Establishment of Sterilization Doses for biomaterial- Products

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Abstract

As medical technology advances there is an increasing demand in new product formulations. Research in the field of biodegradable polymers for biomedical applications has been intense. However, the sterilization capability is a necessary requirement for any material to be used in a medical application. Six biomaterial-based products, four of them based on chitosan, were assessed for sterilization by gamma radiation. The followed methodology was based on the validation of specific procedures to characterize products microbiota. The bioburden knowledge was used to correct the production lines and further on to estimate gamma radiation doses that guarantee products safety and quality. For all the products it was able to validate a Sterilization Dose (D_{min}) that guarantees the probability of a non sterile product item in one million processed items (SAL = 10^{-6}). The estimated D_{min} ranged between 17 and 25 kGy. The applied dose establishment methodology allowed a product specific sterilization dose, reducing potential gamma radiation effects on functional and mechanical properties of the analyzed products. However, for one product the estimated radiation sterilization dose was found to cause considerable structural damage.

Keywords: gamma radiation, sterilization dose, biomaterial products.

Introduction

The traditional methods of sterilization of medical devices include the use of heat, chemicals (e.g.: ethylene oxide) or radiation. Steam sterilization by autoclaving at 121°C is the most widely employed method but might induce hydrolysis and/or melting of the polymer matrix [1]. Chemical sterilization with ethylene oxide gas offers the advantage of effective treatment at ambient temperature. However, its popularity is decreasing due to the well-known toxicity and flammability of ethylene oxide. High-energy radiation sterilization has the advantages of high efficiency, negligible thermal effects, and allows for packing prior to treatment.

The existing number and type of microorganisms present in/on a product is known as the product's bioburden and it is the result from contaminations either from the materials or from the production line: equipments, environment, personnel or/and the final product unit (package). Nowadays, the generally accepted target for sterilization processes is that it assures that the probability of finding a non sterile unit is less than one in one million. That is, the whole process will provide a Sterility Assurance Level (SAL) equal or better than 10^{-6}. The selection of a sterilization dose can be done by different approaches, either it is calculated using determinations of the number of viable microorganisms or information obtained by incremental dose or by substantiation of the sterilization dose (e.g. 15 kGy or 25 kGy) [2].

Chitin is the most abundant natural biopolymer derived from exoskeletons of crustaceans. Chitosan is a product derived

from n-deacetylation of chitin in the presence of hot alkali. Chitosan as a raw material could be used in many applications, in pharmaceuticals, cosmetics, biomedical, biotechnological, agriculture, food and non-food industries as well (water treatment, paper and textile) [3].

This paper focuses the studies developed on the validation of methodologies to evaluate the bioburden of six biomaterial based-products that lead to the estimation of corresponding Sterilization Doses (D_{min}).

Material and Methods

A. Product definition

Six biomedical products (Portugal) were analysed for its suitability to be sterilized by gamma radiation. The tested products are considered individual health care products in its packing system to be used independently in clinical practice. Products 1 to 3 are intended to be used as wound dressing. Their composition is similar and based on chitosan, but the differences are reflected in product presentation (e.g. dry, hidrogel). Products 4 and 5 are injectable bone substitutes, the last one with chitosan in its formulation. Product 6 is a calcium phosphate cement to fill voids in bone.

B. Sampling plan

The Sample Item Portion (SIP) is the portion of the product to be tested. An entire item (SIP = 1.0) was used for testing each product. None of the target products were included within a product family regarding its composition and presentation specificities. Products sampling plans for establishing the sterilization dose were representative of that subjected to routine processing and conditions. Sample product items were selected from final normal products that undergone the packaging process and belonging to the same batch. For each of the target products, the current sampling plan is composed by 30 product items:

- 10 items from one batch for the validation of bioburden determination method (n = 10).
- 10 randomly items from one independent production batch (n = 10) for the determination of bioburden frequency.
- 10 randomly items from one independent production batch (n = 10) for the verification dose experiment.

Considering manufacturer batch definition, and the large number of different products (N = 6) to establish the Sterilization Dose, it was determined to only analyse single batches following the steps directions of Method 1 for a single production batch as defined in [2].

C. Bioburden determination

The validation of bioburden determination methods were performed by artificial contamination of product samples with known concentrations (10 - 100 CFU/ sample) of a bacterial pure culture of Bacillus pumilus E601. This procedure was made to evaluate the efficiency of the method to recover and quantify the microorganisms present in/on the product. The experimental procedures used were based on conventional bacteriologic techniques according to [4].

