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MICROBIAL PROCESS FOR THE SYNTHESIS OF GLUCURONIDES OF ATORVASTATIN AND SIMVASTATIN

Kalpana Divekar*, Eshwar Reddy and Brahmani Priyadarshini S. R.

College of Pharmaceutical Sciences Dayananda Sagar University K.S. Layout, Bangalore 560078.

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***Corresponding Author**
Kalpana Divekar
College of Pharmaceutical Sciences Dayananda Sagar University K.S. Layout, Bangalore 560078.

ABSTRACT

Aim of this study was to investigate a novel method for the synthesis of glucuronides of atorvastatin and simvastatin using microorganism. In the first step of the investigation, some of the fungal species like Aspergillus ochraceous, Aspergillus flavus, Aspergillus niger, Rhizopus stolanifer MTCC 162, Rhizopus stolanifer MTCC 2591 and Saccharomyces cerevisiae were screened for their ability to bring about glucuronidation of the two selected statins. In the second step, the bioconversion was carried out in the presence of surfactant, SLS. In the screening, it was found that Aspergillus flavus and Aspergillus ochraceous were capable of bringing out the glucuronidation of Atorvastatin and Aspergillus niger of Simvastatin. Aspergillus ochraceous, converted atorvastatin to three glucuronide conjugates, whereas with Aspergillus flavus, four different glucuronide conjugates were identified. The study also indicated that the bio conversion was higher when carried out in presence of a surfactant.

KEYWORDS: Glucuronides, atorvastatin, simvastatin, Aspergillus flavus,

INTRODUCTION

Statins, also called HMG-CoA reductase inhibitors, are well established as lipid lowering agents and are used extensively in the management of hyperlipidemia, hypertriglyceridemia and atherosclerotic disease by targeting the rate limiting enzyme, hydroxyl methyl glutaryl CoA enzyme, involved in cholesterol biosynthesis.[1]

Metabolism of drugs are classified into two phases where, phase I involves reactions that increase the polarity of the compound and phase II comprises mainly of conjugation reaction

for removal of reaction group which results in increase of active transport and thereby facilitate excretion of the drug.[2] Glucuronidation is one such important pathway where an endogenous substrate like glucuronic acid conjugates with the drug molecule. Such metabolic activity can result in the formation of toxic or pharmacologically active chemicals. Therefore regulatory agencies including US FDA recommends testing of drug metabolites and the evaluation of their safety in clinical research.[3]

Statins undergo varying degrees of metabolism in both animals and humans, catalyzed primarily by the cytochrome P450 system and followed by β-glucuronidation at the dihydroxy heptanoic or heptenoic acid side chain.[4] Atorvastatin is converted extensively to three glucuronidated forms after oral administration. Three glucuronidated metabolites of Atorvastatin in man are: atorvastatin acyl glucuronide conjugate, lactone ether glucuronide conjugate and ether glucuronide conjugate[5] whereas simvastatin is converted to simvastatin hydroxyl acid acyl glucuronide. Testing of these metabolites require the synthesis of adequate amounts of the metabolites. Chemical preparation of these authentic drug metabolites requires multiple steps and is often cumbersome.[6] Therefore there is an immense need to explore alternative methods for synthesis of drug metabolites.

Microorganisms have been reported to conduct biochemical reactions similar to mammalian metabolism. Glucuronidation, however, is not well documented. A literature search revealed that only some microorganisms being reported to add glucuronic acid to either natural products or synthetic organic compounds.[7] This article describes a novel process for the preparation of glucuronides from Atorvastatin and simvastatin utilizing the microorganisms Aspergillus ochraceous, Aspergillus flavus, Aspergillus niger, Rhizopus stolanifer MTCC 162, Rhizopus stolanifer MTCC 2591 and Saccharomyces cerevisiae.

