



Determination of Papain Activity in Topical Dosage Forms: Single Laboratory Validation Assay

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SUMMARY. Papain is a proteolytic enzyme used in topical formulations for peeling and treatment of burns, wounds and scars. It is also used as progressive depilatory agent and as cutaneous absorption enhancer. This work was aimed at validating a spectrofluorimetric method to quantify the activity of papain in semisolid pharmaceutical dosage forms. Benzyloxycarbonyl-phenylalanyl-arginine 7-amido-4-methylcoumarin was used as substrate in assays performed in microplates. The production of 7-amido-4-methylcoumarin (7-MCA) over different periods of time was used to quantify the concentration of papain in pharmaceutical forms. This method presented a linear relationship between papain concentration and rate of 7-MCA production ($R = 0.9974$). Besides that, the method presented an estimated detection limit (LOD) of 0.040 USP.mL⁻¹, an estimated quantification limit (LOQ) of 0.12 USP.mL⁻¹, a precision (RSD%) ranging from 2.7 to 5.0 and an accuracy (E%) of 95.3 to 96.7. Components of the tested topical formulation did not interfere in the papain detection. Finally, the method is simple and fast.

RESUMEN. "Determinación de la Actividad de la Papaína en Formas Farmacéuticas Tópicas: de la Metodología Analítica". La papaína es una enzima proteolítica usada en formulaciones tópicas para el tratamiento de quemaduras, heridas, cicatrices, exfoliación de la piel mediante depilado, así como agente depilatorio progresivo y para incrementar la absorción cutánea. El presente trabajo tiene como finalidad validar una metodología que cuantifique la actividad de la papaína en formas farmacéuticas semisólidas. Para la validación del método fueron considerados los siguientes parámetros: linealidad, especificidad, límite de detección, cuantificación, precisión y exactitud. Benziloxycarbonil-fenilalanina-arginina 7-amido 4-metilcumarina fue usada como sustrato para el análisis de la actividad enzimática de la papaína, la cual fue determinada por detección espectrofluorométrica de la 7-amido-4-metilcumarina adherida a microplacas. El método fue validado de acuerdo con los siguientes resultados experimentales: coeficiente de correlación ($r = 0,9974$); Límite de detección (LOD = 0,040 USP.mL⁻¹), límite de cuantificación (LOQ = 0,12 USP.mL⁻¹), precisión (RSD % = 2,7 a 5,0), y exactitud (E% = 95,3 a 96,7), demostrando además especificidad para el ensayo realizado en las formulaciones tópicas, siendo este un método simple, rápido y con una adecuada sensibilidad.

INTRODUCTION

Papain is a protease obtained from the latex of leaves and fruits of *Carica papaya* Linné and its nomination is related to the dry raw latex and also to the crystallized enzyme. The papain used in topical formulations consists of an enzyme mixture, involving essentially a combination of papain and chymopapain. The papain enzyme hydrolyses specifically peptide bonds of hydrophobic amino acids at P2 position and,

preferentially, basic amino acids at P1 position. It is usually used in topical formulations for treatment of burns, wounds, scars and peeling. It has been also employed as progressive depilatory agent and absorption enhancer ¹⁻⁷.

This enzyme may be partially and even totally inactivated if stored at room temperature for one month. Therefore, a control of this parameter is important in the development of topical formulations containing papain. Moreover, the

KEY WORDS: Benzyloxycarbonyl-phenylalanyl-arginine 7-amido-4-methylcoumarin, Method validation, Papain, Spectrofluorometry.

PALABRAS CLAVE: Benziloxycarbonil-fenilalanina-arginina 7-amido 4-metilcumarina, Espectrofluorometría, Papaína, Validación de método.

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method used to measure the papain activity must be validated in order to certify the results. The necessity of specific, sensitive and quick methodology for quantification of proteolytic enzymes has prompted the progress of many analytical methods⁸. These methods can be separated in two categories: enzymatic and immunologic methods. The first depend on quantitative hydrolysis of one protein or peptide substrate. The second case is based on the interaction with an antibody. There are a lot of variety, in both categories, that depends on the detection method employed^{9,10}.

Papain enzymatic activity can be determined by measurement of the hydrolysis of natural proteins or synthetic substrates, like esters or low molecular weight amides. Such reactions may release dyes or chromogenic or fluorogenic products, which can be measured by spectrophotometers or fluorimeters¹⁰⁻¹².

Various synthetic substrates containing chromogenic or fluorogenic groups have been studied, including those containing β -naphthylamide, 7-amino-4-methylcoumarin, *p*-nitroaniline and *p*-nitrophenyl. Methods for protein quantification with fluorescent groups like fluorescein and 2-methoxy-2,4-difenilfuran-3-one were also described¹³⁻¹⁵.

