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# I-Number Assay and Erythropoietin Potency: Retrospective Capillary Zone Electrophoresis Data Analysis of the Biological Reference Preparations of Erythropoietin

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## Abstract

The capillary zone electrophoresis data of erythropoietin reference preparations batch 1 (BRP1), batch 2 (BRP2) and batch 3 (BRP3) of three collaborative studies published in 2004, 2007 and 2015 have retrospectively been analyzed by the author via the *I*-number assay, a physicochemical assay that allows potency calculation of erythropoietin samples in a precise and accurate manner. The results are summarized as follows:

The *I*-number assay revealed an inter-laboratory precision of CV <1.0% and an accuracy of 100.0 ± <1.5%. The *I*-number calculated for an EPO sample and the corresponding EPO reference standard may be regarded analytically "indistinguishable" at *I*-number differences of <1.5%, "comparable" at differences of 1.5-3.0% and "different" at differences of >3.0%.

This retrospective analysis by the author of the CZE data of the (candidate) EPO BRPs has revealed that the bioactivity stated for EPO BRP1 (130.0 IU/µg) should have been stated about 5% higher ("BRP1+5%"=136.5 IU/µg) and the bioactivity stated for EPO BRP2 (130.0 IU/µg) should have been stated about 10% higher ("BRP2+10%"=143.0 IU/µg), and the two new potency values are proposed by the author herewith. The potency assigned for EPO BRP3 (141.1 IU/µg) was without any doubt and therefore confirmed.

**Keywords:** Bioassay; Biological reference preparation (BRP); Capillary zone electrophoresis (CZE); Erythropoietin (EPO); Potency; Standard

**Abbreviations:** BRP: Biological Reference Preparation; cBRP: candidate BRP; CRS: Chemical Reference Substance; cCRS: candidate CRS; CV: Coefficient of Variation; CZE: Capillary Zone Electrophoresis; EDQM: European Directorate for the Quality of Medicines; EPO: Erythropoietin; IF: Isoform; *I*-number: Isoform number calculated via the peak numbering in CZE; $i_n$: Individual isoform number shares; IS: International standard; IU: International unit; MV: Mean value; NIBSC: National Institute for Biological Standards and Control; Ph. Eur.: European Pharmacopoeia; $p_n$: Peak area percent shares; ref(s): Reference(s); std.: Standard

## Introduction

Recombinant human erythropoietin is a biotechnologically produced hormone, which stimulates human red blood cell growth and is therefore marketed worldwide for the treatment of anemia. The biological activity of the erythropoietin medicinal products is determined via *in vivo* assays in mice which are known to be highly inaccurate (CV ≈ 25% [1,2], ≈ 20% [3,4]; uncertainty 15-30% [5] as stated by Zimmermann et al. [3]). Therefore, there are ongoing efforts to replace the highly contested (consumption of animals) and highly variable polycythaemic and normocythaemic mouse bioassays in the quality control of erythropoietin by more precise and more accurate physicochemical methods.

In 2006, the *I*-number assay, based on CZE data of EPO samples, has been introduced as a physicochemical test that could be used for the quality control of EPO samples [6-8]. However, the suitability of the assay to predict the bioactivity of EPO samples (proof of principle) has only been demonstrated in 2017 [9].

A second assay, the so-called "Ibio-number assay" that likewise is based on the CZE data of the EPO samples, has been introduced at the same time [7-8], however has only recently been termed as such [10,11], when the assay was applied to calculate the potencies of various EPO drug substance and drug product samples [10] respectively the various (candidate) biological reference preparations of erythropoietin [11].

Whereas the "I-number assay" uses the peak numbering of the EPO isoforms separated in CZE for "I-number" calculation [6-9], the "Ibio-number assay" uses factors that reflect the bioactivity of the EPO isoforms for direct potency calculation [7,8,10,11].

**Note:** It should be mentioned at this stage that the current "I-number" study dealing with EPO (c)BRPs in a way parallels the "Ibio-number" study reported elsewhere [11] as it is based on the same set of CZE data gathered in various collaborative studies designed to establish EPO BRP1 [12], EPO BRP2 [13], EPO BRP3 [14] and the EPO chemical reference substance [15]. Hence, the final potencies calculated via the two assays are quite comparable (mean difference=1.2% [11]), 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.

