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Defending Dynepo detection

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Doping analysis as the detection of prohibited substances in an athlete's bodily specimen is not solely a scientific task; anti-doping scientists are challenged by the athlete's experts and have to defend their results in front of arbitration panels such as the Court of Arbitration in Sports (CAS). Compliance with the internationally accepted standards issued by the World Anti-Doping Agency (WADA) is commonly the main aspect to prove and demonstrate.

Taking the example of four cases of doping with Epoetin delta (Dynepo) in endurance disciplines like marathon, triathlon, and cycling, the challenges and experiences in court and the argumentation lines of the defence experts are discussed. In all cases, doping with Epoetin delta was detected by two methods: isoelectric focusing (IEF) and SDS-PAGE.

Epoetin delta is known to be produced in a human fibrosoma cell line. The slightly more abundant bands alpha and beta result in a short detection window using IEF analysis alone. With the additional complementary information obtained by SDS-PAGE analysis, data interpretation and defence in court is facilitated.

Keywords: CAS; Dynepo; doping control; SDS-PAGE; isoelectric focusing; IEF

Introduction

Detection of doping substances in an athlete's bodily specimen is not only a question of (bio)-analytical chemistry. The defence of the results of doping analysis in front of the court has grown to a task as challenging as the detection itself.

Once informed of an adverse analytical finding in their body fluid, athletes have the opportunity to appeal against any decision of an arbitration panel, mostly starting at national level, aiming to the Court of Arbitration in Sports (CAS) as last level of jurisdiction according to the World Anti-Doping Code (WADC).[1]

Confirmation of the presence of a prohibited substance in an athlete's doping control sample is performed in most cases by mass spectrometry, serving as the gold standard among experts; analyses have to follow commonly accepted rules and technical documents as provided by the World Anti-Doping Agency (WADA).

In case of substances without any mass spectrometric detection method such as selected proteins or peptides, special rules apply: detection follows either guidelines such as those for growth hormone doping,[2] or technical documents as for recombinant erythropoietin (rEPO).

Both guidelines and technical documents reflect state-of-the art knowledge in anti-doping testing and are likewise subject to further development. Knowledge about the pharmacokinetic properties and the detection probabilities of doping substances grows with the amount of research. With a delay in time these new findings are reflected by revisions of technical documents.

Laboratories accredited for anti-doping testing sometimes record analytical data which necessitate further investigation into the nature of these observations. These findings may be a result of the (mis)use of new or altered substances by athletes to circumvent detection with standard methods. Research in these observations leads to extended knowledge and new assays for detection.

Such results might be much harder to defend in front of the court, because they may not necessarily comply with well-known standards.

This paper tries to create awareness on the mechanisms in place after reporting a positive result and shares experience in defending analytical results in front of the court. As a practical example, the scientific defence of a few adverse analytical findings with Dynepo in the years 2008 and 2009 is given.

Burden of Proof

WADC, as the level 1 document, is the basis for anti-doping science. This mandatory document not only defines the procedural rules and responsibilities in doping control, it defines doping and provides the burden and standard of proof. Article 3 reads:

> *The Anti-Doping Organization shall have the burden of establishing that an anti-doping rule violation has occurred. The standard of proof shall be whether the Anti-Doping Organization has established an anti-doping rule violation to the comfortable satisfaction of the hearing panel bearing in mind the seriousness of the allegation which is made. This standard of proof in all cases is greater than a mere balance of probability but less than proof beyond a reasonable doubt.*

Although the WADC does not require proof beyond any reasonable doubt, anti-doping labs are used to designing their detection methods to unequivocally determine the presence of a prohibited substance as indicated in The Prohibited List (TPL).[3] In cases of the detection of exogenous substances, the proof of its mere presence in an athlete's sample is sufficient to constitute

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an anti-doping rule violation; in contrast, the proof of doping with substances naturally occurring in the human body has to include evidence of the exogenous origin.

WADA technical documents are designed to compile state-of-the-art knowledge of their particular topic. Harmonization of testing performance and reporting are the aims of these level 2 documents, which must be incorporated into the laboratory's policies and procedures.[4] The use of accredited methods for detection of doping substances, the compliance with EN ISO/IEC 17025, the WADA International Standard for Laboratories (ISL)[5] and the relevant technical documents constitute a basis sufficient to demonstrate the presence of a prohibited substance in the bodily specimen submitted to analysis.

