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Original research paper

Optimization of amino acid-stabilized erythropoietin parenteral formulation: *In vitro* and *in vivo* assessment

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The aim of this study was to optimize the formulation of erythropoietin (EPO) using amino acids instead of human serum albumin (HSA) and to evaluate its *in vivo* stability in order to avoid the risk of viral contamination and antigenicity. Different EPO formulations were developed in such a way as to allow studying the effects of amino acids and surfactants on the EPO stability profile. The main techniques applied for EPO analysis were ELISA, Bradford method, and SDS gel electrophoresis. The *in vivo* stability was evaluated in a Balb-c mouse animal model. The results showed that the presence of surfactant was very useful in preventing the initial adsorption of EPO on the walls of vials and in minimizing protein aggregation. Amino acid combinations, glycine with glutamic acid, provided maximum stability. Formulation F4 (containing glycine, glutamic acid and Tween 20) showed minimum aggregation and degradation and *in vivo* activity equivalent to commercially available HSA-stabilized EPO (Eprex®).

Keywords: erythropoietin (EPO), amino acids, human serum albumin, protein stability, Bradford, ELISA, gel electrophoresis

Erythropoietin (EPO) is α-glycoprotein hormone of 165 amino acids, with molecular mass of 35 KDa, which is 40 % glycosylated (1, 2). This glycosylated part is a prerequisite for biological activity and has an important role for stabilization of the tertiary structure (3). It is produced by the peritubular capillary endothelial cells in the kidney and a small amount is produced in the liver (4, 5). It stimulates division and differentiation of the precursors of red blood cells (RBCs) in the bone marrow to mature red blood cells and prevent their apoptosis (1).

EPO has various indications, including treatments of anemia associated with kidney diseases, anemia associated with bone marrow transplantation, iron deficiency anemia,

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cancer- or antitumor agent-related anemia, and AIDS-associated anemia (6–9). However, EPO has low stability and degrades in aqueous solutions (10); moreover, it adsorbs to the inner surface of the wall of the syringe, which contributes to its aggregation and denaturation. Denaturation of EPO is generally irreversible. Thus, once denatured, EPO cannot recover its native properties. Further, the denatured protein structure is likely to cause hypersensitivity (11). Under such circumstances, various attempts have been made to increase the stability of EPO in aqueous solution. Some protein formulations solved denaturation by a lyophilization method (12). However, lyophilization has a number of drawbacks, including high manufacturing cost, the need of a large capacity freeze-dryer, and reconstitution prior to injection. Another approach is to incorporate protein stabilizers such as surfactants, like polysaccharides, serum albumin, macromolecules, amino acids and salts (11–14). Kawaguchi and Shimoda (13) introduced the use of stabilizers such as polyethylene glycol, proteins, saccharides, amino acids, and organic and inorganic salts to stabilize both lyophilized and aqueous formulations of EPO. The role of human serum albumin (HSA), dextran, cellulose and lecithin in preventing protein adsorption to the ampoule wall was described also by Kawaguchi and Shimoda (14). The recovery yield of EPO was improved from 16 to 69–98 % after storage for 2 hours at 20 °C (14). Konings *et al.* (15) reported the use of β-cyclodextrin as stabilizer in aqueous, lyophilized and spray-dried powder formulations of EPO, but with a risk of renal toxicity due to the use of cyclodextrin (15). Strickland (16) compared the stabilizing effect of HSA with other stabilizing agents such as benzyl alcohol, parabens, phenol or their combinations in different parenteral EPO formulations. The results showed low stability compared to the EPO formulation with HSA. Yamazaki *et al.* (17) reported the use of an amino acid, such as leucine, serine, glutamic acid, arginine or histidine as a stabilizing agent instead of HSA or purified gelatin; such formulations showed good stability. Stabilization with HSA carries a high risk of viral contamination, in addition to being more susceptible to allergic reactions. In Europe, the use of materials from human and animal sources as pharmaceutical additives is being increasingly restricted (11). For this reason, further studies are required for optimization of stable EPO formulations free from HSA.

The primary aim of this study was to optimize one or more aqueous parenteral EPO formulations free from blood derived products and to prove their stability both *in vitro* and *in vivo*.

EXPERIMENTAL

Materials

Erythropoietin was a gift from SPIMACO (Qassim, Saudi Arabia), Commassie blue was obtained from Merck (Germany), Poloxamer 188 NF, Sodium dodecyl sulphate and glycine were purchased from Sigma-Aldrich (USA), EPO ELISA kits from Roche (Basel, Switzerland). Silver staining kit was obtained from GE Healthcare Life Sciences, Sweden. All other chemicals and reagents were of pharmaceutical grade.

