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New urinary EPO drug testing method using two-dimensional gel electrophoresis[*]

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[*] Part of the data presented in the seminar has been published in Clinica Chimica Acta (2005) [1]. Additional data only are presented in this article.

Abstract

We present a two-dimensional electrophoresis (2DE) method for the detection of the drug, recombinant erythropoietin (rHuEPO) in urine and its separation from endogenous erythropoietin (HuEPO). This method involves a one-step acetonitrile precipitation of the proteins in urine, addition of an internal standard, two dimensional gel electrophoresis (2D PAGE), a single Western blot and chemiluminescent immunodetection.
Results. The 2DE method separates HuEPO and rHuEPO isoforms by both iso-electric point and molecular mass. We have identified several urinary proteins with which the monoclonal EPO antibody used in the current test has non-specific binding. The iso-electric points of these cross-reactive proteins are in the same pI range as the isoforms of HuEPO and rHuEPO however, they separate distinctly by the 2DE method. Alpha- 2-HS-glycoprotein (HSGP) was identified by peptide mass fingerprinting as one of the urinary cross-reacting proteins, and commercially available purified HSGP was chosen to be added into urine samples as an internal standard prior to separation. Software (EpIQ) was specifically developed that applies

four separate criteria to the detection of the migration of rHuEPO and HuEPO relative to the internal standard.

Conclusion. The combination of sample preparation, two-dimensional separation, internal standard, standardized blotting procedures and image analysis software enables the 2DE test for rHuEPO in urine to be performed reproducibly and accurately.

Introduction

We have reported detail results on the urine preparation, immobilised pH gradient (IPG), 2DE, EPO detection method and level of detection, antibody specificity (both primary and secondary antibodies), internal standard, and development of image analysis software (EpIQ) in a publication since this workshop [1]. In this article we describe the comparative analysis of urine samples spiked with rHuEPO by the 2DE method and the current 1D IEF method.

Results

Using the 2DE method, we have analyzed (blind analysis) 11 spiked (+) and 8 unspiked (-) urine samples supplied by the NMI in Sydney. Native HuEPO was detected by the 2DE method in most of the corresponding unspiked samples (Fig.1, b (E-, H-, B -, F-) and d (D-, A-). Although containing bands in the rHuEPO pI range, the unspiked sample A- was determined to be negative by the 1D method, using the current WADA criteria (http://www.wada-ama.org/rtecontent/document/td2004epo_en.pdf). The 2D method also showed the presence of unusually basic EPO isoforms, which, if only the relative pI position was taken into account would be identified as recombinant rHuEPO by the image analysis software EpIQ. However when the other three criteria were applied by the software, the isoforms were identified as native HuEPO. The same native EPO isoforms can be seen in the A+ sample underlying the added rHuEPO isoforms. The added rHuEPO was detected in all but one of the spiked samples (Fig.2d, J+) albeit with only low levels visible in G+ and K+. No samples resulted in a positive detection of rHuEPO when it was not there.

