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Research Article

VALIDATION OF IN VITRO ANALYTICAL METHOD TO MEASURE PAPAIN ACTIVITY IN PHARMACEUTICAL FORMULATIONS

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ABSTRACT

Objectives: The purpose of this paper was to validate an alternative method for the determination of papain activity in pharmaceutical formulations using a low molecular weight substrate called *N*-Benzoyl-DL-arginine-ρ-nitroanilide.

Methods: The assessment of enzymatic activity is based on the amount of ρ-nitroaniline released over time formed during the hydrolysis of this substrate caused the active papain, estimated colorimetrically at 405 nm at pH 6.0 and 40°C. The method was validated according to the United States Pharmacopeia guidelines. Parameters such as accuracy, precision, linearity, limit of detection and limit of quantification were estimated. Prototype - solid, semi-solid and liquid - formulations were prepared and assayed using this technique.

Results: The results revealed linearity established as R= 0.9978, precision values of 1.68% and accuracy of 94.15%. The method also holds a limit of detection and limit of quantification of 123.98 USP.mL^{-1} and 413.27 USP.mL^{-1} respectively. No interference was observed when the prototype formulations were assayed.

Conclusion: The results indicated that such methodology is an alternative, reliable and adequate method to determine the papain activity in pharmaceutical formulations.

Keywords: *N*-Benzoyl-DL-arginine-*ρ*-nitroanilide; Validation; Papain; Pharmaceutical formulation.

INTRODUCTION

The use of medicinal products containing enzymes has increased due to their broad therapeutic potential. Currently such products are being used in the pharmaceutical field for several applications, such as debridement agents [1] and transdermal drug penetration enhancers [2]. Papain (*Carica papaya* Linné) is a proteolytic enzyme isolated from the papaya latex, green fruit and leaves which has been extensively used in wound healing and ulcers treatment [3].

Enzymes in general such as papain require specific environments in order to maintain their bioactivity. Due to its molecular complexity this enzyme in particular may be inactivated by denaturation processes induced by high temperatures and the presence of denaturants as well as inorganic agents among other substances [4]. The assessment of the enzymatic stability is fundamental to any study, research or industrial process involving this particular group of proteins, once it provides data about the bioactivity of these molecules which also indicates the viability or interference of a particular process or reagent.

Most analytical methods for the determination of enzyme activity may be divided into enzymatic, which is the focus of this research, and immunological, according to the mechanism involved in the reaction. The enzymatic methods are based on the hydrolysis undergone by a specific substrate in contact with the enzyme which releases a product of low molecular weight formed during the reaction, proportional to the bioactive enzyme and able to be quantified using colorimetric or fluorimetric methods [5].

The current techniques available in literature related to methods to quantify papain activity are in a short number and often linked to intensive laboratory work, long periods of time, also involving high cost equipment, reagents and substrates. As examples researchers have shown the determination of papain activity using synthetic substrates, e.g. benzyloxcarbonyl-phenylalanylarginine 7-amido-4-methylcoumarin [6] and *N*-Benzoyl-DL-arginine-*ρ*-nitroanilide [5] based on the papain catalyzed conversion of the compound.

In a more specific way the kinetics of the papain catalyzed hydrolysis over *N*-Benzoyl-DL-arginine-*ρ*-nitroanilide was previously evaluated and its potential application for activity quantification assays for papain [5], as well as other trypsin- like enzymes was highlighted [7]. This particular compound is a low molecular weight substrate that releases *ρ*-nitroaniline as product after contact with active papain.

This substrate is of low cost and the product formation may be followed by UV-Vis techniques [5]. However, validation studies must be performed in order to assure the ability of any analytical method to generate reliable and interpretable information, as well as confirm whether the features implemented in the method comply with the required standards for the analytic applications or not [8], through consistently documented evidence established by laboratory studies [9-10].

On this account the validation of an alternative technique to quantify papain proteolytic activity - using the Benzoyl-DL-arginine-ρ-nitroanilide as a substrate - in solid, semi-solid and liquid prototype formulations was performed in order to assure the applicability of the method as well as assure its compliance with technical specifications.

