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ARTIGO

Development and validation of a method for the determination of valproic acid in pharmaceutical formulations by high performance liquid chromatography with diode array detection (HPLC-DAD)

Desenvolvimento e validação de um método para a determinação de ácido valpróico em formulações farmacêuticas por cromatografia líquida de alta eficiência acoplada com detector de conjunto de fotodiodos

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ABSTRACT

Valproic acid (VA) is used as an anticonvulsant and is used in the treatment of bipolar disorder and depression. The official analytical method for this drug in the compendia is gas chromatography, which is unavailable in many quality control laboratories. Here, we report a validated alternative method using high performance liquid chromatography with diode array detection (HPLC-DAD). The optimized parameters and conditions were as follows: C18 column (5 µm; 250 x 4 mm d.i.); flow 1.0 mL.min-1; wavelength: 210 nm; mobile phase: 55% acetonitrile (ACN) in water containing 0.05% v/v trifluoroacetic acid (TFA) (v/v). The analytical parameters that were validated included the selectivity and matrix effects, linearity, repeatability and intermediate, precision, accuracy, recovery and robustness. This method identifies VA unambiguously. In validation, the following results were obtained: good linearity in concentrations between 0.7 and 1.3 mg.mL-1 (r2 = 0.9998), relative standard deviations (RSDs) of 0.68% for repeatability and 1.23% for intermediate precision, a recovery of 99.42 to 101.55% (RSD 0.14 to 0.74%) and an accuracy of 100.68% (RSD = 0.79%). This method is robust to small variations in procedure.

KEYWORDS: High performance liquid chromatography; HPLC-DAD; valproic acid; validation

RESUMO

O ácido valpróico (VA) é utilizado como anticonvulsivante e também na terapêutica da desordem bipolar e depressão. O método de análise deste medicamento nos compêndios oficiais é por cromatografia gasosa e muitos laboratórios oficiais de controle de qualidade não possuem tal equipamento. A proposta deste estudo foi desenvolver e validar um método analítico por cromatografia líquida de alta eficiência acoplada com detector de conjunto de fotodiodos para a análise deste fármaco. Os parâmetros e condições otimizados foram: coluna C18 (5 µm; 250 x 4 mm d.i.), fluxo de 1,0 mL min-1, comprimento de onda 210 nm, fase móvel 55% ACN e 45% solução 0,05% v/v de ácido trifluoracético (v/v). Os parâmetros analíticos avaliados na validação foram: seletividade e efeito matriz, linearidade, repetitividade e precisão intermediária, exatidão e robustez. A metodologia desenvolvida mostrou identificar de forma inequívoca o analito de interesse. No que concerne aos parâmetros de validação foram obtidos: faixa linear nas concentrações de 0,7 a 1,3 mg mL-1 (r2 = 0,9998), repetitividade: desvio padrão relativo (DPR) = 0,68%, precisão intermediária: DPR = 1,23%, recuperação de 99,42 a 101,55% (DPR de 0,14 a 0,74%) e exatidão de 100,68% (DPR = 0,79%). Quando submetido a pequenas variações, a metodologia mostrou-se robusta.

PALAVRAS CHAVE: Cromatografia líquida de alta eficiência acoplada com detector de conjunto de fotodiodos; ácido valpróico; validação

1-Introduction

Valproic acid (VA, 2-propylpentanoic acid, $C_8H_{16}O_2$, Figure 1) is a colorless liquid that is slightly miscible with water and very soluble in organic solvents and has a pKa of 4.6.[1] This substance was first approved as an anticonvulsant in the United States in 1978. Its properties were accidentally discovered when it was used as a vehicle for other anticonvulsants. VA is rapidly absorbed orally, attaining maximal blood levels one to four hours after the administration of 250 mg. It is quickly distributed to tissues and binds strongly to human plasma proteins (90%). Metabolism occurs mainly in the liver (95%); less than 5% is excreted intact.