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| | Lab | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | *I*-numbers | | | | | | | | | | |
| **BRP1** | | **Lab 2** | **Lab 3** | **Lab 8** | **Lab 9** | **Lab 10** | **Lab 11** | | | | |
| | | 522.1 | 519.2 | 515.0 | 522.4 | 519.4 | 514.1 | | | | |
| Data from [6] | **MV** | **518.7** | **±** | **3.5** | **CV=** | **0.7%** | **(n=6)** | | | | |
| **cBRP2 (1)** | | **Lab 2** | **Lab 3** | **Lab 8** | **Lab 9** | **Lab 10** | Lab 11 | **Lab 12** | | | |
| | | 544.4 | 543.0 | 539.7 | 542.9 | 544.4 | (1) | 539.7 | | | |
| Data from ref [6] | **MV** | **542.4** | **±** | **2.2** | **CV =** | **0.4%** | **(n = 6)** | | | | |
| **BRP2** | | **Lab 1** | **Lab 2** | **Lab 3** | **Lab 4** | **Lab 6** | **Lab 7** | **Lab 9** | **Lab 13** | **Lab 15** | **Lab 16** |
| RT uncorrected **(2)** | | 543.0 | 543.4 | 538.6 | 546.3 | 538.0 | 543.0 | 547.1 | 547.5 | 541.2 | 540.3 |
| Data from Table 2 | **MV** | **542.8** | **±** | **3.4** | **CV =** | **0.6%** | **(n = 10)** | | | | |
| **cBRP3 (3)** | | **Lab 1** | **Lab 2** | **Lab 3** | **Lab 4** | **Lab 6** | **Lab 7** | **Lab 9** | **Lab 13** | **Lab 15** | **Lab 16** |
| RT uncorrected **(2)** | | 531.0 | 534.4 | 527.6 | (3) | 531.9 | 539.6 | 529.5 | 531.9 | 526.9 | 530.4 |
| Data from ref [9] | **MV** | **531.5** | **±** | **3.8** | **CV =** | **0.7%** | **(n = 9)** | | | | |
| **BRP3 (4)** | | **Lab 1** | Lab 2 | **Lab 3** | **Lab 4** | **Lab 5** | | | | | |
| RT uncorrected **(2)** | | 531.1 | (4) | 527.2 | 525.6 | 526.3 | | | | | |
| Data not shown **(5)** | **MV** | **527.6** | **±** | **2.5** | **CV =** | **0.5%** | **(n = 4)** | | | | |
| **cCRS** | | **Lab 1** | **Lab 2** | **Lab 3** | **Lab 4** | **Lab 5** | | | | | |
| RT uncorrected **(2)** | | 534.8 | 535.1 | 528.8 | 528.4 | 526.1 | | | | | |
| Data not shown **(5)** | **MV** | **530.6** | **±** | **4.1** | **CV =** | **0.8%** | **(n = 5)** | | | | |

(1) For cBRP2, lab 11 was an outlier according to Grubbs (α = 0.01) as well as according to Dixon (α = 0.002) and was therefore disregarded.
(2) The uncorrected values were used for *I*-number calculation, in order to stay in line with the previously published data of BRP1 and cBRP2 [6].
(3) For cBRP3, lab 4 was an outlier according to Grubbs (α = 0.01) as well as according to Dixon (α = 0.02) and was therefore disregarded.
(4) For BRP3, lab 2 was an outlier according to Grubbs (α = 0.05) as well as according to Dixon (α = 0.02) and was therefore disregarded.
(5) These data have been calculated in analogy to Table 2 and are not presented in details herein.

**Table 1:** *I*-numbers determined for the various (c)BRPs and cCRS and inter-laboratory precision of the assay.

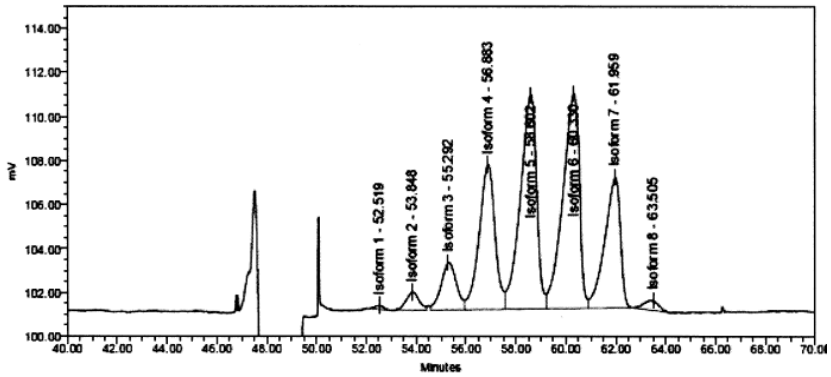


**Figure 1:** CZE electropherogram of EPO cBRP3, taken from the 2007 study [14] with permission by EDQM.

## Materials and Methods

EPO sample preparation and analytical conditions of CZE were as first described in 2002 [16] and kept unchanged up to date [17,18]. EPO BRP1, cBRP2, BRP2, cBRP3, BRP3 and cCRS were as described in collaborative studies published in 1997 [12], 2004 [13], 2007 [14] and 2015 [15], and the CZE data were taken from the latter three studies. In each of these studies, the BRPs consisted in 50:50 (weight / weight) blends of epoetin-alpha and epoetin-beta. The stated potencies of BRP1 and BRP2 were 32,500 IU/vial, each, where each vial contained approximately 250 µg EPO [12,13], equivalent to 130,000 IU/mg or 130.0 IU/µg. The stated potency of BRP3 was 35,280 IU/vial, where each vial contained approximately 250 µg EPO [14], equivalent to 141,120 IU/mg or 141.1 IU/µg.

The *I*-numbers of these EPO materials were calculated as earlier described [6]: "*I* is defined as the sum of the products of the individual CZE peak area percent shares ($p_n$) of the EPO isoforms (n=1-8) and the corresponding individual isoform numbers (*n*)" [6] (Formula 1):

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

The *I*-numbers retrospectively calculated by the author from the CZE data published in these collaborative studies were used to assess the inter-laboratory precision and accuracy of the *I*-number assay. To this, the BRP of the earlier study was taken as the "standard", the candidate sample was taken as the "sample". For example, in the 2007 study [14], designed to establish erythropoietin reference preparation batch 3, BRP2 of the 2004 study [13] was regarded as the "standard", and the candidate sample (cBRP3) was regarded as the "sample".

Outliers according to Grubbs [19] respectively Dixon [20], as retrospectively identified by the author in the collaborative studies upon *I*-number calculation, are specified in footnotes to summary Table 1 and disregarded in the current study.

