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RESEARCH PAPER

Manufacturing of a Lyophilized Parenteral Dosage Form of the Indoloquinone Antitumor Agent EO9 for Phase II Clinical Studies in the Setting of a Hospital Pharmacy

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ABSTRACT

The manufacturing process for 28 batches (5555 vials) of a parenteral lyophilized dosage form of the investigational cytotoxic drug EO9 within the setting of a hospital pharmacy is described. Quality control of lyophilized EO9 indicated that all batches conformed to the specifications for the finished product. Long-term stability data of EO9 finished product are presented. In addition, the antimicrobial activity of EO9 against several microorganisms was determined. The implications of Good Manufacturing Practice (GMP) with respect to such a relatively large production in a small manufacturing site are discussed.

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INTRODUCTION

EO9 (3-hydroxymethyl-5-aziridinyl-1-methyl-2-(1*H*-indole-4,7-dione)-propenol; NSC 382,456; Fig. 1) is the lead compound in a series of novel and fully synthetic bioreductive alkylating indoloquinones with potent antitumor properties (1,2). EO9 is undergoing clinical evaluation in Europe under the auspices of the New Drug Development Coordinating Committee (NDDCC) and the New Drug Development Office (NDDO) of the European Organization for Research and Treatment of Cancer (EORTC). It was selected for clinical study because of its distinct mechanism of activation, its activity against hypoxic cells, its preferential solid tumor activity, and its lack of bone marrow toxicity in animal studies (1). Two phase I and pharmacologic studies have been conducted to determine the toxicity, the maximum tolerated dose (MTD), pharmacology, and antitumor response of EO9 in two dose regimens: a weekly or a 3-weekly schedule (3,4). Furthermore, five phase II clinical trials with EO9 have been performed in patients with breast, colorectal, gastric, pancreatic, and non-small-cell lung cancer, respectively (5).

A stable lyophilized formulation of EO9, intended for use in preclinical and clinical studies, has been designed (6). The prototype, containing 8 mg of EO9, 200 mg of lactose per vial, and sodium hydroxide for pH adjustment, was found to be the optimal formulation in terms of solubility, length of the freeze-drying cycle, stability, and dosing requirements for phase I clinical trials (6). After manufacturing batches of lyophilized EO9 for the phase I clinical trials, specifications for the finished product were drawn up to which each batch of lyophilized EO9 intended for the upcoming phase II trials had to conform.

This paper discusses the manufacturing process for more than 5000 vials of lyophilized EO9 for phase II clinical trials in the pharmacy of the Slotervaart Hospital, which is a 525-bed general hospital. The specifications drawn up for the finished product were prospectively tested and the implications of GMP with respect to such a large production in a small manufacturing site are discussed. Furthermore, the long-term stability of EO9 finished product was assessed. In addition, the antimicrobial activity of EO9 against several microorganisms was determined.

MATERIALS AND METHODS

Chemicals

EO9 was synthesized by Kyowa Hakko Kogyo Co., Ltd. (Tokyo, Japan) and was provided by the EORTC-NDDO (Amsterdam, The Netherlands). Lactose was obtained from BUFA B.V. Pharmaceutical Products (Uitgeest, The Netherlands) and conformed to the European Pharmacopeia (EP) (7). Sodium hydroxide was purchased from Merck (Darmstadt, Germany). Water for injection was manufactured by the Slotervaart Hospital (Amsterdam, The Netherlands) and conformed to the EP (8). Bacto® Tryptic Soy Broth (TSB) was obtained from Difco Laboratories Inc. (Detroit, USA) and the composition conformed to the United States Pharmacopeia (USP 23) (9). *Staphylococcus aureus, Staphylococcus epidermidis, Escherichia coli, Klebsiella pneumonia,* and *Candida albicans* were isolated from human specimens and were provided by the Department of Medical Microbiology (Slotervaart Hospital). Sterile TSB broth was used for the antimicrobial test. All other chemicals were of analytical grade and de-ionized water was used throughout.

