IOSR Journal Of Pharmacy
(e)-ISSN: 2250-3013, (p)-ISSN: 2319-4219
www.iosrphr.org Volume 4, Issue 10 (October 2014), PP. 63-68

Validation Of Radiation Sterilization Dose For Proteases Immobilized On Aldehyde-Containing Textile Carriers

Svetla Maksimova Dyankova[1], Maria Doncheva Doneva[1], Tsvetan Dimitrov Tsvetkov [1]
[1](*Institute of Cryobiology and Food Technologies, Sofia, Bulgaria)*

ABSTRACT: *The main objective of this work is to establish the parameters of radiation sterilization for three proteolytic enzymes (papain, trypsin and subtilisin) immobilized on aldehyde-containing textile carriers in terms of the development of new biomaterials – wound dressings with debridement effect. This paper describes the steps taken to validate a low dose sterilization process by following the Method 2A defined in ISO 11137. The content of aldehyde groups in the used modified cellulose carriers is 0.798 ± 0.002 mmol/g. After immobilization and freeze-drying of the respective enzyme, all experimental variants demonstrated proteolytic activity in the following ranges: Variant 1 (immobilized papain) - 47.64±1.73 U/g; Variant 2 (immobilized trypsin) - 84.57±1.45 U/g; Variant 3 (immobilized subtilisin) - 55.53± 3.46 U/g. The obtained results for enzyme activity made it possible to determine the dose of 30 kGy as the maximum acceptable dose for all experimental variants biomaterials. The minimal doses necessary to achieve sterilization at SAL of 10^{-6} are respectively: variant 1 (immobilized papain) - 16.0 kGy; variant 2 (immobilized trypsin) - 15.2 kGy and variant 3 (immobilized subtilisin) - 12.1 kGy. These low terminal doses provide effective bactericidal coverage and very likely have minimal impact on the properties of the biomaterials. For all three variants the proteolytic activity remains above 90% of the initial value.*

KEYWORDS: *proteolytic enzymes, biomaterials, sterility assurance level, sterilization dose*

I. INTRODUCTION

The intensive investigations of immobilized enzymes for biomedical application began in the early sixties of the 20th century [1]. Besides an increased resistance to various denaturating factors and a possibility for a repeated usage, the enzymes immobilization has a number of additional advantages important for the medical practice: more precise dosing, prolongation of the enzyme activity, increased stability during storage, decreased risk of allergic and immunological reactions. The immobilization of proteolytic enzymes on biocompatible polymer carriers creates a number of possibilities in the development of new dressing materials for wound debridement [2]. Clinical experience and existing research strongly support debridement as a necessary component of wound bed preparation when slough or eschar is present. Enzymatic debriding agents are an effective alternative for removing necrotic material from pressure ulcers, leg ulcers, and partial-thickness burns. They may be used to debride both adherent slough and eschar [3]. Various enzymes have been developed, including bacterial collagenase, plant derived papain/urea, fibrinolysin/DNAse, trypsin, streptokinase-streptodornase combination etc. [4].Traditional dressings of natural cotton fibers have still a major application in medicine as conventional linters and textile dressings that provide for a passive protection of wounds and have a number of advantages: strength, high absorption capacity, accessibility with respect to sources and price, good physiological endurance and others. In order to be used as carriers for enzymes immobilization they should undergo certain chemical and physical modifications [5].

The partial oxidation of the hydroxyl groups is one of the most widely used methods for modification of cellulose materials [6]. The alkaline salts of the periodic acid oxidize specifically the cellulose through a disruption of the glucopyranose ring at C2-C3 and forming of two aldehyde groups per monomer unit - dialdehydecellulose ([6];[7]). Activated cellulose materials, containing aldehyde groups, have been used as carriers for covalent immobilization of trypsin, papain etc. and obtaining of enzyme-containing dressing materials ([8];[9]).According to the existing normative base the wound dressings are medical devices and their development, clinical testing and production is subject to a strict control for ensuring the quality of the respective product. In the European Union the basic requirements to the medical products are subject to Directive 93/42/EEC and Directive 2007/47/EC ([10];[11]). The medical devices designated for application on open wounds or on severe burn injuries should be sterile. The selection of the appropriate sterilization method and the validation of the sterilization process are of main importance for the quality of the finished product.