Briefly, item samples (n = 10/product) were taken aseptically from their package and transferred individually into sterile stomacher bags. To each sample bag was added 50 ml of saline solution with a tensoactive agent (0.9% NaCl + 0.1% Tween 80). Samples were homogenised in a stomacher blender equipment (Stomacher 3500; Seaward, UK) during 15 minutes. With exception of Product 3, all product bioburden were quantified by membrane

filtration technique. Aliquots of products washing solution were filtrated, in triplicate, through cellulose nitrate membrane (0.45 µm; Sartorius AG; Germany) and placed into Tryptic Soy Agar (TSA) petri dishes. For injectable formulations a glass microfiber membrane (1.2 µm; Whatman; USA) was used as pre-filter. The incorporation technique was used to quantify Product 3 microbiota. Aliquots of washing solution were incorporated into double strength TSA medium and let solidify in petri dishes.

Samples petri dishes were incubated at 30 ± 2 ºC during 7 days. Colony forming units per sample (CFU/sample) were estimated for each product.

The natural microbiota of the six products was morphologically and biochemically characterized. Isolates were divided in eleven morphological types based on macroscopic (e.g. pigmentation, texture, shape), microscopic (e.g. cellular morphology, endospores presence/absence) and biochemical characteristics (e.g. gram staining, catalase and oxidase activities). The definition of the eleven morphological types was based on the Bergey's Manual of Determinative Bacteriology [5]. The frequency of each morphological type was calculated based on the number of isolates and its characterization.

D. Establishment of Sterilization Dose

The establishment of Sterilization Dose, i.e. gamma radiation dose necessary to achieve a Sterility Assurance Level (SAL) of 10^{-6}, was based on the Method 1 for one production batch. The verification dose (VD) experiment was conducted with a SAL of 10^{-1}, using 10 product items from one batch (n = 10). Ten product items for each product type were exposed to the estimated VD and each item was submitted individually to a sterility test. The product's irradiations were performed in its original package in a licensed (July 2004) semi-industrial gamma facility located in the ITN campus. The product's absorbed dose was monitorized by calibrated routine dosimeters (Perspex, Harwell) to identify the highest and lowest doses absorbed by the product [6]. The irradiation was performed in a calibrated geometry (dose rate = 2.42 kGy/h) that is comparable to the whole irradiation process in the irradiation chamber. The irradiation geometry was planned in a way that minimized the Dose Uniformity (D_{max}/D_{min}) ≥ 0.90.

The sterility test was performed in a clean room environment (ISO 5) by placing individually the product items in a sterile stomacher bag mimicking the product's clinical use (procedure based on the products Instruction Leaflet). To verify the presence/absence of microorganisms, 100 ml of a nutrient rich liquid medium (Tryptic Soy Broth, TSB) was added to each sample bag. The samples were incubated at 30ºC during 14 days.

Results

Due to the natural characteristics of the analysed products (e.g. bioburden, mechanical, functional), the selected approach to establish the sterilization doses was based on Method 1: dose setting using bioburden information as described in [2], in order to obtain a product-specific dose.

E. Bioburden

The validation of the bioburden determination methods was performed by artificial contamination of all types of product samples with a known concentration (10-100 CFU/sample) of a bacterial culture of Bacillus pumilus E601. This bacterial concentration range was selected based on the preliminary bioburden estimation made by the manufacturer (< 100 CFU/sample).

This procedure allowed the determination of method's recovery efficiencies and the calculation of the respective correction factors. The recovery efficiencies ranged between 84 % – 99% and the correction factors varied from 1.01 to 1.2. Additionally, the tested bioburden determination methods were found adequate for the microorganism' culture and enumeration for the spiked samples and non spiked samples. The detection limits were established in 1 CFU/ sample.

The bioburden determination method for each product was selected based on: i) the highest achieved recovering efficiency (spiked samples); ii) product nature (e.g. absorbing capacity, powder presentation, viscosity) and iii) lower detection limit. In particular, the Product 3 presented a higher viscosity after homogenization, characteristic that makes impracticable the use of membrane filtration system, leading to the application of the incorporation method. The use of pre-filters in the syringe presentations (product 4 and 5) to collect sample powder was chosen since its presence could mask and or inhibit the microbial growth. The introduction of a tensoactive in the washing solution was made to enhance the microflora removing capacity. Furthermore, the reduction of washing solution volumes was a compromise between the reduction of detection limit and the maintenance of method recovery efficiency. The validated methods also permit the employment of isolates' macroscopic and microbial characterization techniques.

