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VALIDATION OF AN ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) KIT FOR THE QUANTIFICATION OF GLIADINS IN MEDICINES AND COSMETICS

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ABSTRACT

Celiac disease is caused by intolerance to gluten and the only current treatment is a gluten-free diet. Gliadins are the alcohol-soluble proteins that make up the majority of gluten and are the ones that have the most harmful effect on patients with celiac disease. In recent years the issue of the presence of gluten in medicines and cosmetics, has become relevant since it is present in many excipients that are used as lubricants or absorbents, to provide mass or volume, give shape, color and consistency. The quantification of gliadins was carried out using the ELISA technique based on the R5 antibody, with a RIDASCREEN® Gliadin kit from R-Biopharm and a microplate ELISA spectrophotometer. To validate the immunological analytical method, the specificity, intermediate precision, relative accuracy and range were measured according to the USP 41 specifications for biologic assays. Recovery percentages of gliadins in positive control samples higher than 65 % were obtained. The relative standard deviation resulting from the amount of gliadins recovered to determine the intermediate precision of the kit was 7,6 %. Then, when determining the relative accuracy, an average absolute error value of 9,5 % was obtained. The range established to accurately and precisely analyze a sample was from 5 mg/kg to 80 mg/kg. The analytical method validated in this project meets performance characteristics such as specificity, intermediate precision, relative accuracy and range, suitable for the determination of gliadin content in drugs and cosmetics.

KEYWORDS: Gliadins, gluten, ELISA, validation, medicines, cosmetics.

INTRODUCTION

Celiac disease is a systemic autoimmune disease, caused by intolerance to gluten that manifests itself as a chronic inflammation of the small intestine mucosa, causing diarrhea and weight loss. This pathology is mainly present in adults, however, cases have been diagnosed in children, adolescents and even the elderly.[1,2]

The first description of celiac disease, according to Francis Adams, was made by the physician Aretaeus of Cappadocia in the second century BC. Between the 1950s and 1970s there were notable advances and discoveries such as that of William Dicke, a Dutch pediatrician, who in 1950 made one of the most important discoveries about this disease, when he discovered the relationship between the intake of certain cereals (wheat and rye) and the manifestation of the disease. This discovery made possible the establishment of gluten-free diets for the treatment of celiac disease.[3,4] Three years later, William Dicke, together with Weijers and Van de Kamer demonstrated that the toxic action of the flour was bound to the protein fraction of the cereals, mainly the wheat, the barley, the rye and the oats, the gluten and more concretely to the gliadin.[3]

Celiac Disease has increased its prevalence in recent years and although it can be treated, this disease still has no cure.[5] As it is not a "gluten allergy", but a "gluten intolerance"; it is defined as a chronic disease,[4] for which the only effective treatment, in force until now, is a strictly maintained gluten-free diet for life.[5,6]

Gluten is a protein of low nutritional value, very used in the elaboration of bread due to its capacity to retain air in the protein matrix, making it easier for the dough to adhere better. Gliadins, rich in glutamine and proline, are the alcohol-soluble fraction of gluten and contain most of the toxic components for patients with celiac disease.[7,8]

In recent years the issue of the presence of gluten in medicines has become relevant since it is present in many excipients that are used as lubricants or absorbents, to provide mass or volume, give shape, color and consistency to tablets and capsules.[9]

To perform the determination of gluten it is necessary to have an analytical method that has high sensitivity (up to concentrations of micrograms per milliliter) and selectivity towards gluten and not other compounds with similar properties. It must also be reproducible in different laboratories and it is also desirable that it has a low cost of analysis in terms of equipment, reagents and

time. Both the analytical method and the extraction method must be validated.[10]

There are different analytical techniques used for the determination of gliadins, including: polymerase chain reaction (PCR), Western Blot, mass spectrometry (MS), immunochromatographic strips and enzyme-linked immunosorbent assay (ELISA).[10] The ELISA technique is one of the most sensitive, versatile and reproducible technologies available. It is an assay based on the immunological principle of antibody recognition and binding to antigens.[11]