MATERIALS AND METHODS

Materials

Papain water-soluble (30000 USP-U/mg) for biochemistry and L-cysteine hydrochloride were purchased from Merck®; *N*-benzoyl-DL-arginine-*ρ*-nitroanilide (BAPA), sodium metabisulfite and imidazolidinyl urea were from Sigma-Aldrich®; acetic acid, ethylenediamine tetraacetic acid (EDTA), propylene glycol, disodium phosphate and dimethyl sulfoxide (DMSO) were acquired from Labsynth®.

Reverse osmosis water was used in all experiments. Polyvinylpyrrolidone K 30 (Kollidon® 30) from BASF®, microcrystalline cellulose (Microcel® MC-101) from Blanver Farmoquímica LTDA, hydroxyethylcellulose (Natrosol™) from Ashland Aqualon. All chemicals were of analytical grade.

Methods

Prototype formulation evaluation

Papain containing prototype formulations were prepared in different pharmaceutical forms according to the compositions described in **Table 1** and submitted to the assay in order to assure the reliability and applicability of the method on such pharmaceutical forms.

Table 1: Prototype formulations for the determination of papain activity

Composition	Liquid Formulation (%)	Semi-solid Formulation (%)	Solid Formulation (%)
Papain	0.2	0.2	0.2
L-cysteine	0.16	0.16	0.16
Disodium phosphate	-	6.8	6.8
Disodium metabisulphite	-	-	0.1
EDTA	-	1.3	1.3
Aristoflex®AVC	-	4.0	-
Polyethylene glycol	-	5.0	5.0
Microcrystalline cellulose	-	-	30.0

Solid formulation

Papain containing pellets were developed by extrusion-spheronization process using the described ingredients. A 10% PVP K30 aqueous solution was used as an agglutinant agent. The extrusion process was performed in a CALEVA® extrusion apparatus model using 1 mm sieves and 16 rpm. The spheronization process was performed in a 250 CALEVA® spheronizer using 1000rpm for approximately four minutes. The samples were then dried in a fluidized bed HÜTTLIN® MICROLAB at 33 ºC and speed of $12m^3.hour^{-1}$ (air entrance) until proper drying of the material was achieved. A physical mixture of the components was performed. The phosphate and L-cysteine were solubilized prior to their addition to the mixture. Polyethylene glycol was added to the mixture and then the agglutinant agent.

Semi-solid formulation

Papain 0.8% phosphate buffer solution (pH = 6) containing L-cysteine was gelified using 2% (w/w) hydroxyethylcellulose. Disodium methabisulphite (0.008%), imidazolidinyl urea (0.5%) and propylene glycol (5%) were added to the formulation.

Liquid Formulation

Papain was diluted in aqueous solution in presence of L-cysteine hydrochloride monohydrate and EDTA in order to reach a final concentration of 1200 $USP.mL^{-1}$.

Sample extraction

An established amount of the solid or semi-solid formulation was diluted in buffer to reach final theoretical biological activity of 1200 $USP.mL^{-1}$. The samples were submitted to sonication for 15 minutes, filtered twice to remove solid and insoluble compounds and then transferred to the wells for quantification.

Quantification of enzymatic activity

Standard papain solution

Papain containing solutions were prepared, in order to reach seven different papain concentrations ranging from 444.0 to 5,333.3 $USP.mL^{-1}$, using phosphate buffer (50mM) pH 6.0 in presence of L-cysteine hydrochloride monohydrate and EDTA.

N-Benzoyl-DL-arginine-p-nitroanilide Solution

N-Benzoyl-DL-arginine-ρ-nitroanilide was diluted in DMSO and aliquots of 400 µL of the substrate was diluted with phosphate buffer pH 6.0 in presence of L-cysteine hydrochloride monohydrate and EDTA in order to reach a substrate final concentration of 0.1 mmol.

Enzymatic assay

Microplate preparation

The solutions were transferred to a 96 wells microplate in the following order: substrate, papain (respective dilution) and acetic acid 30% (v/v) to cease the reaction. During the experiment preparation all solutions were kept on ice and covered with aluminum foil. The wells were filled with 50 µL acetic acid 30% (v/v), 120 µL substrate solution, 100 µL sample (papain dilution or diluted sample).

