Venlafaxine is a novel antidepressant, which acts by inhibition of the reuptake of presynaptic noradrenalin and serotonin. In humans, it is metabolized by CYP450 2D6 to an active metabolite O-desmethyl venlafaxine, which has antidepressant activity similar to that of parent drug. Microorganisms have recently been successfully used as models for drug metabolism studies and for obtaining metabolites that could be developed as new drug entities. In the present investigation venlafaxine was used for producing an active metabolite by microbial model using different microorganisms. For estimation of venlafaxine and its metabolites in microbial biotransformation studies, a rapid, specific and sensitive HPLC method was developed. Linearity was observed over a concentration range of 0.5µg – 10µg/ml. Accuracy (98.15%) was achieved for all quality controls with intra-day and inter-day variation coefficient less than 8%. No endogenous interfering peaks were visible with blank culture media. A metabolite peak was found in the sample of *Saccharomyces cerevisiae* culture among five organisms used. This method was used for estimation of the venlafaxine metabolites in microbial biotransformation studies.

**Keywords:** Venlafaxine, microbial biotransformation, venlafaxine HPLC

**1. Introduction**

Venlafaxine is a novel phenethylamine bicyclic antidepressant (fig.1) which inhibits the reuptake of both noradrenalin and serotonin(1,2,3). In human, venlafaxine is well absorbed and is extensively metabolized to two less active metabolites N-desmethyl and N,O- didesmethyl metabolite and one active metabolite O- desmethyl venlafaxine (fig.1) has antidepressant activity profile similar to that of parent drug (4). Traditionally, drug metabolism studies were conducted on small animal models, perfused organs (5,6) in vitro enzyme systems and in vitro cell cultures. Later microbial models were developed as an alternative methods to study the metabolic fate of the drug with advantages of reducing the use of animals, in the early phases of drug development.

Microorganisms such as bacteria and fungi were used as in vitro models for the prediction of mammalian drug metabolism with successful applications (7,8,9). A systematic examination of microbial hydroxylation on variety of model organic compounds (10) followed by a comparison of O- and N-dealkylation reactions led Smith and Rosazza (8) to propose that a microbial transformation systems could closely mimic most of the phase I transformations of a drug observed in mammals. The use of microorganisms as models of mammalian metabolism has been well documented (11,12,13,14)
for obtaining novel metabolites as new drug entities and also for producing existing metabolites in large amounts.

In the present investigation, different microorganisms were used for evaluating their ability to metabolize venlafaxine. The aim of this study was to identify the microbes that can be used for production of an active metabolite of venlafaxine O-desmethyl venlafaxine in larger quantities for further characterization as well as pharmacological and toxicological evaluation. For that, the estimation of venlafaxine and its metabolites in microbial culture media is essential. The metabolites of drugs formed by microorganisms in culture are identified and confirmed by TLC, HPLC, LCMS or NMR techniques.(15) But the published methods for venlafaxine analysis (16) are only in biological fluids which include mainly solid phase extraction of drugs and are tedious (17). Therefore the present study is aimed at development of a simple, rapid and useful method for identification of the venlafaxine and its metabolites in the microbial culture media.

2. Materials and Methods

2.1. Microorganisms

Cultures were obtained from NCL, Pune, India. The cultures used in the present work were, Proteus vulgaris (NCIM 2027), Pseudomonas aeruginosa (NCIM 2053), Nocardia hydrocarbooxydans (NCIM 2386), Cunninghamella elegans (NCIM 689) and Saccharomyces cerevisiae (NCIM 3090). These were selected from different types of microorganisms i.e. bacteria, fungi, and yeast. Based on the literature few of these were used for different substrates and found that are mimicking human metabolism.

Vidyavathi et al

---

Fig. 1: Mammalian metabolic pathway of venlafaxine
2.2 Chemicals

Venlafaxine was obtained from Vimta labs, Hyderabad, India. All the reagents used in the analysis were of HPLC grade. Acetonitrile and sodium dihydrogen phosphate were purchased from Merck, Mumbai, India. Chloroform, Isopropanol, n-Heptane were obtained from SD. fine chemicals Ltd., Mumbai, India. Culture media was purchased from Himedia, Mumbai, India.