The bioactivity of the "sample" (Bio_sample) was calculated from the *I*-number of the "sample" ($I_{sample}$), the *I*-number of the BRP "standard" ($I_{std.}$) and the stated bioactivity of the BRP "standard" (Bio_std.), as shown in Formula 2.

| Peak No. | Lab 1 | | Lab 2 | | Lab 3 | | Lab 4 | | Lab 6 | | Lab 7 | | Lab 9 | | Lab 13 | | Lab 15 | | Lab 16 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $p_n$ | $i_n$ | $p_n$ | $i_n$ | $p_n$ | $i_n$ | $p_n$ | $i_n$ | $p_n$ | $i_n$ | $p_n$ | $i_n$ | $p_n$ | $i_n$ | $p_n$ | $i_n$ | $p_n$ | $i_n$ | $p_n$ | $i_n$ |
| 1 | 0.1 | 0.1 | 0.0 | 0.0 | 0.5 | 0.5 | 0.0 | 0.0 | 0.3 | 0.3 | 0.0 | 0.0 | 0.1 | 0.1 | 0.0 | 0.0 | 0.2 | 0.2 | 0.1 | 0.1 |
| 2 | 1.1 | 2.2 | 0.4 | 0.8 | 1.5 | 3.0 | 1.0 | 2.0 | 1.3 | 2.6 | 1.1 | 2.2 | 0.7 | 1.4 | 0.6 | 1.2 | 1.1 | 2.2 | 1.0 | 2.0 |
| 3 | 4.8 | 14.4 | 5.8 | 17.4 | 4.9 | 14.7 | 4.2 | 12.6 | 4.9 | 14.7 | 4.5 | 13.5 | 2.7 | 8.1 | 4.3 | 12.9 | 4.8 | 14.4 | 4.6 | 13.8 |
| 4 | 16.7 | 66.8 | 16.8 | 67.2 | 16.9 | 67.6 | 16.1 | 64.4 | 17.4 | 69.6 | 17.5 | 70.0 | 17.4 | 69.6 | 16.6 | 66.4 | 17.0 | 68.0 | 16.9 | 67.6 |
| 5 | 26.8 | 134.0 | 26.6 | 133.0 | 26.4 | 132.0 | 27.5 | 137.5 | 27.3 | 136.5 | 26.9 | 134.5 | 28.8 | 144.0 | 27.5 | 137.5 | 26.4 | 132.0 | 27.5 | 137.5 |
| 6 | 29.8 | 178.8 | 29.7 | 178.2 | 29.6 | 177.6 | 30.2 | 181.2 | 29.5 | 177.0 | 29.2 | 175.2 | 30.0 | 180.0 | 29.8 | 178.8 | 29.7 | 178.2 | 29.7 | 178.2 |
| 7 | 18.9 | 132.3 | 18.8 | 131.6 | 18.4 | 128.8 | 19.4 | 135.8 | 17.9 | 125.3 | 18.8 | 131.6 | 18.5 | 129.5 | 18.9 | 132.3 | 18.6 | 130.2 | 18.1 | 126.7 |
| 8 | 1.8 | 14.4 | 1.9 | 15.2 | 1.8 | 14.4 | 1.6 | 12.8 | 1.5 | 12.0 | 2.0 | 16.0 | 1.8 | 14.4 | 2.3 | 18.4 | 2.0 | 16.0 | 1.8 | 14.4 |
| ***I* =** | **543.0** | | **543.4** | | **538.6** | | **546.3** | | **538.0** | | **543.0** | | **547.1** | | **547.5** | | **541.2** | | **540.3** | |
| **Mean** | **542.8** | | **±** | **3.4** | **CV** | **=** | **0.6 %** | | **(n = 10)** | | | | | | | | | | | |

CZE data derived from Table 3c of Behr-Gross et al. 2007 [14] – Isoform distribution (in %) of BRP2 uncorrected for migration time.

**Table 2:** *I*-number calculation for BRP2 and inter-laboratory precision calculated from the 2007 study [14].

| Standard | Sample | *I*-number (mean value) | MV ± 2SD | Accuracy [%] Sample vs. std. | Difference [%] Sample vs. std. | CZE data origin Year of study [ref], labs | Classification proposed by the author |
|---|---|---|---|---|---|---|---|
| BRP1 | | 518.7 | 525.7 - 511.7 | **104.6%** | **+ 4.6** | 2004 [13], 6 labs | Different (>3.0%) |
| | cBRP2 | 542.4 | 546.7 - 538.0 | | | | |
| cBRP2 | | | | 100.1% | + 0.1 | 2004 [13] and 2007 [14], 16 labs | Indistinguishable (<1.5%) |
| | BRP2 | 542.8 | 549.6 - 536.1 | | | | |
| BRP2 | | | | 97.9% | - 2.1 | 2007 [14], 10 labs | Comparable (1.5 – 3.0%) |
| | cBRP3 | 531.5 | 539.1 - 523.8 | | | | |
| cBRP3 | | | | 99.3% | - 0.7 | 2007 [14] and 2015 [15], 15 labs | Indistinguishable (<1.5%) |
| | BRP3 | 527.6 | 532.5 - 522.6 | | | | |
| BRP3 | | | | 100.6% | + 0.6 | 2015 [15], 5 labs | Indistinguishable (<1.5%) |
| | cCRS | 530.6 | 538.8 - 522.5 | | | | |

**Table 3:** Accuracy of *I*-number of sample versus *I*-number of standard.

$$\text{Bio\_sample} = I_{sample} / I_{std.} * \text{Bio\_std.} \quad (2)$$

Note: For calculations according to Formula 2 in which Bio_std. was Bio_BRP3 = 141.1 IU/µg, $I_{cBRP3}$ = 531.5 was used (instead of $I_{BRP3}$ = 527.6), as $I_{cBRP3}$ was gained from 9 labs [14] (instead of only from 4 labs ($I_{BRP3}$) [15]) (cp. Table 1), which rendered the $I_{cBRP3}$ value more reliable.

