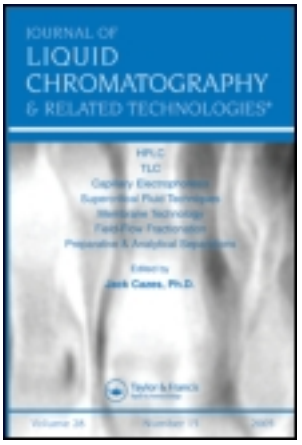

Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/ljlc20

STRESS TESTING OF VALSARTAN. DEVELOPMENT AND VALIDATION OF A HIGH PERFORMANCE LIQUID CHROMATOGRAPHY STABILITY-INDICATING ASSAY

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Available online: 13 Apr 2012

To cite this article: Romina M. Bianchini, Patricia M. Castellano & Teodoro S. Kaufman (2012): STRESS TESTING OF VALSARTAN. DEVELOPMENT AND VALIDATION OF A HIGH PERFORMANCE LIQUID CHROMATOGRAPHY STABILITY-INDICATING ASSAY, Journal of Liquid Chromatography & Related Technologies, 35:8, 1053-1069

To link to this article: http://dx.doi.org/10.1080/10826076.2011.615094

Journal of Liquid Chromatography & Related Technologies, 35:1053–1069, 2012

ISSN: 1082-6076 print/1520-572X online
DOI: 10.1080/10826076.2011.615094

STRESS TESTING OF VALSARTAN. DEVELOPMENT AND VALIDATION OF A HIGH PERFORMANCE LIQUID CHROMATOGRAPHY STABILITY-INDICATING ASSAY

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□ *Stress testing studies were carried out on Valsartan (VAL) under hydrolytic (acidic, basic, and neutral), photolytic, oxidative, and thermal (in the solid state) conditions. Relevant degradation was observed when the drug was exposed to photolytic conditions, where two degradants (DP-1 and DP-2) were produced and when submitted to acid hydrolysis, which yielded one degradation product (DP-3). A high performance liquid chromatography method for the simultaneous determination of VAL and its degradation products was developed, optimized employing experimental design strategies, and validated, employing fully characterized standards of the degradation products. The results proved the stability-indicating property of the method.*

Keywords drug impurities, experimental design approach, HPLC, stability-indicating method, validation, valsartan

INTRODUCTION

Valsartan (VAL, Figure 1) is (*S*)-2-(*N*-((2′-(1*H*-tetrazol-5-yl)biphenyl-4-yl)methyl) pentanamido)-3-methylbutanoic acid. The drug is a synthetic non-peptidic compound with angiotensin receptor blocking activity, useful in the treatment of all grades of hypertension,[1] which has recently acquired official status in the European, United States, and other relevant pharmacopoeias.[2–4]

The wide use of VAL[5] has elicited great interest in developing analytical determinations for the drug in bulk,[6,7] manufacturing process samples,[8–10] pharmaceutical formulations[11–14] and biological fluids.[15–18]

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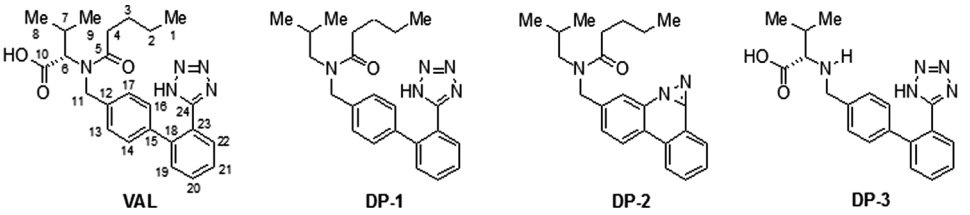

FIGURE 1 Chemical structures of VAL and its degradation products (DP-1, DP-2, and DP-3).

The aim of stress testing is to provide evidence on how the quality of a drug is affected under the influence of different conditions. The rationale behind the tests is that drug decomposition may result in loss of potency and the advent of possible adverse effects due to the formation of degradation products.[19]

Stress tests have been carried out on VAL,[20–25] and several methods for the determination of the drug and its degradation impurities have been developed. However, they suffer from different limitations, which include not being optimized or fully validated, requiring instrumentation not readily available, or demanding excessive chromatographic run times. Other method shortcomings are not having been designed to allow the quantification of the degradation products, employing conditions where the degradants do not elute during the analysis time, being the extent of the degradation merely evaluated in terms of loss of assay, or employing standards of degradation products which have not been duly characterized.[26–28]

Therefore, the outcome of stress testing of VAL, together with the development and optimization of a stability-indicating HPLC method for the quantification of VAL and its relevant degradation products are presented here. For that purpose, fully characterized relevant degradation impurities were used, as well as experimental design and response surface methodologies. The results of method validation and method application are also reported.

EXPERIMENTAL

Instrumentation and Software

The HPLC separations were carried out with a Varian Prostar 210 liquid chromatograph (Varian Inc., Walnut Creek, CA) equipped with two pumps, a Rheodyne injector fitted with a 20 µl loop, a temperature controller, and Varian Prostar 325 variable dual-wavelength UV-Vis detector. The specificity studies were performed on degraded samples and synthetic mixtures of VAL and impurities with a HP 1100 liquid chromatograph (Agilent Technologies, Inc., Wilmington, DE), equipped with a photodiode array detector and controlled by the Chemstation software.

