

Biotransformation of Vincamine using Microbial cultures

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Abstract: Microbial transformation is a complementary tool in the investigation of drug metabolism. In the present investigation, biotransformation of vincamine was studied using microbial cultures. Bacterial, fungal, and yeast cultures were employed to elucidate the metabolism of vincamine. The results indicate that a number of microorganisms metabolized vincamine to various levels to yield three major metabolites, which were identified by HPLC-DAD and LC-MS-MS analyses. HPLC analysis of the extracts of the cultures showed that 16 out of 39 cultures were able to metabolize vincamine to produce one or more metabolites and the biotransformed products are more polar than the substrate vincamine. Out of 16 cultures maximum biotransformation of vincamine was observed with the cultures of *Absidia coerulea* MTCC 1335. The major metabolite was hydroxylated metabolite of vincamine, while the other metabolites were produced by dihydroxylation and methyl hydroxylation which are not seen in mammalian metabolism. The results will support the formation of new metabolites and more polar metabolites which have significance in the improvement of pharmacokinetic parameters of vincamine derivatives.

Keywords: Biotransformation, Microbial Cell Cultures, Vincamine, Metabolites, HPLC, LC-MS-MS

I. Introduction

An important factor in the evaluation of safety and efficacy of any drug is the knowledge of how it is metabolized. Traditionally, drug metabolism studies are conducted on small animal models, perfused organs, cell cultures and enzyme systems [1]. It's a universal fact that microorganisms are sources of many enzymes and they catalyze different types of chemical reactions. Reports [2] indicate that some microorganisms especially fungi are found to possess the cytochrome p450 group of enzymes. The cytochrome p450 enzymes are responsible for a large number of Phase I metabolic pathways. Microbial models may constitute an alternative to the use of animal models, if they can mimic the mammalian metabolism and can give some information about the metabolic fate of the drug. These microbial biotransformation processes are also important to produce newer metabolites which were formed by the introduction of functional groups into unattainable sites of the molecules [3].

Vincamine is an alkaloid obtained from the leaves of *Vinca minor* L.; or *Catharanthus roseus* (*Apocynaceae*). It has been therapeutically used as a vasodilator and antihypertensive agent, particularly in cerebrovascular disorders [4]. Vincamine is used for the prevention and treatment of cerebrovascular insufficiencies and disorders [5]. Vincamine is almost completely metabolized in rats after oral administration. The main urinary metabolites were vincamine conjugates. Radiolabel studies in rats after an oral dose of 10 mg/kg body weight demonstrated that vincamine is hydrolysed by the plasma esterases to vincaminic acid which is quickly decarboxylated and oxidized to eburnamenine. Vincamine is also hydroxylated to 6- β -hydroxyvincamine, which accounts for 40% of the total urinary and biliary excretion, followed by 6- α -hydroxyvincamine (8%), and conjugated 6-keto-vincamine (10%) [6].

The aim of present investigation is to study the metabolites of vincamine formed by microorganisms and to compare them with those produced in mammals. To date there is no report on microbial biotransformation of vincamine. Since vincamine is metabolized by CYP group of enzymes by oxidative pathway, microbial cultures particularly fungi may provide an alternative to produce these mammalian metabolites [7]. Fungi serve as a source of CYP and there are chances of producing new metabolites that possess activity which may increase cerebral blood flow or any other interesting activity. Close structural similarity between the agents which enhances the memory power and the possibility of producing new and unusual metabolites with microorganisms prompted us to investigate the microbial metabolism of vincamine. The importance of introduction of polar groups in enhancing the pharmacokinetics and reducing the toxicity of a semisynthetic vincamines such as vinpocetin and ethyl apovincaminatate [8, 9] are also the reasons for selecting vincamine to study for microbial metabolism. In the present investigation, a screening study was performed by vincamine for its metabolism by 29 fungal, 3 bacterial and 7 yeast cultures.

