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Development and validation of spectrophotometric methodology to determine 5-aminosalicylic acid in drugs assisted with experimental design

Desenvolvimento e validação de metodologia espectrofotométrica para determinação do ácido 5-aminossalicílico em medicamentos com auxílio de planejamento experimental

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ABSTRACT

5-Aminosalicylic acid (5-ASA) is a drug used to treat inflammation in the digestive tract. Among the few analytical techniques that quantify 5-ASA described in the literature, High Performance Liquid Chromatography is the best one, although it has a high cost. Thus, this work aims at developing and validating a spectrophotometric method to determine 5-ASA in pharmaceutical forms based on the reaction of formation of a colored complex between 5-ASA and Fe^{3+} ions. Experimental design and response surface methodology were applied to reduce the number of experiments and obtain the best conditions for the complex absorbance measurement procedures. Experiments were carried out using three factors at two levels, with three replications at the central point, namely: 5-ASA concentration (150 mg/L; 275 mg/L and 400 mg/L), $FeCl_3$ concentration (100 mg/L; 200 mg/L and 300 mg/L) and volume ratio (1:2; 1:1 and 2:1) between the amounts of 5-ASA and $FeCl_3$. Statistical analysis of effects,

interactions, ANOVA, and response surface graphics were obtained by *Minitab 17.0* and *Statistica 8.0* software. The optima conditions obtained from the response surface analysis for the absorbance measurements of 5-ASA/Fe^{3+} formed complex were with the highest concentrations of 5-ASA (400 mg/L) and $FeCl_3$ (300 mg/L) and the lowest volume ratio between these (1:2). The optimized condition of the assay has been validated according to International Conference on Harmonization (ICH) guidelines to confirm linearity, precision and accuracy. The different formulations of drugs containing 5-ASA analyzed recorded values in agreement with those ones of the specifications in formulations. This spectrophotometric methodology was considered of low cost, simple, fast and reliable to determine 5-ASA in pharmaceuticals.

Keywords: 5-ASA; mesalazine; spectrophotometry; factorial design.

RESUMO

O ácido 5-aminosalicílico (5-ASA) é um fármaco utilizado para tratar inflamações no trato digestivo. Dentre as poucas técnicas analíticas de quantificação do 5-ASA descritos na literatura, destaca-se a Cromatografia Líquida de Alta Eficiência, todavia possui custo elevado. Assim, o objetivo deste trabalho foi desenvolver e validar um método espectrofotométrico para determinar o 5-ASA em formas farmacêuticas, baseado na reação de formação de um complexo colorido entre 5-ASA e íons Fe^{3+}. O planejamento experimental e a metodologia de superfície de resposta foram empregados visando reduzir o número de experimentos e obter as melhores condições para os procedimentos de medidas de absorbância do complexo. Foram realizados experimentos utilizando-se três fatores em dois níveis, com três repetições no ponto central, a saber: concentração de 5-ASA (150 mg/L; 275 mg/L e 400 mg/L), concentração de $FeCl_3$ (100 mg/L; 200 mg/L e 300 mg/L) e proporção em volume (1:2; 1:1 e 2:1) entre as quantidades de 5-ASA e $FeCl_3$. A análise estatística dos efeitos, interações, ANOVA, e gráficos de superfície de resposta foram feitos por softwares *Minitab 17.0* e *Statistica 8.0*. As condições ótimas obtidas a partir da análise da superfície de resposta para as medidas de absorbância do complexo formado 5-ASA/Fe^{3+} foram com as maiores concentrações de 5-ASA (400 mg/L) e de $FeCl_3$ (300 mg/L) e menor proporção de volume entre esses (1:2). A condição otimizada do ensaio foi validada de acordo com as diretrizes da Conferência Internacional sobre Harmonização (CIH) para confirmar linearidade, precisão e exatidão. As diferentes formulações de medicamentos contendo 5-ASA analisados registraram valores em concordância com aqueles das especificações nas formulações. Esta metodologia espectrofotométrica mostrou ser de baixo custo, simples, rápida e confiável para se determinar 5-ASA em produtos farmacêuticos.

Palavras-chave: 5-ASA; Mesalazina; Espectrofotometria; Planejamento fatorial

1. INTRODUCTION

The 5-aminosalicylic acid (5-ASA), also known as mesalazine, is a widely ingested drug during the inflammation treatment in digestive system. It can also be taken to treat ulcerative colitis as well as in cases of cancer (Cai et al., 2003; Gotti et al., 2001; Joshi et al., 2005).

