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UV-Derivative Spectrophotometric and Stability-Indicating High-Performance Liquid Chromatographic Methods for Determination of Simvastatin in Tablets

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***SUMMARY*. A stability-indicating high-performance liquid chromatographic (HPLC) and a second-order derivative spectrophotometric (UVDS) analytical methods were validated and compared for determination of simvastatin in tablets. The HPLC method was performed with isocratic elution using a C18 column and a mobile phase composed of methanol:acetonitrile:water (60:20:20, v/v/v) at a flow rate of 1.0 ml/min. The detection was made at 239 nm. In UVDS method, methanol and water were used in first dilution and distilled water was used in consecutive dilutions and as background. The second-order derivative signal measurement was taken at 255 nm. Analytical curves showed correlation coefficients > 0.999 for both methods. The quantitation limits (QL) were 2.41 μg/ml for HPLC and 0.45 μg/ml for UVDS, respectively. Intra and inter-day relative standard deviations were < 2.0 %. Statistical analysis with t- and F-tests are not exceeding their critical values demonstrating that there is no significant difference between the two methods at 95 % confidence level.**

INTRODUCTION

Simvastatin (SV), [lS-[lα,3α,7α,8β(2S*,4S*), 8aβ]]-2,2-dimethylbutanoic acid 1, 2, 3, 7, 8, 8a-hexahydro-3,7-dimethyl-8-[2-(tetrahydro-4-hydroxy-6-oxo-2H-pyran-2-yl)-ethyl] naphthalenyl ester (Fig. 1), is obtained from the fermentation of *Aspergillus terreus*. This compound, a highly effective cholesterol-lowering agent, is being used in the control of hypercholesterolemia. It exhibits a very important hepatic first-pass metabolism, acting by blocking the 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA), reducing thus the low-density lipoproteins. The SV is a potent inhibitor of HMG-CoA reductase, which is a limiting enzyme in cholesterol biosynthesis [1].

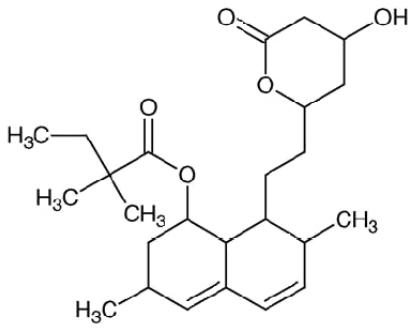

Figure 1. Chemical structure of Simvastatin.

Several methods based on different techniques have been reported for the analysis of SV in biological fluids. These include, high-performance liquid chromatography/mass spectrometry (LC/MS/MS) [2-9] and gas chromatography/mass spectrometry (GC/MS) [10] and high-performance liquid chromatography (HPLC) [11-15]. Although these methods are sensitive to permit their use in determination of SV in urine, plasma or serum, only few methods are reported for assay of SV in pharmaceutical formulations. Among them, HPLC methods have been described using expensive reagents or buffers in the mobile phase [16-23]. Some spectrophotometric methods have been reported in the literature for the determination of SV by complexation with metal ions, using organic solvents or complex reagents [24-29]. These procedures are not adequate for routine applications, and the condi-

KEY WORDS: Simvastatin, Stability-indicating HPLC method; UVDS method.

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tions are quite harmful to the environment. Based on the review of the literature, it was not found any work comparing these two analytical techniques for the determination of SV in tablets. Therefore, the aim of this research was to develop, validate and compare simple, economic and fast analytical methods to be applied in quality control routine analysis for the determination of SV in tablets. The proposed HPLC method can be considered as stability-indicating method because it can separate SV from its degradation products. The proposed UVDS method is inexpensive and low-polluting, so it can be used as an excellent alternative method in routine analysis.

EXPERIMENTAL

Chemicals and reagents

Simvastatin (99.85% purity) and tablets containing 10.0 mg SV/tablet (sample A), 20.0 mg of SV/tablet (sample B) and 40.0 mg SV/tablet (sample C) were supplied by Laboratório Baldacci S.A. Brazil. Placebo was prepared by mixing lactose monohydrate, microcrystalline cellulose, pregelatinized starch, butyl-hydroxyanizol, butylated hydroxytoluene, talc, magnesium stearate, hypromelose, hydroxypropyl cellulose, titanium dioxide, iron oxides, iron III oxide (10 and 20 mg), yellow iron oxide, (5 and 20 mg) and black iron oxide (20 mg). Methanol, acetonitrile (HPLC grade) and methanol (analytical grade) were purchased from Merck® (Darmstadt, Germany). High purity water was prepared by using Milli-Q® Plus (Millipore, Milford, USA) water purification system.

