Scientific paper

Report on Isoelectric Focusing Trial of Erythropoietin Profiling in Two Cancer Patients During Chemotherapy and Darbepoetin Treatment

Klemen Španinger,[1] Simona Borštnar,[2] Erika Matos,[2] Barbara Možina,[2] Arthur J. Sytkowski,[3] Radovan Komel[1] and Nataša Debeljak[1,]*

[1] Institute of Biochemistry, Faculty of Medicine, University of Ljubljana, Slovenia

[2] Institute of Oncology, Ljubljana, Slovenia

[3] Division of Hematology and Oncology, Beth Israel Deaconess Medical Center, Department of Medicine, Harvard Medical School, Boston, USA

** Corresponding author: E-mail: natasa.debeljak@mf.uni-lj.si*

Received: 29-10-2010

Abstract

The level of erythropoietin, main regulator of erythropoiesis, is affected by hypoxia, anaemia, application of recombinant erythropoietin, chemotherapy and others. Isoelectric focusing (IEF) combined with double immunobloting is a method that enables distinct analysis of endogenous and recombinant erythropoietin isoforms.
Aim of our study was to set up analysis of treatment effects on the pattern of endogenous erythropoietin in anaemic breast cancer patient. Urine and blood samples were collected during and after termination of the treatment and analysed by isoelectric focusing. Endogenous erythropoietin was found lower, but still detectable during darbepoetin treatment. Normal shift of erythropoietin isoforms between serum vs. urine, ordinary seen in healthy volunteers, was not observed indicating kidney damage. The patient was suffering from heavy proteinuria and had low Glomerular filtration rate indicating acute renal failure, probably caused by clinical status or cisplatin chemotherapy.
IEF has not yet been used for follow up of erythropoietin profile in cancer patients. It enables to monitor the effects of treatment on the level of endogenous erythropoietin and indirectly indicates kidney function.

Keywords: Erythropoietin, darbepoetin alpha, detection, cancer, cisplatin chemotherapy, isoelectric focusing

1. Introduction

Erythropoietin (Epo), the main regulator of erythropoiesis,[1] is synthesized in kidneys in cortico-interstitial cells. Recombinant human Epo (rHuEpo; epoetins and darbepoetin) can be administered to patients with cancer in order to reduce anaemia, including that associated with chemotherapy.[2] Treatment of anaemia improves the patients' quality of life, as well as the efficacy of oncological treatment. Controversy exists, however, regarding the potential adverse effects of rHuEpo treatment in certain cancers.[3]

Epo is transported by the blood stream to the bone marrow where it binds to the Epo receptor located on erythroid progenitor cells.[4] Epo is responsible for proliferation and differentiation of erythroid burst-forming units (BFU-E) and erythroid colony-forming units (CFU-E) to mature erythrocytes capable of transporting oxygen to tissues. The half-life of this protein is 6 to 8 hours.[5,6]

The main stimulus for Epo production is hypoxia, however it is regulated by many other factors. Epo's up-regulation by hypoxia and anaemia is well understood.[7] It is known that Epo is down-regulated due to kidney damaging chemotherapy, rHuEpo treatment and presence of molecular factors such as TNF and IL-1,[8] however the level of down regulation and detailed mechanisms are not well understood. It was previously noted that the level of endogenous Epo is reduced after two administrations of rHuEpo (dr. F. Lasne, AFLD, personal communication). Cisplatin chemotherapy is known to be cytotoxic to kidneys, inducing acute and chronic renal insufficiency.[9] As kidneys are

the site of endogenous Epo production, any kidney damage may result in reduced endogenous Epo levels.

