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Article

Development and Validation of a Simple and Fast HPLC Method for Determination of Lovastatin, Pravastatin and Simvastatin

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Statins are effective and often-prescribed drugs for the treatment of hypercholesterolemia. This study shows a simple and fast method validation by reversed-phase high-performance liquid chromatography in the linear range 28 to 52 μg/mL to quantify lovastatin, pravastatin sodium or simvastatin in bulk drug or dosage forms. Statins were determined using a C8 endcapped column (250 × 4 mm, 5 μm), isocratic mobile phase of acetonitrile and 0.1% phosphoric acid (65:35), 30°C, ultraviolet–diode array detection at λ 238 nm and 1.5 mL/min flow for lovastatin and simvastatin and 1.0 mL/min for pravastatin sodium. The developed method is fast, simple, reliable and shows appropriate linearity ($r > 0.999$), accuracy (98.8–101.6%), precision (relative standard deviation <2%) and selectivity toward placebo and/or degradation products in very similar chromatographic conditions for all statins.

Introduction

Statins are competitive inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, which catalyzes a previous and limiting stage to biosynthesis of cholesterol. These drugs are well tolerated and effective for the treatment of hypercholesterolemia to reduce plasmatic cholesterol levels (1). The statin class is the most currently prescribed among cholesterol-lowering drugs. Because of the increasing generic pharmaceutical industry market in the world, the development of quantitative methods is relevant to establish official methods to reach uniformity in the assessment and interpretation of analytical results.

Although the structures of lovastatin (LOV), pravastatin sodium (PRV) and simvastatin (SIM) are similar (Figure 1), analytical methods described in the literature differ and have been separately developed for each drug.

Reversed-phase high-performance liquid chromatography (RP-HPLC) techniques have been described for determination of LOV, PRV or SIM by using detection with ultraviolet (UV), visible spectrophotometry, fluorimetry or mass spectrometry (MS). However, a sample pre-treatment is necessary with visible or fluorimetry detection (2, 3). Zhao and colleagues developed a method by HPLC–MS tandem, with a turbo-ionspray interface detection for simultaneous determination of SIM and its hydroxyacid in human plasma using liquid–solid extraction (4). Although the method was sensitive, precise and accurate for biologic matrices, sample handling and clean-up procedure before analysis is laborious. Additionally, the equipment is expensive and not accessible to many analytical laboratories (3, 4). Other methods have been reported, such as oxidation-reduction and complex formation reactions for statin determination in bulk drug and pharmaceutical formulations by visible spectrophotometry (5, 6, 7). These methods require several reagents for chemical reactions and involve cost and time. Derivative UV spectrophotometry has also been described for SIM determination in tablets toward placebo and drug degradation products and compared with HPLC (8, 9). However, none of the eluted degradation peaks were identified or characterized. Although derivative UV is effective to eliminate interferences, it requires an internal software capable of performing derivation calculations. The described methods were developed to quantify one statin at a time.

Monographs of LOV, PRV and SIM have been included in some compendia, such as the British Pharmacopoeia (BP2010), European Pharmacopoeia (EP2010 or EP2009), United States Pharmacopeia (USP32) and Japanese Pharmacopoeia 2006 (JP2006). In the RP-HPLC recommended method for raw material or dosage forms, conditions differ regarding mobile phase and type of reversed-column (octylsilane/RP8 or octadecylsilane/RP18) at the UV detection is performed at λ 238 nm (10–14).

The employed method conditions for LOV determination in USP32, BP2010 and EP2009 are: 1.5 mL/min, RP8 column, acetonitrile and 0.1% phosphoric acid (65:35) in isocratic elution (USP32) (13) or gradient elution for 20 min (BP2010, EP2009) (10, 12). For PRV, a mobile phase constituted of glacial acetic acid, triethylamine, methanol and water (1:1:450:550), isocratic mode, 1.3 mL/min in a RP18 column are recommended in BP2010, EP2010 or JP2006 (10, 11, 14). In addition, USP32 describes a gradient elution during 20 min with variable ratios of 0.08M phosphoric acid pH 5.0 and acetonitrile as mobile phase, 1.0 mL/min (13). Assay methods for SIM raw material described in BP2010, EP2009 and USP32 employ a RP18 endcapped column, 3.0 mL/min and variable ratios of 0.1% aqueous phosphoric acid solution and acetonitrile (50:50) mixed with 0.1% phosphoric acid in acetonitrile as mobile phase in gradient elution for 13 min (10, 12, 13).

