ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Evaluation Of Papain In Pharmaceutical Solid Dosage Forms: Ultraviolet Spectrophotometry Method Validation.

Felipe Augusto NeryNambu, Fúlvio GabrielCorazza, Marcelo Dutra Duque, Patrícia Santos Lopes, Vânia Rodrigues Leite-Silva, and Newton Andréo-Filho*.

Departamento de Ciências Farmacêuticas – Instituto de Ciências Ambientais, Químicas e Farmacêuticas. Universidade Federal de São Paulo – UNIFESP, Brazil.

ABSTRACT

Papain is an enzyme whichhas shown potential activity to enhance the gastrointestinal drug absorption, allowing be explored to improve the bioavailability ofdrugs.Thus, thequantificationof papain using a simple, fast and affordable method is importantin quality control of solid dosage forms containing the enzyme. In this work a spectrophotometric method was developed and validated in four pH mediums to quantify papainin solid dosage forms and dissolution test.The linearity for all pH values was higher than 0.99. Others parameters determined in validation considering lowestand highest values were: detection limit 0.0008 $mg.mL^{-1}$ (pH 5.5)and 0.0059 $mg.mL^{-1}$ (pH 1.2),quantification limit0.0027 $mg.mL^{-1}$ (pH 5.5) and0.0199$mg.mL^{-1}$(pH 1.2), repeatability in terms of RSD0.1% (pH 1.2) and 2.5% (pH 6.8), accuracy in terms of recuperation (R%) 98.0% (pH 6.8)and102.8 (pH 1.2). Specificity was evaluated against an ordinary mixture of solid excipients using three treatments for interferents elimination. Filtration through 0.22 µm pore size reaching low interference (<5.0 %) in papain determination. In conclusion, a simple method for papain determination was achieved,allowing its use for content analysis and dissolutiontest of papainfrom solid dosage forms.

Keywords: papain, spectrophotometry,method validation, solid dosage forms.

https://doi.org/10.33887/rjpbcs/2019.10.6.14

Corresponding author

ISSN: 0975-8585

INTRODUCTION

Papain is an enzyme belonging to Cysteine Protease family. It was the first enzyme of this family isolated and characterized from the latex of leaves and fruit of Carica papaya [1, 2]. Due to its proteolytic activity is used as a debriding agent incutaneous wounds at different phases of cicatrization, as well burned skin and tissues in necrosis process [2, 3, 4]. Additionally, some authors report other uses for papain, such as enhancer of drug skin permeation, antifungal and anti-inflammatory agent [1, 3, 5].

As other enzymes, papain may lose its activity when exposed to an inhospitable environment such as extremes of pH's and temperature, and oxidant compounds. However, despite the inherent instability of enzymes, papain maintains its activity at pH 5.0 to 7.0, and temperature up to 55 °C [6, 7, 8]. Due to such features, papain is just present in some dosage forms for topic application [1, 9].

Despite that, there is a growing interest to use papain for the treatment of diseases in gastrointestinal tract using the oral route, since it might aid on the management of ulcerative processes and might favor the absorption of some drugs as well, improving the bioavailability[10, 11, 12]. Among the solid dosage forms for oral route, tablets are the most used due to the feasibility of manufacturing and lower drug exposition to environmental factors, providing better conditions for enzyme stability [13, 14]. Furthermore, the possibility of coating tablets might allow the delivery to a site-specific, e.g. colonic drug delivery systems, reducing the enzyme exposition throughout the gut [15].

As a common occurrence for others active compounds, medicines conveying enzymes must have well-established quality standards to guarantee that each unit will have efficacy and safety adequate for administration[16]. In this context, the appropriate analytical methods are essential to demonstrate that a medicine unit has the correct content of active compound, an acceptable drug dosage and the adequate way which a drug is released from the dosage form. Methods for quantificationof active compounds, as the UV-vis spectrophotometry, assumes great importance since they could determine small concentrations of active compound in solution, being used in tests such as content uniformity and dissolution. The dissolution test is essential to demonstrate the *in vitro* performance of solid dosage forms [17, 18, 19].

Ideally, the analytical methods should be simple, affordable, and possible to validate[20, 21]. In this sense, UV-vis spectrophotometry is a simple technique, widely spread in academic laboratories and pharmaceutical companies worldwide. Additionally, spectrophotometers are equipment used in routine analysis at the bigger and smaller laboratories, making possible that a specific spectrophotometric method would be widely applied. In this work, our group developed an UV-vis spectrophotometric method aiming to quantify papain in solid dosage forms such as tablets, hard capsules and pellets, and into four buffer solutions used as dissolution medium.

The validation of analytical methods is estimated through quality parameters able to demonstrate in a concise way, using statistical models, that the method proposed is adequate to use. The parameters most used are specificity, linearity, precision, accuracy, robustness, and detection and quantification limits [17, 20, 22]. Depending on the type of analysis, compound, and guide used, the number of tests might vary, but generally, it is applied the ICH recommendations,or the ones described at the guide considered as a reference in the country where the analysis will take place.