## Results

A reference electropherogram is shown in Figure 1.

### Inter-laboratory precision

The inter-laboratory precision data are summarized as follows (Table 1):

The *I*-number calculations for BRP1 std. and cBRP2 sample (2004 study [13]), for BRP2 std. and cBRP3 sample (2007 study [14]), and for BRP3 std. and cCRS sample (2015 study [15]) provided $I_{BRP1}$=518.7 ± 3.5 (CV=0.7%, n=6 labs) [6], $I_{cBRP2}$=542.4 ± 2.2 (CV=0.4%, n=6 labs) [6], $I_{BRP2}$=542.8 ± 3.4 (CV=0.6%, n=10 labs) (Table 2), $I_{cBRP3}$=531.5 ± 3.8 (CV=0.7%, n=9 labs) [9], $I_{BRP3}$=527.6 ± 2.5 (CV=0.5%, n=4 labs) and $I_{cCRS}$=530.6 ± 4.1 (CV=0.8%, n=5 labs).

The *I*-number calculations for BRP1 std. and cBRP2 sample have already been published previously [6], and the *I*-number calculation for cBRP3 is described elsewhere [9]; hence, these data are not again included herein. The *I*-number calculation for BRP2 is exemplarily given in details in Table 2. The *I*-numbers of BRP3 and cCRS have been calculated alike and are not shown in details, however their overall results are included in Table 1.

In summary, the CZE data of the three collaborative studies [13-15] revealed for the *I*-number assay an inter-laboratory precision of CV <1.0% each, ranging from CV=0.4% (2004 study [13]) to CV=0.8% (2015 study [15]) (Table 1).

### Accuracy

**Accuracy of "*I*-number of sample versus *I*-number of standard":** The bioactivity calculated via the *I*-number of the sample was compared with the potency calculated via the *I*-number of the reference standard that was regarded as the "true" potency value (=100%). The accuracy data of "sample versus standard" are summarized as follows (Table 3).

In the 2004 study [13] (designed to establish cBRP2), the sample cBRP2 was measured against reference standard BRP1 (=100%). BRP1 exhibited $I_{BRP1}$=518.7 [6] (=100%); MV ± 2 SD range: 525.7–511.7. cBRP2 sample exhibited $I_{cBRP2}$=542.4 [6] (accuracy=104.6%); MV ± 2 SD range: 546.7–538.0. The two MV ± 2 SD ranges were completely separated.

In the 2004 study [13] and the 2007 study [14], two identical samples have been measured; 2004: cBRP2, herein regarded as std. (=100%; data as before); 2007: BRP2, herein regarded as the sample. BRP2 sample exhibited $I_{BRP2}$=542.8 (accuracy=100.1%); MV ± 2 SD range: 549.6–536.1. The two MV ± 2 SD ranges were almost congruent.

In the 2007 study [14] (designed to establish cBRP3), the sample cBRP3 was measured against reference standard BRP2 (=100%; data as before). cBRP3 sample exhibited $I_{cBRP3}$=531.5 (accuracy=97.9%); MV ± 2 SD range: 539.1–523.8. The two MV ± 2 SD ranges were partially overlapping.

In the 2007 study [14] and the 2015 study [15], again two identical samples have been measured (2007: cBRP3, herein regarded as std. (=100%; data as before); 2015: BRP3, herein regarded as the sample. BRP3 sample exhibited $I_{BRP3}$=527.6 (accuracy=99.3%); MV ± 2 SD range: 532.5–522.6. The two MV ± 2 SD ranges were almost congruent.

In the 2015 study [15] (designed to establish cCRS), the cCRS sample was prepared in analogy to BRP3 and measured against

| Sample designation | | BRP1 | BRP1 + 5 % | cBRP2 | BRP2 | BRP2 + 10 % | cBRP3 | BRP3 | cCRS | Accuracy [%] against stated or proposed potency | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | BRP2 = 130.0 IU/µg | BRP2 + 10 % = 143.0 IU/µg | BRP3 = 141.1 IU/µg |
| ***I*-number** | | **518.7** | | **542.4** | **542.8** | | **531.5** | **527.6** | **530.6** | | | |
| **Stated bioactivity [IU/µg]** [ref] | | **130.0** [12] | | **130.0** [13] | | | **141.1** [14] | | | | | |
| **Newly proposed bioactivity [IU/µg]** | | | **136.5** | | | **143.0** | | | | | | |
| **Bioactivity calculated via Formula 2 [IU/µg]** | cBRP2 ($I_{cBRP2}$ / $I_{BRP1}$* BRP1) | | | 135.9 | | | | | | **104.6** | 95.1 | |
| | cBRP2 ($I_{cBRP2}$ / $I_{BRP1}$* "BRP1+5%") | | | 142.7 | | | | | | 109.8 | **99.8** | |
| | cBRP2 ($I_{cBRP2}$ / $I_{cBRP3}$* BRP3) | | | 144.1 | | | | | | 110.8 | **100.8** | |
| | cBRP3 ($I_{cBRP3}$ / $I_{BRP2}$* BRP2) | | | | | | 127.3 | | | | | 90.2 |
| | cBRP3 ($I_{cBRP3}$ / $I_{BRP2}$* "BRP2+10%") | | | | | | 140.0 | | | | | **99.2** |
| | cCRS ($I_{cCRS}$ / $I_{cBRP3}$* BRP3) | | | | | | | | 140.9 | | | **99.8** |