II. Materials And Methods

2.1 CHEMICALS

Vincamine used in the study was in amorphous form, kindly gifted by Covex Pharma, Madrid, Spain. The solvents were purchased from Ranbaxy Fine Chemicals Ltd., New Delhi and all other chemicals of highest available purity were purchased from Hi Media Laboratories Pvt. Ltd., Mumbai. The organisms capable of producing mammalian metabolites were selected and obtained from Microbial Type Culture Collection and Gene Bank (MTCC), Chandigarh, India and National Collection of Industrial Microorganisms (NCIM), NCL, Pune, India.

2.2 CULTURE PROCEDURE

Procured cultures were revived, sub cultured and stored in refrigerator at 4°C. The bacterial, fungal and yeast cultures were maintained on nutrient agar, potato dextrose agar and MGYB (malt extract, glucose, yeast and peptone) agar slants respectively. The media used for biotransformation studies of fungi cultures is Dextrose broth, supplemented with 0.02% Triton X 100 [10], bacteria is nutrient agar broth and for yeast cultures is MGYB broth. Media was prepared in 50ml conical flasks with 10ml media in each flask. The flasks were cotton plugged, covered with aluminum foil and sterilized by autoclaving at 121°C (15 lb in²) for 15min.

2.3 BIOTRANSFORMATION

The second stage cultures (10ml culture in 50ml capacity conical flask) were added with 2mg each of vincamine (in 100 μ l solvent (mixture of dichloromethane and methanol in the ratio 1: 3)) using a micropipette. Prior to addition, the drug solution was filter sterilized using sterile syringe driven filter unit of 0.45 μ m (Millipore, MA, USA). The final drug concentration in each flask will be 0.2g l⁻¹ culture. The flasks were gently shaken immediately after the addition of drug for its even distribution and minimizing the toxic effects to some cells. The precipitate of vincamine was not visually observed in the culture. 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 drug. The culture controls were added with 100 μ l solvent, 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 ten days, the flasks were taken out, extracted and analyzed.

2.4 EXTRACTION AND SAMPLE PREPARATION OF VINCAMINE AND METABOLITES

The cultures, after ten days of incubation with vincamine, were taken out and extracted with 3 \times 10 ml of ethyl acetate by vortex mixing for 1 min. The combined organic phase (upper ethyl acetate layers) was evaporated under reduced pressure followed by drying in vacuum oven. The dried samples were reconstituted in 1.5ml each of HPLC grade methanol by vortex mixing for 1min. The samples were then taken into micro centrifuge tubes and centrifuged at 12,000x g and 20°C for 20min in micro refrigerated centrifuge. The supernatants were taken out and used for HPLC-DAD and LC-MS-MS analysis.

2.5 HPLC-DAD ANALYSIS

The samples prepared after extraction were analyzed by an isocratic HPLC method for presence of metabolites. HPLC analysis was performed on LC-10AT (Shimadzu Corporation, Kyoto, Japan) system by injecting 20 μ l of sample using Hamilton Rheodyne syringe (Hamilton Bonaduz AG, Switzerland) into syringe loading sample injector (Model 7725i, Rheodyne LP, CA, USA) following the conditions quite close to the method described previously by Dal Bo[11]. The column used was Luna C18, 5 μ , 250 x 4.6mm i.d. (Phenomenex, USA). The mobile phase consisted of a mixture of acetonitrile-methanol-buffer (10mM ammonium acetate pH adjusted to 7.3 with triethylamine) in 20:30:50 ratio. The mobile phase was degassed using ultrasonic bath (Sonorex, Bandelin Electronic, Germany). The analysis was performed isocratically at a flow rate of 1ml/min at ambient temperature. Drug and its metabolites were detected using diode array detector (Shimadzu SPD M10Avp model, Shimadzu Corporation, Kyoto, Japan) at a wavelength of 270 nm. The data analysis was performed by Class M10 software (Shimadzu Corporation, Kyoto, Japan). The UV absorption spectrum of the metabolites was compared with that of vincamine. 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 m/z values of drug and metabolites and their fragmentation ions were recorded by LC-MS-MS using Perkin Elmer Sciex API mass spectrometer set in positive mode. The API-4000 LC-MS/MS was operated under the multiple reaction-monitoring mode (MRM) using the electrospray ionization technique. The separation was performed on X-Terra MS C-18 column (4.6mm x 50mm, 5 μ). The mobile phase consisted of a

