the sterile medium to which same amount of the drug was added and incubated without microorganisms. The incubation was continued under similar conditions for 10 d, the flasks were taken out, extracted and analysed.

### **2.4 EXTRACTION AND SAMPLE PREPARATION**

The cultures, after 10 d of incubation with midazolam, were extracted for midazolam and its metabolites. The flask contents (10 ml) were added with 0.5 ml of 1 mol  $\text{l}^{-1}$  NaOH, shaken vigorously and extracted with 3 x 10 ml of hexane-chloroform mixture (7:3). The combined organic phases were evaporated under reduced pressure and the dried samples were reconstituted in 1.5 ml of methanol-water mixture (1:1). The samples were then centrifuged at 12,000 x g and 20 °C for 20 min and the supernatants were used for HPLC-DAD and LC-MS analysis.

### **2.5 HPLC-DAD ANALYSIS**

For the presence of metabolite(s), the samples were analyzed by slightly modified and revalidated isocratic reversed phase HPLC method reported by Casas et al. [10]. The HPLC analysis was performed on LC-10AT (Shimadzu, Japan) system by injecting 20  $\mu\text{l}$  of sample into syringe loading sample injector. The column used was Luna C18, 5  $\mu$  and 250 x 4.6 mm i.d. (Phenomenex, USA) and the mobile phase consisted of a mixture of methanol and water in 80:20 ratio. The analysis was performed at a flow rate of 0.5 ml  $\text{min}^{-1}$ . Midazolam and its metabolites were detected using diode array detector (SPD-M10Avp, Shimadzu, Japan) at a wavelength of 220 nm. The data analysis was performed by Class M10 software. The UV absorption spectrum of the metabolites was compared with that of midazolam. The metabolites were quantified based on the peak areas and expressed as percentages of metabolites formed. The calculations were performed with respect to the total area of drug and metabolites together taken as 100%.

### **2.6 LC-MS-MS ANALYSIS**

The mass/charge (m/z) values of midazolam and its metabolites and their fragmentation ions were recorded by LC-MS-MS using Perkin Elmer SCIEX API mass spectrometer set in positive mode. The column

used was Inertsil ODS-2, RP, C18 of 250 x 4.6 mm and the mobile phase, acetonitrile/water (pH adjusted to 3.2 with formic acid; 35:65), was pumped at a flow rate of 1 ml min<sup>-1</sup>. Fifty microliters of the sample was injected using an autosampler device. The vaporizer temperature and the discharge current were set at 300 °C and 10 µA respectively. The fragments were scanned in the m/z range of 100 to 500 and the data were processed with Sciex Analyst software. The metabolites were identified basing on pattern of UV spectra in HPLC-DAD and m/z values of the fragmentation products obtained in LC-MS-MS analysis.

## 2.7 INHIBITION OF METABOLISM BY DILTIAZEM

The inhibition of 1'-hydroxy metabolite formation by diltiazem was studied in all the nine cultures. The fungal and actinomycetes cultures were grown in the same media that is used for biotransformation and a two stage fermentation protocol was used for the study. Each 10 ml of the second stage cultures were added with 0.5, 1, 1.5 and 2 mg of diltiazem (in 100 µl of methanol) to get the inhibitor concentrations of 0.05, 0.1, 0.15 and 0.2 g l<sup>-1</sup> culture and incubated at 30 °C for a period of 24 h to inhibit the enzymes. Each culture was studied in quadruplicate while running suitable controls. The incubation time was high enough to achieve maximal inhibition of enzymes, since inhibition of enzymes is a quick process when compared to induction.

The diltiazem inhibited second stage cultures were added with 2 mg each of midazolam (in 100 µl water) to get a final drug concentration of 0.2 g l<sup>-1</sup>. Each culture was studied in quadruplicate while running suitable controls and the incubation was continued under similar conditions for 10 d. The contents of the flasks were extracted and analysed using the procedure similar to that employed for midazolam metabolism by microbial cultures. The percentages of 1'-hydroxy metabolite in the cultures were calculated.