The use of microplates associated to the synthetic substrate benzyloxycarbonyl-phenylalanyl-arginine 7-amido-4-methylcoumarin is an alternative to the standard spectrophotometer method for papain dosage. The hydrolysis of this substrate is catalyzed by papain releasing the fluorescent product 7-amido-4-methylcoumarin (7-MCA), which is detectable in low concentration. This method is potentially applicable as an analytical tool for papain in pharmaceutical forms, because it's higher sensibility and easy manipulation.

The aim of this research work was to validate a method that employs the synthetic substrate benzyloxycarbonyl-phenylalanyl-arginine 7-amido-4-methylcoumarin and microplates for quantification of the papain activity incorporated in different formulations for topical use (gels and emulsions)¹⁵⁻¹⁷.

Experimental

Chemicals

Acetic acid (100.0%), hydrochloric acid (37.0%), ethylenedinitrilotetraacetic acid disodium salt dehydrate (EDTA) (99.5%), dibasic sodium phosphate (99.5%), sodium hydroxide pellets (99.0%) and L-cysteine hydrochloride monohydrate were purchased from Merck (São Paulo,

SP, Brazil). The substrate benzyloxycarbonyl-phenylalanyl-arginine 7-amido-4-methylcoumarin (N-CBZ-PHE-ARG-7-MCA) was purchased from Sigma (São Paulo, SP, Brazil). Dimethylsulphoxide (99.9%) was purchased from LabSynth (São Paulo, SP, Brazil). Secondary standard papain (E.C. 3.4.22.2) 30,000 USP - U.mg⁻¹ was purchased from Merck (São Paulo, SP, Brazil). All reagents were of analytical or biochemical grade. Any further purification was performed with them. Milli QTM water was used throughout the study.

Prototype topical formulations

Topical formulations were developed as oil-in-water (o/w) emulsion and aqueous gel. The qualitative composition of the o/w emulsions (formulations A and B) involved the following raw materials of pharmaceutical grade: dibutyl adipate, decyl oleate, hydroxylated milk glycerides, EDTA, myristyl lactate, mineral oil, isopropyl palmitate, phenoxyethanol (and) methylparaben (and) ethylparaben (and) propylparaben (and) butylparaben, propyleneglycol, cyclopentasiloxane (and) PEG/PPG -18/18 dimeticone, emulsifying wax, papain, L-cysteine hydrochloride monohydrate, fragrance and distilled water. Carboxy vinyl polymer was added in formulation A and ammonium acryloyldimethyltaurate/VP copolymer in formulation B. The qualitative composition of the aqueous gel (formulation C) was carboxy vinyl polymer, propylene glycol, methylparaben, papain, L-cysteine hydrochloride monohydrate, and distilled water. All components were obtained from commercial sources and used as received, without any further purification.

Apparatus

A Packard Fluorocount™ AF 10000 and BF 10000 plate spectrofluorimeter was used with filters giving excitation and emission wavelength maxima at 360 and 460 nm, respectively. 96 wells microplate were used for enzymatic assays. A Sartorius BL 2106 analytical balance, Labsystem pipets (100 -1000 μ L), Brand pipets (10-100 μ L), Nova Ética controlled thermal bath and Milli Q Academic water purification system were also used.

Buffer and substrate solutions

Phosphate buffer pH 6.0, with L-cysteine hydrochloride monohydrate and EDTA

Dibasic sodium phosphate was accurately weighed (3.55 g) and transferred to a 500 mL volumetric flask; EDTA (7.0 g) and L-cysteine

hydrochloride monohydrate (3.05 g) were added and the contents of the flask were diluted with Milli Q water.

Substrate (N-CBZ-PHE-ARG-7-MCA) solution

An aliquot of 400 μL of N-CBZ-PHE-ARG-7-MCA previously dissolved in dimethyl sulfoxide (DMSO) (1mM) was transferred to a 25 mL volumetric flask and diluted with Phosphate buffer pH 6.0, containing L-cysteine hydrochloride monohydrate and EDTA.

