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DRUG SYNTHESIS METHODS AND MANUFACTURING TECHNOLOGY

STERILIZATION OF OCULAR MEDICAL INSERTS WITH IMMOBILIZED PROTEINS

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The influence of sterilization of ocular medicinal inserts with human serum albumin (HSA), lysozyme, papain, and urea on the preservation of their physicochemical properties was studied. It was found that the physicochemical properties of inserts with HSA and lysozyme preserved totally their physicochemical properties after sterilization by γ-irradiation in doses of 10, 17, and 28 kGy. The influence of γ-irradiation on the proteolytic activity of ocular medicinal inserts with papain and urea was dose-dependent and reduced the activity by 85 and 93% and led to its complete loss. Adding antiseptics used in the production of ophthalmologic preparations (nipagin, sodium metabisulfite, benzyl alcohol, benzalkonium chloride, etc.) caused an activity loss of the ocular medicinal inserts with papain and urea and did not provide microbiological purity and sterility. UV irradiation of ocular medicinal inserts with papain and urea produced microbiologically pure inserts that preserved 63.8% of the proteolytic activity. Manufacturing of ocular medicinal inserts with papain and urea under aseptic conditions using sterilizing filtration (79.7% activity preservation) was recommended for sterilization.

Keywords: ocular medicinal inserts, papain, urea, lysozyme, human serum albumin, sterilization.

Ocular dosage forms should be sterile and stable and not contain mechanical contaminants. Favorable conditions for the development and multiplication of microorganisms at all stages of the manufacturing, storage, transportation, and use of the preparation create problems with avoiding microbial contamination of ophthalmologic drugs.

Infection by non-sterile preparations can cause series consequences that sometime lead to sight loss because lacrimal fluid in ocular diseases contains little lysozyme, a hydrolytic enzyme that normally prevents eye infections, and the conjunctiva is unprotected from the negative effect of the microorganisms. The sterilization method selected for developed ophthalmological preparations is of fundamental importance and is based on the stability of the drugs to heating, the action of chemicals (antiseptics, preservatives, stabilizers), the resistance to UV- and γ-irradiation, etc. Regardless of the sterilization method, ocular preparations must be manufactured under aseptic conditions [1, 2].

Previously, we together with V. P. Filatov Institute of Ocular Diseases and Tissue Therapy, NAMS of Ukraine, developed ocular medicinal inserts (OMIs) with human serum albumin (HSA), papain, and urea in addition to lysozyme with anti-inflammatory, burn-treatment, and antibacterial activity immobilized in polyvinylalcohol (PVA) [3 – 7]. However, the sterilization method for the preparations was selected without a sufficient review.

The goal of the present work was to study the influence of sterilization methods for OMIs with HSA, lysozyme, papain, and urea on the preservation of their physicochemical properties.

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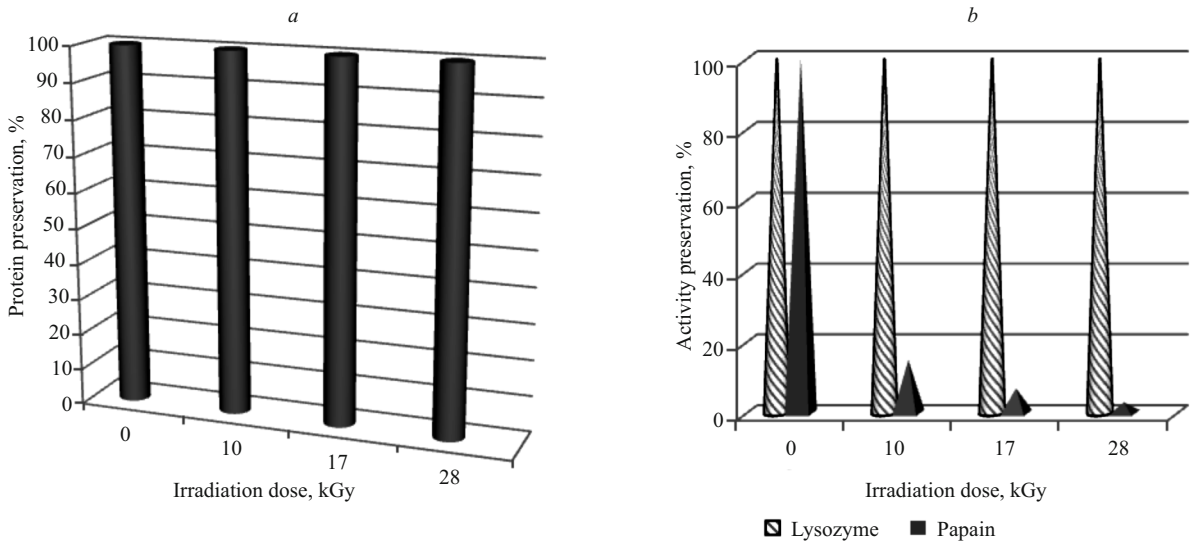

