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Research Article

Validation of Simvastatin Analysis Methods in Human Blood Plasma (*In Vitro*) Using HPLC-UV Detector

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ABSTRACT

The use of simvastatin (SV) increases along with the increasing number of patients with hyperlipidemia and cardiovascular disease risk factors. Consequently, this condition leads to the increasing need of analytical determination of SV in blood plasma. Analysis of SV in human plasma using protein precipitation method and HPLC with UV detector has not been reported. This research was purpose to find out the rapid, accurate, and valid of SV analysis method in human plasma. In this research plasma samples were treated with protein precipitation method. The analyte was then analyzed using HPLC with C_{18} column 250x4 mm and 5 µm of particle size, the mobile phase contained of phosphate buffer 0.01 M (pH 4.0) and acetonitrile 30:70 v/v with flow rate 1 mL/minute, and detected at 239 nm. The analysis method was validated based on some parameters, such as selectivity, accuracy, precision, repeatability, linearity, LOD, LOQ, and system suitability. The result showed selectivity represented by Rs was 2.870, repeatability by its CV< 2%, and linearity by its coefficient correlation (r) 0.9992 for concentration range 0.08-0.32 ppm. Based on chromatogram peak area, LOD and LOQ were 0.0132 and 0.0440 ppm respectively, accuracy and precision were 86.25-89.36% and 0.66-1.81% were obtained. The result of system suitability test from retention time and chromatogram peak area showed by its CV < 2%. The analysis method was proved to be valid for SV analysis in human plasma.

Keywords: HPLC, Simvastatin, Protein Precipitation method, Human plasma.

INTRODUCTION

SV is an antihyperlipidemic drug that works as an inhibitor of hidroksimetilglutaril-CoA (HMG-CoA) reductase[1]. It is often used in the treatment of hypercholesterolemia[2]. In addition, SV can also prevent the onset of cardiovascular disease[3].

Previous studies have reported the analyses of SV in human blood plasma by the method of HPLC using an UV detector[2,4], followed by liquid-liquid extraction (LLE) in LC-MS[5], SPE in UPLC using an MS detector[6], SPE in HPLC using an MS detector[7,8], and by the method of protein deposition in LC-MS[9–11].

Studies regarding SV in human blood plasma by the method of protein deposition and HPLC using an UV detector are yet to be reported. Protein deposition is a method to isolate or separate the compounds from a matrix[12–14]. We chose HPLC combined with a UV detector as is widely preferred by clinical laboratories in Indonesia. Prior to in vivo analyses, a standard procedure is to investigate the action of drugs in vitro on human blood plasma. Moreover, a validation of the in vitro method should be performed to evaluate its compliance with the requirements.

In this study, the in vitro analysis of SV in human blood plasma by protein precipitation and HPLC using an UV detector was validated. The results of this study are expected to serve as a reference for laboratories undertaking investigations of SV in plasma, especially for the purposes of drug monitoring

MATERIAL AND METHODS

Materials

SV (Teva, Belgium, Purity >99%), sodium dihydrogen phosphate: p.a. monohydrate (Merck), acetonitrile HPLC

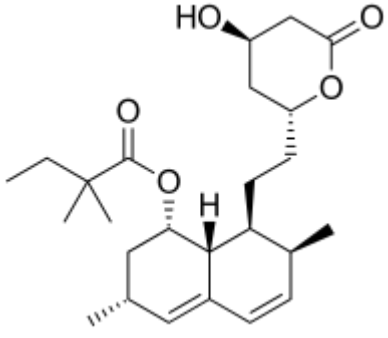

Figure 1: Structure of SV

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Table 1: Calculation of SV molar extinction.

Molar extinction of SV in mobile phase on 239 nm			
Replication	Concentration (M)	Absorbance	Molar extinction ε ($M^{-1}.cm^{-1}$)
1	0.0000119	0.2771	23.198
2	0.0000179	0.4169	23.268
3	0.0000239	0.5645	23.630
Total			70.097
$\bar{X}$			23.366

Table 2: Result of HPLC optimization.