VA is indicated as monotherapy for absence crises, myoclonus, partial and tonic-clonic seizures,[2] and myoclonic juvenile epilepsy. However, some systemic toxicity has been observed.[3] VA is also used as an alternative to lithium in patients with bipolar disorder, as well as in depression, migraine, febrile convulsions and to treat brain tumor (alone or in combination with other anticonvulsants).[4,5]

The analytical technique recommended by the US Pharmacopoeia for analyzing VA capsules is gas chromatography (CG).[6] However, it is important to note that some of these methods require a derivatization step, which is time consuming and has a detrimental effect on performance. Several laboratories use HPLC to analyze anticonvulsants with chemical properties that are very similar to VA. Therefore, this study is intended to develop and validate a rapid, simple and effective alternative method using HPLC-DAD to analyze VA in pharmaceutical formulations.

2- Experimental

2.1 - Reagents

All solutions used in this study were prepared with ultrapure water using a Milli-Q® (Millipore) system. Acetonitrile (for liquid chromatography), sodium hydroxide (Pure Analysis, PA) and boric acid (Pure Analysis, PA) were purchased from Vetec (Rio de Janeiro, Brazil). Trifluoroacetic, acetic and phosphoric acids (all Pure Analysis, PA) were purchased from Merck (Darmstadt, Germany). A reference sample of valproic acid was donated by ABBOTT laboratory (purity 100.17%). VA capsules (250 mg) were used as a reference for methodological and validation studies. Mobile phase components were degassed using sonication under reduced pressure for 10 min before use. All solutions were filtered using Teflon® 0.22 µm membranes.

2.2 - Equipments

The following equipments were used: (1) high performance liquid chromatograph (Young Lin), with an ultraviolet-visible diode array detector (HPLC-DAD; Hogye, Korea), (2) high performance liquid chromatograph (Varian) with ultraviolet-visible detector (HPLC-UV; California, United States), (3) Sonicator (Nova Ética, São Paulo, Brazil), (4) analytical balance (Mettler Toledo A204, São Paulo, Brazil), (5) pH meter (Micronal, São Paulo, Brazil). A Lichrospher® C18 column (5 µm; 250 x 4 mm d.i.) was used during for validation studies, and Hypersil BDS C18 (5 µm; 250 x 4 mm d.i.) was used for robustness tests. Data were acquired with Star Workstation Version 5.5 software (Varian®).

2.3 – Chromatographic Conditions

The chromatographic conditions were based on those reported by Amini.[7] The optimal analytical conditions were a mobile phase of 55% acetonitrile (ACN) in water containing 0.05% v/v trifluoroacetic acid (TFA) (v/v) (pH 2.3); flow: 1 mL.min^{-1}; column Lichrospher® C18 (5µm; 250 x 4 mm d.i.); wavelength: 210 nm; injection volume: 25 µL; and temperature: 25 °C.

2.4 – Preparation of standard and samples

2.4.1- Standard preparation

Stock solutions of VA were prepared in the mobile phase mixture described under "chromatographic conditions" at a concentration of 1.03 mg.mL^{-1}. Working standard solutions were obtained by diluting aliquots of each stock solution in the same mixture. All solutions were sonicated for 30 min before use and were stirred every 5 min during use.

2.4.2- Preparation of blank solution

The blank solution was prepared from all of the substances in the capsule except VA. The quantities of these substances were as specified in each formulation[8]. The blank solution was prepared in the mobile phase described under "chromatographic conditions". The solution was sonicated for 30 min and was then stirred every 5 min. The solution was filtered through Teflon® 0.45 µm membranes and analyzed in triplicate.

2.4.3- Sample preparation

The standard solution of VA was added to the blank solution to give a nominal concentration of 1.00 mg.mL^{-1}.

2.4.4- Preparation of the pharmaceutical formulation

After validating the method, 3 samples containing approximately 1.00 mg.mL^{-1} of VA from 250 mg capsules (medications A (lot 710098F01), B (lot 850758F01) and C (lot 809707)) were analyzed.

