Journal of Pharmaceutical and Biomedical Analysis 51 (2010) 255–259

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Short communication

New screening protocol for recombinant human erythropoietins based on differential elution after immunoaffinity purification

J. Mallorquí[a,b], R. Gutiérrez-Gallego[a,b], J. Segura[a,b], C. de Bolòs[c], J.A. Pascual[a,b,*]

[a] Bioanalysis Group, Neuropsychopharmacology Research Program, IMIM-Hospital del Mar, PRBB, Barcelona, Spain
[b] Department of Experimental and Health Sciences, Pompeu Fabra University, PRBB, Barcelona, Spain
[c] Gastric Carcinogenesis Group, Cancer Research Program, IMIM-Hospital del Mar, PRBB, Barcelona, Spain

ARTICLE INFO

Article history:
Received 12 March 2009
Received in revised form 13 July 2009
Accepted 15 July 2009
Available online 24 July 2009

Keywords:
Erythropoietin
Doping
Screening
Immunoaffinity

ABSTRACT

A screening method able to differentiate recombinant human erythropoietins (rhEPOs) and analogues like CERA from human urinary erythropoietin (uhEPO) is described. The method is based on the discrimination between isoforms observed when the protein is eluted under acidic followed by basic conditions from immunoaffinity microtiter wells. From a comparison with the complex IEF protocol currently applied in anti-doping analysis, the newly developed assay procedure is amenable to wide screening application and presents good resolving power between rhEPOs and uhEPO.

1. Introduction

Erythropoietin is the protein responsible for the production of erythrocytes. Its use is prohibited in sport [1] due to the performance enhancing power resulting from the increased oxygenation capacity. A recombinant pharmaceutical product (epoetin) was available in 1985 and marketed as epoetin alpha (e.g. eprex®). Since then a series of analogues have been marketed with differing pharmacokinetic properties like epoetin beta (e.g. neorecormon®), omega (epomax®), delta (dynepo®), darbepoetin alpha (NESP, aranesp®), pegserpoetin alpha (CERA, mircera®), amongst others.

Their unequivocal identification in biological fluids is very challenging since their concentration is very low (i.e. in the low fM range) and epoetins are structurally very close to the endogenously produced glycoprotein [2–4] thus differentiating them from endogenous EPO is also very difficult. The method currently used in doping control to differentiate between exogenous and endogenous EPO is based on isoelectric focusing separation [5]. However, the method is very labour intensive, expensive and time consuming making it unsuitable for high-throughput screening. As a result, not all samples collected for doping control are analysed for the presence of exogenous EPO.

There have been a number of attempts to develop efficient EPO affinity purification methods to isolate pure endogenous material for structural elucidation and characterisation. Lectins have been used for this purpose and some affinity differences between rhEPO and uhEPO were claimed [6]. Immunopurification approaches with a variety of antibodies have also been tried, but it came out to be difficult with recoveries always far from quantitative [7]. Lately, different techniques have been described that should allow such purification [8,9]. Granting no-isoform discrimination was a critical factor as they were intended for purification prior to the determination of their electrophoretic behaviour.

However it was soon observed [10] that under particular elution conditions there was a consistent selective elution of the more basic bands of the profile. As the profiles of the recombinant products differ from the endogenous urinary EPO, the selectivity in the elution results in changes in the overall recovery, thus being a potential mean to discriminate between them. The aim of the present work was the development of a screening test to differentiate between endogenous and exogenous EPO based on the isoform discrimination observed under certain elution conditions from immunopurification systems.

2. Experimental

2.1. Chemicals and reagents

Recombinant human EPO (rhEPO) standard preparation (equimolar mixture of epoetin alpha and beta) was obtained

* Corresponding author at: Bioanalysis Group, Neuropsychopharmacology Research Program, IMIM-Hospital del Mar, PRBB, Dr. Aiguader, 88, 08003-Barcelona, Spain. Tel.: +34 933 160 500; fax: +34 933 160 467.
E-mail address: jpascual@imim.es (J.A. Pascual).