Scientific Background

The detection and reporting of doping with recombinant erythropoietin (rEPO) is harmonized by a WADA technical document. At the timeframe of the positive cases with Dynepo described herein (the years 2008 and 2009) the 2007 version of the technical document TD2007EPO, entitled *Harmonization of the Method for the Identification of Epoetin alpha and beta (rEPO) and Darbepoetin alpha (Nesp) by IEF-Double Blotting and Chemiluminescent Detection*[6] was in effect. This documents specifically deals with criteria for the detection of three forms of rEPO, namely Epoetin alpha, beta, and Darbepoetin alpha.

Three sets of criteria have to be fulfilled before issuing a positive finding with rEPO: acceptance criteria, identification criteria, and stability criteria. The documentation of the adverse analytical finding has to include all necessary information to demonstrate that the criteria as outlined in the TD2007EPO are met.

The 2007 version of the technical document for EPO detection incorporated changes compared to the 2004 version of the technical document TD2004EPO,[7] especially concerning the identification criteria. Similar to the 2004 document, the 2007 version requires that each of the two most intense bands in the basic area must be more intense than any band in the endogenous area, as measured by densitometry, but adds in brackets an indication of the ratio: approximately twice or more.

As mentioned above, the TD2007EPO is limited to three different forms of rEPO. The appearance of biosimilar forms of EPO, copy-EPO preparations and new generation EPOs like MIRCERA, a pegylated form of Epoetin beta, made it necessary to further develop the technical document and to include and modify criteria to cope with different forms of recombinant erythropoietin as much as possible. The recent issue of the technical document called TD2009EPO, is entitled *Harmonization of the method for the identification of recombinant erythropoietins (i.e. epoetins) and analogues (e.g. darbepoetin and methoxypolyethylene glycol-epoetin beta)*[8] and reflects new developments in EPO detection as well as criteria to identify new forms of rEPO.

In case of adverse analytical findings for rEPO, a second opinion of an experienced expert in rEPO detection, expressing a similar interpretation of the test results, is required before issuing the test result.[8] The second opinion should preferably originate from an author of the technical document.

This paper deals with the detection and especially the legal defence of adverse analytical findings with Dynepo. Dynepo is a solution containing Epoetin delta as an active ingredient. The EMEA protocol[9] defines Epoetin delta as 'a copy of the human hormone, produced by a method known as "genetic engineering": the enzyme is made by a cell in which the gene (the code) for the enzyme is activated so that the cell makes more of the enzyme and it can then be extracted and used'.

Table 1. Band ratio calculations of Epoetin alpha, beta, and delta

	Epoetin alpha	Epoetin beta	Epoetin delta
Band 1/Alpha	No band alpha	12	2.9
Band 2/Alpha	No band alpha	17	3.1

Epoetin delta is made in cultured human cells (human fibrosarcoma cell line HT-1080).[10] The glycosylation pattern is less heterogenous compared to Epoetin alpha/beta, predominantly tetraantennary.[11] The pharmaceutical company Aventis obtained the license in Europe in 2002; to date, Dynepo is no longer authorized in the European Union.

Compared to Epoetin alpha and beta, the isoform pattern of Epoetin delta deriving from isoelectric focusing (IEF) polyacrylamide gel electrophoresis contains a higher intensity of two bands in the endogenous area (see Figure 1). According to the nomenclature of the bands in the TD2007EPO, bands alpha and beta are clearly present on the gel image after IEF.[12]

The calculation of the band ratio of band 1/alpha and band 2/alpha is indicated in Table 1 demonstration the decrease of the diagnostic band ratios starting from Epoetin alpha to Epoetin delta, which is close to a ratio of 3 in the medical preparation, without having passed through the body.

On sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) Epoetin delta produces a very sharp band, which is unusual compared to Epoetin alpha, beta, omega, and human urinary and serum EPO.[13,14]

Thus SDS-PAGE provides valuable complementary information for the unequivocal detection of Dynepo.