Considering that variable conditions for statins determination are found in the literature, a validated single HPLC isocratic method for the determination of LOV, PRV or SIM in bulk drug or dosage forms using similar chromatographic conditions, except for a flow adjustment for PRV, is described. A stress study was performed for LOV and SIM to assess the drug in the presence of degradation products and to evaluate the method's selectivity.

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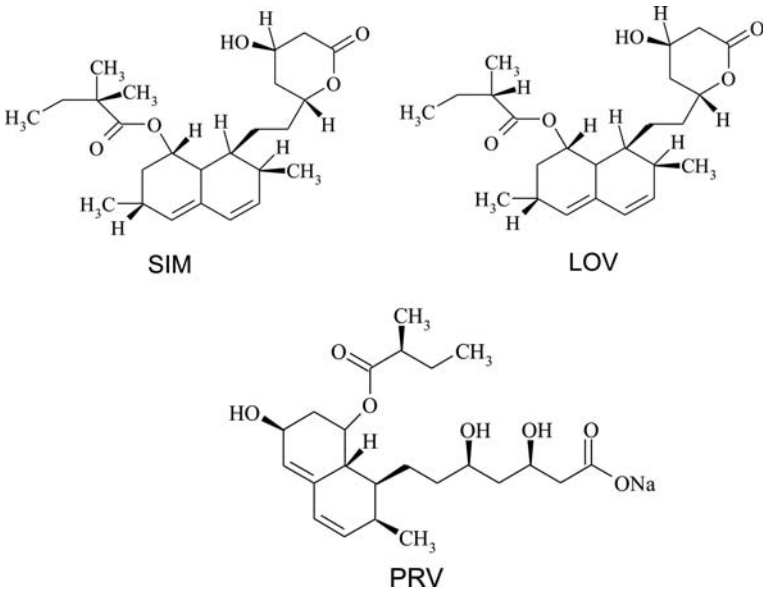

Figure 1. Chemical structures of LOV, PRV and SIM.

Experimental

Material

LOV working standard (100.8% purity, batch 0612002) and LOV raw material (99.9% purity, batch 07061205A) were acquired from Galena Química e Farmacêutica (Campinas, SP, Brazil) and Pharma Nostra Comercial (Rio de Janeiro, RJ, Brazil), respectively. SIM working standard (99.1%, batch 0080/00348) was donated by Medley Pharmaceuticals (Campinas, SP, Brazil) and SIM raw material (99.2% purity, batch 2113002/2007) was donated by Laboratório Globo (São José da Lapa, MG, Brazil). PRV working standard (98.8% purity, batch IF070102) and PRV raw material (99.8% purity, batch 06081546A01) were acquired from Deg (São Paulo, SP, Brazil) and Pharma Nostra Comercial, respectively.

Placebo excipients were microcrystalline cellulose MC 102 Microcel (Colorcon do Brasil; São Paulo, SP); ascorbic acid (Sigma; St. Louis, MO), citric acid, sodium starch glycolate and colloidal silicon dioxide (Henrifarma; São Paulo, SP, Brazil), buthylhydroxytoluene (InduKern do Brasil Química; São Paulo, SP, Brazil), magnesium stearate and directly compressible lactose Tablettose 100 (Ipiranga Química; São Paulo, SP, Brazil); sodium lauryl sulfate (Pharmacopeia Attivos Magistrais; São Paulo, SP, Brazil), polyethylene glycol (PEG) 6000 (Labsynth; Diadema, SP, Brazil) and talc (Proquímios; Rio de Janeiro, RJ, Brazil).

Acetonitrile (J.T. Baker; Phillipsburg, NJ), concentrated hydrochloric acid, sodium hydroxide, phosphoric acid (Merck; Darmstadt, Germany) and hydrogen peroxide (Labsynth) were used. All chemicals and reagents were of analytical or chromatographic grade and used without ulterior purification.

HPLC analysis was performed in a HP1100 chromatograph (Agilent Technologies, Santa Clara, CA) coupled to a quaternary pump, automatic injector, a UV–diode array (DAD) detector and column oven. An ultrasonic cleaner (1400, Unique; Indaiatuba, SP, Brazil), a centrifuge (Jouan B4i; Thermo Scientific; Waltham, MA), a dry oven (306/1, Fanem; São Paulo, SP, Brazil) and a UV light with cabin (ENF-240C, Spectroline; New York, NY) were used.