Three samples from each formulation (A and B) were prepared by weighing and homogenizing ten capsules from each lot. This mixture was weighed; after dilution, the mobile phase contained approximately 1.00 mg.mL^{-1} of VA. The samples were sonicated for 30 min and were then stirred every 5 min. The samples were filtered through Teflon® 0.45 µm membranes and analyzed in triplicate by comparison with the analytical curve (curve 1).

2.5 - *System Adequacy*

To evaluate the adequacy of these parameters, standard and sample solutions were prepared in triplicate in concentrations of 1.03 mg.mL^{-1} and 1.00 mg.mL^{-1}, respectively. The mobile phase was used as solvent as described above. The solutions were sonicated for 30 min and were stirred every 5 min when in use.

2.6 – Stability Study of Sample and Standard Solutions

The standard solution of valproic acid (1.03 mg.mL^{-1}) was analyzed immediately after preparation, after 6, 12, 24, 48, and 72 hours, and finally after one week. The sample solution (1.00 mg.mL^{-1}) was evaluated 1, 2, 3, 4, 5, 6, and 24 hours after preparation. Both solutions were stored at room temperature (25 °C) in the dark.

2.7 – Validation Procedure

As this method falls under category I (quantification of the main component)[10], validation requires evaluating the following parameters: selectivity – matrix effect, linearity, linear work range, accuracy, recovery, precision (repeatability and intermediate precision), and robustness.[9,10] The acceptance criteria and recommendations for most parameters are given in the Results and Discussion sections. All equipment, instruments, and glass utilized in the validation were qualified and/or calibrated according to ISOGUIDE 17025, and standard operating procedures were used.

2.7.1 - Selectivity and matrix effects

Selectivity and matrix effects were evaluated by comparing the chromatograms for the blank and sample solutions (1.00 mg.mL^{-1}) at the retention time (t_R) of the peak observed for the standard VA solution.

2.7.2 – Linear Work Range

The linear work range was established as per Sousa and Junqueira (2005), who stated that the percentage of the analyte contained in the linearity range should vary from 70 to 130% of the theoretical concentration.[11]

2.7.3 - Linearity

Triplicate samples of VA standard solutions (curve 1) and sample solutions (blank fortified with VA, curve 2) of 0.7, 0.8, 0.9, 1.0,1.1, 1.2, and 1.3 mg.mL^{-1} were used. The steps used for linear evaluation were as follows: (i) determining the concentration range of interest, considering the application and that the expected concentrations of the samples should be close to the center of this range, and (ii) preparing an analytical curve using standard solutions at seven concentrations from 70 to 130%, with three independent replicates for each concentration and aleatory injections of these solutions into the HPLC-DAD System.[11]

Evaluating the linearity involved using the ordinary minimum squares method (OMSM), and the regression parameters and treatments of extreme values were evaluated as described below. (i) Determination of chromatographic peak areas relative to the valproic acid peak. (ii) Estimation of the slope, intersect, residuals, variances and r^2. (iii) Exclusion of extreme values using the Jackknife method for standardized residuals.[11] The third step also involved verifying the regression residuals and adjusting the linear model as follows. (i) Residual normality was adjusted by the Ryan-Joiner test; (ii) residual independence was adjusted by the Durbin-Watson test; (iii) residual homoscedasticity was adjusted by the Brown-Forsythe test; and (iv) the significance of the regression was verified and the linear model was adjusted by variance analysis (ANOVA).[11] The software used was Microsoft Office Excel® 2007.

2.7.4 – Precision (Repeatability and Intermediate Precision)

2.7.4.1 - Repeatability

Thirty determinations were made for the sample solution (1.00 mg.mL^{-1}) by the same analyst on the same day using the curve obtained from the standard VA solution (curve 1). The concentration and relative standard deviation were calculated.

