

Quantification of Lovastatin Produced by *Monascus purpureus*

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Abstract: Development of a novel method for the quantification of lovastatin is an interesting problem in the analytical field. In the literature, many reports use spectrophotometric method for the quantification of lovastatin. However, the analysis of fermentation broth containing lovastatin appears to be inaccurate using spectrophotometric method. Hence, the estimation of lovastatin produced by *Monascus purpureus* and pure lovastatin was attempted by UV-visible spectrophotometer as well as HPLC. It was observed that the analogues and/or intermediates of lovastatin synthesized in the fermentation broth and the products of fermentation caused superimposition effect on the absorption spectrum. Phosphate is a medium constituent for the production of lovastatin by the organism which contributed significantly to the superimposition of absorption spectrum. On the other hand, HPLC analysis consistently gave reliable results for the estimation of lovastatin under all the experimental conditions studied.

Keywords: Lovastatin, *Monascus purpureus*, spectrophotometry, HPLC.

1. INTRODUCTION

The conversion of 3-hydroxy-3-methyl-glutaryl coenzyme A (HMG-CoA) into mevalonate is the rate-limiting step in the cholesterol cascade mediated by HMG-CoA reductase (E.C 1.1.1.88). Statins are structural analogues of HMG-CoA and bind with the enzyme at its active site. This alters the conformation of the enzyme. The reversible competitive binding between statin and the enzyme prevents the access of the substrate HMG-CoA to the HMG-CoA reductase [1-6]. Among all statins, lovastatin is the first approved by USFDA for the treatment of hypercholesterolemia in 1987. Lovastatin also serves as a precursor for other important statins like simvastatin and wuxistatin [7, 8].

Development of analytical methods for the estimation of lovastatin is an interesting topic due to the existing problems in the analytical techniques. Pure and fermentation-derived lovastatin were quantified mostly using UV-visible spectrophotometer [9-16], HPLC [17-20], and capillary electrophoresis [21, 22].

The spectrophotometric method of analysis of lovastatin is simple, faster, eco-friendly, and less laborious method of analysis than other analytical techniques. However, the spectrophotometric quantification of lovastatin is not

accurate in fermentation broth as well as for the system having competitive molecules which also absorbs light at 238 nm [2, 3]. Lovastatin has been produced commercially through fermentation route only [1, 6]. Hence, it is important to measure the content of lovastatin accurately in the fermentation broth. Many researchers have used HPLC technique for the quantification of lovastatin in biological samples, but the reason for shifting/moving from the UV-visible spectrophotometer to HPLC has not been explained so far for the estimation of fermentation-derived lovastatin. In the present study, we have used UV-visible spectrophotometer and HPLC techniques for the quantification of lovastatin produced in the fermentation broth. Our objective was to highlight the various forms of interferences in the quantification of lovastatin with necessary experimental proof, which has not been reported so far.

2. EXPERIMENTAL

2.1. Chemicals

Lovastatin (lactone form) was procured from Sigma-Aldrich. All other chemicals were of analytical grade and procured from Sisco Research Laboratories Pvt. Ltd., Mumbai, India. For the assay and preparation of reagents, Milli-Q water was used throughout the study. Lovastatin was dissolved in acetonitrile to get a stock solution of 1 mg/mL. The hydroxy acid form of lovastatin was prepared by hydrolysis as described by Yang and Hwang [23].

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2.2. Organism and Culture Conditions

Monascus purpureus MTCC 369 was obtained from the Institute of Microbial Technology, Chandigarh, India. A suspension containing 10^5 - 10^6 spores/mL was used to inoculate a basal medium having the following composition (in g/L): dextrose, 100; peptone, 10; KNO_3 , 2; $\text{NH}_4(\text{H}_2\text{PO}_4)$, 2; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5; CaCl_2 , 0.1. The pH was adjusted to 6 using 0.1 N NaOH solutions [16]. The culture was incubated at 30°C for 48 h in an orbital shaker maintained at 120 rpm.

The defined production medium had the following composition (in g/L): dextrose, 29.59; NH_4Cl , 3.86; KH_2PO_4 , 1.73; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.86; $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 0.19. The pH was adjusted to 6 [16]. The complex production medium contained (in g/L): rice powder, 34.4; peptone, 10.8; glycerol, 33.2; glucose, 129.2; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1; KNO_3 , 2. The pH was adjusted to 6 [24]. A 500-mL Erlenmeyer flask containing 100 mL of sterile medium was inoculated with 10% (v/v) seed culture and incubated at 30°C for 15 days on a rotary shaker maintained at 120 rpm [18]. Care was taken to restrict evaporation loss during fermentation. Suitable correction factor has been used in this regard.

