DEVELOPMENT AND VALIDATION OF AN HPLC METHOD FOR THE SIMULTANEOUS DETERMINATION OF AMLODIPINE, HYDROCHLOROTHIAZIDE, AND VALSARTAN IN TABLETS OF THEIR NOVEL TRIPLE COMBINATION AND BINARY PHARMACEUTICAL ASSOCIATIONS

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☐ *The novel triple combination between Amlodipine (AML), Hydrochlorothiazide (HCT), and Valsartan (VAL) provides a new option for treating hypertension. The development and validation of an HPLC method for their simultaneous determination in pharmaceutical combinations, employing experimental design strategies, is reported. The drugs were separated on a C18 column at 30° C, using a 38:62 (v/v) mixture of 30 mM phosphate buffer (pH 5.5) and MeOH as mobile phase, delivered at 1.0 mL min^{-1}. Detection was performed at 234 nm.*

Despite the wide difference in analytes' concentrations, the method showed good linearity ($r^2 > 0.995$) in the ranges 7.0–13.0 μg mL^{-1}, 17.6–32.8 μg mL^{-1}, and 226.2–420.2 μg mL^{-1} for AML, HCT, and VAL, respectively, being specific (peak purity > 0.999), accurate (bias of analyte recoveries < 2.0%), and precise (inter- and intra-day variations < 2%). It was also robust to small changes in flow rate (±0.05 mL min^{-1}), pH (±0.1 unit) and proportion of MeOH (±3%) in the mobile phase. The method was applied to the assay of AML, HCT, and VAL in tablets of their novel association and formulations containing the HCT-VAL and AML-VAL binary combinations.

Keywords experimental design, HPLC-DAD, HPLC-UV, RP-HPLC, triple combination, validation

INTRODUCTION

Most of the adverse cardiovascular events are attributed to high blood pressure, hypertension being one of the major health problems in many areas of the world. Hence, modern antihypertensive therapy is designed to

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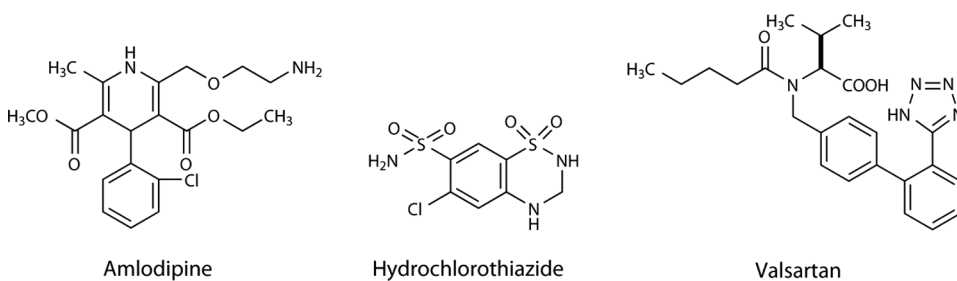
FIGURE 1 Chemical structures of Amlodipine, Hydrochlorothiazide and Valsartan.

reduce the risk of developing cardiovascular complications, which cause high mortality rates among hypertensive patients. Combination therapies are recommended when monotherapy is unable to properly control blood pressure. As multiple therapies are more effective than a drug alone, these therapies have now become a widely used choice to manage hypertension.[1,2]

The 2009 reappraisal of the European Society of Hypertension/ European Society of Cardiology Guidelines, suggested the use of triple-combinations, containing a calcium channel blocker, a thiazide diuretic, and an angiotensin II receptor blocker, as efficient alternatives for the management of hypertensive patients with moderate to very high risk;[3] the benefits of such novel therapeutic strategy have been discussed.[4,5] The association among Amlodipine (AML), Hydrochlorothiazide (HCT), and Valsartan (VAL),[6] with structures as shown in Figure 1, has been recently approved in the light of this new therapeutic paradigm.

