

Research Article

I-Number Assay - A Physicochemical Assay that Predicts the Bioactivity of EPO Samples: Proof of Principle

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Abstract

The I-number assay, based on capillary zone electrophoresis data, is evaluated as a physicochemical assay that enables to calculate the bioactivity of erythropoietin medicinal products. In part 1, the CZE data of the candidate biological reference preparation EPO cBRP3 of a collaborative study of 10 laboratories (published in 2007) are used to define the relationship between the I-number of EPO cBRP3 and the stated bioactivity of EPO BRP3. In this retrospective analysis by the author of the CZE data of this study, the I-number assay revealed an inter-laboratory precision of $CV = 0.7\%$ ($n = 9$ labs, 1 outlier lab excluded). In part 2, the CZE data of an epoetin alfa drug substance secondary standard from Centocor and an artificial epoetin beta concentrated solution sample from Roche (the mean of 40 epoetin beta training batches) were retrospectively analyzed by the author using the I-number assay. Moreover, the hypothetical epoetin alfa / beta 1:1 mixture of the Centocor and the Roche concentrated solution materials was prepared and the I-number compared with the stated bioactivity of EPO BRP3. The results are summarized as follows: The I-number assay applied to Centocor's epoetin alfa secondary standard provided an I-number of $I\alpha = 545.6$, corresponding with a bioactivity of $144.8 \text{ IU}/\mu\text{g}$. The same assay applied to the mean of the 40 epoetin beta training batches from Roche provided an I-number of $I\beta_{40tr} = 509.0$, corresponding with a bioactivity of $135.1 \text{ IU}/\mu\text{g}$. The hypothetical 1:1 mixture of both products yielded an I-number of $I\alpha/\beta_{40tr} = 527.3$, corresponding with a bioactivity of $140.0 \text{ IU}/\mu\text{g}$, which was in accordance with the stated bioactivity of BRP3 of $141.1 \text{ IU}/\mu\text{g}$ (accuracy = 99.2%; difference to the stated bioactivity = -0.8%). This accordance between the potency of the artificial epoetin alfa / beta 1:1 mixture and the stated bioactivity of EPO BRP3 may be taken as a proof of principle that the I-number of an EPO sample allows to precisely calculate its biological activity.

Keywords: Bioassay; Biological Reference Preparation (BRP); Capillary Zone Electrophoresis (CZE), Epoetin, Potency; Standard

Abbreviations

BNM: Bioassay in Normocythaemic Mice; BRP: Biological Reference Preparation; cBRP: Candidate BRP; CV: Coefficient of Variation; CZE: Capillary Zone Electrophoresis; EDQM: European Directorate for the Quality of Medicines; EPO: Erythropoietin; HPAEC: High-performance anion-exchange chromatography;

I-number: Isoform number calculated via the peak numbering in CZE; IF: Isoform; in: Individual Isoform number shares; IU: International unit; MV: Mean value; Ph. Eur.: European Pharmacopoeia; pn: Peak area percent shares; RP-HPLC: Reversed-phase high-performance liquid chromatography.

Introduction

The reduction, refinement and replacement (3Rs) of bioassays by physicochemical or cell based assays or other alternative methods is a common goal of the European Commission, health

care and animal welfare societies, regulatory authorities, expert groups and pharmaceutical companies [1], in particular with erythropoietin [2]. Three physicochemical assays based on capillary zone electrophoresis data have up till now aimed at replacing the mouse bioassay of erythropoietin batch release according to Ph. Eur. [3], i) the “I-number assay” introduced in 2006 for the quality control of EPO medicinal products [4-6] and recently used to calculate and compare the potencies of the biological reference preparations of erythropoietin [7], ii) the “Ibio-number assay” that has likewise been introduced in 2005/2006 [5-6] however only recently been termed as such when the assay was applied to calculate the potencies of various EPO drug substance and drug product samples [8] respectively the various (candidate) EPO BRPs [9], and iii) the “reduced linear regression model” from Roche introduced in 2010/2011 [10-12] that meanwhile has been accepted by the European authorities to replace the Normocythaemic mouse bioassay of epoetin beta drug substance batch release [13]. Whereas the “I-number assay” uses the peak numbering of the EPO Isoforms separated in CZE for “I-number” calculation [4-7], the “Ibio-number assay” uses factors that reflect the bioactivity of the EPO isoforms [5-6] for direct potency calculation [8-9]. And whereas these two assays use all eight EPO Isoforms separated in CZE for bioactivity calculation (where applicable), the Roche assay uses only five Isoforms and adds instead a term reflecting sialylation [10]. The aim of this current study was to prove that the “I-number assay”, like the “Ibio-number assay” [8-9], is suitability to predict the potency of EPO medicinal products with high precision and accuracy.