## III. Results

The drug control did not show any additional peaks. Upon extraction from the media, the recovery of midazolam from the dextrose broth was >92.6%. HPLC analysis of the extracts of the cultures showed that all the cultures studied were able to metabolize midazolam to produce two metabolites (Table 1). Representative HPLC chromatograms were shown in Figure 2. All the organisms studied produced the same two metabolites, but in different relative amounts. The metabolite peaks were identified in HPLC basing on the similarity in UV spectra in the diode array detector.

Midazolam was eluted at 11.3 min and the metabolites produced were designated as M<sub>1</sub> (9.3 min) and M<sub>2</sub> (9.7 min). Control incubations without midazolam added showed no coincidental peaks eluting with identified metabolites. The results pertaining to the metabolite production was depicted in Table 1. M<sub>2</sub> was the major metabolite found in all the cultures studied. *A. coerulea* MTCC 1335 produced 56.78% of this metabolite whereas *C. echinulata* NCIM 691 produced only 8.24% of this metabolite.

In positive mode of LC-MS-MS analysis, midazolam and its metabolites M<sub>1</sub> and M<sub>2</sub> gave their protonated molecular ions at m/z values 326, 342 and 342 respectively. LC-MS spectrum of M<sub>2</sub> was shown in Figure 3. M<sub>1</sub> and M<sub>2</sub> gave protonated molecular ions [(M+H)<sup>+</sup>] at m/z 342. M<sub>1</sub> upon fragmentation in positive mode gave fragment ions at m/z 297, 276, 256, 222 and 109 whereas M<sub>2</sub> gave fragment ions at m/z 324, 297, 276, 261, 234, 203, 177, 168, 141 and 109. Based on the fragmentation pattern, M<sub>1</sub> and M<sub>2</sub> were identified as 4-hydroxy and 1'-hydroxylated metabolites of midazolam. The retention times, m/z values of parent ions (M+H)<sup>+</sup>, predicted molecular formulae and metabolic reactions for various metabolites are shown in Table 2.

Among the nine cultures studied some of them have shown potential for inhibition of midazolam metabolism by diltiazem (table 3). *C. blakesleana* NCIM 688, *C. echinulata* NCIM 693, *C. elegans* NCIM 690 and *Cunninghamella* sp. NCIM 1184 showed positive for drug interaction. Diltiazem has shown clear concentration dependent inhibition of midazolam metabolism in these cultures. Figure 4 shows the percentages of M<sub>2</sub> formed in presence of various concentrations of diltiazem in these four cultures.

## IV. Discussion

The drug control has not shown any additional peaks in HPLC analysis indicate that the substrate midazolam was stable in the media. HPLC analysis of the extracts of the cultures showed that all the cultures studied were able to metabolize midazolam to produce two metabolites evidenced from the absence of these new peaks in drug control as well as culture controls (Table 1). All the organisms studied produced the same two metabolites, but in different relative amounts. The production of metabolites by microbial cultures may be due to the presence of enzymes expressed naturally or induced by the drug or media component(s). Both the metabolites produced were eluted before the drug indicating that they are polar than midazolam. The metabolite peaks were identified in HPLC basing on the similarity in UV spectra in the diode array detector. All these new peaks had shown similar UV spectral pattern (from 190 to 370 nm) with that of midazolam indicates that the drug has undergone only minor structural changes. The UV λ<sub>max</sub> of the metabolites were within a narrow range with that of midazolam which indicates that their extinction coefficient at 220 nm was not significantly different from that of the parent drug.

The metabolites were quantified basing on their areas and expressed as percentages of metabolites formed with respect to the total area of drug and metabolites together taken as 100%. From table 1, it can be observed that a significant variation was observed in the quantitative metabolite production among various cultures. The varying production may be attributed to many reasons viz., expression of varying levels of enzymes required for this reaction, substrate/product inhibited biotransformation, non-optimal conditions (media and incubation) for biotransformation, etc.