MATERIALS AND METHODS

Chemicals: Simvastatin and atorvastatin were kind gift samples from Dr Reddy's, Hyderabad. Inorganic salts and buffer salts were obtained from Qualigens. Growth media compounds were procured from Hi-Media (Mumbai, India). All other chemicals used were of analytical grade.

Microorganisms: Saccharomyces cerevisiae MTCC 174, Aspergillus niger MTCC 961, and Rhizopus stolonifer MTCC 2198 were obtained from MTCC, Chandigarh.

Aspergillus niger, Aspergillus flavus, and Aspergillus oryzae species were cultured and isolated in the microbiology department of Dayananda Sagar College of Biological sciences, identified and authenticated at Bangalore University.

Media: The organisms were maintained on MRBA media containing Dextrose 10.0g, Peptone 5.0g, Potassium dihydrogen phosphate 1.0g, Magnesium sulphate 0.5g, Rose Bengal 0.0035g, Agar 20.0g, Distilled water 1000 ml and Streptomycin 0.03g.

METHODOLOGY

The experimental work was divided into three phases:

1. Cultivation of the microorganism for obtaining substantial biomass.
2. Bioconversion of the substrate.
3. Extraction and analysis of the product.

The spores from the maintenance culture was inoculated onto sterilized potato dextrose medium (PDB) containing potato 200.0g, dextrose 5.0g, and distilled water 1000ml. The pH of the medium was adjusted to 6.0. The inoculated medium was incubated at 25 °C for 5 days to get sufficient biomass.

After sufficient growth of the bioconversion medium, the substrate (Simvastatin and Atorvastatin) was added to the medium aseptically to obtain the final concentration 0.5mg/ml. The flasks were then incubated at 30^0C, 160rpm for 72hrs.

After 72 hrs the broth was filtered at pump. Then the filtrates were analyzed by LC/MS for the presence of glucuronidated products.

Bioconversion with surfactant

About 4 mg of substrate (statins) dissolved in dimethyl sulphoxide (DMSO), was mixed with 12 mg of sodium lauryl sulphate (SLS) and stirred for 10min.[8] The solvent was then removed under reduced pressure and the solid obtained was used for bioconversion as mentioned earlier.

LC/MS analysis

LC/MS analyses was carried out using a Micromass (Beverly, MA, USA) Quattro 1 triple quadrupole mass spectrometer. The ionization method used was Atmospheric pressure

Chemical ionisation in both the positive and negative ion mode. The LC equipment comprised of a SHIMAZDU, Japan HPLC system consisting of C18 Phenomenex cartridge with dimensions 250mmx4.6mm x 0.5µm. The solvent system was methanol/ water (90/10) at a flow rate of 0.2 ml min^{-1}. The injected volume was 5µl. The detector was a UV photodiode array (PDA) detector with detection at 254 nm.

RESULTS AND DISCUSSION

The glucuronidation process was carried out in two stages. In the screening, it was found that *Aspergillus flavus* and *Aspergillus ochraceous* were capable of bringing out the glucuronidation of Atorvastatin and *Aspergillus niger* of Simvastatin. The LC/MS spectra of atorvastatin and simvastatin is shown in figure 1 and 2 respectively. For Atorvastatin, among the two, *Aspergillus flavus* showed maximum conversion (figure 3). *Aspergillus ochraceous,* converted atorvastatin to three different glucuronide conjugates. The three glucuronide conjugates were identified as protonated ions of Atorvastatin lactone ether glucuronide with the loss of aniline moiety, Atorvastatin lactone ether glucuronide and Atorvastatin acyl β-D-glucuronide with m/z values 625, m/z 717 and m/z 735 respectively. Whereas with *Aspergillus flavus*, four different glucuronide conjugates were identified. Three of the glucuronides have been reported earlier whereas one was identified as protonated ions of atorvastatin acyl β-D- glucuronide moiety with the loss of aniline moiety with m/z value 642.[9]