0731-7085/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2009.07.019

from the European Pharmacopoeia Commission, Biological Reference Preparation (BRP) batch no. 2. Epoetin delta (dynepo™) was obtained as the pharmaceutical preparation (syringe containing 3000 IU in 0.3 mL solution) from Shire Pharmaceuticals. Darbepoetin alpha or NESP (aranesp™) was obtained as the pharmaceutical preparation (syringe containing 10 μg in 0.4 mL solution) from Amgen Europe B.V. Pegserpoetin alpha or CERA (mircera™) was obtained as the pharmaceutical preparation (syringe containing 200 μg in 0.3 mL solution) from Roche. Human urinary EPO (uhEPO) was purchased from the National Institute form Biological Standards and Control (NIBSC). Protease-free Tris, glycine, phosphate-buffered saline (PBS), tween 20 and bovine serum albumin (BSA) were from Sigma. Complete™, protease inhibitor cocktail, was from Roche. Steriflip filters (0.22 μm), Amicon Ultra-15 and Ultra-4 (MWCO 30 kDa), Durapore (0.65 μm) and Immobilon-P (0.45 μm) membranes were from Millipore. ELISA assay kit (Quantikine IVD Erythropoietin) and anti-hEPO mAb (clone AE7A5) were from R&D Systems. EPO Chemiluminescent Immunoassay (Immulite 1000 EPO) and diluent were from DPC. Urea, gelbond film, electrode paper and DTT were from Amersham-Pharmacia. Acrylamide-Bis (97:3, w/w) and SDS were from Merck. Ampholytes Servalyt 2–4 and 4–6 were from Serva. TEMED was from BioRad. Supersignal West Femto and biotin-conjugated goat-antimouse IgG (H + L) were from Pierce. Streptavidin–HRP was from Biospa. All other chemicals were of the highest purity commercially available.

2.2. Standard solutions

Working solutions in PBS at 400 mIU/mL of rhEPO (BRP standard), epoetin delta or uhEPO and 0.4 μg/mL for NESP or 0.8 μg/mL for CERA were prepared by serial dilutions of the original stock preparations and stored at 4 °C.

2.3. Calibration samples

Standard mixtures of rhEPO and uhEPO were prepared at different proportions, simulating the possible content of a real sample after administration of the recombinant substance. These mixtures were taken through the assay procedure. Five calibration mixtures were prepared all containing a total of 400 mIU/mL in PBS with different proportions of rhEPO and uhEPO: 100%/0%, 75%/25%, 50%/50%, 25%/75% and 0%/100% of rhEPO and uhEPO, respectively. The solutions were stored at 4 °C until used.

In order to test for a potential matrix effect, the urine from a volunteer not using any EPO product (blank urine) was concentrated by ultrafiltration. The pooled retentates were quantified for the EPO content and appropriate volumes spiked with rhEPO in amounts to obtain proportions rhEPO with respect to total EPO detectable by the current IEF method (i.e. 90%, 87% and 71%). Final mixtures were diluted in PBS in order to obtain a final total EPO concentration equal to the original retentate (sample without rhEPO). The samples were submitted to the newly proposed screening procedure as well as to the current IEF method.

2.4. Urine samples and excretion studies

Thirty urine samples were obtained from healthy volunteers not using rhEPO to establish the range of population (negative) values. Samples were concentrated by ultrafiltration as described below.

Excretion studies of epoetin alpha (eprex, Amgen) and epoetin beta (neorecormon, Roche) were performed consisting of 3 subcutaneous injections (every 48 h) of 50 and 20 IU/kg respectively. Morning urine samples were obtained daily, analysed by the current IEF method and different aliquots for each study pooled to get a reference urine showing the content of rhEPO ("positive urine").

The studies were approved by the local ethics committee at IMIM-Hospital del Mar. Urine aliquots were concentrated by ultrafiltration as described below. The final retentates were used to test the proposed assay as well as submitted to the current IEF method.