Fig. 1. Preservation of protein and enzyme activity of OMI as functions of γ-irradiation dose: human serum albumin (*a*) and papain and lysozyme (*b*).

EXPERIMENTAL PART

Papain (320 U/g, Merck, Germany), urea (analytically pure, Reakhim, Ukraine), chicken egg white lysozyme (EC 3.2.1.17, Applichem, Belgium), HSA solution (10%, blood transfusion station, Mariupol, Ukraine), and PVA (MW 30,000, OAO Vitaminy, Uman, Ukraine) were purchased commercially.

TABLE 1. Characteristics of OMI with Proteins

Properties	OMI with		
	serum albumin	papain and urea	lysozyme
Protein:PVA mole ratio*	1:4	1:1.2	1:2.4
Activity, U/g	–	389 ± 9	
Protein preservation, %	100	100	100
Optimum pH	-	5 – 8	
Optimum temp.	-	30 – 65	
Area, cm^2		0.64 ± 0.02	
Thickness, mm		0.35 ± 0.01	
Average mass, mg	11.5 ± 0.5	8.1 ± 0.4	10.4 ± 0.5
Color		cream	
Solubility in water, normal saline, min		20.0 ± 1.0	
Shelf life, yr	2	1.5	2

* Protein:PVA mole ratio below which protein is immobilized by inclusion in the polymer

Proteolytic activity of free and immobilized papain was determined using a modified Anson method [8] and Hammerstein-grade casein as the substrate. The amount of enzyme that catalyzed casein cleavage in 1 min at 37°C to products that were not precipitated by trichloroacetic acid was taken as the unit proteolytic activity. The content was expressed in micromoles of tyrosine.

Protein content was monitored by the Lowry–Hartree method [9]; urea, by the Michon–Arnaud method [10]. The immobilization was performed using the published procedure [11].

Hydrolytic activity of lysozyme was determined by a bacteriolytic method using acetone powder of *Micrococcus lysodeikticus* (strain 2665) as substrate. The amount of lysozyme that reduced the optical density of a cell suspension by 0.001 in 1 min at 55°C was taken as the unit activity [12].

Sterilization by γ-irradiation was carried out at OAO Gemoplast (Belgorod-Dnestr, Ukraine) according to GOST R ISO 11137 – 2000 "Sterilization of Medical Products". The source of γ-radiation was ^{60}Co. Sterilization at different doses of 10, 17, and 28 kGy was used to study the influence of γ-irradiation on preservation of OMI proteolytic activity. The doses were selected due to validation and process control requirements.

A Philips UV-irradiator at wavelength 220 – 280 nm and power 0.6 W/m^2 was used for the UV-sterilization of OMIs with papain and urea with and without nipagin. Exposures of 60, 90, and 120 min were used. Each side of the OMI was irradiated for the given time. Then, the OMI was placed on the surface of soy-casein agar in a Petri dish and incubated at 30 – 35°C for 5 d.

