DEVELOPMENT OF SPECTROPHOTOMETRIC METHOD FOR SIMULTANEOUS ESTIMATION OF PRODUCTS ACCUMULATED DURING CHOLESTEROL BIOCONVERSION BY PSEUDOMONAS PUTIDA MTCC 1259

Kirti Pawar1∗, Megha Bhatt1 and Sambhaji Pawar2
1Ashok and Rita Patel Institute of Integrated Study and Research in Biotechnology and Allied Sciences, Anand, Gujarat, India.
2A R College of Pharmacy, Vallabh Vidya Nagar, Anand, Gujarat, India.
∗Corresponding author’s E-mail: drkirtipawar@rediffmail.com

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ABSTRACT

Bioconversion of cholesterol was carried out by growing cells of Pseudomonas putida MTCC 1259. Accumulation of two bioconversion products namely cholestenone and 9α-hydroxy androstadienedione was observed in presence of n-propanol as steroid ring cleavage inhibitor. A spectrophotometric method for simultaneous estimation of the two bioconversion products was developed using sulphuric acid chromogens. The method can be utilized to follow the progress of bioconversion during side chain cleavage of cholesterol by Pseudomonas putida MTCC 1259.

Keywords: Cholesterol Side chain cleavage; Simultaneous estimation; Steroid bioconversion; Cholestenone.

INTRODUCTION

Simultaneous estimation of precursor and product steroids has always been a problem during steroid bioconversions since these compounds show similar absorption patterns under UV light. These compounds form chromogens that show overlapping absorption profiles in visible range. Hence an appropriate estimation method for determination of precursor and product concentrations in the fermentation extracts has been a difficult task. A large number of methods based on HPLC, GLC have been routinely used for the simultaneous estimation of steroids1. Patil et al., successfully developed a UV method for simultaneous estimation of precursor and product steroids during bioconversion of Androstenedione2. Pawar et al., developed a spectrophotometric simultaneous estimation method for hydrocortisone succinate and its bioconversion product3. Sulphuric acid chromogens have been effectively used for simultaneous determination of steroidal compounds on thin layer chromatography. Pseudomonas putida MTCC 1259 was found to accumulate two bioconversion products identified to be cholestenone and 9α-hydroxy androstadienedione (9α-OH-AD) during cholesterol bioconversion in presence of n-propanol as inhibitor for steroid ring cleavage4. Further, the two products were the only steroids detected in the fermentation medium after about 72 hrs of bioconversion in presence of n-propanol. It was decided to prepare sulphuric acid chromogens of the side chain degradation products of cholesterol and study their absorption spectra with a view to develop a simultaneous estimation method for the two bioconversion products.

MATERIALS AND METHODS

Pseudomonas putida MTCC 1259 was grown in a medium containing (g l−1): Peptone, 5; Yeast extract, 2; Beef extract, 1 and Sodium Chloride, 5; pH of the medium was adjusted to 7.0; dispensed (20 ml) in a series of 100 ml conical flasks and sterilized by autoclaving at 121°C for 15 min and allowed to cool to room temperature. The flasks were inoculated with actively growing culture of the organism in the same medium, allowed to grow overnight at 30°C on a shaking platform at 100 rpm and cholesterol (100 µg) dissolved in 0.2 ml acetone was added. After 24 hr of addition of substrate cholesterol, n-propanol was added to the bioconversion flasks to achieve a 5 % concentration. Samples were drawn at specified intervals and extracted twice with equal volumes of ethyl acetate. The organic layer was decanted, dried over sodium sulphate and solvent evaporated by keeping the tubes in a boiling water bath. The bioconversion products were determined qualitatively by Thin Layer Chromatography.

For isolation of the steroidal fermentation products, the contents of the flasks were extracted with twice the volume of ethyl acetate, separated by preparative TLC5, and re-crystallized from acetone or methanol and used for further studies. The purified bioconversion products (30 µg) were separately dissolved in 3 ml concentrated sulphuric acid, chromogens developed by heating the tubes in boiling water bath for 30 minutes and absorption spectrum of the compounds determined in the wavelength range of 200 to 400 nm.

[Graph of Overlay spectra of Cholestenone and 9α-OH AD]
RESULTS AND DISCUSSION

It is clear from the above figure that both the chromogens show significant absorbance at all the wavelengths selected for drawing the spectrum. It was decided to make equimolar solutions of the compounds and determine the absorptions of the respective sulphuric acid chromogens to determine their difference in absorptivities. Using the above graph, absorption maxima of 9α-OH-AD was at 280 nm & that of cholestenone was chosen as 260 nm. Simultaneous estimation equations for cholestenone and 9α-OH-AD were developed using the method described for simultaneous estimation of solasodine and solasonine. Following simultaneous equations were used for developing equations for estimation of 9α-OH-AD and cholestenone in binary mixture.

\[ X_1 = C_d \alpha_1 + C_n \beta_1 \]  \hspace{1cm} (1)  
\[ X_2 = C_d \alpha_2 + C_n \beta_2 \]  \hspace{1cm} (2)

Where, \( X_1 \) = absorbance of binary mixture at 280 nm; \( X_2 \) = absorbance of binary mixture at 260 nm; \( C_d \) = % concentration of cholestenone (cholestenone \( \lambda_{\text{max}} \) = 260 nm); \( C_n \) = % concentration of AD (AD \( \lambda_{\text{max}} \) = 280 nm); \( \alpha_1 \) = Mass Absorptivity of AD at 280 nm = 886; \( \beta_2 \) = Mass Absorptivity of cholestenone at 280 nm = 27.3; \( \alpha_2 \) = Mass Absorptivity of AD at 260 nm = 873.33; \( \beta_1 \) = Mass Absorptivity of cholestenone at 260 nm = 167

Absorptivity of the 30 µg/ml solutions of the two compounds was determined, values put in the above equations and following equations were developed for determination of concentration of the two compounds in the binary mixtures.

\[ C_n = 0.00713824 \times A_{280} - 0.00703544 \times A_{260} \]  \hspace{1cm} (A)  
\[ C_d = 0.0013457 \times A_{280} - 2.1994416 \times A_{260} \]  \hspace{1cm} (B)

Where, \( A_{280} \) = Absorption of binary mixture at 280 nm and \( A_{260} \) = Absorption of binary mixture at 260 nm. A simultaneous estimation method for the two bioconversion products of cholesterol by was successfully developed.

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