The structure elucidation of the metabolites was carried out from the m/z values of the protonated molecular ion and fragment ion peaks obtained in LC-MS-MS analysis. The structures of the metabolites were proposed from the m/z values of the protonated molecular ions and fragment ions, HPLC retention times, chromatographic elution order and comparison with previous reports. In positive mode of LC-MS-MS analysis, both the metabolites ( $M_1$  and  $M_2$ ) gave the same protonated molecular ion at m/z 342, that is, 16 higher than midazolam. This suggests that these might be hydroxylation products of midazolam. Since these two metabolites produced the same protonated molecular ion, they might be positional isomers having same molecular formula. The structures of these two metabolites were assigned from fragmentation patterns in LC-MS-MS analysis and previous reports.  $M_1$  upon fragmentation in positive mode gave fragment ions at m/z 297, 276, 256, 222 and 109 whereas  $M_2$  gave fragment ions at m/z 324, 297, 276, 261, 234, 203, 177, 168, 141 and 109. Similar fragments for 4-hydroxy and 1'-hydroxy metabolites of midazolam was reported [11]. Based on the fragmentation pattern,  $M_1$  and  $M_2$  were identified as 4-hydroxy and 1'-hydroxylated metabolites of midazolam. The retention times and elution order of metabolites in HPLC has given an idea on the polarity and thus helped in the structural elucidation.

The retention times, m/z values of parent ions ( $M+H$ )<sup>+</sup>, predicted molecular formulae and metabolic reactions for various metabolites are shown in Table 2. From Table 1 it was found that  $M_2$  was the major metabolite produced in all the cultures studied. Hence it can be concluded that 1'-hydroxy metabolite, which was eluting at 9.7 min in HPLC analysis, is the major metabolite. In the present investigation, nine cultures were screened for their ability to metabolize midazolam. In the initial screening studies all these microbial cultures has shown to possess good metabolizing ability of midazolam. The results also indicated that the 1'-hydroxy midazolam was the major metabolite produced by all the microorganisms used.

Diltiazem was found to be a potent inhibitor of CYP3A4 responsible for the metabolism of midazolam. In the present study diltiazem was incubated with all the microbial cultures expressing midazolam metabolizing enzymes. All the nine cultures were employed in the inhibition study, because all these cultures produced good amounts of metabolite  $M_2$ . Among the nine cultures studied some of them have shown potential for inhibition of midazolam metabolism by diltiazem. Diltiazem is a CYP3A4 specific inhibitor and the present study reveal the evidence for the functional role of the enzymes in these cultures was similar to CYP3A4 in their metabolic nature. Few cultures did not shown any decrease in midazolam metabolism since those enzymes responsible for metabolism were not inhibited by the diltiazem which indicates that the enzymes are functionally different from CYP3A4.

Among the cultures tested, *C. blakesleana* NCIM 688, *C. echinulata* NCIM 693, *C. elegans* NCIM 690 and *Cunninghamella* sp. NCIM 1184 showed positive for drug interaction. Diltiazem has shown clear concentration dependent inhibition of midazolam metabolism in these cultures. The inhibition is of particular significance in *Cunninghamella* sp. NCIM 1184. Our previous study has shown a significant inhibition of diclofenac metabolism by clopidogrel in four fungi viz., *C. blakesleana* NCIM 687, *C. blakesleana* NCIM 688, *C. elegans* NCIM 690 and *C. echinulata* NCIM 691 [9].

## V. Conclusions

*In vitro* studies have become a means of rapid identification of potential drug-drug interactions. The *in vitro* methods are useful in predicting the inhibition of drug metabolizing enzymes but may not predict the magnitude of interactions *in vivo*. The same may be applicable to microbial cultures for drug metabolism as well as drug interactions.