This drug takes part of non-steroidal anti-inflammatory drugs, which are widely used to treat inflammatory bowel disease since they have milder adverse effects when compared to corticosteroids (Gasche & Campregher, 2011)

Levels of 5-ASA in tablets, enemas and biological matrices such as blood and secretions, are traditionally measured by chromatographic techniques (Nobilis et al., 2006; Palumbo et al., 1995). HPLC (High-Performance Liquid Chromatography) method is granted in the American Pharmacopoeia, since it is based on mobile phase containing tetrabutylammonium hydrogen sulfate as an ion-pairing agent, however, there is a decrease in column lifetime (Usp, 2000).

Besides, the chromatographic method is an expensive method and the analyses performance by HPLC is not feasible for some areas. Literature has also shown few pharmaceutical quantification methods of 5-ASA. As an example, the Brazilian pharmacopoeia does not describe any procedure to determine 5-ASA in pharmaceuticals (Brasil, 2010).

However, the determination of salicylate levels can be obtained by a low-cost, accurate and precise analytical method, known as Trinder method, according to the spectrophotometry technique. This technique is based on a colored complex generation between salicylate and ferric ions. Furthermore, it is important to highlight that the studied salicylate concentration is directly associated to the violet color magnitude of the produced complex (Moreau, 2013).

Thus, the development and validation of an analytical method are directly related to which statistical model will be chosen and that also answers to the desired requirements, in order to document evidences that the method accomplishes what is demanded (Ruela, Araújo & Pereira, 2009). Consequently, some analytical parameters should be defined for both development and validation of a method. And, some of them can be highlighted such as: specificity, linearity, precision, limit of detection, limit of quantification and accuracy (Anvisa, 2017).

Features such as method optimization, cost reduction, reduced waste generation and shorter runtime are increasingly substantial on researching and developing analytical methodologies (Silva et al., 2008). So, the use of statistical tools such as experimental design becomes interesting, since this design prospects experimental conditions of the system with the minimum of possible experiments. It also foments the methodology optimization and increases its sensitivity (Barros Neto, Scarminio & Bruns, 2010).

The experimental design is a useful analytical strategy and its main application consists on selecting the most relevant factors of a given analytical system. After this sorting process, the most significant variables, experiments are carried out to allow refinement and a better knowledge of the system under study (Montgomery, 1991).

The selected variables are optimized by statistical software that will generate mathematical models. Finally, a mathematical equation is established that relates the variables to be optimized with the analytic response (response surface). Therefore, this trial aimed at developing and validating a simple, fast, cheap and effective analytical methodology to determine 5-ASA in pharmaceutical products, using a spectrophotometric technique based on the reaction of colored complex formation with metal ion Fe^{3+}.

Experimental design and response surface methodology (RSM) were applied to study the effect of 5-ASA and $FeCl_3$ concentrations and their volume ratio on 5-ASA/Fe^{3+} formed complex absorbance.

2. METHODOLOGY

The 5-ASA was prepared using a secondary standard from Purifarma, whose technical report reinforced 100.14% of purity content. All the described spectrophotometric analyses herein were carried out using a SP-220 Bio-spectrum spectrophotometer. A 1.0 cm glass cuvette of optical path was used in all measurements.

2.1 EXPERIMENTAL DESIGN

Experimental design prospects the system conditions with the least number of experiments. It puts forward the methodology optimization and increases its sensitivity. This is a useful analytical strategy to carry out the screening of the most relevant variables of such system. Thus, a three-factor (X_1, X_2, and X_3) and three-levels (-1, 0, and +1) experimental design (DOE) was used in this trial in order to achieve maximum information about 5-ASA/Fe^{3+} formed complex absorbance. The studied factors (independent variables) were 5-ASA concentration (X_1, mg/L), $FeCl_3$ concentration (X_2, mg/L), and volume ratio among 5-ASA and Fe^{3+} amounts (X_3) to evaluate complex absorbance response. Each variable was coded at one of three levels, -1, 0, and +1. The minimum and maximum levels (Table 1) given to each factor were chosen based on preliminary experiments. Each experiment was performed in triplicate and the average values were taken as the response (Y). The trials were carried out randomly, and defined by draw, in order to avoid systematic errors.

Table 1 - Coded and uncoded levels of the independent variables used in a 2^3 factorial design.

		Coded levels		
Independent variable	**Factor**	**-1**	**0**	**+1**
5-ASA concentration (mg/L)	X_1	150	275	400
$FeCl_3$ concentration (mg/L)	X_2	100	200	300
volume ratio (5-ASA: $FeCl_3$)	X_3	1:2	1:1	2:1

The 2^3 factorial design was designed with 11 trials that included triplicates at the central point. The main effects, variables interactions, their respective coefficients for the mathematical model, as well as the analysis of variance (ANOVA) were calculated to determine the model validity. So, the effects of those variables were described with the difference between average response at the highest level and average response at the lowest one.