Methods

Preparation of standard erythropoietin formulation. – Thirteen EPO parenteral formulations were prepared aseptically (Table I). All formulations contained 40 μg of EPO and 5 mg mannitol to adjust the tonicity. The components of each formulation were dissolved in a

small volume of sterile phosphate buffer saline pH 7 (PBS). Then, the volume was completed to give ingredient concentrations per one mL using PBS as shown in Table I. The final solution was sterilized by membrane filtration using a 0.22-μm cutoff cup filter. Volume of 1mL of each formulation was filled in a sterile standard glass vial. Sixty vials were prepared for each formulation. Factors such as the effects of amino acid and surfactant and their type on the stability profile of EPO were investigated, resulting in various compositions of formulations. A formulation containing 2.5 mg HSA alone with 40 μg EPO (F13) was included for comparison, since it has a similar composition to that of the currently marketed EPO formulation (Eprex®, 1000 IU/0.5 mL, Janssen-Cilag Company, Canada). The formulation contains HSA (0.25 %), sodium chloride, sodium citrate, citric acids, and benzyl alcohol (0.9 %) in injection water. One IU is equivalent to 7.9 ng EPO.

Accelerated stability testing. – Thirty vials from each formulation were stored for two months at 25 and 40 °C in stability cabinets thermostatically controlled to ± 1 °C. Samples of five vials were taken from each formulation at each of the storage temperatures at time intervals of 0, 15, 30, 45 and 60 days. The samples were evaluated to detect EPO stability using the following analytical methods.

Bradford method. – This is a colorimetric analytical method applied for the quantitative determination of protein concentration, principally based on color transfer of a Coomassie dye from green or red (unbound) to blue (bound with protein). The intensity of color is proportional to the protein concentration (18). Simply, 1 mL of Bradford reagent was added to 100 μL of the sample and measured at λ_{max} 595 nm.

Enzyme linked immunosorbent assay (ELISA). – EPO-ELISA kit was used to detect EPO concentration according to the protocol provided by the following procedures. A standard EPO stock solution was prepared at a concentration of 1000 ng mL^{-1}. Then, serial dilutions were made to prepare concentrations of 10, 5, 2.5, 1.25 and 0.6 ng mL^{-1}. All standard solutions and EPO samples were incubated overnight in an ELISA plate pre-coated with rabbit polyclonal anti-hEPO capture antibody at 4 °C. After rinsing, the EPO-antibody complex bound to the plate surface was detected with the peroxidase-conjugated secondary antibody (HRP). After washing, tetra-methylbenzidine (TMB) was added for 10 minutes untill blue color started to come out. Finally, TMB solution was added to stop the reaction and absorption was measured at 450 nm.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). – SDS gel electrophoresis was used to detect any aggregation or degradation of EPO. Briefly, samples were electrophoresed with freshly prepared 12 % polyacrylamide gel using an electrophoresis system (Hoefer SE 280 gel electrophoresis unit, Hoefer Inc., USA). Gel sheets were silver stained as previously described (19). First, the gel was prepared to eliminate the interfering substances and thus avert the possibility of high background and/or poor contrast. Then, gels were sensitized to increase the sensitivity and/or contrast. Consequently, the gel was impregnated with the silvering agent. Images were developed by adding a dilute formaldehyde solution. Finally, the reaction was stopped to prevent overdevelopment.

Western-blotting. – It is a technique used to determine a specific protein by marking it with a proper antibody. Initially the samples were electrophoresed as described before, then the gel was blotted electrophoretically onto a polyvinylidene fluoride membrane and

assayed with rabbit polyclonal anti-hEPO capture antibody as the first antibody. Then the detection was done using peroxidase-conjugated secondary antibody. The bands were visualized by adding TMB as enzyme substrate.

In vivo *bioassay*. – The studies were carried out using 8-week old male Balb/c mice, weighing from 16–18 g, obtained from the animal care house, Faculty of Pharmacy, King Saud University (Riyadh, KSA). All the animals were healthy and used for the first time as experimental animals. The animals were housed in suitable cages with continuous food and water supply. The room temperature was adjusted to 20 ± 2 °C with twelve hours dark/light intervals.

The animal experiments complied with the United States National Institute of Health (US NIH) Guide for the Care and Use of Laboratory Animals (NIH Publication No. 18–23, 1985). The study protocol was approved by the Animal Care and Use Committee, College of Pharmacy. King Saud University (Riyadh, KSA).

The animals were divided into four groups, each containing 6 mice. A parallel study was performed where each group received one treatment by subcutaneous injection. Group one was a negative control group receiving sterile PBS. Groups 2, 3, and 4 received 150 IU of EPO solution in PBS, Eprex® and F4, respectively. Four days after injection, 5 μL blood samples were collected from the tail vein and mixed with 1 mL Retic-count reagent. The ratio of reticulocytes to RBCs was measured using a flow-cytometer (Becton Dickinson FACSCalibur, Biosciences, USA). Data were analyzed using the BD CellQuest software.