2.3. Analysis of Products

2.3.1. Pure Lovastatin

Working standards of lactone- and hydroxy acid- forms of lovastatin were prepared and the absorbance was read at 238 nm using a UV-visible spectrophotometer (JASCO V-630, Essex, UK). The zero-order absorption spectra were obtained over the wavelength range of 200-600 nm using the appropriate dispersion medium in a quartz cuvette (1 cm path length) at 1.5 nm slit width ($\Delta\lambda$).

The limit of quantification (QL) and detection limit (DL) was found using the equation given below,

$$DL; QL = \frac{F \times (SD)}{a}$$

where F value for DL and QL are $3.3 \mu\text{g}$ and $10 \mu\text{g}$, respectively. SD is the standard deviation of the blank and 'a' is the slope of the standard plot [25].

Quantitative HPLC was performed using a gradient HPLC (Shimadzu, Prominence HPLC, Kyoto, Japan) equipped with a photodiode array detector and Luna C18 column (250×4.6 mm, $5 \mu\text{m}$). The mobile phase was composed of acetonitrile and acidified water (0.1% H_3PO_4) (60:40, v/v) which was filtered through a $0.22\text{-}\mu\text{m}$ membrane filter. The flow rate of solvent and column temperature was maintained at 1 mL/min and 40°C , respectively. A $20 \mu\text{L}$ sample was injected into the column for analysis. The working standards of lactone- and hydroxy acid- forms of lovastatin were analyzed by HPLC at 238 nm.

2.3.2. Lovastatin Produced by *Monascus Purpureus*

The sample (cell suspension) was homogenized by ultrasonication for 20 min followed by extraction with an equal volume of ethyl acetate at 60°C for 30 min with intermittent shaking. The upper organic layer was isolated from the aqueous layer and dried under vacuum in a rotary

evaporator. The dry residue was resuspended in pure acetonitrile. This suspension was filtered through a $0.22\text{-}\mu\text{m}$ nylon filter paper (Pall Trincor[®], USA) before analysis by UV-visible spectrophotometer and HPLC [17].

Thin layer chromatography (TLC) (using dichloromethane/ethyl acetate (70:30, v/v)) was done to confirm the presence of lovastatin [26].

Inductively-coupled plasma optical emission spectrometer (ICP-OES, Perkin-Elmer Optima 5300 DV, Shelton, CT) was used to measure potassium ($\lambda_{\text{max}} = 766.5$ nm) and phosphorus ($\lambda_{\text{max}} = 213.62$ nm) present in the suspension and also released during ultrasonication. The flow rates of the auxiliary, plasma, and nebulizer were maintained at 0.2, 15, and 0.8 L/min and that of the sample at 1.5 mL/min, respectively. A radiation source of 40 MHz and RF power of 1300 were used in the system. Potassium and phosphorus were measured using the axially-viewed plasma set-up.

3. RESULT AND DISCUSSION

3.1. Characterization of Pure Lovastatin Using Separately UV-visible Spectrophotometer and HPLC

Pure lovastatin has three different absorption maxima at 232, 238, and 247 nm in UV-visible spectrophotometry (*cf.* Fig. 1A), which suggests better identification of lovastatin from other compounds. The characteristics of the peaks could be due to the presence of diene [27]. The absorption spectra of both lactone form and hydroxy acid form of lovastatin in a mixture appear similar (*cf.* Fig. 1). The regression analysis of the individual forms of lovastatin and molar extinction coefficient (ϵ) are given in Table 1. The molar extinction coefficients for the lactone and hydroxy acid forms of lovastatin are $4.89 \times 10^4 \pm 21.8$ and $5.99 \times 10^4 \pm 36 \text{ M}^{-1}\text{cm}^{-1}$, respectively. This is valid for the estimation range of lovastatin between $2 \mu\text{g}$ and $20 \mu\text{g}$. Values of DL and QL for lovastatin are $0.39 \mu\text{g}$ and $1.16 \mu\text{g}$, respectively.