The aim of this work was to develop and validate an analytical method using UV-vis spectrophotometry to quantify papain in pharmaceutical solid oral dosage forms and four different buffer solutions used as dissolution medium.

MATERIAL AND METHODS

Material

Papain analytical standard (Papain *Carica Papaya*, EMD Chemiclas Inc, San Diego, USA) was used to build analytical curves. Hydrochloric acid, sodium hydroxide, glacial acetic acid, heptahydrate sodium dibasic phosphate, all in analytical grade, were purchased from LabSynth (Labsynth Ltda, Diadema, Brazil). The excipients, microcrystalline cellulose (Microcel™ 102), sodium glycolate starch (Explosol™) were kindly donated

by Blanver (Blanver Ltda, Cotia, Brazil). Magnesium stearate (Labsynth Ltda, Diadema, Brazil) and colloidal silicon dioxide (Aerosil™ 200 Pharma, Evonik Corporation, Essen, Germany) were provided by UNIFESP.

Analytical Curves

An ultraviolet-visible (UV-vis) spectrophotometric method was developed for evaluation of papain. Analytical curves were built according to the Validation of Analytical Procedures: text and methodology Q2(R1) guideline from International Conference on Harmonization (ICH) and the National Health Surveillance Agency (ANVISA) from Brazil[20, 22].

Initially, the adequate wavelength to build the analytical curves was determined by scanning papain solutions, in four different buffers (HCl 0.01M pH 1.2, acetate buffer 0.05M pH 4.5, phosphate buffers 0.05M pH 5.5 and 6.8), in a spectrophotometer (Thermo, model Evolution 201, São Paulo, Brazil) at wavelength ranging from 200 to 400 nm, using 10.0 mm quartz cuvettes. Those buffer solutions were used as solvent in papain analytical curves.

Six levels of papain concentration ranging from 0.019 to 0.285 $mgmL^{-1}$ were prepared for sequential dilutions using automatic pipette (Thermo, mod. Finnpipette ®F1, São Paulo, Brazil). The solutions were prepared in triplicate. All dilutions of papain were analyzed at the established wavelength and the absorbances were recorded. For each analytical curve, the correspondent buffer solution was used as blank.The gathered data were used for the construction of analytical curves, which were used for determination of validation parameters, linearity, range, detection and quantification limits. Besides that, solid oral dosage forms were used to evaluate the specificity of the method in the presence or absence of sodium glycolate starch, a superdesintegrant excipient. Precision and accuracy were determined using three levels of papain concentration (low, medium and high) as well.

Linearity

Linearity for four analytical curves of papain in different buffer solutions were determined by data analysis of the absorbances values recorded from six concentrations levels (in triplicate) of papain. Therefore, statistical analysis was performed to determine correlation (r) and determination (r^2) coefficients, y-intercept, the slope of the regression line, residual sum of squares, evaluation of the significance of angular coefficient and data homoscedasticity. The data statistic evaluation was performed on software OriginPro 2017 (64-bit) (Student Version, OriginLab Corporation, Northampton USA).

Specificity

The specificity of an analytical method might be demonstrated by its capability to identify or quantify the interest substance without the interference of other substances, impurities or matrix constituents (as excipients), which can be present in a sample for analysis. In this way, the specificity evaluation is directly dependent on the formulation and indirectly dependent on processing since it might promote changes in the active compound due to destabilization.

Specificity was evaluated using excipients regularly used in tablet formulations (microcrystalline cellulose 32 %w/w, pre-gelatinized starch50 % w/w, sodium glycolate starch 3 % w/w, colloidal silicon dioxide 3 % w/w and magnesium stearate 2 %w/w). Special attention was given to sodium glycolate starch, since in aqueous medium it acquires a negative charge, which might be incompatible with papain a positively charged enzyme. Therefore, fourtablet formulations (F1 – 0% w/w PPN and F2 – 10 % w/w PPN, both containing sodium glycolate starch; F3 – 0% w/w PPN and F4 – 10 % w/w PPN, both without sodium glycolate starch)have been tested.

For the test, all excipients were separately weighted on an analytical scale and put in a glass mortar. The compounds were homogenized and samples of 90 mg(F1 and F3) and 100 mg (F2 and F4) were taken off for analysis. The samples were previously dispersed in 40 mL of one of the four different buffer solutions and kept by 30 minutes on ultrasound bath for extraction/dissolution of papain. The volume was completed to 50 mL in a volumetric flask. Afterward, the samples were divided into two halves, where one half was filtered through a polycarbonate membrane (0.22 µm pore size) whereas the other half was centrifuged at

5000 rpm for 10 minutes. The supernatant was taken and divided into two halves, where one half was filtered through polycarbonate membrane.

All final solutions were analyzed by spectrophotometry at the same test conditions applied for papain standard solution. The values of absorbance for quantification of papain at specific wavelength were recorded for all samples.The percent ratio of absorbances between samples with and withoutpapain were calculated. The method was considered complying when the presence of excipients did not cause variation in papainabsorbance higher than 5 %.