Statistical analysis

All the obtained data were statistically analyzed using one-way analysis of variance (ANOVA), followed by a *post hoc* test (Bonferroni test). The value of $p \leq 0.05$ was taken as the criterion for a statistically significant difference.

RESULTS AND DISCUSSION

Effect of surfactant on erythropoietin stability

Effect of the surfactant presence. – The effect of the presence of surfactant can be elucidated by comparing the results obtained with both F4 and F12. Both formulations were of the same composition (glycine and glutamic acid) except that F4 contained 0.1 mg Tween 20 while F12 did not contain any surfactant at all (Table I).

The initial EPO concentrations were 38.0 ± 1.9 and 33.1 ± 1.8 μg mL^{-1} for F4 and F12, respectively (Table II and Fig. 1). After two months of storage at 25 and 40 °C, F12 exhibited statistically significant ($p > 0.05$) reduction in EPO concentration compared to F4. The results determined by ELISA (Table III) also confirmed this finding. This can be attributed to the anti-adsorptive effect of the surfactant (Tween 20), which is in agreement with many reports in the literature (17, 21).

The SDS-gel images are shown in Fig. 2. A summary of the results is presented in Table IV. Neither aggregation nor degradation was seen for F4 at 25 °C. When stored at 40 °C, both F4 and F12 showed aggregation after 30 days and only F4 showed degradation after 60 days.

Table I. The exact composition of each of the prepared EPO parenteral formulations

	F1[a]	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11	F12	F13
EPO (μg)	40	40	40	40	40	40	40	40	40	40	40	40	40
Mannitol (mg)	5	5	5	5	5	5	5	5	5	5	5	5	
Glycine (mg)		6	6	6	6	6	6	6			6	6	
Leucine (mg)			1.5		1.5	0.5	1.5						
Arginine (mg)		1.5			0.5	1.5	0.5						
Glutamic acid (mg)				1.5			0.5		1.5	8	1.5	1.5	
Tween 20 (mg)		0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1			
Poloxmer NF 188 (mg)											0.1		
HSA (mg)													2.5

[a] The volume of each formulation was adjusted to 1 mL with phosphate buffer saline (PBS).

Table II. The remaining EPO concentration determined by the Bradford method in EPO formulations, at different time intervals, stored at 25 and 40 °C

Time	Day								
	0	15		30		45		60	
Formu-lation		Temperature (°C)							
		25	40	25	40	25	40	25	40
F1 (C)	[a]28.0±1.8	28.2±0.6	28.1±1.7	28.6±1.7	26.8±0.3	28.9±0.9	25.9±1.2	22.3±2.1	19.8±2.9
F2	37.9±1.7	35.7±3.1	32.9±1.3	35.9±0.1	29.0±0.3	35.9±1.0	29.8±3.1	33.3±0.8	24.0±2.8
F3	41.6±0.9	37.1±0.9	35.5±1.2	37.2±0.4	34.8±1.9	42.8±2.0	34.0±0.5	38.7±1.5	33.4±2.9
F4	38.0±1.9	36.3±1.9	33.5±0.4	38.3±0.8	32.5±3.4	40.6±0.6	35.2±0.8	40.9±3.2	34.0±1.5
F5	42.9±1.1	42.2±1.9	36.6±1.6	42.3±1.0	36.4±2.5	40.5±3.2	34.6±2.7	32.6±1.7	26.4±1.3
F6	37.5±0.5	41.8±2.0	32.1±0.8	36.8±1.1	32.8±0.9	36.3±1.5	30.0±0.5	28.3±1.6	22.3±3.2
F7	40.3±0.8	42.8±1.0	42.8±2.1	41.6±0.6	40.2±1.4	40.5±1.6	36.9±1.9	31.1±0.5	25.1±1.2
F8	41.2±0.7	39.0±2.5	42.2±1.7	39.0±2.5	40.9±0.3	39.6±0.7	35.9±0.4	39.6±1.3	30.1±0.7
F9	39.0±0.6	44.0±0.7	39.3±0.6	40.6±2.3	29.0±3.0	35.3±0.3	26.3±2.8	37.5±2.3	25.3±0.1
F10	34.1±2.7	35.2±2.3	31.2±2.2	33.9±0.3	23.5±0.9	32.2±1.8	23.5±0.8	32.1±0.9	24.8±0.3
F11	34.0±0.9	34.1±0.9	36.7±1.6	39.1±0.5	35.2±0.2	39.2±0.4	29.8±1.2	40.8±1.5	29.7±1.5
F12	33.1±1.8	30.5±1.6	27.8±2.3	35.3±2.0	27.8±2.7	31.9±1.3	25.3±0.8	30.6±3.0	26.5±2.2

[a] The remaining EPO concentration (μg mL^{-1}), mean ± SD, $n = 5$.