Note: It should be mentioned at this stage that the current “I-number” study in a way parallels the “Ibio-number” study reported elsewhere [8] as it is based on the same set of CZE data. Hence, the final potencies calculated via the two assays are quite comparable (mean difference = 1.2%), and the arguments in the two papers are likewise very similar (if not identical). But the “I-number assay” deserves its own presentation in a separate paper, as potency calculation according to the “I-number assay” is based on the peak numbering in CZE of the EPO Isoforms, whereas potency calculation according to the “Ibio-number assay” is based on the bioactivities of these Isoforms.

Materials and Methods

Materials

a) EPO cBRP3 was as described in a collaborative study published in 2007 [14], and the CZE data were taken from this study. EPO cBRP3 consisted in “a 50:50 (weight / weight) blending of 2 erythropoietin preparations corresponding to products currently available on the European market (erythropoietin-alfa and erythropoietin-beta), with each vial containing approximately 250 µg erythropoietin” [14] – with “a potency of 35,280 IU/vial” [14], i.e. 141,120 IU/mg or 141.1 IU/µg, used throughout herein.

b) Epoetin alfa secondary standard was from Centocor as described elsewhere [15], and the CZE data were taken from this study. Briefly, the epoetin alfa sample from Centocor consisted in epoetin alfa obtained from Johnson & Johnson Ortho Biotech and was used to prepare a secondary standard that has been calibrated against EPO BRP1 [15].

c) An artificial epoetin beta material was hypothetically constructed from a pool of 40 epoetin beta training batches from Roche, “derived from several variants of a well-defined manufacturing procedure which is tightly controlled within defined limits” [10]. The mean isoform percentages measured by Roche in CZE of these 40 epoetin beta training batches were used to calculate the mean I-number (IB_40tr) of this hypothetical epoetin beta mixture. Noteworthy, in these 40 epoetin beta training batches “batches were also included with markedly low biological potencies” [10], determined in Normocythaemic mice.

d) A hypothetical epoetin alfa / beta 1:1 mixture was constructed from Centocor’s epoetin alfa secondary standard [15] and the mean of Roche’s 40 epoetin beta training batches [10].

Methods

EPO sample preparation and analytical conditions of CZE were as first described in 2002 [16] and kept unchanged up to date [3]. The I numbers were calculated as earlier described [4-6]. Thus, “the overall isoform number I of EPO is the sum of the products of the individual CZE peak area percent shares (pn) and the corresponding individual isoform numbers (n)” [4] (Formula 1)

$$I = p_1 \times 1 + p_2 \times 2 + p_3 \times 3 + p_4 \times 4 + p_5 \times 5 + p_6 \times 6 + p_7 \times 7 + p_8 \times 8 \text{ (Formula 1)}$$

The bioactivity of a sample (Bio_sample) was calculated from the I-number of the sample (I_{sample}), the I-number of cBRP3 (I_{cBRP3}) and the stated bioactivity of BRP3 (expressed by the author as 141.1 IU/µg), as shown in (Formula 2).

$$\text{Bio_sample} = I_{\text{sample}} / I_{\text{cBRP3}} * \text{Bio_BRP3} \text{ (Formula 2)}$$

The I-number of cBRP3 (I_{cBRP3}) was retrospectively calculated by the author according to Formula 1 from the CZE data given in the collaborative study published in 2007 [14]. This I_{cBRP3} -number was correlated with the stated bioactivity of EPO BRP3. Note: The outlier according to Grubbs [17] and according to Dixon [18] revealed by the author in the course of this retrospective I-number calculation was disregarded, which is specified in the footnote to (Table 1). The I number of Centocor’s epoetin alfa secondary standard (I_{α}) was retrospectively calculated by the author according to Formula 1, using the CZE data published by Centocor [15]. The mean I-number of Roche’s 40 epoetin beta training batches (IB_40tr) was retrospectively calculated by the author according to Formula 1, using the mean CZE data published by Roche [10]. Thus, the average peak area values of isoform 1-8 (numbering ac-

ording to Ph. Eur. [3,16]) respectively IF_8 – IF_1 (numbering by Roche [10]) of these 40 epoetin beta training batches were regarded as the values of an artificial epoetin beta concentrated solution pool material. Note: Compared with the Roche papers [10-12], the isoforms are numbered in reverse order in this paper, which uses the order of the Ph. Eur. monograph [3,16] (Figure 1). The I-number of the hypothetical epoetin alfa / beta 1:1 mixture (I α /I β _40tr) was gained from the I-number of Centocor’s epoetin alfa secondary standard (I α) and the mean I-number of Roche’s 40 epoetin beta training batches (I β _40tr) as the arithmetic mean (Formula 3).