Precision

Precision expresses the closeness of agreement between the results obtained through analysis using samples prepared in the way described in the analytical method to be validated. Thus, complying the range of concentration in analytical curves, papain solutions in four different buffer solutions were prepared in three different levels, low, medium and high (0.038, 0.130 and 0.230 mg mL^{-1}, respectively). These solutions were analyzed as described previously for analytical curves. To express the precision of the method the parameters mean, standard deviation, and relative standard deviation of the papain solutions were calculated. All tests were conducted in triplicate.

Accuracy

Accuracy assesses the closeness of experimental data of analyte concentration to the true concentration of the analyte in a solution of known concentration. Accuracy was run after established specificity and linearity. For this purpose, the same data collected for the precision test was used since the parameters for accuracy are the same, differing only in the data analysis. While the precision is focused on data dispersion, in the accuracy attention is given to closeness to the true value. Thus, the mean values of absorbance collected for samples of low, mean and high concentrations of papain for different buffer solutions were applied on correspondent analytical curves for determination of papain concentration. The obtained values were related to the true values as shown in Equation 1. The recovery value (R%) must be equal or higher than 95 % of papain concentration and not higher than 105 %, according to ICH[20].

$$R(\%) = \frac{\text{Mean papain concentration from experimental data}}{\text{True value of papain concentration}} \times 100 \qquad \text{Equation 1}$$

Detection and Quantification Limits

Detection and quantification limits were calculated for four different buffer solutions based on the standard deviation of the y-intercept values and respective slopes from three equations of analytical curves. Equations2 and 3 were used for determining detection and quantification limits.

$$DL = \frac{3.3\sigma}{S} \qquad \text{Equation 2} \qquad\qquad QL = \frac{10\sigma}{S} \qquad \text{Equation 3}$$

Where:
DL is the detection limit
QL is the quantification limit
σ is the standard deviation of the y-intercept from threeanalytical curves
S is the mean of the slope from three analytical curves

RESULTS AND DISCUSSION

Validation is an important step on the development of analytical method necessary to certify that the measurements done with a specific technique can provide reliable data, with adequate accuracy and precision for a given purpose [23].

Papain is a proteolytic enzyme and is rising the attention of researchers and pharmaceutical companies that seek to apply this type of molecule as an active compound in medicines. Despite that interest, there are not analytical methodologies to quantify papain in a fast, not expensive and simple way. Instead, the methods available are based on enzymatic activity, normally involving complex, time-prolonged and expensive

ISSN: 0975-8585

procedures or through the interaction of antibodies and the enzymes to immunoassays methods[24,25, 26].In this sense, it was proposed a simple method, based on UV spectrophotometric absorption of papain for quantification in solid oral dosage forms of medicines formulations. This type of assay allows to quantify the enzyme concentration during a manufacturing process, like mixture step, or quality control concerning content uniformity and dissolution test. Despite that, it must be demonstrated that papain maintains its biological activity into formulations of solid dosage forms, what is related with the capability of interacting with specific substrates for papain, as α-benzoyl-DL-arginine p-nitroanilide hydrochloride or casein, generating the metabolites [24, 27].

Initially, papain solutions were prepared using four different buffer solutions and the absorption spectra were recorded to identify the appropriate wavelength for papain analysis. It was verified that papain does not present a well-defined wavelength of absorption (a characteristic peak with good response of absorbance *vs* concentration). Instead, papain presented a peak absorption near to 278 nm, notwithstanding with a low absorptivity (ε_{278}=17086.5±2312 $Lmol^{-1}cm^{-1}$; RSD = 13.5%). For instance, it was selected the wavelength of 230 nm to run the analysis since the absorptivity for this wavelength was ε_{230}=62129±2341 L mol^{-1} cm^{-1} (RSD = 3.8%), about 3.6 folds higher than absorptivity at 278 nm. Papain spectra are shown in Figure 1.