Results from the spectrophotometric analysis gave the total amount of lovastatin (hydroxy acid plus lactone forms) rather than the amount of the individual forms of lovastatin, while distinct peaks were obtained for the hydroxy acid and lactone forms of lovastatin by HPLC technique (*cf.* Fig. 1B). Hence, the individual forms of lovastatin cannot be distinguished in both pure and fermentation derived samples by UV-visible spectrophotometer. Therefore, the spectrophotometric method of quantification of lovastatin could lead to a confusion in clinical studies, since the individual forms of lovastatin have specific properties, viz., pharmacological activity, solubility, lipophilic nature, and transportation characteristics.

The lactone form of lovastatin is more lipophilic than the hydroxy acid form. For this reason, the lactone form is accepted by the hepatic cells. This behavior determines the bioavailability of lovastatin. In liver, the diffused lactone form of lovastatin is converted into hydrolyzed forms including the hydroxy acid form by cytochrome P450 3A4 [28]. Hence, the quantification of the individual forms of lovastatin as well as hydrolyzed/metabolized products of

Table 1. Assay parameters of UV-visible spectrophotometric measurements.

Nature of Compound Treated	Test Sample	Regression equation, $Y = aX + b$		Correlation Coefficient (r^2)	Molar absorption coefficient ($\epsilon_{\lambda_{238}}$), $M^{-1}cm^{-1}$
		Slope, μg^{-1} (a)	Intercept (b)		
For a pure compound	Lactone form of lovastatin standard	0.0404	-0.0032	0.999	$4.89 \times 10^4 \pm 21.8$
	Hydroxy acid form of lovastatin	0.0426	0.0218	0.9985	$5.99 \times 10^4 \pm 36$
In the presence of competitive molecules	Lovastatin + KH_2PO_4	0.0182	-0.0576	0.849	$1.55 \times 10^4 \pm 61.5$
	Lovastatin + Unautoclaved chemically defined medium	0.0208	-0.0539	0.9343	$1.80 \times 10^4 \pm 53$
	Lovastatin + Autoclaved chemically defined medium	0.0146	-0.0372	0.8971	$1.34 \times 10^4 \pm 36$
	Lovastatin + Unautoclaved complex medium	0.0156	-0.0098	0.9215	$1.71 \times 10^4 \pm 43$
	Lovastatin + Autoclaved complex medium	0.0139	0.0275	0.8227	$2.1 \times 10^4 \pm 55$

where Y is the absorbance value at 238 nm and X is the amount of lovastatin (μg). The molar absorption coefficients are expressed as mean \pm standard deviation for the sample size of $n=5$. The detection limit (DL) and quantification (DQ) are found 0.39 μg and 1.16 μg , respectively from the equation, $DL, QL = \frac{F \times (SD)}{a}$, where F value for DL and QL are 3.3 μg and 10 μg and SD is the standard deviation of the blank. 'a' is the slope of the standard plot [25]. The regression model for HPLC data is $y = 2 \times 10^6 x$ for all the cases (remained same), 'y' is the detector response (AU) and 'x' is the amount of lovastatin (μg).

lovastatin is necessary to determine the metabolic rate in controlled delivery systems. Several reports use the spectrophotometric method of quantification of *in vitro* and *in vivo* drug release characteristics [29-35]. A few studies have reported the controlled release characteristics of lovastatin *in vitro* by measuring lovastatin using UV-visible spectrophotometer and *in vivo* release characteristics of lovastatin measured by HPLC technique [29, 36].

3.2. Effect of Phosphate and Potassium on Lovastatin Estimation

The phosphate and potassium contents were estimated in the culture sample of *M. purpureus* grown in defined as well as in complex production medium, using ICP-OES. The samples were subjected to ultrasonication before estimation. Samples were collected every 24 h for 15 days. Significant amounts of phosphorus were released compared to