Method

Chromatographic conditions for the determination of LOV, PRV and SIM included an RP8 endcapped column (250 × 4 mm; 5 μm, LiChrospher Merck), acetonitrile and 0.1% phosphoric acid (65:35), λ 238 nm UV–DAD detection, 30°C with 1.5 mL/min for LOV and SIM, and 1.0 mL/min for PRV. All solutions were diluted in acetonitrile, except PRV, for which the first dilution was in water. All solutions were filtered through 0.45-μm pore cellulose membrane (Sartorius; Goettingen, Germany) and a 10-μL volume was injected into the chromatograph. All measurements were performed in triplicate.

Validation

Method validation was performed for each drug. Calibration curves were separately prepared by transferring 3.5, 4.25, 5.0, 5.75 and 6.5 mL aliquots of LOV, PRV and SIM stock standard solutions (40 mg% in acetonitrile for LOV and SIM or water for PRV) to a 50-mL volumetric flask. Final concentrations obtained for each statin were 28, 34, 40, 46 and 52 μg/mL in acetonitrile. Linear regression was performed using the least-squares method of the concentration versus the average of absolute peak areas. Statistical analysis was accomplished by analysis of variance (ANOVA) of the regression at 5% significance level.

The precision of the method was evaluated through the repeatability (intra-assay) and the intermediate precision (inter-assay) at three levels of concentration (28, 40 and 52 μg/mL, in triplicate) (15–17). The requirements were met if the relative standard deviation (RSD) for each concentration did not exceed 2% for repeatability or 5% for intermediate precision in two days (15, 17, 19).

Limits of quantitation (LOQ), the lowest LOV, PRV and SIM concentrations that can accurately be determined, were calculated by the equation $LOQ = 10\ s_a/b$ (where s_a is the standard deviation of the curve intercept, a; b is the slope of the calibration curve) (17, 20).

The accuracy of the method was evaluated by the standard addition method. Standard solutions of LOV, PRV or SIM standard (40 mg%) were added to the placebo, diluted with 35 mL of acetonitrile for LOV and SIM or water for PRV, sonicated for 15 min and completed with acetonitrile (LOV and SIM) or water (PRV). Solutions were centrifuged (4 min, 4,000 rpm) and aliquots withdrawn from the supernatant were transferred to 50-mL volumetric flasks. Final concentrations corresponded to 70, 100 and 130% of the working concentration (40 μg/mL). The results were expressed in percentage of recovery of drug. The average recovery of analyte must be within 98.0–102.0% at each level (15, 20).

Placebo constituents and their percentages were those commonly used in tablet manufacture: PEG6000 (12.5%), ascorbic acid (0.05%), citric acid (0.8%), colloidal silicon dioxide (0.5%), butylhydroxytoluene (0.5%), microcrystalline cellulose (43%), magnesium stearate (0.5%), sodium starch glycolate (2.0%), lactose (36.15%), sodium lauryl sulfate (0.5%) and talc (1.0%).

The selectivity was demonstrated by suitable separation of all potential interfering substances, placebo or degradation products toward the peak of interest within a specified resolution, R_s, usually ≥ 2 (17, 20). The purity of the peak of interest in degraded sample was determined by the UV–DAD scanning. Selectivity was accomplished by evaluating possible interference of degradation products, yielded by stress degradation, for LOV and SIM in different conditions to verify whether the method can be considered a selective or a specific stability-indicative assay method (18). The selectivity of the method for PRV was evaluated in the presence of excipients.

The robustness of the method for the determination of LOV and SIM was deliberately evaluated by modifying chromatographic conditions and verifying their influence in the results. Six sample solutions of LOV, PRV and SIM and their reference solutions were separately prepared in the working concentration 40 μg/mL. The intentionally modified parameters were organic solvent amount (± 2%), temperature (± 5°C) and flow rate (± 0.1 units) (13, 19). The results obtained after the modification of parameters were compared with those obtained in the established (nominal) chromatographic conditions by ANOVA and Tukey test.

Stress degradation

Accurately weighed amounts of LOV and SIM were separately transferred either to volumetric flasks (containing 10% of acetonitrile, for drug dissolution, and 50% of adequate medium) for neutral, acid and alkaline hydrolysis, oxidation or to Petri dishes for dry heat and UV exposure.