There are different types of ELISA assays, the most used in gluten detection being the sandwich assays and the competitive assays. In the sandwich, two antibodies are used, the primary antibody and the secondary antibody, attached to the enzyme. In this assay, the direct bonding of gluten to the two antibodies is established, leaving the antigen "trapped" between the two.[10] This type of assay does not detect gluten in hydrolyzed and/or highly processed foods such as starches, alcoholic beverages, syrups, etc. in which the gluten, due to thermal or enzymatic treatments as part of the food manufacturing process, is fragmented into fractions incapable of being recognized by the antibodies. This fragmentation can originate small peptides that are not recognized by the antibodies and nevertheless they can contain sequences of the harmful epitopes for the celiac patients. [12]

In the competitive ELISA, the sample is incubated with the antibody and then this preparation is added to an antigen coated surface (e.g. wheat gliadins) so that the free antibody not bound to the gluten in the sample is attached to the surface. Finally, the amount of free antibody is detected; the more free antibody is detected, the less gluten the sample contains.[10] The problem is that, until now, it has not been possible to establish a clear relationship between the amount of peptide quantified and the total amount of gluten in food.[12]

There is a method that is based on the R5 antibody, which recognizes a fragment of 5 amino acids widely repeated in the gluten. This method allows the detection of gluten in wheat, rye and barley, even after heat treatment of the food. To detect oat gluten, the same group has developed a specific ELISA assay for oats. It is a very sensitive method, being therefore particularly appropriate for the analysis of gluten in the low concentration range, (20-200) mg/k. This method has obtained Type I recognition from the Codex Alimentarius Committee on Methods of Analysis and Sampling. Several commercial houses distribute gluten detection kits based on this method.[10,12,13]

MATERIALS AND METHODS

Equipment

An ELISA microplate spectrophotometer, an analytical balance, a 50 mL tube centrifuge, a 50 °C water bath, a homogenizing mill, a (100-1000) µL micropipette, a 1 to 5 mL micropipette and a multichannel micropipette were used.

Reagents and materials

Yellow tips from (50-200) µL, blue tips from (100-1000) µL, conical tubes of 50 mL, a patented cocktail and three RIDASCREEN® Gliadin kits (R-Biopharm AG, Darmstadt, Germany) from different batches (13496 and 13467) were used. Two gliadin-containing oat positive controls (batch T27168AQC) and two gliadin-free oat negative controls (batch T27168BQC), of FAPAS (Proficiency testing from Fera Science Ltd.) were used as reference standards.

The RIDASCREEN® Gliadin Kit (R7001), is a commercial enzyme sandwich immunoassay for the quantitative analysis of prolamins from wheat (gliadin), rye (secalin) and barley (hordein) in a variety of foods. With each test kit it is possible to perform 96 determinations and each kit includes all the necessary reagents for enzyme immunoassay, including the standards. It has a detection limit of 1,5 mg/kg gliadins or 3 mg/kg gluten, plus a limit of quantification of 2,5 mg/kg gliadins or 5 mg/kg gluten. A microtiter plate spectrophotometer is required for the quantification. [14]

Standards preparation

The gliadin curve standards were assembled according to the RIDASCREEN® Gliadin kit protocol to obtain concentrations of (5, 10, 20, 40 and 80) µg/mL.

Sample preparation

For sample preparation 5 g of sample were homogenized and mixed. About 0,25 g of sample was accurately weighed on an analytical balance, 2,5 mL of patented cocktail was added and shaken. It was incubated for 40 minutes at 50 °C. Following the RIDASCREEN® Gliadin kit protocol, it was extracted with a patented solution from the kit and centrifuged. The supernatant was used for the analysis.

Samples were also prepared with the addition of 0,25 g of gliadin positive control. In addition, 0,25 g of powdered milk was added, as recommended by the manufacturer of the commercial kit, and the sample preparation procedure for gliadin extraction was continued.

Procedure

The planning and execution of the gliadin determination were carried out according to the indications of the commercial RIDASCREEN® Gliadin kit manufacturer and the validation was carried out according to the USP 41, with respect to the specifications for biological analysis.

The wells were assembled with each of the samples and with the added sample in the microplate. They were incubated, the substrate was added and the time was taken for 30 minutes at room temperature in the dark.