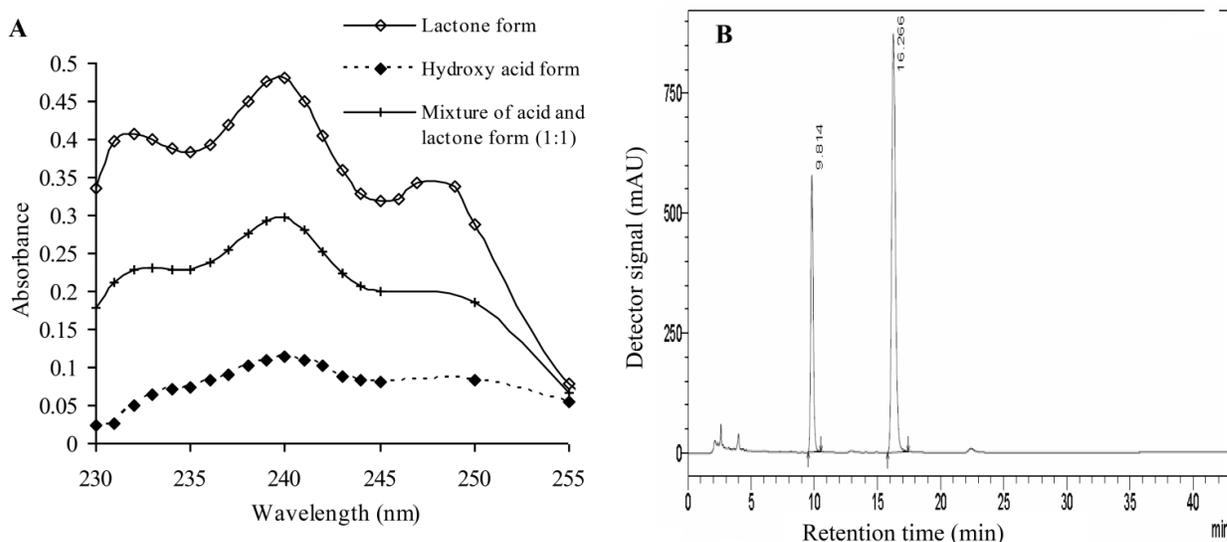


Fig. (1). UV-visible spectrophotometric and HPLC methods of estimation of pure lovastatin. (A) The lactone and hydroxy acid forms of lovastatin exhibited absorbance maxima (λ_{max}) at 232, 238, and 247 nm individually as well as in mixture (1:1) by UV-Visible spectrophotometer. (B) Both the forms of lovastatin quantified separately using HPLC. The retention times for hydroxy acid and lactone forms were 9.8 and 16.3 min, respectively.

potassium during the ultrasonication of the culture broth. The total amount of phosphorus in the defined medium was more than that in the complex medium. The maximum total amount of phosphorus (equivalent to KH_2PO_4 concentration) already present in the broth and released during ultrasonication was found to be 1.807 g/l and 0.0175 g/l for the defined and complex production medium, respectively. The effect of phosphate (equivalent to KH_2PO_4 concentration) in the estimation of a known quantity of pure lovastatin (lactone form) is shown in Figs. (2A) and (2C).

Jerotskaja *et al.* [37] measured some of the constituents in the spent human blood samples, after dialysis, using UV-visible spectrophotometer and quantified the levels of potassium and phosphate at 227 and 237 nm, respectively. It

suggests that the phosphate and potassium already present in the medium as well as that released from cells after sonication led to errors in lovastatin estimation by UV-visible spectrophotometer. The presence of diene groups in the phosphate and its related compounds might absorb at 238 nm. This could contribute to inaccurate measurement of this drug by the spectrophotometric method [27, 38].

It is pertinent to mention here that this study is relevant to a problem associated in the assay of drug delivery system, where the drug is dispersed in the phosphate buffer. The study is with respect to *in vitro* and *in vivo* release of lovastatin. The phosphate alone appears to be a potential interfering component as observed from the present study. Several reports suggested spectrophotometric measurement