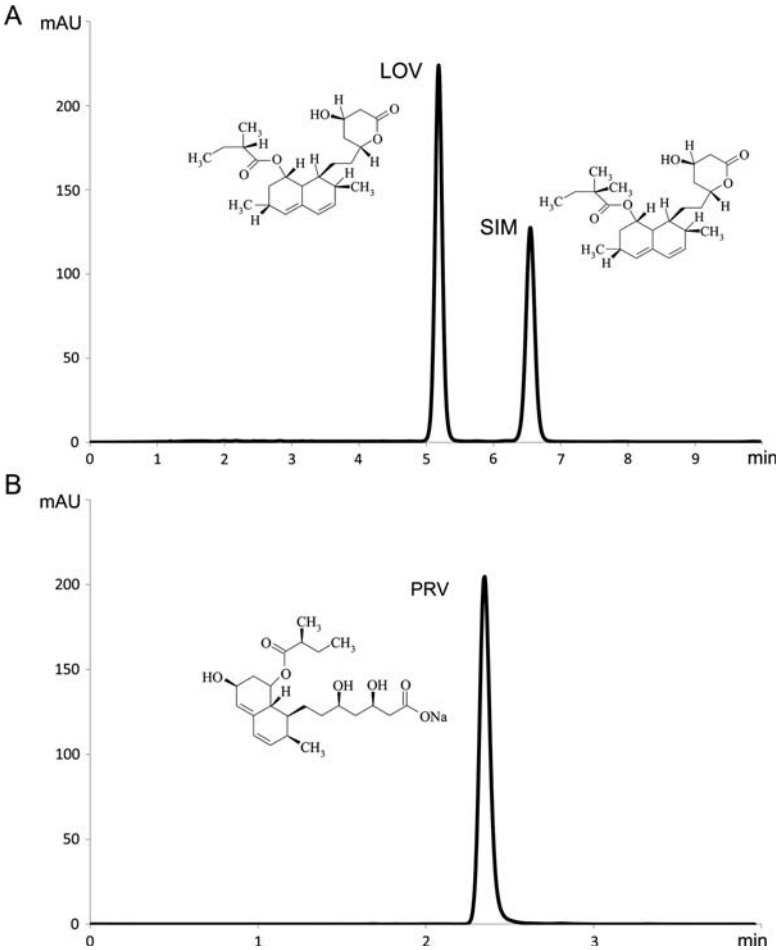

Figure 2. Chromatograms: mixture of LOV (t_R 5.1, T 0.98) and SIM (t_R 6.5, T 0.97) with R_s 6.73 (A); PRV (t_R 2.3, T 1.18) (B). Conditions: RP8-e column, mobile phase 65:35 acetonitrile and 0.1% v/v phosphoric acid, UV detection λ 238 nm, concentration 40 μg/mL in acetonitrile, 1.5 mL/min (LOV and SIM) and 1.0 mL/min (PRV).

All samples submitted to stress degradation (neutral, acid and alkaline hydrolysis, oxidation, dry heat and UV exposure) were prepared at 300 μg/mL and evaluated by the chromatographic method.

Neutral, acid and alkaline hydrolysis

For neutral hydrolysis, LOV and SIM were separately heated (steam bath, 4 h) in volumetric flasks in aqueous medium. Samples were cooled and the volume was completed with acetonitrile. The same procedure was repeated for acid or alkaline hydrolysis, except that 1 N HCl or 1 N NaOH medium was added, respectively.

Table I
Statistical Results for Calibration Curves of LOV, PRV and SIM by HPLC*

Parameters	LOV	PRV	SIM
Equation	$y = 22.31x - 7.98$	$y = 28.11x + 0.73$	$y = 21.594x - 5.02$
Correlation coefficient (r)	0.9998	0.9991	0.9994
RSD (%)	0.40	0.93	0.82
Intercept p value†	0.093	0.958	0.579
Slope p value‡	2.66×10^{-24}	2.03×10^{-19}	3.67×10^{-20}

*Note: critical value $\alpha = 0.05$; conditions are shown in Figure 2.
†Intercept not significant if $P > 0.05$.
‡Slope significant for $P < 0.05$.

