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Erythropoietin Biosimilar Products and Immunogenicity: A Pharmacovigilance Study

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Abstract

Aim: The aim of the present study was to evaluate the immunogenicity of a generic product of erythropoietin registered in Jordan, by detecting the presence of anti-recombinant human erythropoietin antibodies in the serum via an ELISA technique.

Materials and Methods: Briefly, polystyrene micro-titer plates (96-well) were coated with rhEPO (the generic product) at 10 µg/1mL. Goat anti-human IgG:HRP or rabbit polyclonal to human IgM:HRP was added to the wells and incubated. A prepared substrate solution was then added to each well. The absorbance was measured with a microplate reader after green color development (n=3). The sera of 95 patients were tested for the presence of IgM or IgG antibodies.

Results: Antibodies were detected in 26.3% of the population; where 16.8% were found to have only IgG antibodies, 7.4% had only IgM antibodies, and 2.1% had both antibodies. Cigarette smoking correlated significantly with the development of IgG antibodies. Moreover folate administration correlated inversely with decreasing the risk of developing IgM antibodies**.**

Conclusions: this study proves the immunogenic effect for this product.

Keywords: Recombinant human erythropoietin, anti-erythropoietin antibodies, ELISA, immunogenicity, pharmacovigilance.

1. Introduction

Anemia is one of the most common complications of chronic renal failure (CRF) [1-2].The primary cause of such condition is insufficient production of endogenous erythropoietin (EPO) by the kidney, which is known to be the principal regulator of erythropoiesis [3]. Since the introduction of recombinant human EPO (rhEPO) in late 1980s, it has been used successfully and safely to treat anemia in patients with CRF, chemotherapy and malignancy induced anemia [4-5]. It is one of the biologically active biopharmaceuticals and has the potential to evoke an immune response [6-7]. Post-authorization pharmacovigilance can prove crucial in minimizing the risk of developing unwanted outcomes such as immunogenicity [8]. Several factors were known to affect rhEPO immunogenic reaction. Principally the presence of impurities in the product, structural modifications due to the manufacturing process and storage conditions can effectually increase immunogenicity [9]. Besides, intravenous route of administration was found less immunogenic than intramuscular or subcutaneous routes of administration [11-12].

One prominent example was Eprex® (epoetinalfa) associated immunogenicity, where a minor change in the formulation resulted in the development of neutralizing antibodies (Abs) in some patients [11-12]. Basically the interaction between the uncoated rubber stoppers and the addition of new stabilizer (polysorbate 80) to the formulation in the pre-filled syringes was involved [13, 14, 16]. Recently anti-rhEPO Abs has been detected in CRF patients who developed sudden anemia or pure red cell aplasia (PRCA), along with loss of response to rhEPO. Henceforth it is highly recommended to test for anti-rhEPO Abs in any patient who develops sudden, unexplained anemia following treatment with any type of rhEPO products [4-5]. Taken together the aim of the present study was to evaluate the immunogenicity of an erythropoietin generic product that is registered in Jordan and given to patients with CRF who are on regular hemodialysis, by detecting the presence of anti-rhEPO Abs in their serum using the enzyme-linked immunosorbent assay (ELISA).

2. Materials and Methods

2.1 Materials

Native Human IgG andgoat Anti Human IgG: HRP were purchased from ABD Serotec Inc. (UK); Goat polycolonal to Rabbit IgG- H&L (HRP) and rabbit polycolonal to Human IgM (HRP) were obtained from Abcam Inc. (UK); Anti-human EPO antibody were from R&D System Inc. (Europe); Bovine serum albumin, substrate (2, 2 azino-bis (3-ethyl benzothiazoline-6-sulfonic acid) and Tween 80 were procured from Sigma-Aldrich Inc. (USA)

2.2 Serum samples

Serum samples were collected from patients with end stage renal disease (ESRD) who are on regular hemodialysis three times weakly and administered regular rhEPO of the same generic product with a fixed dose of 4000 IU twice weekly. Blood samples were collected (5-7ml each) in sterile serum clot activator test tubes. Patient's data were collected from their records in the hemodialysis units of five different hospitals in Amman / Jordan. The data included personal and medical information (e.g. age, gender, disease duration, cause of the disease, other diseases/medications used, cigarette smoking, hemoglobin readings and others). Negative controls are prepared by pooling sera from six healthy individuals including three males and three females who did not take any rhEPO products. Positive controls were not included in this study due to the lake of confirmed positive samples for that specific generic product. Since this research involves humans, an IRB approval had been sought and an informed consent form was signed by each patient. The serum was harvested by centrifuging the coagulated blood samples at a speed of 3000 rounds/min for 5 minutes at a temperature of 4^{o}C. Then the serum samples were labeled and stored as aliquots at -20°C until analysis. Exclusion criteria: immunosuppressant agents.