2.3 Fermentation procedure

The experiments were carried out using their respective growth media consisting of the following composition: For bacteria: Peptone 1 g, sodium chloride 0.5 g, beef extract 1 g, distilled water 100 ml and pH adjusted to 7.0-7.2. For fungus: Potato extract, dextrose 2 g, yeast extract 0.3 g, peptone 0.5 g, distilled water 100 ml. For yeast: Malt extract 0.3 g, glucose 1 g, yeast extract 0.3 g, peptone 0.5 g, distilled water 100 ml. pH adjusted to 6.4-6.8. Stock cultures were stored on agar slants prepared according to the above composition at 4°C, and transferred for every 2 months to maintain viability. The media were sterilized in an autoclave for 20 min. at 121°C and 15 lb/sq.in. Microbial metabolism studies were carried out by shake flask cultures in an incubator shaker, operating at 120 rpm at 32°C. The experiments were carried out in conical flask (250 ml) containing 50 ml. growth medium. Fermentations were carried out according to standard protocol. In brief, the substrate (venlafaxine) was prepared as a 1% (w/v) solution in methanol and added to the culture medium of selected organisms at a concentration of 10 μg/ml of medium in samples and incubated in shaker. The study also maintained the substrate control to which substrate was added and incubated without microorganisms and culture control consisted of fermentation blanks in which the microorganisms were grown under identical conditions without the substrate. The incubation was continued for 48 h.

2.4 HPLC analysis of extracts of microbial samples

2.4.1. Extraction procedure:

The pre incubated medium was heated on water bath at 50°C for 30 min. and centrifuged at 4000 rpm for 10 min. at 37°C (Remi instruments Pvt. Ltd., Mumbai, India). A clear supernatant liquid was collected and extracted by mixture of chloroform, isopropanol, n-heptane (HPLC grade, Ranbaxy Fine Chemical Ltd., Delhi, India) at a ratio of 60:14:26(15). The upper organic layer was collected from two immiscible layers and was dried. The extract was reconstituted with 1ml. acetonitrile (HPLC grade, Ranbaxy Fine Chemical Ltd., Delhi, India) and centrifuged at 13000 rpm for 8 min. at 37°C in Biofuge fresco centrifuge (Heraeus, Germany). 20 µl portions were injected into the HPLC. Calibration standards were prepared in the range of 1.0 to 250μg/ml.

2.4.2. Chromatographic conditions:

High performance liquid chromatography (HPLC) analysis was conducted using a HPLC system (Shimadzu, Kyoto, Japan) consisted of LC-8A solvent delivery module and SPD-10AVP UV-Visible spectrophotometric detector and a Wakosil II5c-18rs-100a 5UM, 4.6X 250 mm SS column (SGE, Japan). Sensitivity was set at 0.001 augs. Mobile phase consisted of acetonitrile and 0.05 M disodium hydrogen phosphate buffer of pH 3.8 (25:75 v/v) with a flow rate of 1 ml/min. Elute was monitored using a UV/Vis detector set at 200 nm.

2.4.3. Standard solutions

Stock solution of 1mg/ml of venlafaxine was prepared in methanol and stored at 4°C. Appropriate dilutions of venlafaxine were made in methanol to produce working stock solutions of 50.0, 10.0, 1.0 μg/ml. these dilutions were used to spike in culture media in the preparation of calibration curves. Calibration samples were prepared by spiking 200µl of media with the appropriate amount of the drug on the day of
analysis. Samples for the determination of recovery, precision and accuracy were prepared by spiking control media in bulk at appropriate concentrations (1, 10, 50 µg/ml) and stored at -20°C.

3. Results and Discussion

3.1. Chromatography

Typical chromatogram corresponding to blank media and sample media obtained after adding 200µl of 10 µg/ml venlafaxine in sample of *Cunninghmela elegans* culture are shown in (figure 2 a,b) respectively. No endogenous (broth) interfering peaks were visible in blank media at retention time of venlafaxine confirming the specificity of the analytical method. System suitability parameters for the method were as follows: theoretical plates for venlafaxine were 2024, tailing factors were less than 1.25.

3.2. Quantification

A representative calibration graph of peak area versus venlafaxine concentration in the range of 0.5 µg to 10 µg resulted in regression equation $y = 252773X + 57656$ ($r^2 = 0.9993$) (fig.3) the lowest concentration with relative standard deviation (RSD) <20% was taken as lower limit of quantification (LLOQ) and was found to be 0.05 µg/ml. The RSD and S/N ratio at LLOQ were found to be 15% and 6% respectively.