Stop solution was added. The absorbance at 450 nm was read within 30 minutes of the addition of the stop solution.

Analytical method validation

Specificity

For this purpose, seven medicines with different active principles were analyzed, of which it was known by its formulation that they do not contain gluten. In addition, fifteen cosmetics were analyzed. The amount of gluten present in the medicines and cosmetics was analyzed, and samples of the twenty-two products were taken and a known amount of gluten was added to them. To the seven medicines, 20 mg of gluten was added; to the cosmetic gel, evanescence cosmetic and to the cosmetic cold cream 19,9 mg and to the other cosmetic products 45,22 mg.

As acceptance criteria, it is established that the method is specific if at least 65% of the added gliadin standard is recovered.

Intermediate precision

To measure this parameter the assay was performed following the analytical procedure described by the kit manufacturer in three different days using two batches of RIDASCREEN® Gliadin kit and samples with the positive control added. As an acceptance criterion, the coefficient of variation of the percentages of recovery obtained must be less than 10 %.

Relative accuracy

This parameter was measured using the data obtained in the evaluation of the intermediate precision. The mean absolute error was calculated for each level of concentration. As an acceptance criterion it was established that the value of the mean absolute error of the amount of gliadins recovered must be less or equal to 10 %.

Range

The range was determined according to the data obtained in the analysis of intermediate precision and relative accuracy of the method.

RESULTS AND DISCUSSION

Specificity

This parameter was evaluated to check that the analytical method used allows the quantification of gliadins in an unequivocal way in the presence of other substances as impurities or degradation products and other matrix components that may be present in the sample. [15]

Table 1: Data obtained for the evaluation of method specificity in medicines.

Active principle of the medicine	Gliadins recovered in samples (mg/kg)	Gliadins recovered in positive control samples (mg/kg)	Recovery percentage (%)
Deflazacort	< 5,00	13,0	65
Amlodipine/Valsartan	< 5,00	16,2	81
Atorvastatin	< 5,00	13,0	65
Montelukast	< 5,00	14,1	70
Quinapril	< 5,00	18,0	90
Metformin HCl	< 5,00	16,7	84
Mean	**< 5,00**	**15,2**	**76**

Table 2: Data obtained for the evaluation of method specificity in cosmetics.

Cosmetic	Gliadins recovered in samples (mg/kg)	Gliadins recovered in positive control samples (mg/kg)	Recovery percentage (%)
Facial cleansing gel	< 5,00	42,9	95
Facial mask	< 5,00	31,1	69
Facial cream	< 5,00	54,5	121
Drop control	< 5,00	38,8	86
Heat shield	< 5,00	31,1	69
Argan oil shampoo	< 5,00	40,7	90
Capillary treatment	< 5,00	41,1	91
Hair gel (1)	< 5,00	42,2	93
Cold cream (1)	< 5,00	54,1	120
Hair gel (2)	< 5,00	41,4	92
Cold cream (2)	< 5,00	35,5	79
Evanescence cream	< 5,00	42,4	94
Cosmetic gel	< 5,00	20,4	102
Evanescence cosmetic cream	< 5,00	26,6	133
Cold cream cosmetic	< 5,00	26,3	132
Mean	**< 5,00**	**37,9**	**98**

To evaluate the specificity, the gluten content was determined in seven medicines with different active ingredients and fifteen cosmetic products. In all products, a gluten content lower than 5 mg/kg was found.

It was observed, table 3, that the amount of gluten recovered in the positive control samples is not affected by the presence of the active principles, or matrices evaluated, since it was possible to recover at least 65 % of the standard of added gliadin, with a percentage of average recovery of 76 % in medicines and 98 % in cosmetics. With these results obtained, which meet the acceptance criteria, it is demonstrated that there is an absence in the interference of the components of the Matrix, so the method proves to be specific in the detection of gliadins in medicines and cosmetics.

Intermediate precision

This parameter is used to assess the degree of agreement between individual test results when the procedure is applied repeatedly to multiple samples. [15]

Table 3: Data obtained for the evaluation of the intermediate precision of the analysis.