Composition of Mobile Phase (phosphate buffer pH 4.0: ACN)	Flow rate (mL/minute)	Time retention of SV (minute)	Resolution (Rs)
40: 60	0.8	18.467	2.967
	1.0	15.600	3.167
35: 65	0.8	12.983	2.344
	1.0	10.717	2.435
30: 70	0.8	9.692	2.141
	1.0	7.967	2.870

Table 3: Results of the recovery extraction SV based on Area chromatogram (n = 3).

Recovery extraction SV (%)		
Replication	Concentration of SV (ppm)	
	0.16	0.32
1	99.8250	97.7758
2	100.0692	97.7943
3	99.8252	97.7211
$\bar{X}$	99.91	97.76
%CV	0.14	0.04

Table 4: Test results of repeatability SV based on retention time and Area chromatogram (n = 6).

Concentration of SV (ppm)	Retention time (minute)	Area chromatogram
0.32	8.183	6521
0.32	8.200	6432
0.32	8.192	6518
0.32	8.183	6493
0.32	8.175	6682
0.32	8.183	6562
$\bar{X}$	8.186	6534.67
%CV	0.11	1.28

grade (JT Baker), phosphoric acid pro analysis (Merck), ethanol pro analysis (Merck), aqua bides (Ika Pharmindo), human blood plasma was obtained from the Red Cross Indonesia Bandung).

Tools

A set of high-performance liquid chromatography instrument (Shimadzu LC-10 ATVP) equipped with UV-Vis detector SPD, Shimadzu auto injector System Controller SCL-A, column HPLC (LiChroCART); length 250 mm, particle size 5 μm, Spectrophotometer UV-Vis (analytical Jena, Specord 200), pH meter (Ohmeter), ultrasonic bath (NEY 1510), tube centrifugation, analytic scales (Sartorius) sensitivity of 0.1 mg, vacuum filter with porous filter 0.4-0.45 μm

Methods

Preparation of mobile phase

As many as 0.6900 grams of sodium dihydrogen phosphate monohydrate weighed thoroughly, put in a 500 ml measuring flask, diluted with aquabidest until final volume was reached, phosphoric acid is added to adjust the pH to 4.0 using the pH meter. The solution is filtered to use Millipore 0.45 μm with the help of a vacuum. Then a solution was vacuumed by an ultrasonic bath for 15-20 minutes. Acetonitrile was vacuumed using ultrasonic bath until air bubbles are appeared.

Measurement of wavelength.

Accurate weight of 50 mg SV, then dissolved into a measuring flask 10 mL with ethanol to mark boundaries to obtain the final concentration of 5000 ppm. Take of 20 μL of solution, put in 10 mL measuring flasks, diluted with phase motion (acetonitrile and phosphate buffer) so the obtained concentrations of 10 ppm. Then the solution is analyzed by ultraviolet spectrophotometer at a wavelength of 220-320 nm so that the retrieved spectrum absorption maximum wavelength and SV.

Determination of molar extinction

A SV solution with each concentration 11.9; 17.9; μM and its absorbance measured at 23.9 on maximum wavelength of SV, and calculated the value of the molar extinction.

Optimaztion of HPLC condition

A SV solution with a concentration 0.16 ppm was injected as many as 10 μl into a tool of comparison with HPLC mobile phase the composition of phosphate (0.01 pH 4.0 M) and 40:60 acetonitrile v/v, 35:65 v/v, 30:70 v/v on the flow rate 0.8 and 1.0 mL/min. Retention time and the separation of the peak of SV with peak plasma components that are generated from each condition HPLC observed. The most excellent conditions selected for later use on the next stage of analysis.

Sample preparation and determination of recovery extraction.

In five tube centrifugations, put as many as 500μl plasma containing SV with a final concentration of 0.08; 0.12; 0.16; 0.24; and 0.32 ppm. Afterward, acetonitrile is added as much as 1 ml. The mixture is then being homogenized by a vortex mixer for two minutes. The fifth of these tubes are inserted into the centrifugation and centrifuged for three minutes with a speed of 3000 rpm. The results of the extraction solution in the form of injected as many as 10 μl to optimum conditions. The efficiency of extraction (% recovery) was obtained by comparing plasma chromatogram area that have a spike concentration with SV 0.16 ppm to 0.32 ppm against area chromatogram motion phases that have spike the same concentration with SV.