HPLC and UVDS instrumentation and experimental conditions

The HPLC method was performed using a liquid chromatograph model CG 480C with an isocratic pump, a UV-VIS detector CG 435, an injector fitted with a 20 µl loop and an integrator CG 200 (Scientific Instruments®, São Paulo, Brazil). The method was carried out on a LiChrospher® C18 column (125 x 4.6 mm, 5 µm). The mobile phase was constituted of methanol:acetonitrile:water (60:20:20 v/v/v); the flow rate was 1.0 ml/min. The UV detection was made at 239 nm, and all analyses were done at 25 ± 1 °C. The mobile phase was prepared fresh each day and vacuum-filtered through a 0.45 µm pore filtration membrane (Millex® HV, Millipore, Milford, USA). The UVDS method was performed on a UV-VIS spectrophotometer model UV-1601, and the output signal was monitored and processed using UVPC v3.91 Personal Spectroscopy Software (Shimadzu® Corporation, Tokyo, Japan). The method was performed using methanol and distilled water in the first dilutions and distilled water as solvent in posterior dilutions and as blank. The second-derivative $^2A/^2\lambda$ signals were taken at 255 nm using a 1.0 cm quartz cell. The Δ^2A values were measured at 0.2 nm with scanning speed of 370 nm/min over a range from 360 to 200 nm. The second-derivative spectra were obtained by plotting λ against $\Delta^2A/\Delta^2\lambda$ (-0.35 to 0.35). The dilutions were made using three piston-operated pipette (0.02-0.1 ml, 0.1-1.0 ml and 0.5-5.0 ml) Transferpette® from Brand® (Werthein, Germany).

Methods validation

The methods were validated according to the USP 31th ed. 2008 [16] and ICH Guidance for Industry [30]. Tests of t and F were used to compare the proposed methods [31].

System suitability

Standard solution containing 9.0 µg/ml of SV was prepared by dilution in the mobile phase. System suitability was determined from six replicate injections of standard solution.

Selectivity

The selectivity of methods was evaluated through the analyses of the placebo solutions, which were prepared with the excipients of the pharmaceutical formulation. Adequate dilutions were made to obtain solutions equivalent to 9.0 µg/ml of SV. For HPLC method, triplicate determinations were made with solutions of each flask, and the chromatograms were compared with those obtained with standard and sample solution containing 9.0 µg/ml of SV. For UVDS method, triplicate measurements were made with solution of each volumetric flask, and the second-derivative spectra were overlaid with those obtained with standard and sample solutions containing 9.0 µg/ml of SV.

Analytical curves

The stock solutions were prepared by weighing separately and exactly 10.0 mg of standard SV in duplicate and transferring to 50 ml volumetric flasks. Approximately 43 ml of mobile phase was added for HPLC method. For UVDS method 20 ml of methanol was added to dissolve SV and approximately 20 ml of distilled water was also added. Both mixtures were soni-

cated for 10 min. The volumes were completed with mobile phase for HPLC and distilled water for UVDS. Aliquots of 0.05, 0.25, 0.45, 0.65 and 0.85 ml were transferred to 10 ml volumetric flasks. The volumes were completed with mobile phase for HPLC and distilled water for UVDS. The final concentrations were 1.0, 5.0, 9.0, 13.0 and 17.0 µg/ml for both methods. The analytical curves were constructed by plotting mean response (n = 3) versus respective concentrations of SV.

Detection limit (DL) and quantitation limit (QL)

The DL and QL for HPLC and UVDS methods were determined based on standard error and slope of the analytical curves [30].