Data analysis was evaluated using *Minitab 17.0* and *Statistica 8.0* software. The fit quality on polynomial equation was evaluated by R^2 determination coefficient. The optimal values were obtained from the selected variables and analyzed by the response surface methodology. Thus, the F test was applied as a criterion for validating the statistical significance of the obtained models at 95% confidence interval.

2.2 WAVELENGTH SCANNING OF 5-ASA/FE^{3+} COMPLEX

The complex formation reaction was carried out using the following concentrations from stock solutions of $FeCl_3$ and 5-ASA, both at 2 g/L: $FeCl_3$ (300 mg/L) and 5-ASA (400 mg/L). The sample was prepared at 1:2 volume ratio, as it follows: 1.0 mL 5-ASA 400 mg/L and 2.0 mL 300 mg/L $FeCl_3$ were pipetted and transferred to a glass cuvette. After homogenization, the measurement of maximum absorption wavelength on 5-ASA/Fe^{3+} complex was carried out by scanning range from 400 nm to 800 nm. A reading of the solution with only $FeCl_3$ (300 mg/L) and H_2O was also recorded (blank).

2.3 EFFECT OF REACTION TIME ON THE COMPLEX STABILITY

This test aimed at evaluating the stability of formed 5-ASA/Fe^{3+} complex over a period of time by measuring its absorbance values. The sample preparation was the same as described above and, after homogenization, a reading in spectrophotometer at the best absorbance wavelength was carried out. The test was carried out from 0 to 5-minute terms at 30-second

intervals from one reading to the other and after the first 5 minutes, the test occurred at 6, 8, 10, 15, 20, 25 and 30 minutes, with a total of 18 readings.

2.4 SPECTROPHOTOMETRIC ANALYSIS OF PHARMACEUTICAL PRODUCTS

The analytical curve of 5-ASA was obtained from the preparation of 5-ASA solutions at different concentrations (from 150 to 450 mg/L) and mixed with fixed concentration of $FeCl_3$ solution (300 mg/L). Then, 1 mL aliquots of each 5-ASA concentration and 2 mL of $FeCl_3$ solution were transferred to a glass cuvette to be homogenized, and absorbance was measured the at the best wavelength in a spectrophotometer.

Five different formulations of pharmaceutical products containing 5-ASA products were analyzed: the reference tablet (Mesacol®), generic tablet (Gnr), manipulated capsule (Mpl), sachet (Sch) and enema (Ene) were analyzed by spectrophotometric methodology. Solutions of each drug were prepared at the best concentration obtained by the experimental design. Then, solutions were transferred to the glass cuvette with an optimized volume ratio of 5-ASA and $FeCl_3$ and, after homogenization, each absorbance was measured at the best wavelength.

In order to compare the developed spectrophotometric methodology, volumetric analyses were carried out in the same five pharmaceuticals containing 5-ASA. This volumetric analysis was done according to the British Pharmacopoeia, whose titration was with NaOH 0.1 mol/L.

3. RESULTS AND DISCUSSION

The maximum absorbance wavelength of 5-ASA/Fe^{3+} complex was 520 nm, which was chosen for subsequent readings.

3.1 EXPERIMENTAL DESIGN

It can be observed the matrix of a coded factorial design and the concentration values of the three studied variables as well as absorbance values, obtained by spectrophotometric reading of 5-ASA/$FeCl_3$ complex (Table 2). The values obtained by the essays (9, 10 and 11) at the central points showed small variations, indicating a good reproducibility.

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Table 2 - Coded and real values of the 2^3 factorial design matrix with results for absorbance.

Essays	**Random order**	**5-ASA concentration**†	**$FeCl_3$ concentration**†	**Volume ratio**	**Absorbance**§
		X_1	X_2	X_3	Y
1	5	-1 (150)	-1 (100)	-1 (1:2)	0.143 ± 0.008
2	1	1 (400)	-1 (100)	-1 (1:2)	0.158 ± 0.007
3	8	-1 (150)	1 (300)	-1 (1:2)	0.732 ± 0.006
4	4	1 (400)	1 (300)	-1 (1:2)	0.892 ± 0.008
5	11	-1 (150)	-1 (100)	1 (2:1)	0.082 ± 0.010
6	2	1 (400)	-1 (100)	1 (2:1)	0.117 ± 0.008
7	3	-1 (150)	1 (300)	1 (2:1)	0.452 ± 0.009
8	10	1 (400)	1 (300)	1 (2:1)	0.533 ± 0.009
9	6	0 (275)	0 (200)	0 (1:1)	0.373 ± 0.007
10	9	0 (275)	0 (200)	0 (1:1)	0.361 ± 0.007
11	7	0 (275)	0 (200)	0 (1:1)	0.380 ± 0.005

† Concentration in mg/L

§ Results are average of triplicate analysis for 5-ASA/Fe^{3+} complex absorbance ± standard deviation.