2.3 Antibodies detection using ELISA Assay

An ELISA for the detection of Abs was developed and modified on the lab based on previous work [17]. Briefly, 96-well polystyrene micro-titer plates were coated with rhEPO (the generic product) at 10 µg/1mL PBS (pH 7.4), and then incubated overnight at 37ºC. The plates were washed five times with PBS containing 0.5 mL/L Tween 80 to remove the free unbound rhEPO. After that the plates were coated with PBS containing 3 g/mL bovine serum albumin (BSA) for 4 h at room temperature (R.T). The contents of the wells were removed and 100µL of serum dilution (1:50) was added to each well and incubated for 1 h at RT. Plates were then washed five times as mentioned above. After that 100 µL of goat anti-human IgG:HRP or rabbit polyclonal to human IgM (HRP) was added to the wells and incubated for 1 h at RT in a dark place. Post washing, 100 µL of already prepared substrate solution (2, 2 azino-bis (3-ethyl benzothiazoline-6-sulfonic acid) were added to each well and allowed to stay at RT in a dark place. The absorbance was measured with a microplate reader at 405nm at 10, 20, 30 min after developing the green color (Bio-Tek Instruments, USA). Sera from six healthy blood donors were used as control group. All experiments were performed in triplicate (Fig. 1).

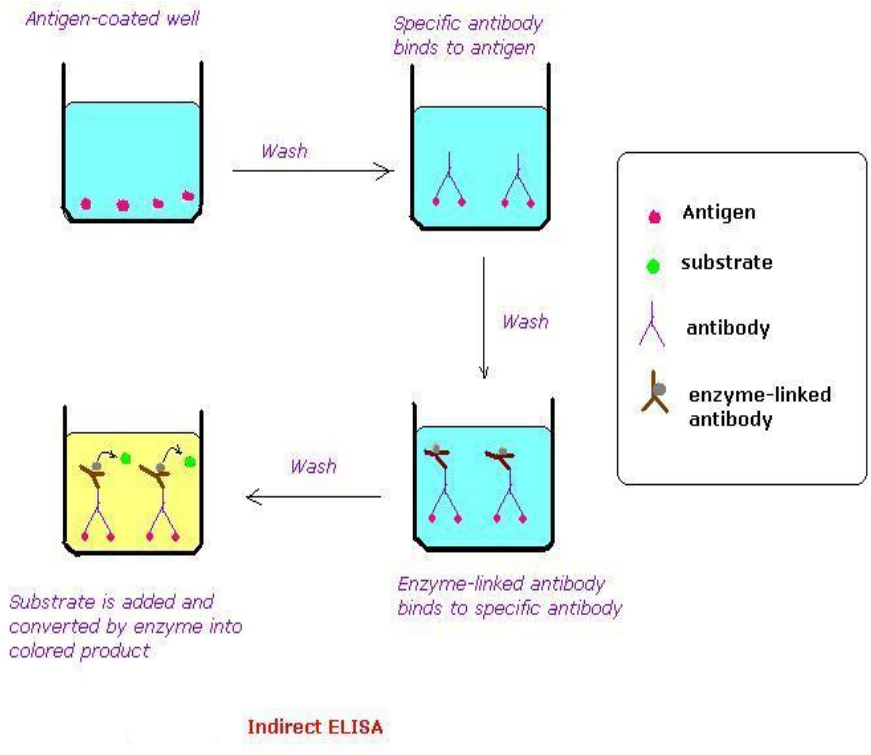

Figure 1: The principle of the test

2.4 Method optimization

There are different types of assays that have been used for the detection of anti-rhEPo Abs, which include ELISA, radio-immunoprecipitation (RIPA), and the BIAcore biosensor assay [18]. In early studies, screening was primarily performed using RIPA [19-20]. However, as RIPA requires the use of radioactive material and is difficult to perform, this led to the development of double antigen-bridging ELISA demonstrating advantages such as high specificity and good practicality over other immunoassay formats for the analysis of anti-rhEPO Abs [21]. High sensitive assays decrease the probability of false-negative findings [18]. While a high specific assay has a very low probability of measuring false positives resulting from nonspecific binding of Abs. Most assays use blocking reagents that reduce or eliminate nonspecific Abs binding [18]. Each assay has its own advantages and disadvantages (Table 1).