2.5. Urine ultrafiltration

Urine samples were concentrated by ultrafiltration as previously described with small modifications [5,2]. Briefly, 2 mL of Tris–HCl (pH 7.4, 3.75 M) and 0.4 mL of Complete™ solution were added to 20 mL urine samples. After centrifugation at 2700 × *g* and 20 °C for 10 min, the supernatant was microfiltered using a 0.22 μm Steriflip device. The filtrate was then submitted to a first ultrafiltration using Amicon Ultra-15 at 3350 × *g* and 20 °C for 10–15 min. The retentate was then washed with 15 mL of Tris–HCl (pH 7.4; 50 mM) and 0.4 ml of Complete™ solution on the same filter and then centrifuged again under the same conditions. The retentate (about 100–150 μL) was taken through the assay procedure.

2.6. Assay procedure

The assay procedure consists of two steps, namely, isoform selective immunopurification and quantification. A well plate of an ELISA assay kit was used for immunopurification (Quantikine IVD, R&D). The EPO Immulite® chemiluminescent assay was used for quantification.

2.6.1. Selective immunopurification

On each well of the 96-well plate, 100 μL of assay diluent followed by 100 μL of sample (urine retentate or analyte solution) were applied and incubated for 1.5 h at room temperature with shaking. Supernatant (unbound) was transferred to an eppendorf vial for IEF analyses. Wells were washed with 300 μL PBS containing 0.1% tween 20 (PBS–tween). A first (acidic) elution was performed by incubation with 250 μL of 0.7% acetic acid (pH 2.8) for 2 min with shaking. Wells were then washed twice with 300 μL PBS–tween and a second (basic) elution was performed by incubation with 250 μL of glycine buffer (0.4 M; pH 11.3) containing urea (6 M). First (acidic) and second (basic) eluates were filtered through a preconditioned Amicon Ultra-4 containing 1 mL water and centrifuged at 3000 × *g* and 20 °C for 6 min to obtain a final volume between 100 and 150 μL. Final retentates were diluted to 350 μL with Immulite assay diluent (final pH between 7 and 7.5), vortex mixed and used for quantification.

2.6.2. Quantification

Final retentates were transferred to the assay sample cups and directly quantified in an Immulite 1000 instrument.

2.7. Analysis by isoelectric focusing (IEF)

Analyses by IEF, when needed, were performed as described elsewhere [2]. Urine fractions after application of the present protocol, i.e. immunopurification supernatants (unbound fraction) and elution fractions (acidic and basic), once concentrated as described above, were further ultrafiltered through an Amicon Ultra-4 to obtain a final volume below 40 μL. From this final retentate, 20 μL were applied to the IEF gel.

3. Results and discussion

Developing an efficient immunopurification method for EPO from urine and plasma or serum has always had great interest for identification and/or quantification purposes but also from the point of view of the structural elucidation and characterisation of the glycoprotein isoforms. From the various attempts to develop

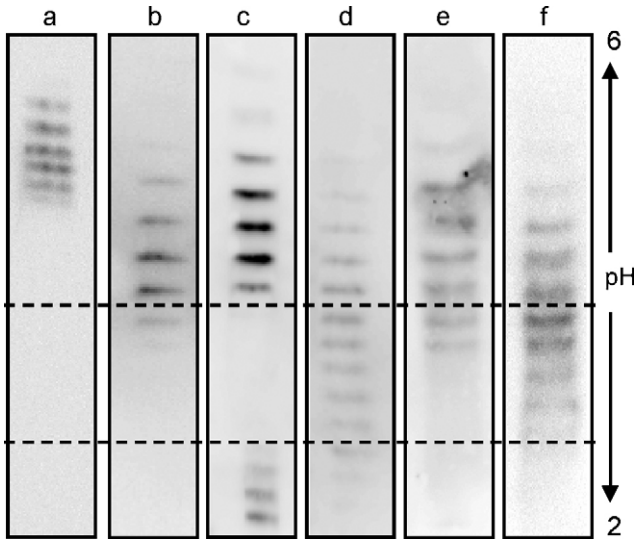


Fig. 1. Isoelectric focusing (IEF) profiles of different recombinant EPO products and immunopurified fractions of human urinary EPO standard. (a) Pegserpoetin alpha (Mircera) from Roche; (b) epoetin delta (Dynepo) from Shire Pharmaceuticals; (c) mixture of the Biological Reference Preparation (BRP) of recombinant EPO; (d) human urinary EPO preparation from NIBSC; (e) acidic elution of the urinary EPO standard; (f) basic elution of the urinary EPO standard. The horizontal lines differentiate basic (upper), endogenous (middle) and acidic (lower) areas according to the WADA's Technical Document TD2007EPO.