Preparation of samples and standard solutions for intermediate precision determination

Twenty tablets were weighed separately. The mean weight was determined followed by trituration to fine powder. For HPLC and UVDS methods, two separate amounts, equivalents to 1.5 mg of SV were weighed and transferred to two 50 ml volumetric flasks. For HPLC method, approximately 35 ml of mobile phase was added. For UVDS method, approximately 20 ml of methanol was added until solubilization, then approximately 20 ml of distilled water was added. The mixtures were sonicated for 10 min. The volume of each volumetric flask was completed with mobile phase (HPLC) and distilled water (UVDS), and the final solutions were filtered through Whatmann's filter paper Nº 1. The solutions were stored refrigerated until use. Ten separate aliquots of 3.0 ml for each method were transferred to 10 ml volumetric flasks, and the volumes were completed with mobile phase for HPLC and with distilled water for UVDS. Solutions for HPLC were filtered through 0.45 µm, (Millex®HV, Millipore, Milford, USA), before injection into the chromatographic system. For the two methods, the final concentrations were approximately 9.0 µg/ml of SV. Standard solutions containing 9.0 µg/ml of SV were prepared by dilution in mobile phase and distilled water for HPLC and UVDS methods, respectively. Triplicate determinations were made using the solutions of each flask during three consecutive days using HPLC and UVDS methods.

Preparation of samples and standard solutions for accuracy determination

The accuracy of the methods was assessed by determining the agreement between the measured analyte concentrations of the fortified and unfortified sample and the known amount of analyte added to fortify the sample. For the HPLC and UVDS methods, standard and sample solutions were prepared separately to obtain solutions containing 200.0 µg/ml of SV. Three aliquots of 0.125, 0.325 and 0.525 ml of standard solution for each method were used to fortify 0.125 ml of sample solution in three separate 10 ml volumetric flasks. The final SV concentrations of these fortified solutions were 5.0, 9.0 and 13.0 µg/ml. For HPLC method, all solutions were filtered through 0.45 µm, (Millex®HV, Millipore, Milford, USA), before injection into the system.

Preparation of standard solutions for robustness determination

Robustness was assessed by testing the susceptibility of measurements to deliberate variation of the analytical parameters. For HPLC method, the mobile phases used were constituted of methanol:acetonitrile:water (59.5:21:19.5, v/v/v) and (60.5:19:20.5, v/v/v), at a flow rate of 0.9 and 1.1 ml/min. The volume of injection was fixed at 20 µL, and UV detections were made at 234 and 243 nm. All analyses were done at 25 ± 1 °C. The mobile phases were prepared fresh and vacuum-filtered through a 0.45 µm, Millex®HV (Millipore, Milford, USA). For UVDS method, first dilutions were made with methanol and water, and second dilutions were made with distilled water. Standard solutions were prepared separately as previously described to obtain solutions containing 9.0 µg/ml of SV, and this test was made in triplicate for each method. Instrumental responses were evaluated for the robustness variability.

Preparation of samples and standard solutions for stability determination

For HPLC and UVDS methods, standard and sample solutions were prepared separately as described above to obtain solutions containing 9.0 µg/ml of SV. Triplicate measurements were made during three days (intermediate precision).

Stress testing

Neutral hydrolysis was made with water, chemical oxidations with 3% H_2O_2, acid hydrolysis with 1.0 mol/l HCl and alkaline hydrolysis with 1.0 mol/l NaOH. The solutions were heated at 80 °C during four hours. Aliquots resulting from neutral hydrolysis and chemical oxidation

were adequately diluted before HPLC analysis. The solutions obtained after acid and alkaline hydrolysis were transferred to volumetric flasks and neutralized before HPLC analysis. The final concentrations of solutions were 9.0 µg/ml of SV, and the volumes were completed with mobile phase.

Comparison between HPLC and UVDS

The F-test was applied to determine whether one population is more variable than another in relative standard deviation (RSD) of intermediate precision assay. The t-test was applied to determine whether there is or there is not a significant difference between the means of two proposed methods [31].

RESULTS AND DISCUSSION

Selection of the HPLC and UVDS conditions

In order to develop an efficient and simple stability-indicating HPLC method for quantitative determination of SV, different mobile phases were tested to achieve efficient separation of SV, excipients and degradation products formed under stress conditions. The objective of this research was to obtain rapid and reliable chromatographic separation with good peak symmetry using simple mobile phase without the presence of buffers, acids or other ionic substances, since the use of this kind of substances demand too much special care with chromatographic system, and can damage the column in routine analyses [32]. The proposed method was selective and specific for the separation and quantitative determination of SV in a short chromatographic run 4.70 min (Fig. 2).