Epo is a heavily glycosylated protein with molecular weight of 30–34 kDa containing three N-linked glycans on asparagines (N24, N38 and N83) and one O-linked glycan on serine (S126). Carbohydrate represents approximately 35–40% of the Epo molecular mass and is important for its biological activity.[10] The differential detection of endogenous (eEpo) and exogenous (rHuEpo) Epo is very complex due to their high protein similarity. Isoelectric focusing (IEF) combined with double immunobloting, developed by dr. Francoise Lasne and used for the first time as an Epo doping test during the Sydney Olympic 2000,[11] is as powerful method able to distinguish specific eEpo and rHuEpo isoforms. The method is based on the fact that Epo isoforms differ in glycosylation resulting in differential migration in the pH gradient. The position of the isoforms on the gel is determined by isoelectric point (pI). Compared to eEpo, darbepoetin is more glycosylated as it has two additional N glycosylation sites,[12] while glycosylation pattern in epoetins is different due to production in a cell line. More glycosylated (darbepoetin) isoforms have pI in acidic region of the IEF gel, epoetin isoforms have pI in the more basic region, while eEpo isoforms are found in basic and endogenous region of the gel. Recently the method was modified enabling detection of Epo in blood and not just urine.[13] IEF is regularly used in anti-doping procedures in the aim to detect rHuEpo misuse in sport.[11] Epo specific IEF has never been used before to follow the effect of treatment in cancer patients, probably due to a very complex method of detection and limited access to the technology. Other methods used for Epo characterization are capillary electrophoresis,[14] two dimensional electrophoresis (2D)[15] and MAIIA (Membrane Assisted Isoform ImmunoAssay).[16] The problems that can occur when using 2D method are interferences of several proteins which overlap with the rHuEpo pI.[17] This is not the case when using 1D IEF method combined with double immunobloting. MAIIA is an ultra-sensitive method, but it can not analyse Epo isoforms separately. Therefore IEF is the only method that can be used for semi-quantification of specific isoforms of Epo.

The contemporary effects of factors having opposite effect on endogenous Epo regulation in cancer patients have never been addressed before. We used IEF method in the aim to address the influence of cisplatin and rHuEpo administration on the endogenous Epo pattern in anaemic cancer patient.

2. Experimental

2. 1. Patients Status and Sample Collection

In 2003 60-year old female was diagnosed with breast cancer. Histological examination confirmed invasive lobular carcinoma with numerous positive axillary lymph nodes, highly positive hormonal receptor status (estrogen and progesterone receptors) and negative HER2 status. Shortly after surgery, bone metastases were found. She was treated with different systemic therapies. In July 2008 progression of metastases in the abdominal organs was noted, including both ureters, causing bilateral hydronephrosis. Nephrostomy tubes were introduced and renal function improved. Platinum based chemotherapy (cisplatin and gemcitabine) was delivered (10/11.07.2008, 07/08.08.2008, 28/29.08.2008, 18/19.09.2008, 20/21.10.2008, 13/14.11.2008, 04/05.12.2008). Due to anaemia darbepoetin (500 mcg s.c.) was administered (18.09.2008, 21.10.2008, 13.11.2008, 04.12.2008) (Fig. 1).

Control patient (without rHuEpo treatment) was 51-year old female patient with primary metastatic bladder cancer with metastases in bones, peripheral, retroperitoneal and mediastinal limphnodes. Otherwise she was healthy without other diseases, with normal renal and liver function and with normal protein levels. She was treated with first line therapy in combination of cisplatin and gemcitabine, which is a standard chemotherapy option in metastatic bladder cancer. The study was approved by Slovenian Ethics Committee (39/02/06, 39/08/07).

During treatment haematological and urine parameters were followed. Urine was analysed for protein with semi-quantitative test on strips for urine analysis. In venous blood samples standard haematology parameters (white blood cells, red blood cells, Haemoglobin, Haematocrit, MCV, MCH, MCHC and Platelets) were measured.

Urine and blood samples for IEF analysis were obtained during and after chemotherapy/rHuEpo administration: 27.10.2008 (sample A), 27.01.2009 (B) and 24.02.2009 (C) (Fig. 1). Samples of the control patient were obtained during 3rd cycle of chemotherapy. All samples were kept at –70 °C until analysis. Total proteins were measured with coomassie Plus Protein Assay Reagent, total Epo by ELISA. Endogenous Epo (eEpo) during darbepoetin treatment was estimated by IEF analysis (semi-quantification using Aida software: Advanced image data analyzer 1D, Raytest), GFR (glomerular filtration rate) according to Wright.[18]