$$I\alpha/I\beta_{40tr} = (I\alpha + I\beta_{40tr}) / 2 \text{ (Formula 3)}$$

The bioactivity of this hypothetical epoetin alfa / beta 1:1 sample was again calculated via I β BRP3 and the stated bioactivity of BRP3 (cp. Formula 2).

Results

A reference electropherogram is shown in (Figure 1).

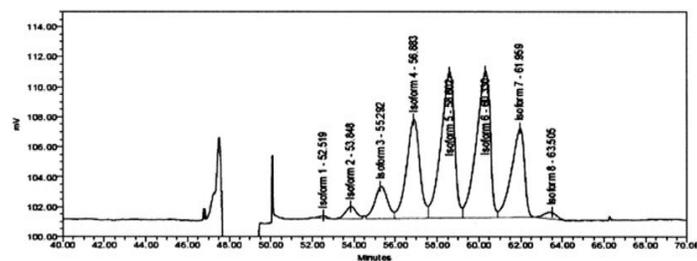


Figure 1: CZE electropherogram of EPO cBRP3, taken from the 2007 study [14] – with permission by EDQM.

The I-number calculation for cBRP3 from the CZE data published in the 2007 study [14] provided I β BRP3 = 531.5 ± 3.8 (inter-laboratory precision CV = 0.7%; n = 9 labs) (Table 1), which was related to the stated bioactivity of EPO BRP3 [14], expressed as 141.1 IU/μg. Centocor’s epoetin alfa secondary standard [15] provided I α = 545.6 ± 0.6 (n = 4 CZE runs) which, calculated via Formula 2, corresponded with a bioactivity of 144.8 IU/μg (Table 2).

Peak No.	Lab 1		Lab 2		Lab 3		Lab 4		Lab 6		Lab 7		Lab 9		Lab 13		Lab 15		Lab 16	
	p _n	i _n	p _n	i _n	p _n	i _n	p _n	i _n	p _n	i _n	p _n	i _n								
1	0.2	0.2	0.6	0.6	0.4	0.4	0	0	0.4	0.4	0	0	0.5	0.5	0	0	0.7	0.7	0.6	0.6
2	1.7	3.4	1.6	3.2	2.4	4.8	0.7	1.4	1.5	3	1	2	1.6	3.2	1.6	3.2	2	4	1.2	2.4
3	5.5	16.5	5.3	15.9	5.3	15.9	3.4	10.2	5.5	16.5	4.7	14.1	5.4	16.2	5.9	17.7	5.7	17.1	5.5	16.5
4	18	73.2	17	68.8	18	73.2	15	60.4	18	72	18	71.2	18	72.8	18.1	72.4	18.6	74.4	17.5	70
5	28	139	27	136	28	139	28	138	28	138	28	141	28	141	27.9	140	27.2	136	28.5	143
6	28	170	29	171	28	168	32	191	29	172	29	173	28	168	28.1	169	27.6	166	28.7	172
7	17	117	17	122	16	115	20	139	17	120	18	123	17	116	16.7	117	16.5	116	16.2	113
8	1.5	12	2.2	17.6	1.5	12	1.6	12.8	1.4	11.2	1.9	15.2	1.6	12.8	1.7	13.6	1.7	13.6	1.6	12.8
I _{bio} =	531		534.4		527.6		552.8		531.9		539.6		529.5		531.9		526.9		530.4	
Mean	533.6		±	7.6					(n = 10)											
					CV = 1.4 %															
Mean	531.5		±	3.8	CV = 0.7 %				(n = 9)		(Outlier Lab 4 disregarded)									

Note: Lab 4 proved to be an outlier according to Grubbs ($\alpha = 0.01$) and according to Dixon ($\alpha = 0.02$) and was therefore disregarded. I β BRP3 = 531.5 is related to the stated potency of BRP3 [14], expressed as 141.1 IU/μg.