The equation regarding the mathematical model was obtained based on the general equation of the linear model to predict the response variables at 95% confidence level:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 \quad (1)$$

Where, Y represents the expected response; β_0 is the interception; β_1, β_2 and β_3 are linear coefficients; β_{12}, β_{13} and β_{23} are coefficients of interaction. Accuracy and overall capacity of the above model were evaluated using the determination coefficient (R^2).

The effects table shows the significant variables, either positively or negatively, according to the obtained absorbance results. Thus, it can be observed that variable 1 (5-ASA concentration) is significant and influences positively absorbance increase (Table 3). Similarly, variable 2 ($FeCl_3$ concentration) is also significant and has a positive effect; besides, it increases absorbance. However, variable 3 (volume ratio) has a significant effect, but in a negative way, consequently it decreases absorbance.

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Table 3 - Effect table of 2^3 factorial design.

	Effect	**standard error**	**t(4)**	**p**
Average	0.3837	0.0068	56.3078	0.0000 *
(1) 5-ASA concentration	0.0728	0.0160	4.5520	0.0104 *
(2) $FeCl_3$ concentration	0.5273	0.0160	32.9899	0.0000 *
(3) volume ratio	-0.1853	0.0160	-11.5911	0.0003 *
5-ASA x $FeCl_3$	0.0478	0.0160	2.9877	0.0404 *
5-ASA x volume ratio	-0.0148	0.0160	-0.9229	0.4082
$FeCl_3$ x volume ratio	-0.1348	0.0160	-8.4313	0.0010 *

*Statistically significant at 95% confidence.

The interaction $FeCl_3$ x volume ratio (2x3) was significant, because when there is some change on $FeCl_3$ concentration, it causes a negative change in the response, in other words, an absorbance decrease. On the other hand, 5-ASA x volume ratio (1x3) interaction was not significant, because, when there was some change on 5-ASA ratio, the response was statistically the same. Ultimately, 5-ASA x $FeCl_3$ (1x2) interaction is significant, because when there is some change on 5-ASA, there is also a positive change on the response, and consequently an increase in the absorbance result.

The interaction graphs were drawn using the *Minitab 17.0* software for the significant interactions, in order to illustrate absorbance values variation due to the levels changes. In Figure 1, it can be observed the interaction between 5-ASA and $FeCl_3$ concentrations (1x2 interaction).

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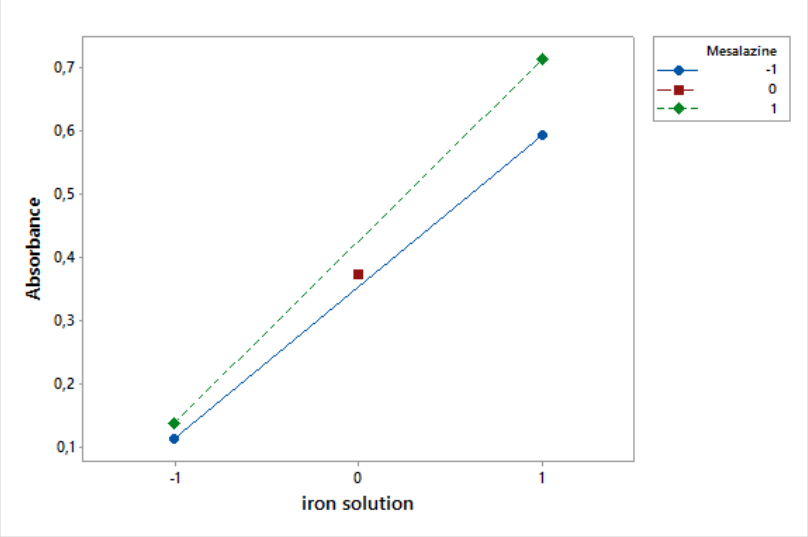
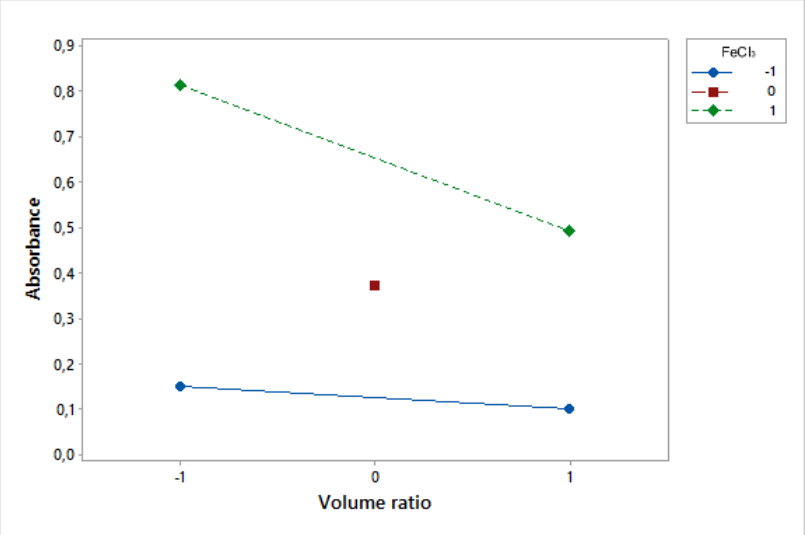
Figure 1. Graph of the interaction between both concentrations of 5-ASA and $FeCl_3$.



