TO SYNTHESIZE AND CHARACTERIZE METABOLITE OF SIMVASTATIN: PKPD AND TOXICITY PREDICTIONS BY COMPUTATIONAL APPROACHES

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ABSTRACT:

Simvastatin is a member of the statin drug class, used to reduce cholesterol levels in the bloodstream. The current research work emphases on the synthesis, characterization, and optimization of RP-HPLC method for determination of simvastatin and its synthesized metabolite β-hydroxy acid as an impurity simultaneously with high accuracy, precision, and sensitivity. In the present paper properties such as ADME and toxicity of the BHA were predicted through different ADME database like SWISS ADME and molesoft. The metabolite BHA of simvastatin was synthesized in the laboratory and characterized by UV, MS, NMR and FT-IR spectroscopy.

The method was developed using Kinetex C18, 4.6 mm x 250 mm, 5 µm as the stationary phase, flow rate 1.0 mL/min, volume 20 µL with a run time of 10 minutes at 244 nm wavelength using Methanol (80%) : Phosphate buffer 20mM pH 3 (20%) as mobile phase. The HPLC analytical method, which includes the detection of the simvastatin metabolite, underwent validation according to the ICH Q2B (R1) guidelines. Analysis revealed that the findings were in the region of 98.57% to 101.86% for all analytes with satisfactory accuracy and precision. The developed method can be used for detection and quantification of metabolite in bulk or formulations of simvastatin.

Further optimization of the method for quantification of BHA in biological fluids could make it useful for clinical and bioequivalence studies. Assessment of toxicity by using computational tools like Swiss tool is reported with the hope of reducing threats.

Keywords: Simvastatin, β-hydroxy acid, RP-HPLC, ADME database

INTRODUCTION:

Globally, cardiovascular disease (CVD) is at present the main factor contributing to transience. The 17 million premature deaths (under the age of 70) brought on by non-communicable diseases in 2019; it was responsible for 38%..[1]

Simvastatin is a medication that belongs to a class of drugs known as statins, which are used to lower cholesterol levels in the blood. It works by blocking an enzyme called HMG-CoA reductase, which is involved in the production of cholesterol in the liver. By reducing the amount of cholesterol produced in the liver, simvastatin helps to lower the total amount of cholesterol and other fats in the blood.

The starting material for simvastatin synthesis is a compound called diethylallylmalonate, which is reacted with an aldehyde to produce a compound called β-hydroxyethyl-β-methylglutarylthiophenyl ether (HMG-Th). This reaction is catalyzed by a base such as sodium hydride or potassium tert-butoxide.[2]

The International Conference on Harmonization (ICH) guidelines require that all impurities in drug substances or drug products be identified and characterized. The ICH defines impurities as any component of a pharmaceutical product that is not the intended active ingredient or excipient.

The guidelines set strict limits on the levels of impurities that are permitted in pharmaceutical products, and they require that impurities present at levels above 0.1% be recognized and qualified. Identification and certification are necessary even at lower concentrations if impurities are anticipated to be extremely hazardous.[3]

For pharmaceutical products to be safe and effective, impurities must be properly identified and characterized.

One of the areas that is of present pharmaco-economic and clinical importance is the synthesis and characterization of impurities and clinically important metabolites of novel medications using the least amount of time and money possible. The primary concerns of a medical researcher in such work are always adequate separation, selectivity, sensitivity of detection, and correct quantification.

The evaluation of absorption, distribution, metabolism, and excretion (ADME) is essential for drug discovery. Many drugs suffer from off-target relationships as well as significant metabolic pathways, which prevent them from achieving their intended target association in blood brain permeation, efficacy, as well as safety. Early assessment of ADME during the drug development phase aids in spotting possible pharmacokinetic-related errors or preventing them in later clinical phases. For ADME prediction, in silico models have developed into a strong alternative to experimental methods. Through the SwissADME database and molesoft, which is publicly accessible at http://www.swissadme.ch, attributes such as ADMET of the synthesize metabolite of simvastatin that is β hydroxy acid were also anticipated in the work.[4]

Simvastatin was predicted to have a considerable number of impurities which were evaluated using a variety of analytical methods such HPLC and LC-MS.[5,6] Simvastatin and its metabolite, BHA, may both be measured simultaneously in bodily fluids using a number of techniques that have been documented. [7-10]

The specific objective of this study is to synthesis and characterization of metabolite of Simvastatin (SIM) that is β Hydroxy acid (BHA). Also the use of non-compendial reference standards is stressed in this communication when chromatographic procedures like HPLC are used to quantify synthesized metabolite.

Second part of the study is in silico models have nurtured an effective substitute to experimental procedures for predictions of ADME. In the work, properties such as ADME and toxicity of the synthesized metabolite BHA were predicted through different ADME database like SWISS ADME and molesoft.

MATERIAL AND METHODS:

Chemicals and Reagents:

The working standard and pure Simvastatin were received as gift samples from Cipla Pvt Ltd India. The tablets Simlip TAB (Cipla PVT Ltd) were purchased from a local medical shop. Other analytical reagents including methanol (HPLC grade), Potassium dihydrogen orthophosphate, Dipotassium hydrogen orthophosphate (HPLC grade) and ortho-phosphoric acid were used for an analytical grade. High purity Milli-Q water was purchased from Merck (India). All other chemicals used were AR grade.