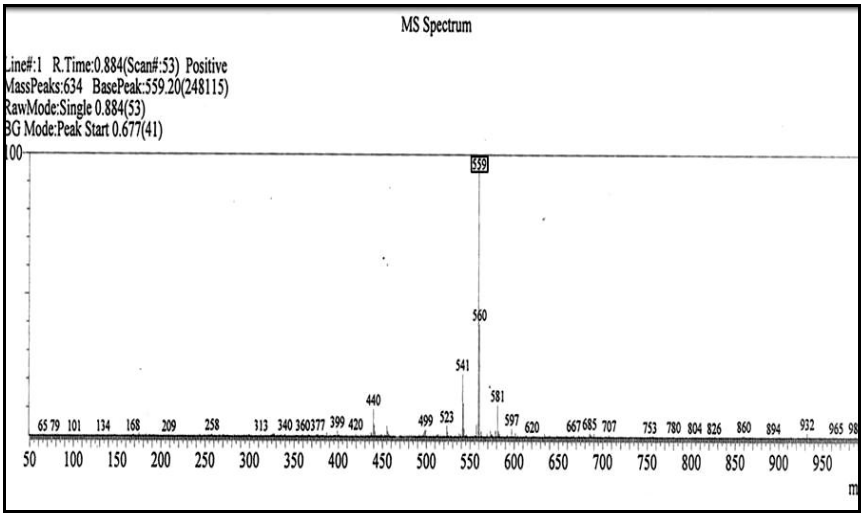

Figure 1: LC/MS Spectra of atorvastatin.

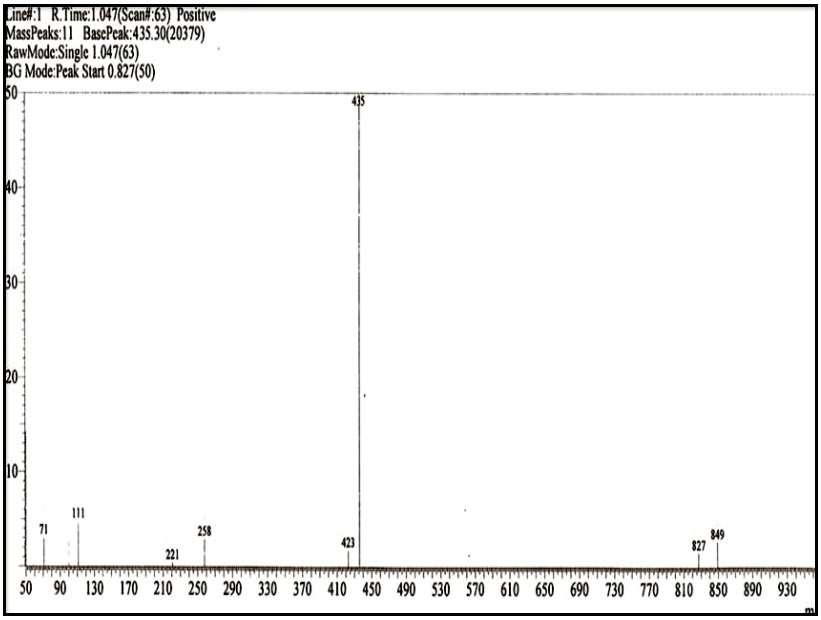

Figure 2: LC/MS Spectra of simvastatin.