Quality Control of EO9 Bulk Drug and Excipients

EO9 bulk drug was supplied with a certificate of analysis from the manufacturer. In addition, at our laboratory the identity of the bulk drug was reconfirmed by infrared (IR) spectroscopy, high-performance liquid chromatography (HPLC) analysis, and ultraviolet/visible (UV/VIS) spectrophotometry. Furthermore, the appear-

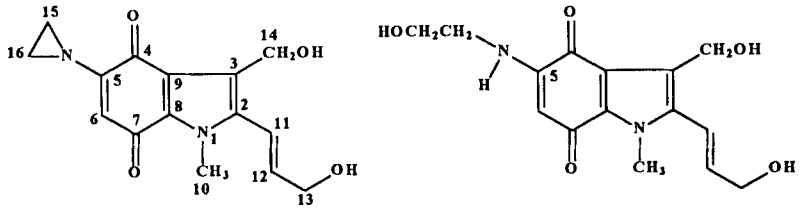

Figure 1. Chemical structures of EO9 (left) and EO5A (right), the major degradation product of EO9.

ance of the raw material and the state of solution of a 1 μg/ml solution in methanol were checked by visual inspection, the purity was determined by HPLC analysis, the melting point was determined by the capillary glass tube method, and the moisture content in the bulk drug was determined by the Karl-Fischer titration method.

Lactose and sodium hydroxide were both delivered with a certificate of analysis from the supplier. The identity and the purity of these excipients were reconfirmed before use.

Formulation and Quality Control of EO9 Finished Product

The formulation process of EO9 has been previously described (6). All 28 batches of lyophilized EO9 intended for the phase II clinical studies were manufactured according to that particular formulation process. All manipulations took place, as far as possible, under laminar downflow conditions in a Model MKV 183 II biological safety cabinet (Class 100; Interflow B.V., Wieringerwerf, The Netherlands) in a Class 10,000 clean room (Interflow B.V.). Before use, the glass vials, lyophilization stoppers, and all the glassware were washed thoroughly with distilled water and then sterilized by autoclaving for 20 min at 120°C. The freeze-dryer was disinfected by a solution containing *n*-propanol/isopropanol/water (420:280:200, v/v/v).

Several in-process control tests were performed, for example, determination of the EO9 content in the formulation solution before and after membrane filtration by UV/VIS spectrophotometry, and determination of the pH of the formulation solution.

Quality control was performed by visual inspection of the appearance and the color of the pharmaceutical product. HPLC analysis and UV/VIS spectrophotometry were used to determine the content and purity of the product. The residual moisture content in the lyophilized product was determined by the Karl-Fischer titration method. Furthermore, a limulus amoebocyte lysate test for the presence of endotoxins and a sterility test were performed. These test methods have been described in detail previously (6). For the production of the phase II clinical batches, the number of test methods was extended. In addition, the reconstitution of the finished product and any foreign insoluble matter after reconstitution were checked by visual inspection. Furthermore, the pH after reconstitution was determined and the weight variation was determined according to the weight variation test in the Japanese Pharmacopeia (JP XII) (10).

Long-Term Stability of EO9 Finished Product

The results of a shelf-life study with lyophilized EO9, after storage for 1 year at 2°-8°C, were previously reported (6). This stability study has been extended up to 30 months. The content and purity of lyophilized EO9 were determined by means of HPLC analysis after storage for 18, 24, and 30 months, respectively, at 2°-8°C in a dark environment. Furthermore, the appearance and color of lyophilized EO9 were registered, and after reconstitution the pH and the clarity of solution were established.