Different clinical trials demonstrated that this triple combination effectively reduced blood pressure in hypertensive patients,[7–9] being a safe, efficacious, appropriate, and highly convenient option for patients who have had difficulty reaching their blood pressure goals on dual antihypertensive combinations.[10]

Valsartan and Hydrochlorothiazide have been simultaneously determined in combined tablets by derivative UV-spectroscopy,[11,12] HPLC,[13–15] and capillary electrophoresis,[16] whereas AML and VAL were quantified in their mixtures by chromatographic (TLC and HPLC),[17–19] spectrophotometric,[20] and spectrofluorimetric[21] means, as well as under chemometric assistance.[22] On the other hand, the HPLC-MS determination of VAL and HCT in human plasma and forensic samples[23] have been reported,[24–26] and the HPLC quantification of AML and HCT in their formulations has also been informed.[27,28]

However, a literature search revealed the lack of a validated HPLC method for the simultaneous determination AML, HCT, and VAL in their pharmaceutical association. Therefore, the aim of this study was to rationally develop and validate an HPLC assay procedure, suitable for the analysis

of the drugs in their novel triple combination, as well as related binary associations.

EXPERIMENTAL

Chemicals

Pharmaceutical grade amlodipine besylate, hydrochlorothiazide, and valsartan were obtained from Droguería Saporiti (Buenos Aires, Argentina). Methanol was HPLC grade (J. T. Baker, Mexico); other chemicals employed were of analytical grade (Merck, Darmstadt). Double distilled water was used for the preparation of the mobile phase. Commercial tablets (AML 5 mg, HCT 12.5 mg, and VAL 160 mg; AML 5 mg and VAL 160 mg; HCT 12.5 mg and VAL 160 mg) were acquired at local pharmacies. All dilutions were performed in volumetric flasks and liquids were filtered through 0.45 μm nylon filters before use.

Apparatus and Software

The UV spectra were acquired with a Shimadzu UV-1601 PC UV-VIS spectrophotometer. Chromatographic analyses were carried out with a Varian Prostar 210 high performance liquid chromatograph equipped with two isocratic pumps, a 20 μl loop, a column oven, a Varian 325 spectrophotometric detector, and the automation system software Star 6.41. Specificity experiments were carried out in HP 1100 liquid chromatograph equipped with a photodiode array detector. The experimental designs were prepared and evaluated with Design Expert v. 7.1 (State-Ease, Inc., Minneapolis, MN). Statistical analyses were carried out with OriginLab 8.5 (OriginLab Corp., Northampton, MA).

Chromatographic Conditions

In the optimized procedure, the separation was performed with a mobile phase containing 62% MeOH and 38% phosphate buffer solution (30 mM, pH 5.5), pumped at a flow rate of $1.0 \, \text{mL} \, \text{min}^{-1}$ through a Luna C18 column (250 × 4.6 mm i.d., 5 μm particle size), thermostatized at 30°C. The analytes were detected at 234 nm.

Preparation of Standard Solutions

The standard stock solutions of AML ($0.050 \, \text{mg} \, \text{mL}^{-1}$) and HCT ($0.126 \, \text{mg} \, \text{mL}^{-1}$) were prepared by separately transferring accurately

weighed amounts of each drug to 25-mL volumetric flasks and completing to their marks with MeOH, then transferring accurate volumes of each solution to 25-mL volumetric flasks and completing to volume with the mobile phase. The standard stock solution of VAL ($1.62 \, \text{mg mL}^{-1}$) was prepared by dissolving an accurately weighed amount of the drug with mobile phase, in a 25-mL volumetric flask. The standard solutions could be stored at 4°C for up to three mo protected from light with no evidence of decomposition. Solutions containing mixtures of the analytes were prepared daily in volumetric flasks by mixing appropriate volumes of the corresponding standard solutions and completing to the mark with mobile phase.

Preparation of the Calibration Curves

Standard calibration curves were prepared with five calibrators over a concentration range of 7.0–$13.0 \, \mu\text{g mL}^{-1}$ for AML, 17.6–$32.8 \, \mu\text{g mL}^{-1}$ for HCT, and 226.2–$420.2 \, \text{mg mL}^{-1}$ for VAL. The solutions were injected in triplicate and chromatographed under the optimized chromatographic conditions. The peak area counts were plotted against the concentration of the corresponding analyte and the regression equation was adjusted.

Preparation of Pharmaceutical Samples

In each case, twenty units were weighed and their average weight was calculated. The tablets were crushed to a homogenous powder, and an amount equivalent to the average weight of one unit was accurately weighed and transferred into a 25-mL volumetric flask to which MeOH (12.5 mL) was added. After mechanically shaking the flask for 10 min, the mixture was diluted to the mark with MeOH and mixed. An aliquot (10 mL) was taken, centrifuged 10 min at 3000 rpm and 1.0 mL of the supernatant was transferred to a 10-mL flask, completing to the mark with mobile phase. For HPLC analysis, the samples were filtered through a 0.20-μm nylon filter. The contents of the analytes were obtained from the regression equation of the corresponding calibration curve.