The infrared spectrum of DP-3 was obtained in a Perkin Elmer Spectra One spectrophotometer (Perkin Elmer Co., Yokohama, Japan). The ^{1}H

and ^{13}C NMR spectra were acquired with a Bruker Avance 300 spectrometer (Bruker BioSpin GmbH, Karlsruhe, Germany). The melting point of DP-3 was recorded on an Ionomex hot stage apparatus (Ionomex SRL, Buenos Aires, Argentina) and its specific optical rotation was measured with a Jasco DIP 1000 digital photopolarimeter (Jasco Co., Ltd. Tokyo, Japan).

The photolytic stress studies were carried out in a photostability chamber fitted with four daylight fluorescent lamps (Philips F4T5/D, 6500 K, Philips Lighting, Turnhout, Belgium) and four short-wavelength UV-lamps (Philips G4T5); the samples were also submitted to radiation from a halide lamp (Philips HPA 400), rich in radiation between 320 and 400 nm, covering options 1 and 2 of the ICH photostability testing guidelines.[29]

Standards of DP-1 and DP-2 were obtained as previously reported,[30] and the standard of DP-3 was prepared by acid hydrolysis of VAL in refluxing 1 N HCl.[22] The degradation impurities were characterized by infrared and NMR (^{1}H and ^{13}C) spectroscopy and mass spectrometry.

The pH of the solutions was determined with a model 125 Corning pH-meter (Corning, Inc., Elmira, NY) fitted with a Corning combined glass electrode.

The experimental designs were prepared and analyzed with Design expert 7.0 (Stat-Ease, Inc., Minneapolis, MN). Statistical treatment of the data was performed with the SPSS v. 15 application software (SPSS, Inc., Chicago, IL).

Chemicals and Materials

The experiments were performed with pharmaceutically-certified VAL and analytical grade reagents (Merck & Co., Darmstadt, Germany). Double distilled water and HPLC-grade solvents (J. T. Baker, Mexico) were employed for the chromatographic analyses and for sample preparation. Phosphate solutions were prepared according to the USP 32,[3] in double distilled water. Liquids were filtered through 0.20 μm nylon filters before use.

Forced Decomposition of VAL

The studies were carried out under hydrolytic, photolytic, oxidation and dry heat conditions. Samples were taken at regular intervals. For the HPLC analyses, aliquots of the stressed samples were neutralized when required and diluted with mobile phase to a target concentration of VAL (0.30 mg mL^{-1}, assuming no degradation). For comparison, forced decomposition samples were chromatographed along with non-stressed standard samples.

Hydrolytic and Oxidative Conditions

Acidic, neutral and alkaline hydrolytic studies were carried out by submitting solutions of VAL (3.0 mg mL^{-1}) in HCl (0.1–1 N), distilled water

and NaOH (0.1–1 N), respectively, to an 8 h reflux period. The oxidative stress studies were carried out with H_2O_2 (0.3% and 1.0%), *tert*-butyl hydroperoxide (TBHP, 3.0 equiv.), and magnesium monoperoxy phthalate (MMPP, 1.2 equiv.) as oxidizing agents, on solutions of VAL (3.0 mg mL^{-1}).

Photolytic Studies

Solutions of VAL (6 mg mL^{-1}) in a water:^{t}BuOH mixture (2:1, v/v) were placed in Pyrex (visible and long wavelength UV-light) or quartz (short wavelength UV-light) vessels, at 15 cm from the light sources. For chromatographic analyses, 0.5 mL samples were periodically withdrawn and diluted to 10 mL with MeOH.

Dry Heat and Humidity Degradation Studies

The powdered drug was spread in a flat-bottomed tube to give a homogeneous layer not more than 5 mm thick and exposed to dry heat (80°C) or 65% relative humidity during 60 days. For periodic analyses, specimens (3 mg) of the solid were transferred to a 10-mL volumetric flask and dissolved with MeOH.

Solutions

Stock standard solutions of VAL (3.76 mg mL^{-1}) and degradation products DP-1 (0.334 mg mL^{-1}), DP-2 (0.252 mg mL^{-1}), and DP-3 (0.398 mg mL^{-1}) were prepared in MeOH, and stored in light-resistant containers.

Solutions for analyses containing mixtures of the analytes were prepared immediately before use, by appropriate dilution of the stock solutions or degraded samples with mobile phase. The solutions were stored in a refrigerator, where they demonstrated to be stable at least 90 days at 4°C, and were left to attain room temperature before use. All dilutions were performed in volumetric flasks. The solutions were protected from light throughout the experiments.

Chromatographic Conditions

The HPLC determinations were carried out at 30°C. In the optimized procedure, a 40:60 (v/v) mixture of acetonitrile and potassium phosphate (20 mM, pH = 3.0), was employed as mobile phase, pumped at a flow rate of 1.0 mL min^{-1}. The separation was performed on a 250 mm × 4.6 mm on a cyano column (Luna, 5 μm particle size), using 226 nm as detection wavelength. The chromatograms were recorded and analyzed employing Varian Galaxie v. 6.0 software.