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**Table 1. Midazolam metabolism by microbial cultures**

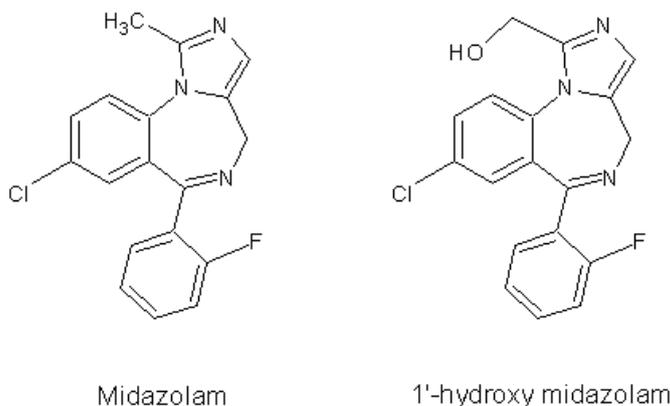
Culture	%M <sub>1</sub>	%M <sub>2</sub>
<i>Absidia coerulea</i> MTCC 1335	2.67 ± 0.29	56.78 ± 4.21
<i>Cunninghamella blakesleana</i> NCIM 687	2.97 ± 0.25	11.40 ± 0.78
<i>Cunninghamella blakesleana</i> NCIM 688	7.86 ± 0.63	19.41 ± 0.91
<i>Cunninghamella echinulata</i> NCIM 691	3.68 ± 0.28	8.24 ± 0.52
<i>Cunninghamella echinulata</i> NCIM 693	8.11 ± 0.59	19.82 ± 1.21
<i>Cunninghamella elegans</i> NCIM 690	5.31 ± 0.41	16.05 ± 1.26
<i>Cunninghamella</i> sp. NCIM 1184	25.01 ± 2.11	51.72 ± 3.93
<i>Rhizopus arrhizus</i> NCIM 997	6.54 ± 0.61	10.69 ± 0.61
<i>Streptomyces rimosus</i> NCIM 2213	9.68 ± 0.61	32.58 ± 1.91

**Table 2. Retention times, m/z values and predicted reactions involved in the biotransformation of midazolam by microbial cultures.**

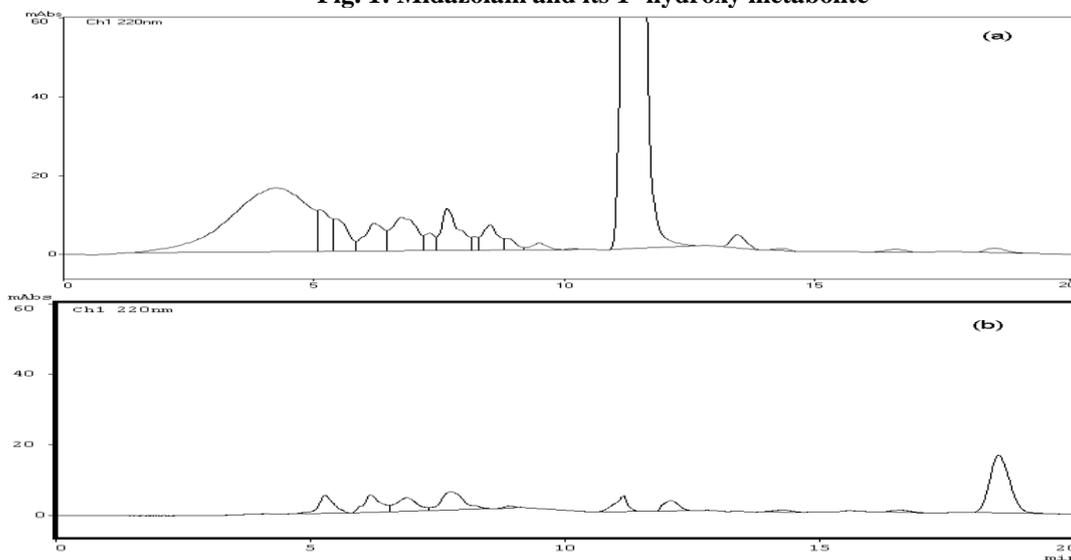
Metabolite	R <sub>t</sub>	[M+H] <sup>+</sup>	Predicted Molecular formulae	Predicted reaction
Midazolam	11.3	326	C <sub>18</sub> H <sub>13</sub> ClFN <sub>3</sub>	-
M <sub>1</sub>	9.3	342	C <sub>18</sub> H <sub>13</sub> OCIFN <sub>3</sub>	4-hydroxylation
M <sub>2</sub>	9.7	342	C <sub>18</sub> H <sub>13</sub> OCIFN <sub>3</sub>	1'-hydroxylation