mixture of acetonitrile: methanol: buffer (10mM ammonium acetate pH adjusted to 7.3 with triethylamine) in 20:30:50 ratio, was isocratically pumped at a flow rate of 1ml/min. 50 μ l of the sample was injected using auto sampler device. The vaporizer temperature and the discharge current were set at 300°C and 10 μ A respectively. The fragments were scanned in the m/z range of 50 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.

III. Results

There was no significant change in the medium pH after the addition of the drug. In all the three drug controls (i.e., drug in three different media) studied, none has shown any additional peaks in HPLC analysis indicating that the substrate vincamine was stable in the media. The % recoveries of vincamine from these media were > 92.3%.

HPLC analysis of the extracts of the cultures showed that 16 out of 39 cultures were able to metabolize vincamine to produce one or more metabolites evidenced from the absence of these new peaks in drug control as well as culture controls. Out of 16 microbial cultures the maximum biotransformation of Vincamine was observed with the cultures of *Absidia coerulea* MTCC 1335. Representative HPLC chromatograms were shown in “Fig. 1 and Fig. 2”.

Vincamine was eluted at 21min and the metabolites produced were designated as M1, M2 and M3 were eluted at 4.9min, 7.8min and 8.4min respectively. Control samples with and without vincamine showed no coincidental peaks eluting with identified metabolites. The results pertaining to the metabolite production was shown in Table 1. All the new peaks had shown nearly similar UV spectral pattern from 190 to 370nm

A large variation was observed in the quantitative metabolite production among various cultures. M3 was the metabolite found in most of the cultures. *Absidia coerulea* MTCC 1335 produced 31.76% of this metabolite whereas *Rhizopus stolonifer* NCIM 880 produced only 7.93% of this metabolite.

The metabolites M1, M2 and M3 had shown the protonated molecular ions at m/z values 387.2, 401.2 and 371.3 respectively. The parent drug Vincamine has shown protonated molecular ion at m/z 355.3. Table 2 represents the retention times, m/z values of parent ions (M+H)⁺ and predicted molecular formulae and metabolic reactions for various metabolites. The ionization spectrum of the m/z 355.3 pertaining to vincamine showed product ions at m/z 337.3, 308.4, 294.2, 280.3 and 236.3. Sequential losses of H₂O (18), C₂H₅ (29), CH₂ (14), CH₂ (14) and CO₂ (44) was observed.

HPLC retention time of M1 is 4.91min. Fragmentation pattern of M1 by LC-MS-MS was shown in Figure 3. M1 gave the protonated molecular ion at m/z 387.2 that is 32 higher than the parent drug giving a clue that hydroxylation took place twice. The ionization spectrum of the m/z 387 showed product ions at m/z 369.2, 353.2, 336.2, 308.4, 294.2, 280.3 and 236.1. Sequential losses of H₂O (18), O (16), OH (17), C₂H₄ (28), CH₂ (14), CH₂ (14) and CO₂ (44).

HPLC retention time of M2 is 7.36min. Fragmentation pattern of M2 by LC-MS-MS was shown in Figure 4. M2 gave the protonated molecular ion at m/z 401.2 that is 46 higher than the parent drug. The ionization spectrum of the m/z 401 showed product ions at m/z 383.3, 366.3, 338.3, 324.3, 308.3, 294.8, 280.4 and 236.1. Sequential losses of H₂O (18), OH (17), C₂H₄ (28), CH₂ (14), O (16), CH₂ (14), CH₂ (14) and CO₂ (44).