Preparation and Characterization of DP-3

A solution of VAL (1000 mg) in 1 N HCl (35 mL) was refluxed in the dark, until TLC analysis [$CHCl_3$:MeOH (60:40, v/v)] indicated complete consumption of the drug. Then, the solution was treated with 4 N NaOH until pH = 5, concentrated to 10 mL under reduced pressure and left overnight at 4°C. Careful removal of the solvent by filtration, followed by washing of the residue with distilled water (3×4 mL) and final drying of the crystals afforded DP-3 (962 mg, 96%), as a white solid m.p. 177–180°C. $[\alpha]_D^{20} = +4.3$ ($c = 1.3$, MeOH). IR (KBr, ν): 3610–2420 (COOH), 3426, 2980, 2626, 1626 (C=O), 1582, 1574, 1482, 741 and 728 cm^{-1}; ^1H NMR ($DMSO_{d6}$, δ): 0.88 and 0.94 ($2 \times$ d, $2 \times 3H$, $J = 6.9$, H-8 and H-9), 2.05–2.15 (m, 1H, H-7), 2.51 (bs, 1H, $w_{1/2} = 6$, NH), 3.18 (d, 1H, $J = 3.8$, H-6), 3.70 (bs, 1H, $w_{1/2} = 17$, CO_2H), 3.89 (d, 1H, $J = 12.9$, H-11), 4.02 (d, 1H, $J = 12.9$, H-11), 7.12 (d, 2H, $J = 8.1$, H-2′ and H-16), 7.37 (d, 2H. $J = 8.1$, H-13 and H-17), and 7.50 (d, 1H, $J = 7.2$, H-19), 7.54 (dd, 1H, $J = 6.9$ and 7.2, H-21), 7.63 (d, 1H, $J = 6.9$, H-22), and 7.64 (t, 1H, $J = 7.2$, H-20); ^{13}C NMR ($DMSO_{d6}$, δ): 18.8 and 19.3 (C-8 and C-9), 29.9 (C-7), 50.9 (C-11), 66.4 (C-6), 124.9 (C-23), 128.2 (2C, C-13 and C-17), 129.3 (C-21), 129.5 (2C, C-14 and C-16), 131.0 (2C, C-22 and C-19), 131.1 (C-20), 134.8 (C-15), 139.8 (C-12), 141.5 (C-18), 156.3 (C-24), and 172.0 (C-10).

RESULTS AND DISCUSSION

Forced Degradation of VAL

The degradation behavior of VAL when submitted to forced degradation, under hydrolytic, photolytic, oxidizing, and dry heat (solid) conditions is summarized in Table 1. Under neutral and basic conditions, the solutions of VAL demonstrated to be stable, even under reflux; however, exposure to refluxing HCl resulted in generation of DP-3 (Figure 1) as the sole degradation product, the amount of which depended on the time of exposure and acid concentration. The impurity, which is also a synthetic intermediate of the drug,[8] was unequivocally characterized by analysis of its spectral data (Figure 2).

Treatment of VAL with hydrogen peroxide resulted only in little and irrelevant degradation,[23] while exposure of the drug to milder oxidizing agents, such as magnesium monoperoxy phthalate (MMPP) and *tert*-butyl hydroperoxide (TBHP) gave no degradation products, making evident the stability of VAL to oxidizing conditions.[21]

On the other side, VAL proved to be stable to visible light, while irradiation with the halide lamp resulted in the formation of DP-1 and

TABLE 1 Stress Testing Conditions for VAL

Stress Condition	Stressing Agent (Concentration)	Exposure Condition	Time (days)	Results
Hydrolysis				
Acid	HCl (0.1 N)	reflux	0.50	DP-3 (92%)
	HCl (0.1 N)	reflux	0.125	DP-3 (32%)
	HCl (1.0 N)	reflux	0.20	DP-3 (100%)
	HCl (0.5 N)	reflux	0.063	DP-3 (71%)
	HCl (0.7 N)	40	0.05	DP-3 (1.0%)
	HCl (0.5 N)	r.t.	1.50	DP-3 (<LOQ)
Basic	NaOH (0.1 N)	reflux	0.50	Stable
	NaOH (1.0 N)	reflux	0.50	DP-3 (<LOQ)
Neutral	H_2O	reflux	0.50	Stable
Oxidation	H_2O_2 (0.3%)	r.t.	1.50	Irrelevant degradation
	H_2O_2 (1.0%)	reflux	0.30	Irrelevant degradation
	H_2O_2 (0.3%)	reflux	0.50	Irrelevant degradation
	MMPP (1.3 equiv.)	r.t.	10	Stable
	TBHP (3.0 equiv.)	r.t.	30	Stable
Photolysis				
Visible	H_2O	35°C	30	Irrelevant degradation
UV-Long wavelength	H_2O	35°C	60	Irrelevant degradation
Halide lamp	H_2O	40°C	1.0	DP-1 (5.8%) and DP-2 (0.42%)
UV-Short wavelength	H_2O:^{t}BuOH	35°C	1.0	DP-1 (4.4%) and DP-2 (1.1%)
Solid state				
Dry heat		70°C	60	Stable
Humidity		65% RH	60	Stable
Halide lamp		25°C	1.0	DP-1 (5.9%) and DP-2 (0.71%)