Validation of the Manufacturing Process

The manufacturing process of EO9 is validated, in terms of aseptic working procedures, every 6 months. The aseptic media fill was performed in the same manner as the formulation process of EO9 (6) except that sterile TSB was used instead of the EO9 formulation solution. After preparation of the broth, it was sterilized by autoclaving for 20 min at 120°C. Even though a sterile broth solution was used, the broth was again sterilized by membrane sterilization through a 0.22-μm filter (Milli-fil GS™; Millipore, Bedford, MA, USA) in order to mimic the formulation process as closely as possible. Aliquots (40 ml) of TBS were filled into 50-ml colorless glass vials (type I; Aluglass B.V., Uithoorn, The Netherlands). The recovery of each media fill was approximately 200 vials/run. Lyophilization closures (grey butyl V9032; Helvoet Pharma N.V., Alken, Belgium) were partially inserted into each vial and subsequently the vials were loaded into a Lyovac GT 4 freeze-dryer (Finn Aqua Santasalo-Sohlberg, Hürth, Germany) at room temperature. The freezing stage was carried out at -40°C for 10.5 hr. After establishing a vacuum (10^{-1} mbar), the lyophilization cycle was stopped and the vials were stoppered pneumatically in a vacuum and subsequently capped with aluminum caps (Helvoet Pharma N.V.).

Half of the vials were incubated at 37°C and the other half at room temperature (23°-23.5°C). After 1 and 2 weeks of incubation, every vial was inspected visually for microbial growth.

Antimicrobial Activity of EO9

The antimicrobial activity was determined against *Staphylococcus aureus, Staphylococcus epidermidis,*

Escherichia coli, Klebsiella pneumonia, and *Candida albicans*. A vial of lyophilized EO9, containing 8.0 mg of the active substance, was reconstituted with 8.0 ml of sterile TSB broth. A dilution series in sterile broth was then prepared in the concentration range of 0.5 to 6×10^{-5} mg/ml in sterile test tubes. Subsequently, the microorganisms, with an inoculum of 10^5 colony-forming units per milliliter, were added to each test tube. The test tubes were incubated by 37°C for 24 hr. The antimicrobial activity of EO9 against each microorganism was assessed by visual inspection; turbidity of the solution indicated bacterial growth. The lowest concentration of EO9 which still inhibits bacterial growth is defined as the minimal inhibitory concentration (MIC).

RESULTS

Quality Control of EO9 Bulk Drug

Table 1 shows the test methods and their corresponding specifications of EO9 bulk drug. The lot of EO9 bulk drug which was used for the production of lyophilized EO9 for the phase II clinical trials conformed to the specifications listed.

Formulation and Quality Control of EO9 Finished Product

In total, 28 batches (5555 vials) of EO9 finished product have been manufactured at our formulation facilities for phase II clinical trials. The net recovery of the formulation process was 88%. For example, 667 of the 5555 vials have been used for the following purposes: registration of the product temperature during the lyophilization process by means of Pt-100 resistance thermometers, in-process control tests, and quality control tests; and of each manufactured lot, 6 vials have been kept in storage for stability studies.

Several in-process control tests were performed, for example, determination of the EO9 content in the formulation solution before and after membrane filtration by UV/VIS spectrophotometry, and determination of the pH of the formulation solution. The theoretical concentration of EO9 in the formulation solution was 0.2 mg/ml. The mean measured concentrations of EO9 in the formulation solution before and after membrane filtration were 0.208 ± 0.005 and 0.203 ± 0.003 mg/ml, respectively, indicating 2.5% loss of EO9 during filtration ($p < 0.00001$). In an earlier study, the chemical stability of EO9 was investigated and it was shown that EO9 was most stable in the pH region 8–9 (11). The mean pH of the formulation solution was 8.5 ± 0.5, by which the amount of degradation of EO9 during the manufacturing process was minimized.

Table 2 shows the test methods and their corresponding specifications for the EO9 finished product. All batches of finished product conformed to the specifications drawn up for this particular investigational cytotoxic drug. Figure 2 shows the deviation in the EO9 content, as determined by HPLC analysis, of the 28 manufactured batches. The lines represent the upper and lower limits of the content.