The change in absorbance values is observed when there is some change on (-1 to 1) levels; therefore, it can be state that such interaction is significant. At -1 level, the absorbance value is low and points are close to each other. Therefore, there is an increase in the variable-response (absorbance) as (-1 to 1) levels change and the distance between the points increases, with emphasis on this interaction significance.

The interaction between $FeCl_3$ concentration and volume ratio is presented in Figure 2.

Figure 2. Graph of interaction between $FeCl_3$ concentration and volume ratio (5-ASA: $FeCl_3$).



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When the (-1 to 1) levels change, there is a change on absorbance values, in other words, it is observed a significant interaction. At -1 level, the absorbance value is high and the points are far from one another. However, there is a decrease in response variable (absorbance) as there is a change in (-1 to 1) levels and the distance between the points is reduced, although there is a highlight on the significance of this interaction. Table 4 presents regression coefficients of 2^3 experimental design.

Table 4 - Regression coefficients of 2^3 experimental design.

	Regression Coefficients	**Standard Errors**	**t(4)**	**p**
Averages	0.3837	0.0068	56.3078	0.0000 *
(1) 5-ASA concentration	0.0364	0.0080	4.5520	0.0104 *
(2) $FeCl_3$ concentration	0.2636	0.0080	32.9899	0.0000 *
(3) volume ratio	-0.0926	0.0080	-11.5911	0.0003 *
5-ASA x $FeCl_3$	0.0239	0.0080	2.9877	0.0404 *
5-ASA x volume ratio	-0.0074	0.0080	-0.9229	0.4083
$FeCl_3$ x volume ratio	-0.0674	0.0080	-8.4313	0.0011 *

*Statistically significant at 95% confidence.

Equation 2 shows the linear model that was obtained to predict absorbance regarding both 5-ASA and $FeCl_3$ concentrations as well as volume ratio between them.

$$Y = 0.384 + 0.036X_1 + 0.264X_2 - 0.093X_3 + 0.024X_1X_2 - 0.067X_2X_3 \quad (2)$$

Table 5 shows the analysis of variance (ANOVA) regarding the experimental design.

Table 5 - ANOVA test for 2^3 factorial design.

	sum of squares	**degree of freedom**	**Medium Square**	**Calculated-F value**	**Critical-F value**	**P**
regression	0.6761	5	0.1352	272.77	5.05	<0.01
residue	0.0025	5	0.0005			
lack of adjustment	0.0023	3	0.0008	8.28	19.16	0.11
Pure error	0.0002	2	9.2333			
Total	0.6810	10				

R^2= 0.9970

It should be highlighted that, based on the F test, the calculated F value (272.77) is 54 times greater than the critical F (5.05) with $p < 0.01$. This result implies a satisfactory representation by the linear model. The lack of adjustment was not significant because the calculated F (8.28) was lower than the critical F (19.16), which means that the experimental data are included in the obtained model. The coefficient of determination R^2 (0.9970) suggests that the adjusted model can explain 99.70% variability of the experimental data, which confirms the quality of adjustment.

The response surface can be observed in Figure 3, regarding the following studied variables: $FeCl_3$ concentration in relation to 5-ASA concentration and $FeCl_3$ concentration in relation to volume ratio (5-ASA: $FeCl_3$). The highest peak is highlighted in red, which indicates the highest point of absorbance obtained for the complex regarding the concentration of both solutions (Fig. 3a). Therefore, there is some evidence of a better optimization when the maximum concentration of iron solution (300 mg/L) and the maximum concentration of 5-ASA solution (400 mg/L) were applied. 5-ASA concentration showed that it interferes little in the absorbance of the formed complex. It can be observed also that the maximum absorbance point occurred when it was used the highest concentration of $FeCl_3$ solution (300 mg/L), and the highest (1:2) volume ratio of 5-ASA and $FeCl_3$ for reading (Fig. 3b).