Day No.	RIDASCREEN® Gliadin kit batch	Positive gliadins control content (mg/kg)	Average (mg/kg)	Standard deviation (mg/kg)	Relative standard deviation (%)
1	13496	16,8	18,3	1,4	7,6
		16,8			
		18,0			
2	13467	18,7			
		17,9			
		16,8			
3	13467	19,5			
		19,6			
		20,6			

For this parameter the gluten content of positive controls was measured on three different days, using two different batches of the RIDASCREEN® Gliadin kit. The average obtained was 18,3 mg/kg, from each data of the positive control sample and the average of them the standard deviation was calculated, which was 1,4 mg/kg, finally the percentage of relative standard deviation was obtained. As can be seen in table 3, the relative standard deviation was 7,6 %, so it meets the specification as it is less than 10 %. This allows us to determine that the method used to quantify gliadins in medicines and cosmetics is precise.

Relative accuracy

This parameter allows to assess the closeness of the average value obtained from a series of test results and the accepted reference value. [15]

Table 4: Absolute error data obtained for accuracy evaluation.

Day No.	RIDASCREEN® Gliadin kit batch	Absolute error (%)	Mean absolute error (%)
1	13496	15,6	9,5
		15,6	
		9,8	
2	13467	4,6	
		0,1	
		6,3	
3	13467	9,0	
		9,6	
		14,9	

The data obtained in the intermediate precision evaluation were used to calculate the absolute error at each concentration level. As it can be seen in table 4, the value of the mean absolute error of the amount of gluten recovered was 9,5 %, less than 10 %, so it meets the acceptance criterion and it is determined that the method to quantify gliadins in medicines and cosmetics is accurate.

Range

The range is defined as the known potencies of an analyte that has been demonstrated to be determined with intermediate precision and relative accuracy using the established method. [15]

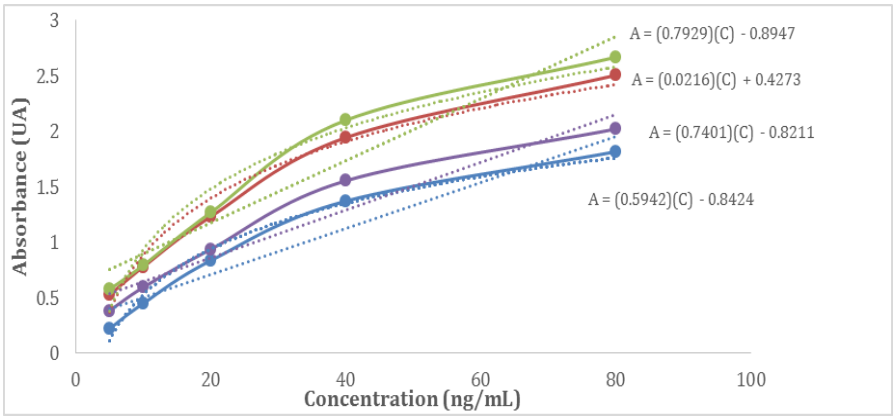

Figure 1: Relationship between absorbance and gliadin concentration.

The evaluated range was from 5 mg/kg (detection limit) up to 80 mg/kg (value up to which the method was optimized). Four calibration curves, Fig. 1, were obtained in separate experiments using two different batches.

The calibration curves for the independent analysis were made with two different batches of RIDASCREEN® Gliadin kits. As can be seen in Fig. 1, the curves remained similar in all four cases. The lowest curve (blue) showed lower absorbances than the other three curves. However, this behavior is expected as indicated in the RIDASCREEN® Gliadin certificate of analysis, which explains that the absorbance decreases during the life span of the kit. For the curves shown in Fig. 1, the remaining life of the kits are in decreasing order of absorbances.

CONCLUSION

The analytical method validated in this project meets performance characteristics such as specificity, intermediate precision, relative accuracy and range, suitable for the determination of gliadin content in drugs and cosmetics. The results obtained in the validation of the method allow us to conclude that it can be used in the quality control of medicines and cosmetics. For this reason, the Laboratorio de Análisis y Asesoría Farmacéutica (LAYAFA®), official drug quality control laboratory of Costa Rica, included the service of analysis of gliadins in medicines and cosmetics, being the only country in the region that carries out these analyses.

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