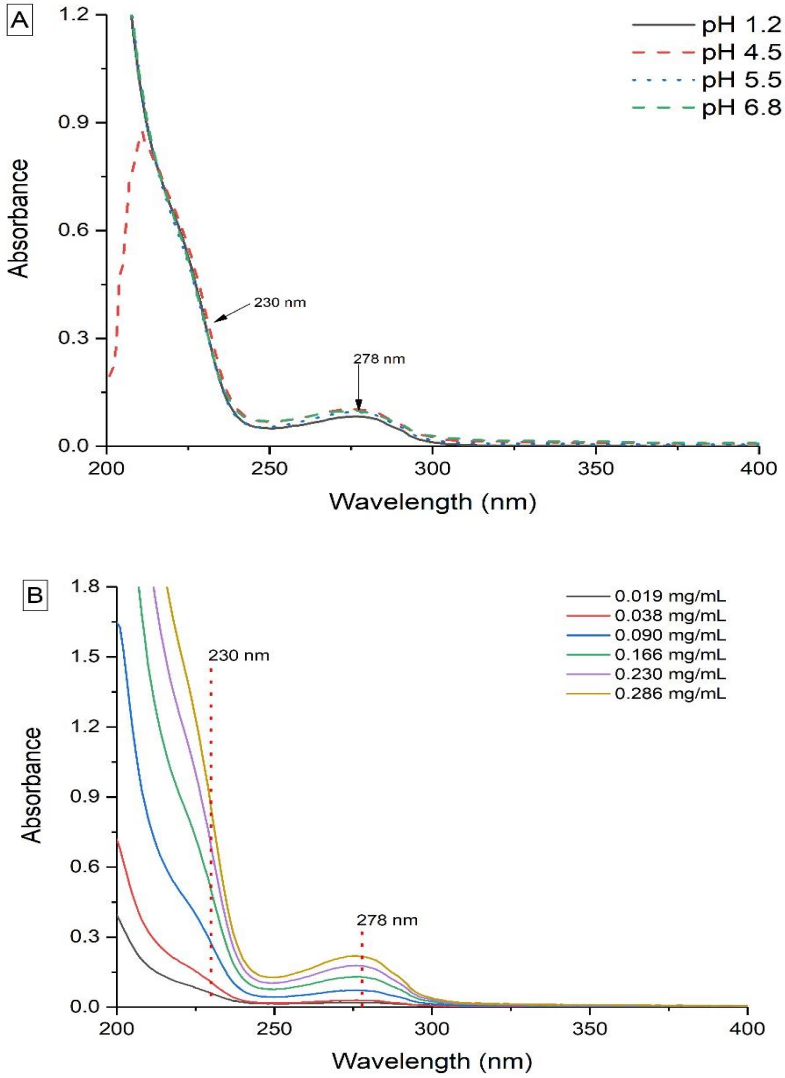

Figure 1: Absorbance spectra of papain solutions at different buffer solutions for UV-vis range spectrum from 200 to 400 nm. A- The arrows point the wavelength where papain presented a defined absorption peak (278 nm) and wavelength chosen for quantifying papain (230 nm); B- The plot shows the increase of absorbance response due to rising the papain concentration in pH 5.5 buffer solution.

ISSN: 0975-8585

Specificity

On spectrophotometry, once the analysis wavelength was established, it is crucial to verify if the chosen wavelength allows quantifying the analyte without the interference of other components, present accidentally or not in the medium of analysis.

The specificity parameter must be determined during the method validation since it is essential to demonstrate that the analytical method does not suffer interference of impurities, excipients or matrix of analysis, such as the dissolution medium [20, 28]. Drug products regularly are complex mixtures where several components are used with specific roles in a dosage form. Diluents, lubricants, disintegrants, and binders are some classes of excipients used in tablet formulation[29]. Specifically, for minitablets obtained by direct compression, the formulation must show excellent flow and compaction properties, that regularly implies low drug loading[29, 30]. In this context, tablet formulations containing 10 % w/w of papain and diluents such as microcrystalline cellulose and pre-gelatinized starch, sodium glycolate starch as disintegrant compound, and colloidal silicon dioxide and magnesium stearate as lubricants, were obtained. Lubricants were used in proportion higher than the usual, about 5 % w/w. This was done to obtain formulations with adequate flow properties for minitablets manufacturing. Besides, two formulations (F3 and F4) were prepared without sodium glycolate starch to evaluate a probable incompatibility of papain (positive charge) and the superdesintegrant (negative charge).

For specificity test, about 100 mg of tablet formulations containing 10 mg papain or 90 mg of formulation without papain were used as samples, dispersed in buffer solutions and submitted to three different treatments: filtration through a membrane with 0.22 µm pore size or centrifuged at 5000 rpm for 10 minutes or centrifuged and filtered in the same conditions. Such treatments were applied due to the presence of small and insoluble particles in suspension into analysis solution. These particles came from insoluble materials present in the formulation, especially colloidal silicon dioxide and magnesium stearate. Despite these compounds do not absorb at 230 nm, the particles in suspension are able to disperse UV light. In this sense, it is essential to remove those particles from the sample to avoid interference in the quantification of the analyte. The results of specificity test are shown in Table 1.

Table 1: Data of absorbance at 230 nm for specificity test using different treatments to eliminate insoluble particles from the analysis samples addressing the quantification of papain by spectrophotometry.