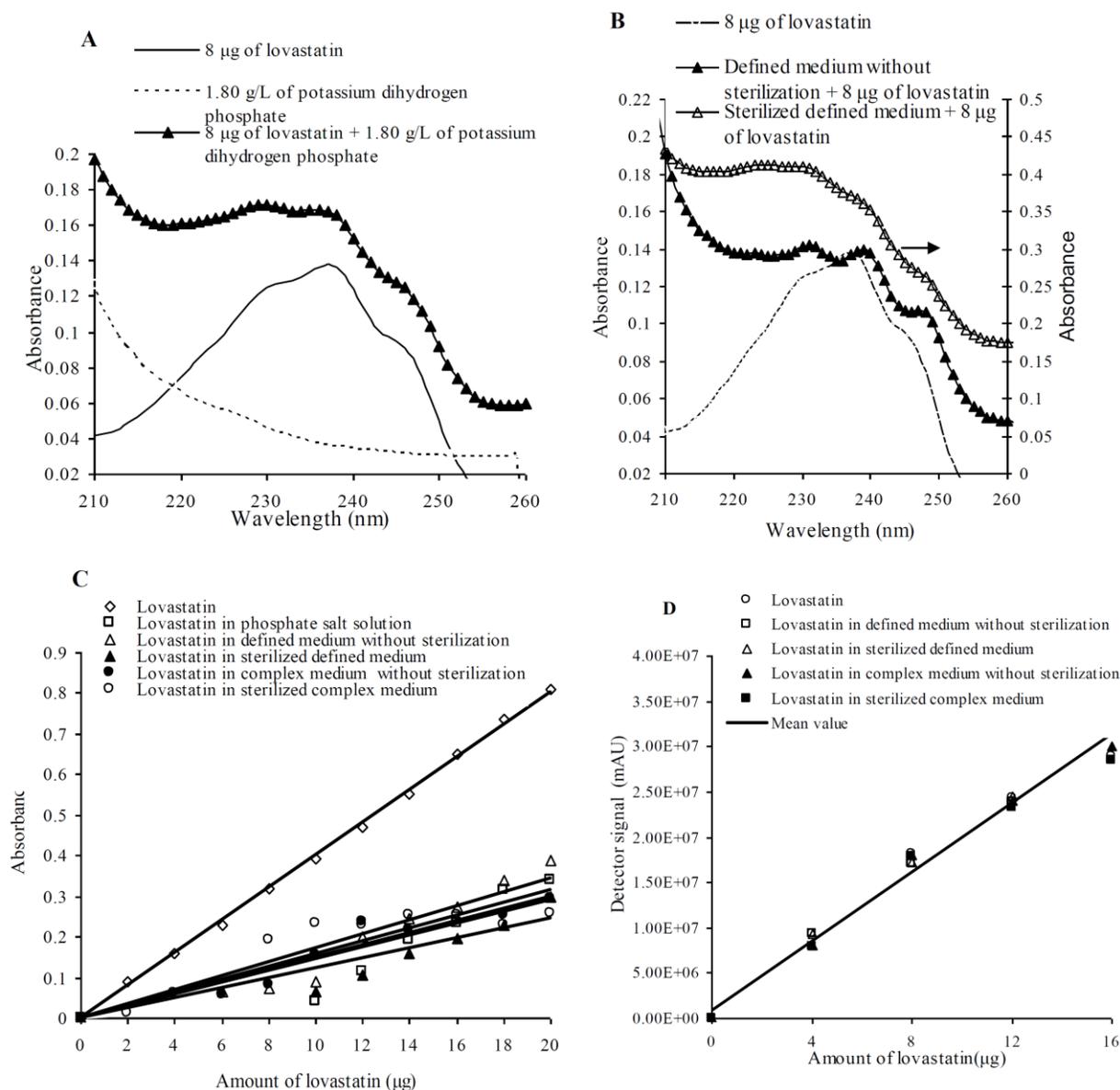


Fig. (2). Inaccuracy in lovastatin estimation by UV-visible spectrophotometry caused by phosphate and medium constituents. (A) Effect of maximum equivalent phosphate present in the medium as well as released from cells during sonication in defined medium (1.80 g/L) on the lovastatin absorption spectrum. (B) Effect of sterilization of defined medium constituents on lovastatin absorption spectrum. (C) Effect of medium constituents and phosphate on the absorbance versus amount of lovastatin plots measured by UV-visible spectrophotometry. (D) Effect of medium constituents on the standard plot of lovastatin obtained by HPLC technique.

for those *in vitro* and *in vivo* release characteristics of the drug [29-35]. Also, a few articles reported the release characteristics of the drug *in vitro* by UV-visible spectrophotometry and *in vivo* release characteristics using HPLC analysis, considering the competitive molecules in the serum [29, 36].

In the development of drug delivery systems, it is important to know the stability of lovastatin and its metabolism. The lactone form of lovastatin is metabolized or converted into hydrolyzed product including the acid form of lovastatin in the liver. Thus, the hydrolyzed product concentration is more important for the drug delivery and release characteristics of the drug. This change in concentration cannot be effectively determined by UV-visible spectrophotometer [39]. Jacobson *et al.* (1999) [40] also measured the hydrolyzed products of lovastatin in addition to lovastatin itself by HPLC at 239 nm. There are examples of lovastatin analogues and hydrolyzed products measured around 238 nm which cannot be distinguished in the spectrophotometric method.