HPLC retention time of M3 is 8.44min. Fragmentation pattern of M3 by LC-MS-MS was shown in Figure 5. M3 gave the protonated molecular ion at m/z 371.3 which is 16 units higher than vincamine. The ionization spectrum of the m/z 371.3 showed product ions at m/z 353.3, 336.4, 308.4, 294.2, 280.3 and 236.1. Sequential loss of H₂O (18), OH (17), C₂H₄ (28), CH₂ (14), CH₂ (14) and CO₂ (44). Figure 6 represents the proposed biotransformation pathway of vincamine by *Absidia coerulea* MTCC 1335.

IV. Discussion

The effect of drug addition on the pH of the media was studied since pH is an important factor in the growth and physiology of the microorganism and also in the biotransformation. All the bacterial and yeast cultures produced fine suspensions in liquid media, but the fungi studied produced matt forming cultures. To avoid matt formation Triton X 100 was added to the medium [10]. This also improved the permeability and thus increased the uptake vincamine.

During the first stage, all the bacterial, yeast and fungal cultures produced fine suspensions in liquid media and attained good growth within 48hours. Second stage culture was used for the biotransformation study. Many organisms produced the same range of 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). All the metabolites produced were eluted before the drug indicating that they are more polar than vincamine. The relative polarity of the metabolites also helped in their characterization.

In HPLC method development, the retention time of the drug was set at around 21min so that the metabolites, being more polar in general, will elute before the drug. 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 370nm) with that of vincamine indicating that the drug has undergone only minor structural changes in the process of metabolism by microorganisms. This also indicates that only some groups have been added from the structure and this observation reveals that there was no loss of aromaticity, ring fission or ring fusion since any of these changes would be expected to lead to a high alteration of the metabolites' UV spectra. The UV λ_{\max} of the metabolites were within a narrow range with that of vincamine which indicates that their extinction coefficient at 270nm was not significantly different from that of the parent drug. Cha reported that the fungi *Cunninghamella elegans* was able to N-demethylate malachite green, where the metabolites showed a slight decrease (618 to 608nm) in absorption maxima (visible λ_{\max}) than the substrate malachite green [12].

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%. Zhang in their study on the fungal metabolism of cyclobenzaprine, quantified the metabolites basing on their areas by expressing as a percent of the drug added originally in the drug control [13]. Hansen Jr. calculated the single metabolite formation based on the initial dose of triprolidine added after 10 days of incubation, and found that the drug and its metabolites were accounted for 23 and 55% respectively [14]. They assumed that the remaining 22% of the initial dose (may contain both drug and its metabolite) might be adsorbed by the fungal cells.

The low production of M3 in *Rhizopus stolonifer* NCIM 880 may be attributed to many reasons such as expression of low 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 fragmentation results obtained in LC-MS-MS analysis. The structures of the metabolites were proposed from the m/z values of the fragmentation ions and HPLC retention times.

Basing on the mass fragmentation and HPLC retention time, M3 was assumed as a hydroxyl metabolite of vincamine, where the hydroxylation took place on the terminal methyl of ethyl side chain of vincamine. This metabolite was not reported in mammals so far. Hansen Jr. reported the formation of hydroxymethyl metabolite of triprolidine using the fungi *Cunninghamella elegans*, where the $-\text{CH}_3$ group present on the benzene ring was hydroxylated to $-\text{CH}_2\text{OH}$ and this reaction was mediated by CYP [14]. Cheng found that *Streptomyces griseus* was able to perform hydroxylation of methyl group at C-30 position of quinovic acid 3-O- β -D-quinovopyranoside and reported that this is a new oxidized metabolite which was difficult to achieve by chemical means [15]. In the present study, 12 cultures have produced this metabolite. Since this metabolite formation occurs by direct oxidation of methyl group on the vincamine molecule, no specific pathway is involved in this reaction. This suggests that M3 might be hydroxylation product of vincamine.