The anti-adsorbent effect induced by the presence of surfactant was attributed to the rivalry between the protein and the surfactant on the interface, leading to smaller driving force for protein surface adsorption (22). This was also pointed out by Chen and Dickinson (23). They showed that gelatin was displaced from the oil/water interface by the addition

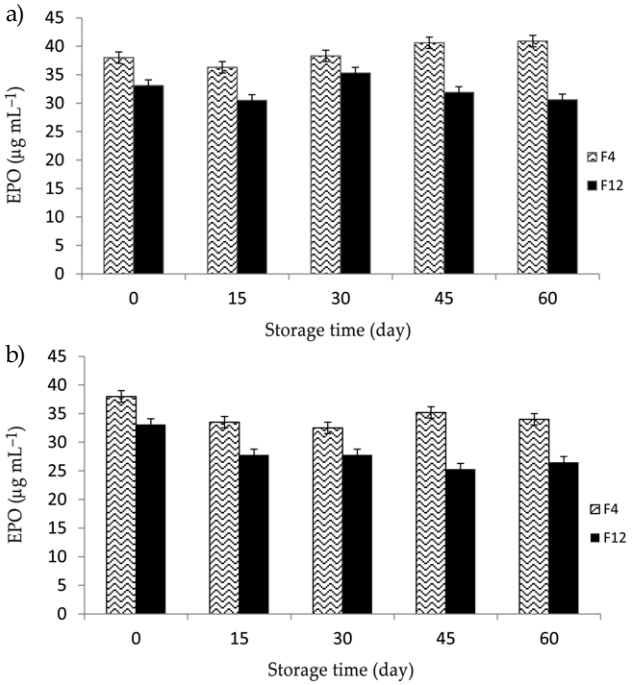


Fig. 1. Comparison between the remaining EPO concentrations for F4, F12 stored at: a) 25 °C, b) 40 °C for 60 days, determined by the Bradford method. Values represent mean ± SD, $n = 5$.

of a non-ionic surfactant (Tween 20). This effect also led to reduction in protein degradation as the surface tension was decreased. Surfactant concentration is vital for reducing both protein adsorption and damage. Randolph and Jones (22) recommended a surfactant concentration fairly higher than the critical micellar concentration (CMC) to provide protection against the harm induced by the protein adsorption on the interface.

Effect of surfactant type. – The effect of surfactant type on EPO stability was determined by comparing F4 (containing Tween 20) with F11 (containing poloxamer). Fig. 3 compares F4 and F11 regarding the amount of EPO remaining after the two-month storage at both 25 and 40 °C. The initial EPO concentration for F11 (34.0 ± 0.9 μg mL^{-1}) was significantly lower ($p > 0.05$) than that obtained with F4 (38.0 ± 1.9 μg mL^{-1}). For samples stored at 25 °C, EPO concentrations determined for both formulations at all time points were close, with statistically non-significant differences. For samples stored at 40 °C, among all the samples of both formulations, only F11 samples, taken after 45 and 60 days suffered a significant loss of a magnitude of 29.8 ± 1.2 and 29.7 ± 1.5 μg mL^{-1}, respectively. Gel images showed aggregation for all F11 samples stored at both temperatures but no degradation was detected. In the case of F4, neither aggregation nor degradation was noticed for samples stored at 25 °C, while at 40 °C, aggregation was detected for 30, 45, and 60 days samples and degradation was observed only for the 60-day sample.

Table III. The remaining EPO concentration determined by ELISA in EPO formulations, at different time intervals, stored at 25 and 40 °C

Time	0 Day	15 Days		30 Days		45 Days		60 Days	
Formulation		Temperature (°C)							
		25	40	25	40	25	40	25	40
F1 (control)	34.9±0.9[a]	34.2±0.9	26.3±1.3	34.4±0.8	22.7±3.8	25.9±1.2	19.1±1.2	25.9±1.3	23.7±1.0
F2	35.7±0.3	41.9±0.9	23.0±2.0	37.0±0.3	15.7±0.3	30.3±1.1	14.0±0.7	33.3±4.2	14.7±0.6
F3	36.2±1.8	40.6±0.1	18.5±1.9	37.8±1.8	11.0±2.9	29.0±2.2	12.8±1.4	34.8±2.8	10.5±1.6
F4	39.8±2.7	42.4±0.6	19.5±2.4	39.8±2.7	16.4±2.2	33.9±1.4	15.1±0.8	34.6±4.1	14.5±0.5
F5	39.2±1.3	36.2±2.2	14.7±1.7	38.0±1.5	12.0±0.7	24.1±0.1	6.3±0.2	20.9±1.2	3.8±0.1
F6	37.5±0.1	40.0±3.8	19.8±1.6	38.7±1.7	13.0±1.0	27.0±0.2	14.7±1.3	21.7±0.8	8.6±0.6
F7	42.1±0.1	37.0±1.3	17.4±0.8	38.2±1.0	9.2±0.1	26.0±0.3	9.0±0.6	21.5±0.5	3.6±0.1
F8	44.2±4.1	37.0±0.3	15.5±1.2	38.9±1.0	6.7±0.3	29.1±0.5	5.9±0.1	22.1±0.9	2.8±0.2
F9	39.3±2.0	27.8±0.5	9.9±0.4	32.9±1.4	7.7±0.4	27.2±4.7	2.9±0.1	27.1±4.6	3.5±0.4
F10	35.6±2.5	26.7±0.7	10.9±1.3	29.6±1.1	7.5±0.2	27.8±1.3	3.9±0.2	27.4±1.2	3.8±0.8
F11	34.9±3.3	33.7±1.2	28.3±2.3	31.2±1.5	22.0±1.6	28.5±2.2	10.3±1.0	10.3±1.0	10.0±1.5
F12	31±0.9	27.1±1.6	19.1±2.4	24.1±1.2	16.1±1.6	23.4±2.5	13.5±0.7	24.3±2.1	9.5±0.1
F13	33.0±1.7	26.6±3.1	20.9±0.1	28.2±3.5	21.8±0.7	24.4±0.9	13.9±0.3	13.3±0.9	12.2±2.8