Due to the presence in product formulations of chitosan and/or hydroxyapatite, and recognizing their antimicrobial proprieties [7], [8] it was expected to obtain low bioburden products. In a first approach, the obtained bioburden results pointed out to higher dispersions intra batch, for the majority of the products (data not shown). These findings indicated that the products production lines were not uniform. Based on the characterization of products microbiota, some corrective actions were suggested to the manufacturer to be implemented in order to lower, and homogenise the products' bioburden. Namely, the use of non-pyrogenic water in the production of the hidrogel dressing (product 1) and autoclavation of raw material used in the production of injectables due to their high bioburden. The obtained bioburden values, after lines production corrections, are presented in Table 1.

Table 1: Average bioburden values, respective standard error and contamination peak for one batch of the six analysed product (n = 10/product).

	(CFU/sample)	
Product	**Average bioburden[a] ± standard error**	**Maximum bioburden value (contamination peak)**
Product 1 - Hidrogel	5 ± 1	15
Product 2 – lyophilized wound dressing	2.0 ± 1.8	16
Product 3 – dry wound dressing	1.5 ± 0.8	13
Product 4 – non-chitosan injectable	$(2 \pm 1) \times 10^5$	8×10^5
Product 5 – chitosan injectable	1.0 ± 0.8	8
Product 6 - cement	$(5 \pm 1) \times 10^2$	1.3×10^3

[a] corrected values using the calculated correction factor.

Although the mentioned actions were also for Product 4, 30% of the analysed items (3/10) presented bioburden ranging 10^5CFU/sample. For the other seven items, the bioburden was found to be lower than 1000 CFU/sample. This high bioburden heterogeneity intra batch could be related with an insufficient sterilization (no uniformity of heat/stem process) of raw material. Other factor that could justify the observed high bioburden values is concerning product nature, namely the absence of the antimicrobiotic chitosan that could permit bacterial growth if there are available nutrients. The bioburden of wound dressing products and product 5 could be integrated in a lower contamination class of < 1-30 CFU. In opposite, the bioburden of products 4 and 6 were mainly included in a higher contamination class of > 30-300 CFU.

The natural microbiota of the six products (n = 2046) was morphologically and biochemically characterized. Based on the results, products isolates were divided in pre-defined eleven morphological types to establish phenotypic typification profiles. Six of the eleven morphological types were found among products isolates, being product 6 the one that presented the highest microbial diversity. The morphological type cocci, gram-positive, catalase-positive was found to be the most frequent type between the isolates from the products 5 (67%) and 6 (29%). The products 1, 3 and 4 presented predominantly isolates belonging to the group of non-spore forming gram positive rods (relative frequency of 56%; 41% and 83% respectively). The fungi were the major (67%) contaminant of the lyophilized form of wound dressing (product 2).

Considering the diversity of morphological types found in the products, a preliminary production line study was made concerning the environment of production room. Briefly, five sedimentation TSA culture media plates were settled in the production room during four hour of operation. The most frequent air isolates (n = 23) were phenotipically characterized and subdivided in morphological types, as products isolates. The most frequent morphological types found were cocci, gram-positive, catalase-positive (30%) and non-spore forming gram-positive rods (26%). This result could suggest the production room environment as a potential contamination transmission route, since the same morphological types profiles were found among products microbiota. Nevertheless, a detailed production line study with the collection of samples (e.g. air, water, personnel, and equipments) and comparison with the products isolates is needed to confirm a correlation between contaminations links.

F. Sterilization Dose

As mentioned the selected approach to establish the Sterilization Dose, i.e. gamma radiation dose necessary to achieve a Sterility Assurance Level (SAL) of 10^{-6}, was the Method 1 for single batches as described in [2]. In practice, the estimation is made of the average bioburden or contamination peak that is used to select a Verification Dose (VD) for a defined SAL. In the present study it was selected a SAL of 10^{-1} for the Verification Dose experiment using a validated software tool [9]. This choice was made to reduce the number of items (n = 10/product) to subject individually to a test of sterility, and was in agreement with the recommendations of International Pharmacopeia (e.g: USP, European) for sterility test sampling. If there is not more than one positive test the Sterilization Dose to achieve a SAL of 10^{-6} could be extrapolated. However, as mentioned in [10] a practical proof of the required level of sterility assurance of 10^{-6} is not possible. Moreover, the attainability of this condition could be fundamentally