Table II
Precision and Accuracy Results for LOV, PRV and SIM by HPLC*

		Precision (%RSD)					
		LOV		PRV		SIM	
Concentration (μg/mL)	Day	Intra-day	Inter-day	Intra-day	Inter-day	Intra-day	Inter-day
28	1	1.10	0.88	0.69	0.64	0.69	1.06
	2	0.71		0.72		1.35	
40	1	0.44	0.53	1.20	1.21	0.98	1.32
	2	0.48		1.47		1.46	
52	1	0.23	0.18	0.97	1.18	0.84	0.99
	2	0.07		1.55		1.00	
		Average recovery (%)					
28		101.6		100.1		99.7	
40		101.2		100.2		99.6	
52		101.5		99.8		100.3	

*Note: Conditions are shown in Figure 2.

Oxidation

Accurately weighed amounts of LOV and SIM were separately transferred to volumetric flasks containing 10% of acetonitrile, 50% of distilled water and 10% of hydrogen peroxide (30% v/v). After heating (steam bath, 4 h), the samples were cooled and the volumes were completed with acetonitrile.

Dry heat and UV exposure

Accurately weighed amounts of LOV and SIM were separately transferred to Petri dishes. Samples were left in a dry oven (105°C, 4 h) or exposed to UV radiation in a short wavelength (λ 254 nm, 4 h). The samples were quantitatively transferred to volumetric flasks and the volume was completed with acetonitrile.

Results and Discussion

In Figure 2A, profiled chromatographic results are retention time (t_R), 5.1 ± 0.2 min and retention factor (k), 4.18 for LOV; t_R 6.5 ± 0.2 min and k 5.51 min for SIM. The R_s between the peaks was 6.73. The PRV chromatogram resulted in t_R 2.3 ± 0.2 min and k 1.30 (Figure 2B). Found k values obtained were satisfactory and met the recommended range ($0.5 < k < 20$), as described by Snyder and colleagues (19). All peak tailing factors (T) were below 1.2, compared to the United States Food and Drug Administration's recommended limit of less than 2 (20).

The linearity of the method for all statins was evaluated in the concentration range 28–52 μg/mL ($n = 5$) in triplicate (Table I). Correlation coefficient (r) values greater than 0.999 (20) and significant slope at 5% of significance level were verified.

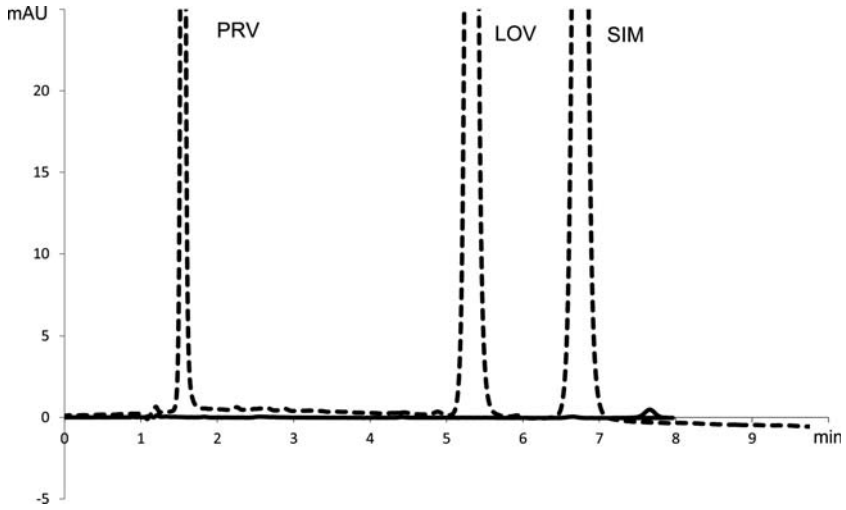

Figure 3. Overlaid chromatograms for placebo (full line) and a mixture of PRV, LOV and SIM reference solutions (hatched line). Conditions are the same as those shown in Figure 2, except for flow rate, 1.5 mL/min.