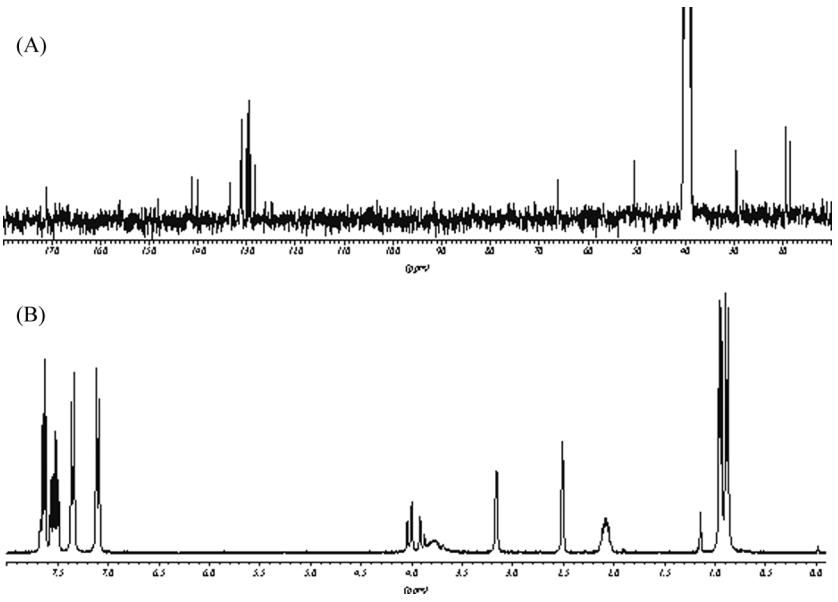

FIGURE 2 (A) Proton decoupled ^{13}C NMR spectrum of DP-3; (B) ^{1}H NMR spectrum of DP-3.

DP-2, which were also found upon exposure of the drug to short wavelength UV-light.

In the solid state, irradiation of with the halide lamp also resulted in formation of DP-1 and DP-2, while the drug demonstrated to be stable when stored during two months at room temperature and 65% relative humidity, and when subjected to dry heat (70°C) for 60 d. Interestingly, we were unable to repeat a previous observation regarding the presence of a product resulting from hydrolysis of the amide linkage,[29] suggesting that perhaps traces of humidity present in the solid were responsible for the reported result.

Method Development

The stability-indicating method for the separation and quantification VAL and its impurities was developed and validated employing spectroscopically characterized standards of the degradation products.

Selection of the Detection Wavelength

The UV spectra of VAL and its degradation products in the 220–300 nm region are shown in Figure 3A. The analytes, which share a substituted biphenyl type chromophore, do not exhibit a common absorption maximum and their absorptivities are higher at lower wavelengths.

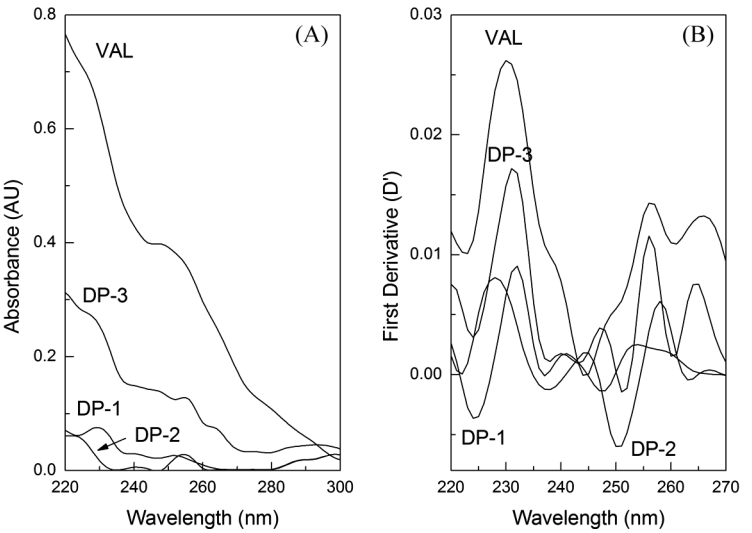

FIGURE 3 (A) UV spectra of VAL (0.194 mg mL^{-1}), DP-1 (4.07 μg mL^{-1}), DP-2 (3.18 μg mL^{-1}), and DP-3 (4.82 μg mL^{-1}) in the mobile phase, in the region between 220 and 300 nm. (B) First derivative of the UV spectra of VAL and its degradation impurities.

Therefore, the first derivatives of their UV spectra were examined (Figure 3B), searching for minimum variation of the analytes' absorption with changes in the detection wavelength. It was observed that the first derivatives of the spectra of VAL, DP-1, and DP-3 exhibit a minimum around 226 nm, where sensitivity of their determination should be favored by their good absorption (Figure 3A). At longer wavelengths, especially above 235 nm, sensitivity of the method markedly decreases mainly for DP-1 and DP-3 making difficult their quantification. Therefore, 226 nm was selected as detection wavelength.