To develop and to validate the UVDS method, different solvents were tested including methanol and ethanol. The objective was to develop sensitive, inexpensive and efficient method. Methanol was used for solubilization of SV, and after its solubilization, distilled water was used for consecutive dilutions. Second-order derivative spectra were used to annul the interference of excipients. The wavelength interval to perform Δ^2A measurements was 0.2 nm; thus, higher values increased the signal/noise ratio. Good results of spectra were obtained at a scanning speed of 370 nm/min. It was also observed that when the scanning rate is higher, the signal/noise ratio increases and vice-versa, fact that lead to non reliable results in the analysis. The direct spectrum was obtained between +1.0 and 0.0, and the second-order derivative spectrum was obtained between +0.350 and -0.350 (Fig. 3). The quantitative determination was made through the zero peak method, thus it allowed the annulment of the excipients interference. The method was found to be rapid, low-polluting and inexpensive when compared with other methods found in literature. The proposed method can be used for routine analysis of SV in quality control of tablets.

Methods validation

The objective of validation of an analytical procedure is to demonstrate that the method is adequated for its intended purpose. Parameters like selectivity, linearity, precision, accuracy, robustness, stability and specificity were determined.

System suitability

System suitability test is an integral part of liquid chromatographic method. It is used to verify that the resolution and reproducibility of the chromatographic system are adequated for the analysis to be done [16]. Standard solution was injected, and the RSD amongst values for each parameter were determined. In all cases RSD values were below 2.0%, which proves the reliability and effectiveness of the method for proposed applications (Table 1).

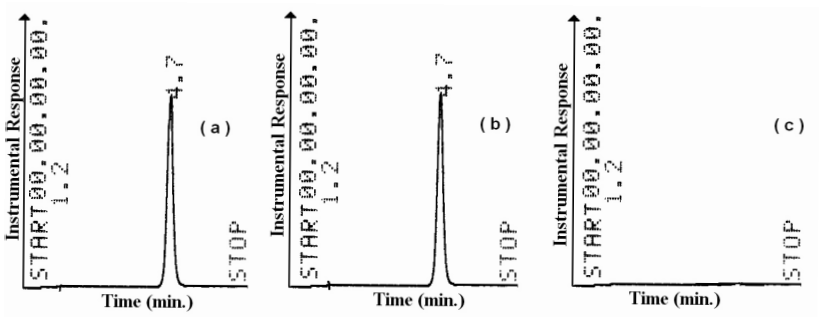

Figure 2. Representative chromatograms of SV standard (**a**), sample A (**b**) and placebo (**c**). Concentration: 9.0 µg/ml of SV. Chromatographic conditions: column: LiChrospher® C18 (125 x 4.6 mm, 5 µm), mobile phase: methanol:acetonitrile:water (60:20:20 v/v/v), flow rate: 1.0 ml/min, UV detection at 239 nm and temperature of 25 ± 1 °C.

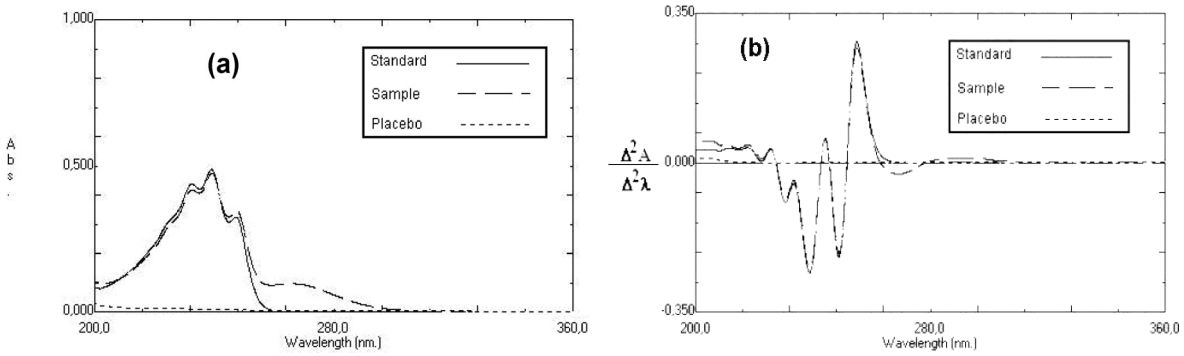

Figure 3. Representative direct UV spectra (**a**) and second-order derivative spectra (**b**) absorption of solutions containing 9.0 µg/ml SV in distilled water: standard (——), sample A (– – –) and placebo (- - -).