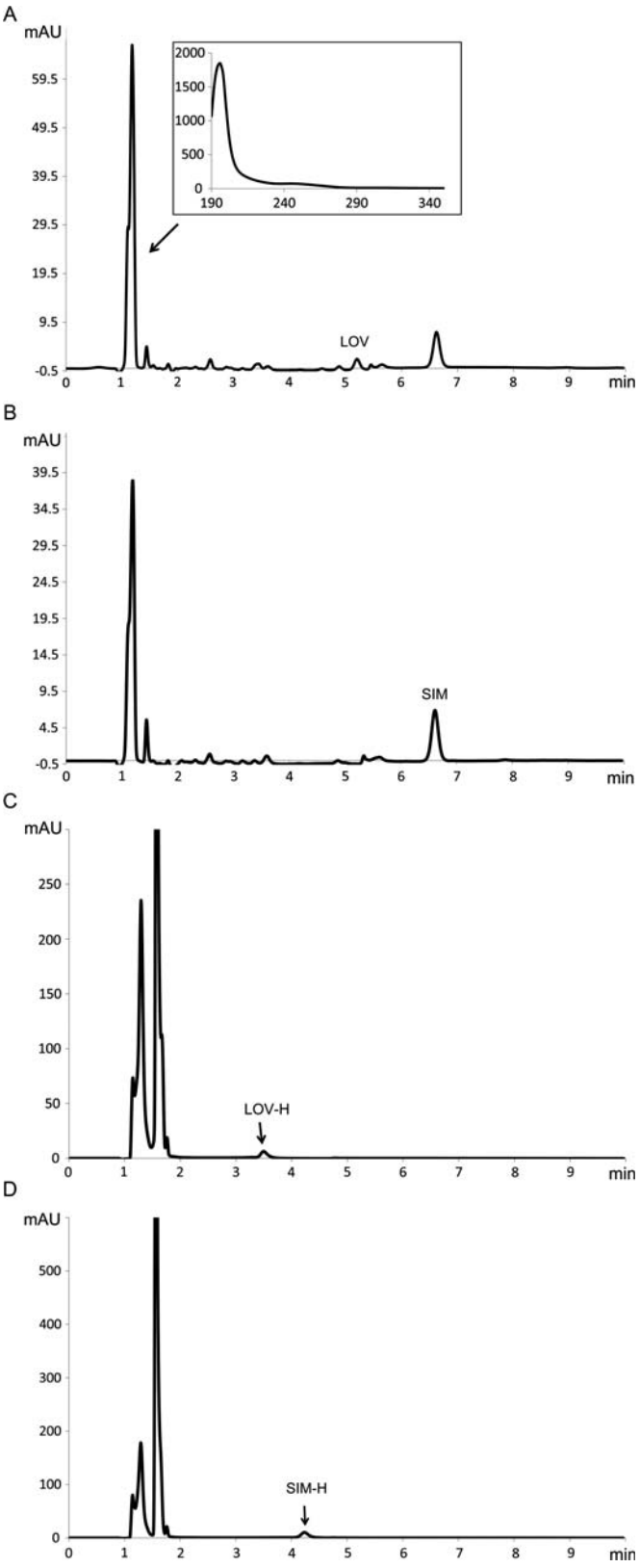

Figure 4. Chromatograms obtained after: acid hydrolysis of LOV (t_R 5.20 min) (insert: UV–DAD spectrum of unidentified degradation product) (A); acid hydrolysis of SIM (t_R 6.61 min) (B); alkaline hydrolysis of LOV (C); alkaline hydrolysis of SIM (100°C, 4 h, 10% acetonitrile plus 1 N HCl or 1 N NaOH) (D). Arrows show hydroxyacids LOV-H and SIM-H.

The precision of the method was expressed by the RSD values of a series of measurements in two days by different analysts. In Table II, the results are summarized for repeatability (intra-day) and intermediate precision (inter-day) for three levels of concentration (triplicate). RSD values obtained for repeatability and intermediate precision were satisfactory, not exceeding the maximum acceptable value of 2% (15, 17, 19).

LOQ values found by the equation $LOQ = 10s_a/b$ were 1.97 μg/mL for LOV, 4.81 μg/mL for PRV and 4.08 μg/mL for SIM.

The accuracy, evaluated by the standard addition to placebo, was expressed as the recovery percentage (Table II). The average recovery was within the range (98.0–102.0%) recommended by Green (15) for all statins at each concentration level.

Selectivity to excipients was attested (Figure 3) by the superposed chromatograms of the placebo with the LOV, PRV and SIM reference mixture solution. The presence of UV-absorbing interferences in the same retention time of LOV, PRV or SIM was not detected. The peak purity for LOV, PRV and SIM was proven by the UV–DAD detector scanning, which presented similarity indices (SI) greater than 99.0%.