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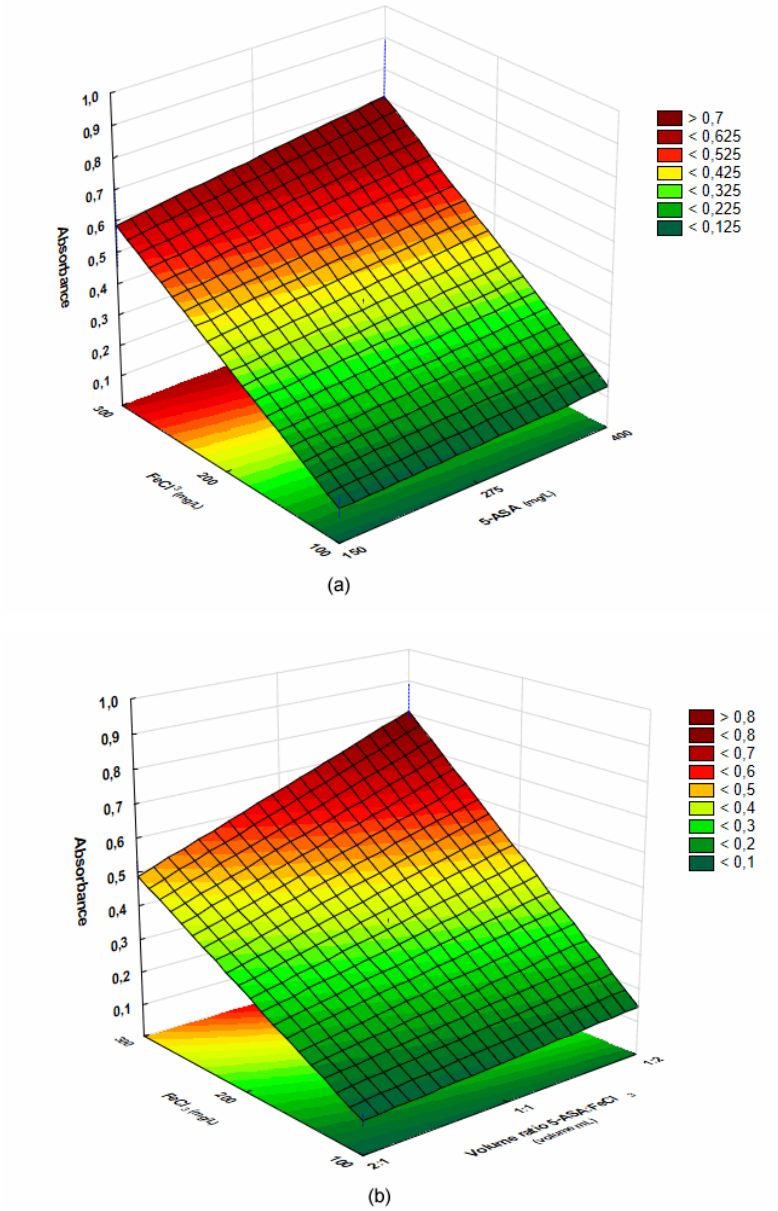
Figure 3. Response surface plots for the effect of *(a)* 5-ASA and $FeCl_3$ concentrations (mg/L) and *(b)* $FeCl_3$ concentration (mg/L) and volume ratio (5-ASA:$FeCl_3$) on absorbance of the complex formed between them.



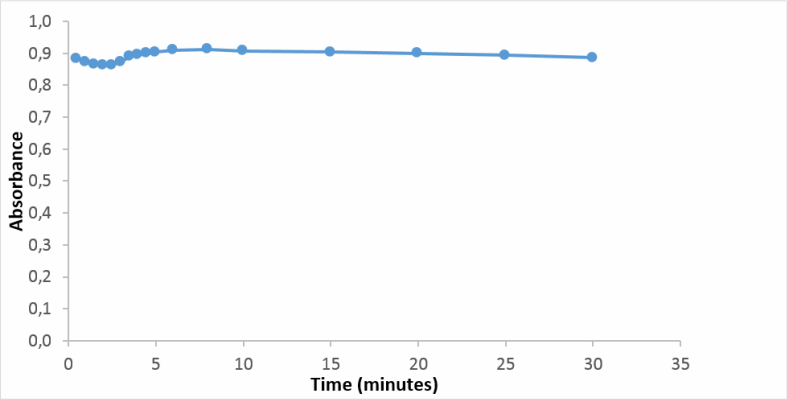
Therefore, it can be concluded that the ideal concentrations to optimize this method are those ones with maximum concentrations of 5-ASA and $FeCl_3$ solutions (400 and 300 mg/L, respectively) and the lowest volume ratio between them (1:2).