immunoaffinity purification systems, it became evident that isoform discrimination was a risk to be avoided or at least checked for. Recent publications [8,11] have shown the use of different immunopurification approaches that seemed not to alter the isoelectric profile of EPO. However, other works have shown significant isoform discrimination that may result from the use of particular antibodies or the elution conditions [10].

Using ELISA microplates from R&D (Quantikine™) as immunopurification system, elution at acidic pH (e.g. pH ~3) produced a significant selectivity towards the bands corresponding to the basic area (as defined in the WADA's technical document for the analysis of EPO, TD2009EPO [12]) while elution at a basic pH (e.g. pH ~11) produced no discrimination (Fig. 1). We had already reported a previous experience of getting a similar behaviour under acidic conditions using a sepharose 4B immunoaffinity column prepared with a different antibody (clone AE7A5 also from R&D systems). Plates with the antibodies used by StemCells Inc. for their ELISA kit (clones 16F1H1 and 26G9C10) were also tested since they are widely described as used prior to SDS-PAGE analysis [9,13]. The combination (1:1) of both antibodies produced no discrimination when eluting at acidic pH. However, the fact that a mixture of two different antibodies is used may have played a role. The complexity of systematically studying these facts considering the many available antibodies and possible supports and binding methods, however, was out of the scope of the present work.

Given the differences in isoelectric profile between rhEPOs and uhEPO with higher proportion of their bands located in the basic area for the recombinant products, the test conditions described herein should show higher relative recoveries (amount of EPO in the acidic elution fraction with respect to the overall amount recovered in both acidic and basic fractions). This difference may be exploited to differentiate them.

To test this hypothesis, standard solutions of uhEPO, rhEPO, epoetin delta (dynepo™), CERA and NESP were subject to the assay procedure described quantifying the content of the two elution fractions (acidic and basic). Mixtures with different proportions between rhEPO and uhEPO were also tested in order to simulate the situation encountered in real urine samples. Results are shown

Table 1
Relative recoveries in the two elution fractions after immunopurification (%A for the acidic elution and %B for the basic elution) as well as the ratio QA defined as the quotient between the value of %A obtained for each particular compound and the value obtained for uhEPO taken as reference.

Substance	Relative recoveries		Ratio QA (±sd)
	%A	%B	
CERA ($n=3$)	96.4	3.6	1.51 (±0.011)
Dynepo™ ($n=3$)	89.4	10.6	1.40 (±0.018)
rhEPO ($n=10$)	86.5	13.5	1.34 (±0.125)
Mix rhEPO:uhEPO (75:25) ($n=3$)	83.5	16.5	1.31 (±0.006)
Mix rhEPO:uhEPO (50:50) ($n=3$)	79.0	21.0	1.24 (±0.014)
Mix rhEPO:uhEPO (25:75) ($n=3$)	74.7	25.3	1.17 (±0.032)
uhEPO ($n=10$)	61.7	38.3	1 (reference)
NESP ($n=8$)	38.0	62.0	0.60 (±0.082)

in Table 1. As can be seen, relative recoveries in each of the two fractions progressively varied from 96.4% in the acidic fraction for CERA (having the whole isoelectric profile in the "basic area") according to TD2009EPO [12] down to 38.0% for NESP, with its whole isoelectric profile in the "acidic area". Changes in those relative recoveries could also be observed when proportions rhEPO to uhEPO were changed. In order to minimize the potential day to day variations, a ratio between the relative recovery in the acidic fraction for any sample with respect to the same value obtained for the analysis of uhEPO standard, run in parallel, was calculated (ratio QA). Those ratios varied from 1.51 for CERA (more basic) to 0.60 for NESP (more acidic) in correlation with the basicity or acidity of the IEF profiles of the compounds (see Table 1). To test the stability of uhEPO and rhEPO under the acidic and basic elution conditions the compounds were incubated in those media and analysed by IEF. No changes were observed in their profiles (data not shown) ruling out its potential contribution to the observed selectivity.