**Table 3. Effect of diltiazem on midazolam metabolism**

Treatment	uced								
	<i>Absidia coerulea</i> MTCC 1335	<i>Cunninghamella blakesleana</i> NCIM 687	<i>Cunninghamella blakesleana</i> NCIM 688	<i>Cunninghamella echinulata</i> NCIM 691	<i>Cunninghamella echinulata</i> NCIM 693	<i>Cunninghamella elegans</i> NCIM 690	<i>Cunninghamella</i> sp. NCIM 1184	<i>Rhizopus arrhizus</i> NCIM 997	<i>Streptomyces rimosus</i> NCIM 2213
Control	56.78± 4.21	11.40 ± 0.81	19.41 ± 1.31	8.24 ± 1.41	19.82 ± 1.41	16.05 ± 1.21	51.72 ± 3.99	10.69 ± 1.07	32.58 ± 2.21
+ Diltiazem (0.005%)	55.55 ± 5.11	11.11 ± 0.82	18.51 ± 1.32	8.12 ± 1.28	16.54 ± 1.31	12.29 ± 1.01	42.52 ± 3.81	10.52 ± 1.11	31.25 ± 2.52
+ Diltiazem (0.01%)	56.52 ± 4.93	11.21 ± 0.99	16.24 ± 1.32	8.21 ± 1.29	12.52 ± 0.99	8.56 ± 1.11	33.22 ± 3.01	10.26 ± 1.21	30.25 ± 2.23
+ Diltiazem (0.015%)	54.26 ± 5.12	11.16 ± 1.01	12.29 ± 1.21	8.16 ± 1.21	9.29 ± 0.82	5.29 ± 0.59	20.52 ± 2.19	10.01 ± 1.05	30.11 ± 2.01
+ Diltiazem (0.02%)	55.35 ± 5.22	10.86 ± 0.78	8.56 ± 1.01	8.15 ± 1.09	5.52 ± 0.55	3.28 ± 0.51	10.05 ± 1.51	10.12 ± 1.39	30.62 ± 2.83



**Fig. 1: Midazolam and its 1'-hydroxy metabolite**



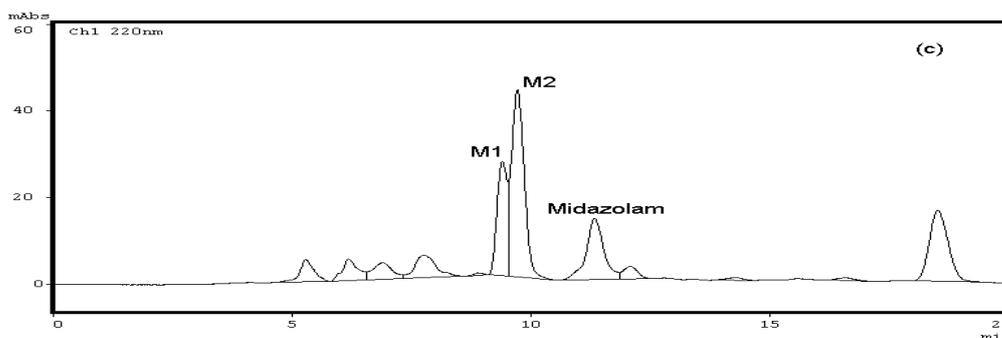


Fig. 2: HPLC chromatograms of a) midazolam drug control, b) culture control and c) metabolites of *Cunninghamella* sp. NCIM 1184