Optimization of the Composition of the Mobile Phase

Degradation of VAL by decarboxylation and deacylation yielded products with widely different polarities. Exploratory experiments using C-8 and C-18 columns (at 30°C, employing MeOH-phosphate and acetonitrile-phosphate mixed solvents and a flow rate of 1.0 mL min^{-1}) failed to provide short, acceptable separations and proper peak shapes under different conditions. However, when a cyano column was tested, it was observed that acetonitrile-phosphate buffer mixtures gave separations with satisfactory peak shapes.

In order to optimize the composition of the mobile phase, the influence of pH of the buffer and the proportion of the organic solvent were systematically investigated, employing a two-factor, three level (3^2) full factorial experimental design. The pH domain was between 2.0 and 4.0, while the proportion of the organic solvent was studied in the range from 30 to 50%. Several responses were recorded, including the resolution between the analytes, the capacity factor for the least retained analyte and the retention time of the last eluting analyte.

It was observed that decreasing the amount of acetonitrile as well as increasing the pH of the aqueous phase increased all retention times. Degradation product DP-3 appeared to be the least sensitive to changes. On the other side, DP-1 and DP-2 were more influenced by the proportion of acetonitrile in the mobile phase than by variations in the pH of the aqueous phase. Since the selected responses were not affected in the same manner by changes in experimental parameters, a multicriteria optimization methodology was employed by means of Derringer's desirability function.[31]

The goals set were to maximize the retention time of the least retained peak (DP-3) and simultaneously minimize the retention time of the last eluting peak (DP-2), while maximizing the critical resolution between VAL and DP-1 and keeping the resolution between other pairs of peeks over 2.5. The solvent composition maximizing the global desirability function (*D*), a 40:60 (v/v) mixture of acetonitrile and phosphate buffer (pH 3.00, 0.02 M), was selected as the optimum mobile phase (Figure 4A).

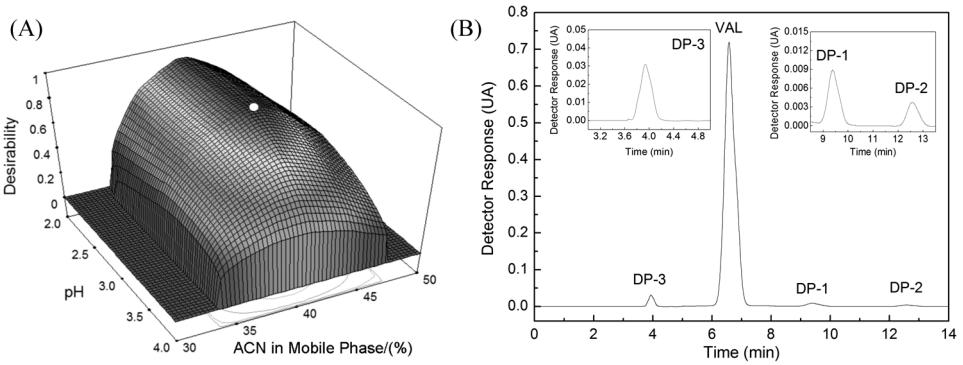

FIGURE 4 (A) Response surface plot of Derringer's desirability as a function of percentage of organic phase and pH of the aqueous buffer for the separation of VAL and its degradation products. (B) Typical HPLC separation of VAL and its degradation products DP-1, DP-2, and DP-3 under the optimized chromatographic conditions.

A representative chromatogram run under these conditions ($D = 0.92$) is presented in Figure 4B, where the retention times of DP-3, VAL, DP-1, and DP-2 were 3.9, 7.2, 9.2, and 12.5 min, respectively, and resolution between adjacent peaks was clearly achieved.

Method Validation

Method validation was carried per ICH[32] and pharmacopoeial indications.[3]

System Suitability Test

The test was performed in agreement with USP 32[3] and BP 2011[4] indications in order to verify the system performance. The RSD values of the peak-areas of five replicate injections of the mixed standard solution were 1.0%, 1.1%, 0.9%, and DP-3 for VAL; DP-1, DP-2 and DP-3, respectively, being considered satisfactory (no more than 2.0%). Other system suitability parameters, including capacity (k, calculated employing an experimentally determined t_0 value of 2.6 min) and selectivity (α) factors, theoretical plates count, USP resolutions (R_s), and tailing factors (T_f) were also determined for the four analytes. The results, depicted in Table 2, were considered acceptable.

Specificity

Homogeneity of all peaks was established using a photodiode array detector (Figure 5A) and performing placebo runs as well as chromatographs of VAL alone (Figure 5B). No interferences were observed at the

TABLE 2 System Suitability Parameters

Parameter	VAL	DP-1	DP-2	DP-3
System precision (RSD, %)[a]	1.0	1.1	0.9	2.0
Capacity factor (k)	1.99	2.95	4.37	0.41
Selectivity factor (α)[b]	1.00	1.48	2.20	4.87
Resolution (R_s)[c]	3.75	5.08	–	8.28
Tailing factor (T_f)	1.49	1.40	1.29	1.27
Efficiency ($N\,m^{-1}$)	10360	13160	24800	6450

[a]Data from five replicate injections.
[b]Regarding the peak of VAL.
[c]Relative to the next eluting peak.

retention times of VAL and its degradation products. Well-separated peaks, with resolution $R_s > 2$ to the closest eluted peak, indicated full compliance with the requirements for specificity.