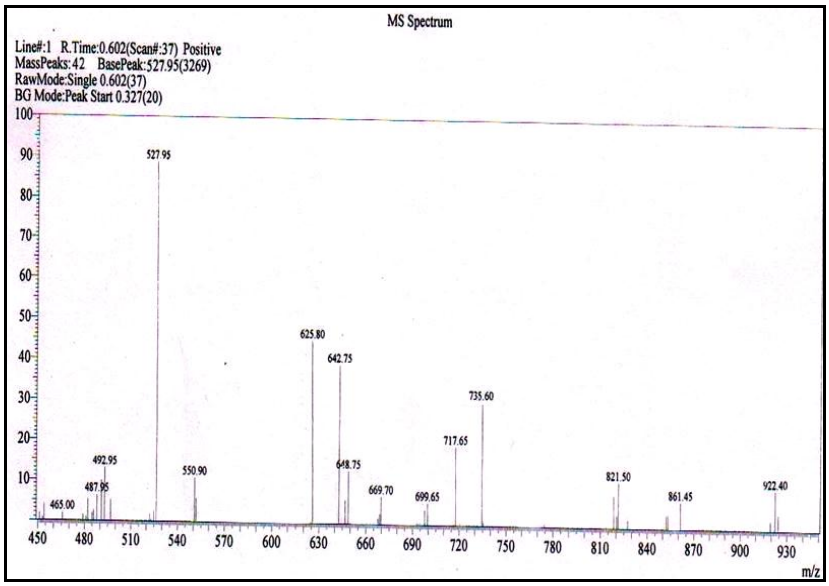

Figure 3: LC/MS spectra of Atorvastatin with *Aspergillus flavus*.

Simvastatin was converted to, two glucuronide conjugates of Simvastatin by *Aspergillus niger* (Figure 4). They were protonated ion of 1-propionic acid derivative of Simvastatin hydroxy acid glucuronide and Simvastatin acyl β-D- glucuronide with m/z values 525 and 613 respectively. Apart from these two, one more rare metabolite was identified i.e., the protonated ion of dihydrodiol Simvastatin hydroxy acid with m/z value 471.[0]

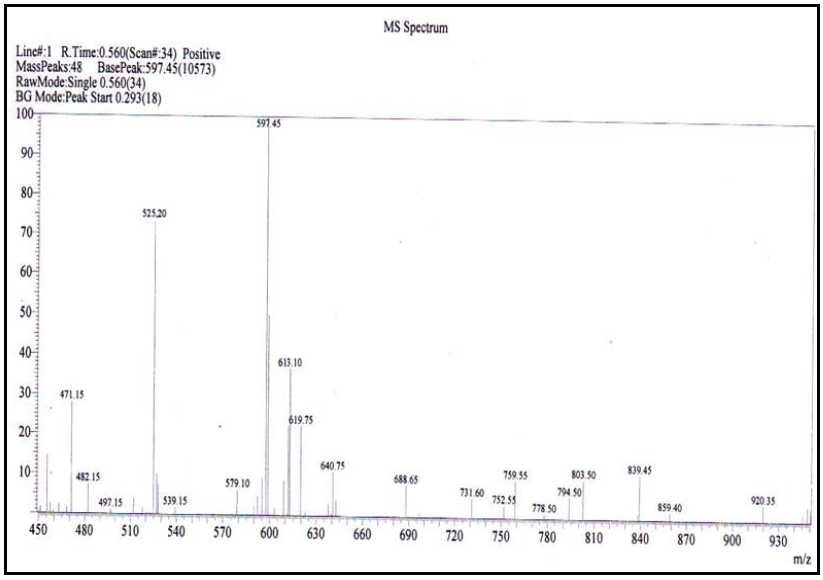

Figure 4: LC/MS Spectra of simvastatin with *Aspergillus niger*.

One of the reasons for low concentration of the products in microbial fermentation process may be the solubility of the substrate in the culture media. Therefore to enhance the solubility, the glucuronidation was carried in presence of sodium lauryl sulphate. Surfactants act by reducing the surface tension at the interface forming, micro emulsion in which the hydrophobic substrate is solubilized, thereby increasing its solubility.[11] In presence of surfactant, statins exhibited the same glucuronide metabolites but with increased concentrations along with the reactant peaks i.e., atorvastatin and simvastatin with m/z values 559 and 435 respectively as shown in figures 5 and 6 respectively.