**Table 4:** Accuracy of bioactivity of sample (calculated via Formula 2) versus stated or proposed bioactivity.

| a) Calculated potency of "sample" versus calculated potency of "standard" (= 100 %) | | | b) Calculated potency of "sample"versus stated or proposed potency (= 100 %) | | |
|---|---|---|---|---|---|
| | **Difference (cp. Table 3)** | **Judgement** | | **Difference (cp. Table 4)** | **Judgement** |
| BRP1 | - | - | BRP1 vs. stated BRP1 | +4.6 % | Different |
| | | | BRP1 vs. proposed "BRP1 + 5 %" | -0.4 % | Indistinguishable |
| cBRP2 sample vs. BRP1 std. | +4.6 % | Different | cBRP2 vs. stated BRP2 | +10.8 % | Different |
| | | | cBRP2 vs. proposed "BRP2 + 10 %" | +0.8 % | Indistinguishable |
| cBRP3 sample vs. BRP2 std. | - 0.7 % | Indistinguishable | cBRP3 vs. stated BRP3 | -0.8 % | Indistinguishable |
| cCRS sample vs. BRP3 std. | +0.6 % | Indistinguishable | cCRS vs. stated BRP3 | -0.2 % | Indistinguishable |

**Table 5:** Calculated, stated and proposed potencies (Summary Table).

reference standard BRP3 (=100%; data as before). cCRS sample exhibited $I_{cCRS}$=530.6 (accuracy=100.6%); MV ± 2 SD range: 538.8–522.5. The two MV ± 2 SD ranges were almost congruent.

**Accuracy of "*I*-number of sample versus stated bioactivity":** The bioactivities of the (c)BRPs and the cCRS, as calculated from the *I*-numbers according to Formula 2, are summarized in Table 4 and compared with the stated potencies of the BRPs, which were regarded as the "true" potency values (=100%, each). The accuracy data of "sample versus stated bioactivity" are summarized as follows (cp. Table 4).

**cBRP2**: Based on the stated bioactivity of BRP1 (130.0 IU/µg) and the *I*-numbers of cBRP2 sample ($I_{cBRP2}$=542.4 [6]) and BRP1 std. ($I_{BRP1}$=518.7 [6]), the bioactivity of cBRP2 sample was calculated to 135.9 IU/µg, which differed from the stated bioactivity of BRP2 (130.0 IU/µg) by +4.6% (accuracy=104.6%). It differed likewise from the proposed "BRP2+10%" value (143.0 IU/µg) by -4.9% (accuracy=95.1%) (Table 4).

Based on the proposed "BRP1+5%" value (136.5 IU/µg), the bioactivity of cBRP2 sample was calculated to 142.7 IU/µg, which differed from the stated bioactivity of BRP2 (130.0 IU/µg) by +9.8% (accuracy=109.8%), however fitted well with the proposed "BRP2+10%" value (143.0 IU/µg) (difference=-0.2%; accuracy=99.8%).

If the bioactivity of cBRP2 was calculated via $I_{cBRP3}$ and the stated bioactivity of BRP3 (141.1 IU/µg), a potency value of 144.1 IU/µg was obtained, which differed from the potency value stated for BRP2 (130.0 IU/µg) by +10.8%, but fitted well to the proposed "BRP2+10%" value (143.0 IU/µg) (accuracy 100.8%; difference= +0.8%).

**cBRP3**: Based on the stated bioactivity of BRP2 (130.0 IU/µg) and the *I*-numbers of cBRP3 sample ($I_{cBRP3}$=531.5 [9]) and BRP2 std. ($I_{BRP2}$=542.8; cp. Table 2), the bioactivity of cBRP3 sample was calculated to 127.3 IU/µg, which differed from the stated bioactivity of BRP3 (141.1 IU/µg) by -9.8% (accuracy=90.2%) (Table 4).

If the proposed "BRP2+10%" value (143.0 IU/µg) was used instead, the bioactivity of cBRP3 sample was calculated to 140.0 IU/µg, which differed from the stated bioactivity of BRP3 (141.1 IU/µg) by -0.8% (accuracy=99.2%).

**cCRS**: Based on the stated bioactivity of BRP3 (141.1 IU/µg) and the *I*-numbers of cCRS sample ($I_{cCRS}$=530.6; cp. Table 1) and cBRP3 std. ($I_{cBRP3}$=531.5; cp. Table 1), the bioactivity of cCRS sample was calculated to 140.9 IU/µg, which differed from the stated bioactivity of BRP3 (141.1 IU/µg) by -0.2% (accuracy=99.8%).

The accuracy data of "sample versus standard" and of "sample versus stated potency" respectively "sample versus proposed potency" are finally summarized in Table 5.

The calculated potency of BRP1 std. was "different" from the stated potency of BRP1 (difference= +4.6%) (mismatch), but fitted well to the proposed "BRP1+5%" value (difference= -0.4%).