Synthesis and characterization of BHA

Procedure:

Accurately weighed 50 mg of simvastatin was added in 50 mL methanol contained in a 100 ml round bottom flask. 25 mL of 2N sodium hydroxide was added to the above solution. It was then left to reflux over a water bath for 2 hours. The solution was then cooled and was made acidic using strong hydrochloric acid. Confirmation of the acidity of solution was done with methyl orange. This solution underwent three extractions using 10 mL of HPLC-grade chloroform. Layer of chloroform was separated. Petri dish was used to pour the solution for evaporation. BHA was separates out from the solution and recrystallized from optimum methanol.

Characterization:

BHA have been characterized by spectroscopic techniques by UV, FT-IR, MS, and NMR. Uv spectra of synthesized compound was recorded on JASCO V-730 Double beam UV/Vis spectrophotometer.

IR spectra were recorded and the absorption band assigned to vibration of different bands on JASCO FTIR-4600.

Characterization of synthesized metabolite was done by ^{1}H NMR spectroscopy. The compound was checked for solubility testing in $CDCl_3$, DMSOd6, D_2O. The compound was then sent to SAIF, Chandigarh for further NMR analysis for confirmation of structure.

A Simadzu QP 3010 mass spectroscopy interfaced with gas chromatography via electron impaction source was used for mass analysis and detection. A flow rate of 1 mL/min was used for sample analysis using Helium as mobile phase. The electron impaction source temperature was maintained at 280 nm. The detector used was gas chromatographic real analyzer. The column used was having 300 m length with 0.2 mm internal diameter.

HPLC Method Development:

Chromatographic conditions and instrument:

The analytical method development and validation were performed by using RP-HPLC [Schimadzu Model LC-2030 PLUS (IND)] with UV detector consisting of Kinetex C18 column 4.6 mm x 250 mm, 5 µm column. The flow rate of 1.0 mL/min, injection volume of 20 µL with a run time of 10 minutes, and a wavelength of 244 nm were optimized with mobile phase (gradient mode) consisting of Methanol (80%) : Phosphate buffer 20mM pH 3 (20%). Other instruments used in the validation like electronic balance (Mettler Toledo), ultra sonicator (Pci- Analytics), and pH meter (Mettler Toledo) were calibrated.

Preparation of buffer solution:

About 0.299 g of disodium hydrogen phosphate and 1.625 g of potassium dihydrogen phosphate were weighed and transferred in 550 ml of the distilled water and dissolved under continuous stirring. The buffer solution was filtered through 0.45 µm millipore membrane filter.

Preparation of mobile phase:

The methanol and phosphate buffer solution were filtered and degassed in the ratio of (80:20 % v/v) used as mobile phase, diluent, and blank solution. The pH of the mobile phase was adjusted to 3 by the addition of ortho-phosphoric acid.

Preparation of stock and standard solutions:

Accurately weighed 10 mg of SIM and BHA was dissolved in a sufficient quantity of mobile phase in a 100 mL calibrated volumetric flask respectively. The solution was sonicated for 10 minutes to dissolve the drug and the final volume of 100 mL was adjusted by the addition of the mobile phase. This resulting solution was considered as a stock solution (100 µg/mL). From these both stock solutions, 1-7 mL solutions for SIM were transferred in to 10 ml volumetric flask to get the final concentration ranging from 10 to 70 µg/mL and 0.1-1mL solutions for BHA were transferred in to 10 ml volumetric flask to get the final concentration ranging from 10 to 10 µg/mL respectively.

Preparation of sample solution:

About 20 tablets (Simplip) of simvastatin were weighed accurately, crushed & mixed properly. The powder equivalent to 10 mg of Simvastatin was weighed accurately and transferred in a 100 mL volumetric flask and a sufficient amount of diluents was added. The solution was sonicated for 20 minutes and volume was adjusted to 100 mL using the same diluents. This solution was considered as stock solution and further dilutions were made as per the requirement to get the desired SIM concentration.

Method Validation:

The developed analytical method was validated by performing system suitability, specificity, precision (system and method), accuracy, linearity, ruggedness, the limit of detection (LOD), and limit of quantitation (LOQ) as per ICH guidelines. [3]

System suitability study:

System suitability parameters were analyzed on freshly prepared standard stock solution SIM and BHA. Both the drug and its metabolite were injected into the chromatographic system under the optimization of chromatographic conditions. Parameters that were studied to evaluate the suitability of the system were number of theoretical plates, resolution factor, retention time, and selectivity, limit of detection and limit of quantitation.

Specificity:

The specificity of the analytical method was assessed by injecting standard and test solutions of the SIM. The samples were checked for interference peaks in the chromatogram. Chromatogram of standard and test should be identical with near retention time.