RESULTS AND DISCUSSION

Method Development and Optimization

Detection Wavelength
Figure 2 exhibits the spectra of the analytes, dissolved in mobile phase, between 220 and 290 nm. The most suitable detection wavelength was rationally selected employing a multi-objective optimization strategy, which
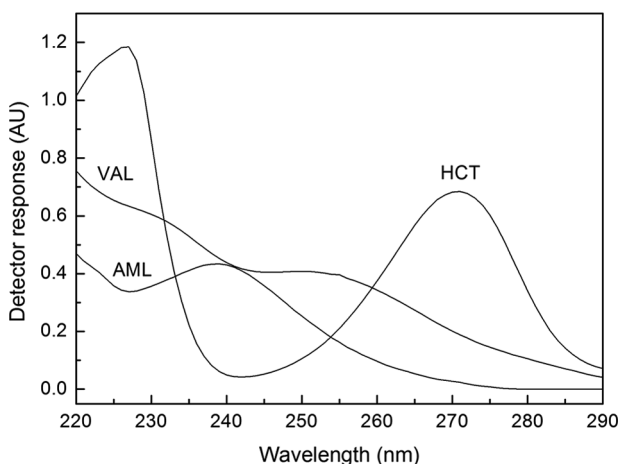
FIGURE 2 UV-Vis spectra of AML $(10.0\,\mu g\,mL^{-1})$, HCT $(10.0\,\mu g\,mL^{-1})$, and VAL $(65.0\,\mu g\,mL^{-1})$, dissolved in the mobile phase.

consisted in building and evaluating a desirability function upon the UV-Vis spectral data of the analytes. In this methodology, the desirable combination of k factors is obtained through a function, which transforms the estimated response variable $\hat{y}_i$ of each factor into a partial desirability value d_i. According to the optimization objectives, the values of d_i range from zero for a completely undesirable response, to one for a fully desired response. Assuming the response variables have the same weight on the global optimal solution, the overall desirability (D) is obtained as the geometric mean of the corresponding partial desirabilities $[D = (d_1 \times d_2 \times \ldots \times d_k)^{1/k}]$. [29,30]

Considering the relative concentrations of the analytes in the tablets, the desirability function was built with the goals of maximizing the absorbances of AML and HCT, while simultaneously minimizing the absorption of VAL, the most abundant analyte. Under these conditions, the optimum detection wavelength was 234 nm ($D = 0.58$).

Composition of the Mobile Phase

Taking into account that the chemical structures and acid-base properties of the analytes are widely different, preliminary screening experiments were carried out on a C18 column in an ample experimental domain. The performance of MeOH-phosphate buffer mixtures was evaluated at 25 and 30°C, after changing the pH (3.1–6.2) and concentration (10–30 mM) of the buffer, and the amount of MeOH (50–70%) in the mobile phase. Chromatographies were performed at $1.0\,mL\,min^{-1}$, which provided separations in a reasonable time.

It was observed that within the tested ranges, the temperature had no significant effect on the separations, that these were unsatisfactory at the lower pH levels, and that the 30 mM buffer yielded improved peak shapes while increasing the column capacity for HCT. Therefore, column temperature was set at $30 \pm 0.2°C$, the buffer concentration was established at 30 mM, and a two-factor three-level experimental design (3^2) was chosen for performing the experiments aimed to optimize the pH and proportion of MeOH in the mobile phase in a more restricted domain (pH between 4.4 and 6.2 and MeOH between 54 and 70%).

The system responses (retention times and peak resolutions) were evaluated by means of a response surface methodology with the aims of maximizing peak resolution and the retention time of the first eluting compound, while establishing 12 min as the duration of the chromatography. The mobile phase composition better complying with these goals was a 38:62 (v/v) mixture of 30 mM phosphate buffer (pH 5.5) and MeOH (Figure 3A). Typical chromatograms of the mixed standard solution and commercial tablet formulations are shown in Figure 3B.