The fact that Dynepo shows bands in the endogenous area leads to a reduced time window for its detection in comparison to Epoetin alpha/beta, if IEF is solely used for identification and the criteria of the TD2007EPO are applied. After the application of a therapeutic dose of Dynepo, the diagnostic ratio of approximately 'two or more' is exceeded only for a few hours.[15] SDS-PAGE analysis is able to widen the window of opportunity for the detection of Dynepo.[14]

Case-Related Information and Court Activities

As mentioned above, five cases of Dynepo doping were detected in our laboratory in the years 2008 and 2009. All of these cases showed abnormal profiles with ratios of band 2 in the recombinant area to band alpha as the most abundant band in the endogenous area in proximity to two.

SDS-PAGE analyses showed in all the cases sharp bands, typical for Dynepo (see, for example, Figure 2, lane 1).

At the time of the analysis of these samples only the IEF detection method, but not the SDS-PAGE method, has been under the scope of ISO accreditation. So the SDS-PAGE method was used as additional evidence, but the results were defended using the IEF data.

For all of these cases, B-sample analysis was requested by the athletes; and for all of these cases the analytical findings in the B-samples confirmed the findings in the corresponding A-samples.

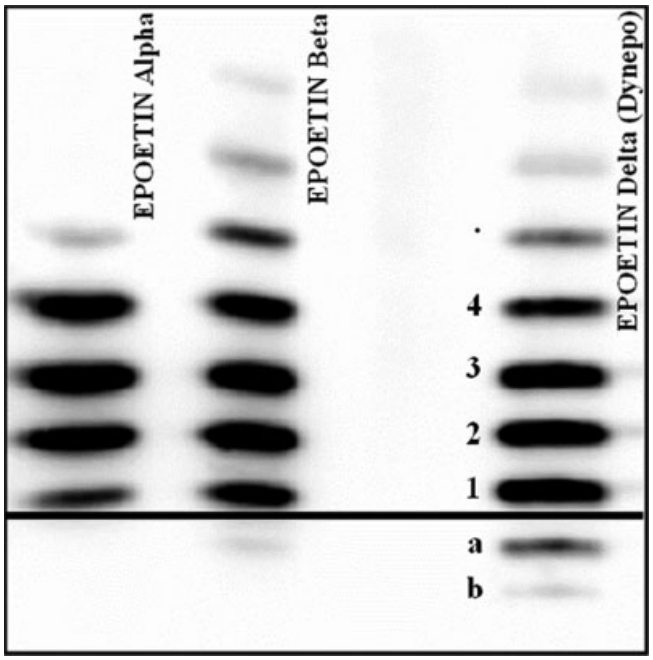

Figure 1. Comparison of the IEF-PAGE patterns of Epoetin alpha, beta, and delta.

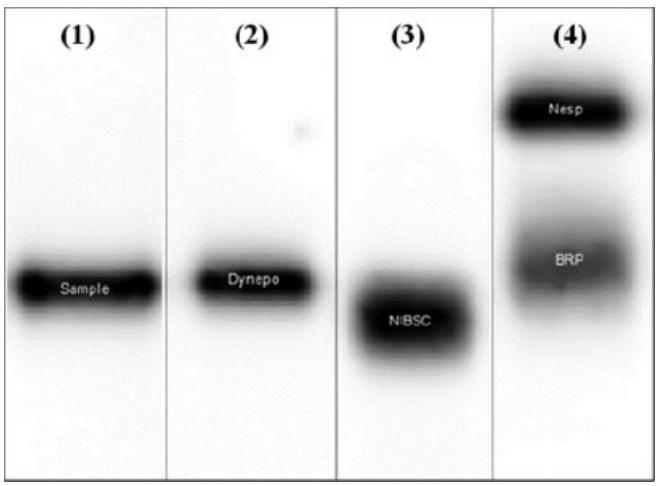

Figure 2. Behaviour of Epoetin delta (Dynepo) on SDS-PAGE: (1) athlete's sample; (2) Dynepo standard from a syringe; (3) National Institute for Biological Standards and Control (NIBSC) standard of endogenous urinary EPO; and (4) Biological reference preparation (BRP) standard from the European Pharmacopeia, a mixture of Epoetin alpha and beta.