Linearity:

From the stock solution of SIM 1, 2, 3, 4, 5, 6,7 and 8 mL solutions were transferred into 10 ml volumetric flask and diluted with mobile phase to get the final concentration ranging from 10 to 80 µg/mL and BHA 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1 mL were transferred into 10 ml volumetric flask and diluted with mobile phase to get the final concentration ranging from 1 to 10 µg/mL and injected (20 µL) at a flow rate of 1.0 mL/min for 10 minutes. The samples were analyzed at a wavelength of 244 nm. The calibration curve was plotted for SIM and BHA between concentration and peak area obtained.

Precision:

The repeatability of the method was confirmed by performing the precision study. System and method precision was assessed.

System Precision:

The standard solution of SIM was injected five times into the system as per protocol. The RSD for peak area should be NMT than 2.0%.

Method precision:

The standard solution of SIM was injected six times individually using a single as per test method. The assay of SIM should be not less than 90.0 % and not more than 110.0%.

LOD/ LOQ:

The limit of detection was calculated as the minimum concentration which can be detected by the system and LOQ was estimated as the lowest possible amount which can be accurately and precisely quantified. From the linearity data, LOD and LOQ were calculated using the following formula.

$$LOD = \frac{3.3\sigma}{S}$$

σ = standard deviation of the response

S=slope of the calibration curve of the analyte.

$$LOQ = \frac{10\sigma}{S}$$

σ = standard deviation of the response

S = slope of the calibration curve of the analyte.

Accuracy:

The assay was performed in triplicate as per the test method with the equivalent amount of SIM concentration at 80%, 100%, and 120% of the labeled amount. The accuracy was determined against the standard solution as per their strength. The average % recovery of SIM was calculated. The mean % recovery of the SIM at each spike level should be not less than 90.0% and not more than 110.0%.

Robustness:

The robustness of the developed method was validated by intentional deviation in flow rate. The standard solution was prepared as per the test method and injected into the HPLC system at flow rates of 0.7 mL/min, 0.8 mL /min, and 0.9 mL/min. The system suitability parameters were evaluated.

PKPD predications

In order to reduce the cost, time and late failure of compound and gain early estimation of true positive results. We have performed *in-silico* based ADME analysis. For the assessment of ADME, toxicity and drug likeness of BHA using online server SWISS ADME and molesoft has been used. Compound BHA was subjected to Swiss-ADME online server for their drug likeness and toxicity prediction.

RESULT AND DISCUSSION:

After extensive literature survey sodium hydroxide selected for synthesis of BHA from SIM shown in Fig. 1. The BHA have been characterized by spectroscopic techniques like UV, IR, NMR and GC/MS. The UV, FT-IR, NMR and mass spectra, are reported in Fig. 2, Fig. 3, Fig. 4 and Fig 5 respectively. The analysis of spectral data confirmed the synthesis of product with desired purity and quality.

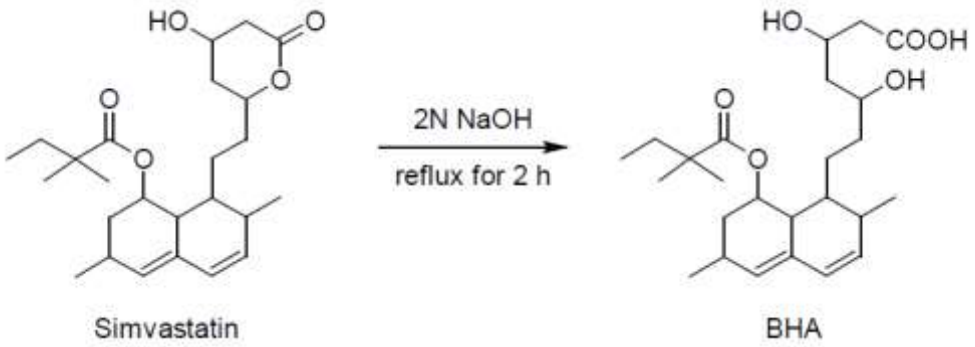


Fig. No 1: Synthesis procedure for metabolite β hydroxy acid (BHA)