Table 1

Specifications of EO9 Bulk Drug (6)

Test Method/Test Item	Specification
Visual inspection	
1. Appearance	Dark-red crystalline powder
2. State of solution	A 1-μg/ml solution in methanol is a clear, dark-red solution
IR spectroscopy	
3. Identification	IR spectrum bulk drug = IR spectrum reference standard
HPLC analysis	
4. Identification	R_t bulk drug = R_t reference standard
5. Purity	>98%
6. Impurities	Total (EO5A + others) < 2%
UV/VIS spectrophotometry	
7. Identification	λ_{max}: 269–273, 311–315, and 505–512 nm
Capillary glass tube method	
8. Melting point	184°–189°C (uncorrected)
Karl-Fischer titration method	
9. Moisture content	<2%

Table 2

Specifications for EO9 Finished Product

Test Method/Test Item	Specification
Visual examination	
1. Appearance	Pink/red lyophilized cake
2. Reconstitution	A clear, dark-red solution
3. Foreign insoluble matter (JP XXII)	A clear, dark-red solution, free from foreign insoluble matter
Mathematical claculations	
4. Osmotic pressure	Isotonic with blood
HPLC analysis	
5. Identification	R_t bulk drug = R_t reference standard
6. Content	8.0 ± 0.4 mg
7. Purity	>98%
8. Impurities	Total (EO5A + others) < 2%
UV/VIS spectrophotometry	
9. Identification	λ_{max}: 269–273, 311–315, and 505–512 nm
10. Content	8.0 ± 0.4 mg
pH measurements	
11. pH after reconstitution	6.5–9.5
LAL test	
12. Pyrogens	<3.3 EU/vial
Sterility test (USP XXII <71>) (6)	
13. Sterility	Sterile
Karl-Fischer titration	
14. Residual moisture content	<1%
Weight variation test (JP XII) (7)	
15. Weight variation	Deviation < 7.5%

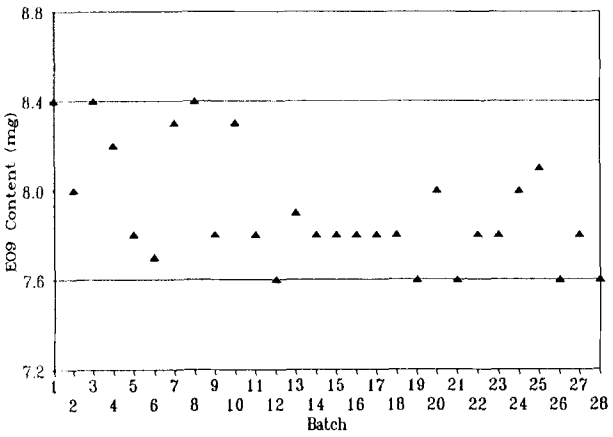

Figure 2. EO9 content (milligrams) of the 28 manufactured batches of EO9 finished product as determined by HPLC analysis. The lines represent the specification for the content in the finished product.

Long-Term Stability of EO9 Finished Product

Table 3 shows the results of the long-term stability study of lyophilized EO9. The stability data up to 1 year have been previously published (6). It was decided, however, to extend the stability study of lyophilized EO9. It seems that EO9 finished product is stable for at least 30 months when stored at 2°–8°C in a dark environment. The appearance, reconstitution, clarity of solution, content, purity, and pH after reconstitution still conformed to the specifications drawn up for the finished product.

Validation of the Manufacturing Process

Half of the vials containing TSB broth were incubated at 37°C and the other half at room temperature (23°–23.5°C). After 2 weeks of incubation, the broth

Table 3

Long-Term Stability of Lyophilized EO9 at 2°–8°C in a Dark Environment

Storage Time (Months)	Content ± *SD* (%)	pH After Reconstitution	Number of Vials
6	101.0 ± 3.4	8.6	10
12	98.2 ± 2.2	8.1	4
18	102.4 ± 2.1	7.9	4
24	95.0 ± 1.0	7.6	3
30	97.5 ± 1.8	7.7	3

in all vials was still clear; thus no bacterial growth had occurred. In order to test the capacity of the TBS broth to allow bacterial growth, it was challenged with several designated microorganisms. After 1 day of incubation at 37°C, the broth was turbid, indicating extensive bacterial growth.