2. 2. Reagents

The rHuEpo used as a treatment was Aranesp (Darbepoetin α), Amgen, USA. For the reference standards (Ref) biological reference preparation (BRP – biological reference preparation, equimolecular mixture of recombinant Epoetins α and β, standard from the European Pharmacopoeia) and Darbepoetin α were used. Tris and glycine were from Acros Organics, NaCl was from Merck, Sucrose was from Alkaloid, ampholytes (2–4, 4–6 and 6–8), Acrylamide/Bis solution 29:1 and Gel-Fix for PAG were from Serva, PBS (phosphate-buffered saline) and BSA (bovine serum albumin) were from Sigma, DTT (ultra pure dithiothreiol) was from Invitrogen, Tween 20 protein

grade 10% and Urea plus One were from Calbiochem. Complete, protease inhibitors, was from Roche. Steriflip, Centricon plus 20, Centricon YM 30, Immobilon-P and Durapore membranes were from Millipore. ELISA for human Epo, monoclonal mouse anti-human Epo (AE7A5)[19] and anti-human Epo (9C21D11) antibodies were from R&D. Chemiluminescent substrate SuperSignal West Femto and Coomassie Plus Protein Assay Reagent were from ThermoFisher Scientific. Biotinylated goat anti-mouse IgG was from Thermo Scientific. Streptavidine peroxidase complex was from Invitrogen. Glass econo columns, Affi-gel Hz hydrazide gel, Affi-gel oxidizer Sodium periodate and Affi-gel Hz 10x coupling buffer were from Bio-rad.

2. 3. Sample Preparation

Urine was thawed (18 ml) and 200 μl of Complete with EDTA and 1.8 ml of 3.75M Tris/HCl pH 7.4 were added. It was centrifuged, filtered trough Steriflip device using vacuum and ultrafiltrated with Centricon Plus 20 (MWCO 30.000). Retentate was buffer washed with 50 mM Tris/HCl pH 7.4 with Complete and loaded on to the immunoaffinity column containing anti-human Epo antibody (9C21D11). Eluate was recovered and loaded on the same column for 5 times. Column was rinsed with 0.3 M NaCl/PBS and eluted with buffer containing 0.4 M Glycine/NaOH pH 11, 0.01 M NaCl, 6 M urea, 0.01% Tween 20 and 0.02% BSA buffer. Eluate was ultrafiltered with Centricon Plus 20 (MWCO 30.000) and Micronon YM30 (MWCO 30.000) to obtain final volume of 20–80 μl. Epo concentration in the final retentate was assayed with Epo ELISA. Serum samples was loaded directly to immunoaffinity column; the following steps are identical to urine preparation.[20,21]

2. 4. Isoelectric Focusing

Retentates were diluted if necessary to obtain final Epo concentration of 800 IU/l (determined by ELISA) and 15–18 mIU were loaded on the gel. The standards of rHuEpo (Ref) were prepared in 1% BSA/Tris/HCl containing 800 IU/l of BRP and 600 IU/l of darbepoetin supplemented with 10% v/v of Tween 20.

IEF gel was prepared using 16.8 g urea, 1.95 g Sucrose, 15 ml water and 2 ml of each servalites. Prefocusing was performed at 250 V for 30 min, afterwards samples were loaded (20 μl) using rectangular pieces of filter paper. Electrophoresis was run on Multiphor II Electrophoresis at 1 W/cm of the gel length till 3600 Vh were reached.[20,21]

2. 5. Immunoblotting

Gel was submitted to semidry transfer in 25 mM Tris/182 mM Glycine at 1 mA/cm^2 for 30 min. Membrane was removed and incubated in 5 mM DTT for 45 min at 37 °C, followed by blocking in 5% non-fat milk (LFM) in PBS for 45 min. The membrane was incubated with 1/1000 dilution of primary AE7A5 anti human Epo antibody in 1% LFM/PBS for 1 h. Subsequently the membrane was washed with 0.5% LFM/PBS and briefly rinsed with PBS. Second semidry transfer was performed using 0.7% acetic acid at 0.8 mA/cm^2 for 10 min. Membrane was again blocked with LFM and incubated in 1/1600 dilution of biotinylated goat antimouse IgG in 1% LFM/PBS over night at 4 °C. Membrane was washed 6 times with PBS, incubated in 1/1200 dilution of streptavidin:bitinylated peroxidise complex for 1h, washed and covered with chemiluminiscent solution and analysed with camera LAS 1000 FujiFilm. The electropherograme was analysed using Aida (Advanced image data analyzer 1D, Raytest) software.[20,21]

3. Results and Discussion

The haematological parameters were followed during the course of treatment (Fig. 1). A drop of red blood cells (RBC) and haemoglobin (Hb) levels after the second cycle of chemotherapy treatment was observed, resulting in normocytic anaemia. Darbepoetin was administered at the fourth cycle of chemotherapy until the end of the treatment, leading to improved Hb and RBC levels.