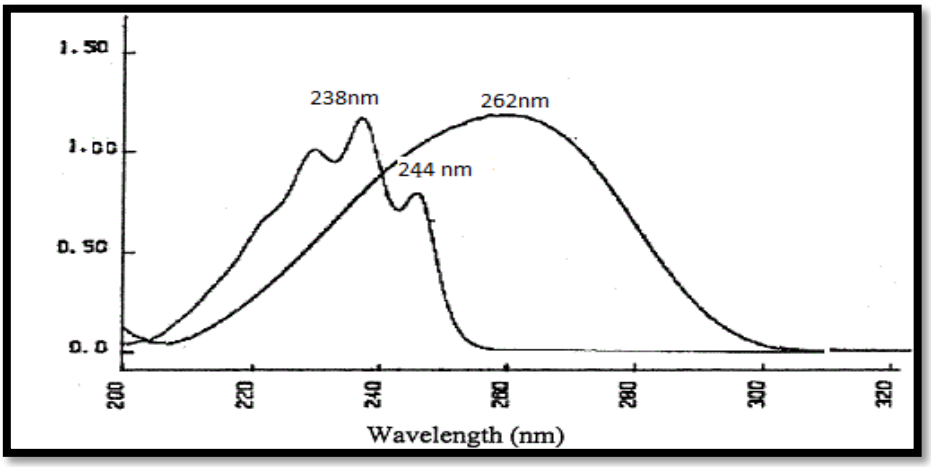


Fig. No. 2: Overlain UV Spectrum of SIM and BHA

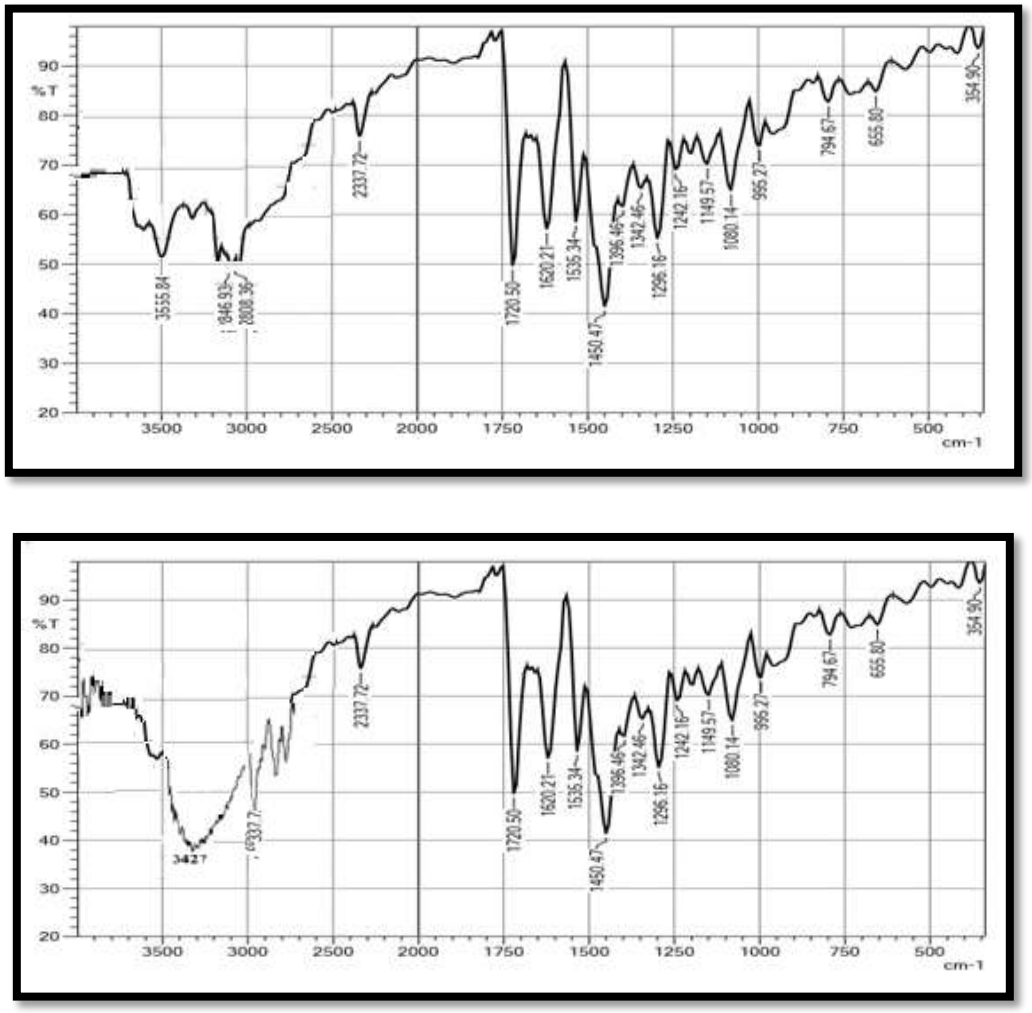


Fig. No. 3: Infrared Spectrum of SIM and BHA

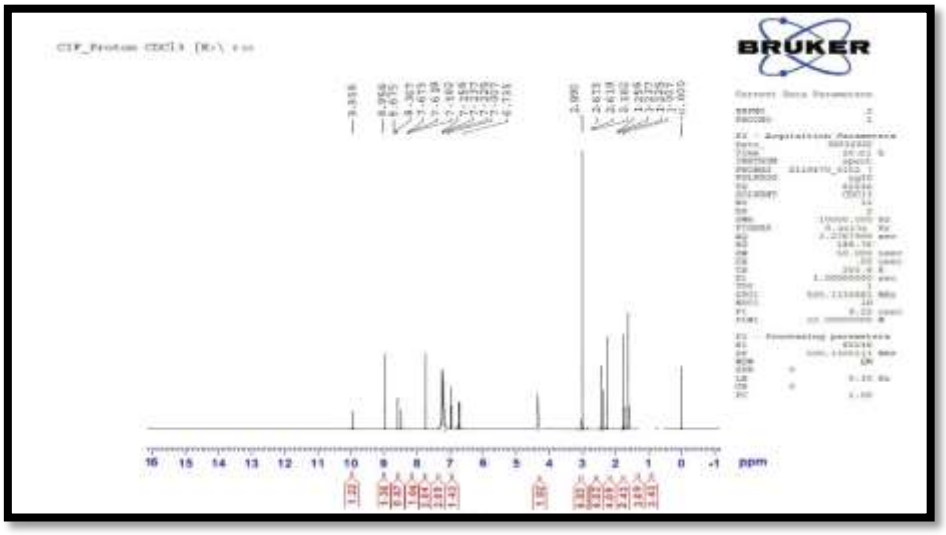


Fig. No. 4: NMR of BHA

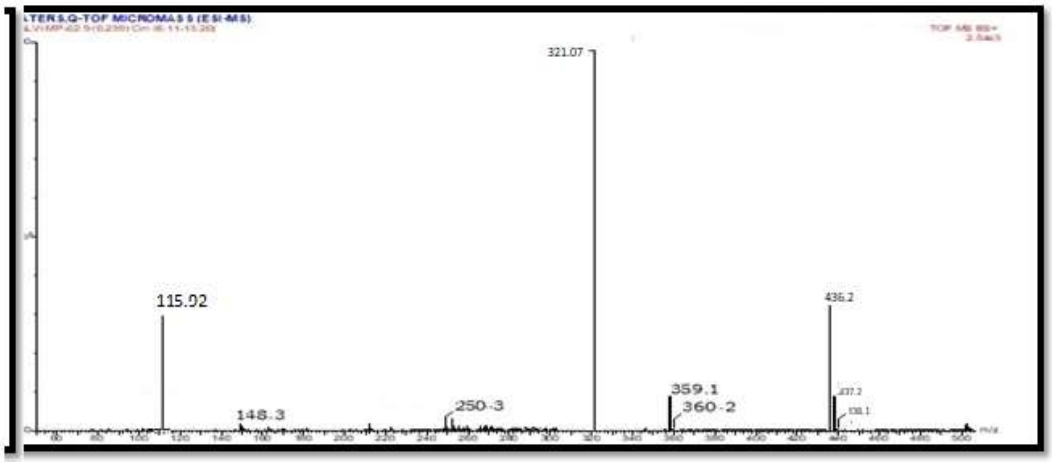


Fig. No. 5: Mass spectra of BHA

During analytical method development, the selection of stationary phase is very crucial and it mainly depends on the molecular weight and solubility of the molecule. In this study, the C18 column was selected based on the prior knowledge regarding the SIM to be best studied in RP-HPLC. Different concentrations of the methanol and buffer were studied and optimized to get the symmetric peak of the drug with a shorter run time. The proper separation well resolved, and excellent symmetrical peaks were observed with Methanol (80%) : Phosphate buffer 20mM pH 3 (20%). The retention time of the SIM and BHA were observed at 2.82 and 5.65 min respectively**.** Table 1 describes the various mobile phases tried with observations. The optimized chromatographic conditions are presented in Table 2. The chromatograms of the standard and test solutions are presented in Fig. 6. The method is validated by using ICH Q2R1 (Q2B) guidelines.

Table No. 1: Chromatographic behavior of SIM and BHA in mobile phase of various compositions

Sr No.	Mobile Phase	Concentration (% *v/v*)	Retention Time (min)	
			SIM	BHA
1	Acetonitrile: Water	80:20	2.98	Peak not obtained up to 15 min
2	Methanol: Water	95:5	3.93	Peak not obtained up to 15 min
3	**Methanol: Phosphate Buffer 20mM pH 3**	**80:20**	**2.82**	**5.65**
4	Methanol: Phosphate Buffer 20mM pH 3	90:10	3.40	3.86

Table 2: **Optimized chromatographic conditions**

Sr. No.	Parameters	Details
1	Flow rate	1.0 mL/min
2	Column	Kinetex C18, 4.6 mm x 250 mm, 5µm column
3	Detector wavelength	244 nm
4	Injection volume	20 µl
5	Run time	10 min
6	Retention time	2.82 and 5.65 min