The same 19 urine samples were also analyzed by NMI using the French 1D IEF method [2] (Figs. 1 a and c; Figs. 2 a and c; and Table 1). A summary of the comparative results of the methods is shown in Table 2. The 1D IEF method failed to detect rHuEPO in three samples (Tables 1 and 2) using the criteria currently defined by the WADA.
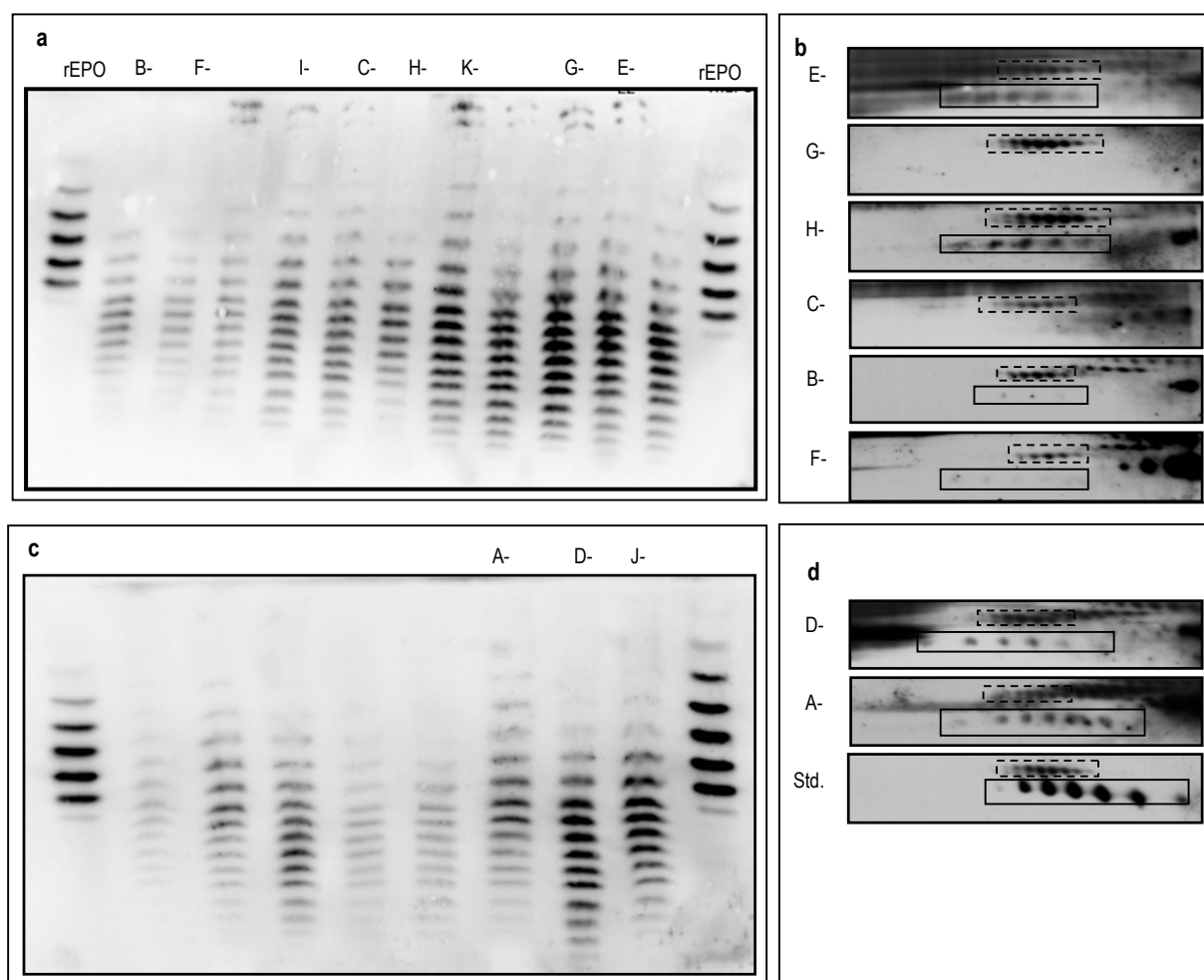
Figure 1. Comparison of 1D (**a** and **c**) and 2DE (**b** and **d**) methods for the blind analysis of EPO in unspiked (-) urine samples. Corresponding urine samples were spiked with rHuEPO and are shown in **Figure 2.** Un-spiked samples I, J and K were not supplied to us because of limited amounts. In Fig. d, std. corresponds to a standard image that includes an internal standard (HSGP) and rHuEPO. Images in **b** and **d** are zoomed in images obtained from the full images of 2D blots. Approximate pH range is 3.4 to 4.8 and mass range is 48 to 30 kDa (top to bottom). Dotted line boxes represent the added internal standard HSGP isoforms and solid line boxes represent either native HuEPO or recombinant rHuEPO.