The time between the analysis of the A-samples and the B-samples was accompanied by media work in favour of the athletes, claiming analytical errors or errors in results interpretation. Documentation provided by the laboratory to the arbitration panels appeared in the media.

Four cases went to arbitration.

Case 1: A marathon runner was cleared by the national federation from any doping allegation first; the international federation concerned passed the case to CAS. The athlete was banned for two years.

Case 2: A marathon runner tested positive for Dynepo twice within a period of one month. The first national arbitration panel (the legal commission of the National Anti-Doping Agency) banned the athlete for 2 years; the ban was confirmed by the second national arbitration panel. The athlete refused to go to CAS.

Case 3: A triathlete was banned by the first national arbitration panel. Due to an additional attempt of bribery by this athlete, the case went to the criminal court and the athlete was punished with conditional confinement.

Case 4: A cyclist was banned for life due to the second anti-doping rule violation at the first national arbitration commission; the ban was reduced to 20 years by the second national arbitration commission.

Arbitration panels and expert witnesses

Expert witnesses are expected to be properly educated and experienced to provide opinions on technical matters beyond the average person's understanding.[4] Their role is to assist the panel in understanding the technical aspects. Expert witnesses shall be independent from both parties involved.

Detection technology develops with every challenge of new forms of doping. And technology is developing in a more complex direction, rather than the opposite way. So to assist a panel of laypersons, mostly lawyers, in a clear and understandable way defines a challenge for the laboratory expert.

Main Objectives of the Defence and Panel Decisions

Most of the above-mentioned athletes were accompanied by their own experts during B-sample analysis as well as in front of the court. These experts compiled written counter expertise for the defence of the athlete. The main challenges as well as the response from the laboratory are listed:

Specificity of the antibody

Detection of rEPO according to the technical document requires the use of a defined monoclonal antibody (mAB) clone AE7A5 (R&D Systems of Minneapolis, MN, USA). This antibody is considered a critical reagent and shall not be changed.

In some publications of research groups other than the accredited anti-doping laboratories, doubts regarding the specificity of this mAB were raised.[16,17,18] Consequently, these arguments were used by the athlete's defence.

The CAS award 2008/A/1608[19] clearly pointed out, that the 'direct detection method' as codified by the WADA technical document TD2007EPO is validated by CAS in several decisions. The CAS panel confirmed the reliability of the detection method to find the presence of rEPO in a urine sample.

Smears, spots, and bubbles

The technical document TD2007EPO defines three sets of criteria. The first one, the acceptance criteria, deals with the quality of the gel image as a subject for densitometry. If the acceptance criteria are not fulfilled, identification criteria are not to be applied.

Section 1 of the acceptance criteria says:

> *Spots, smears, areas of excessive background or absent signal in a lane that significantly interferes with the application of the identification criteria shall invalidate the lane.*

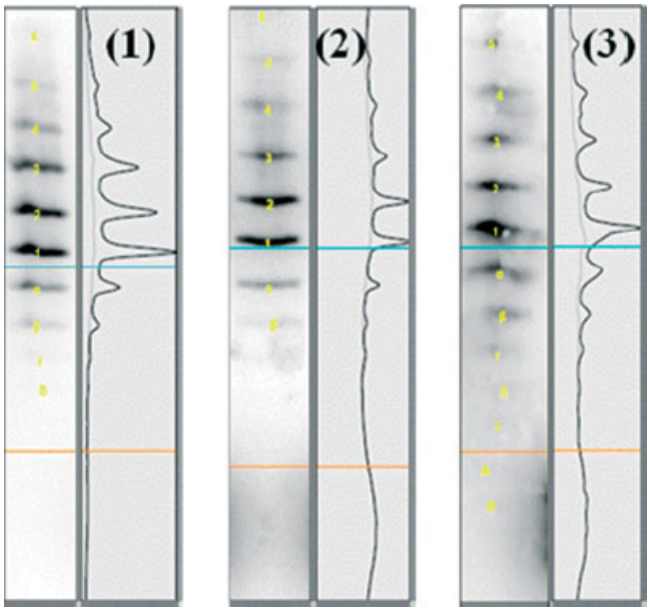

Figure 3. Typical IEF – patterns of a urine sample reported positive for rEPO. (1) confirmation analysis of the A-sample; (2) confirmation analysis of the A-sample; and (3) confirmation analysis of the B-sample. The band ratios of band 2/alpha are around 2.