Enzymatic assay

Papain activity was determined using N-CBZ-PHE-ARG-7-MCA as substrate. On 96 well microplates were added the following solutions: EDTA-cysteine buffer (55.0 μL); N-CBZ-PHE-ARG-7-MCA substrate (125.0 μL); papain (sample and/or standard) or formulations (containing or not papain) (20.0 μL); and acetic acid 30% (v/v) specifically to promote the reaction interruption (40.0 μL). During the preparing of the microplate scheme, all solutions were kept in ice bath, including the plate, and, after, the microplate was put in water bath at 40.0 ± 0.5 °C for 45 minutes and the reaction was interrupted in intervals of 15 minutes with acetic acid 30% (v/v) solution addition, initiating at time zero and finalizing at 45 minutes. Fluorescence was registered with filters giving excitation and emission wavelength at 360 and 460 nm and a curve of 4 time values was constructed for each sample aiming the determination of the increase velocity of fluorescence in function of papain concentration. The experiment was performed in replicates of three. The velocity increase of fluorescence units by minute, for each concentration, was obtained by plotting time in function of fluorescence increment^{15,17}.

Analytical curve and linearity

The linearity of the method was studied over concentrations of secondary standard papain (30000 USP.mg⁻¹) ranging from 0.45 to 3.75 USP.mL⁻¹. Three replicated were used. Optimal conditions for calibration curve were determined by the analysis of the coefficient correlation (*r*), precision (*R.S.D.*, %) and accuracy (*E*, %) ¹⁸⁻²⁰.

Specificity

An analytical method is specific when it is capable to provide truthful measurements of the compound in study in the presence of other components, impurities, degradation products

and components of the matrix^{21,22}. About 3.125 g of formulations A, B and C, without papain, were weighed and dispersed, separately in Phosphate buffer pH 6.0 with L-cysteine hydrochloride monohydrate and EDTA. These samples were used in enzymatic assays as described above.

Precision

Precision, calculated as RSD (%) (**Eq. 1**) was achieved employing ten replicates of the formulations A, B and C, which were submitted to enzymatic assay as described above. The concentration of papain detected on each of them was calculated for 100.0 mL of sample²¹⁻²³.

$$\text{Precision} = \text{RSD} (\%) = \frac{SD \times 100}{C} \quad \text{Eq. 1}$$

where *SD* is standard deviation and *C* is the mean of the calculated concentrations.

Accuracy/recovery

To achieve accuracy/recovery by **Eq. 2**, aliquots of A, B and C were diluted in phosphate buffer and a secondary standard papain (30,000 USP.mg⁻¹) was added to a known theoretical final concentration^{22,24}. The papain concentration was calculated as describe in the item *Enzymatic assay*.

$$\text{Accuracy} = E (\%) = \frac{C \times 100}{TC} \quad \text{Eq. 2}$$

where *C* is the mean of the calculated concentrations and *TC* the theoretical concentration.

Detection limit (LOD) and quantification limit (LOQ)

LOD and LOQ were estimated by the slope and mean of y-intercept of analytical curve, using the **Eq. 3** and **4**^{24,25}.

$$\text{LOD} = \frac{3.3 \sigma}{S} \quad \text{Eq. 3}$$

where *LOD* is the estimated detection limit (USP. mL⁻¹), σ is mean standard deviation, and *S* is the slope of the calibration curve.

$$\text{Quantification limit} = \text{LOQ} = \frac{10 \sigma}{S} \quad \text{Eq. 4}$$

where *LOQ* is the estimated quantification limit (USP. mL⁻¹), σ is mean standard deviation, and *S* is the slope of the analytical curve.

RESULTS AND DISCUSSION

The correlation between velocity of 7-MCA production from N- CBZ-Phe-Arg-7MCA *versus* papain concentration is linear over the range employed, with *r* equal to 0.9974 (19,20). Moreover, it was observed a direct relationship between the concentrations of the standard papain and the increment of the velocity of 7-MCA production. The equation for this relationship was $y = 6.6991x + 0.6515$, where *y* = velocity of 7-MCA (unit per minute), and *x* = concentration of secondary standard papain (USP.mL⁻¹). Data obtained to the analytical curve are reported in Table 1 and Fig. 1.

Concentration (USP.mL ⁻¹)	V	SD	RSD (%)	E (%)
0.450	4.05	0.105	2.59	112.78
0.750	5.44	0.127	2.33	95.34
1.500	10.23	0.193	1.88	95.32
3.000	21.40	0.388	1.81	103.25
3.750	25.44	0.497	1.95	98.67

Table 1. Standard curve data for enzymatic activity of papain (Merck 30000, USP.mg⁻¹). *V* is the mean of the fluorescence increment versus time of three replicates, *SD* is the standard deviation of *V*, *RSD* is precision (%) and *E* is accuracy (%).