2.7.4.2 – Intermediate Precision

Ten analyses of the same sample solution (1.00 mg.mL^{-1}) were performed by three analysts in the same laboratory using the same method on different days and different equipment, using the curve obtained from the standard VA solution (curve 1), prepared recently. The obtained results were evaluated using the intermediate precision standard deviation (Spi).

2.7.5 - Accuracy

Samples were prepared by adding the standard VA solution to the blank solution, giving concentrations of approximately 0.7, 0.8, 0.9, 1.0, 1.1, 1.2, and 1.3 mg.mL^{-1}, in triplicate. These solutions were analyzed using the analytical curve (curve 1). Accuracy and RSD averages were calculated.

2.7.6 – Recovery

Capsules containing 250 mg of VA were analyzed using the calibration curve (curve 1: 0.7, 0.8, 0.9, 1.0, 1.1, 1.2, and 1.3 mg.mL^{-1} of VA); after this, the medication was added to the standard solution of VA and diluted in the same concentrations using this curve. Recovery and RSD were calculated for each concentration.

2.7.7 – Robustness

Eight combinations were assayed (Table 1) during method development, with seven parameters varied: time in the ultrasonic bath (20 or 30 min), column manufacturer (Merck or Supelco), mobile phase composition (55:45 or 60:40 of acetonitrile and TFA 0.05%, respectively), flow (1.0 or 1.2 mL.min^{-1}), column oven temperature (25 or 30 °C), pH (2.3 or 2.6), and stirring (present or absent).

The effect on the adequacy parameters of the chromatographic system (i.e., the number of theoretical plates and the asymmetry factor) was calculated for each of the seven variables in Table 1. The analyses were performed under normal and modified conditions. The Youden test was applied to evaluate the influence of each variation.[9]

Table 1- The eight combinations of conditions evaluated for robustness using the Youden test

Robustness test				
Parameters	Condition 1	Condition 2	Condition 3	Condition 4
Time at ultrasound	30 min	30 min	30 min	30 min
Column	Merck	Merck	Supelco	Supelco
Mobile phase	55:45	60:40	55:45	60:40
Flow	1.0	1.0	1.2	1.2
Temperature	25 °C	30 °C	25 °C	30 °C
pH	2.3	2.6	2.6	2.3
Stirring	Present	Absent	Absent	Present
	Condition 5	Condition 6	Condition 7	Condition 8
Time at ultrasound	20 min	20 min	20 min	20 min
Column	Merck	Merck	Supelco	Supelco
Mobile phase	55:45	60:40	55:45	60:40
Flow	1.2	1.2	1.0	1.0
Temperature	30 °C	25 °C	30 °C	25 °C
pH	2.3	2.6	2.6	2.3
Stirring	Absent	Present	Present	Absent

3- Results and Discussions

3.1 – Method Development and Validation

Figure 1 shows the typical HPLC-DAD chromatograms obtained for the standard VA solution and a pharmaceutical preparation. In both cases, a well defined chromatographic peak was obtained (t_R 5.7 min). The total run time was 10 min.

The retention times of the sample (1.00 mg.mL^{-1} VA) and standard solutions (1.03 mg.mL^{-1} VA) in triplicate were 5.70 min (RSD 0.06%) and 5.73 min (RSD 0.04%), respectively. The asymmetry factors obtained for the sample and standard VA peaks (1.07 (RSD 0.2%) and 1.06 (RSD 0.5%), respectively) are within the limits described in the recent literature. The number of theoretical plates (5397 (RSD 0.1%) and 5619 (RSD 1.2%), respectively) are also well above the minimal value of 2000 theoretical plates, thus confirming the efficiency of the chromatographic system. The RSD values for the comparison of the VA peak areas (0.35% and 0.43%, respectively) indicate that repeatability falls within the specifications of the 2% test.[12] These values indicate that the parameters used were adequate for analytical purposes; i.e., this method is suitable for the determination of VA in pharmaceutical formulations.