Range and Linearity

A series of mixtures containing different concentrations of each compound were analyzed. Seven levels were studied, ranging between 10 to 150% of the target value for VAL (considering $0.30\,mg\,mL^{-1}$ VAL as 100%) and 0.05–1.30% (with regard to VAL) for the degradation products. This selection was performed taking into account that typical tolerance levels for degradation products currently range between 0.1 and 1.0% of the main analyte.

Triplicate injections were performed. The observed correlation coefficients (r) were 0.9998 for VAL and 0.9995, 0.9997, and 0.9987 for DP-1, DP-2, and DP-3, respectively, exceeding the requirements for assessing linearity for active principles ($r > 0.9990$) and impurities in bulk drugs ($r > 0.98$).[32] Furthermore, the plots of the residuals exhibited random

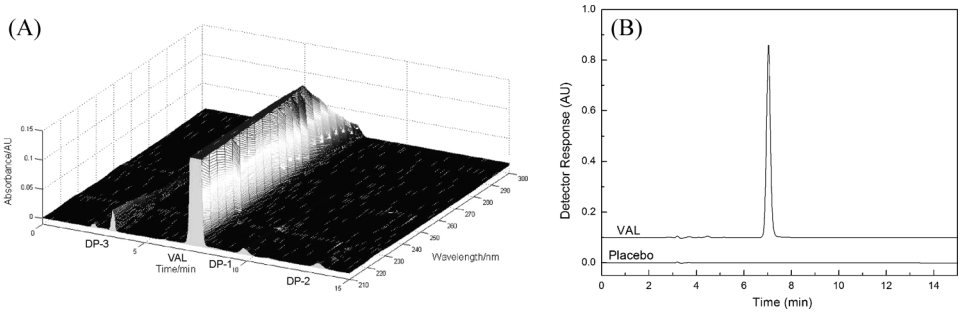

FIGURE 5 Demonstration of method specificity. (A) HPLC-DAD analysis of degradation products DP-1 ($4.07\,\mu g\,mL^{-1}$, 1.4%), DP-2 ($3.18\,\mu g\,mL^{-1}$, 1.1%), and DP-3 ($4.82\,\mu g\,mL^{-1}$, 1.6%) in the presence of VAL ($0.19\,mg\,mL^{-1}$). (B) Chromatographic runs of (1) Placebo and (2) VAL.

patterns, with the residuals passing the normal distribution test ($p < 0.05$), all of which evidenced that the method was linear in the studied range (Table 3).

Precision

Intra- and inter-day precision were evaluated. In order to verify intra-day variation, six independent samples containing the analytes at three concentration levels were injected in triplicate, at random, by the same operator in the same day. The observed RSD values for the different analyte levels (Table 3) were in the range 0.2–0.6% for VAL and 0.5–2.7% for the degradation products, complying with general acceptance criteria ($\leq$2.0% for VAL and $\leq$10% for the degradation products).[33]

The inter-day precision of the method was assessed by three different analysts working in three successive days and employing independently prepared samples containing mixtures of the analytes. The analyte recoveries and RSD values for VAL and its impurities are displayed in Table 3. A two-way ANOVA of these results revealed no statistically significant differences between days and between analysts, confirming the precision of the method.

Accuracy

Method accuracy was evaluated by determination of the recovery of VAL and its degradation products, using the standard addition method on a pre-analyzed sample containing 0.13 mg mL^{-1} VAL. The results, expressed as method bias [analyte recovered (%) – total analyte in the sample (%)], are shown in Table 3. Acceptable bias data (<2% for VAL and <5% for the degradation products) confirmed that the method enables the accurate determination of the analytes.

Robustness

The effects of deliberate variations around the optimized settings in the pH (2.9 and 3.1) and proportion of acetonitrile in the mobile phase (39 and 41%), temperature (29 and 31°C), and flow rate (0.95 and 1.05 mL min^{-1}) on analyte recoveries, were evaluated with the aid of a Plackett-Burman design.[34]

For each run, analytes' recoveries were determined and the effects were calculated by subtracting the mean responses from the low factor levels ("-" levels) from the mean responses obtained from the upper factor levels ("+" levels). Positive effects indicate that an increase of the value of the factor would result in an increase in the recovery of the analyte.

It was observed that flow rate evidenced the largest effect, being negative for all the analytes (Table 4). The pH of the buffer had negative effect

TABLE 3 Range and Linearity; Precision and Accuracy Parameters of the HPLC Method for the Determination of VAL, DP-1, DP-2, and DP-3; and LOD and LOQ Values for the Degradation Products