Radiation sterilization is a treatment of a material to be sterilized by gamma-rays or high energy electron, either packaged or in bulk for a specific time to achieve the sterility assurance level (SAL) of 10^{-6}. Radiation sterilization dose (RSD) depends on the number of contaminant microbes (bioburden), their types and their resistance to radiation. The radiation process can be used to sterilize products in the final package [12].
Validation of RSD for health care products such as medical devices and biological products has to follow the ISO document No 11137:2006 [13].The main objective of the investigation is to establish the parameters of radiation sterilization for three proteolytic enzymes, immobilized on aldehyde-containing textile carriers, in terms of the development of new biomaterials – wound dressings with debridement effect. This paper will describe the steps taken to validate a low dose sterilization process by following the Method 2A defined in ISO 11137.

II. MATERIALS AND METHODS

Materials : Proteolytic enzymes: papain (EC 3. 4. 22. 2) from Carica papaya L., and trypsin (EC 3.4.21.4) from porcine pancreas were purchased from Merck; subtilisin (EC 3.4.21.14) from Bacillus subtilis was obtained from Biovet (Bulgaria).Activated textile carriers: gauze compresses containing pure cotton fibers preliminary oxidized with 0.1 M solution of sodium periodate.
All reagents and chemicals used were of p.a. quality (Fluka).

Analyses of the textile carriers: Content of aldehyde groups in the oxidized cellulose –according TAPPI, T430 [14].

Immobilization of proteolytic enzymes : The activated textile carriers and the respective enzyme (prepared with a buffer solution) were incubated, at 15ºC, for 24 h and, finally, washed with distilled water, until the washing solution was free of protein, which was confirmed with a spectrophotometric assay at λ280 nm (Spectrophotometer UV/VIS, SHIMADZU UV-1800). The washed textile materials with incorporated enzymes were frozen and dried through lyophilization (freeze-dryer LIO-5, 5PASCAL), after which they were packed in heat sealed three-layer aluminum foil bags.

Proteolytic activity assay : The proteolytic activity of free and immobilized enzymes was assessed by the increase of tyrosine concentration using the Anson's modified method (casein substrate, at specific temperature and pH-value for the respective enzyme).

Microbiological validation of sterilization process (according to ISO 11137, Method 2A) The ISO Standard 11137 Method 2A describes a series of five stages, which are required in order to identify an appropriate terminal sterilization dose for each product. Each stage must be completed successfully in order to advance to the next stage. The five stages and methods used to complete each are described below.Stage 1 - SAL selection and sample acquisition: The selected SAL for this study was 10^{-6}. Experimental series of the three enzymes (papain, trypsin and subtilisin) immobilized on textile carriers were divided in three equal batches of 300 pcs each. Radiation measurements were taken by multiple dosimeters placed among the samples within the container with an accuracy of ±15%.Stage 2 - incremental dose experiment: The purpose of stage 2 is to assess the level of the microbial bioburden of products. The bioburden was assessed after using the incremental dose experiment as defined in the standard. Three batches of each immobilized enzyme were sent for incremental dosing, 60 samples per dose, 20 from each batch, in 2 kGy increments. The samples were irradiated on gamma-irradiation installation "Gamma-1300", with radiation source Cs^{137} and dose rate - 1.75 Gy/min. Following sterilization, the samples were tested for sterility by complete immersion in soybean casein digest broth (National Centre of Infectious and Parasitic Diseases, Bulgaria) at 30±2°C for 14 days. A positive culture was defined as being turbid upon observation and therefore not sterile. Upon completion of the sterility test, all positive cultures were used to calculate the verification dose.