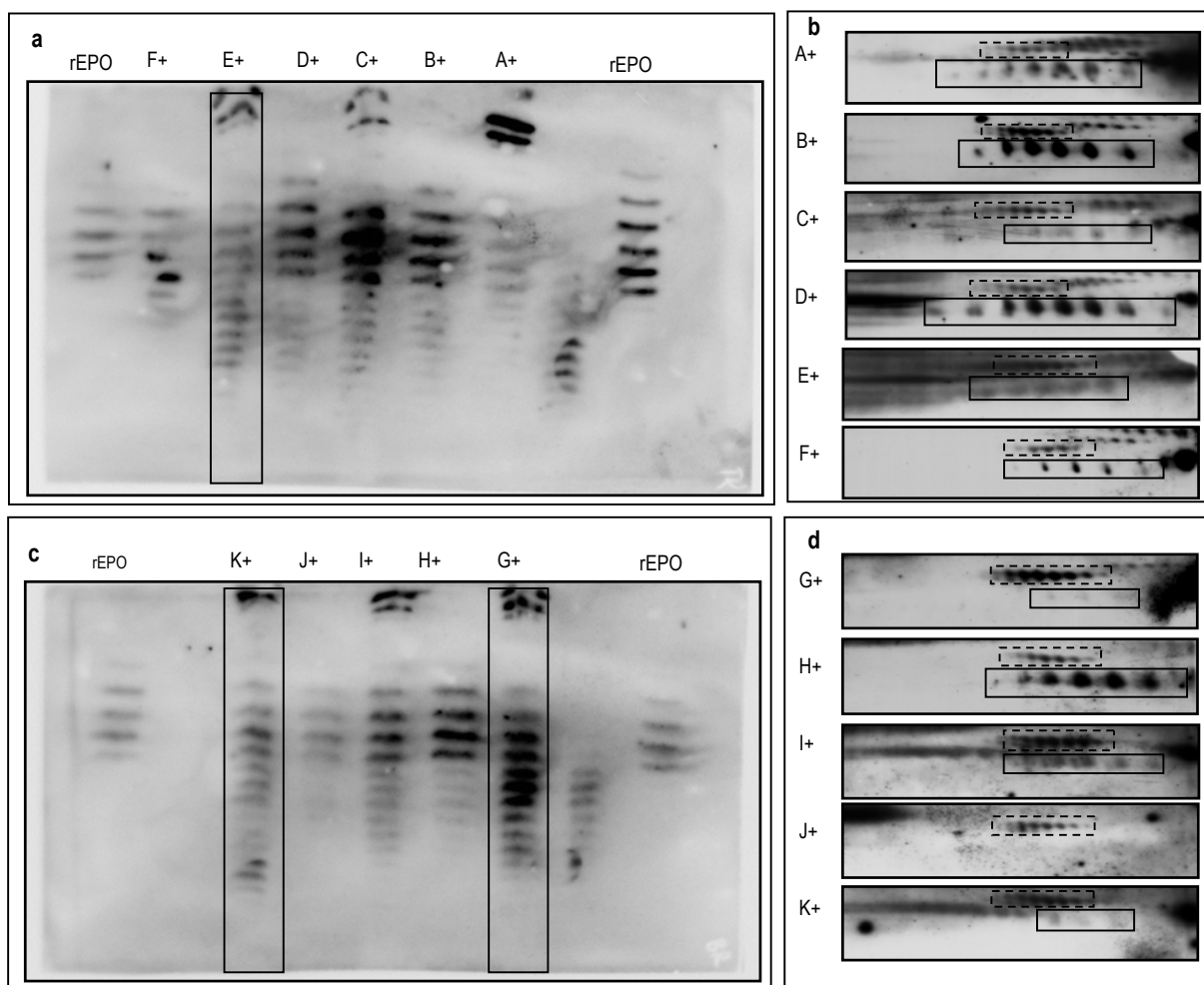
Figure 2. Comparison of 1D (a and c) and 2DE (b and d) methods for the blind analysis of EPO in urine samples spiked (+) with rHuEPO. Images of the corresponding unspiked urine samples are shown in **Figure 1**. Boxed lanes in Figs. a and c (samples E+, G+ and K+) were spiked with rHuEPO but the 1D IEF method failed to produce conclusive results. The 2DE method separated all the EPO isoforms distinctly and EpIQ software diagnosed them correctly except in the case of J+. Images in b and d are zoomed in images obtained from the full images of 2D blots. Approximate pH range is 3.4 to 4.8 and mass range is 48 to 30 kDa (top to bottom). Dotted line boxes represent the added internal standard HSGP isoforms and solid line boxes represent either native HuEPO or recombinant rHuEPO.