The robustness varied conditions (mobile phase composition, temperature and flow rate) tested and compared by means of the Tukey test at 5% significance level were: 63 and 67% acetonitrile; 25 and 35°C; 1.4 and 1.6 mL/min for LOV and SIM, 0.9 and 1.1 mL/min for PRV. The average values obtained with different conditions did not differ for LOV and SIM; hence the method is robust in all tested conditions. On the other hand, the average peak areas obtained for PRV determination were statistically different after flow rate change to 0.9 or 1.1 mL/min. These results indicate that the flow rate is critical for PRV determination using the proposed method, because the highly polar sodium salt drug is less interactive with the column and quickly eluted.

Stress degradation

Figures 4 to 6 show the chromatograms obtained after stress degradation for LOV and SIM. PRV stress degradation was not performed because the drug and its possible degradation products are considered to have low retention times. As a confirmation of this hypothesis, Önal and Sagirli (21) conducted a forced degradation study with PRV in acidic, alkaline, neutral and oxidative conditions (80°C, 1 h) and thermal (105°C, 5 h) and photolytic stresses in PRV solution (10 h, λ 366 nm). The study was performed with a RP18 column, methanol −0.02 M phosphate buffer, 57:43, pH 7. PRV showed complete degradation after acid hydrolysis. For all conditions, the degradation products eluted before the PRV elution time (t_R approximately 5 min), which is evidence of the method's selectivity in the presence of degradation products.

In the present work, all generated degradation products and tested excipients from the placebo (Figure 3) were satisfactorily separated from statin peaks with $R_s \geq 2$. After acid hydrolysis (10% acetonitrile plus 1 N HCl, 100°C, 4 h) LOV and SIM were still detected in the presence of their degradation products (Figures 4A and 4B). However LOV and SIM were more sensitive to alkaline hydrolysis (Figures 4C and 4D), yielding

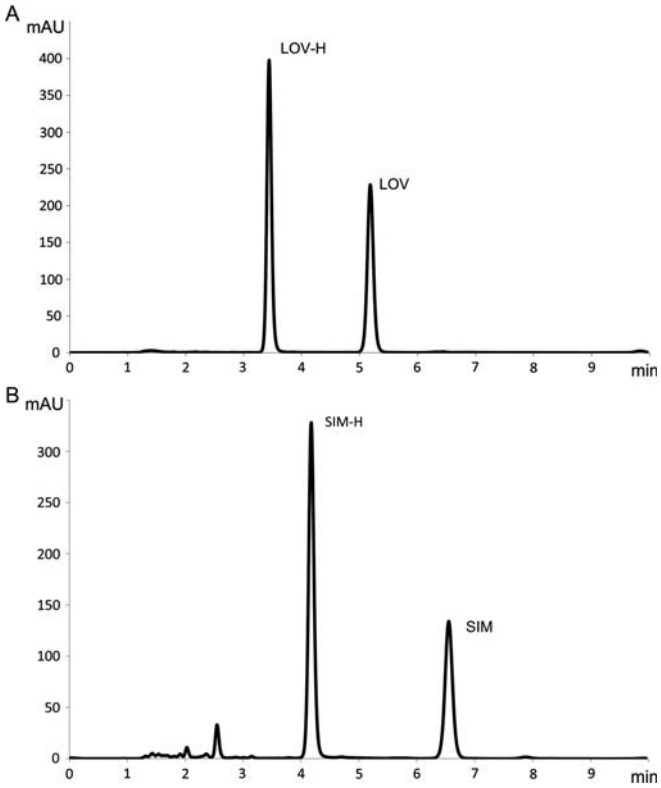

Figure 5. Chromatograms obtained after neutral hydrolysis (10% acetonitrile, 100°C, 4 h) for LOV (t_R 5.18 min) (A); SIM (t_R 6.55 min) (B). LOV-H and SIM-H are hydroxyacid forms of LOV and SIM, respectively.