The application of the proposed method to the detection of the presence of recombinant EPO products in urine samples requires the determination of population values for the chosen marker (i.e. ratio QA) as well as the establishment of a cut-off value. A set of 30 urine samples from volunteers not using any EPO product (blank urine samples) were analysed following the assay procedure. Again uhEPO was also processed in parallel as reference for the marker.

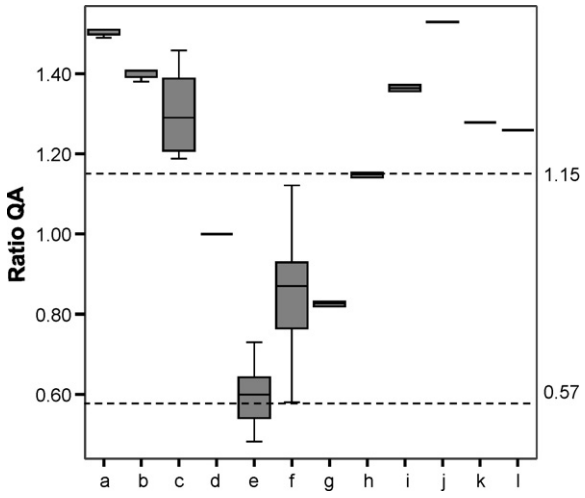
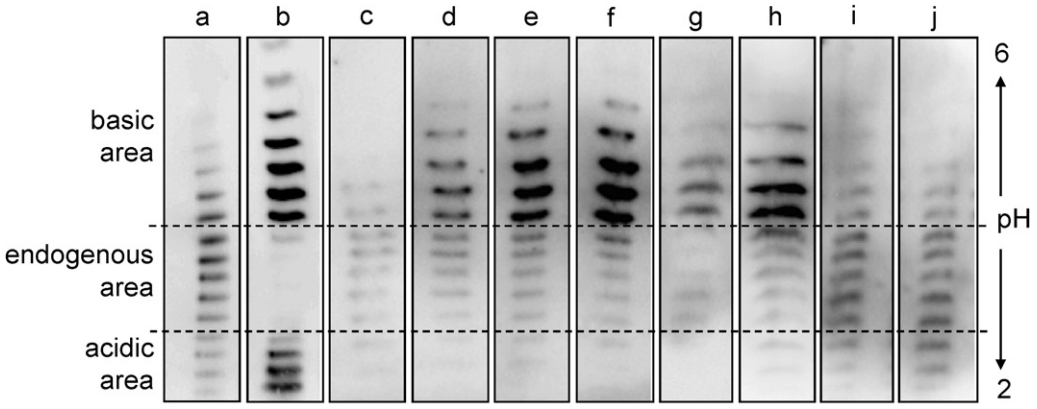


Fig. 2. Box plot of the ratio QA values obtained for the different samples analysed. Dotted lines indicate the limits of the 95% confidence interval for the negative samples. (a) CERA ($n=3$); (b) dynepo ($n=3$); (c) rhEPO BRP std ($n=10$); (d) human urinary EPO, NIBSC std ($n=10$); (e) NESP ($n=8$); (f) blank urine samples ($n=30$); (g) blank urine sample chosen to spike ($n=3$); (h–j) blank urine sample spiked with 71%, 87% and 90% rhEPO BRP std ($n=3$ each) respectively; (k) sample from an excretion study of epoetin alpha (eprex); (l) sample from an excretion study of epoetin beta (neorecormon).