Table 1: cBRP3 I-number calculation and inter-laboratory precision

Basis: Table 3d of Behr-Gross et al. 2007 [14] - Isoform distribution (in %) of cBRP3 uncorrected for migration time.

Roche’s 40 epoetin beta training batches provided a mean I-number of I β _40tr = 509.0 which, calculated via Formula 2, corresponded with a bioactivity of 135.1 IU/μg (Table 3).

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Peak No.	Run 1		Run 2		Run 3		Run 4	
	pn	in	pn	in	pn	in	pn	in
1	2.1	6.3	2.2	6.6	2.2	6.6	2.1	6.3
2	18.9	75.6	19.0	76.0	18.7	74.8	18.9	75.6
3	30.5	152.5	30.6	153.0	30.3	151.5	30.4	152.0
4	30.2	181.2	30.2	181.2	30.2	181.2	30.2	181.2
5	17.2	120.4	17.0	119.0	17.3	121.1	17.2	120.4
6	1.3	10.4	1.2	9.6	1.3	10.4	1.2	9.6
I α =	546,4		545,4		545,6		545,1	
Mean	545.6		± 0.6		CV = 0.1%		(n = 4)	

Bioactivity calculation via **Formula 2**:

$$\text{Bio}_\alpha = I_\alpha / I_{\text{cBRP3}} * \text{Bio_BRP3} = 545.6 / 531.5 * 141.1 \text{ IU}/\mu\text{g} = 144.8 \text{ IU}/\mu\text{g}$$

Table 2: Epoetin alpha secondary standard, I-number calculation

Basis: CZE data from Table 2 of Zhang et al. [15]

Peak No.	Run 1		Run 2		Run 3		Run 4	
	pn	in	pn	in	pn	in	pn	in
1	2.1	6.3	2.2	6.6	2.2	6.6	2.1	6.3
2	18.9	75.6	19	76	18.7	74.8	18.9	75.6
3	30.5	152.5	30.6	153	30.3	151.5	30.4	152
4	30.2	181.2	30.2	181.2	30.2	181.2	30.2	181.2
5	17.2	120.4	17	119	17.3	121.1	17.2	120.4
6	1.3	10.4	1.2	9.6	1.3	10.4	1.2	9.6
I α =	546,4		545,4		545,6		545,1	
Mean	545.6		± 0.6		CV = 0.10%		(n = 4)	

Bioactivity calculation via **(Formula 2)**

$$\text{Bio}_\beta_{40\text{tr}} = I_{\beta_{40\text{tr}}} / I_{\text{cBRP3}} * \text{Bio_BRP3} = 509.0 / 531.5 * 141.1 \text{ IU}/\mu\text{g} = 135.1 \text{ IU}/\mu\text{g}$$

Table 3: 40 Epoetin beta training batches, I-number calculation

Basis: CZE data from Table 2 of Zimmermann et al. [10].

Note: As mentioned in the “Methods”, the peak numbering used by Roche [10-12] was inverse, compared to the peak numbering according to Ph. Eur. [3,16] respectively the I β -number assay [8-9], and the isoform numbers had to be considered accordingly (Table 3). Centocor’s epoetin alfa secondary standard and Roche’s mean of the 40 epoetin beta training batches, when theoretically mixed 1:1, yielded a mean I $\alpha/\beta_{40\text{tr}}$ = 527.3 which, calculated via Formula 2, corresponded with a bioactivity of 140.0 IU/ μg that matched the stated bioactivity of BRP3 with an accuracy of 99.2% (difference = 0.8%) (Table 4).

Material	I-number		Calculated bioactivity [IU/ μg]	Remarks
Epoetin alfa secondary standard (Centocor)	I α =	545.6	144.8	cp. Table 2
Epoetin beta, mean of 40 training batches (Roche)	I $\beta_{40\text{tr}}$ =	509.0	135.1	cp. Table 3
Epoetin $\alpha/\beta_{40\text{tr}}$	I $\alpha/\beta_{40\text{tr}}$ =	527.3	140.0	Accuracy against the stated bioactivity of BRP3 = 99.2%

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(1:1 mixture)				
cBRP3	IcBRP3 =	531.5		
(n = 9 labs)				
BRP3, stated bioactivity [IU/μg]		141.1		