Table 1. Results of unknown urine samples analyzed by NMI using the 1D IEF method.

Sample No.	Percent Basic Isoforms	Largest Basic Peak Area B1	Second Largest Basic Peak Area B2	Largest Endo. Peak Area A1	Ratio 1 B1/A1	Ratio 2 B2/A1	Sample comments	All four blots were clear and well distributed with minimal blemishes - Diagnosis
A+	86.7	120550	72603	13969	8.63	5.20	3IU/L spike	Weak signal except for some very basic material - negative on quality grounds despite the numeric data.
A-	53.6	644361	502798	684847	0.94	0.73		Clear negative
B+	86.0	260700	181818	28833	9.04	6.31	3IU/L spike	Clear positive
B-	35.6	362921	247895	550571	0.66	0.45		Clear negative
C+	78.8	272390	132476	35453	7.68	3.74	6IU/L spike	Clear positive
C-	36.8	568766	383728	710071	0.80	0.54		Clear negative
D+	84.8	168144	159299	28521	5.90	5.59	3IU/L spike	Clear positive
D-	22.0	787303	341127	1512164	0.52	0.23		Clear negative
E+	45.6	69019	55757	62170	1.11	0.90	3IU/L spike	Negative according to numeric data, and isoform intensity is distributed biphasically across endogenous and basic areas
E-	34.0	1097798	805658	1555884	0.71	0.52		Clear negative
F+	88.3	145062	82025	12756	11.37	6.43	3IU/L spike	Clear positive
F-	35.1	177391	102723	358318	0.50	0.29		Clear negative
G+	45.1	117316	83363	105577	1.11	0.79	3IU/L spike	Negative- numerically and the isoform distribution across basic and endogenous areas does not support a positive
G-	27.5	1378104	884141	2013235	0.68	0.44		Clear negative
H+	88.4	175204	143687	23481	7.46	6.12	3IU/L spike	Clear positive
H-	42.1	889614	433077	1010678	0.88	0.43		Clear negative
I+	80.6	122063	120550	19233	6.35	6.27	3IU/L spike	Clear positive
I-	45.4	971105	650518	677543	1.43	0.96		Clear negative
J+	83.7	55401	40832	8395	6.60	4.86	3IU/L spike	Clear positive
J-	34.3	1113384	691321	1337208	0.83	0.52		Clear negative
K+	57.9	90214	47753	25000	3.61	1.91	3IU/L spike	Negative- numerically and the isoform distribution across basic and endogenous areas does not support a positive
K-	42.5	1645109	1117876	1705133	0.96	0.66		Clear negative

Table 2. Summary of the comparison of 1D and 2DE methods on the analysis of unknown urine samples[1].

Sample	1D IEF	2DE
A+	Correct	Correct
A-	Correct	Correct
B+	Correct	Correct
B-	Correct	Correct
C+	Correct	Correct
C-	Correct	Correct
D+	Correct	Correct
D-	Correct	Correct
E+	False negative	Correct
E-	Correct	Correct
F+	Correct	Correct
F-	Correct	Correct
G+	False negative	Correct
G-	Correct	Correct
H+	Correct	Correct
H-	Correct	Correct
I+	Correct	Correct
J+	Correct	False negative
K+	False negative	Correct

[1] 1D IEF method was carried out by NMI; 2DE method was carried out by Proteome Systems, sample information was not known to us until the results were compared.

Discussion

The 2DE method for the analysis of EPO in urine has addressed most of the WADA recommendations [3] for the improvement of the test. In this discussion we will focus on the comparison of 1D IEF and 2DE method for the analysis of unknown urine samples supplied by the NMI.

We have used the 2DE method to identify urines which had been spiked with 3IU/L (except sample C+, which was spiked at 6IU/L) rHuEPO and detected native HuEPO in most of the corresponding unspiked urine. The 2DE system separates EPO and urinary proteins in two-dimensions, with separation of proteins according to their iso-electric points in the first dimension and further separation according to their molecular masses in the second dimension. This approach separates each protein isoform as an individual component on the gels. Additionally we have included an internal standard in each sample that allowed correct localization of the EPO isoforms for scoring the results by the image analysis software. This

combination of 2D separation and image analysis software allowed the correct identification of all but one of the urines spiked with rHuEPO.