Selectivity

All excipients were used to prepare placebo. The resulting solutions were analyzed using the proposed methods. Results were compared with those obtained in the analyses of standard and sample solutions at the same concentration level. The HPLC and UVDS methods are selective for determination of SV in tablets (Figs. 2 and 3).

Analytical curves

Analytical curves for HPLC and UVDS methods were evaluated and checked by analyzing standard solutions at five concentration levels ranging from 1.0 to 17.0 µg/ml of SV, respectively. The correlation coefficients *(r)* were > 0.999. Therefore, the methods presented good linearity (Table 2). The analytical curves were established by least square linear regression [30]. *F* test was applied and values of 8159.54 (HPLC) and 8810.88 (UVDS) were obtained for proposed methods, respectively ($F_{(0.05;\ 1,3)}$ = 10.13). The data provide conclusive evidence of linearity between concentration and instrumental response [31].

Parameters	Mean	RSD (%)
Retention time	4.70	0.27
Capacity factor	2.70	1.12
Theoretical plates	6435.20	1.43
Area	19368.50	1.27
Asymmetry	1.29	0.39

Table 1. Results of system suitability test of HPLC method.

Detection limit (DL) and quantitation limit (QL)

The DL and QL were determined based on the standard deviation amongst response and slope of the curve [30]. The obtained theoretical values for QL were actually prepared and were cross checked by analysis using proposed methods. The DL and QL were 0.72 and 2.41 µg/ml for HPLC and 0.13 and 0.45 µg/ml for UVDS methods, respectively (Table 2). These low values indicated good sensitivity of the methods.

Intermediate precision

The precision had been determined by the repeatability (intra-day) and expressed as RSD (Table 3) [30]. Inter-day data were evaluated by ANOVA analysis. One-way ANOVA was used to compare the variability amongst responses on three consecutive days, and it is expressed as

Statistical parameters	HPLC	UVDS
Concentration range (µg/ml)	1.0 – 17.0	1.0 – 17.0
Regression equation	y = 2501.69 ± (109.998) x – 768.03 ± (1169.296)	y = 0.0325 ± (0.00012) x + 0.0004 ± (0.00123)
Correlation coefficient *(r)* *	0.9994	0.9999
Detection limit (µg/ml)	0.72 ± 0.01	0.13 ± 0.01
Quantitation limit (µg/ml)	2.41 ± 0.03	0.45 ± 0.01

Table 2. Linear regression data in the analysis of SV using HPLC and UVDS methods. * n = 5.

	HPLC			UVDS		
	Sample A	**Sample B**	**Sample C**	**Sample A**	**Sample B**	**Sample C**
Intra-day*						
Day 1	10.05±0.11	20.22±0.26	40.16±0.34	10.05±0.08	20.38±0.18	40.35±0.20
RSD	1.47	1.79	1.19	1.18	1.26	0.70
Day 2	10.20±0.15	20.06±0.28	40.32±0.64	10.21± 0.13	20.40±0.26	40.21±0.27
RSD	2.00	1.98	1.33	1.79	1.76	0.95
Day 3	10.10±0.15	20.07±0.29	40.31±0.22	10.36±0.09	20.42±0.20	40.46±0.38
RSD	1.96	1.95	0.76	1.18	1.37	1.31
Inter-day**	10.15±0.01	20.12±0.03	40.27±0.06	10.21±0.05	20.40±0.03	40.34±0.01
RSD	0.37	0.40	0.42	1.43	0.46	0.07

Table 3. Results of quantitative determination of intermediate precision and statistical data obtained in the analysis of SV in tablets. *Arithmetic mean value (n = 10); **Mean value after statistically treated with ANOVA (n = 30); Sample A = declared amount 10.0 mg/tablet; Sample B = declared amount 20.0 mg/tablet; Sample C = declared amount 40.0 mg/tablet.