Stage 3 - verification dose experiment: The purpose of stage 3 is to demonstrate that the dose calculated in stage 2 is appropriate and effective. Additional 100 samples were irradiated with the dose calculated in stage 2 and the samples were submitted for sterility testing as previously described.Stages 4 and 5 - consideration of results and establishing the sterilization dose: The results obtained from the previous stages were used to calculate the final sterilization dose to be used in an operational setting (refer to the standard for a detailed derivation of the calculations).

III. RESULTS

Establishing the maximum acceptable dose : The content of aldehyde groups in the used modified cellulose carriers is 0.798 ± 0.002 mmol/g. After incorporating of the respective enzyme and freeze-drying, all experimental variants demonstrated proteolytic activity in the following ranges: Variant 1 (immobilized papain) - 47.64±1.73 U/g; Variant 2 (immobilized trypsin) - 84.57±1.45 U/g; Variant 3 (immobilized subtilisin) - 55.53± 3.46 U/g.

The data for the residual proteolytic activity of the initial and immobilized enzymes, before and after irradiation, are shown in Table 1.

Table 1. Residual activity of the initial and immobilized enzymes before and after irradiation with different doses

Enzyme	Preparation	Residual activity (%)				
		0 kGy	10 kGy	15 kGy	25 kGy	30 kGy
Papain	Enzyme powder	100	73.07	57.63	29.90	11.80
	Variant 1	100	97.71	93.90	90.74	91.20
Trypsin	Enzyme powder	100	74.26	71.05	69.74	68.20
	Variant 2	100	98.05	96.81	91.90	85.37
Subtilisin	Enzyme powder	100	91.97	90.80	73.31	65.20
	Variant 3	100	99.08	93.79	90.20	89.14

The results show that gamma-rays irradiation with doses up to 30 kGy leads to a gradual reducing of the proteolytic activity of the studied enzymes. A considerable higher tolerance of the enzymes to gamma irradiation, after immobilization on modified cellulose carriers, can be seen. This tendency is most notably observed for the enzyme papain. After irradiation with a 30 kGy dose, the residual enzyme activity of the immobilized papain is 91.20%, while for the enzyme powder the reduction of the catalytic activity reaches 88.20% of the initial value.

IV. MICROBIOLOGICAL VALIDATION OF STERILIZATION PROCESS

Results of incremental dose experiment : Following the selection and acquisition of samples at stage 1, the incremental dose experiment was performed to assess the bioburden levels on final products. The actual values delivered for each target dose, ranging from 1.8 to 15.8 kGy, are shown in Table 2.

Table 2. Actual doses delivered during the incremental dose experiment

Enzyme Product	Nominal incremental dose (kGy)						
	2 kGy	4 kGy	6 kGy	8 kGy	10 kGy	12 kGy	14 kGy
Variant 1	1.8-2.3	3.6-4.5	5.2-6.4	6.9-9.2	8.7-10.9	10.8-13.2	12.5-15.8
Variant 2	1.9-2.2	3.5-4.2	5.4-6.5	7.2-8.4	9.4-11.2	11.5-12.9	13.2-14.9
Variant 3	1.8-2.1	3.8-4.4	5.7-6.7	7.6-8.7	9.7-10.9	11.3-12.6	13.6-14.8

Referring to Table 2, the actual delivered doses varied slightly from the target values. There are minimum and maximum doses for each target and a slight difference for each variant. These differences are a function of the multiple irradiation runs needed to perform the experiment, not of the differences in the enzyme products. The sterility testing results are shown in Table 3, along with the target and actual dose for each incremental setting.