Every spot on the gel image was indicated by the defence experts, without further reflecting its significance for the analytical result.

Besides the fact that the analytical results would have been interpreted in the same manner with or without the spots indicated by the defence experts, the random nature of the spots was demonstrated by the fact that at least three independent analyses were done on every single sample (screening, A-sample confirmation, B-sample confirmation). Single spots never appeared on the same place. So finally these artefacts did not significantly interfere with the application of the second set of criteria, the identification criteria.

Protein aggregate precipitation

A single gel image of a B-sample confirmation had a few abundant spots, two of them located in and close to the acidic area of the lane, where the sample of the athlete was placed (Figure 4).

According to the opinion of the defence experts these artefacts derive from precipitated proteins of the athlete's urine sample. The artefact might have altered the band pattern on its way through the zone of the artefact; consequently the results can no longer be regarded as valid.

The panel accepted that the artefacts are not from the urine sample (different shape, appearance between lanes, randomly on the gel image, no appearance on the A-sample gels and comparable band distribution for A- and B-sample) and there has no influence on the band pattern, because the migration of the bands is towards the artefact, and not through the artefact.

Finally, the panel concluded that the defence expert's 'opinion . . . offers only a possible explanation, without proper scientific foundation for the appearance and the effects of smears'.[19]

Appearance of endogenous bands

The defence experts claimed that the band patterns of the athletes show endogenous bands (Figure 3). They concluded

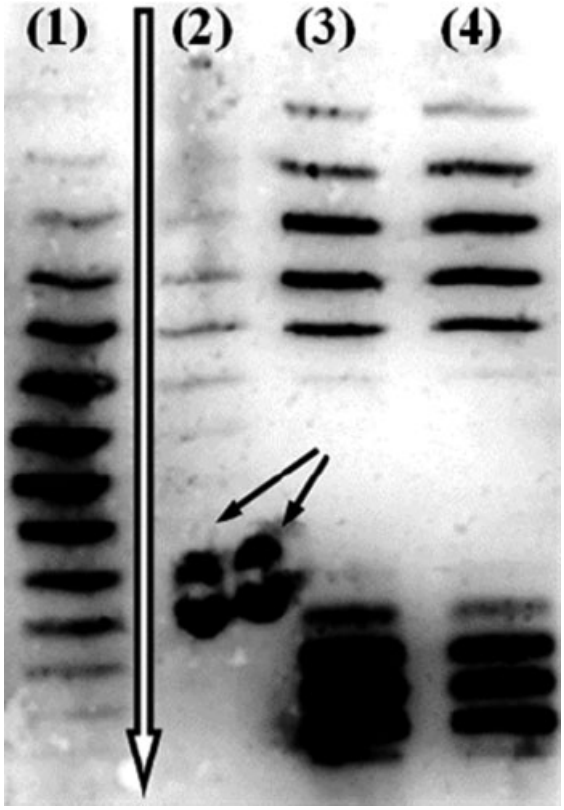

Figure 4. IEF-gel image of a B-sample confirmation. (1) negative control standard (NIBSC); (2) athlete's sample; (3) stability test; and (4) BRP/Darbepoietin alpha mixture. The small arrows indicate an artefact on the athlete's lane; the big arrow indicates the direction of migration of the protein bands.

that the presence of endogenous bands clearly proves that the origin of the EPO detected in the athlete's urine samples derives from endogenous production. The pattern of the urines samples concerned does not fit perfectly to the example in the TD2007EPO, which does not show any endogenous band.

The panel was informed that the term 'endogenous band' does not exist according to the TD2007EPO. The document defines basic, endogenous, and acidic areas, where bands are located. Even some of the recombinant products, without passing through the body and being the subject of metabolism or dilution with endogenously produced EPO, show bands in the endogenous area (e.g. Dynepo, Figure 1). And finally the TD2007EPO supposes the existence of bands in the endogenous area for the calculation of band ratios.

Identification criteria are not fulfilled

The most prominent argument of the athlete's defence was the fact that some of the analysis made showed band ratios of band 2/alpha below 2. This leads to the question of an authentic interpretation of the terms 'approximately 2 or more'. According to the athlete's defence, this means more than approximately two. Table 2 shows an example of case 1.