[a] The remaining EPO concentration (μg mL^{-1}), mean ± SD, $n = 5$.

The remaining concentrations determined by ELISA were much lower for F11 (poloxamer) compared to F4 (Tween 20) at both storage temperatures, reaching a magnitude close to 10 μg mL^{-1}. The increase in F11 EPO concentrations determined by the Bradford method upon storage may be attributed to the possibility of interaction between the hydrophilic blocks of poloxamer with Coomassie giving an overestimated EPO concentration. This is evidenced by much lower concentrations obtained with ELISA for F11.

It was found that the beneficial effects of Tween 20 were superior to that of poloxamer 118. This may be attributed to the difference in CMC between the two surfactants. The reported CMC of poloxamer 118 is 1 mg mL^{-1} while it is 0.0987 mg mL^{-1} for Tween 20 (24). The concentration of surfactant used in all formulations was 0.1 mg mL^{-1}, which corresponds to Tween 20 CMC but is lower than that of Poloxamer 118. This can explain the superiority of Tween 20, since the use of surfactants at a high concentration is not recommended as it may alter protein stability (25).

Effect of amino acids on EPO stability

Effect of the amino acid presence. – It was evaluated by comparing the remaining EPO concentration of F1 (control with no amino acids and surfactants) with F12 (no surfactants but containing glycine and glutamic acid). Bradford results showed that F1 exhibited significantly lower ($p > 0.05$) EPO concentration than F12 at all time points. However, the re-

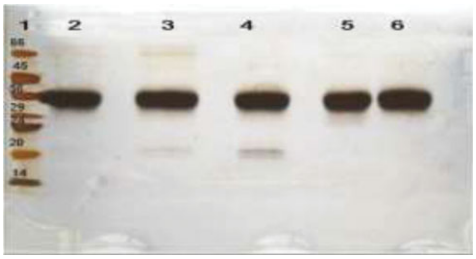


Image of SDS gel electrophoresis
Lane 1: Molecular mass marker
Lane 2: F4 stored for 15 days at 40 °C
Lane 3: F3 stored for 15 days at 40 °C
Lane 4: F2 stored for 15 days at 40 °C
Lane 5: F1 stored for 15 days at 40 °C
Lane 6: F1 stored for 15 days at 25 °C

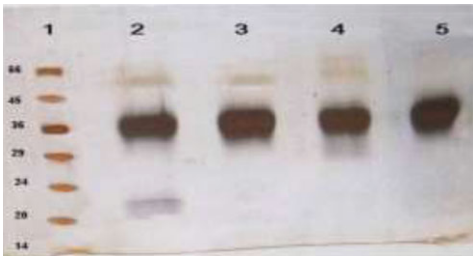


Image of SDS gel electrophoresis
Lane 1: Molecular mass marker
Lane 2: F2 stored for 30 days at 40 °C
Lane 3: F2 stored for 30 days at 25 °C
Lane 4: F1 stored for 30 days at 40 °C
Lane 5: F1 stored for 30 days at 25 °C

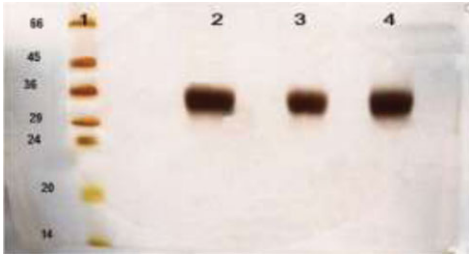


Image of SDS gel electrophoresis
Lane 1: Molecular mass marker
Lane 2: F4 stored for 15 days at 25 °C
Lane 3: F3 stored for 15 days at 25 °C
Lane 4: F2 stored for 15 days at 25 °C