Experimental Conditions

The experiment was kept in a MARCONI® Model MA 159BB water bath at 40 ºC for 45 minutes at pH 6.0. The reaction was interrupted with acetic acid 30% v/v addition in the wells of the subsequent lines every 15 minutes for 45 minutes. The UV-Vis analyses were performed and a linear curve was constructed to determine the increase in absorbance depending on the concentration of papain over time. The enzyme activity was measured according to the ρ-nitroaniline absorbance values estimated at λ=405 nm using a LGC® model LM-LGC microplate reader.

Validation of analytical methodology

Validation was performed following the parameters of United States Pharmacopeia [8]. The analytical characteristics analyzed were linearity, precision, accuracy, limit of quantification and limit of detection.

Linearity and precision

Linearity was determined by evaluation of seven different papain concentrations in order to estimate the linear correlation coefficient. The correlation coefficient was calculated by the analytical curve or calibration curve. Precision was evaluated trough coefficient of variation calculated according to the equation bellow based on ten absorbance determinations at 1777 $USP.mL^{-1}$ papain concentration. Precision is expressed as relative standard deviation or coefficient of variation (CV%) according to **EQ 1**:

Equation 1 RSD = SD.100/ACD

RSD = relative standard deviation, SD = standard deviation, ACD = average concentration determined (n=10)

Accuracy

The accuracy can be expressed by **EQ 2**:

Equation 2 A =EMC. 100/TC

A = Accuracy, EMC = experimental mean concentration, TC = theoretical concentration

A recovery test was performed using a known enzyme concentration of 2666.6 USP.mL-1 and the amount of enzyme solution was quantified by the enzymatic assay.

Limit of detection and Limit of quantification

The limit of detection (LOD) was established by the construction of a calibration curve with papain concentrations and is expressed by **EQ 3**:

Equation 3 LOD = SDa.3/IC

SDa = standard deviation of the intercept with y axis of the calibration curve (n= 3); IC = slope of calibration curve.

The limit of quantification (LOQ) was established by analyzing decreasing concentrations of papain. Calibration curve was developed based on such values and the results were applied according to **EQ 4**:

Equation 4 LOQ = SDa.10/IC

SDa = standard deviation of the intercept with y axis of the calibration curve (n= 10); IC = slope of calibration curve.

RESULTS AND DISCUSSION

Validation of analytical methodology

The enzyme activity was measured based on the enzyme ability to cleave an amide bond in N-Benzoyl-DL-arginine-ρ-nitroanilide. The ρ-nitroaniline formed during the reaction of hydrolysis of the product substrate can be followed colorimetrically. Thus, the amount of substrate hydrolysed proportional to papain activity was calculated according to the absorbance of ρ-nitroaniline at 405 nm depending on the reaction time [11]. The analytical standard curve obtained from the reaction of the enzyme with the substrate as a function of time is shown in **Figure 1**.

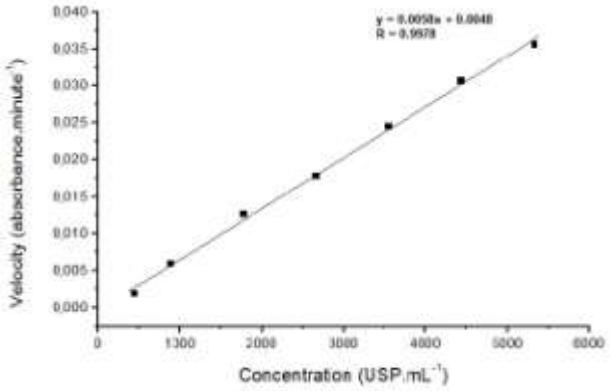

Fig. 1: Calibration curve of papain activity

According to **Figure 1** there was a proportional relation between papain concentration and the formation rate of the colorimetric compound (*ρ*-nitroaniline) as a function of time. In other words, the ratio of hydrolysis observed in the experiment was proportional to the concentration of enzyme which is essential for quantification and the purpose of this work.

The linear correlation coefficient was established as 0.9978 which is consistent with literature [12-13] and international standards [8] thus demonstrating satisfactory linearity of the analytical method. The equation for this curve is y = 0.0058x + 0.0048 where y is the amount of *ρ*-nitroaniline released and x is papain activity (USP.mL^{-1}).