The A-sample underwent confirmation analysis twice before reporting it as adverse analytical finding. The band ratios of band 1/alpha are clearly above 2 in each case, the band ratios of band 2/alpha beyond two, with 1.54 as the lowest value.

Table 2. Band ratio calculations of multiple analyses of a urine sample reported positive for rEPO

Ratio	A-Sample analysis 1. Confirmation	A-Sample analysis 2. Confirmation	B-Sample analysis
Band 1/alpha	3.09	2.32	2.78
Band 2/alpha	2.63	1.54	1.91

The panel concluded that 'the 2/α ratio of 1.91 in the B-sample analysis is to be considered approximately a ratio of 2, and therefore confirms the presence of rEPO'. And further: 'Such a conclusion is not contradicted by the 2/α ratio of 1.54 reported with respect to the second analysis of the A sample.'[19]

Complete fulfilment of all the criteria of the TD2009EPO

In a different case[20] dealing with the detection of rEPOs different to Dynepo, the question was raised, whether all the criteria for positivity of the respective technical document have to be fulfilled. In this particular case the two most intense bands in the basic area were not consecutive, mainly due to the low concentration of rEPO in the sample.

The panel awarded that 'even though the second criteria, namely that the two most intense bands measured by densitometry in the basic area must be consecutive, is not completely met, such circumstance does not prevent the Panel to rely on the convincing evidence provided by [lab expert 1 and lab expert 2] to conclude that the sample of the Athletes clearly establish an AAF.'

Effort urine vs. hypoxia vs. Dynepo excretion

The defence experts claimed that urines with band ratios close to 2 or below are a result of effort and proteinuria as published by Lasne *et al*.[21] This was undermined by the fact that urine samples are taken post-competition after strenuous exercise or after an intense training session.

Alternatively hypoxia was outlined as the possible reason of a shift of the band pattern towards the basic area.[22] The use of hypoxic chambers by some of the athletes was claimed.

However the panel agreed that the criteria of the technical document are regarded as an accepted standard to distinguish between an adverse analytical finding and a shifted profile due to strenuous exercise. As long as an accredited laboratory can demonstrate that the criteria for identification as outlined in a valid technical document are fulfilled, this was regarded sufficient evidence to report the presence of rEPO for a particular sample.

Interpretation monopoly at WADA laboratories

The analysis and the interpretation of the data deriving from anti-doping testing in general, and for testing for rEPO in particular was claimed to be a monopoly of WADA-accredited laboratories. Even the support of adverse analytical findings by a second opinion of an independent expert derives from the community of anti-doping laboratories.

This argumentation does not take into account that accreditation of WADA laboratories requires accreditation by ISO/IEC 17025 confirming the competence of an analytical laboratory for the type of analyses performed. This is comparable to the situation of specialized physicians in cancer diagnosis, where second opinions are requested from competent and experienced specialists. In addition, WADA laboratories are challenged with different types of external quality assessment samples: double-blind test samples, blind samples, as well as educational samples. The performance of the laboratories within this quality assessment scheme is a key indicator for the maintenance of its accreditation, meaning that accreditation can be revoked if the performance is not satisfying.

Each laboratory reaccreditation audit has to include a WADA-trained assessor for checking compliance with the ISL in its latest version. And finally WADA accredited laboratories have to provide a full documentation package in support of an adverse analytical finding challenged by an athlete. The package has to be in a format that in the absence of the analyst, another competent analyst could evaluate what tests had been performed and interpret the data.[5] Thus every competent analyst can review the quality of the data and the chain of custody information.

In addition it has to be mentioned that the existence of a sealed aliquot of the original sample, the B-sample, and the right of the athlete to attend the B-sample analysis accompanied by an external and experienced expert[5] of his/her choice constitutes a backup security for the laboratory and the athlete. During B-sample analysis WADA laboratories demonstrate in a transparent way the validity of the results of the A-sample as well as the compliance with WADA standards.