RSD < 2.0%. The low intra and inter-day RSD values corroborate the intermediate precision of the proposed methods, respectively (Table 3).

Accuracy

According to ICH Guidelines the standard solution addition should be done in a range from 80 to 120% of the nominal concentration (mean concentration of curve) [30]. The accuracy of the methods was evaluated at three concentration levels. Triplicate determinations were made at each concentration level using HPLC and UVDS methods. The accuracy is expressed as percentage of standard recovered from sample matrix [30]. The mean recoveries of SV were found to be in the range of 101.51% and 99.75% indicating good accuracy for HPLC and UVDS methods (Table 4).

Robustness

The robustness of an analytical procedure is a measurement of its capacity to remain unaffected by small and deliberate variations in method parameters and provides an indication of its reliability during normal usage [30]. Upon deliberate changes in the analytical parameters, no significant changes were observed in the instrumental responses, and the RSD was calculated showing results < 1.0% for both methods (Table 5). Thus, the proposed methods can be considered robust.

Stress testing

Forced degradation is an important part of the process of drug product development. The stress testing is defined as a stability testing performed in more drastic conditions than those

Sample	HPLC			UVDS		
	Amount added (µg/ml)	**Amount found (µg/ml)**	**Recovery (%)**	**Amount added (µg/ml)**	**Amount found (µg/ml)**	**Recovery (%)**
A	2.50	2.47	98.75	2.50	2.52	100.68
	6.50	6.74	103.18	6.50	6.52	100.24
	10.50	10.66	101.54	10.50	10.43	99.30
B	2.50	2.57	102.90	2.50	2.57	102.76
	6.50	6.66	102.14	6.50	6.61	101.44
	10.50	10.40	99.07	10.50	10.60	100.94
C	2.50	2.58	103.04	2.50	2.44	97.59
	6.50	6.66	102.14	6.50	6.31	97.40
	10.50	10.59	100.86	10.50	10.23	97.42

Table 4. Results obtained in the recovery of SV standard solution added to sample A, sample B and sample C analyzed by the proposed HPLC and UVDS methods.

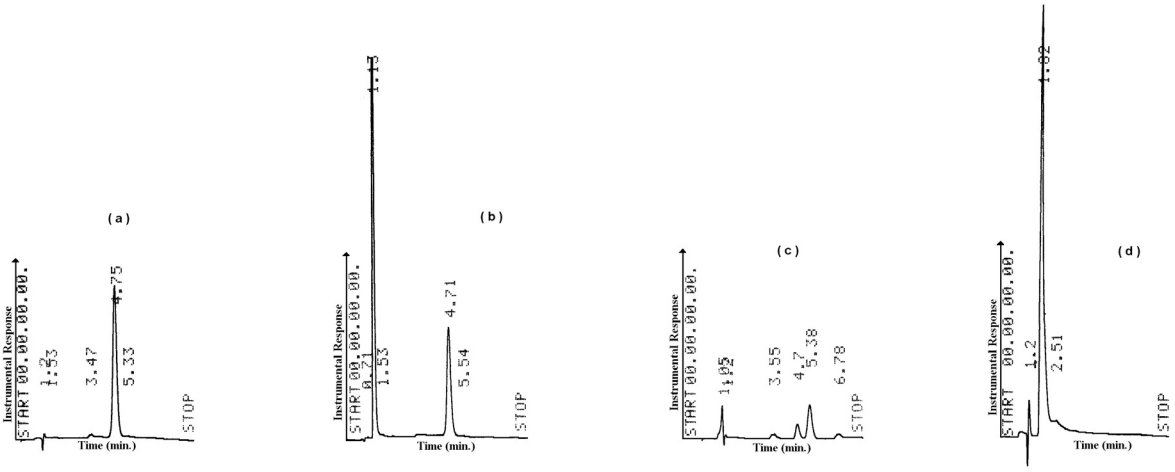

Figure 4. Representative chromatograms after stress conditions. Concentration: 9.0 µg/ml of SV. Chromatograms (**a**) neutral hydrolysis, (**b**) chemical oxidation, (**c**) acid hydrolysis and (**d**) alkaline hydrolysis. Chromatographic conditions: column: LiChrospher® C18 (125 x 4.6 mm, 5 µm), mobile phase: methanol: acetonitrile:water (60:20:20 v/v/v), flow rate 1.0 ml/min, UV detection at 239 nm, and temperature of 25 ± 1°C.