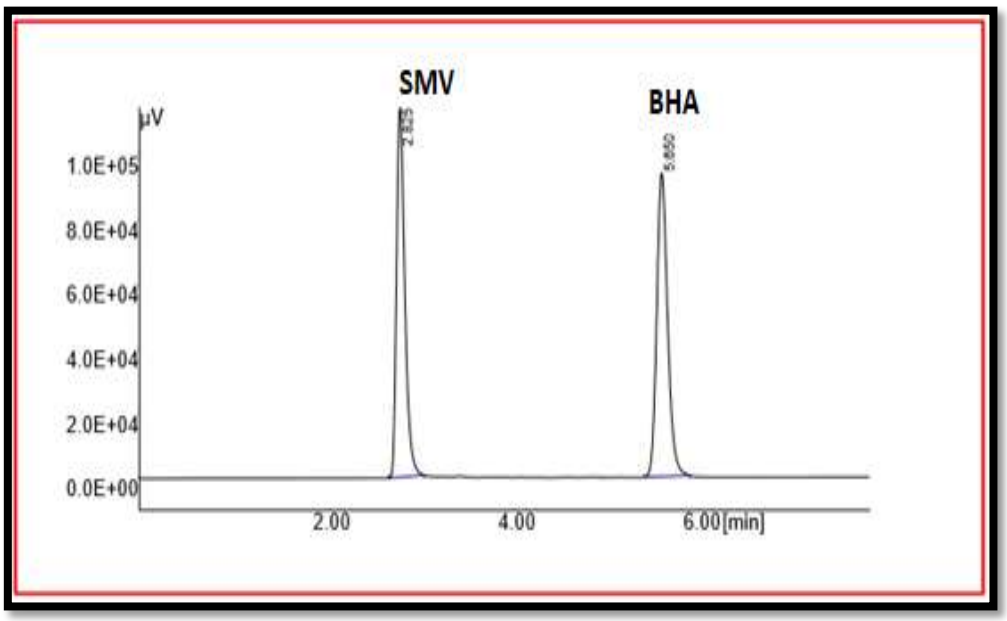


Fig. No. 6: **Chromatogram of SIM and BHA in Methanol: Phosphate Buffer (80:20% *v/v*).**

Linearity has been studied by plotting calibration curves for SIM and BHA. Calibration curve SIM and BHA were found linear between the concentration range of 10 to 100 µg/mL and 1 to 10 µg/mL respectively. The Fig. 7 and 8 represents linearity graph of SIM and BHA. The least square line was obtained by plotting the values of mean peak area versus concentrations

Table 3. As the value of correlation coefficient is 0.9980 and 0.9996 for SIM and BHA respectively, it indicates that method was linear.

Table No. 4: Linearity studies on SIM and BHA

Sr. No.	Sample			
	SIM		BHA	
	Conc^n (μgmL^{-1})	Area (N=3)	Conc^n (μgmL^{-1})	Area (N=3)
1	10	470629.41	1	49467.75
2	20	829510.75	2	90453.25
3	40	1617806.65	4	174892.5
4	60	2471759.04	6	258851
5	80	3147179.50	8	333405.12
6	100	3803640	10	413057
Straight line equation	Y=37621x+11302		Y=40442x+11072	
R^2	0.9980		0.9996	
SD	2026.52		2113.57	
Slope	39414		37277.06	

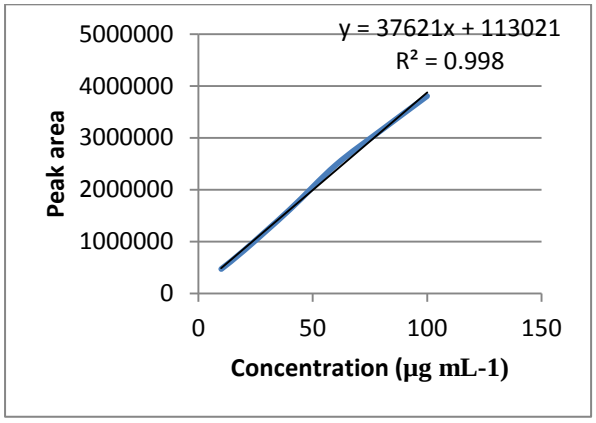


Fig. No. 7: Linearity graph of SIM

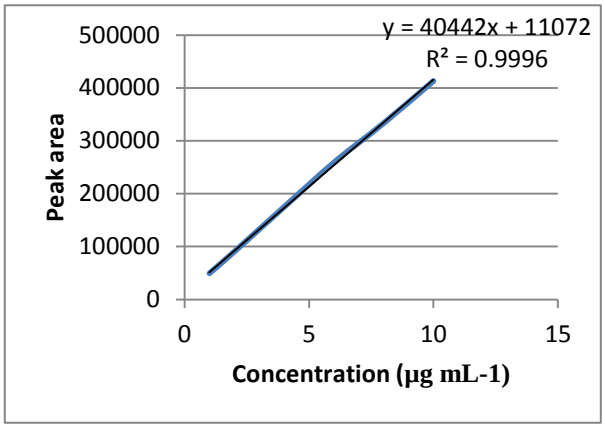


Fig. No. 8: Linearity graph of BHA

The recovery studies of BHA were found to be in range of 98.57% to 101.86% and shows %RSD ≥1.97, proving accuracy of the developed method Table 5. Intra and inter day precision studies proves the repeatability and reproducibility of method Table 6.

Table No. 5: Accuracy studies on SIM and BHA

Sample	Drug in formulation (mg)	Spiked level	Conc^n added (mg)	Area (N=3)	Conc^n found (mg)	% recovery	SD	%RSD
SIM	20	80%	108	3842204	106.46	98.57	54891.03	1.42
	20	100%	120	4295722	119.18	99.31	49508.15	1.15
	20	120%	132	4798773	133.29	100.97	34282.55	0.71

BHA	2	80%	10.8	401831.1	10.86	100.58	8560.058	1.97
	2	100%	12	478625.7	12.22	101.86	6126.373	1.27
	2	120%	13.2	515439.3	13.07	99.02	4467.501	0.87

***Acceptance criteria < 2.0.**

Table No. 6: Intra and inter day variations of SIM and BHA

Details of Parameter		Sample	
		SIM	BHA
Day 1	Mean (N=3)	2829398	260244.3
	SD	32789.25	3179.506
	%RSD	1.15	1.22
Day 2	Mean (N=3)	2839139	248366.6
	SD	34561.79	4384.592
	%RSD	1.21	1.76
Day 3	Mean (N=3)	2860359	272641.5
	SD	34656.01	4533.262
	%RSD	1.21	1.66

Specificity:

No interference was observed at the retention time of the SIM and BHA in both standard and test solutions. The retention time of both standard and test solutions was found to be identical and meet the acceptance criteria of the specificity test. Fig. 9.