Parameters	VAL	DP-1	DP-2	DP-3
Linear concentration range[a]	0.030–0.450	0.05–1.30	0.05–1.30	0.05–1.30
Linearity - Regression equations[b]				
Slope (±standard deviation, $b \pm SD_b$)	6.43×10^8 (2.9×10^6)	1.74×10^6 (1.4×10^4)	1.069×10^6 (5.9×10^3)	1.79×10^6 (2.1×10^4)
Intercept (±standard deviation, $a \pm SD_a$)	1.5×10^5 (7.9×10^5)	-1.5×10^4 (1.0×10^4)	2.4×10^3 (4.2×10^3)	6.8×10^4 (2.6×10^4)
Correlation coefficient (r)[c]	0.9998	0.9995	0.9997	0.9987
Intra-day Precision[d]				
Low Level (Recovery, % ±SD)	100.8 ± 0.2	99.9 ± 1.1	101.3 ± 0.8	102.0 ± 1.7
Medium Level (Recovery, % ±SD)	100.3 ± 0.6	99.8 ± 0.7	100.2 ± 0.7	98.1 ± 1.1
High Level (Recovery, % ±SD)	100.1 ± 0.5	99.0 ± 0.5	98.9 ± 0.7	101.9 ± 2.7
Inter-day Precision[d]				
Low Level (Recovery, % ±SD)	101.5 ± 0.5	100.6 ± 0.9	101.6 ± 1.2	102.0 ± 1.9
Medium Level (Recovery, % ±SD)	100.4 ± 0.4	99.8 ± 0.9	100.5 ± 1.0	99.5 ± 1.6
High Level (Recovery, % ±SD)	100.3 ± 0.7	99.0 ± 0.5	98.9 ± 0.6	101.4 ± 2.2
Accuracy[e]				
Low Level (Bias, %)	+1.3	+0.0	−0.4	−0.7
Medium Level (Bias, %)	−0.2	−0.6	−0.7	−2.2
High Level (Bias, %)	+0.8	−1.4	−1.1	−1.6
Detection limit (LOD)[a]	–	0.006	0.011	0.019
Quantification limit (LOQ)[a]	–	0.018	0.036	0.063

[a]Concentration expressed in mg mL^{-1} for VAL and in % for the degradation products for 0.30 mg mL^{-1} of VAL, taken as 100%.

[b]$Y = a + bC$, where Y is the peak area and C is the concentration (in mg mL^{-1} for VAL and in % for its degradation products).

[c]$n = 21$; $p < 0.05$.

[d]Low, Medium, and High Levels were for VAL: 0.21 mg mL^{-1}(70%), 0.30 mg mL^{-1} (100%) and 0.39 mg mL^{-1} (130%); for the degradants: 0.54%, 0.81% and 1.10% (for 0.30 mg mL^{-1} VAL).

[e]Low, Medium, and High Levels were, for VAL 18.1, 28.6, and 39.1 mg mL^{-1}. For the degradants (for 0.30 mg mL^{-1} VAL taken as 100%): 0.67, 0.93 and 1.22% for DP-1; 0.68, 0.95, and 1.20% for DP-2 and 0.76, 1.05, and 1.34% for DP-3. Samples were injected in triplicate.

TABLE 4 Results of the Plackett-Burman Experimental Design for the Robustness Test: Factors, Responses, and Effects

	Factors[a]				Analyte Recoveries (%)			
Run	pH	Organic Phase (%)	Temperature (°C)	Flow Rate (mL min^{-1})	VAL	DP-1	DP-2	DP-3
1	−	−	−	+	94.0	93.8	94.0	99.7
2	−	−	−	−	105.7	105.7	107.9	102.8
3	+	−	+	+	96.3	95.1	95.3	91.3
4	+	+	−	−	105.4	101.4	107.3	102.0
5	+	−	+	+	97.0	95.3	100.0	91.1
6	+	+	−	+	96.7	93.3	95.4	96.0
7	+	+	+	−	108.1	98.5	101.8	99.2
8	−	−	+	−	106.1	106.5	107.4	100.1
9	+	−	−	−	106.0	100.7	107.8	101.8
10	−	+	−	+	95.2	92.7	93.8	100.9
11	−	+	+	−	105.6	104.6	106.4	100.0
12	−	+	+	+	96.0	93.2	95.4	96.9
			Effects					
VAL	1.15	0.32	1.02	−10.28				
DP-1	−2.03	−2.23	0.93	−9.00				
DP-2	0.45	−2.05	0.02	−10.78				
DP-3	−3.16	1.38	−4.10	−5.01				

[a]Factor levels. pH: 2.9 (-) and 3.1 (+); Organic phase: 39% (-) and 41% (+); Temperature: 29°C (-) and 31°C (+); Flow rate: 0.95 mL min^{-1}(-) and 1.05 mL min^{-1} (+).

only on DP-3 and DP-2, while an increase in the proportion of acetonitrile lowered the recovery rates of DP-1 and DP-2 and increasing column temperature resulted in diminished recoveries of DP-3. However, their magnitudes were below the significance limit. In all cases, resolutions between neighboring peaks were found to be higher than 2.0 and the tailing factors were less than 1.6, being considered satisfactory. Furthermore, for the purposes of this assay, all runs gave satisfactory separations and the differences between the recoveries under the different conditions and the intra-day precision mean results (Table 3) were found to be below 10%.[35]

Limits of Detection and Quantification

The limit of quantification (LOQ) is the lowest analyte concentration that can be determined with a given RSD, which may vary between 10 to 20%.[36] The LOQs of the degradation impurities were determined by the procedure recommended by Épshtein,[37] by plotting the RSD values of repeated determinations of the analyte in the neighborhood of the LOQ against their respective concentrations (Figure 6).