Antimicrobial Activity of EO9

The antimicrobial activity of EO9 against *Staphylococcus aureus, Staphyloccus epidermidis, Escherichia coli, Klebsiella pneumonia*, and *Candida albicans* was determined. EO9 did not inhibit growth of the latter three, not even at the highest concentration. EO9 showed, however, antimicrobial activity against both tested *Staphlyococcus* microorganisms. The MIC values were 9.8×10^{-4} (*S. aureus*) and 4.9×10^{-4} mg/ml (*S. epidermidis*), respectively.

DISCUSSION

Medicinal products intended for research and development trials are not at present subject to either marketing or manufacturing European Community (EC) legislation (12). However, when adopting Directive 91/356/EEC on GMP for medicinal products for human use (13), it was agreed that EEC Member States may require compliance with the principles of GMP during the manufacture of products intended for use in clinical trials, which is in agreement with earlier suggestions in an EC Discussion Paper (III/3044/91) of January 1991. It was, therefore, agreed to prepare an annex to the EC guide to Good Manufacturing Practice for Medicinal Products (12). This annex specifically addresses those practices which may be different for investigational products, as they are usually not manufactured under routine conditions, and with possibly incomplete characterization of the product at initial stages of clinical development. The major differences in the manufacturing proces between licensed and investigational medicinal products are that the production processes may not be validated to the extent necessary for a routine production operation, and that the specifications (for packaging materials, and intermediates, bulk, and finished products) may vary during development (12). Specifications for both active and nonactive ingredients should be periodically reassessed. It may not be necessary to produce master formula and processing instructions, but for every manufacturing operation or supply, there should be clear and adequate written instructions and written records. All the information necessary to draft the detailed written instructions on processing, packaging, quality control testing, batch release, storage conditions, and/or shipping should be kept together in a product specification file. Packaging instructions of investigational products are based on the order; contrary to large-scale manufacturing of licensed medicinal products, batches of investigational products may be subdivided into different batches and packaged in several operations over a period of time. Although it is likely that the number of staff involved will be small, separate people should be responsible for production and quality control. During the manufacture of investigational products, it may be that different products are handled in the same premises and at the same time, and this reinforces the need to eliminate all risks of contamination. For sterile products, there should be no reduction in the degree of validation of sterilizing equipment. Validation of aseptic processes may present problems when the batch size is small (12). To further structure the requirements for the manufacture of sterile medicinal products, a directive on GMP and certification of starting materials for the industrial manufacture of medicinal products has just been presented (III/5808/94—Draft 4) (14). Up until now all starting materials have to be checked against their specifications before being

released for use in "pharmaceutical" manufacture. However, this is not in line with the general principle of quality assurance, which is that the quality should be built into the product and not checked on the end product (14). For this reason, manufacturing of starting materials should be submitted to a quality system adapted to that very stage of production.

The formulation laboratory in our hospital pharmacy was especially designed for the pharmaceutical development and small-scale manufacturing of investigational cytotoxic drugs. The formulation laboratory (dimensions 4.5 × 3.8 m) is a class 10,000 clean room containing a class 100 weighing area, a class 100 biological safety cabinet, and a class 100 downflow facility which has access to a Lyovac GT-4 freeze-dryer. To enter the clean room one has to pass through a class 100,000 sluice (dimensions 1.4 × 3.8 m), which also acts as a changing facility. The biological safety cabinet is a laminary downflow work station, which is suitable for the aseptic preparation and filling process. The air which is removed from the biological safety cabinet is first filtered through HEPA filters and, for safety reasons, is then directly led through a channel to the roof of the hospital. The clean room is validated every 6 months in terms of the presence of particles, the quality of the filters is tested, and aseptic working procedures are checked by performing media fills. Even though the formulation laboratory is small, it meets most of the GMP requirements. The air pressure in a clean room is normally positive relative to surrounding areas of a lower air grade. However, the recommendations regarding air pressure differentials may need to be modified in case of pathogenic, highly toxic, and other dangerous materials or products (14). Since our formulation facility is meant solely for the manufacturing of cytotoxic drugs, it was decided to keep the pressure of the clean room negative relative to the surrounding areas in order to prevent cytotoxic contamination to the surrounding environment. The pressure in the entrance sluice is positive relative to both the clean room and the surrounding areas.