Sample treatment	Absorbance 230 nm		Matrix Influence |%|	Absorbance 230 nm		Matrix Influence (%)
	F1	F2		F3	F4	
HCl 0.01M pH 1.2						
Filtration	0.0182	0.4544	4.00	-0.0082	0.4679	1.75
Centrifugation	0.0370	0.5687	6.51	-0.0050	0.5406	0.92
Centrifugation and Filtration	0.0172	0.5108	3.37	-0.0053	0.5083	1.04
Acetate buffer 0.05M pH 4.5						
Filtration	0.0183	0.3315	5.52	0.0326	0.5036	6.48
Centrifugation	0.0290	0.4143	7.01	0.0297	0.5726	5.18
Centrifugation and Filtration	0.0062	0.3865	1.61	0.0315	0.5158	6.11
Phosphate buffer 0.05M pH 5.5						
Filtration	-0.0077	0.3451	2.24	-0.0155	0.4235	3.67
Centrifugation	0.0276	0.3611	7.65	0.0698	0.5477	12.75
Centrifugation and Filtration	-0.0270	0.3348	8.05	-0.01827	0.4383	4.17
Phosphate buffer 0.05M pH 6.8						
Filtration	-0.0064	0.2806	2.28	-0.0110	0.4019	2.73
Centrifugation	0.0311	0.3779	8.24	0.0634	0.5155	12.30
Centrifugation and Filtration	-0.0202	0.3344	6.04	-0.0195	0.4259	4.59

For all buffers studied, it was possible to observe that centrifugation process was the worst to eliminate the influence of the matrix in simulated formulations containing papain. Furthermore, the simulated formulations containing the super disintegrant sodium glycolate starch showed greater matrix influence in papain analysis. This fact indicates a possible interaction between the papain and that excipient, which might be attributed to opposite charges of each other. Once papain is a positively charged protein and the super disintegrant is negatively charged, the interaction can take place when, in presence of water, some part of the compounds begins to dissolve and dissociate leading to the attraction of the counter-ions. This interaction exert influence in papain dissolution from solid dosage forms since the associated counter-ions could reduce or even inactivate the enzyme. In this way, it is suggested that poly-anions compounds, like sodium glycolate starch, must be avoided in formulations containing papain.

On the other hand, simulated formulations without sodium glycolate starch showed adequate specificity for both sample treatment, filtrationand centrifugation plus filtration, since matrix influence values were lower than 5%. An exception must be done for acetate buffer with matrix influence values of 6.48 and 6.11 %, respectively to filtration and centrifugation plus filtration.

As early mentioned, specificity parameter is dependent on the matrix which the analyte is inserted. In this work, the approach for specificity test was to verify if excipients regularly used for compounding solid oral dosage forms, especially tablets, would be able to interfere in papain analysis when using the spectrophotometric method at 230 nm. This wavelength is so close to the beginning of UV range, where several organic compounds might absorb UV light. Besides, some excipients with reduced particle size, named lubricants and glidants, are used for tablets compounding. Despite the insolubility of these compounds in an aqueous medium and the incapability to absorb at 230 nm, they can stay in suspension in analysis medium leading to the light spreading phenomenon during the measurement, resulting in a false data for sample absorption. Thus, as demonstrated in this work, it is essential to remove those particles from the samples before analysis.

Linearity

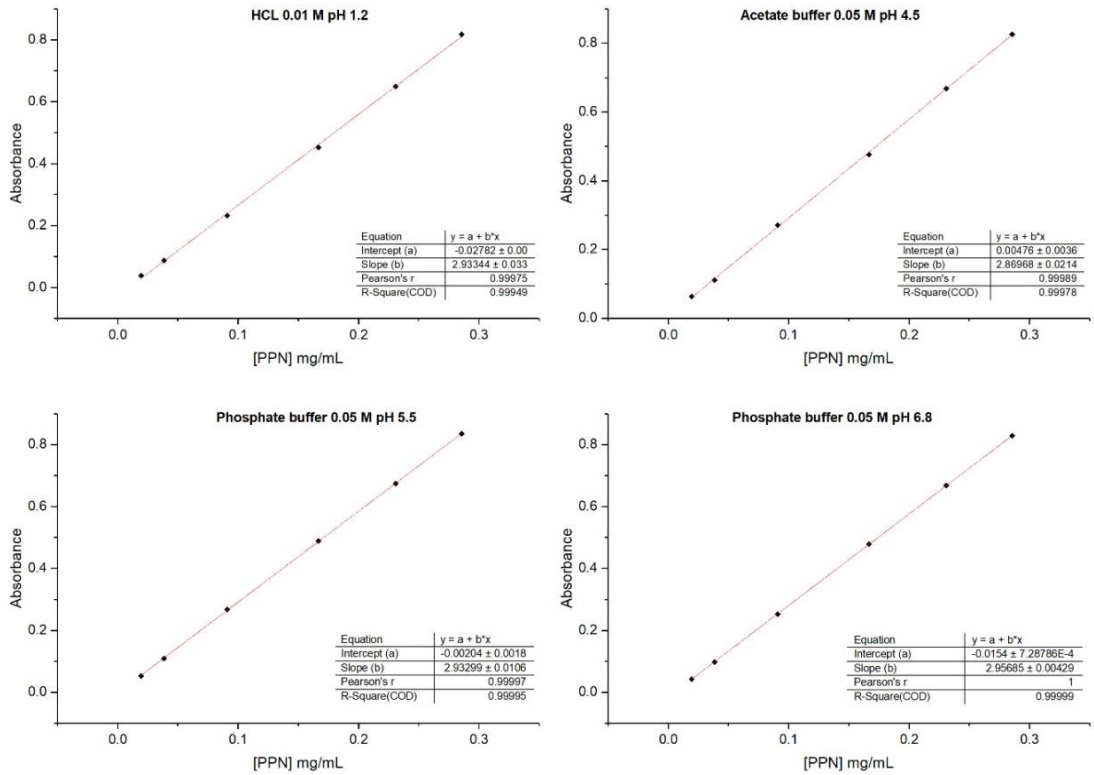

Figure 2: Analytical curves of papain (range from 0.019 mg/mL to 0.285 mg/mL) obtained for four buffer solutions in different pHs values.