Table 3. Results derived from incremental dose experiments: doses delivered, and number of positive test of sterility

Variant	Batch No		Incremental doses delivered (kGy) and No of positives at each dose						
			2	**4**	**6**	**8**	**10**	**12**	**14**
1	1	Delivered dose (kGy)	2.3	4.5	5.2	8.3	8.7	13.2	12.5
		Number of positives	2	0	0	0	0	0	0
	2	Delivered dose (kGy)	2.1	3.6	5.8	6.9	9.5	12.6	13.7
		Number of positives	1	0	0	0	0	0	0
	3	Delivered dose (kGy)	1.8	4.2	6.4	9.2	10.9	10.8	15.8
		Number of positives	4	0	0	0	0	0	0

2	1	Delivered dose (kGy)	2	4.2	5.4	7.8	9.4	12.9	13.2
		Number of positives	1	0	0	0	0	0	0
	2	Delivered dose (kGy)	2.2	3.8	6.1	7.2	10.7	11.5	14.4
		Number of positives	0	0	0	0	0	0	0
	3	Delivered dose (kGy)	1.9	3.5	6.5	8.4	11.2	12.3	14.9
		Number of positives	0	0	0	0	0	0	0
3	1	Delivered dose (kGy)	2.1	4.4	5.7	8.1	9.7	12.6	13.6
		Number of positives	0	0	0	0	0	0	0
	2	Delivered dose (kGy)	2.4	4	6.3	7.6	10.1	11.8	14.1
		Number of positives	0	0	0	0	0	0	0
	3	Delivered dose (kGy)	1.8	3.8	6.7	8.7	10.9	11.3	14.8
		Number of positives	0	0	0	0	0	0	0

For the Variant 1 (immobilized papain) were established seven positive sterility tests – two in batch 01, actual dose – 2.3 kGy; one in batch 02, actual dose – 2.1 kGy and four in batch 03, actual dose – 1.8 kGy.

For Variant 2 (immobilized trypsin) was established one positive sample in batch 01, actual dose - 2.0 kGy.

All sterility tests for the batches of Variant 3 (immobilized subtilisin) were negative.

Variants 1 and 2 meet the standard requirement "the lowest dose at which one positive occurs, immediately preceded and followed by doses where all tests are negative". After determining of d* for each batch (d* - the first incremental dose at which 1 positive test of sterility occurs, and followed by all negative tests of sterility), the following values of D* (the median of the three batch d*s) were calculated for these variants: Variant 1 - 4.2 kGy and Variant 2 - 3.8 kGy.Variant 3 meets the standard requirement "the lowest dose of two consecutive doses where all tests are negative, followed by no more than one further positive test". For this variant was calculated the following value for D* - 2.1 kGy.

V. RESULTS OF VERIFICATION DOSE EXPERIMENT

Hundred pieces of samples from each variant were irradiated with the preliminary established doses (D*), based on the calculations at stage 2. The summarized results of the verification dose experiment are shown in Table 4.

Table 4. Results and calculation of verification dose experiment

Variant	Term	Value
1 (immobilized papain)	$(D^*)^a$	4.2 kGy
	delivered doses range	3.8-4.5 kGy
	$(DD^*)^b$	4.5 kGy
	$(CD^*)^c$	0
	FNP = DD* (when CD* ≤ 2)	4.5 kGy
2 (immobilized trypsin)	D*	3.8 kGy
	delivered doses range	3.5-4.0 kGy
	DD*	4.0 kGy
	CD*	0
	FNP = DD* (when CD* ≤ 2)	4.0 kGy
3 (immobilized subtilisin)	D*	2.1 kGy
	delivered doses range	1.8-2.3 kGy
	DD*	2.3 kGy
	CD*	1
	FNP = DD* (when CD* ≤ 2)	2.3 kGy

$(D^*)^a$ - from Stage 2 experiment;
$(DD^*)^b$ - the dose delivered at the Stage 3 experiment (the highest value of the dose range is delivered);
$(CD^*)^c$ - the number of positive tests of sterility observed at the Stage 3 experiment.