HPLC			UVDS		
Analytical conditions	**Recovery (%)**	**RSD (%)**	**Analytical conditions**	**Recovery (%)**	**RSD (%)**
Mobile phase (59.5:19.5:21, v/v/v)	99.31	0.29	Methanol:distilled water (60:40, v/v)	98.21	0.16
Mobile phase (60.5:20.5:19, v/v/v)	100.40	0.35	Methanol:distilled water (70:30, v/v)	98.34	0.07
Flow rate of 0.9 ml/min	100.35	0.16	Scanning speed fast (2200 nm/min)	100.44	0.53
Flow rate of 1.1 ml/min	101.58	0.11	Scanning speed slow (260 nm/min)	99.79	0.15
UV detection at 234 nm	98.79	0.94	Δ^2A measures interval 0.5 nm	99.46	0.26
UV detection at 243 nm	99.54	0.74	Δ^2A measures interval 1.0 nm	98.53	0.68

Table 5. Results obtained in the robustness test for HPLC and UVDS methods.

used for accelerated stability tests [33,34]. In the HPLC method, after neutral hydrolysis and chemical oxidation occurred a slight increase in the SV retention time (Figs. 4a and 4b), respectively. New peaks were observed after acid hydrolysis (Fig. 4c) and alkaline hydrolysis (Figure 4d).

Table 6 indicates the extent of degradation of SV under stress conditions. These results indicate that the unknown peaks are due to the presence of degradation products. The proposed method was appropriate for quantitative determination of SV in the presence of its degradation products, since all these products could be separated, as it can be observed in the chromatograms. For this reason this method is specific, and it can be used as a stability-indicating method.

Stress condition	**Degradation %**
Neutral hydrolysis	2.1
Chemical oxidation	0.0
Acid hydrolysis	77.5
Alkaline hydrolysis	100.0

Table 6. Results of forced degradation of SV determined by HPLC.

Stability of solutions

In order to obtain reliable experimental results, it is essential to evaluate the stability of standard and sample solutions. Standard and sample solutions were stored refrigerated during three consecutive days (intermediate precision), and the obtained results were compared with freshly prepared solutions. No differences were observed in the instrumental responses under described conditions, so the SV was stable under the above mentioned conditions.

Day	*F*-test [a]			*t*-test [b]		
	Sample A	Sample B	Sample C	Sample A	Sample B	Sample C
1	1.58	2.01	2.89	-1.62	1.12	1.06
2	1.25	1.22	3.44	0.13	2.01	-0.37
3	2.88	2.17	2.99	3.35	2.07	0.76

Table 7. Statistical results obtained in the comparison between HPLC and UVDS methods. Tabled [a] value at 95% confidence 4.03 (n = 10); Tabled [b] value at 95% confidence 2.10 (n = 10).

Comparison between HPLC and UVDS methods

The results are shown in Table 7. Variability comparison (*F*-test) was done in each day on three consecutives days. It was observed that the results obtained for samples A, B and C were found to be less than the critical value at 95% confidence level (4.03). These data show that the variances do not differ statistically. The t-test was applied to determine whether it is not a statistically significant difference between the means of the two proposed methods. Samples A, B and C give results lower than the critical value (2.10) in all days. This fact demonstrated that there is not significant difference between the means.

CONCLUSION

The proposed HPLC and UVDS methods for quantitative determination of SV in tablets are simple and economic in comparison with other published methods. The excipients of the commercial samples did not interfere in the analysis proving that the methods present selectivity. The proposed HPLC method is specific, and it can be used as a stability-indicating method. The UVDS method can be used as an excellent alternative method for routine analysis in quality control. Statistically there is not significant difference between HPLC and UVDS methods with 95% confidence level.

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