Table 1: Advantages and disadvantages of the different Abs assay methods [18]

Assay	Advantages	Disadvantage	Susceptibility to false positive or false negative results
RIP	Highly sensitive, common usage, wide experience, multiple parameters, inexpensive, versatile and/or different types	Radiometric, rapid decay of radio labeled antigens, non-automated procedure requiring centrifugation or microfiltration, not easily scaled up, may not detect all Ab isotypes especially IgM	Susceptible to interference with IgM rheumatoid factor or high backgrounds with some sera
ELISA	Highly sensitive, common usage, wide experience, multiple parameters, scalable and automated, non radiometric, Ab isotype analysis, inexpensive, versatile and/or different types	High background readings in some assays, toxic enzyme substrate reagents, does not always measure low affinity Abs	Susceptible to interference with IgM rheumatoid factors (false positive) or naturally occurring Abs, competition among Ab isotypes can hamper isotype analysis (false negative), nonspecific binding can cause high background readings and reduce sensitivity
BIAcore	Real time binding and competitive analysis of both low and high affinity Abs and isotypes, epitope mapping, ligand purification analysis, Ab affinity measurements, semi automated, sequential testing of samples on single sensor chip	High cost, poor access to instrument, lack of expertise among researchers	Remarkably non-susceptible to contaminating matrices and interfering factors
Bioassays	Only measures neutralizing Abs	Only moderately sensitive, can be made automated, growth dependent assays can be time consuming, different cell lines used among labs, not easily scalable	Serum neutralizing factors arising from existing morbidities could result in false positive for neutralizing Abs against therapeutic proteins, serum factors other than Abs can results in false positive, so bioassay results must be interpreted in light of immunoassay results

To date, there are no definite recommendations for which assay should be used as the standard method for the screening of anti-rhEpo Abs. This study used indirect ELISA assay after being standardized and properly validated in the lab. It is highly sensitive, and one of the most commonly used methods, non- radiometric, inexpensive, versatile and adaptable [22-23]. The first variable to be standardized was rhEPO coating concentration, Urra *et al.*, [17] used rhEPO at a concentration of 10µg/ml. Two different concentrations were tested in the lab, 10µg/ml and 20µg/ml; no significant differences in the absorbance readings were detected, so to decrease the amount of the antigen (rhEPO) used in the coating process, 10µg/ml concentration were used in the study. The second variable, incubation conditions including: incubation time and temperature; four different incubation conditions were tested : the plates were incubated for one night at 4°C, two days at 4°C, six days at 4°C, and for one night at 37°C.

The results showed a significant improvement in the detection of the Abs using one night incubation at 37°c; this is similar to what was done by Gross *et al.*, [24]. According to the manufacturer recommendations, the concentration of anti-human EPO Abs ranges between 0.5µg/ml and 1µg/ml, during optimization process different concentrations were tested: 0.8, 0.4, 0.2, 0.1, and 0.05µg/ml, we went down in the concentration to check for the sensitivity level, this was proved and 0.4 µg/ml concentration were used during the standardization process. The manufacturer recommendation is to use Goat polyclonal to Rabbit IgG-HRP at 1:120000 dilutions. Two different dilutions: 1:120000 and 1:40000 were tested; the absorbance readings were within the detectable range of the microplate reader (0.2-1.2) at the 1:40000 dilutions.