Microbiological purity was studied by membrane filtration and by direct subsurface seeding.

60 min (6 CFU) 90 min (4 CFU) 120 min (0 CFU)

Fig. 2. Microbiological purity of inserts with papain and urea after UV irradiation.

Membrane filtration of solution (0.1 mL) for manufacturing OMIs used sterile nitrocellulose 0.45 μm membrane filters (Sartorius). The filter was placed onto soy-casein agar after the filtration and incubated at 30 – 35°C for 5 d.

Direct subsurface seeding used the solution (0.1 mL) for manufacturing OMIs and Petri dishes with soy-casein agar. The dishes were incubated for 5 d.

Single-use Minisart syringe cartridge filters (Sartorius) with 0.2 μm cellulose acetate membranes were used for sterilizing filtration of the solutions used to prepare the OMIs.

The whole volume of solution containing all components for manufacturing OMIs was filtered through a syringe cartridge filter into a sterile vial. Then, the microbiological purity was assayed using membrane filtration and direct seeding in order to confirm that the filtered solution was sterile. The activity and protein content were checked using the aforementioned methods.

RESULTS AND DISCUSSION

Immobilization of papain, HSA, and lysozyme in PVA by inclusion in the gel showed that the proteins formed a complex with the carrier and could be incorporated mechanically [13]. Table 1 presents the principal characteristics of the studied OMIs.

γ-Irradiation is widely used to sterilize protein preparations in the pharmaceutical and biotechnological industries and, among others, to sterilize single-use medical instruments (catheters, internal infusion systems), dressings, drugs from plant raw material, etc. [1].

Test batches of polymeric inserts (100 each) that were sterilized at OAO Gemoplast were produced in order to study the influence of γ-irradiation on the protein content and enzyme activity. The results (Fig. 1*a* and 1b) showed that γ-irradiation at doses of 10 – 28 kGy did not influence the protein content and the proteolytic activity of the OMI with HSA and lysozyme.

Table 2 presents the OMI characteristics after irradiation. The principal parameters of the inserts with albumin and lysozyme did not change after sterilization at the maximum dose of 28 kGy. The fully preserved protein content and hydrolytic activity after storage for 1 yr, the insert mass, and the dissolution time in H_2O and normal saline were interesting.

The OMI with papain and urea lost 85% of its proteolytic activity already after irradiation at a dose of 10 kGy. Increasing the irradiation dose further led to a complete loss of

TABLE 2. Characteristics of OMI with Immobilized Human Serum Albumin/Lysozyme After Sterilization by γ-Irradiation at a Dose of 28 kGy

Parameter	After albumin/lysozyme immobilization	Shelf life of OMI after sterilization, months				
		0	1	6	12	
Preservation of protein/activity in insert, %, $M \pm m$	100.0/100.0 ± 4.8	100.0/100.0 ± 5.2	99.7/99.5 ± 4.6	98.5/97.8 ± 4.3	100.0/99.3 ± 4.9	
S, cm^2, $M \pm m$	0.64 ± 0.02					
Color	cream					
Mass, mg, $M \pm m$	8.0 ± 0.1	7.8 ± 0.1	7.5 ± 0.1	7.6 ± 0.1	7.5 ± 0.1	7.9 ± 0.1
Dissolution time in water, normal saline, min, $M \pm m$	20 ± 0.7					

TABLE 3. Influence of Preservatives and Antiseptics on Papain Proteolytic Activity