Robustness

Method immunity against small variations from the optimum conditions in buffer pH (± 0.1 pH units), amount of MeOH in the mobile phase ($\pm 2\%$) and changes in flow rate (± 0.05 mL min^{-1}), was tested with experiments selected according to a 2^3 factorial design strategy. The resolutions (R_s), tailing factors (T_f), and column efficiency (N) for the analytes

(A)   (B)
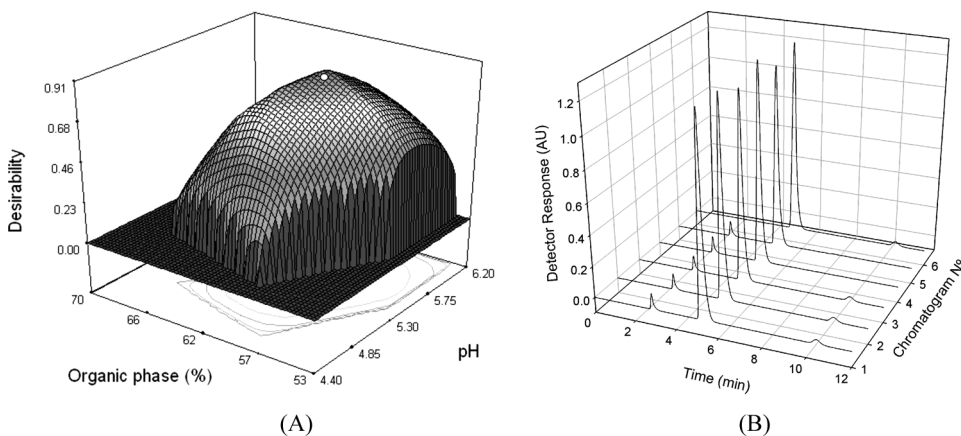
FIGURE 3 (A) Response surface for the determination of the optimum composition of the mobile phase. (B) Typical chromatograms of the separation of AML, HCT and VAL. Solution samples: (1) Mixture of standards at the 100% level for each analyte; (2) and (3) Tablets containing the triple combination; (4) and (5) Tablets containing HCT and VAL; (6) Tablets of VAL and AML.

were recorded as responses and the effects were visualized as Pareto plots. A factor was considered to be statistically significant at the 5% level if the 'size of effect' was greater than the corresponding critical t value. The method was found to withstand these variations; the analytes were always adequately resolved ($R_s > 2.0$), the order of peak elution was unchanged, and the tailing factors remained below 1.6. In addition, drug recoveries were $100.1 \pm 2.1\%$ for AML, $99.8 \pm 1.6\%$ for HCT, and $99.8 \pm 1.0\%$ for VAL. ANOVA tests indicated that the analyte recoveries were not influenced in a statistically significant manner by the different separation conditions [$F_{AML} = 0.72$; $F_{HCT} = 0.66$; $F_{VAL} = 0.49$; $F_{(0.95,3,4)} = 6.59$].

In addition, inter-column differences in the retention times of the analytes, under the optimal conditions, were within 0.2 min when a new column of the same brand was employed. Based on these findings, it was concluded that the method is robust within the recommended operating ranges for the parameters examined in this study.

System Suitability Test

The system suitability test (SST) is an integral part of chromatographic methods development which is used to verify that the system is adequate for the analysis to be performed. It has been shown that system suitability limits can be defined from the results of a robustness test.[31,32] Within this strategy, when no significant effects are found,[33] the extremes of the confidence intervals ($Y_{up/low}$) around the nominal values (b_0) of SST parameters can be employed as upper/lower worst cases for setting the proper test limits, according to [$Y_{up/low} = b_0 \pm t_{0.05,\ n-1}(s/n^{0.5})$], where s is the standard deviation of the n experiments. Therefore, the corresponding SST limits were determined (Table 1) employing the resolution, tailing factor, and theoretical plate responses of the previously employed 2^3 factorial design ($n = 8$).

In addition, five replicates of a combined standard solution containing $10.0 \, \mu g \, mL^{-1}$ AML, $25.2 \, \mu g \, mL^{-1}$ HCT, and $323.2 \, \mu g \, mL^{-1}$ VAL were analyzed, yielding RSD values of 0.88%, 0.41%, and 0.15% for AML, HCT, and VAL, respectively (Table 1). These results met the general compendial requirements ($<2\%$) and were considered satisfactory.

TABLE 1 System Suitability Parameters

Analyte	Resolution (R_s)	Tailing Factor (T_f)	Plate Number (N)	RSD (%) ($n = 5$)
HCT		1.51	3920	0.41
VAL	6.2	0.87	1270	0.15
AML	10.5	1.20	6620	0.88

Method Validation

The developed method was validated[34] with regard to specificity, linearity, range, precision, and accuracy.