The linear relationship between a range of analyte concentrations and the measured responses must be assessed addressing to establish mathematically how much the independent variable (concentration) might interfere in the dependent variable (absorbance)[20, 22]. In this study, for all buffer solutions tested the method showed adequate linearity since for all analytical curves the correlation coefficient was higher than 0.99 (Figure 2). This parameter is important to reveal a proportionality relationship between papain concentration and the absorbance at 230 nm.

Despite adequate linearity obtained from analytical curves, for all buffer solutions it was observed that the point of lower concentration (0.019 mg/mL) showed relative standard deviation above the acceptable value (RSD > 5%), addressing that for papain diluted samples there was greater variation of absorbance on UV at 230 nm (RSD =6.22 %at pH 4.5 and RSD = 9.83 %at pH 5.5). In this sense, considering the response variation and ICH guideline [20] which recommends using at least five different levels of concentration to build analytical curves, new curves were built using only the five greater concentrations of papain. The linear regression equation, linearity data, and relative standard deviation are presented in Table 2.

Table 2: Data of analytical curves from equations of linear regression (y = bx + a) obtained for different buffers using five or six levels of papain concentration (n=3).

Buffers	Levels of [PPN]	Equation terms (y=bx+a)		R^2	DL*	QL*
		b = slope	a = y-intercept			
HCl 0.01 M pH 1.2	6	2.9334±0.0331	-0.0278±0.005	0.9994	0.0059	0.0199
	5	2.9624±0.0718	-0.0341±0.008	0.9996	0.0086	0.0288
Acetate Buffer 0.05 M pH 4.5	6	2.8696±0.021	0.0047±0.003	0.9997	0.0034	0.0114
	5	2.8787±0.0424	0.0028±0.008	0.9997	0.0087	0.0292
Phosphate buffer 0.05 M pH 5.5	6	2.9329±0.01	-0.0020±0.00	0.9999	0.0008	0.0027
	5	2.9254±0.0359	-0.0004±0.003	0.9999	0.0037	0.0125
Phosphate buffer 0.05 M pH 6.8	6	2.9568±0.0042	-0.0154±0.0007	1.0000	0.0021	0.0072
	5	2.9582±0.0209	-0.0157±0.0019	1.0000	0.0019	0.0065

* DL is the detection limit and QL is the quantification limit

It was obtained three analytical curves for each buffer solution using five and six levels of papain concentration. The terms “a” and “b” of each curve were compared using Student’s t Test to evaluate if there were differences due to avoiding the lower concentration level in the curves with five points. There were no significant differences between terms “a” and “b” for HCl 0.01 M (p = 0,3080; p = 0.1759), acetate buffer 0.05 M pH 4.5 (p = 0.3958; p = 0.3839), phosphate buffer 0.05 M pH 5.5 (p = 0.3813; p = 0.2576) and phosphate buffer 0.05 M pH 6.8 (p = 0.4686; p = 0.4257), respectively. Therefore, the analytical curves built with six concentration levels were considered acceptable.

Detection and quantification limits

Detection and Quantification limits were calculated using the standard deviation of y-intercept from analytical curves and the mean of the slopes for the same curves (Table 3)[20]. For all data, the detection limit was under the lower level of concentration used to build the analytical curves. In fact, detection limits were close to zero, revealing the reliability of the method to detect tiny papain amounts.

On the other hand, the quantification limits were very closeto the lower level of concentration in HCl and acetate buffers. It suggests that the minor level of concentration (0.019 mg/mL) of the analytical curves should be avoided. For those buffers, with the aim of executing a secure analysis it is recommended to use concentration from 0.038 mg/mL or over, as suggested in Range parameter as follow. For phosphate

buffers (pH 5.5 and 6.8), the quantification limits were lower than the more diluted level of concentration in the analytical curves, suggesting that the inferior limit in the curves might be considered in the analysis range.

Range

According to ICH, the range is normally derived from linearity studies and it is dependent on the application desired for the procedure [20]. In this study the method was developed to reach two specific aims, quantify papain in different buffer solutions simulating the dissolution media at different pHs, and quantify papain in solid oral dosage forms as minitablets. Considering these two matrixes for analysis, the analytical curve must cover, preferentially, all range of absorbances which provide a secure analysis according to Lambert-Beer law[31].