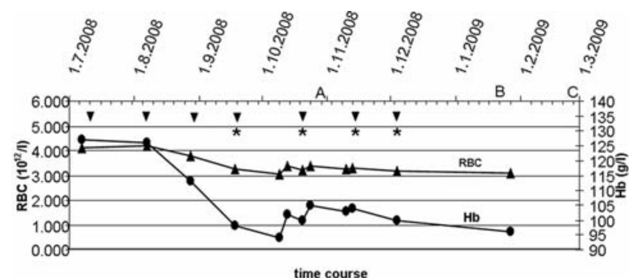

Fig. 1 Haemoglobin (Hb) and red blood cell (RBC) levels during the time course of treatment. Chemotherapy treatment is marked with ▼, darbepoetin with * and IEF sampling with A, B and C.

To study the effects of patient status and treatment regime on the level of endogenous Epo (eEpo) several blood and urine samples from breast cancer patient were collected and analysed with IEF (Fig. 2). IEF analysis of samples A, collected 6 days after 2nd darbepoetin administration, gives strong darbepoetin isoforms in acidic region of the gel and low level of several eEpo isoforms in endogenous region. In samples B and C, collected 8 and 12 weeks after the termination of the chemotherapy/darbepoetin treatment as well as in the control patient, only the eEpo isoforms are present. It is interesting to point out that even though sample A was collected after 2nd darbe-

poetin administration, eEpo is still present. This could lead to a conclusion that rHuEpo administration was not necessary. However the haemoglobin levels rose after the rHuEpo treatment showing that treatment was useful and successful (Fig. 1). It may be possible, that the observed endogenous Epo does not derive from kidney, but from liver[22] or breast cancer tissue.[23,24] We could assume that synthesis of Epo in cancer tissue is not regulated in the same manner as in kidneys.

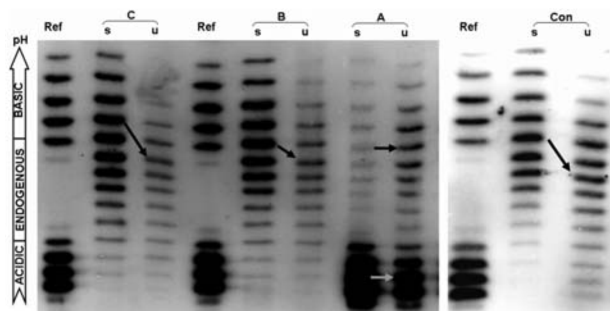

Fig. 2 Isoelectric focusing profile of Epo from serum (s) and urine (u) samples of darbepoetin treated (A,B,C) and control (Con) patient. Arrows show shift of Epo isoforms between serum and urine samples. A, B and C are IEF sample collections pointed out in Fig. 1, Ref is Epo reference.

Total Epo concentration in serum of sample A is elevated due to presence of rHuEpo, eEpo is estimated to 47 IU/l (Table 1). In sample B and C only the endogenous isoforms are present as rHuEpo was cleared from the system, the level of eEpo increased to 271 and 526 IU/l, respectably.

Table 1 Protein analysis of IEF samples. Epo concentration in the sample A is very high as it is obtained during darbepoetin treatment. Epo concentration droped in sample B and C, however not yet to the physiological values. Epo concentration in blood is higher than in urine, as expected. Protein levels in all serum samples are in physiological level, serum creatinin is in normal ranges. Total proteins in urine are increased and GFR diminished in all samples from darbepoetin treated patient

Sample	Biological Medium	Total Epo (IU/l)	Estimated eEpo (IU/l)	Total proteins (g/l)	Serum creatinin (μmol/l)	GFR (ml/min)
Normal values	Serum		>10	60–85	44–90	>90
	Urine		>1	<0.08		
A	Serum	1121	47	72.68	82	64
	Urine	100.2	24.8	1.3		
B	Serum	271	271	79.6	88	60
	Urine	76.9	76.9	1.25		
C	Serum	526	526	73.27	82	64
	Urine	64.9	64.9	0.62		
Con	Serum	54.2	54.2	/	55	126
	Urine	1.3	1.3	/		