It has been reported that the pI of urinary HuEPO isoforms sometimes shifts to become more basic if rHuEPO is administered in an individual [4]. However, we have seen in the 2DE method that some basic isoforms with apparent slightly higher molecular masses than the EPO (either HuEPO or rHuEPO) on the gels are reactive to the EPO antibody which is present even when the native and recombinant EPO isoforms are also present in their usual pI ranges (Fig. 3b). These basic isofroms are also observed in the same pI ranges when separated by the 1D IEF method (Fig. 3a) using the same urine as used for 2DE analysis. Attempts were made to identify this basic protein from the 2D gels by peptide mass fingerprinting but were unsuccessful because of the very low amounts of proteins present in the gel. This suggests that the level of this protein in urine is very low but has very high affinity to the EPO antibody. It is not known whether these basic isoforms are the break down products of HuEPO or another cross reactive protein (other than those already reported, ref: 1) however, further study is needed to confirm the identity of this protein.

Conclusion

The 2DE method presented has dealt with most of the WADA recommendations for a new EPO test [3] and provides a sensitive and accurate detection of the EPO drug in urine. In the blind urine analysis, out of 19 samples, the 2DE method correctly detected rHuEPO with one false negative, in contrast to the current 1D IEF method which diagnosed three false negatives. Neither method identified a false positive.
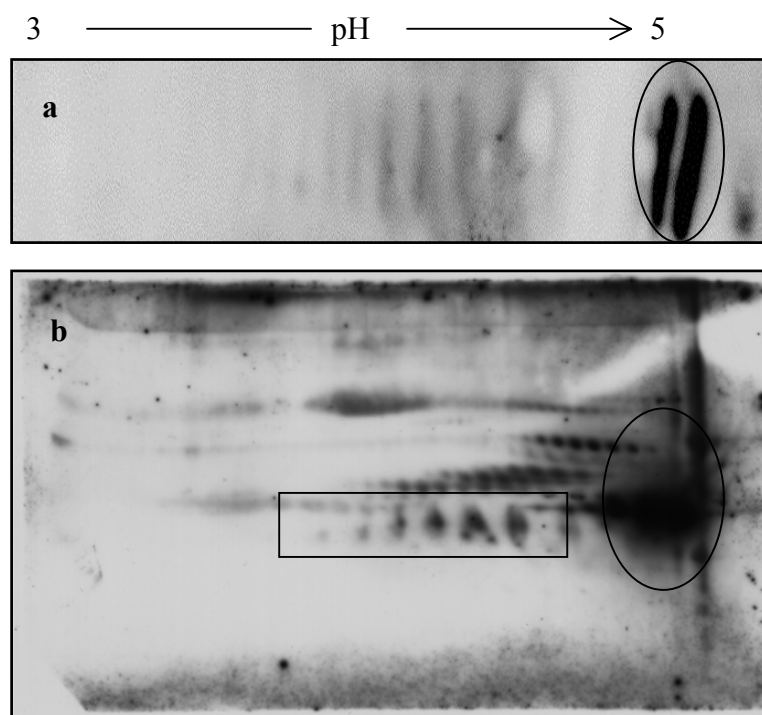
Figure 3. Basic proteins are detected by the antibody while EPO isoforms are at their usual pI. Sample A+ analyzed by 1D IEF (a) and 2DE (b) methods show basic proteins (circled) while EPO isoforms are present in their usual position (boxed). This data shows that EPO isoforms and unknown protein(s) separated in the "basic" area of pH 3-5 are detected by the EPO antibody.

References

[1] Khan A, Grinyer J, Truong ST, Breen EJ, Packer NH. New urinary EPO drug testing method using two-dimensional gel electrophoresis. Clin Chim Acta, 2005; 358: 119-130.

[2] Lasne F, Martin L, Crepin N, de Ceaurriz J. Detection of isoelectric profiles of erythropoietin in urine: differentiation of natural and administered recombinant hormones. Anal Biochem 2002;311:119-126.

[3] Peltre G, Thormann W. Evaluation report of the urine EPO test. Paris and Bern: Council of the World Anti-Doping Agency (WADA), 2003;March 11.

[4] Nissen-Lie G, Birkeland K, Hemmersbach P, Skibeli V. Serum sTfR levels may indicate charge profiling of urinary r-hEPO in doping control. Med Sci Sports Exerc 2004;36:588-93.