Basing on the mass fragmentation and HPLC retention time, M1 was assumed as a dihydroxyl metabolite of vincamine where the hydroxylations took place on the terminal methyl group of ethyl side chain and on the eburnamenine ring of vincamine. The favorable position for the hydroxylation on eburnamenine ring is either 6 α or 6 β . Formation of this metabolite may take place from M3, which underwent hydroxylation again to produce the dihydroxylated metabolite M1. This metabolite was also not reported in mammalian metabolism. Vigne reported this type of double regiospecific hydroxylation of exo-3-pinanyl-N-phenyl carbamate and exo-3-pinanyl-N,N-methylphenyl carbamate where one hydroxylation occurs on terminal methyl group of the pinanyl moiety and a second hydroxylation takes place on the aromatic ring [16]. The study also reported that, when a compound possess an aromatic ring and an alkyl group, the hydroxylation first occurs on the alkyl group and that the alcohol thus formed serves as a substrate to undergo second hydroxylation on the aromatic ring leading to the dihydroxylated product. Hence, in the present study, it can be assumed that the formation of M1 might take place from M3. This suggests that M1 might be dihydroxylated product of vincamine.

Basing on the mass fragmentation and HPLC retention time, M2 was assumed as a methoxy and hydroxyl metabolite of vincamine where the hydroxylation took place on the terminal methyl group of ethyl side chain and methylation on the hydroxyl (M1) of eburnamenine ring of vincamine (O- methylation) . Hosny reported the methylation of 3' and 4' hydroxyl of fistein, 3' O-methylation of catechin and O-methylation of quercetin by *Streptomyces gresius* [17]. Similarly Hosny and Rosazza reported O-methylation of genistein by *Streptomyces* [18]. Hence, it is assumed that the formation of this metabolite may take place from M3, which underwent hydroxylation again to produce the dihydroxylated metabolite M1 and the 2nd hydroxyl group i.e., the hydroxylation on eburnamenine ring of vincamine underwent methylation to form M2. This metabolite was also not reported in mammalian metabolism.

Some of the cultures employed in the present study did not produce any metabolites for vincamine. This may be attributed to the absence of substrate specific enzymes required for biotransformation. It may also

be due to the substrate not reaching the site of biotransformation, i.e., lack of membrane transport processes involved to get the substrate into the site where the enzymes are known to be located.

V. Conclusion

At the outset, three microbial metabolites were identified from the microbial cultures screened. All the three metabolites were not reported in mammals. It was suggested that the metabolite M3 (Hydroxy metabolite) is formed first which underwent further hydroxylation to form more polar metabolite M1 (dihydroxylated metabolite). The third metabolite M2 is formed by the methylation of the second hydroxy group and was found to be less polar when compared to M1 but it is a little more polar when compared to M3. These observations were confirmed by the retention times of the metabolites by RP-HPLC and LC-MS-MS analysis in which M1 being more polar of the three metabolites was eluted first at 4.9 min., M2 being intermediately polar was eluted at 7.8 min. and M3 being the least polar of the three metabolites was eluted at 8.4 min.

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FIGURE LEGENDS

Figure 1. Representative HPLC chromatogram of vincamine in media (Control 1)

Figure 2. Representative HPLC chromatogram of vincamine biotransformation by *Absidia coerulea* MTCC 1335

Figure 3. Fragmentation pattern of metabolite M1 by LC-MS-MS

Figure 4. Fragmentation pattern of metabolite M2 by LC-MS-MS

Figure 5. Fragmentation pattern of metabolite M3 by LC-MS-MS

Figure 6. Proposed biotransformation pathway of vincamine by *Absidia coerulea* MTCC 1335.