3.3. Effect of Medium Constituents in Lovastatin Estimation

The constituents of the complex medium possibly contribute to error in lovastatin estimation and the corresponding absorbance values are higher than those obtained using the defined medium (*cf.* Fig. (2B)). The sterilized complex production medium showed a higher absorbance value than the same medium before sterilization. In the case of defined production medium, autoclaving of medium increased the absorbance which resulted in errors during lovastatin estimation (*cf.* Fig. (2B)). These results confirmed that the composition of the production medium (either defined or complex) and sterilization of the medium contribute to the error in the lovastatin assay (*cf.* Fig. (2B) and (2C)). The magnitude of error increased with the degree of complexity of the medium and the concentration of the

medium constituents. These results suggest that the spectrophotometric measurements are not suitable for quantification of lovastatin.

Pham *et al.* (2011) [41] reported a similar phenomenon for the estimation of xylose, where the availability of a rapid, inexpensive, and sensitive method like the spectrophotometric method is often limited by the complex nature of the medium. Some of the medium constituents, time of heating of medium, and trace elements in the medium were the reasons for inaccurate estimation of xylose.

3.4. Quantification of Lovastatin Produced by *Monascus sp.* Using UV-visible Spectrophotometric and HPLC Methods

The absorbances of aliquots of culture filtrate and of ethyl acetate-extract of culture filtrates obtained from the fermentation of *Monascus purpureus* and of *Monascus ruber* were separately measured against a suitable blank. Absorbance values were very high. There is no clear maximum peak value at 238 nm, which probably suggest that the presence of a number of compounds could possibly contribute to the absorbance value. Results described here exclusively for *Monascus purpureus*.

(a) Effect of Lovastatin Analogues and Its Intermediates

Batch fermentation was carried out for lovastatin production by *Monascus purpureus*. The presence of lovastatin was confirmed by TLC in the fermentation broth in the entire fermentation cycle. Lovastatin was measured in the ethyl acetate extract of culture filtrate separately by HPLC technique and on a spectrophotometer. Maximum production of lovastatin was between the 6th and 10th day of fermentation. The absorbance read on the spectrophotometer show equivalence to statin in the mg/l range, whereas the concentration of lovastatin measured by HPLC technique is

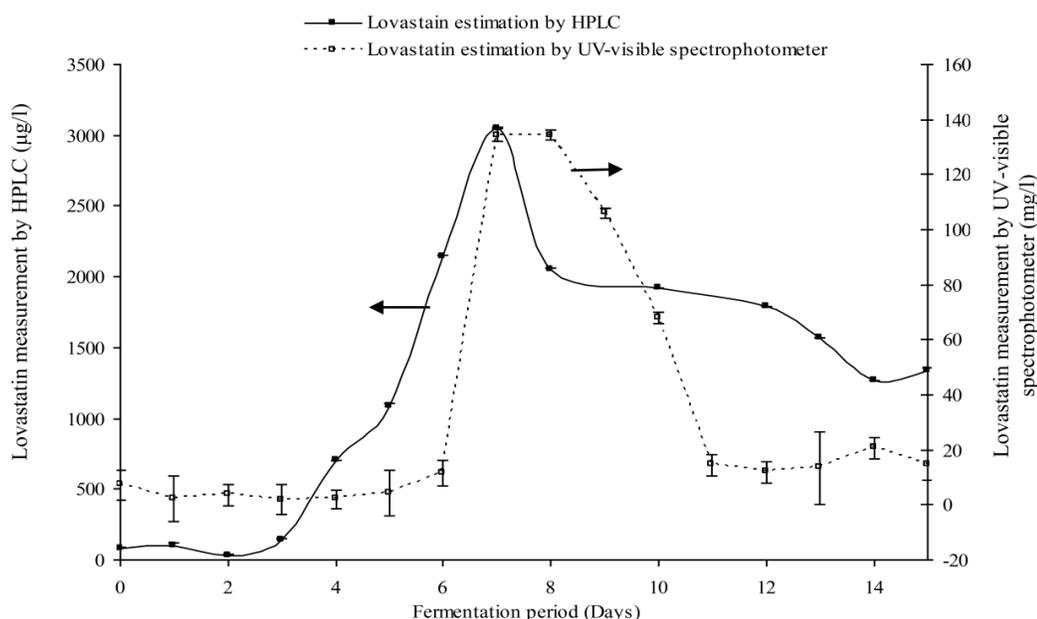


Fig. (3). Concentration profiles of lovastatin production analyzed by UV-visible spectrophotometry and HPLC.