Table 2: Dosimetric results obtained in the verification dose experiments for each of the six products (n = 6/product)

Product	Estimated VD (kGy) SAL 10^{-1}	Product items absorbed dose (kGy)			Dose Uniformity (kGy)
		Average	Highest	Lowest	
Product 1	3.5	3.3	3.4	3.2	1.1
Product 2	3.5	3.5	3.7	3.3	1.1
Product 3	3.0	3.1	3.2	2.9	1.1
Product 4	8.0	7.9	8.2	7.5	1.1
Product 5	2.6	2.7	2.8	2.6	1.1
Product 6	8.0	7.9	8.7	7.4	1.2

dubious. The inherent problem with this requirement is that evaluating the success of such sterilization by means of a final inspection is impossible, since contamination rates on the order of 10^{-6} cannot be recorded in experiments [10].

Statistically the variance of batch results was found to be heterogeneous ($P<0.05$), and to our point of view it was not accurate to consider average batch as products bioburden. Therefore, the bioburden values used to estimate the Sterilization Doses corresponded to the product contamination peaks (see Table I), assuring like this a more conservative and prudent approach ("worst case" scenario). An exception was made for product 4 since it was assumed 1000 CFU/sample as bioburden value for verification dose experiment (in fact this will correspond to a substantiation of 25 kGy as sterilization dose). This decision was made since if the 10^5 CFU/sample contamination peak was assumed the corresponding sterilization dose would be as high as 36 kGy, and product properties could be lost. Moreover, a guarantee was assumed by the manufacturer that none product batch will pass Quality Control with a bioburden higher than 100 CFU/sample.

The products samples' absorbed doses were monitorized by calibrated routine dosimeters (n = 6/product; Perspex, Harwell) to identify the highest and lowest doses absorbed by product [6]. Dosimetric and verification dose experiment results are presented in Table II and III, respectively.

According to [2], the product samples' highest absorbed dose should not exceed VD by more than 10%. Consulting the results presented in Table II this requisite was fulfilled for the six products. Other mandatory stipulation is that the mean of the highest and lowest doses delivered to the product samples' must be > 90% VD, this requirement was also achieved. In addition, no microbial growth was detected in any of the performed sterility tests during the 14 days of incubation (see Table III).

Table 3: Verification dose experiment sterility test results and extrapolated Sterilization Dose for each of the studied products.

Product	Sterility test results (positive/ negative)	Sterilization Dose (kGy) SAL 10^{-6}
Product 1	0/10	18.2
Product 2	0/10	18.3
Product 3	0/10	18.0
Product 4	0/10	25.0
Product 5	0/10	17.3
Product 6	0/10	25.0

Based on the data obtained the verification dose experiments for the six products were accepted and the 10^{-6} extrapolated doses presented in the Table III could be established as Sterilization Dose for each of the corresponding biomaterial based products.

G. Conclusions

In order to guarantee a high level of microbiological safety, the entire production or preparation process, and the steps preceding the sterilization treatment, would have to be taken into account to ensure the microbiological safety of the final product [10]. Corrective actions were effectively implemented in order to lower and homogenise the products' bioburden. Nevertheless, a detailed production line study must be done in order to accurately determine the production critical control points and products contamination transmission routes. Moreover, a quality system plan must be developed based on these data.

Although the radiation dose of 25 kGy could be adequate to guarantee a SAL of 10^{-6} based on the results obtained (e.g. dressing products) and a dose substantiation approach perhaps simpler. We decide to select the Method 1 in the establishment of the Sterilization Dose, since it could permit lower required radiation doses (D_{min}) and better guarantee the specific mechanical and functional properties of the target products (increase the interval between product D_{min} and D_{max}). The applied dose establishment methodology allowed a product specific sterilization dose, reducing potential gamma radiation effects on functional and mechanical proprieties of the analyzed products. Further studies indicated that one of the wound dressing products could not stand the estimated sterilization dose, leading to a complete loss of its structure resistivity. Chitosan could degrade into fragments under gamma rays irradiation, but this apparent setback could be used favorably as cited in by [11], in order to upgrade the development of byproducts for medical and other applications. A strict collaboration with manufacturer continues to improve the stability to ionizing radiation of this product and/or to develop an alternative sterilization method, like the one mentioned in [12].

An optimized strategy must be adjusted to each specific product to ensure the highest possible level of infection-prevention safety. This will enable the manufacturer and the user "to build sterility into a product as opposed to building a product and testing it for sterility" [10].

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