The calculated potency of cBRP2 sample was "different" from the BRP1 std. (difference= +4.6%) as well as from the stated potency of BRP2 (difference= +10.8%) (double mismatch), but fitted well to the proposed "BRP2+10%" value (difference= +0.8%).

The calculated potency of cBRP3 sample was "indistinguishable" from the BRP2 std. (difference = -0.7%) as well as from the stated potency of BRP3 (difference= -0.8%) (double consistency).

The calculated potency of cCRS sample was "indistinguishable" from the BRP3 std. (difference= +0.6%) as well as from the stated potency of BRP3 (difference= -0.2%) (double consistency).

## Discussion

The data summarized herein may be regarded as a retrospective analysis by the author of CZE data of three collaborative studies [13-15], dealing with EPO BRPs, with respect to "inter-laboratory precision" (same material, different labs, different studies) and "accuracy"–i.e., i) accuracy of "sample versus standard" and ii) accuracy of "sample versus stated bioactivity".

### Inter-laboratory precision

The high inter-laboratory precision of the assay has already been shown in the previous calculation (CV=0.7%, n=6 labs) [6], using the CZE data of the 2004 collaborative study [13]. The inter-laboratory precision calculated from the CZE data of the 2007 [14] and the 2015 [15] collaborative study was likewise high, resulting in CV<1.0% per study (Table 1). This precise set of data primarily relies on the precision and accuracy of CZE. But exactly for that reason, this data simultaneously reflects the bioactivity of the EPO samples in CZE in a likewise precise manner [9]. Thus, CZE of EPO samples provides a means for EPO bioactivity determination of previously unmet precision. The question was whether these precisely determined potency values are likewise accurate, which is discussed in the next section.

### Accuracy

Once the accuracy data had been summarized, thus allowing an overview (cp. Table 5), inconsistencies have been revealed by the author between the bioactivities calculated via the *I*-number assay for "sample versus standard" (CZE data, each), on the one hand side, and between the bioactivities calculated via the *I*-number assay for the "samples" (CZE data) versus the stated bioactivities of the BRPs, on the other hand side. The results suggested that the bioactivities of BRP1 and BRP2 have been stated about 5% and about 10% too low, respectively.

In a first attempt to check the accuracy of the assay, the potencies calculated via the *I*-number assay for the cBRP "samples" versus the BRP "standards" were compared (CZE data, each). As can be seen from summary Table 5a, the potency calculated for cBRP2 sample did not fit to the potency calculated for BRP1 std. (2004 study [13]; difference= +4.6%), whereas the potency calculated for cBRP3 sample versus BRP2 std. fitted well (2007 study [14]; difference= -0.7%). Likewise, the potency calculated for cCRS sample versus BRP3 std. fitted well (2015 study [15]; difference = +0.6%). Why was there such a clear fitting for cBRP3 sample versus BRP2 std. and cCRS sample versus BRP3 std. and such a clear mismatch for cBRP2 sample versus BRP1 std.? – This question is examined in the next section.

In a second attempt to check the accuracy of the assay, the potencies calculated via the *I*-number assay for the (c)BRPs (CZE data, each) and the stated potencies of the BRPs (mouse bioassay, each) were compared. As can be seen from summary Table 5b, the potency calculated via the *I*-number assay for EPO BRP1 std. (from the CZE data of the 2004 study [13]) did not fit to the stated potency of EPO BRP1 (difference= +4.6%). Likewise, the potency calculated via the *I*-number assay for cBRP2 sample (from the CZE data of the 2004 study [13]) did not fit to the stated potency of EPO BRP2 (difference= +10.8%). In contrast, the potency calculated via the *I*-number assay for cBRP3 sample (from the CZE data of the 2007 study [14]) fitted well with the stated bioactivity of EPO BRP3 (difference= -0.8%). And likewise, the potency calculated via the *I*-number assay for cCRS sample (from the CZE data of the 2015 study [15]) fitted well with the stated bioactivity of EPO BRP3 (difference= -0.2%), Why was there such a clear fitting for calculated cBRP3 versus stated BRP3 and calculated cCRS versus stated BRP3 and such a clear mismatch for calculated BRP1 versus stated BRP1 and calculated cBRP2 versus stated BRP2? – This question is examined in the next section

**Accuracy of "*I*-number of sample versus *I*-number of standard":** As can be seen from Table 3, the *I*-number of BRP1 ($I_{BRP1}$=518.7) differed from the *I*-number of cBRP2 ($I_{cBRP2}$=542.4), which was most likely attributable to the difference in the two reference preparations documented in the study [13], which is further commented in next section. The other *I*-numbers were quite comparable ($I_{cBRP2}$=542.4, $I_{BRP2}$=542.8, $I_{cBRP3}$=531.5, $I_{BRP3}$=527.6, $I_{cCRS}$=530.6).

Note: The outliers according to Grubbs [19] respectively Dixon [20], retrospectively identified by the author in these studies upon *I*-number calculation, were disregarded (see footnotes of Table 1).

In the 2004 study [13], sample cBRP2, as measured against reference standard BRP1, revealed an accuracy of only 104.6% (difference= +4.6%) (Table 3). Thus, these two preparations proved to be "different" (difference >3.0%; see Table 3 and summary at the end of this section), and this reflects the differences in the two reference preparations documented and commented in the 2004 study [13]. The MV ± 2 SD ranges were completely separated, thus likewise proving that the bioactivities of these two products were "different".