Bioactivity calculation via **Formula 2:**

$$\text{Bio}_{\alpha/\beta_40\text{tr}} = I_{\alpha/\beta_40\text{tr}} / I_{\text{cBRP3}} * \text{Bio}_{\text{BRP3}} = 527.3 / 531.5 * 141.1 \text{ IU}/\mu\text{g} = 140.0 \text{ IU}/\mu\text{g}$$

Table 4: Epoetin alfa/beta_{40tr} 1:1 mixture, I-number calculation, accuracy determination

Discussion

Although the I-number assay has been published more than 10 years ago [4], no data has been provided in the literature thus far that shows that the I-number assay allows to calculate the potency of EPO medicinal products, as suggested [4-6], and this was the aim of the current study. To this, the bioactivity of the samples was calculated via the I-number of cBRP3 and the stated bioactivity of BRP3 [14], which laid the basis for the potency calculations of the other samples (cp. Formula 2). This basis is justified by the facts that i) the I-number of cBRP3 could be determined from the data of the 2007 collaborative study of 10 participating laboratories with an inter-laboratory precision of CV = 0.7% (Table 1) and ii) the potency stated for BRP3 was without any doubt – in contrast to the potencies stated for EPO BRP1 and EPO BRP2, as shown elsewhere [7]. This precise set of data primarily relies on the precision and accuracy of CZE. But exactly for that reason, the precision of the I-number determination of EPO samples simultaneously reflects the bioactivity of the samples in CZE in a likewise precise manner [8], which was particularly apparent from the hypothetical epoetin alfa / beta 1:1 mixture that met the stated bioactivity of EPO BRP3 with an amazing accuracy of 99.2% (Table 4). Attempts to calculate the bioactivity of EPO samples via the stated potency of BRP2 and the I-number of BRP2 (cp. Formula 2) have not been satisfactory, most likely due to the fact that the bioactivity of EPO BRP2 has been stated about 10% too low [7,9]. And attempts to compare the calculated bioactivity of Centocor’s secondary standard (144.8 IU/μg; cp. Table 2) with the bioactivity given by Centocor [15] were deliberately omitted as Centocor’s epoetin alfa secondary standard has been calibrated against EPO BRP1, the stated bioactivity of which has been stated about 5% too low [7,9].

As already mentioned elsewhere [8], “the mean biological activity determined in Normocythaemic mice (BNM) of Roche’s 40 epoetin beta training batches was communicated as 196 “IU/μg”. Although not explicitly mentioned by Roche, this value must be read as 196 “IU/μg protein”, concluded by the author from reference [20], and must therefore be multiplied by 0.60 in order to get “IU/μg EPO” glycoprotein, reflecting the protein content of the erythropoietin glycoprotein”[8]. Thus, the mean epoetin beta sample ended up with a BNM of 117.6 “IU/μg EPO” that differed from the bioactivity calculated via the I-number assay (135.1 IU/μg = 100%; cp. Table 3) by -14.9%. This difference may be due to

the fact that in these 40 epoetin beta training batches “batches were also included with markedly low biological potencies” [10]. This difference may, however, also (and more likely) be attributable to the fact the majority of the 40 epoetin beta training batches (if not all) have been calibrated against EPO BRP2, the stated potency of which has been questioned [7,9]. Considering these inconsistencies revealed for Centocor’s epoetin alfa secondary standard (due to its calibration against EPO BRP1) respectively for Roche’s mean of the 40 epoetin beta training batches (due to their likely calibration against EPO BRP2), it is quite amazing that the bioactivity of the Centocor’s epoetin alfa 1:1 mixture calculated via the I-number assay (140.0 IU/μg) was in accordance with the stated bioactivity of EPO BRP3 (141.1 IU/μg) (difference = -0.8%; Table 4), which may be taken as a proof of principle that the bioactivity of EPO samples may be precisely calculated via the I-number assay as previously suggested [4].

Conclusion

The data presented in this retrospective analysis by the author of the CZE data of epoetin Alfa and epoetin beta samples has shown that the “I-number assay” - like the “Ibio-number assay” [8] – is suitable to assess the bioactivity of EPO medicinal products with high precision and accuracy, which should be confirmed in further studies comprising in particular samples from EPO batch release. In combination with other physicochemical assays such as RP-HPLC, peptide mapping and HPAEC, the “I-number assay” – like the “Ibio-number assay” [8] – offers an opportunity to replace the highly contested (consumption of animals) and highly inaccurate mouse bioassay of EPO batch release.

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