If on one hand, in dissolution test it is necessary to be able to measure small amounts of drug dissolved in the dissolution medium, on the other hand, assay of drug products and content uniformity claim a range between 70 to 130 % of the labeled drug. For upper limit, a simple dilution procedure gets putting the papain concentration on the range of analytical curves. Instead, for dissolution test, in case of low release for the first collect time points, the values of concentration can be below 0.019 $mgmL^{-1}$, which can lead to a coefficient of variation higher than 5% as demonstrated in the linearity test, then inadequate to analysis with the purposed method. However, adding in the dissolution medium at zero time a known amount of papain, previously dissolved, might lead the taken samples for the range of linearity of the analytical curve. Therefore, the range for the analytical method was considered from 0.038 to 0.285 $mgmL^{-1}$.

Accuracy

Samples used for the precision test were also used for determining the accuracy of the method. The samples used were of low, medium and high concentration (0.038, 0.130 and 0.231 $mgmL^{-1}$, respectively). In this test, the scope was to determine how close the experimental concentration (found value) is to the true concentration (reference value). The ratio between experimental and true concentrations is named recovery (R%). Considering 3 levels of concentrations, we used it in triplicate and, it was possible to determining nine recovery values. Papain recovery in buffer solutions is shown in Table 3.

All results of accuracy were satisfactory since recovery ranged between 98 and 103%. One-way analysis of variance was run to evaluate the existence of differences between the recovery values for each concentration level. For all analysis p-value was greater than 0.05, indicating the absence of difference between recovery values.

Precision

Table 3: Accuracy in terms of recovery (R%) and repeatability data for the precision test of the analytical method for papain quantification. RSD (%) = relative standard deviation.

Theoretical [PPN] mg mL^{-1}	Experimental [PPN] mg mL^{-1} Mean (n=3)	Standard deviation	RSD (%)	Recovery (%)
HCl 0.01M pH 1.2				
0.0384	0.0395	0.002	5.7	102.8
0.1304	0.1282	0.002	2.0	98.3
0.2307	0.2318	0.000	0.1	100.4
Acetate buffer 0.05M pH 4.5				
0.0384	0.0385	0.005	13.2	100.2
0.1304	0.1302	0.000	0.7	99.8
0.2307	0.2308	0.001	0.7	100.03
Phosphate buffer 0.05M pH 5.5				
0.0384	0.0378	0.000	2.0	98.3
0.1304	0.1316	0.001	1.2	100.9
0.2307	0.2301	0.002	1.2	99.7
Phosphate buffer 0.05M pH 6.8				

0.0384	0.0377	0.000	2.5	98
0.1304	0.1317	0.000	0.5	101
0.2307	0.2300	0.001	0.7	99.7

The precision of an analytical method is usually expressed as the variance, standard deviation or relative standard deviation of a series of measurements. In this work precision was evaluated in terms of repeatability. Data variability and relative standard deviation were determined for three levels of concentration, low, medium and high as stated in accuracy section. The concentrations used were in the range of analysis. Table 3 summarizes the data for repeatability for four different buffer solutions.

The repeatability data were completely satisfactory for phosphate buffer at pH 5.5 and 6.8 since the RSD was lower than 5.0 % for all analysis. On the other hand, the results for the lower concentration of papain for HCl and acetate buffers were unsatisfactory since the RSD were 5.7 and 13.2. Despite that, medium and high concentrations of papain in those buffers provided an excellent RSD lower than 2.5. Furthermore, the correlation coefficient was determined for all analysis in different buffer solutions, which were higher than 0.999, confirming the excellent correlation between independent and dependent variables. The low stability of the papain in acid mediumcould explain the higher response variability for the enzyme when in lower concentration.

CONCLUSION

In this work it was proposed developing and validating a spectrophotometric method for papain determination in solid oral dosage forms and four different buffer solutions used in dissolution tests. Papain showed just one peak of absorbance, however with low molar absorptivity. Thus, the wavelength of 230 nm was used due to the good response sensitivity when the concentration was changed. At this wavelength, the method was challenged against formulations of tablets to evaluate its specificity. Filtration with membranes of 0.22 µm pore size was essential to remove tiny particles providing clear solutions for analysis. Also, it was verified a possible interaction between papain and poly-anion excipient as sodium glycolate starch, suggesting that such excipients should be avoided. About linearity parameter, for the four buffer solutions tested the method showed linearity higher than 0.99, from 0.019 to 0.286 mg/mL of papain. However, the range recommended for analysis was from 0.038 to 0.286 since RSD for the minor concentration was greater than 5%. The method developed might be considered adequate for accuracy and precision. While the accuracy parameter ranging 98 to103 % for all buffer solutions tested, the RSD values were lower than 5% for most of buffer solutions and concentrations, exception for HCl and acetate buffer for the lower papain concentration. In summary, the developed method has shown adequate performance for use in content determination and dissolution tests applicable for solid dosage forms.

ACKNOWLEDGMENTS

The authors are thankful for financial support from Foundation of Research Support of São Paulo State, Brazil (FAPESP, processes n° 2013/04513-2 and n° 2016/22916-5); and for Blanver Ltda – Cotia, Brazil for donating the excipients used in this work.