3.3. Precision

Precision of assay was determined by analyzing media samples containing venlafaxine at three different concentrations. Samples for precision study were obtained by spiking blank media with the analytic solution at each concentration in bulk and the aliquots were stored in ependroff tubes at -4°C. The intra-day precision was determined by analyzing six spiked media samples at each concentration on the same day. For the determination of inter-day precision, fortified samples were analyzed on four different days. The inter-day relative standard deviation

Fig. 2.0  HPLC chromatograms of venlafaxine and its metabolite
(a) Typical HPLC chromatogram of blank culture media (solvent peak)
(b) Typical HPLC chromatogram of venlafaxine (solvent peak and drug peak).
(c) HPLC chromatogram of venlafaxine and its metabolite in *Saccharomyces cerevesiae* culture media (M – Metabolite; D- Drug) (solvent, metabolite and drug peak).
(RSD) ranged from 1.02 to 3.73 at 1.0 µg/ml, 1.16 to 5.03 at 10.0 µg/ml and 3.33 to 5.01 at 50.0 µg/ml. The intra-day RSD were 1.03, 2.16 and 1.95 for 1, 10 and 50 µg/ml, respectively. These values are within the limits (table1) (<15%) specified for inter and intra day precision.

### 3.4. Recovery and accuracy

The extraction recovery of venlafaxine was estimated at 1, 10, 50 µg/ml concentrations. Media samples (in six replicates) containing venlafaxine were extracted and analyzed. Six samples containing similar concentrations of the

#### Table 1. Inter and Intra day variation of venlafaxine analysis in culture media

<table>
<thead>
<tr>
<th>spiked concentration µg/ml variation</th>
<th>µg/ml</th>
<th>day</th>
<th>Measured concentration*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Inter day variation</strong></td>
<td></td>
<td></td>
<td>S.D</td>
</tr>
<tr>
<td>1.0</td>
<td></td>
<td>0</td>
<td>0.98</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>1.02</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>1.35</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>0.96</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
<td>0.99</td>
</tr>
<tr>
<td>10.0</td>
<td></td>
<td>0</td>
<td>10.23</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>9.87</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>10.53</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>10.27</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
<td>9.06</td>
</tr>
<tr>
<td>50.0</td>
<td></td>
<td>0</td>
<td>50.13</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>50.95</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>50.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>49.27</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
<td>48.97</td>
</tr>
<tr>
<td><strong>Intra day variation</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.0</td>
<td></td>
<td>0</td>
<td>0.97</td>
</tr>
<tr>
<td>10.0</td>
<td></td>
<td>0</td>
<td>10.62</td>
</tr>
<tr>
<td>50.0</td>
<td></td>
<td>0</td>
<td>49.63</td>
</tr>
</tbody>
</table>

Microbial Biotransformation Studies
Table 2  Recovery and accuracy of determination of venlafaxine in culture media

<table>
<thead>
<tr>
<th>Concentration (µg/ml)</th>
<th>Absolute recovery (mean ± S.D. n=6)</th>
<th>Accuracy (% (mean ± S.D. n=6)</th>
<th>Range (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>92.49±1.34</td>
<td>99.67±1.97</td>
<td>0.92 - 1.23</td>
</tr>
<tr>
<td>10.0</td>
<td>98.97±1.07</td>
<td>98.32±1.32</td>
<td>9.78 – 10.32</td>
</tr>
<tr>
<td>50.0</td>
<td>99.12±2.31</td>
<td>97.01±0.99</td>
<td>48.90 – 51.23</td>
</tr>
</tbody>
</table>

(compound in mobile phase were directly injected and peak areas were measured. Absolute recovery was calculated by comparing the peak areas for direct injection of pure venlafaxine in methanol with those obtained by media samples containing same amount of venlafaxine. The range of absolute recoveries was from 92.49 to 99.12 (table 2). The accuracy of the method was verified by comparing the concentrations measured for venlafaxine spiked in media with the actual added concentrations. The results (table2) indicate that accuracy of the method was 97.01 to 99.67%. Thus this method is quite simple, sensitive and accurate.

3.5. Metabolite identification in microbial cultures

In the HPLC analysis of the culture extracts of selected organisms, the obtained peaks were compared with controls. An additional peak at 6min. was found in sample of *Saccharomyces cerevesiae* culture extract when compared with its controls. (fig.2 c). It indicates metabolite of venlafaxine was formed by *Saccharomyces cerevesiae*.

4. Conclusions

The HPLC method developed is quite simple, sensitive and accurate and can be adopted for estimation of venlafaxine and its metabolites in the microbial culture media in metabolism studies. It was also found that the *Saccharomyces cerevesiae* is able to metabolise the venlafaxine among the tested microbes.

**References**


Microbial Biotransformation Studies