Table 1. Metabolites of vincamine produced by microbial cultures

Culture	Class	M1 (4.9min)	M2 (7.8min)	M3 (8.4min)
<i>Absidia coerulea</i> MTCC 1335	Fungi	38.21	13.57	31.76
<i>Absidia glauca</i> MTCC 982	Fungi	12.17	-	28.13
<i>Aspergillus flavipes</i> NCIM 1209	Fungi	-	-	-
<i>Aspergillus flavus</i> NCIM 554	Fungi	-	-	-
<i>Aspergillus flavus</i> NCIM 557	Fungi	-	-	-
<i>Aspergillus niger</i> NCIM 1006	Fungi	-	-	-
<i>Aspergillus niger</i> NCIM 589	Fungi	-	-	-
<i>Aspergillus niger</i> NCIM 620	Fungi	-	-	-
<i>Aspergillus ochraceus</i> NCIM 1140	Fungi	-	-	-
<i>Aspergillus parasiticus</i> NCIM 898	Fungi	-	-	-
<i>Beauveria bassiana</i> NCIM 1216	Fungi	-	-	-
<i>Cunninghamella blakesleana</i> NCIM 687	Fungi	-	-	22.10
<i>Cunninghamella blakesleana</i> NCIM 688	Fungi	-	-	12.29
<i>Cunninghamella echinulata</i> NCIM 691	Fungi	-	-	16.17
<i>Cunninghamella echinulata</i> NCIM 693	Fungi	-	-	18.32
<i>Cunninghamella elegans</i> NCIM 689	Fungi	-	-	-
<i>Cunninghamella elegans</i> NCIM 690	Fungi	-	-	14.89
<i>Cunninghamella sp.</i> NCIM 1184	Fungi	-	-	12.16
<i>Curvularia lunata</i> NCIM 716	Fungi	-	-	-
<i>Fusarium oxysporum</i> NCIM 1008	Fungi	-	-	-
<i>Mucor plumbeus</i> NCIM 984	Fungi	10.6	-	11.3
<i>Mucor rouxii</i> MTCC 386	Fungi	7.5	-	15.8
<i>Pencillium brevicompactum</i> MTCC 549	Fungi	-	-	-
<i>Pencillium chrysogenum</i> NCIM 733	Fungi	-	-	-
<i>Pencillium chrysogenum</i> NCIM 738	Fungi	-	-	-
<i>Rhizopus arrhizus</i> NCIM 997	Fungi	8.52	-	9.14
<i>Rhizopus stolonifer</i> NCIM 880	Fungi	3.54	-	7.93
<i>Thamnostylum piriforme</i> NCIM 974	Fungi	-	-	-
<i>Trichothecium roseum</i> NCIM 1147	Fungi	-	-	18.15
<i>Bacillus subtilis</i> MTCC 619	Bacteria	-	-	-
<i>Escherichia coli</i> MTCC 118	Bacteria	-	-	-
<i>Pseudomonas putida</i> NCIM 2782	Bacteria	-	-	-
<i>Rhodotorula rubra</i> NCIM 3172	Yeast	-	-	-
<i>Saccharomyces cerevesiae</i> NCIM 3090	Yeast	-	-	-
<i>Streptomyces griseus</i> NCIM 2622	Yeast	12.39	9.46	16.15
<i>Streptomyces griseus</i> NCIM 2623	Yeast	11.15	8.18	13.35
<i>Streptomyces lavendulae</i> NCIM 2827	Yeast	-	-	-
<i>Streptomyces rimosus</i> NCIM 2213	Yeast	12.68	-	21.32
<i>Streptomyces sp.</i> NCIM 2214	Yeast	-	-	-

Note: Values indicate the percentage of metabolites' area in HPLC analysis. The value is the percentage of a metabolite with respect to the total area of drug and all the metabolites formed.