INTEREST CONFLICT

There are none interest conflict involving this work and its publication.

REFERENCES

[1] Pinto CASO, Lopes PS, Sarruf FD, Polakiewicz B, Kaneko TM, Baby AR, Velasco, MVR.Brazilian Journal of Pharmaceutical Sciences 2011; 47:751-60.
[2] Cordara G, Eerde AV, Grahn EM, Winter HC, Goldstein IJ, Krengel U. PLoS One 2016; 11:1-19.
[3] Manohar CM, Prabhawath V, Sivakumar PM, Doble M. PLoS One 2015; 10:1-17.
[4] Verma S, Dixit R, Pandey, KC. Front Pharmacol 2016; 7:1-12.
[5] Qadeer A, Zaman M, Khan RH. Biochemistry (Moscow) 2014; 79:785-96.
[6] Sasmito T, Demeester J, Bracke M. Pharm. Tidschr. Belg 1982; 59:149-58.
[7] Sumner IG, Harris GW, Taylor MAJ, Pickersgill RW, Owen AJ, Goodenough W. European Journal Biochemistry 1993; 214:129-34.

[8] Choudhury D, Biswas S, Roy S, Dattagupta JK. Protein Eng Des Sel 2010; 23:457-67.
[9] Sim YC, Nam YS, Shin YH, Shin E, Kim S, Chang IS, Rhee JS. Pharmazie 2003; 58:252-6.
[10] Grabovac V, Schmitz T, Föger F, Bernkop-Schnürch A. Pharm Res 2007; 24:1001-6.
[11] Efiana NA, Phan TNQ, Wicaksono AJ, Bernkop-Schnürch A. Colloids Surf B Biointerfaces 2018; 161:228-35.
[12] Menzel C, Bernkop-Schnürch A. Adv Drug Deliv Rev 2018; 124:164-74.
[13] Kristó K, Bajdik J, Kleinebudde P, Pintye-Hódi K. Pharm Dev Technol 2010; 15:354-9.
[14] Varca GHC, Lopes PS, Ferraz HG. Drug Dev Ind Pharm 2014; 41:430-35.
[15] Sharma M, Sharma V, Panda AK, Majundar DK. The Pharmaceutical Society of Japan 2011; 131:697-709.
[16] Chaudhary A, Singh A, Verma PV. Org Med Chem Lett 2014; 4:1-6.
[17] Ribeiro FAL, Ferreira MMC, Morano SC, Silva LR, Schneider RP. Quim Nova 2008; 31:164-71. [In Portuguese, English abstract]
[18] Fonseca LB, Labastie M, Sousa VP, Volpato NM. AAPS Pharmscitech 2009; 10:1145-52.
[19] Alves LDS, Rolim LA, Fontes DAF, Rolim-Neto PJ, Soares MFR, Sobrinho JLS. Quim Nova 2010, 33:1967-72. [In Portuguese, English abstract]
[20] ICH, International Conference on Harmonization. Validation of Analytical Procedures: Text and Methodology Q2(R1). 2005.
[21] Silva JTP, Silva AC, Geiss JMT, Araújo PHH, Becker D, Bracht L, Leimann FV, Bona E, Guerra GP, Gonçalves OH. Food Chem 2017; 230:336-42.
[22] BRASIL, Agência Nacional de Vigilância Sanitária (ANVISA). R. E, nº 899 de 29 de maio de 2003 – Guia para validação de métodos qualitativos e bioanalíticos. Available at http://e-legis.anvisa.gov.br/leisref/public/showAct.php?mode=PRINT_VERSION&id=15132. Accessed March 13, 2018.
[23] Parr MK, Schmidt AH. J Pharm Biomed Anal 2018; 147:506-17.
[24] Llerena-Suster CR, José C, Collins SE, Briand LE, Morcelle SR. Process Biochem 2012; 47:47–56.
[25] Bayramoglu G, Senkal BF, Yilmaz M, Arica MY. Bioresour Technol 2011; 102:9833–7.
[26] Ganapathy R, Manolache S, Sarmadi M, Denes F. J. Biomater. Sci. PolymerEdn 2001; 12:1027–49.
[27] Erlanger BF, Kokowsky N, Cohen W. Arch. Biochem. Biophy 1961; 95:271-8.
[28] Belouafa S, Habti F, Benhar S, Belafkih B, Tayane S, Hamdouch S, Bennamara A, Abourriche A. Int J Metrol Qual Eng 2017; 8:1-10.
[29] Conceição J, Estanqueiro M, Amaral MH, Silva JP, Lobo JMS. American Journal of Medical Sciences and Medicine 2014; 2:71-76.
[30] Mitra B, Chang J, Wu SJ, Wolfe CN, Ternik RL, Gunter TZ, Victor MC. Int J Pharm 2017; 525:149-59.
[31] Maikala RV. Int J Ind Ergon 2010; 40:125–34.