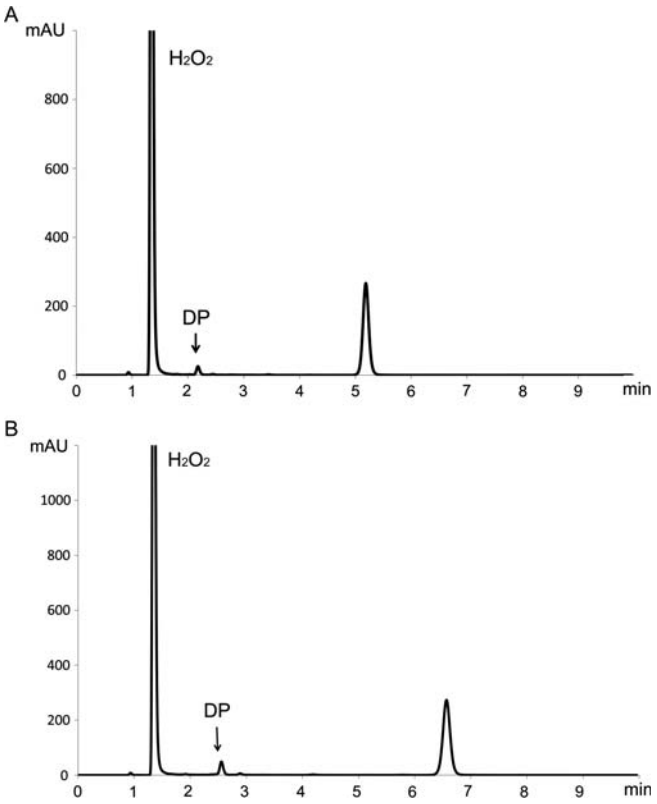

Figure 6. Chromatograms obtained after oxidation for LOV (t_R 5.19 min) (A); SIM (t_R 6.57 min) (10% H_2O_2, 100°C, 4 h) (B). Arrows show degradation products (DP).

complete degradation. These results are in accordance with previous studies (22, 23).

Alvarez-Lueje and colleagues (22) evaluated the stability of LOV under different media (simulated gastric medium without pepsin, 0.06M or 0.1M HCl; phosphate buffer pH 7.4 with or without sodium laurylsulphate) and temperatures (37, 60 and 80°C). They reported that the LOV degradation product keeps UV signal similarity to the parent drug, which is evidence that the chromophore structure remains unaltered. A rank-order LOV stability in different media was: simulated gastric medium without pepsin > 0.06M HCl > 0.1M HCl > phosphate buffer pH 7.4 with sodium laurylsulphate > phosphate buffer pH 7.4.

In the work conducted by Yang and Hwang (23), it was reported that the statins' lactone forms converse to their corresponding hydroxyacid forms by the ring opening. This conversion can be delayed in acidic medium, but occurs almost completely in alkaline solution (0.1 N NaOH prepared with 25 or 50% acetonitrile, 45°C for 1 h) for which the statin peak was not detected, but peaks of hydroxyacid were evident. The conversion is reported to occur in a lower proportion in neutral medium (70% acetonitrile, up to 48 h, room temperature or, in water, up to 2 h, 100°C).

Therefore, the observed peaks at t_R approximately 3.5 and 4.2 min in the chromatograms obtained after alkaline and neutral hydrolysis (Figures 4C, 4D, 5), whose UV spectra were identical to that of LOV and SIM can be identified as their hydroxyacid forms (LOV-H and SIM-H), respectively.

Nevertheless, in the present work, a further conversion of the LOV and SIM corresponding hydroxyacid forms was observed after alkaline hydrolysis, compared to the milder conditions reported by Yang and Hwang (23). Because the hydroxyacid forms (LOV-H and SIM-H) resulted in very small signals after the drastic alkaline hydrolysis (100°C, 4 h, 10% acetonitrile plus 1 N NaOH), they must have been further degraded or yielded non-detectable products by HPLC–UV–DAD.

Following LOV and SIM oxidation, degradation product peaks were detected at t_R 2.18 and 2.55 min, respectively (Figure 6), and did not interfere around retention times t_R 5.2 min (LOV) or 6.5 min (SIM). In addition, the statins showed no interfering degradation after heating at 105°C or UV exposure in the solid state during 4 h. LOV and SIM chromatographic profiles after heating and UV exposure were similar to that obtained in Figure 2A.

Conclusion

The validation of a single developed method for the determination of LOV, SIM (flow rate 1.5 mL/min) or PRV (flow rate 1.0 mL/min) was accomplished by RP-HPLC, showing acceptable linearity, precision, accuracy and selectivity towards placebo and degradation products from the stress study. Moreover, the developed method was adequate because it provided satisfactory results regarding resolution, peak symmetry and time-course of analysis in very similar chromatographic conditions for all statins. The statins were quantified in the presence of the drugs' degradation products and/or formulation excipients; hence, it can be considered a specific stability-indicative assay method, according to Bakshi and Singh (18).

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