The sample and standard solutions were freshly prepared, and the VA peak areas were determined over time. The sample solution was analyzed at a concentration of 1.0 mg.mL-1 over 24 hours. The initial area was 766 mV.s, and the range over this time was from 759 to 772 mV.s, with an average of 764.8 mV.s (DPR 0.57%). The standard solution was analyzed at a concentration of 1.03 mg.mL-1 over 7 days. The initial area was 826 mV.s, and the range was from 816 to 823 mV.s: a difference of 1.2% existed between the lowest and the initial values. The average over the week was 821 mV.s (DPR 0.44%). Sharbir (2003)[12] reported a variation of up to 2.0% compared to the freshly prepared sample. These results show no significant decrease in the peak area of the standard solution over 7 days. Thus, the solution can be stored for this period without affecting the reliability of the results.

To evaluate the selectivity, the UV absorption spectra of the sample and standard were recorded (Figures 2A-2C). Comparing the spectra before (A), during (B), and after (C) the retention time of VA, it is possible to verify that the UV spectra obtained during elution (B) are very similar. There is also no detectable co-eluting compound present in the sample (A and C).

Confirming the absence of any matrix interference or interference from other constituents of the pharmaceutical preparation, no evidence of co-elution was observed (Figure 3). A comparison of the chromatograms of the blank to the reference formulation unequivocally shows that the other signals are due to excipients and that they are not coincident with the VA signal. However, this applies only to the tested matrix (e.g., corn oil, propylparaben, methylparaben, glycerin, deionized water, titanium dioxide, gelatin, yellow dusk dye and mineral oil). Other matrices may exhibit different chromatographic behaviors.[13]