Ratio QA	0.82	1.15	1.37	1.53	1.26	1.28	0.76
TD2009EPO	0.27	1.83	3.02	5.60	2.8	2.05	0.44

Fig. 3. IEF analysis of different urine samples and values of their band intensities according to the WADA's Technical Document TD2009EPO (quotient of intensities between the second most intense band in the basic area and the most intense band in the endogenous area) compared to their ratio QA values. (a) Human urinary EPO NIBSC std; (b) mixture of the rhEPO BRP std and NESP; (c) blank urine, not containing any exogenous EPO; (d–f) blank urine spiked with 71%, 87% and 90% of rhEPO BRP std respectively; (g) urine from an excretion study of epoetin alpha (eprex); (h) urine from an excretion study of epoetin beta (neorecormon); (i, j) blank urine and blank urine unbound fraction respectively.

The mean value obtained for the ratio QA was 0.86 with a standard deviation of 0.145. As can be seen, the value found resulted below 1 showing either a matrix effect for urine or potential differences in behaviour of endogenous urinary EPO with respect to the uhEPO (NIBSC standard). This behaviour made the values obtained for blank urine samples better distinguishable from rhEPO, dynepo or CERA. Conversely, it resulted in a lower discriminating power for NESP.

From the values obtained for blank urine samples, a cut-off value for the ratio QA covering the 95% confidence interval would be 1.15 (upper boundary) and 0.57 (lower boundary). While pure rhEPO/uhEPO mixtures indicate that proportions as low as 25% of rhEPO will be detectable, NESP will only be picked up when present in higher proportions.

In order to study the urine matrix effect for the exogenous substances and compare the sensitivity of the proposed screening approach to the current IEF method, increasing amounts of rhEPO were added to a blank urine retentate and taken through the procedure. These amounts covered the range from 70% to 90% rhEPO with respect to the total EPO content and were chosen as they are detectable by the IEF method. Fig. 2 shows the results obtained as compared to the blank urine samples and standards. Results clearly show that urine samples with a 90% or 87% rhEPO were well detected by the newly developed screening with QA values of 1.53 and 1.37, far above the cut-off of 1.15. Those samples were also detected following the IEF method where, as required by the WADA's technical document TD2009EPO [12], the two most intense bands of the basic area showed intensities more than twice the intensity found for the bands in the endogenous area (Fig. 3). The sample containing 71% rhEPO resulted in a QA value of 1.15 right on the cut-off value while replicate determinations by the IEF method showed intensity ratios under two (1.6–1.8), thus very close but below detectability according to TD2009EPO. So the newly proposed procedure resulted in a slightly better sensitivity than the current IEF method for rhEPO.

The analysis of the unbound fraction (supernatant) of negative samples was also analysed by IEF. No-isoform discrimination was detectable in that fraction (Fig. 3, lane j). Thus, the binding step appears not to create any discrimination. This is important since the unbound fraction may be used for further pre-confirmation tests by IEF or to gather additional evidence through the application of an SDS-PAGE analysis [9,13].

Urine samples from excretion studies of epoetin alpha and beta were also analysed using this procedure. The pooled samples corresponding to the administration of epoetin alpha or beta showed an IEF profile (Fig. 3, lanes g and h) clearly complying with the identification criteria for the presence of recombinant EPO alpha or beta according to TD2009EPO. Using the proposed selective elution procedure, the values obtained for the ratio QA were 1.28 and 1.26 respectively, again clearly above the proposed cut-off value.

The ratio QA correlates with the proportion of the intensity of the IEF profile in the basic area. Any modification of the profile due to the presence of other substances (e.g. NESP) would affect the QA value obtained. Analogously, shifted profiles obtained after certain particular effort conditions (atypical profiles) or after degradation (active urines) would also influence the QA result as they would affect the IEF profile.

4. Conclusions

Elution after immunoaffinity binding using ELISA microplates from R&D (Quantikine^{TM}) has been found to selectively discriminate between isoforms of EPO. As the more basic isoforms of EPO were predominantly eluted at acidic pH, those substances with a more basic profile (i.e. recombinant EPOs, including dynepo, and CERA show a higher relative recovery in that fraction than urinary EPO with a profile shifted towards more acidic p*I* values.