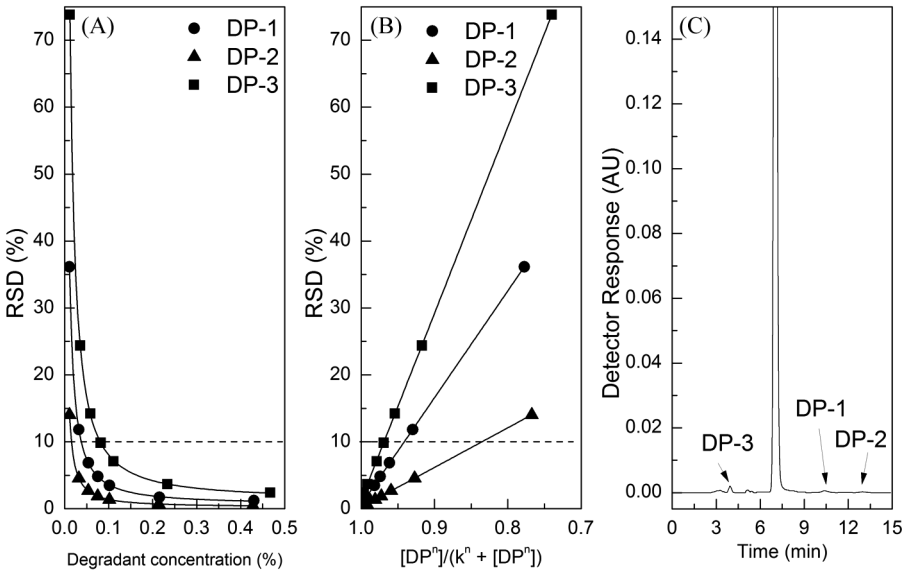

FIGURE 6 Determination of the LOQ values for the degradation products DP-1, DP-2, and DP-3 in the presence of VAL (0.30 mg mL^{-1}). (A) Plot of RSD (%) vs. degradant concentration (in %). (B) Linearized Hill plot. (C) Chromatogram of VAL and its degradation products DP-1, DP-2, and DP-3 at their LOQ value.

Employing linearized Hill plots and a maximum RSD specification of 10%, LOQ values were found to be 0.06 μg mL^{-1}, 0.11 μg mL^{-1}, and 0.19 μg mL^{-1} for DP-1, DP-2, and DP-3, respectively, which represent values lower than 0.1% relative to VAL. Accordingly, detection limits (LOD) were estimated these analytes as 0.018 μg mL^{-1}, 0.036 μg mL^{-1}, and 0.063 μg mL^{-1} (Table 3). Figure 6C shows a chromatogram of the degradation impurities at their LOQ, in the presence of VAL.

Stability of Solutions

The stability of the analytes in the standard solutions (*ST*) was assessed in samples stored 14 days at 4°C, according to Épshtein,[33] from the area under the peak of the analyte at the test time t (S_t) and the initial area S_0, as $ST = 100\ S_t/S_0$. The solution is considered stable if the difference $|100 - ST|$ does not exceed the relative error of the determination of the analyte. The observed differences, $ST = 0.01$, 0.06, 0.42, and 0.31 for VAL, DP-1, DP-2, and DP-3, respectively, were below the corresponding RSD values found for the intra-day precision of the method (Table 3), thus confirming the stability of the solutions. In addition, neither extra peaks, nor changes in the chromatographic pattern were observed.

Application: Quantification of Degradants Formed Under Stress Conditions

The degradation products formed under the different stress conditions were quantitated in properly diluted stress samples employing the validated method, with the results collected in Table 1. Quantifiable amounts of the degradants were formed under photolytic and acid hydrolytic conditions, while basic hydrolysis furnished only traces of DP-3, below its LOQ. DP-1 was found to be formed in the solid (5.9%) and in solution (5.8%) after exposing the samples to the halide lamp for 1 day; under these conditions, DP-2 was formed in 0.71 and 0.42%, respectively. On the other hand, more than 30% DP-3 was observed after refluxing a solution of VAL in 1 N HCl for 3 hr.

CONCLUSIONS

Stress degradation studies were carried out on Valsartan, which allowed access to detailed information regarding its behavior against hydrolytic, oxidation, photolytic, and thermal stimuli. Analyses of stressed samples revealed the formation of two degradants (DP-1 and DP-2) under photolysis and an additional degradation product (DP-3) under acidic conditions. Extending the stress periods allowed access to preparative amounts of the degradation products, which were isolated and unambiguously characterized by FT-IR and NMR techniques.

An HPLC method for the separation and quantification of VAL and its degradation products was optimized and validated with the assistance of experimental designs, demonstrating to be accurate, linear, and precise for the determination of the analytes in their corresponding working ranges, as well as selective and robust. Limits of detection and quantification of the degradation products were below 0.1% with regards to the main analyte. The proposed validated HPLC method has stability-indicating properties and is amenable for routine quality control work.

ACKNOWLEDGMENT

The authors thank National University of Rosario (UNR), Argentine National Research Council (CONICET) and Secretary of Science, Technology and Innovation (SECTeI) for financial support. Thanks are due to Dr. D. Casas (Laboratorios Lazar, Argentine) for the kind provision of valsartan.

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