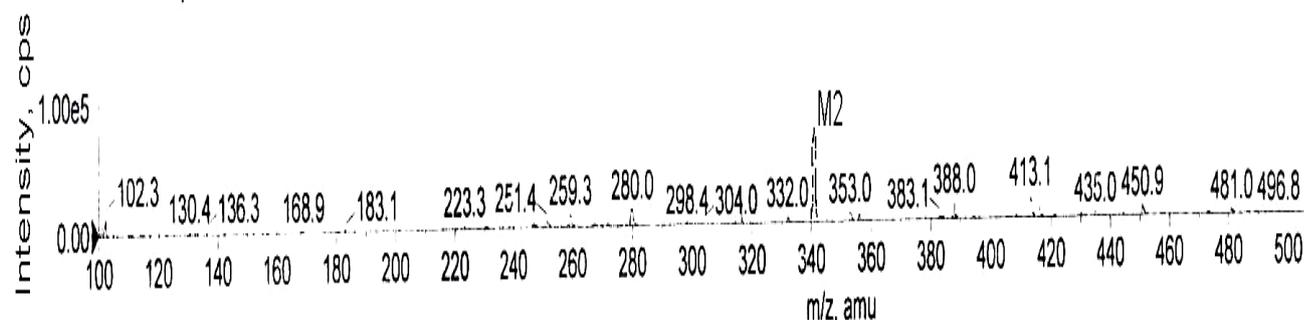


Fig. 3: LC-MS spectra of 1'-hydroxy midazolam

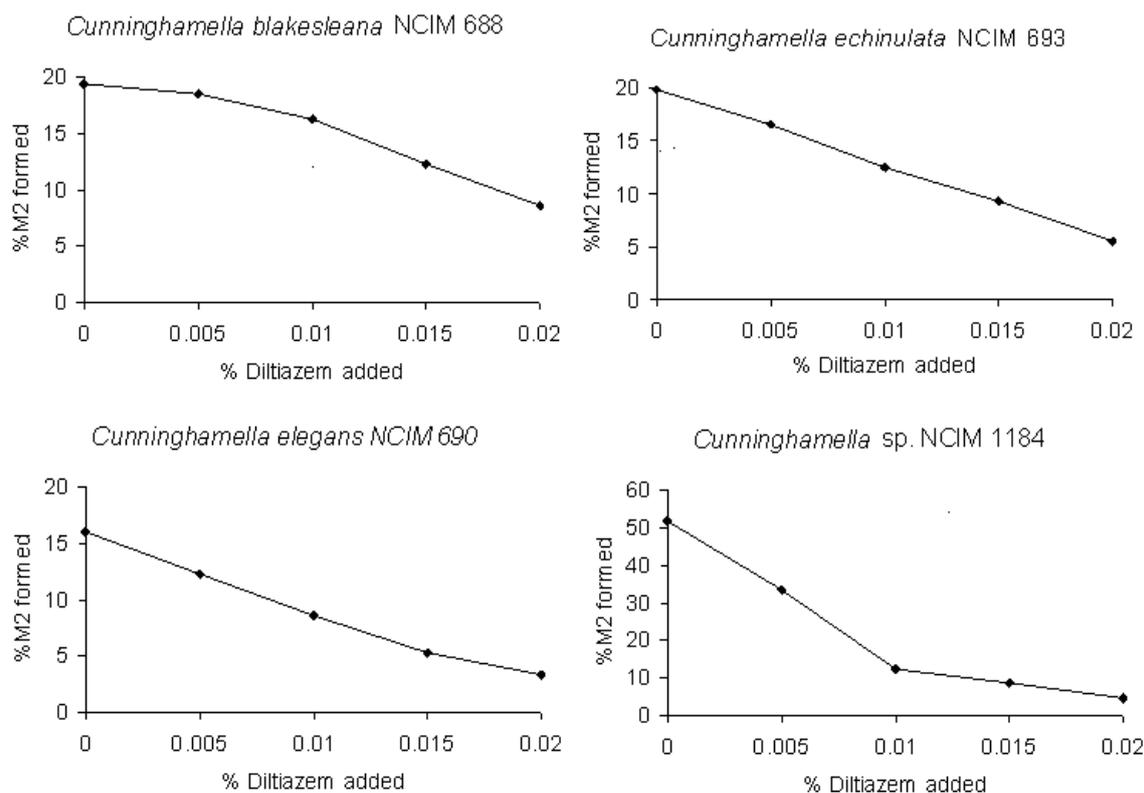


Fig. 4: Effect of diltiazem on the formation of M2 in selected fungal cultures