According to GMP guidelines, media fills are, at defined time intervals, required to validate an aseptic filling process (12). Successfully validating a process may reduce the dependence upon intensive in-process and finished product testing. Microbial contamination can occur during the various stages of manufacture; for example, the bulk drug may become contaminated from the starting materials, the manufacturing equipment, environment, and manufacturing personnel. In addition, when the formulation solution is filled into the final containers, it may be contaminated from the filling equipment, the environment, the container, or the personnel involved in the filling operation. Then, in the hospital the finished product may be further contaminated as a result of dilution and preparation of the infusion for the individual patient. Probably the greatest potential sources of contamination are the operators, as this factor is the least controllable (15). An occupied room will always contain skin fragments in the dust and air. It is known that a large proportion of these skin fragments bear bacteria. It is, for example, estimated that up to 30% of the population may act as permanent or transient carriers of *Staphyloccus aureus* (15). It is therefore of the utmost importance that the personnel involved in the manufacturing process of pharmaceuticals, in particular aseptic preparations, perform at regular time intervals media fills simulating the process to be performed. The production process of EO9 is, therefore, simulated every 6 months with sterile TSB broth. The broth was incubated at two temperatures (i.e., 37°C and room temperature) in order to optimally detect the presence of bacteria, and yeast and molds, respectively (15). In addition, personnel working in the clean room should wear appropriate clothing and work according to the written instructions (standard operating procedures).

The antimicrobial activity of lyophilized EO9 against *Staphyloccus aureus* and *Staphylococcus epidermidis, Escherichia coli* and *Klebsiella pneumonia,* and *Candida albicans* was determined. These microorganisms were selected because they are designated species of gram-positive bacteria, gram-negative bacteria, and yeast, respectively. We were interested in the antimicrobial activity of EO9 both at a concentration of 0.5 mg/ml, which is the EO9 concentration in the infusion fluid, and at 5×10^{-4} mg/ml, which is the mean maximum plasma concentration (C_{max}) reached in the patients after a bolus injection of 22 mg/m^2 of EO9 (4). EO9 showed antimicrobial activity against *Staphylococcus aureus* and *Staphylococcus epidermidis*. The MIC values were around the C_{max} value reached in patients' plasma.

After manufacturing of batches lyophilized EO9 for the phase I clinical trials, specifications for the finished product were drawn up to which each batch of lyophilized EO9 intended for the upcoming phase II trials had to conform. The specifications drawn up for the finished product were prospectively tested. In total, 28 batches of EO9 finished product have been manufactured at our formulation facilities for phase II clinical trials. All 28 batches conformed to the specifications of the finished product and the interbatch variability was

small. Thus, the manufacturing process for lyophilized EO9 in our hospital pharmacy is a reliable and robust process, yielding a finished product of adequate quality. Furthermore, long-term stability experiments have shown that lyophilized EO9 is a stable product when stored at 2°–8°C in a dark environment.

CONCLUSIONS

The manufacturing process for 28 batches (5555 vials) of the pharmaceutical product of the investigational cytotoxic drug EO9 in a general hospital pharmacy environment, has been evaluated. Even though the manufacturing area is relatively small for such a large production, we were able to conform to the specifications of EO9 finished product and we have shown that the interbatch variability is small. The implications of Good Manufacturing Practice (GMP) with respect to such a large production in a small manufacturing site have been discussed and it seems that it is possible to carry out such a large production process in the setting of a hospital pharmacy according to GMP guidelines. Furthermore, long-term stability data indicated that EO9 finished product is stable for at least 30 months when stored at 2°–8°C in a dark environment. In addition, the antimicrobial activity of EO9 against several microorganisms was determined and it showed antimicrobial activity against *Staphylococcus aureus* and *epidermidis*.

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