The use of methods outside the scope of accreditation

For each of these cases, a second method for the analysis of rEPO in urine samples was used together with the accredited IEF method, namely SDS PAGE. This method for the detection of Dynepo was first published by Kohler *et al*.[13] and thus subject to peer-reviewed publication. At the timeframe of the analysis of the positive cases for the marathon runners and the triathlon athlete, the SDS-PAGE method was outside the scope of accreditation.

The data deriving from SDS-PAGE analysis were incorporated into the full documentation package as additional evidence for the adverse analytical finding.

The SDS-PAGE results were challenged by the athlete's defence as being outside the accreditation scope and therefore cannot be used to provide any kind of evidence.

The Panel remarks that the SDS-PAGE analysis was used only as supporting evidence (mainly to identify as Dynepo the kind of rEPO detected) to confirm the results obtained on the basis of the 'direct detection method' codified by TD2007EPO. Indeed, even without the performance of the SDS-PAGE analysis, the analytical findings indicated the presence of rEPO, and therefore were to be reported as positive. This conclusion, therefore, made it irrelevant for the Panel to verify whether the SDS-PAGE analysis is a reliable method for the detection and identification of rEPO and its forms.[19]

Most recent state-of-the-art technology

Technical documents follow recent awareness and guide to a standardization and harmonization of testing and testing performance. Consequently it lies in the nature of a technical document that there is a period where knowledge and experience with new scientific developments already exist and are subject to peer-reviewed publication, but the technical document dealing with this topic is not in force yet. This holds for technical documents on new topics as well as for new versions of already released documents.

In case 4, the athlete's defence challenged that the A-sample analyses were done on the basis of a technical document which

was not in effect at the time of the analysis, thus invalidating the results of the A-sample. Consequently the B-sample analysis results were claimed to be not valid, although the B-sample analysis was performed after the effective date of the relevant TD2009EPO.

On one hand, the response indicated that the technical document in force for the analysis of rEPO at the time of A-sample analysis, the TD2007EPO, only provided criteria for three different forms of rEPO, Epoetin alpha, beta, and Darbepoetin alpha, but not for Dynepo. So this document was not fully applicable to the analysis concerned. On the other hand, a recent CAS decision followed the view of the WADA Science Direction that most recent state-of-the-art technology and knowledge has to be used to identify prohibited substances and methods.[20]

Self-declared innocence

Regarding the point of self-declared innocence from a doping offence by the athlete, the panel concluded that 'this argument cannot seriously support the decision, ... that the currency of denial is devalued by the fact that it is the common coin of the guilty as well as of the innocent[23] and that oral testimony as to innocence, however impressively given, cannot trump scientific evidence as to guilt'.[19,24]

Summary of other defence statements of minor importance for the trial

The format of the second opinions from authors of the TD2007EPO was challenged and along with the fact that there is no standard for providing a second reading. The method itself is no direct proof, but provides only a pattern recognition procedure to distinguish between endogenous and recombinant erythropoietin. The blotting procedure uses a semi-dry blotting and not a wet blotting; incomplete transition of the proteins during the blotting procedure cannot be excluded.

Due to the fact that none of the points summarized in this section constitutes a deviation from the technical document concerned and the defence experts could not support their statements with experimental data, the panels did not further ask for more data.

Perspective

From the experience of national and international court cases dealing with the defence of adverse analytical findings with Dynepo, it can be concluded that anti-doping science continues to drift away from pure laboratory work to challenges of the system itself and the results in particular. Nevertheless experts defending their results should still adhere to the maxim of Tacitus in his *Annales* and defend analytical data *sine ira et studio*.

In addition, scientists have to understand the position of judges and lawyers; they have in general a more formal and 'standards'-dependent view of analytical data.

Methods under the scope of ISO accreditation can hardly be challenged in court. Second opinions from experienced and internationally recognized experts are useful in demonstrating independence in data interpretation; conclusions are no longer regarded as private opinions of scientists defending their own results.

Complementary methods like IEF and SDS-PAGE to detect doping with rEPO are useful; recent research on the development of LC-MSMS based methods[25] possess the potential to further improvement of rEPO detection.

The position of WADA as the accrediting body and the responsible organization for the establishment of globally valid standards is an important contributor to the authentic interpretation of the standards and the correctness of laboratory results.

Finally, court appearance of the scientist responsible for the results is important for the panel.

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