Pinto et al. [6] validated the use of a synthetic substrate to determine papain activity. Although this method offers high sensibility and is potentially applicable, the substrate is indeed expensive, requires fluorimetric equipment accurate handling and performance. Alternatively *N*-Benzoyl-DL-arginine-*ρ*-nitroanilide was identified as a low molecular weight and as potential substrate for papain and other proteolytic enzymes [5] in which the product formation can be easily monitored by UV techniques as stated above.

Table 2: Precision data

Determination (Absorbance)	Papain concentration (*USP.mL^{-1}*)
0.811	1542.9
0.817	1554.33
0.796	1514.15
0.795	1512.26
0.786	1494.94
0.792	1506.49
0.811	1542.9
0.78	1483.15
0.786	1494.94
0.776	1475.85
Average	1512.19
SD (standard deviation)	25.41
RSD % (relative standard deviation)	1.68

Precision values were obtained from the analysis of ten repeated measurements for papain at defined concentration of 1777.0 USP.mL^{-1} and the results are shown in **Table 2**. According to the results, the precision value corresponded to 1.68% and was in accordance with the specifications [8], considering that it was lower than 5% (specified limit). This analytical parameter is essential to assure proper reproducibility and reliability of the assay as pointed out by other researchers [8-9,13].

The recovery test, performed to evaluate the accuracy of the technique, corresponded to 94.15% (**Table 3**) and thus indicated a highly accurate technique. Additionally such value provides an evidence of possible deviation between the estimated values and their theoretical concentrations. The limits of detection (LOD) and quantification (LOQ) estimated using the slope of the calibration curve was equal to 0.0058 revealing a LOD of 123.98 USP.mL^{-1} and a LOQ of 413.27 USP.mL^{-1}, indicating the exact reliable concentration range of the method and confirming the sensitivity of the substrate for the determination of papain enzymatic activity. All parameters combined provided experimental evidence to assure the applicability of the method in further experiments as well as defined limitations and features of the technique.

Table 3: Results of the recovery test

Theoretical conc. (*USP.mL^{-1}*)	Estimated value (*USP.mL^{-1}*)	Waste *(%)*	Accuracy *(%)*
2666.6	2508.26	5.85	94.15

*Estimated values - mean concentration recovered from 6 replicates; Theoretical concentration - added concentration.

The seek for alternative ways to quantify such proteases from distinct sources as well as other compounds of pharmaceutical interest in different pharmaceutical forms [14-17] with precision is mandatory and theme of research from distinct authors. [18-20] Specifically for enzymes, several articles report the development of such methodologies based on natural or synthetic substrates [21-22].

Although robustness of an analytical procedure is fundamental to many validation studies, according to specific literature, in the case of biological methods ruggedness is more applicable, as it can be defined as the intrinsic resistance to the influences exerted by operational and environmental variables on the results of the method [23].

In our experiments the ruggedness were obtained as a variety of normal test conditions, employing different analysts, laboratories, and reagent lots, and in this method no relevant deviations were observed between the assayed parameters highlighting suitable ruggedness of the method.

Another import parameter to be evaluated is the possible interference of the quantification method and its components and formulation excipients [24-25]. On this account as an attempt to identify incompatibilities among the compounds involved in the method and chemicals commonly applied for papain formulation, three prototype formulations **(Table 4)** were developed and assayed for their bioactivity.

Table 4: Activity determination of the prototype formulations

Formulation Type	Measured activity (USP.mL^{-1})	Relative Activity (%)
Solid	999.6	83.3
Liquid formulation	1176.0	98
Semi-solid formulation	1152.0	96
Control formulation	1200.0	100

The formulations assayed presented retained bioactivity and no problems were identified as the analyses were carried out. The results are described in **Table 4**. Macroscopically, no physical changes were observed, such as color, turbidity or precipitate formation that would impair or lead to false estimations, as the prototype formulations were assayed.

Thus the experiment was indeed effective by means of excluding possible incompatibilities among the formulation compounds and the reagents involved in the technique, assuring the applications of the technique in the assayed pharmaceutical forms.

CONCLUSION

The method validated for papain bioactivity quantification presented satisfactory accuracy, precision, linearity, limit of detection and of quantification values and was in accordance with the international standards, comprising an *in vitro* alternative, quick, reliable and effective method to quantify papain activity, which may be performed in papain containing solid, semi-solid and liquid formulations.

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ABBREVIATTIONS

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