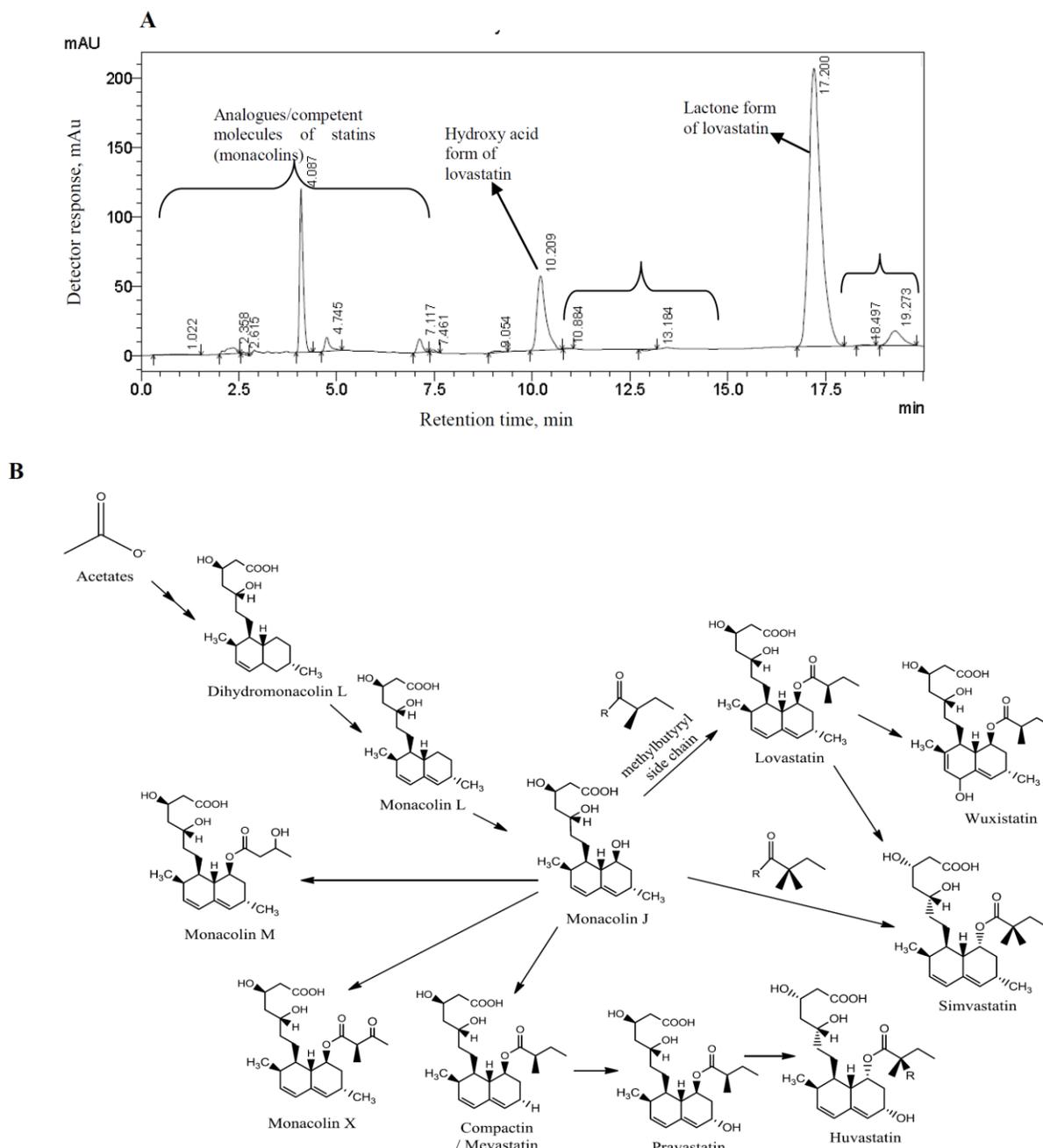


Fig. (4). Possible analogues/competent molecules present in the fermentation broth of *Monascus* sp. **(A)** Chromatogram (measured at 238 nm) of *Monascus purpureus* fermentation samples contains individual forms of lovastatin along with its competent/analogues (monacolins). **(B)** Biosynthesis pathway of lovastatin and its analogues from acetate [6, 7, 44-48]. The possible compounds might be accumulated in fermentation broth, which could showed HPLC chromatogram having different analogues eluted at different retention time. These compounds has tri-substituted heteroannular diene chromophore which absorb at 238 nm [42, 44, 49].