After gamma irradiation with the established doses, for variants 1 and 2 all samples corresponded to the test of sterility. One positive test was observed for variant 3 (CD*=1).

Stages 4 and 5: establishing the sterilization dose:

Applying the calculations as described in the standard and using the data generated from the previous stages, the operational sterilization doses were calculated to be:

Variant 1 - 16.0 kGy ;

Variant 2 - 15.2 kGy;

Variant 3 - 12.1 kGy.

VI. DISCUSSION

The gamma rays irradiation is one of the proven methods for effective reduction of the microbial contamination with a satisfactory preservation of the products and materials qualities. Like all methods of sterilization, gamma-irradiation involves a compromise between inactivation of the contaminating microorganisms and damage to the product being sterilized. The selection of the irradiation dose is determined by many factors – material type, sensitivity to irradiation, degree of contamination with microorganisms and others.

For that reason, before assuming this sterilization method for a given product the following two main groups of requirements should be kept to:

1) the product should remain stable for the delivered dose;

2) the gamma irradiation with the selected dose should provide for a satisfactory degree of sterility.

The gamma-rays irradiation is an appropriate method for sterilization of enzyme products and biomaterials because of their thermolabile nature. The various enzymes show different radiation sensitivity that is additionally affected by the enzyme form: powder, solution or immobilized on various carriers. The experimented proteases are built of one polypeptide chain, which makes them less sensitive to various denaturation agents. Compared to the enzymes with quaternary structure, they preserve to a greatest extent a structural and catalytic stability under different impacts, including radiation treatment. The proteolytic activity preservation is a basic criterion for the enzymes radiation stability and for establishing of the maximum acceptable dose thereof.

The obtained results for enzyme activity made it possible to determine the dose of 30 kGy as the maximum acceptable dose for all the three variants biomaterials with immobilized proteases. Compared to the immobilized enzymes, the enzyme powders are inactivated to a much higher degree after irradiation with the same dose. For trypsin and subtilisin in powder form the maximum acceptable dose is 25 kGy and for papain – 15 kGy. Furuta et al., also report for a considerable loss of enzyme activity of the native papain after radiation treatment. They point out that irradiation of papain water solutions at standard conditions and 15 kGy dose leads to a total inactivation while after enzyme immobilization on activated chitosan beds, the radiation stability increases up to 2 times compared to the solution ([15];[16]). The similar results are found for papain entrapped into a silicone polymeric matrix and irradiated with 25 kGy [17].Because the sterilization dose is a direct function of the level of microorganisms in the product, it is natural to follow up the microbiological purity at each stage of its obtaining. The applying of the necessary steps for limiting of the microbial contamination (including work in a controlled medium), leads to a strong reduction of the microbial population in the materials and a considerable decrease of the risk of contamination of the finished products with microorganisms. According to standard ISO 11137, the sterilization radiation dose is no more fixed to 25 kGy and it is possible to assume a lower sterilization dose, but only after proving by microbiological and statistical methods that the respective dose provides for SAL= 10^{-6}.

For all the three experimented variants the total number of microorganisms in the products before sterilization was from 0 to 100 CFU/g. Low bioburden levels were demonstrated among all the batches, allowing the incremental doses to be reduced to seven 2kGy increments. After fulfillment of the stages formulated in the standard, the terminal sterilization doses for the three variants were calculated in the range from 12.1 kGy to 16.0 kGy. Nevertheless that these values are considerably lower than the widely applied dose of 25 kGy, they provide for an effective sterilization of the experimented biomaterials with immobilized enzymes. The lower radiation doses have a minimal impact on the enzyme activity which remains above 90% of the initial activity for all the three variants.