Validasi Metode Analisis

Validation of analytical methods include some parameters, namely selectivity, repeatability, linearity,

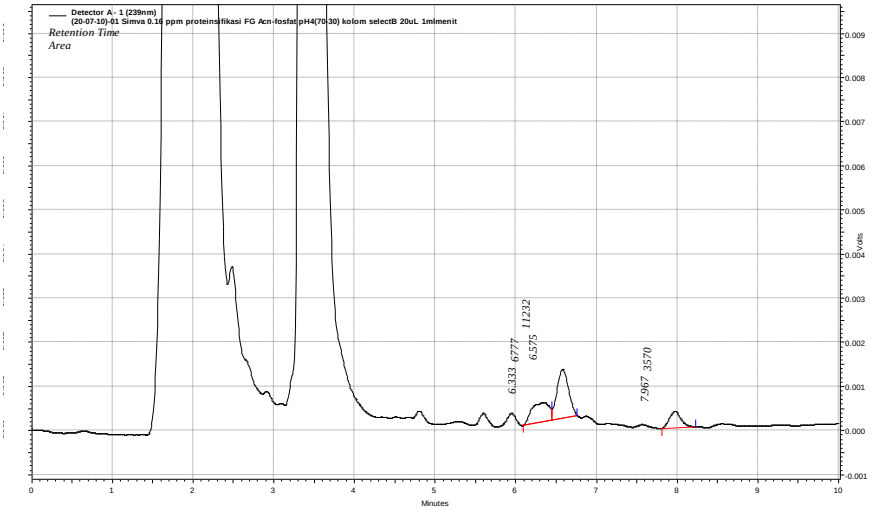

Figure 2: Chromatogram of optimization HPLC condition of SV.

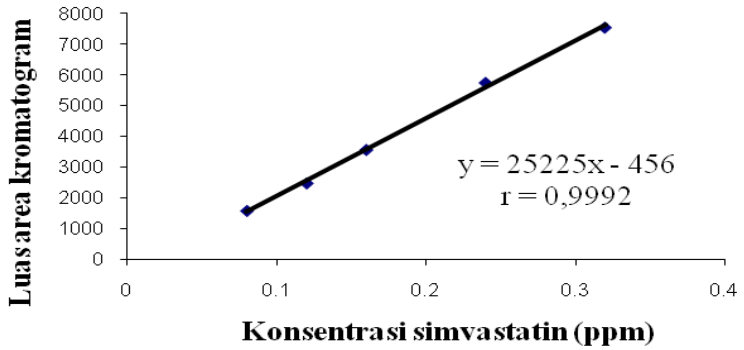

Figure 3: Linearity curve of SV base on area of chromatogram.

LOD (limit of detection), LOQ (limit of quantitation, precision, accuracy, and suitability of the system.

Determination of selectivity

Selectivity is determined by looking at the chromatogram SV HPLC separation results, then determined the value of the resolution.

Repeatability test

Repeatability is determined by making a solution of SV 0.32 ppm in blood plasma, and then extracted using protein precipitation methods. Analytic is injected as many as 20 µl into optimum condition HPLC tool. The experiment is repeated six times then calculated coefficients of variation.

Linearity Evaluation

Linearity is determined by making a series of five raw curve concentration SV (0.16 0.12 0.08; and 0.32 0.24 ppm) in blood plasma. SV in blood plasma is extracted by using the method of deposition of protein. Analytics is injected as many as 20 µ L into the optimum conditions HPLC tool. The experiment was repeated three times. The equation of a calibration curve with a correlation coefficient of best value used to set the sample rate.

LOD and LOQ determination

The value of LOD and LOQ was calculated based on the calibration curve SV that has equations of lines with the best correlation coefficient.

Test of accuracy and precision

Accuracy and precision is determined by making a solution of 0.1 SV samples and 0.3 ppm in blood plasma was then extracted by methods of deposition of protein. Analytics is injected as many as 20 µ L into the optimum conditions HPLC tool. The experiment was repeated three times then percent accuracy (recovery) and precision (% CV) is calculated.

Suitability system test

Test the suitability of the system performed against SV 0.32 ppm in a sample of blood plasma, then extracted using protein precipitation methods. analytics is injected as many as 20 µ L into the optimum conditions HPLC tools, conducted as many as six repetitions. The coefficient of variation of the retention time and area chromatogram calculated.