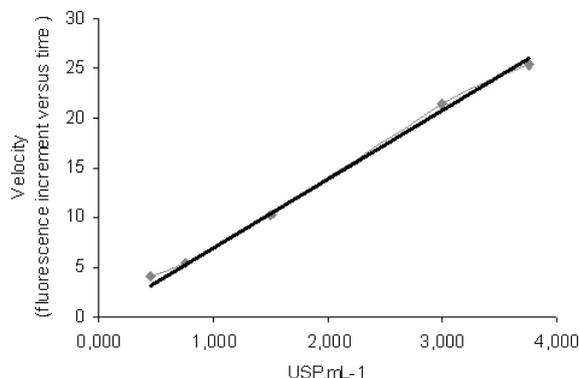


Figure 1. Standard curve data for enzymatic activity of papain (Merck 30000, USP.mg⁻¹). Velocity is the mean of the fluorescence increment versus time of three replicates.

Figure 2 shows that no relevant interference of the formulation excipients was observed. The low velocity of fluorescent compound development (unit of per minute) confirmed the specificity of this method.

Precision, calculated as RSD (%) and accuracy values (E%) are reported in Table 2. The low RSD (%), not exceeding the limit of 5.0%, confirmed the reproducibility of the spectrofluorimetric method for papain detection. Precision

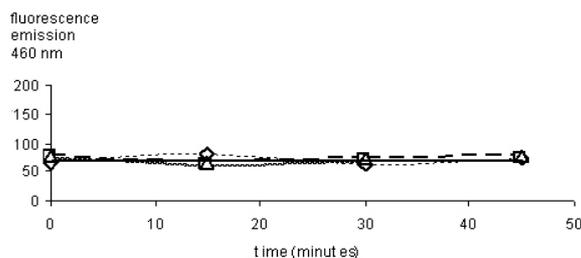


Figure 2. Production of 7-MCA in assays using formulations A (◆), B (■) and C (▲) without papain. Substrate was N- CBZ-Phe-Arg-7-MCA.

Assays	Samples		
	A	B	C
	(USP.mL ⁻¹)		
1	289.9	279.2	256.1
2	298.6	306.0	258.5
3	284.1	304.7	257.5
4	284.2	288.4	257.3
5	301.1	270.0	253.0
6	262.7	300.5	257.3
7	281.4	276.2	241.9
8	283.5	272.5	254.8
9	304.7	276.2	270.4
10	285.1	302.3	253.9
M ± SD	287 ± 12	287 ± 14	256 ± 6
R.S.D. %	4.2	5.0	2.7
E %	119,8	119,8	106,7

Table 2. Precision assays for samples A, B and C. Intra-run assay evaluated in one day. The theoretical concentration of standard papain is 240 USP.mL⁻¹. *M* is the mean concentration of ten replicates; *SD* is the standard deviation of the concentrations; *RSD* is precision (%). *E* is accuracy (%).

must not exceed 5% of the RSD (%) ²², as a result, spectrofluorimetric method provided to generate closeness of responses between the fluorometric measurements. By data obtained experimentally, the method demonstrated high degree of agreement of results ¹⁸.

The recovery is the proportion of the substance of interest already presente or spiked in known concentrations on the samples (formulations) that could be extracted and quantified precisely and accurately. Accuracy and recovery presented calculated values in close proximity to 100%. Recovery mean values were 95.3, 98.7 and 96.7% for samples A, B and C, respectively (Table 3). These accuracy values indicate that differences between the amounts of papain present on the formulations and the theoretical or nominal concentrations of samples are not significant ²².

Formulation	TC	C (USP.mL ⁻¹)	E (%)
A	1,5	1,43	95,3
B	1,5	1,48	98,7
C	1,5	1,45	96,7

Table 3. Accuracy/recovery results for samples A, B and C. *E* (%) is accuracy; *C* is the standard concentration recovered from three replicates; *TC* is the theoretical concentration added to these samples.

LOD is the minimum concentration value of the substance in analysis that is detected com adequate level of confiability; however, it is not able to be quantified with acceptable precision and accuracy, and LOQ is the minimum concentration value that can be determined precisely and accurately by the analytical method. LOD and LOQ values were 0.040 and 0.12 USP.mL⁻¹, respectively. Based on these values, the present method has sensitivity to detect the papain concentrations greater than 0.040 USP.mL⁻¹, differentiating it from the equipment noise and to quantify it with adequate precision and accuracy concentrations up to 0.12 USP.mL⁻¹.

CONCLUSION

A sensitive, precise and reliable spectrofluorimetric method was validated for assay of papain enzymatic activity present in topical formulations. Method advantages were the lack of sample extraction, rapidity, specificity, direct responses and results and equipment convenience. The experimental results have provided that analytical parameters and the single laboratory validation process were precise, accurate and sensitive according to adequate linearity, specificity, recovery, repeatability and reproducibility, LOD and LOQ.

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