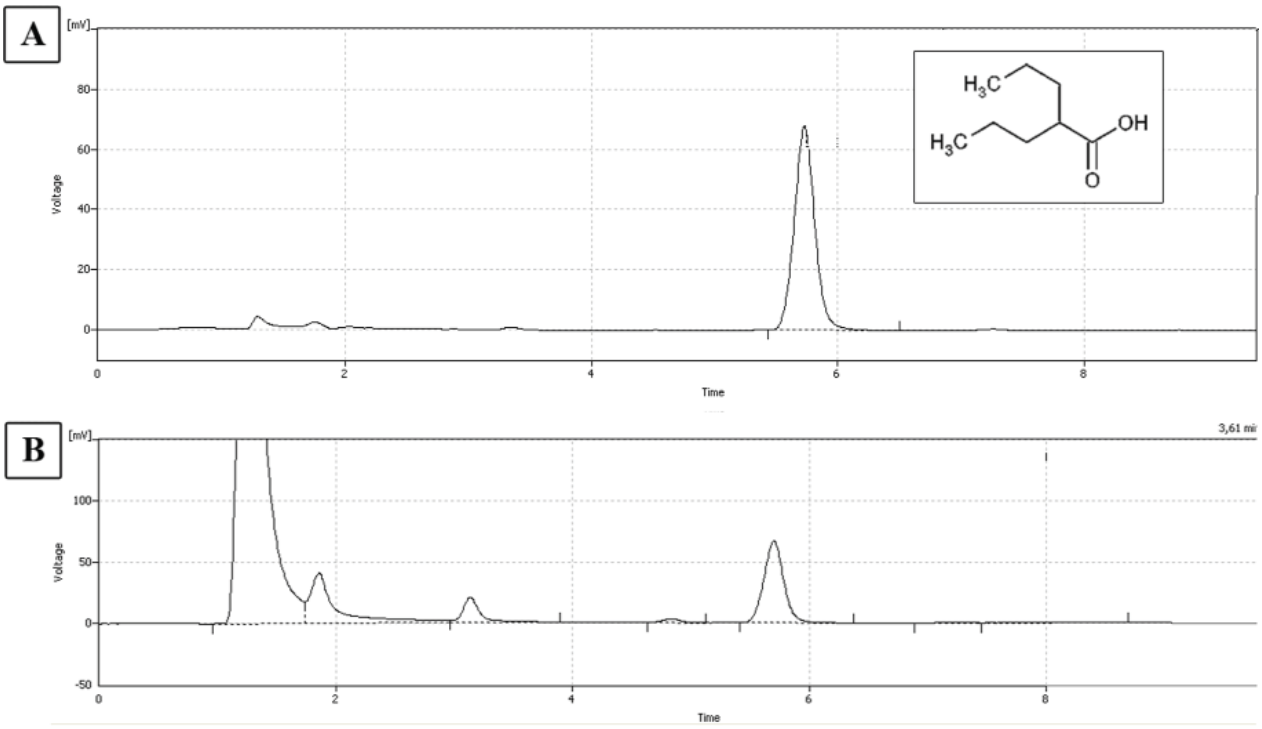

Figure 1 – HPLC-DAD chromatograms of VA at (A) 1.03 mg.mL^{-1} in the standard solution and (B) 1.00 mg.mL^{-1}, prepared using the sample solution. Analytical conditions: Lichrospher C18 column (5 μm; 250 x 4 mm d.i.), mobile phase: 45:55 [aqueous trifluoroacetic acid 0.05% v/v (pH 2.3):acetonitrile], flow: 1.0 mL.min^{-1}, wavelength: 210 nm, temperature: 25 ºC, injection volume: 25 μL.

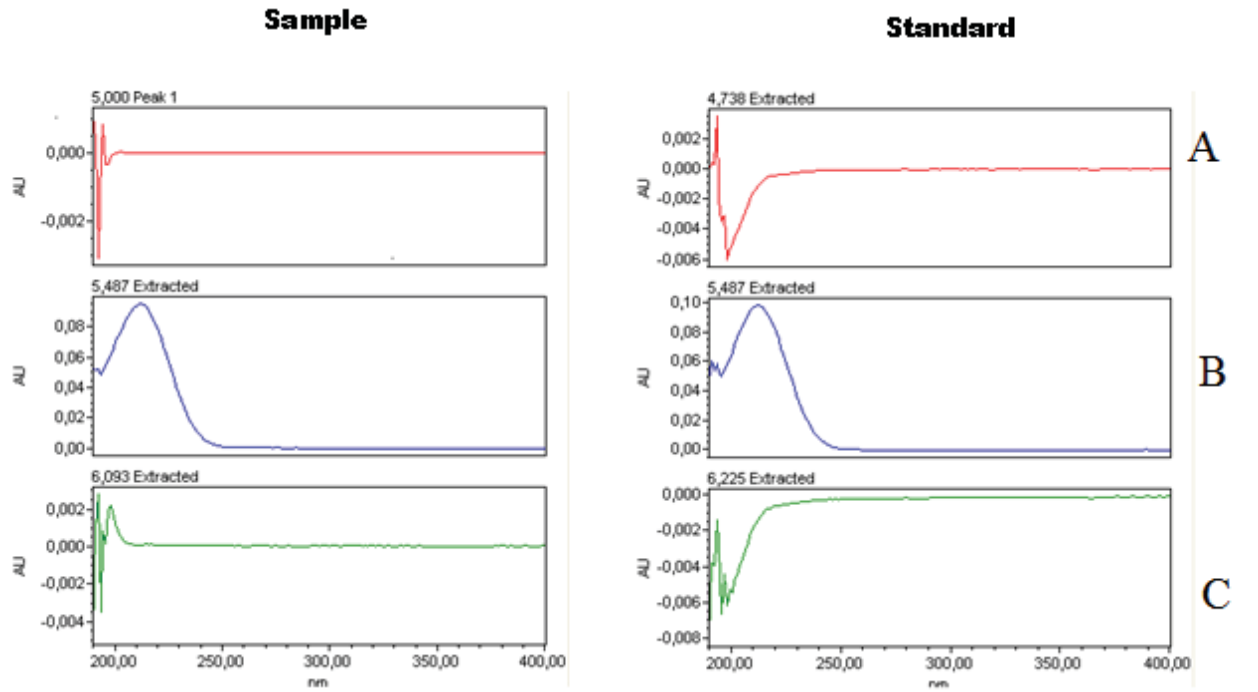

Figure 2 - Absorption spectra in the ultraviolet region of the (I) sample solution (1.00 mg.mL^{-1}) and (II) standard VA solution (1.03 mg.mL^{-1}) before (A), during (B), and after (C) the retention time of VA.