3.2 REACTION TIME EFFECT ON COMPLEX STABILITY

Observing Figure 4 below, it can be seen that the greatest variation in the absorbance values of the formed complex occurs up to 10 minutes. Although, this variation is not important, since the difference in absorbance was only 0.051. After the initial 10 minutes, the

absorbance remained almost steady until the end of 30 minutes. Thus, it is perceived that the complex is stable and so it will be throughout the whole time required to make a test on a commercial sample. So, it is worth emphasizing that this answer is relevant to this trial.

Figure 4. Effect of time on stability of 5-ASA/$FeCl_3$ complex.



3.3 SPECTROPHOTOMETRIC ANALYSIS OF PHARMACEUTICAL PRODUCTS

3.3.1. Validation of the analytical method

Validation methodology followed the rules required by the National Agency of Sanitary Surveillance (ANVISA), a governmental agency that regulates these requirements in Brazil. Parameters such as linearity, precision, intermediate precision, limits of detection and quantification and accuracy were recorded.

Linearity refers to how an analytical methodology demonstrates that the obtained results are directly proportional to the analyte concentration in a sample within a given range. Thus, it is recommended that linearity be determined by analyzing at least five different concentrations (Anvisa, 2017). The minimum acceptable criterion of a linear correlation coefficient (r) shall be 0.99.

Linearity of this method was verified by analytical curve, whose linear regression equation was $y = 0.0008x + 0.5766$ and linear correlation coefficient (r) was 0.9954. According to these values, it was observed that the method showed a good linearity from 150 to 450 mg/L for 5-ASA concentration.

Precision is the evaluation of proximity regarding results obtained in a series of measurements of a multiple sampling of a same sample. The repeatability analysis was carried out in order to evaluate precision, based on six readings of 5-ASA/Fe^{3+} complex, whose applied 5-ASA concentration was the one with mean value (300 mg/L). Then, there were three

repetitions in the same day. This validation parameter aims the correlation among results in a short time with the same analyst and the same instrumentation.

Absorbance values and their respective averages are in Table 6. It is also observed that the method does not show substantial variations when used for analysis in different periods of time, although the conditions and analyst were the same, since the results for relative standard deviation were all less than 1.5% (Anvisa, 2017; Inmetro, 2016).

Table 6 - Absorbance values obtained for the repeatability parameter by the same analyst.

Samples	Absorbance							RSD† (%)
	1	2	3	4	5	6	Average	
Time 01	0.846	0.835	0.841	0.828	0.822	0.825	0.833	1.14
Time 02	0.851	0.859	0.836	0.846	0.831	0.850	0.846	1.22
Time 03	0.849	0.841	0.827	0.851	0.848	0.833	0.842	1.15

† RSD = Relative Standard Deviation

The intermediate precision test detected possible changes during the analyses, and it evaluated when the changing conditions are greater for accomplishment. Thus, the analyses were carried out in different days and with different analysts. The absorbance values obtained for 5-ASA/Fe^{3+} complex, in this test, ranged from 0.827 to 0.859, whose average was 0.843. The RSD value (1.41%) was also lower than 1.5%, which shows good intermediate precision and that there is no significant difference between the two analysts at 95% confidence level.

Thus, both repeatability and intermediate precision showed RSD values lower than the recommended ones by literature and regulatory agency, which is 5%, indicating that such method showed good precision (Anvisa, 2017; Inmetro, 2016).

Limit of detection (LOD) is the smallest amount of analyte to be detected and present in a sample, but not necessarily quantified under the established experimental conditions. LOD is stated by analyzing solutions of known and decreasing concentrations of an analyte until the lowest detectable level (Anvisa, 2017). Its determination can be recorded by visual method, signal-to-noise ratio, based on blank determination or analytical curve parameters, taking into account peculiarities of the used analytical method (Inmetro, 2016). LOD by visual methods is determined by the lowest concentration for which the expected visual effect can be detected,

while by the signal-to-noise ratio is for instrumental methods, whose its value must be higher than or equal to 2:1. And if determination is based on analytical curve parameters, LOD can be calculated by the following equation (3):

$$LOD = \frac{3\ x\ standard\ deviation\ of\ intercept\ with\ Y\ axis}{calibration\ curve\ slope} \qquad (3)$$

Limit of quantification (LOQ) is the smallest amount of analyte in a sample that can be determined with acceptable precision and accuracy. So, for visual methods, LOQ is recorded by the lowest concentration for which the expected visual effect can be observed, while based on analytical curve parameters, it can be calculated by the equation (4):

$$LOQ = \frac{10\ x\ standard\ deviation\ of\ intercept\ with\ Y\ axis}{calibration\ curve\ slope} \qquad (4)$$

Values obtained for LOD and LOQ were 0.0202 and 0.0673, respectively. These parameters are not predicted for determination according to category I classified by ANVISA, but were calculated additionally for the developed method (Anvisa, 2017).