In fact, the isoform composition of the epoetin alfa/beta 1:1 mixture had changed, as one of the products, epoetin alfa or epoetin beta (or both), had changed between the 1997 [12] and the 2004 [13] study. This was, however, not found too dramatic as the bioactivity finally stated for BRP2 from the data of the 2004 study [13] was the same as initially stated for BRP1 from the data of the 1997 study [12] (130.0 IU/µg, each). Nevertheless, the 2004 study [13] reported "some slight differences between BRP1 and cBRP2, which will require amendment to the Ph. Eur. monograph:

• isoform 3 is on average below the requirements of the Ph. Eur. Monograph 1316,

• isoform 7 is within, but very close to the upper limit of the Ph. Eur. specification" (quote from [13]).

This was supported by the CZE data of isoform 3 and isoform 7 of BRP1 and cBRP2 (cp. Table 1 and Table 2 of ref [6] respectively Table 3 of ref [13]). Hence, the specification for the isoform content (per cent) had to be lowered for isoform 3 and increased for isoform 7 (Ph. Eur. 2002 [16]: isoform 3=5-20%; isoform 7=0-20%; Ph. Eur. 2008 [17]: isoform 3=1-20%; isoform 7=5-25%). The lower content ($p_n$) of isoform 3 concomitant with the higher content ($p_n$) of isoform 7 in BRP2 is clearly reflected in the increased *I*-number of cBRP2, compared to BRP1 (Table 3), which was, however, not reflected in the stated bioactivity of 130.0 IU/µg, each.

The fact that the CZE assay is more reliable than the mouse bioassay (which is generally accepted), and the *I*-numbers provide reliable bioactivity values, as shown elsewhere [9], the comparison of the *I*-numbers of BRP1 ($I_{BRP1}$=518.7) and cBRP2 ($I_{cBRP2}$=542.4) (difference= +4.6%; Table 3) made the author suggest that the bioactivity stated for BRP1 (130.0 IU/µg) should most likely have been stated about 5% higher. Hence, a bioactivity value of "BRP1+5%" =136.5 IU/µg is herewith proposed by the author to replace the stated bioactivity of BRP1.

In the 2004 study [13] and the 2007 [14] study, two identical samples have been measured (2004: cBRP2 std.; 2007: BRP2 sample), revealing an accuracy of 100.1% respectively a difference of +0.1% (Table 3), which rendered these two samples analytically "indistinguishable" (difference

<1.5%; for this classification see summary at the end of this section). The MV ± 2 SD ranges were almost congruent, thus likewise proving that the bioactivities of these two samples were "indistinguishable".

In the 2007 study [14], sample cBRP3, as measured against reference standard BRP2, yielded $I_{BRP2}$=542.8 (=100%) and $I_{cBRP3}$=531.5, revealing an accuracy of 97.9% (difference=-2.1%), which rendered these two samples "comparable" (difference 1.5–3.0%; for this classification see summary at the end of this section). The MV ± 2 SD ranges were partially overlapping, thus likewise proving that the bioactivities of these two products were "comparable".

In the 2007 study [14] and the 2015 study [15], again two identical samples have been measured (2007: cBRP3 std.; 2015: BRP3 sample), revealing an accuracy of 99.3% (difference= -0.7%) (Table 3), which rendered these two samples "indistinguishable". The MV ± 2 SD ranges were almost congruent, thus likewise suggesting that the potencies of these two products were "indistinguishable".

In the 2015 study [15], sample cCRS, as measured against reference standard BRP3, yielded an accuracy of 100.6% (difference= +0.6%), which rendered these two samples "indistinguishable". The MV ± 2 SD ranges were almost congruent, thus likewise proving that the potencies of these two products were "indistinguishable".

In summary, the retrospective analysis by the author of the "accuracy" of the *I*-number of "sample versus reference standard" were judged by the author (cp. Table 3) as being analytically

• **"indistinguishable"**

if the difference between sample and standard was <1.5% – valid for BRP2 sample *vs.* cBRP2 std. (+0.1%), BRP3 sample vs. cBRP3 std. (-0.7%) and cCRS sample *vs.* BRP3 std.(+0.6%),

• **"comparable"**

if the difference between sample and standard was 1.5–3.0% – valid for cBRP3 sample *vs.* BRP2 std. (-2.1%),

• **"different"**

if the difference between sample and standard was >3.0% – valid for cBRP2 sample vs. BRP1 std. (+4.6%).

Based on this classification, the difference between cBRP2 sample and BRP1 std. of the 2004 study [13] was about 5-fold higher than for "indistinguishable" samples and more than twice as high as for "comparable" samples, proving that the product composition had changed between 1997 (BRP1) and 2004 (cBRP2), which has already been discussed above and is further addressed in the next section.

The question now was why the *I*-number difference between BRP2 sample and cBRP2 std., as well as between BRP3 sample and cBRP3 std. as well as between cCRS sample and BRP3 std. was <1.5%, each ("indistinguishable"), whereas the difference between cBRP2 sample and BRP1 std. was about 5 times higher (+4.6%), and thus clearly "different". A measuring error associated with cBRP2 sample in the 2004 study ($I_{cBRP2}$=524.4) could be excluded, as the same material used in the 2007 study provided an identical *I*-number ($I_{BRP2}$=524.8) (Table 3). Thus, it was the stated bioactivity of BRP1 that had to be questioned.

In an attempt to answer this question, the potencies calculated via the *I*-number assay were further compared with the stated bioactivities of the samples (see next section).