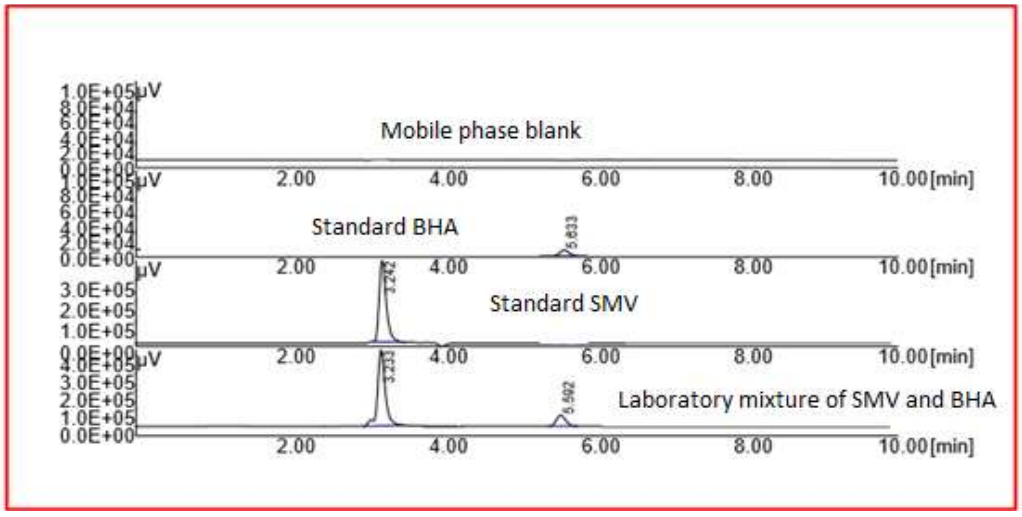


Fig. No. 9: Specificity of the method

LOD/ LOQ:

The LOD for was found to be 0.187 µg/mL for BHA and 0.169 µg/mL for SIM. The LOQ was observed 0.56 µg/mL for BHA and 0.51 µg/mL for SIM. The method showed excellent LOD and LOQ sensitivity.

Robustness:

The robustness of the method was studied by varying the flow rate from 0.7 mL to 0.9 mL and it was observed to be within limits. These changes produced no significant impact on

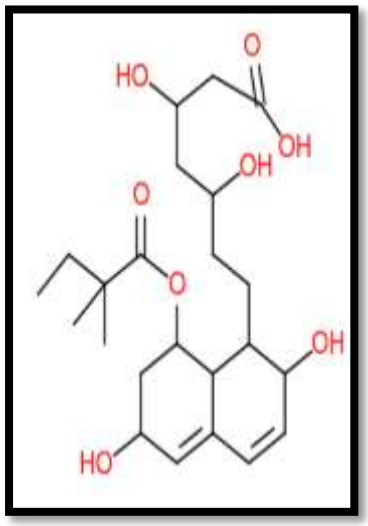
percentage recoveries of drugs. The results of the robustness study indicated that the developed method is robust and is unaffected by small variations in the chromatographic conditions. Table 7 summarizes the effects obtained due to changes of parameters.

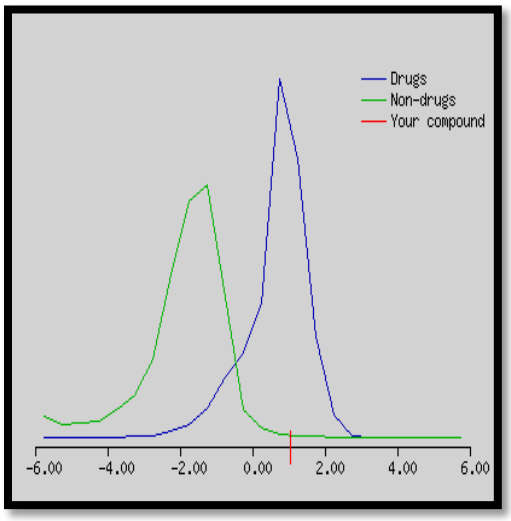
Table No. 7: Variations in flow rate

Sr. No	Flow rate (mL/min)	SIM		BHA	
		RT (min)	Peak area	RT (min)	Peak area
1	0.7	3.271	2222245.22	5.65	334160.0
2	0.8	3.280	2234764.22	5.72	245266.25
3	0.9	3.181	2265922.12	4.567	197041.50

PKPD predications

For the assessment of ADME, toxicity and drug likeness of BHA using online server SWISS ADME and molesoft has been used. Assessment of drug-likeness of the compound CDA is carried out using ***molsoft*** online server package Fig No. 10.