Accuracy of an analytical method is the nearness of the results obtained by the studied method to the true value and can be calculated as a percentage of recovery related to the known amount of analyte added to the sample. In this method, it was determined regarding linear interval of analytical curve, with three concentrations (low, medium and high) and three replications each, totalizing 9 (nine) determinations (Anvisa, 2017; ICH, 2005) and was expressed by equation (5):

$$Accuracy = \frac{experimental\ average\ concentration}{theoretical\ concentration}\ x\ 100 \qquad (5)$$

The values recorded for the accuracy parameter are shown in Table 7.

Table 7 - Experimental values obtained for 5-ASA concentration in the accuracy test.

5-ASA Theoretical Concentration (mg/L)	5-ASA Experimental Concentration (mg/L)				Accuracy (%)
	01	02	03	Average±SD†	
150	148	139	153	147 ± 7.09	97.83
300	331	303	314	316 ± 14.10	105.17
450	449	471	448	456 ± 13.00	101.22

† SD = Standard Deviation

It was observed that accuracy showed values according to the acceptance criteria preconized by ANVISA, that is, from 80 to 120% (Anvisa, 2017), indicating that the proposed spectrophotometric method is accurate, since linearity and precision were suitable.

So, it was possible to highlight that all the tests required to validate this method were carried out and the values were according to what is recommended by governmental agency.

3.3.2 5-ASA analyses in drugs

After the validation procedure has been concluded, the values obtained for 5-ASA determination in samples of different pharmaceutical products, using the developed spectrophotometric methodology, are presented in Table 8.

Table 8 - Values of 5-ASA obtained by spectrophotometry from the studied formulations.

Formulations (400 mg/L)	average concentration±RSD† (mg/L)	% average recovery
Mesacol®	381 ± 3.28	95.3
Gnr	368 ± 2.12	92.0
Mpl	343 ± 3.10	85.8
Sch	337 ± 4.86	84.0
Ene	348 ± 4.23	87.0

† RSD = Relative Standard Deviation

It was observed that the recovery percentage for all formulations was within at least 80% (Anvisa, 2017; Inmetro, 2016), with values ranging from 84.0 to 95.3%. Thus, these results also indicated that RSD was less than 5% for all studied formulations. Values obtained

for 5-ASA analyses of the same formulations by the volumetric methodology described in British Pharmacopoeia (2009) are shown in Table 9.

Table 9 - 5-ASA values obtained by titration in the studied formulations.

Formulations (400 mg/L)	**average concentration±RSD† (mg/L)**	**% average recovery**
Mesacol®	379 ± 4.36	94.8
Gnr	374 ± 2.08	93.5
Mpl	328 ± 2.81	82.0
Sch	337 ± 3.47	84.3
Ene	332 ± 2.87	83.0

† RSD = Relative Standard Deviation

It was also observed that the recovery percentage for all formulations was within at least 80% (BP, 2009), with values ranging from 82 to 95%, approximately, and with RSD values less than 5% for all studied formulations. The F-test was applied to compare the results obtained between the spectrophotometric and volumetric method to verify if there was a significant difference between both methods. However, there was no significant difference between both methods, since the value obtained from F calculated (0.48) was lower than tabulated F value at 95% confidence level (6.39).

It is noteworthy to say that there is no procedure described by the Brazilian Pharmacopoeia for 5-ASA determination regarding pharmaceutical drugs (Brasil, 2010). On the other hand, the American Pharmacopoeia adopts a method that uses High Performance Liquid Chromatography with a mobile phase containing tetrabutylammonium hydrogen sulfate as an ion-pairing agent, but this reduces the useful life of the column (Palumbo et al., 1995).

The volumetric methodology according to British Pharmacopoeia (2009) registered values close to the ones obtained by the spectrophotometric methodology in order to analyze the same drugs, whose goal was to compare the obtained results by the developed method.

4. CONCLUSIONS

The spectrophotometric method was validated to quantify 5-ASA in pharmaceutical formulations, since it showed suitable values for all required parameters. Thus, this methodology complied with the guidelines of the current legislation.

The experimental design and RSM were important tools to optimize and determine the best 5-ASA/Fe^{3+} complex absorbance conditions, revealing more efficiency with higher 5-ASA and $FeCl_3$ amounts and smaller volume ratio between them.

All five different pharmaceutical products containing 5-ASA were able to be analyzed by the proposed spectrophotometric methodology and showed 5-ASA concentration values in agreement with those ones contained in labels and reported by the manufacturer.

It is a low-cost, fast and easy method, which can be used safely and reliably. So, it is practicable both for analyses in a pharmaceutical industry and in a magistral pharmacy.

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