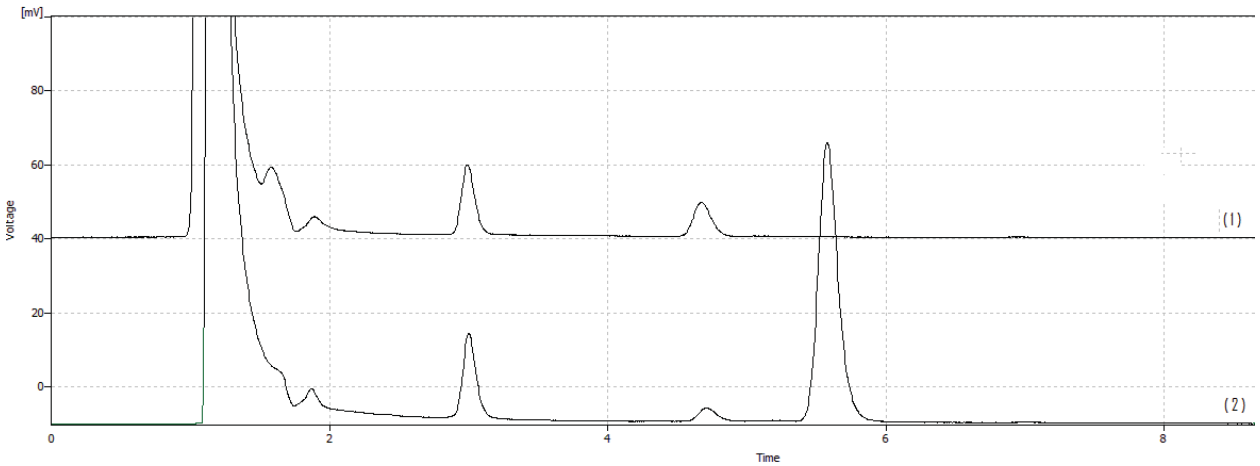

Figure 3- Chromatograms of (1) blank and (2) sample solution (1.00 mg.mL^{-1} of VA). Analytical conditions: Lichrospher C18 column (5 μm; 250 x 4 mm d.i.), mobile phase: 45:55 [aqueous trifluoroacetic acid 0.05% v/v (pH 2.3):acetonitrile], flow: 1.0 mL.min^{-1}, wavelength: 210 nm, temperature: 25 °C, injection volume: 25 μL.

Regarding linearity, the results show that the calibration curve for VA is linear from 0.7 to 1.3 mg.mL^{-1} (y (mAU) = 766 x (mg.mL^{-1}) + 2.08, r^2 0.9998).

In applying the Jackknife test, no aberrant value was identified. The Ryan-Joiner test was utilized to verify the normal distribution of the residuals, which was confirmed (Req 0.99 > Rcrit (α= 0.05) 0.95). The Levene *t* statistic showed homoscedastic behavior (p= 0.849 > 0.05). The Durbin-Watson test confirmed the independence of regression residuals [d (calculated)= 2.15 > dU (Upper limit - α= 0.05)= 1.42].[11]

Repeatability was confirmed over 30 determinations (average 0.998 mg.mL^{-1}, repeatability relative standard deviation (rRSD) 0.68%). According to Sharbir,[12] the maximal acceptable rRSD limit value is 2.0%. The values that we obtained were significantly lower than this criterion.

The intermediate precision standard deviation (Spi) was 1.23%, proving the method's internal reproducibility. According to Horwitz,[14] the maximal value for Spi as a function of the drug concentration is 5.6%. The results obtained for ten analyses of the same solutions (1.00 mg.mL^{-1} of VA) by three different analysts, using the curve for the standard VA solution (curve 1), are shown in Table 2.