**Accuracy of "*I*-number of sample versus stated bioactivity":** The bioactivity of **cBRP2** sample, calculated according to Formula 2 via the stated bioactivity of BRP1 std. (130.0 IU/µg), yielded 135.9 IU/µg, which differed from the stated bioactivity of BRP2 by +4.6% (accuracy=104.6%; Table 4), rendering the two values "different". This calculated cBRP2 potency value (135.9 IU/µg) differed likewise from the proposed "BRP2+10%" value (143.0 IU/µg) by -4.9% (accuracy=95.1%; Table 4), thus questioning the stated bioactivity of BRP1.

In fact, if the bioactivity of cBRP2 was calculated via "BRP1+5%" (136.5 IU/µg), a bioactivity of 142.7 IU/µg was obtained (Table 4), which differed from the stated bioactivity of BRP2 by +9.8% (accuracy=109.8%), which questioned the stated bioactivity of BRP2. However, this 142.7 IU/µg potency value of cBRP2 fitted well with the proposed "BRP2+10%" value (143.0 IU/µg), providing an accuracy of 99.8% (Table 4) respectively a difference of -0.2%, which rendered these two values "indistinguishable".

And if the bioactivity of cBRP2 was calculated via $I_{cBRP3}$ and the undoubted potency value of BRP3 (141.1 IU/µg), a bioactivity of 144.1 IU/µg was obtained, which differed from the potency value stated for BRP2 (130.0 IU/µg) by +10.8%, but fitted well to the proposed "BRP2+10%" value (143.0 IU/µg) (accuracy 100.8%; difference= +0.8%) (Table 4).

If the bioactivity of **cBRP3** sample was calculated (via Formula 2) via the stated bioactivity of BRP2 (130.0 IU/µg), a bioactivity of 127.3 IU/µg was obtained, revealing a difference to the stated bioactivity of BRP3 (141.1 IU/µg) of -9.8% (accuracy=90.2%; Table 4), rendering the two values "different". If, however, "BRP2+10%" (143.0 IU/µg) was used instead, a potency for cBRP3 of 140.0 IU/µg was obtained, corresponding with a difference of only -0.8% (accuracy=99.2%; Table 4), which rendered the two values again analytically "indistinguishable". Therefore, also this second accuracy determination clearly confirmed the proposed "BRP2+10%" value.

Last but not least, the bioactivity of cCRS, calculated via Formula 2 via the stated bioactivity of BRP3 (141.1 IU/µg), yielded a bioactivity of 140.9 IU/µg, which was analytically "indistinguishable" from the stated bioactivity of BRP3, as expected (difference=-0.2%; accuracy 99.8%) (Table 4), as these two materials were prepared similarly from similar starting materials but with different contents.

These results are clearly in favor of the "BRP1+5%" and "BRP2+10%" values proposed by the author herein. This proposal is supported by the quite surprising observation that in the 2007 collaborative study [14] it was found that "direct calibration of BRP2 against the WHO 2nd IS yielded, in all laboratories, results that were systematically higher than the potency of 32,500 IU/vial assigned by direct calibration against WHO 2nd IS in the former study" [14, Abstract]. "UV spectroscopy analysis of the vial contents suggests an increase of 9.5% between BRP2 and cBRP3. Assuming a constant specific activity of 130,000 IU/mg for erythropoietin, this would suggest a predicted activity of 35425 IU/vial" [14]. This would yield for BRP2 a bioactivity of 141.7 IU/µg, which is close to the author's bioactivity calculation for cBRP2 via "BRP1+5%" that resulted in 142.7 IU/µg (Table 4). This thought experiment supports the newly proposed bioactivity "BRP1+5%"=136.5 IU/µg and demonstrates the efficacy of the *I*-number assay in predicting the bioactivity of EPO samples.

The stated bioactivities of BRP2 (130.0 IU/µg) and BRP3 (141.1 IU/µg) differed by ~8%, which is close to the difference of 9.5% mentioned in ref [14] respectively above, whereas the corresponding *I*-numbers ($I_{BRP2}$ and $I_{cBRP3}$) differed by only -2.1% (Table 3), which rendered these values "comparable". If the bioactivity proposed by the author for BRP2, i.e.,"BRP2+10%" =143.0 IU/µg, was compared with the stated

bioactivity of BRP3 (141.1 IU/µg), the difference was reduced to 1.3%, which rendered these two values "indistinguishable".

Thus, the differences revealed between the potencies calculated via the *I*-number assay and the stated potencies were overcome, using the "BRP1+5%" and the "BRP2+10%" values as the reference values (Table 5).

In summary, the comparison of the *I*-numbers and the stated bioactivities has revealed that the bioactivity of BRP1 should have been stated about 5% higher (value proposed by the author herewith: "BRP1+5%" =136.5 IU/µg), and the bioactivity of BRP2 should have been stated about 10% higher (value proposed by the author herewith: "BRP2+10%" =143.0 IU/µg).

## Conclusion

The data presented in this retrospective analysis by the author of the CZE data of various (c) BRPs and the cCRS has shown that the *I*-number assay enables assessment of the potency of EPO reference preparations with high precision and accuracy, paralleling and confirming the results obtained with the Ibio-number assay [11]. Thus, the very broad criteria for EPO identification via CZE according to the Ph. Eur. [18] (which is based on broad ranges defined for the various EPO isoforms) could be replaced by a single and quite narrow I-number range, which would provide a significant increase in assay precision and accuracy and hence in drug safety. Moreover, the I-number assay could be a candidate physicochemical assay to replace the mouse bioassay in the quality control of EPO batch release.

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