RESULT

The maximum absorbance of SV was obtained at 239 nm based on the maximum wavelength of the UV detector. The average molar extinction obtained was 23.366 $M^{-1}cm^{-1}$

Optimization of HPLC analysis condition was presented in table 2.

Result of recovery extraction in human plasma was exhibited in table 3.

Validation of SV method Analysis

Selectivity

Table 5: Test result accuracy and Precision of the sample Chromatogram Area based on SV.

Replication	Nominal concentration of SV (ppm)	Recovery of SV (ppm)	%Accuracy	Presisi (%CV)
1		0.08646	86.46	
2	0.1	0.08908	89.08	1.81
3		0.08936	89.36	
1		0.25875	86.25	
2	0.3	0.26117	87.06	0.66
3		0.26208	87.36	

Table 6: System Suitability results of SV analysis (n = 6)

Parameters	Cooficient of variation (%CV)
Retention time	0.11
Area chromatogram	1.28

Selectivity was showed in the figure 2.
Result of linearity was showed in figure 3
Result of accuracy and precision was presented in table 5.
Result of System Suitability test showed in table 6

DISCUSSION

The maximum absorbance of SV was obtained at 239 nm based on the maximum wavelength of the UV detector. The average molar extinction obtained was 23.366 $M^{-1}cm^{-1}$. This shows that SV has a chromophore system that is detectable using an UV detector in HPLC [15, 16]. The value of the molar extinction SV can be observed in table 1.

Optimization conditions for HPLC included chromatography retention time and resolution (Rs) of the composition at various phases of motion. A phase composition of phosphate buffer 0.01 M (pH 4.0) and acetonitrile (30:70) with a flow rate of 1.0 mL/min was chosen because it gives a resolution of 2.870 (≥1.5) and a peak retention time. Data about the conditions of the HPLC are shown in Table 2.

The process of extraction and Recovery Results using the method of Extraction of Protein Deposition.

Plasma protein deposition was performed by adding acetonitrile as a precipitating agent at a ratio of 1:2 so that the plasma proteins could precipitate out [14]. The filtrate was obtained and about 20 μL was injected into the HPLC tool. Based on the area of spike against the phase of motion, the value of % recovery was obtained. The results of the recovery of SV are presented in Table 3. The extraction efficiency was in the range of 80–120% [15.]

Analysis Validation Methode

The selection of the method was validated based on the peak value of resolution (Rs) of SV and the culmination of other components. Figure 1 shows a peak with a retention time of 7.967 minutes and a peak value of resolution of 2.870. This value meets the criterion for selectivity of the resolution, i.e. >1.5[16].

A test of repeatability based on the retention time provides the value of the coefficient of variation (CV) <2%, concurring with the value for validation prescribed in literature[16]. Results of repeatability are shown in Table 4.

A linear regression line equation with the best correlation coefficient is used to set the sample rate for the calculated value then SV accuracy and precision. A linear regression line equation based on area chromatogram which is used to set the levels of SV is y =25225x – 456 with r = 0.9992. The value of the correlation coefficient obtained was the validation criteria for the linearity parameter has a value because > 0.99. This indicates the existence of a linear relationship between the increases in the concentration of SV with response tool. The SV has a calibration curve equation of lines with the best correlation coefficient is shown in Fig. 2.

Based on the chromatogram, the values of LOD and LOQ were found to be 0.0132 ppm and 0.0440 ppm, respectively. The LOQ value obtained using this method suggests that the method is suitable for the purpose of drug monitoring. Based on the chromatogram, the % recovery obtained in this study was in accordance with that prescribed for biological liquid samples[15,16]. The precision (% CV) was also in agreement with the standard requirement, namely <10%[15,16]. The accuracy and precision data of the study are shown in Table 5.

the results of the suitability test of the system indicated by repeatability measurement methods, indicating that the method of analysis used has met the criteria for compliance of the system with the value of CV of retention time and area chromatogram < 2%[15,16]. A system suitability test results are listed in table 6.

CONCLUSION

The optimum conditions for the extraction of SV from human blood plasma and the validity of experimental method considered are described in this article. Based on the results of repeatability, linearity, selectivity, accuracy, and precision, the method employed here fulfills the requirements for detection of SV in the range of 0.08 – 0.32 ppm.

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CONFLICT OF INTEREST

No conflict of interest associated with this research.

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