Table 2 - Results obtained for ten analyses of the same solutions (1.00 mg.mL^{-1} of VA) by three different analysts.

Analysis	Analyst A (mg.mL^{-1})	Analyst B (mg. mL^{-1})	Analyst C (mg.mL^{-1})
1	0.9965	1.0003	0.9955
2	1.0004	1.0015	0.9902
3	0.9884	0.9911	1.0024
4	0.9884	1.0034	0.9975
5	0.9868	0.9940	1.0008
6	0.9962	0.9858	0.9922
7	0.9897	0.9887	1.0002
8	0.9862	0.9959	0.9994
9	0.9912	0.9993	0.9975
10	0.9898	0.9952	0.9971
Average (mg.mL^{-1})	0.9904	0.9955	0.9973
RSD (%)	0.46	0.57	0.38

The average accuracy was 100.68% (RSD 0.79%). Recovery varied from 99.42 to 101.55%, and the RSD varied from 0.14 to 0.74%. According to Horwitz,[14] the recovery limit may vary between 95 and 105% of the value claimed by the manufacturer (Table 3).

Table 3 - Recovery and RSD for theoretical VA concentrations of 0.7 to 1.355 mg.mL^{-1} and for experimental concentrations of triplicate samples from 0.7 to 1.3 mg.mL^{-1} using the calibration curve for VA (y (mAU) = 766 x (mg.mL^{-1}) + 2.08, r^2 0.9998).

Theoretical concentration (mg.mL^{-1})	Experimental concentration (mg.mL-1)	Recovery (%)	RSD (%)
0.700	0.700	100.00	0.738
0.809	0.805	99.42	0.564
0.918	0.922	100.30	0.245
1.028	1.037	100.80	0.318
1.137	1.144	100.53	0.503
1.246	1.266	101.46	0.209
1.355	1.377	101.55	0.138

Robustness was evaluated according to the system adequacy parameters. The influence of each variation on area repeatability, tail factor, number of the theoretical plates, and retention factor was verified.

Relative standard deviations for 8 combinations varied from 0.11 to 1.44%, which was satisfactory (the maximum permissible value for this parameter is 2%).[12]

Tail factors varied from 1.05 to 1.48. Because these values were all below 2, symmetry was within acceptable limits.[6]

Retention factors were between 2.8 and 4.7; column dead volume differentiation was thus satisfactory. According to Harris,[15] acceptable values are between 2 and 10.

The number of theoretical plates varied from 4181 to 6680, which was higher than the value recommended by Sharbir[12] (N>2,000). This suggests that the column used was adequate. Thus, this method is robust to the variations listed in Table 1.

3.2 – Analysis of Pharmaceutical Formulations

The results of contents for the analysis of pharmaceutical preparations A, B, and C were: 99.82% (RSD 0.34%), 104.58% (RSD 0.34%) and 101.68% (RSD 0.48%), respectively. Compared with the recommendations of USP 33 (90 to 110% of the value claimed by the manufacturer), we conclude that these formulations are satisfactory.

4 - Conclusions

This validated method permits the rapid, efficient and robust analysis of VA. Validation demonstrates that the method permits the reliable and unambiguous identification and quantification of VA. The method also exhibits satisfactory selectivity, linearity, precision (repeatability and intermediate precision), accuracy, recovery and robustness. The validation procedures extended over 3 months. Throughout this period, all of the reagents gave satisfactory results. This technique could also be applied for the determination of sodium valproate in syrup, provided the samples were acidified with 1 mL of aqueous trifluoroacetic acid (0.05% v/v). The VA capsules and syrup tested met UPS 33 specifications. This method will be useful in the quality control of medications containing VA because most official quality control laboratories do not have the gas chromatograph required for the United States Pharmacopoeia (USP) method and thus use liquid chromatography instead.

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