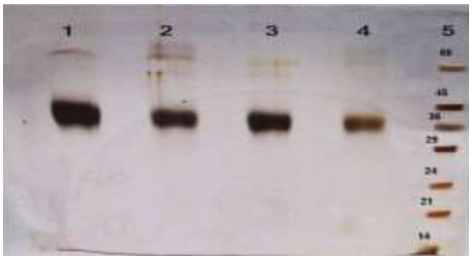


Image of SDS gel electrophoresis
Lane 1: F7 stored for 30 days at 25 °C
Lane 2: F7 stored for 30 days at 40 °C
Lane 3: F8 stored for 30 days at 25 °C
Lane 4: F8 stored for 30 days at 40 °C
Lane 5: Molecular mass marker

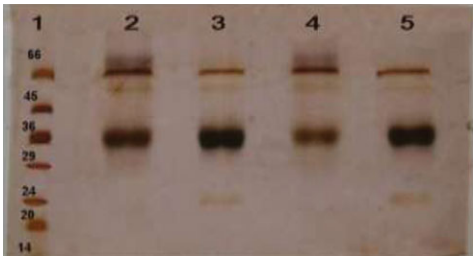
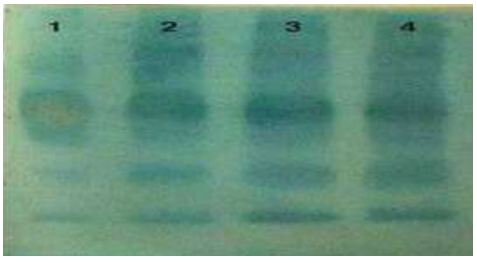


Image of SDS gel electrophoresis
Lane 1: Molecular mass marker
Lane 2: F9 stored for 60 days at 25 °C
Lane 3: F9 stored for 60 days at 40 °C
Lane 4: F10 stored for 60 days at 25 °C
Lane 5: F10 stored for 60 days at 40 °C



Western-blot image
Lane (1): F1 stored for 60 days at 40 °C.
Lane (2): F2 stored for 60 days at 40 °C.
Lane (3): F3 stored for 60 days at 40 °C.
Lane (4): F4 stored for 60 days at 40 °C

Fig. 2. Gel images of some EPO formulations.

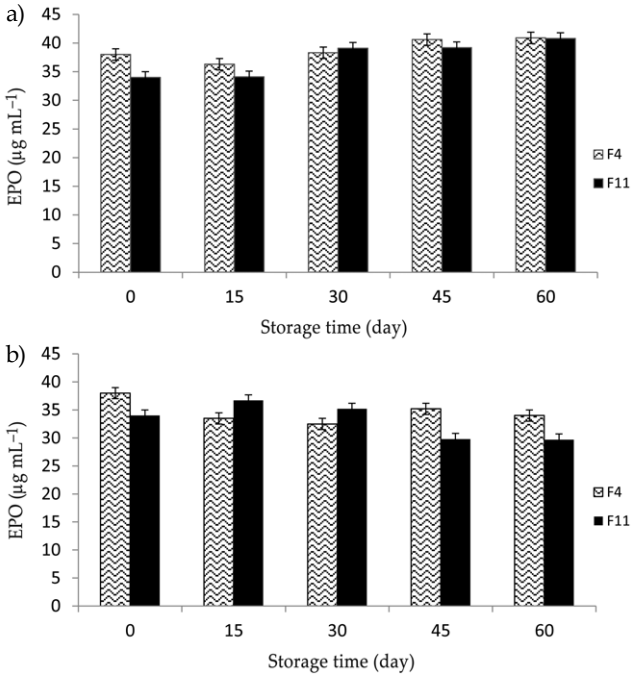


Fig. 3. Comparison between the remaining EPO concentrations for F4 and F11 stored at: a) 25 °C, b) 40 °C, for 60 days, determined by the Bradford method. Values represent mean ± SD, $n = 5$.

sults obtained by ELISA showed significant by lower ($p > 0.05$) concentration for F12 at a number of time points. The SDS gel images revealed aggregation only for samples stored at 40 °C for both formulations and degradation bands were reported only for F1 samples when stored at 40 °C.

Effect of the amino acid type. – Formulation development followed a number of sequences in order to explore the possible role of the amino acid type in the stabilization of EPO. The effect of glycine was studied initially since it is extensively used as a stabilizer in a number of EPO formulation patents (21, 26).

F4 and F9 were selected to evaluate the effect of glycine, since F9 is of the same composition as F4 except for the absence of glycine. Both Bradford and ELISA results revealed no significant difference in most points between the two formulations when they were stored at 25 °C, while for samples stored at 40 °C, F4 generally had significantly higher ($p > 0.05$) EPO content than F9. The gel electrophoresis images showed that the presence of glycine in F4 resulted in resistance to aggregation and degradation at 25 °C and delay of both of them at 40 °C compared to F9.