VII. CONCLUSION

ISO 11137 Method 2A was used for validating of a terminal sterilization dose for gamma-irradiation of three variants biomaterials with immobilized proteolytic enzymes. The enzyme activity for all biomaterials is preserved to a considerable degree after irradiation with doses up to 30 kGy. In comparison to the immobilized enzymes, the enzyme powders are much more unstable when irradiated with the same doses.The minimal doses necessary to achieve sterilization at SAL of 10^{-6} are respectively: variant 1 (immobilized papain) - 16.0 kGy; variant 2 (immobilized trypsin) - 15.2 kGy and variant 3 (immobilized subtilisin) - 12.1 kGy.These low terminal doses provide effective bactericidal coverage and very likely have minimal impact on the properties of the biomaterials. All experimented variants preserved above 90% of the initial enzyme activity.

REFERENCES

[1] A. Esquisabel, R. M. Hernandez, A. Rodriguez and J. L. Pedraz, In: J. M. Guisan (Ed.) *Immobilization of Enzymes and Cells*, (New Jersey: Humana Press Inc., 2006) 283-294.

[2] T. Yudanova and I. Reshetov, Modern wound dressings: Manufacturing and properties. *Pharmaceutical Chemistry Journal, 40 (2)*, 2006, 430-434.

[3] J. Ramundo and M. Gray, Enzymatic wound debridement, *Journal of Wound Ostomy and Continence Nursing, 35 (3)*, 2008, 273-280.

[4] D. Steed, Debridement, *The American Journal of Surgery, 187 (5A)*, 2004, 71-74.

[5] J. W. Phillip, N. R. Bertoniere, D. P Thibodeaux., B. A. Triplett and A. D French, *Cotton Fiber Chemistry and Technology*, (New York: CRC Press, 2007) 70-104.

[6] D-S. Hon, Cellulose and its derivatives: structures, reactions, and medical uses, in S. Dumitriu (Ed.), *Polysaccharides in medicinal applications*, (NewYork, USA: Marcel Dekker, 1996) 87–105.

[7] U. Kim, S. Kuga, M. Wada, T. Okano and T. Kondo, Periodate oxidation of crystalline cellulose. *Biomacromolecules, 1 (3)*, 2000, 488-492.

[8] A. Belov, N. Kazanskaya, V. Filatov and Ye. Belova, Change in proteolytic activity of trypsin immobilized on aldehyde-containing textile carriers in the course of immobilization and storage, *Vestnik Moskovskogo universiteta. Seria 2. Himia, 47 (2)*, 2006, 87-90.

[9] I. Seabra and M. Gil, Cotton gauze bandage: a support for protease immobilization for use in biomedical applications, *Brazilian Journal of Pharmaceutical Sciences, 43*, 2007, 535-542.

[10] Council Directive 93/42/EEC of 14 June 1993 concerning medical devices, European Commission

[11] Council Directive 2007/47/EC of the European Parliament and of the council of 5 September 2007, European Commission.

[12] B. Marciniec and K. Dettlaff, Radiation sterilization of drugs, in *Trends in Radiation Sterilization of Health Care Products*, (Vienna: The International Atomic Energy Agency, 2008) 187–230

[13] International organization for standardization, *Sterilization of Health Care Products Radiation*, Part 2: Establishing the Sterilization Dose, ISO 11137, Geneva, 2006.

[14] *TAPPI Standard Methods*, T430 om-94, (Atlanta:TAPPI Press, 1994).

[15] M. Furuta, I. Ohashi, M. Oka and T. Hayashi, Irradiation effects on hydrases for biomedical applications, *Radiation Physics and Chemistry. 57 (3-6)*, 2000, 455–457.

[16] M. Furuta, I. Ohashi, M. Oka and T. Hayashi, Radiation sterilization of enzyme hybrids with biodegradable polymers. *Radiation Physics and Chemistry, 63*, 2002, 323-325.

[17] G. Zulli, P. Lopes, M. Velasco, M. Alcantara, S. Rogero, A. Lugao and M. Mathor, Influence of gamma radiation onto polymeric matrix with papain, *Radiation Physics and Chemistry, 79*, 2010, 286–288.