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

Metabolite	Rt	m/z value	Predicted reaction	Predicted Molecular formulae
Vincamine	21.2	355.3	-	C ₂₁ H ₂₇ N ₂ O ₃
M1	4.9	387.2	Dihydroxylation	C ₂₁ H ₂₇ N ₂ O ₅
M2	7.8	401.2	Hydroxylation and Methoxylation	C ₂₂ H ₂₉ N ₂ O ₅
M3	8.4	371.3	Hydroxylation	C ₂₁ H ₂₇ N ₂ O ₄

Figure 1. Representative HPLC chromatogram of vincamine in media (Control 1)

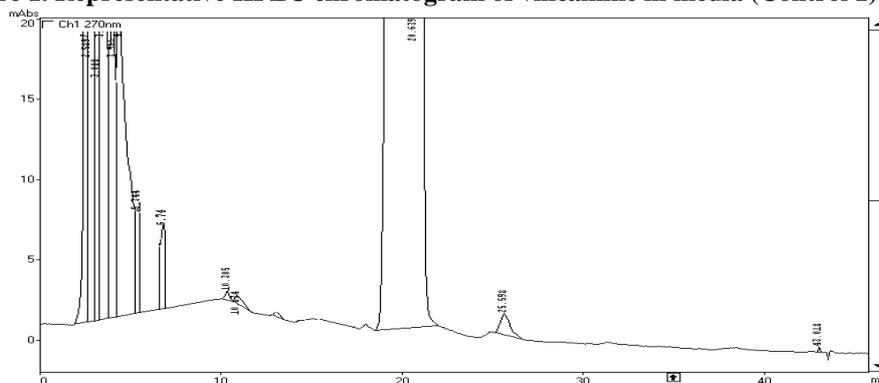


Figure 2. Representative HPLC chromatogram of vincamine biotransformation by *Absidia coerulea* MTCC 1335