actually in the range of $\mu\text{g/l}$ (*cf.* Fig. (3)). These results also suggest that some intermediates produced by the organism during fermentation might absorb at 238 nm (*cf.* Fig. (4)).

It has been reported that the presence of diene groups in lovastatin and some of its intermediates were responsible for this typical interference. Some of the intermediates of lovastatin, *viz.*, monacolin J, X, L, and M were found to have a maximum absorbance at 238 nm [27, 42]. In particular, monacolin L, J, and X were found to exhibit three maximum

absorption peaks similar to the individual forms of pure lovastatin (*cf.* Fig. 1A). However, some of the degraded compounds, *viz.*, methyl esters, anhydro, methoxy, and acetate ester forms of lovastatin and simvastatin could absorb at 238 nm due to the diene groups [43]. These compounds appear as probable interferences in the quantification of lovastatin using a spectrophotometer.

Also, the pleiotropic applications of statins vary among the statins as well as the intermediates [19]. On comparison

between the fermentation-derived statins and the synthetic statins, monacolin J (hydrolyzed product of lovastatin) show better prevention of neurodegenerative condition due to its potential blood-barrier penetration and cholesterol lowering effects on neurons [19]. Thus, the determination of analogues of statins produced in the fermentation broth is necessary, especially, when the compound is used for the preparation of nutraceuticals, fermented food etc. By the spectrophotometric method, it could not be possible to estimate the intermediates and their analogues accurately. Using spectrophotometer one can measure monacolins with relative competitive molecules at 238 nm (*cf.* Fig. 4). On the other hand, HPLC system can be used to measure the intermediates along with lovastatin with greater accuracy (*cf.* Fig. 4). It is possible that more statins could be produced including lovastatin such as compactin, pravastatin, and monacolin J by *Monascus purpureus*. This speculation is almost equivalent to the observation by Manzoni *et al.* [44] for *Monascus sp.*

Jaivel and Marimuthu determined the concentration of lovastatin by spectrophotometer, showing higher estimate for lovastatin in the range of mg/l [9]. Studies on the applications of fermentation-derived lovastatin have used the spectrophotometric measurement for its quantification [15]. The quantification of lovastatin by spectrophotometer in the presence of intermediates also led to errors in the determination of the lethal dose concentration (LDC) in clinical studies. Osman *et al.* have reported the use of spectrophotometric assay method for lovastatin and found a maximum lovastatin production of 188.3 mg/l using *Aspergillus terreus* [10]. The high concentration of lovastatin produced could be the result of nutritional improvement of a complex medium. In this context, several reports are available for lovastatin biosynthesis using different organisms. Those reports have indicated the spectrophotometric method for the quantification of fermentation-derived lovastatin [9-13].

Xie *et al.* reported the accumulation of monacolins especially, monacolin J, inhibited lovastatin biosynthesis [45]. This suggests that there is a possibility of accumulation of monacolin J during the fermentation. Similarly, some of the intermediates of the lovastatin biosynthesis pathway and structural analogues of lovastatin could absorb light at 238 nm.

High absorbance values (> 0.8 after dilution) and the absence of a clear maximum at 238 nm could be attributed to the medium constituents, their complex nature, and the presence of secondary metabolites.

4. CONCLUSION

Lovastatin was estimated by UV-visible spectrophotometry and HPLC techniques. The interference of medium constituents, intermediates, and products of fermentation which led to inaccuracies during the estimation of lovastatin by spectro-photometry was discussed. The spectrophotometric method is suited for the quantification of pure lovastatin alone while HPLC analysis is the most suitable and reliable method for the estimation of lovastatin under all the experimental conditions heretofore studied.

CONFLICT OF INTEREST

The authors confirm that this article has no conflicts of interest.

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