All the above mentioned standardization procedures were done before starting using the serum samples of the patients and the control group. The next step of optimization process was done using the serum samples. The antigen (rhEPO) coated at a concentration of 10µg/ml and incubated for one night at 37°c, as these two factors were previously optimized. Different serum dilutions: Serums as such, 1:4, 1:10, 1:50, 1:100, 1:200 and 1:400 were screened. The best

results were obtained at the 1:50 serum dilutions, at which the absorbance readings were within the acceptable range of the microplate reader (0.2-1.2), when measured at 10, 20 and 30 minutes. This resembles what was done by Urra *et al.*, [17] in which they used different serum dilutions ranging from 1:50 to 1:800. The suggested dilution for Goat anti human IgG: HRP by the manufacturer ranges from 1:4000 to 1:8000, in this study 1:3000, 1:4000, and 1:8000 dilutions were tested, and the most reasonable absorbance readings were obtained at the 1:8000 dilutions with minimal interferences. Rabbit polyclonal to human IgM (HRP) at 1:1000 dilutions were used as recommended by the manufacturer and a reasonable absorbance reading were obtained. Native human IgG at 1:5000 and 1:100000 dilutions were used to eliminate any possibility of non-specific binding of any IgG Abs present in serum samples, this step used to test for high specificity of the assay. The last step in the standardization process was coating some wells with native human IgG instead of rhEPO, it was done to make sure that the enzyme linked Goat anti human IgG: HRP would bind with the coated IgG.

2.5 Statistical Analysis

Data were analyzed using SPSS© software (version 17.0). A (P-value $\leq$ 0.05) was considered statistically significant. Continuous variables were presented as mean ± SD, and categorical variables were presented as frequencies and percentages. Unpaired sample t-test and nonparametric Mann–Whitney test were used for continuous variables. Fisher exact test was used to compare categorical variables.

3. Results and Discussion

3.1 Baseline patients' characteristics

There are many generic rhEPO products registered in the Jordanian market and used for the management of anemia in patients with CRF who require regular hemodialysis. Since these products are considered biosimilars for the originally marketed product (Eprex®), the evaluation of their immunogenicity is of great importance. Many factors influence the immunogenicity of protein-based therapeutics. Thus, the detection and characterization of Abs against protein-based therapeutics is a considerable task. Only clinical studies and careful monitoring of the market can be used to conclusively demonstrate rates of immunogenicity in humans for protein therapeutics [14]. Fully validated, reliable, and robust *in vitro* assays, in combination with sensitive bioassays, are much needed to address these issues. The serum samples of 95 patients with ESRD were collected from the hemodialysis units in different hospitals. These patients were received the same rhEPO generic product under investigation. The ethical committees of the hospitals have approved the protocol of this study. **Table 2** shows the baseline characteristics of the studied population.

Table 2: Participants' baseline characteristics

Patient characteristic	(Frequency) %
Age of patients (mean ± SD)	53.6 ± 16.2 (years)
Min.	14
Max.	85
Gender	
Females	(43) 45.3%
Males	(52) 54.7%
Smoking status	
Smoker	(16) 16.8%
None smoker	(76) 80%
Diabetes mellitus (DM)	(44) 46.3%
Hypertension (HTN)	(56) 58.9%
Ischemic heart disease (IHD)	(15) 15.8%

3.2 Development of antibodies against rhEPO

At present, it is inconceivable to manufacture a biopharmaceutical without the risk of adverse effects. Authorization of biosimilar products is one of the substantial topics in the pharmaceutical industry. However, post-authorization pharmacovigilance is warranted to reduce the risk of developing unwanted outcomes of the biosimilar product such as immunogenicity and to guarantee its post-marketing quality, safety and efficacy. In this study, the immunogenicity effect of the rhEPO product was investigated. The serum samples of 95 patients were tested for the presence of IgM or IgG Abs; twenty five out of 95 patients were found to have Abs against this product with a prevalence of 26.3%. Explicitly 16 patients (16.8%) had IgG Abs only, 7 patients (7.4%) were found to have IgM Abs only, 2 patients (2.1%) had both Abs, and the remaining 70 patients (73.7%) were antibody-free. **Table 3** shows the four groups divided according to their IgG and IgM status.

Table 3: Immuniglobulin (Ig) status in study participants

Ig Status	Frequency (%)
IgG	16 (16.8%)
IgM	7 (7.4%)
Both Ig	2 (2.1%)
Neither Ig	70 (73.7%)

3.3 The effect of different patient characteristics on the immunogenicity

The effect of some patient characteristics; as in gender, hypertension, diabetes, Ischemic Heart Disease, smoking, drugs, weight, and the duration of using rhEPO, on development of IgG and IgM were investigated. Impressively, only smoking significantly associated with developing IgG Abs with a *P*-value of 0.035. Other studied characteristics did not correlate significantly (**Table 4**). Noteworthy, none of these studied patient characteristics correlated to IgM Abs development. Thus smoking might be among the prominent risk factor for developing a chronic immunologic response. However, further studies are still required to confirm this conclusion.