A new screening method amenable to high throughput analysis has been developed based on the differential elution of the EPO isoforms under acidic and basic conditions. It has been shown to differentiate between recombinant and endogenously produced EPO with at least the same sensitivity of the currently used IEF method, although its discriminant power is lower for hyper acidic compounds like NESP. The method is less laborious, more cost-efficient than the IEF procedure and produces solid evidence within a few hours. It appears very useful for the systematic screening of urine samples to suspect abuse of rhEPO or analogues. As for any other anti-doping method, a second step by a confirmatory method will results in a definitive evidence for adverse findings. The fraction of the sample not bound to the antibody during the immunopurifica-

tion step can be further used to apply the current IEF method or gather further evidence using additional procedures.

Acknowledgements

This project has been carried out with the support of WADA and the Spanish Ministry of Science and Education (DEP2006-56207-C03-03).

References

[1] World Anti-Doping Agency (WADA). Available at http://www.wada-ama.org/rtecontent/document/2009_Prohibited_List_ENG_Final_20_Sept_08.pdf, 2009 (accessed 10 March 2009).
[2] V. Belalcazar, R. Gutiérrez Gallego, E. Llop, J. Segura, J.A. Pascual, Assessing the instability of the isoelectric focusing patterns of erythropoietin in urine, Electrophoresis 27 (2006) 4387–4395.
[3] E. Llop, R. Gutiérrez-Gallego, V. Belalcázar, G.J. Gerwig, J.P. Kamerling, J. Segura, J.A. Pascual, Evaluation of protein N-glycosylation in 2-DE: erythropoietin as a study case, Proteomics 7 (2007) 4278–4291.
[4] E. Llop, R. Gutiérrez-Gallego, J. Segura, J. Mallorquí, J.A. Pascual, Structural analysis of the glycosylation of gene-activated erythropoietin (epoetin delta, Dynepo), Anal. Biochem. 383 (2008) 243–254.
[5] F. Lasne, L. Martin, N. Crepin, J. de Ceaurriz, Detection of isoelectric profiles of erythropoietin in urine: differentiation of natural and administered recombinant hormones, Anal. Biochem. 311 (2002) 119–126.
[6] L. Franco Fraguas, J. Carlsson, M. Lönnberg, Lectin affinity chromatography as a tool to differentiate endogenous and recombinant erythropoietins, J. Chromatogr. A 1212 (2008) 82–88.
[7] V. Skibeli, G.A. Nissen-Lie, A. Noreau, P. Torjesen, P. Hemmersbach, K.I. Birkeland, Immuno-affinity extraction of erythropoietin from human serum by magnetic beads, in: W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck (Eds.), Recent Advances in Doping Analysis, Sport verlag Strauss, Köln, 1998, pp. 313–331.
[8] F. Lasne, L. Martin, J.A. Martin, J. de Ceaurriz, Isoelectric profiles of human erythropoietin are different in serum and urine, Int. J. Biol. Macromol. 41 (2007) 354–357.
[9] M. Kohler, C. Ayotte, P. Desharnais, U. Flenker, S. Lüdke, M. Thevis, E. Völker-Schanzer, W. Schänzer, Discrimination of recombinant and endogenous urinary erythropoietin by calculating relative mobility values from SDS gels, Int. J. Sports Med. 29 (2008) 1–6.
[10] V. Belalcázar, J. Segura, C. de Bolòs, S.H. Peng, R. Gutiérrez-Gallego, E. Llop, J.A. Pascual, Poster, 15th IFCC-FESCC European Congress of Clinical Chemistry and Laboratory Medicine, Euromedlab2003, Barcelona, 2003.
[11] Y. Dehnes, M. Borgen, M. Lonnberg, P. Hemmersbach, Shifted isoelectric profiles of endogenous erythropoietin possibly due to different physiological origin, in: W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck (Eds.), Recent Advances in Doping Analysis, Sport verlag Strauss, Köln, 2008, pp. 359–362.
[12] World Anti-Doping Agency (WADA). Available at http://www.wada-ama.org/rtecontent/document/td2009eop_en.pdf, 2009 (accessed 9 July 2009).
[13] C. Reichel, R. Kulovics, V. Jordan, M. Watzinger, T. Geisendorfer, SDS-PAGE of recombinant and endogenous erythropoietins: benefits and limitations of the method for application in doping control, Drug Test Anal. 1 (2009) 43–50.