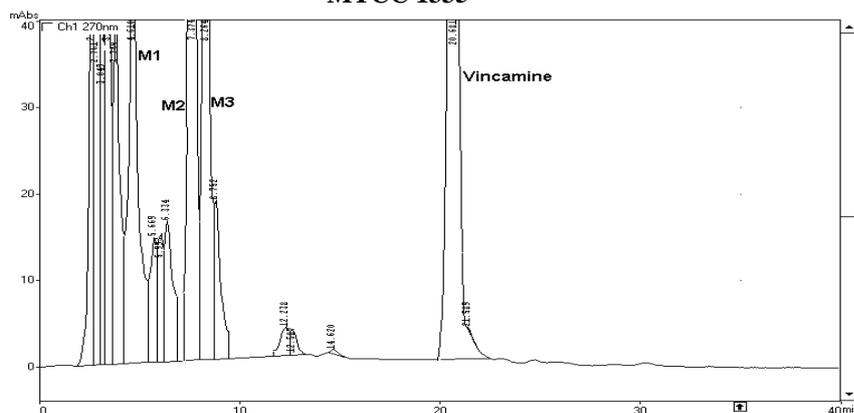


Figure 3. Fragmentation pattern of metabolite M1 by LC-MS-MS

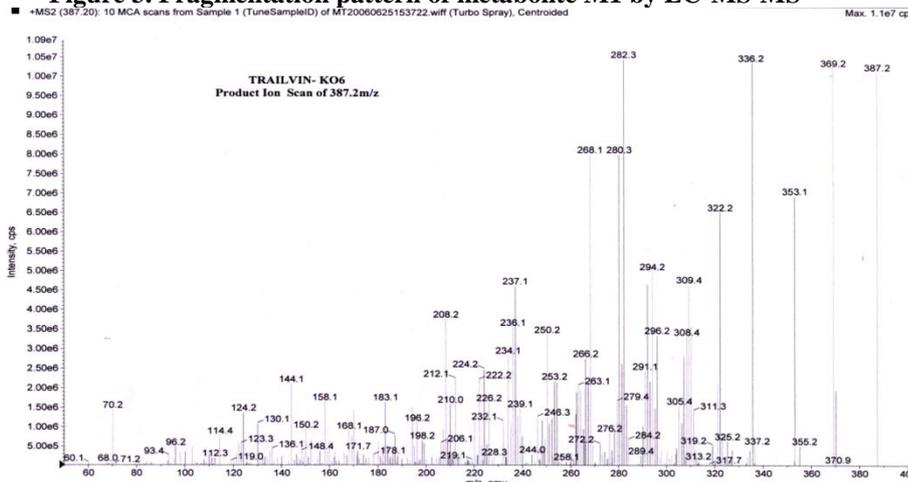


Figure 4. Fragmentation pattern of metabolite M2 by LC-MS-MS

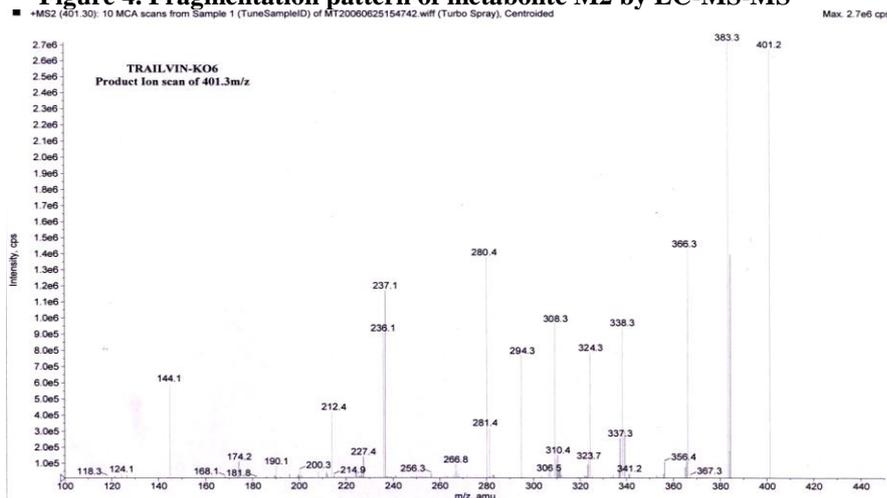


Figure 5. Fragmentation pattern of metabolite M3 by LC-MS-MS

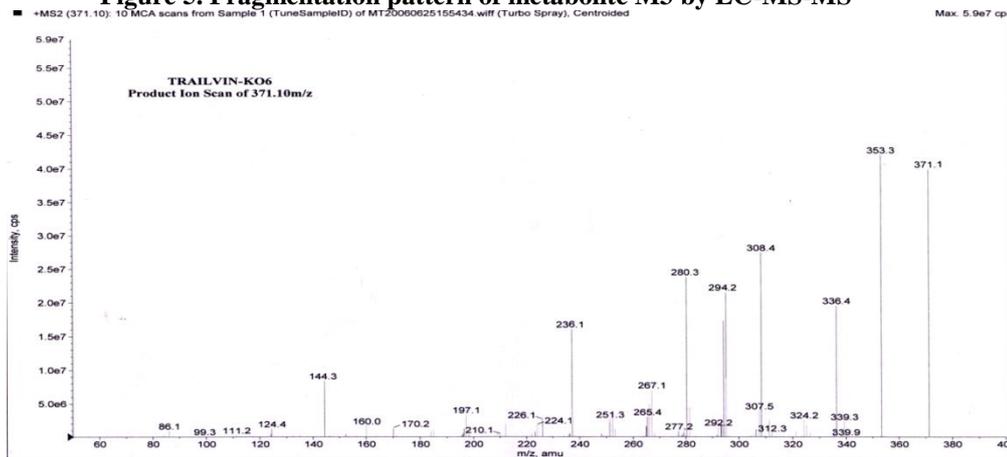


Figure 6. Proposed biotransformation pathway of vincamine by *Absidia coeruleae* MTCC 1335.