Specificity

The chromatogram of a sample of the triple combination was recorded employing a diode-array detector (Figure 4) and the peak purity indices of the analytes were determined. These were better than 0.999, indicating the absence of co-eluting interferents and confirming method specificity. Thus the proposed method may be useful for the quantitative determination of the analytes in their pharmaceutical formulations.

Linearity and Range

Linearity was assessed with independently prepared combined working solutions of AML ($7.0-13.0\,\mu g\,mL^{-1}$), HCT ($17.6-32.8\,\mu g\,mL^{-1}$), and VAL ($226.2-420.2\,mg\,mL^{-1}$) at five concentration levels, covering the range 70–130% of the expected concentration of the analytes in the tablet samples. The combined standards were injected in triplicate and linear relationships were established by plotting analyte concentrations against the corresponding peak areas. Linear regression analysis of the data gave the results shown in Table 2. Correlation coefficients were >0.995 and the confidence intervals of the intercepts included the value of zero. These results confirmed that the method is linear for the three analytes.
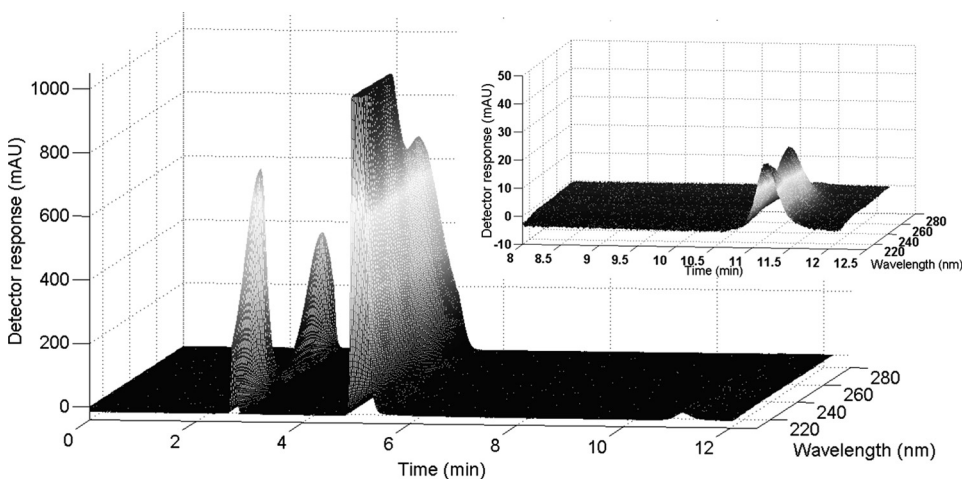
FIGURE 4 Demonstration of method specificity. 3D-elution profile of a sample of the triple combination, containing AML ($10\,\mu g\,mL^{-1}$), HCT ($25\,\mu g\,mL^{-1}$), and VAL ($320\,\mu g\,mL^{-1}$).

TABLE 2 Results from the Determination Range, Linearity, Precision and Accuracy Parameters of the HPLC Method for the Determination of AML, HCT, and VAL

Parameters	AML	HCT	VAL
Linear concentration range ($\mu g \, mL^{-1}$)	7.0–13.0	17.6–32.8	226.2–420.2
Linearity – Regression equations[a]			
Slope ($b \pm SD_b, \times 10^8$)	4.27 ± 0.05	3.98 ± 0.07	5.20 ± 0.10
Confidence interval of the intercept ($\times 10^6$)	−2.82–0.02	−4.4–1.8	−7.0–4.6
Correlation coefficient (r)	0.999	0.997	0.996
Precision			
Repeatability [RSD (%)][b]	0.9	0.6	0.4
Intermediate Precision			
Low Level (Recovery, % ±SD)	102.3 ± 0.6	101.5 ± 0.9	102.2 ± 1.0
Medium Level (Recovery, % ±SD)	100.9 ± 0.5	99.5 ± 0.6	102.5 ± 0.8
High Level (Recovery, % ±SD)	102.0 ± 1.0	102.3 ± 0.4	102.2 ± 0.3
ANOVA – Between days *F*-value[c]	1.871	1.282	1.660
ANOVA – Between analysts *F*-value[c]	1.284	2.523	0.366
Accuracy			
Low Level (Bias, %)	+0.8	−0.05	+0.1
Medium Level (Bias, %)	−0.2	−0.2	−0.01
High Level (Bias, %)	−0.1	−0.2	−0.01

[a]$n = 15$.