Drug Likeness score : 1.07

Fig. No. 10: Graphical data drug-likeness score of BHA

Smile: O=C(O)CC(O)CC(O)CCC2=C(C)C=CC1=CC(C)CC(OC(=O)C(C)(C)CC)C12

It is evident from the graphical data drug-likeness score of the compound BHA is 1.07 and thus can be considered as good drug candidate (Pharmacokinetic consideration).

The compound BHA revealed a (XlogP3) lipophilicity of 3.14; Molecular weight 434.57; Total polar surface area (TPSA) 104.06; ESOL (Log S) value -3.79. Considering the flexibility of structure, the compound exhibited a 11 rotatable bonds followed by maximum 6 h-bonds acceptors and 3 hydrogen bond donors exhibits that the designed compounds could possess a good oral bioavailability score of 0.56 with no violations in the drug likeness activity (Lipinski rule of five).

GI-absorption is high, not permeable to Blood Brain Barrier hence poor chance of crossing the CNS system. Compound BHA is predicted to be a substrate to (P-glyco protein) Pgp, hence chance of efflux out is high. Table 8.

Table No. 8: PKPD analysis of BHA from Swiss-ADME server

Sr. No.	Parameter	Predication
1	GI absorption	High
2	BBB permeant	No
3	Pgp substrate	Yes
4	CYP1A2 inhibitor	No
5	CYP2C19 inhibitor	No
6	CYP2C9 inhibitor	No
7	CYP2D6 inhibitor	No
8	CYP3A4 inhibitor	Yes
9	Lipinski #violations	0
10	Ghose #violations	0

Further assessing the metabolic factor here we identified that compound BHA is predicted as a substrate to CYP250 (CYP1A2, CYP2C19, CYP2C9, CYP2D6) class of enzymes, except for CYP3A4 is predicted as inhibitor, hence the selected compound could be mildly difficult to metabolized and can be delayed in excreted out of the system.

Due to inhibition of the selective CYP450 class of enzyme following pathways and metabolism can be affected they are: Linoleic acid metabolism, Steroid hormone biosynthesis, Retinol metabolism, Metabolism of xenobiotics by cytochrome P450, Bile secretion, Drug metabolism and Chemical carcinogenesis data shown in Fig. 11.

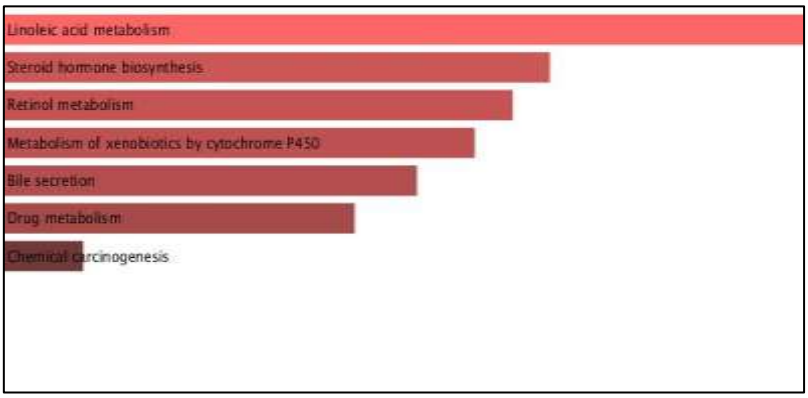


Fig. No. 11: Due to CYP450 (CYP3A4) class of enzyme inhibition following pathway may affect

Further toxicity of compound BHA was analyzed using ADMET SAR version 2 as follows;

Table No. 10: ADMET TOX Prediction using ADMETSAR tool box

Sr. No.	Parameter	Prediction	Probability
1	EYE corrosion	-	0.6300
2	Eye irritation	+	0.067
3	Carcinogenicity	-	0.6733
4	Hepatotoxicity	+	0.4300
5	Acute oral toxicity	+	1.36

6	Ames Mutagenicity	-	0.7300
7	Respiratory toxicity	-	0.600
8	Nephrotoxicity	-	0.5632

The ADMET property table includes classification and regression results. For classification, the columns of the table are endpoint, value and probability, respectively. The value is the predict labels. For example, for toxicity endpoints, the value "+" means Positive/Toxic while "-" means Negative/Nontoxic. The probability is related to the value, and it is generally higher than 50% because if the probability was less than 50%, it should have been predicted as the other result. For the regression models, the columns are endpoint, value and unit.

CONCLUSION:

The developed method is simple, accurate, precise sensitive and selective for estimation of simvastatin and its laboratory synthesized and characterized metabolite BHA. Developed method can be used for estimation of BHA as an impurity if often present in in bulk drug and formulations of simvastatin, further optimization of the method for quantification of BHA in biological fluids could make it useful for clinical and bioequivalence studies. Assessment of toxicity by using computational tools like Swiss tool is reported with the hope of reducing threats.

CONFLICT OF INTEREST:

The author declares no conflict of interest for the present manuscript.

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