During the whole period of treatment, the patient's diuresis was maintained through nephrostomy tubes. The semi-quantitative test of urine taken from nephrostomy catheter showed proteinuria and a slightly decreased GFR indicating 2nd stage of chronic kidney disease.[25] Proteinuria was also confirmed in all three IEF urine samples (Table 1). Proteinuria and low GFR are indicators of damaged kidney function. Kidney damage in our patient may be a consequence of hydronephrosis or cisplatin therapy. Cisplatin is known to induce acute renal insufficiency due to cytotoxic effects on the kidney.[9] The level of total protein dropped notably in the sample C, indicating that the kidneys function was being restored after termination of cisplatin treatment and/or regeneration after hydronephrosis damage. Reduction of proteinuria and increase of eEpo is synchronised, indicating that kidney function and eEpo production are correlated. Interestingly, no kidney damage was observed in the control patient even though being treated with the same cisplatin based chemotherapy.

When comparing serum and urine samples in the control patient, normal shift in Epo isoform distribution was detected. In normal conditions, the Epo isoforms in serum are more basic than those from urine.[13] Proximal tubule cells absorb majority of basic isoforms making urine Epo more acidic.[26] The shift of 2–3 isoforms in eEpo and 1 isoform in rHuEpo was seen in healthy volunteers, while no shift was noted in patients with chronic renal failure and after strenuous physical exercise.[13] Note that no shift in isoform distribution was seen in sample A of darbepoetin treated patient, all isoforms (eEpo and darbepoetin) are perfectly aligned (marked with arrow, Fig. 2). Renal processing in our patient may have been ineffective due to acute renal damage, probably connected with hydronephrosis or nephrotoxic effect of cisplatin therapy.[9] Kidney function seems to be improved after the termination of chemotherapy and darbepoetin treatment, the shift of approximately 1 isoforms in sample B and 2 isoforms in sample C is indicated with arrow (Fig. 2). Increased eEpo level, improved filtration process together and decreased proteinuria indicates that kidney function is being restored.[26] Note, that in the eEpo of the control patient being on the same chemotherapy regime, the shift of 2 isoforms was observed (Con, Fig. 2). The shift is not as big as expected for healthy persons, probably due to cisplatin chemotherapy. The proteinuria status of control patient is not known, GFR is normal.

In the future the method will be used to analyse larger number of cancer patients. Several samples form patients being on different chemotherapy regime and/or rHuEpo form will be collected.

4. Conclusions

Epo specific isoelectric focusing can be used at cancer patients in the aim to follow the pattern of endogenous

Epo during the time course of chemotherapy and rHuEpo treatment. This method provides a tool to investigate the effect of different rHuEpo forms on the level of the endogenous Epo. Furthermore, the effect of chemotherapy and rHuEpo co-treatment on the endogenous Epo production can be monitored.

5. Acknowledgement

IEF was performed in laboratory of dr. Francoise Lasne, AFLD, France. The work was supported by grant J3–0124 to N. D. and Young Researcher Grant to K. Š., both from the Slovenian Research Agency.