Effect of amino acid combinations. – The second step was to examine the effect of using different amino acid combinations. Three amino acids, leucine, arginine and glutamic acid, were selected to represent neutral, alkaline, and acidic amino acid, respectively. F2

Table IV. Summary of SDS-gel electrophoresis results for formulations stored at 25 and 40 °C

Time	15 Days		30 Days		45 Days		60 Days	
Formulation	Temperature (°C)							
	25	40	25	40	25	40	25	40
F1	–	–	–	A, D	A	A, D	A	A, D
F2	–	D	A	A, D	A	A, D	A	A, D
F3	–	A, D	A	A, D	–	A	–	A, D
F4	–	–	–	A	–	A	–	A, D
F5	–	–	A	A	A	A	A	A
F6	–	–	A	A	A	A	A	A
F7	–	–	A	A	–	A	A	A
F8	–	–	A	A	A	A	A	A
F9	A	A	A	A	A	A, D	A	A, D
F10	A	A	A	A	A	A	A	A, D
F11	A	A	A	A	A	A	A	A
F12	–	–	A	A	A	A	A	A

A – aggregation, D – degradation, (–) – refers to the absence of aggregation or degradation.

(glycine and arginine), F3 (glycine and leucine), F4 (glycine and glutamic acid), F8 (glycine alone) were selected. The values of the remaining EPO concentrations based on the ELISA technique were generally lower than those determined by the Bradford method for the four formulations, especially for samples stored at 40 °C, but they followed the same trend. Only with F4, neither aggregation nor degradation bands were noticed when stored at 25 °C while the other three formulations showed aggregation sometimes accompanied with degradation at various stages. In general, the results indicated that the amino acid type had a role in protecting against aggregation and the combination of glycine with glutamic acid (F4) was much better than glycine alone or its combination with leucine or arginine.

The third strategy was to study the effect of applying a combination of more than one amino acid type with glycine. In this regard, three formulations F5 (leucine + arginine, 3:1), F6 (leucine + arginine, 1:3) and F7 (leucine + arginine + glutamic acid, 3:1:1) were formulated and tested. The results of the Bradford method showed that all the three formulations suffered significant loss in the EPO concentration at the last time points (after 60 days at both storage temperatures) and F6 comprised the lowest EPO magnitude among them. The results obtained by the ELISA method were close to those obtained by the Bradford method for 25 °C samples while they were extremely lower for 40 °C samples. The gel images showed that all the three formulations showed aggregation bands after 30 days of storage at both temperatures but no degradation bands appeared for all the tested samples. Compared with F4, it is clear that F4 was superior to the three formulations, exhibited higher

EPO concentrations at both temperatures and showed higher resistance against aggregation. However, the three formulations were more protected against degradation.

The fourth step was to explore the effect of using different concentrations of glutamic acid alone. Two formulations, F9 (1.5 mg mL^{-1}) and F10 (8 mg mL^{-1}), were prepared. It is clear that significant loss in the EPO concentration initially occurred in the case of F10 and both formulations suffered higher rates of EPO loss compared to F4 at both temperatures. Gel images showed early detection of aggregation bands, starting from the first sample (day 15) while no degradation bands were seen in the samples, except for F9 after 45 days and F10 after 60 days of storage at 40 °C. It is concluded that glutamic acid alone was not useful and did not provide reasonable protection against adsorption and aggregation.

Human serum albumin versus *amino acids.* – F13, containing HSA, was not evaluated by the Bradford method and SDS-gel electrophoresis due to the interference caused by HSA with EPO. The molecular size of HSA would interfere with the band referring to aggregation in the case of the gel electrophoresis method and with color change induced by EPO in case of the Bradford method. The results obtained by ELISA showed that the initial EPO concentration (0 day sample) was significantly lower than in most of the formulations (F4, F5, F6, F7, F8 and F9) and very close to F1 (control). The remaining EPO concentration decreased with the increase in storage time at 25 °C, reaching a value of 13.3 ± 0.9 μg mL^{-1}. All the determined concentrations were lower than the corresponding concentrations obtained with F1. After storage at 40 °C, the determined concentrations shifted to lower values closing at 12.2 ± 2.8 μg mL^{-1}.

F1 (control), F2 (glycine and arginine), F3 (glycine and leucine) and F4 (glycine and glutamic acid) stored for 60 days at 40 °C were analyzed using the Western-blot method. These samples were chosen because their SDS images showed clear aggregated and degraded bands. Full polyvinylidene difluoride (PVDF) membrane images (Fig. 2) showed clearly bands for aggregated, degraded and pure EPO.