[b]Six replicates of a combined standard solution at the 100% level.

[c]$F_{(2, 49, 0.95)} = 3.187$.

Precision

Repeatability and intermediate precision of the analytical procedure were verified. The repeatability (intra-day precision) was assessed by the analysis of six independently prepared combined standard solutions containing the three analytes at the 100% level. The RSD (%) values of the peak areas were calculated for the analytes, being 0.88%, 0.59, and 0.41% for AML, HCT, and VAL, respectively.

Intermediate precision (inter-day precision) was determined by triplicate analysis of independently prepared combined standard solutions of the analytes at the levels 70, 100, and 130%, injected by three analysts during three different days. A two-way ANOVA revealed that there was no statistically significant difference between the results obtained during the different days or by the different analysts (Table 2). In all cases, the relative standard deviations were less than 2% for the three drugs; these results confirmed that the analytical procedure is precise.

Accuracy

Method accuracy was assessed on samples a pre-analyzed tablet sample containing the analytes at the 80% level, employing the standard addition method. Three different analyte levels were evaluated and analyte recoveries were determined after adding standards of the analytes to final sample

concentration levels of 90, 100, and 110%. The bias of the results [Analyte recovered (%) – Analyte in the sample (%)] was always below 2%, satisfying the acceptance criteria for the study and indicating that the method enables the highly accurate simultaneous determination of the three drugs (Table 2).

Stability of Solutions

The stability of the standard solutions was verified during three months in samples stored at 4°C and inferred from the peak-areas of the analytes at the test time t (S_t) and at the initial time (S_0), as $ST = 100S_t/S_0$.[35] During this time, the solutions were analyzed periodically and no unexpected peak appeared which might indicate degradation. The solutions were considered stable since the differences $|100-ST| = 0.13$, 0.09 and 0.07 for AML, HCT, and VAL, respectively, did not exceed the RSD values found for method repeatability (Table 2). This indicated that the samples were stable at least for 3 mo.

Applications: Assay of Pharmaceutical Formulations

The validated HPLC method was employed for the simultaneous determination of AML, HCT, and VAL in tablets of five commercial brands of their triple combination and dual association dosage forms. Figure 3B shows chromatograms indicating well resolved peaks of the analytes.

Three independently prepared samples per brand were analyzed, with the mean results, expressed as a percentage of the label claim, and their

TABLE 3 Assay of Different Lots of Commercial Combined Formulations of the Analytes[a]

Brand No	Analyte	Sample No 1	Sample No 2	Sample No 3	Average Content (%)	RSD (%)
1	AML	101.2	99.3	99.2	99.9	1.1
	HCT	98.2	98.7	99.4	98.8	0.6
	VAL	102.6	102.1	101.4	102.0	0.6
2	AML	98.0	100.7	100.6	99.8	1.5
	HCT	95.2	95.1	95.5	95.3	0.2
	VAL	96.3	98.7	98.8	97.9	1.4
3	HCT	96.9	95.4	96.9	96.4	0.9
	VAL	107.8	105.8	107.8	107.2	1.2
4	HCT	100.9	98.7	100.3	99.9	1.1
	VAL	100.4	99.5	97.9	99.3	1.3
5	AML	99.6	98.9	97.9	98.8	0.8
	VAL	100.3	99.5	99.8	99.9	0.4

[a]Independently prepared samples were analyzed. Contents are informed in %, relative to the label claim.

corresponding RSD values, listed in Table 3. The obtained results indicated that the amount of each drug in the analyzed tablets is close to the declared content, complying with the general compendial requirements (90–110% of their corresponding label claim). In all cases, data dispersion among the determinations was below 2% and no interference by the excipients was observed. Therefore, the proposed analytical procedure can be used for assaying the analytes during the quality control of their combined tablet formulations. Interestingly, analysis of the HCT-VAL binary association was performed within 6 min, which favorably compared with previously reported methods aimed to the same purpose.[13,14]

CONCLUSIONS

A convenient and reliable HPLC method was developed for the simultaneous determination of AML, HCT, and VAL in their combined pharmaceutical tablet samples. Operational conditions were optimized with the assistance of experimental designs. The method was validated, exhibiting good linearity, accuracy, precision, and specificity. It was also successfully applied for the analysis of tablets of the triple combination as well as pharmaceutical samples of binary associations containing HCT-VAL and AML-VAL, demonstrating its feasibility for quality control purposes.

ACKNOWLEDGMENTS

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