Preservative/antiseptic	Papain proteolytic activity with added preservative, %, $M \pm m$		
	papain in solution	papain + urea in solution	OMI with papain and urea
Control	100.0 ± 5.0	141.0 ± 7.0	156.0 ± 7.8
Nipagin, 0.15%	79.1 ± 3.9	128.4 ± 6.4	46.2 ± 2.3
Sodium metabisulfite, 0.5%	87.5 ± 4.4	83.0 ± 4.2	93.4 ± 4.8
Levomycetin 0.2% + boric acid 2%	99.5 ± 5.0	102.8 ± 5.2	7.8 ± 0.3
Benzyl alcohol, 0.9%	33.6 ± 1.7	102.1 ± 5.1	23.8 ± 0.9
Benzalkonium chloride, 0.01%	145.2 ± 7.2	100.7 ± 4.9	15.6 ± 0.9

insert proteolytic activity; apparently, because of irreversible destructive changes in the enzyme.

Loss of papain activity after sterilization was recently reported. However, data given by various researchers are contradictory. Thus, it was shown [14] that irradiation of papain at various doses (5 – 35 kGy in steps of 5 kGy) decreased the enzyme activity by 5.4 – 38.0%. It was confirmed [15] that papain was radio-resistant and that its activity was preserved after irradiation at a dose of 25 kGy.

Japanese researchers noted only 10% preservation of papain activity after irradiation at a dose of 15 kGy [16]. This agreed with our results (Fig. 1). They proposed using the radioprotectors polyglycosyloxyethylmethacrylate and α-L-glutamate in order to preserve the enzyme activity (40 – 70%).

The mechanisms of radiation inactivation of papain included both the distortion of its conformation due to the destruction of tyrosine and tryptophan residues, with the tyrosines located near the active center (Tyr-61 and Tyr-67) being preferentially destroyed, and the degradation of the active center imidazole-thiol pair (Cys-25 and His-159) through the action of water radiolysis products (OH radicals) [17].

The influence of preservatives/antiseptics, UV-irradiation, and sterilizing filtration on activity preservation, microbiological purity, and, in several instances, sterility was investigated in order to find an effective sterilization method for OMIs with immobilized papain and urea.

Antiseptics and preservatives that can keep a preparation sterile for its whole useful life are known to be added during the manufacturing of eye drops in order to prevent the growth and multiplication of microorganisms. The most effective preparations against pathogenic microorganisms are merthiolate (0.005%), chlorobutanol hydrate (0.5%), benzalkonium chloride (0.01%), cetylpyridinium chloride (0.01%), nipagin and nipasol (0.15%), and levomycetin (0.2%) in combination with boric acid (2%) [2].

The influence of several preservatives/antiseptics on papain activity in solutions with and without urea and in manufactured OMIs was studied in order to select effective ones for OMIs with papain and urea (Table 3).

Preservatives that had the least effect on papain activity (sodium metabisulfite and nipagin) according to the results were selected.

The microbiological purity of inserts with nipagin and sodium metabisulfite and without them did not differ during investigations of OMIs manufactured with preservatives. This indicated that the bacteriostatic activity of the preservatives/antiseptics was insufficient.

A study of the influence of UV-irradiation on OMIs with papain and urea showed that the number of colony-forming units (CFU) of microorganisms decreased with time from 4 – 6 CFU (60 – 90 min) to 0 (120 min) (Fig. 2).

The proteolytic activity was 63.8% preserved after UV-irradiation of OMIs with papain and urea.

Sterilizing filtration of a solution for manufacturing OMIs that contained all required components had less of an influence on the activity (310.0 U/g or 79.7% of the initial activity). The results confirmed that the initial microbe loading of the solution for manufacturing OMIs was 750 – 1000 CFU whereas a check of the solution after filtration showed that it was microbiologically pure.

Thus, the physicochemical properties of OMIs with HSA and lysozyme were fully preserved after sterilization by γ-irradiation at doses of 10, 17, and 28 kGy. These same doses had a negative influence on preservation of papain proteolytic activity, up to its complete loss. Therefore, OMIs should be manufactured under aseptic conditions using preliminary sterilizing membrane filtration of the manufacturing solutions in order to assure that the preparation is sterile and has highly preserved proteolytic activity.

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