The effect of the amino acid properties on the stabilization of EPO was explored here for the first time by using different combinations of neutral, anionic and cationic amino acids. The results showed that the use of combinations was much better than using single amino acids. F4 (combination of glycine and glutamic acid) showed higher stability than F8 (glycine alone) and F9 and F10 (glutamic acid alone). Only a few patents are available in the literature for the use of different amino acids as EPO stabilizers. Choi *et al.* (27) introduced a stable EPO formulations containing Tween 20, glycine, propylene glycol and mannitol. They claimed stability of the formulation after 5 weeks of storage at 25 and 37 °C using size exclusion chromatography. The recovery was 93 % after storage at 37 °C for 5 weeks, with no detection of dimmers. However, this study did not evaluate the degradation of EPO. Another EPO formulation, containing L-histidine and Tween 80, was presented by Yamazaki *et al.* (17). They showed that a recovery of 99 % was achieved when the formulation was stored for 2 weeks at 40 °C and 6 months at 25 °C using RP-HPLC and SDS-gel electrophoresis. The authors claimed no EPO degradation for samples stored at 40 °C, but nothing was said about degradation of samples stored at 25 °C, and the stability of the formulation against aggregation was never addressed. The application of as elevated temperature of 40 °C for accelerated stability testing appeared to be very harsh for EPO as all the formulations showed either aggregation or degradation and some formulation showed both.

The discrepancy between the results obtained by ELISA and Bradford can be explained by the principle of the ELISA test that depends upon the reaction between the specific EPO antibody and certain epitope in the EPO molecule. In the case of aggregation, some of the epitopes will be covered and not disclosed to the antibody, resulting in smaller amounts in the determined concentration compared to the corresponding concentrations determined using the Bradford method. Since their gel electrophoresis images showed aggregation bands, this explains the significantly lower concentrations determined by ELISA for F5-F12 stored at 25 °C compared to the corresponding concentrations determined by Bradford method. On the other hand, the absence of aggregation bands for F4 (glycine and glutamic acid) was accompanied by closer concentrations determined by both methods at 25 °C. The tremendously lower ELISA-determined concentrations exhibited for all the formulations when stored at 40 °C are attributable to the presence of aggregation bands at all time points. However, in the case of degradation, the ELISA-determined concentrations may not be affected as long as the epitope is exposed. The results obtained by Western blot allowed this conclusion, since the specific EPO antibody used for ELISA reacted with both the aggregation and degradation bands as well as with the original EPO band. Higher ELISA determined concentration for F1 (control) after 60 days at 40 °C compared with the corresponding concentration obtained by the Bradford method can be explained by the degradation proven by gel electrophoresis leading to the over-expression of EPO concentration. Thus, one can conclude that ELISA cannot be considered as a stability indicating assay and, probably, this is the reason for not including it in the EPO analytical tests recommended by the *European Pharmacopoeia* (20).

The optimum formulation

It was clear that among all the tested formulations F4 (containing glycine and glutamic acid together with Tween 20) was the best formulation, since it retained a high concentration of EPO with minimum loss after a two-month storage at 25 °C. Their SDS-gel images showed neither aggregation nor degradation bands and the ELISA determined concentrations were comparable with those determined by the Bradford method. In comparison with F13 (containing HSA), F4 was superior, with significantly higher concentrations at all time points for samples stored at 25 °C.

In vivo *bioassay*

F4 was selected for this part because it was previously shown as the most stable among all the prepared formulations. The average reticulocytes to red blood cells ratio obtained with the negative control group (received only PBS) was 0.13 ± 0.02, while values of 0.33 ± 0.06 and 0.27 ± 0.03 were achieved with both positive control groups receiving pure EPO solution in PBS and Eprex®, respectively. The average reticulocytes to red blood cells ratio obtained with the animal group receiving F4 was 0.33 ± 0.04. Statistical analysis showed that there was no significant difference between F4, pure EPO solution and Eprex®, at $p > 0.05$. This indicates that F4, the formulation showing high *in vitro* stability, has proven to be active *in vivo* and the constituents of the formulation did not affect the physiological activity of EPO.

CONCLUSIONS

The study revealed that the presence of surfactants is important to enhancing EPO stability by reducing adsorption on the surfaces of containers and the tendency of aggregation. However, they may also enhance the degradation rate. Further, the surfactant type is a considerable factor in the formulation of an EPO stable formulation, so Tween 20 is more effective than Poloxamer 118 in the protection of EPO against adsorption and aggregation, while Poloxamer 118 may provide more protection against degradation than Tween 20. Also, the presence of amino acids may reduce the magnitude of EPO adsorption and the amino acid type has a role in the protection of EPO, since the combination of glycine with glutamic acid was much better than glycine alone or its combination with leucine or arginine.

Another point is that ELISA cannot be regarded as a stability indicating assay because degraded protein can be determined. The diligence of the storage condition at 40 °C as elevated temperature for accelerated stability testing appeared to be very harsh for EPO as all the formulations showed either aggregation or degradation, while some formulations showed both. Finally, there is no single analytical technique that can be used for the evaluation of protein in general and the use of gel electrophoresis is highly recommended for explaining the significance of ELISA results.

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