Table 4: The effect of different participants' characteristics on the development of immune response

Variable	IgG positive patients (n=18)	IgG and IgM negative patients (n=70)	*P*-value
Gender (male)	(12) 66.7 %	(38) 54.3 %	0.42
Hypertension	(8) 44.4 %	(44) 62.9 %	0.185
Diabetes	(6) 33.3 %	(34) 48.6 %	0.296
Ischemic heart disease	(2) 11.1 %	(13) 18.6 %	0.726
Smoking	(7) 38.9 %	(9) 13.2%	0.035*
Weight (kg)	68.29 ± 14.19	67.43 ± 22.07	0.827
Duration of using rhEPO (years)	4.57 ± 2.98	3.90 ± 2.73	0.518

3.4 The effects of different medications and supplements

The effects of different medications and supplements commonly used by patients with ESRD, like calcium, vitamin D, iron, folic acid, furosemide, beta blockers, aspirin, statins, calcium channel blockers, isosorbide dinitrate, insulin, ACEI and proton pump inhibitor were also investigated. Results showed that only folic acid use could have a nearing significant protective effect (*P*= 0.051) against the development of IgM. These results agree with other previous studies that render the folate deficiency among the risk factors in the development of rhEPO resistance [6]. Nevertheless none of the administered medications and supplements, even folic acid, had a prophylactic effect against IgG development.

3.5 Hemoglobin (Hgb) readings and IgG development

The means of the Hgb readings for five months between the different groups of patients were also investigated (Table 5). Importantly no significant variations were recognized between any of the five Hgb readings. Interestingly the Hgb means of the IgG positive-patients had the tendency to drop over time when compared to IgG and IgM-negative patients' respective values. In fact, the mean of Hgb values dropped by 0.9 g/dL in five months.

Table 5: The difference in the means of Hgb readings over five months between the patients who had a chronic immune response against and those who did not have any immune response

Hgb readings	IgG Positive patients	IgG and IgM Negative patients	*P*- value
1 (oldest)	10.1 ± 1.9	9.9 ± 1.0	0.892
2	10.1 ± 1.6	10.2 ± 1.7	0.926
3	9.5 ± 1.8	10.0 ± 1.6	0.550
4	9.3 ± 0. 9	9.9 ± 1.5	0.192
5 (newest)	9.2 ± 1.1	9.9 ± 1.5	0.059

4. Conclusions

This study proves the immunogenic effect for this product, by the detection of anti-rhEpo Abs in the serum samples. Further investigation required to evaluate the neutralization effect of the detected anti-rhEpo Abs using bioassays to confirm the negative impact resulted from blocking the rhEpo pharmacological activity (Abs mediated PRCA). Also a strong association between smoking and the development of chronic immune response (IgG Abs) is displayed. Markedly folate supplementation in preventing the development of an acute immune response (IgM Abs) was substantiated.