6. References

1. W. Fried, *Exp Hematol* **2009**, 37, 1007–15.
2. I. Wauters, J. Vansteenkiste, *Expert Opin Biol Ther Expert Opin Biol Ther* **2009**, *9,* 221–30.
3. A. J. Sytkowski, *Sci STKE* **2007**, 2007, pe38.
4. N. Debeljak, A. J. Sytkowski. EpoR. *UCSD-Nature Molecule Pages* **2007:** A000863.
5. J. W. Adamson, *Am J Med* **1996**, *101,* 4S–6S.
6. J. A. Pascual, V. Belalcazar, C. de Bolos, R. Gutierrez, E. Llop, J. Segura, *Ther Drug Monit* **2004**, *26,* 175–9.
7. M. Nangaku, R. Inagi, T. Miyata, T. Fujita, *Nephron Exp Nephrol* **2008**, *110,* e1–7.
8. M. R. K. C. Nowrousian, C. Oberhoff, U. Essers, R. Voigtmann, W. Gallasch, O. Quarder, in: M. Dekker (Ed.): Pathophysiology of cancer-related anemia, New York Basel Hong Kong, **1996**.
9. P. A. Wood, W. J. M. Hrushesky, *J Clin Inv* **1995**, *95,* 1650–9.
10. P. H. Lai, R. Everett, F. F. Wang, T. Arakawa, E. Goldwasser, *J Biol Chem* **1986**, *261,* 3116–21.
11. F. Lasne, J. de Ceaurriz, *Nature* **2000**, *405,* 635.
12. S. Elliott, J. Egrie, J. Browne, T. Lorenzini, L. Busse, N. Rogers, I. Ponting, *Exp Hematol* **2004**, *32,* 1146–55.
13. F. Lasne, L. Martin, J. A. Martin, J. de Ceaurriz, *Int J Biol Macromol* **2007**, *41,* 354–7.
14. J. Zhang, U. Chakraborty, A. P. Villalobos, J. M. Brown, J. P. Foley, *J Pharmaceut Biomed* **2009**, *50,* 538–43.
15. W. Schlags, B. Lachmann, M. Walther, M. Kratzel, C. R. Noe, *Proteomics* **2002**, *2,* 679–82.
16. M. Lonnberg, M. Drevin, J. Carlsson, *J Immunol Methods* **2008**, *339,* 236–44.
17. O. P. Rabin, F. Lasne, J. A. Pascual, M. Saugy, F. J. Delbeke, P. Van Eenoo, *Clinica Chimica Acta* **2006**, *373,* 186–7; author reply 88.
18. J. G. Wright, A. V. Boddy, M. Highley, J. Fenwick, A. McGill, A. H. Calvert, *Brit J Cancer* **2001**, *84,* 452–9.
19. A. J. Sytkowski, J. W. Fisher, *J Biol Chem* **1985**, *260,* 14727–31.
20. F. Lasne, *J Imminol Methods* **2001,** *235,* 125–31
21. F. Lasne, L. Martin, N. Crepin, J. de Ceaurriz, *Anal Biochem* **2002,** *311,* 119–26
22. M. Lonnberg, M. Andren, G. Birgegard, M. Garle, J. Carlsson, *Epo Isoform Determination* in: H.P. Bruch, W. Kühnel, Th. Martinetz, P. Schmucker (Eds.) Focus Uni Luebeck – 8th international Luebeck conference »Pathophysiology and Pharmacology of Erythropoietin and other Hemopoietic Growth Factros«, Luebeck, Germany, **2009**, 39
23. M. O. Arcasoy, K. Amin, A. F. Karayal, S. C. Chou, J. A. Raleigh, M. A. Varia, Z. A. Haroon, *Lab Invest* **2002**, *82,* 911–8.
24. G. Acs, P. J. Zhang, T. R. Rebbeck, P. Acs, A. Verma, *Cancer* **2002**, *95,* 969–81
25. C. Bauer, M. L. Melamed, T. H. Hostetter, *J Am Soc Nephrol* **2008**, *19,* 844–6.
26. S. Lamon, L. Martin, N. Robinson, M. Saugy, J. Ceaurriz, F. Lasne, *Clin J Sport Med* **2009**, *19,* 311–5.

Povzetek

Eritropoetin je glavni regulator eritropoeze. Na njegovo količino vplivajo hipoksija, slabokrvnost, uporaba rekombinantnega eritropoetina, kemoterapija in drugo. Izoelektrično fokusiranje (IEF) v povezavi z dvojnim prenosom je metoda, ki omogoča analizo izooblik endogenega in rekombinantnega eritropoetina.
Namen naše raziskave je bil postavitev analize vpliva zdravljenja na vzorec izooblik endogenega eritropoetina pri anemičnih bolnicah z rakom dojke. Urin in vzorci krvi so bili zbrani med in po končanem zdravljenju in analizirani z izoelektričnim fokusiranjem. Količina endogenega eritropoetina med zdravljenjem z darbepoetinom je bila znižana, vendar kljub temu še vedno merljiva. Zamika izooblik eritropoetina med vzorci seruma in urina, navadno vidnega pri zdravih prostovoljcih, ni bilo. To lahko kaže na okvaro ledvic. Bolnica je imela močno proteinurijo in zmanjšano glomerularno filtracijo. Stanje nakazuje akutno odpoved ledvic, po vsej verjetnosti zaradi kliničnega stanja ali kemoterapije s cisplatinom.
Metoda IEF še ni bila uporabljena v namen analize profila eritropoetina pri bolnikih z rakom. Metoda omogoča spremljanje učinkov zdravljenja na nivo endogenega eritropoetina in indirektno nakazuje funkcijo ledvic.