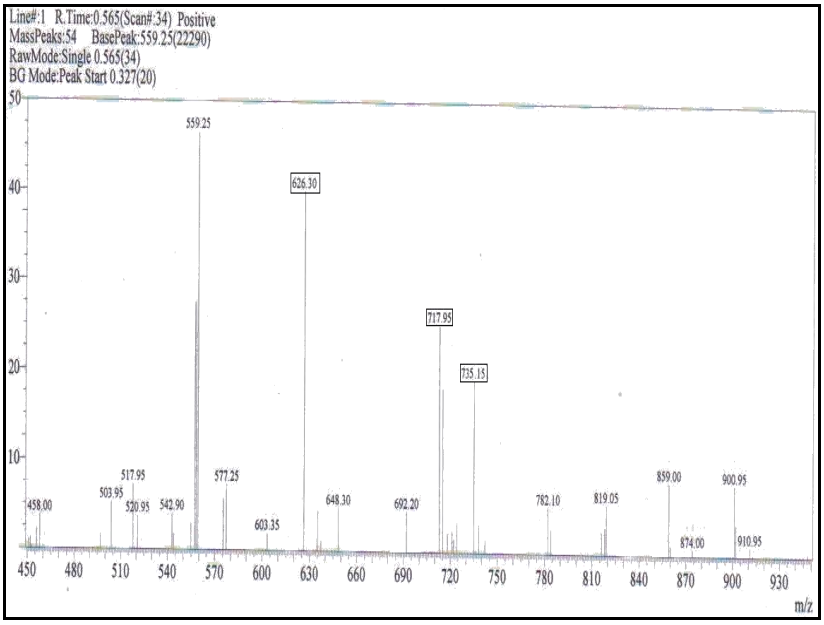

Figure 5: LC/MS Spectra of simvastatin with *A. niger* in presence of surfactant.

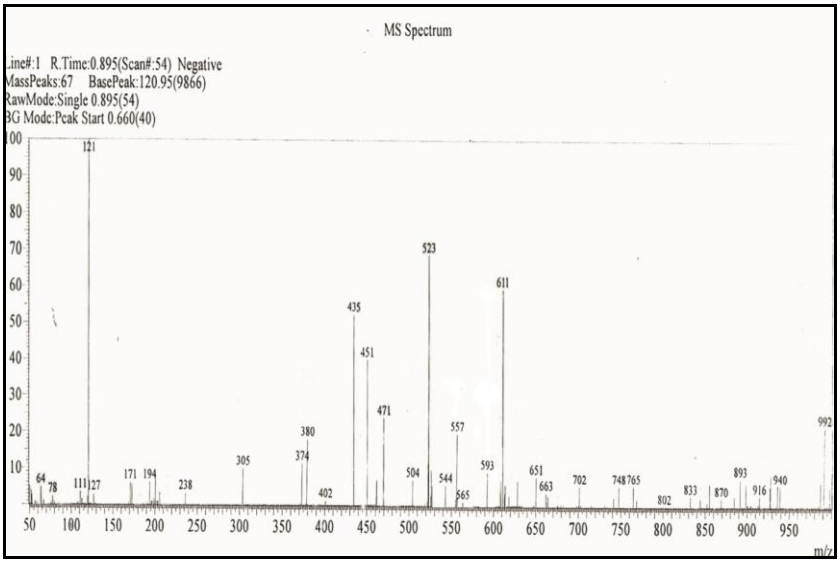

Figure 6: LC/MS Spectra of atorvastatin with *A. flavus* in presence of surfactant.

CONCLUSION

The work presented here describes a microbial bioconversion process for the glucuronidation of atorvastatin and simvastatin., as an alternative route for synthesis of drug gluronides, The metabolites were identified by LC MS data. Among the few species screened for bioconversion, *Aspergillus flavus* and *Aspergillus ochraceous* for atorvastatin and *Aspergillus niger* for simvastatin was capable of carrying out these reactions. Addition of a surfactant had a positive impact on the yield of the product.

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