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References

[1] Astor BC, Muntner P, Levin A, Eustace JA, and Coresh J, Association of kidney function with anemia: the third national health and nutrition examination survey (1988–1994), *Archiv Intern Med*, 2002; 162:1401-1408.
[2] Hsu CY, Mcculloch CE, and Curhan GC. Epidemiology of anemia associated with chronic renal insufficiency among adults in the United States: results from the third national health and nutrition examination survey, *J Amer Soc Nephrol*, 2002; 13: 504-510.
[3] Fehally J, Floege J and Johnson R. Comprehensive clinical nephrology, Mosby Elsevier, 2003; 853–60.
[4] Casadevall N, Nataf J, Viron B, Kolta A, Kiladjian JJ, Martin-Dupont P, Michaud P, Papo T, Ugo V, Teyssandier I, Varet B and Mayeux P. Pure red-cell aplasia and anti- erythropoietin antibodies in patients treated with recombinant erythropoietin, *New Engl J Med*, 2002; 346:469–475.
[5] Tacey A, Greway A, Smiell J, Power D, Kromminga A, Daha M, Casadevall N, Kelley M. The detection of anti-erythropoietin antibodies in human serum and plasma: Part I. Validation of the protocol for a radio immunoprecipitation assay. *J Immunol Meth*, 2003; 283:317-329.
[6] Roger S, Mikhail A. Biosimilars: Opportunity or cause for concern? *J Pharm. Pharm. Sci*. 2007; 10: 405-410.
[7] Committee for medicinal products for human use. Guideline on similar biological medicinal products. CHMP/437/04 2005.
[8] Locatelli F, Roger S. Comparative testing and pharmacovigilance of biosimilars, *Nephrol Dial Transplant*, 2006; 21 (Supp. 5): v13-v16.
[9] Kessler M, Goldsmith D, Schellekens H. Immunogenicity of biopharmaceuticals, *Nephrol. Dial. Transplant*. 2006; 21 (Supp 5):v9-v12.
[10] Schellekens H, Casadevall N, Immunogeicity of recombinant human proteins: causes and consequences, J. Neurol. 2004; 251 (Supp 2):II4-II9.
[11] SCHELLEKENS H. Immunologic mechanisms of EPO-associated pure red cell aplasia, *Best Pract. Res. Clin. Haematol.* 2005; 18:473-480.
[12] Casadevall N. Antibodies against rHuEPO: native and recombinant, *Nephrol. Dial. Transplant.* 2002; 17 (Supp 5):42-47.
[13] Mckoy JM, Stonecash RE, Cournoyer D, Rossert J, Nissenson AR, Raisch DW, Casadevall N, Bennett CL. Epoetin-associated pure red cell aplasia: past, present, and future considerations, *Transfusion* 2008; 48 (8): 1754-1762.
[14] Kromminga A, Schellekens H. Antibodies against erythropoietin and other protein-based therapeutics: an Overview, *Ann. NY Acad. Sci*. 2005; 1050: 257-265.
[15] Schernthaner G. Immunogenicity and allergic potential of animal and human insulins, *Diabetes Care*, 1993; 16:155-165.
[16] Kruger A, Eckardt KU. Pure red cell aplasia induced by antibodies against human recombinant erythropoietin, *Trans Med Hemother*, 2005; 32:97-100.
[17] Urra J, Torre M, Alcazar R, and Peces R. Rapid method for detection of anti-recombinant human erythropoietin antibodies as a new form of erythropoietin resistance, *Clin Chem*, 1997;43:848-849.
[18] Thorpe R, and Swanson SJ. Current Methods for Detecting Antibodies against Erythropoietin and Other Recombinant Proteins, *Clin Diagn Lab Immunol*, 2005; 12: 28–39.
[19] Casadevall N, Nataf J, Viron B, Kolta A, Kiladjian JJ, Martin-Dupont P, Michaud P, Papo T, Ugo V, Teyssandier I, Varet B, and Mayeux P. Pure red-cell aplasia and anti- erythropoietin antibodies in patients treated with recombinant erythropoietin, *New Eng J Med*, 2002; 346:469–475.
[20] Tacey A, Greway A, Smiell J, Power D, Kromminga A, Daha M, Casadevall N, and Kelley M. The detection of anti-erythropoietin antibodies in human serum and plasma: Part I. Validation of the protocol for a radio immunoprecipitation assay, *J Immunol Meth*, 2003; 283:317.
[21] Hoesel J, Gross J, Moller R, Kanne B, Wessner A, Mueller G, Mueller A, Gromnica-Ihle E, Fromme M, Bischoff S, and Haselbeck A. Development and evaluation of a new ELISA for the detection and quantification of anti erythropoietin antibodies in human sera, *J Immunol Meth*, 2004; 294:101.
[22] Swedler W, Wallman J, Froelich CJ, and Teodorescu M. Routine measurement of IgM, IgG, and IgA rheumatoid factors: high sensitivity, specificity, and predictive value for rheumatoid arthritis, *J Rheumatol*, 1997; 24:1037–1044.
[23] Svojanovsky SR, Egodage KL, Wu J, Slavik M, and Wilson GS. High sensitivity ELISA determination of taxol in various human biological fluids, *J Pharmaceut Biomed Anal*, 1999; 20: 549–555.
[24] Gross J, Moller R, Henke W, and Hoesel W. Detection of anti-EPO antibodies in human sera by a bridging ELISA is much more sensitive when coating biotinylated rhEPO to streptavidin rather than using direct coating of rhEPO, *J Immunol Meth*, 2006; 313:176–182.