Recombinant erythropoietin found in seized blood bags from sportsmen

During an anti-doping investigation, the Spanish Guardia Civil confiscated blood bags from elite sportsmen. A novel immuno-purification method demonstrated that plasma samples with elevated erythropoietin (EPO) contained recombinant material (rEPO). This shows that rEPO is used before autologous blood transfusions and that rEPO analysis in plasma can be reliably addressed.

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A network of blood transfusion for doping purposes was detected in Spain in 2006. The so-called *Operation Puerto* (OP) led to the seizure of samples of whole blood, plasma, and red blood cell concentrates for autologous blood transfusion, allegedly belonging to elite sportsmen from different countries. The juridical proceedings were dismissed according to the Spanish law applicable at that time. For the moment, in Spain, the indictment has been shelved.

High (supra-physiological) concentrations of erythropoietin (EPO) were found in some plasma samples. EPO is available as a recombinant pharmaceutical product (rEPO) and included as a doping agent, together with its analogue NESP (darbepoetin alfa), in the *prohibited list* of the World Anti-Doping Agency (WADA). A method based on isoelectric focusing (IEF) can detect rEPO in urine[1] but its direct application to plasma is not feasible due to substantial matrix effects.

We investigated whether the OP plasma samples with elevated EPO concentrations contained rEPO to trigger red blood cell production before storing blood for future transfusion, as clinically used.[2] To disclose the origin of the elevated EPO, a new method allowing its purification was applied.

Bags of human plasma from *Operation Puerto* (OP) were received together with an official juridical request for their analysis. In addition, urine and plasma samples from healthy control subjects were used as control.

EPO concentrations in plasma were determined using a chemiluminescence immunometric assay (Immulite 1000 EPO; Diagnostic Products Corporation (DPC), USA) and confirmed by an ELISA assay (Quantikine IVD Erythropoietin; R&D Systems Inc, USA).

An immuno-purification method using microtiter plates (Corning Incorporated, USA) was developed. A 10 µg/mL solution of a monoclonal anti-human erythropoietin antibody (clone 9C21D11; R&D Systems, USA) in 50 mM NH_4HCO_3 (100 µL/well) was applied for 1 hr. at 37°C. Plates were then washed three times with 0.01M Phosphate-Buffered Saline (PBS), pH 7.4 and blocked with 1% polyvinylpirrolidone in PBS overnight at 4°C with shaking. After aspiration, plasma samples (4-8 mL) were applied (100 µL/well) and incubated overnight at 4°C with shaking. Wells were washed twice with 0.01M PBS. EPO was eluted with 100 µL/well of 0.7% acetic acid, the eluates were frozen in liquid nitrogen and lyophilized. Finally, samples were re-suspended in 18 µl of H_2O and applied to the IEF gel as previously described[3] with additional modifications.[4]

Eight of the samples from the OP (Figure 1, lower panel) were found to have EPO concentrations higher than the normal population range (3.7-29.5 IU/L; Immulite 1000 brochure), while samples from volunteers (6.4-18.5 IU/L, mean 10.5 IU/L) had concentrations in that range. However, no discrimination could be made between endogenous and exogenous EPO on the basis of only those values. Alternatively, as rEPO glycosylation is distinct from its endogenous counterpart, the charge differences are picked-up by the IEF approach developed for urine.[3] The method has been thoroughly examined for its specificity (cross-reactivity of the primary antibody with urine components)[5,6] and stability of EPO in urine (sensitive to the presence of proteases).[7] However, it cannot be applied to plasma because of the high protein load.

We designed a microplate immuno-purification method to isolate EPO from blood plasma. With this protocol, blank samples showed that the IEF profile of

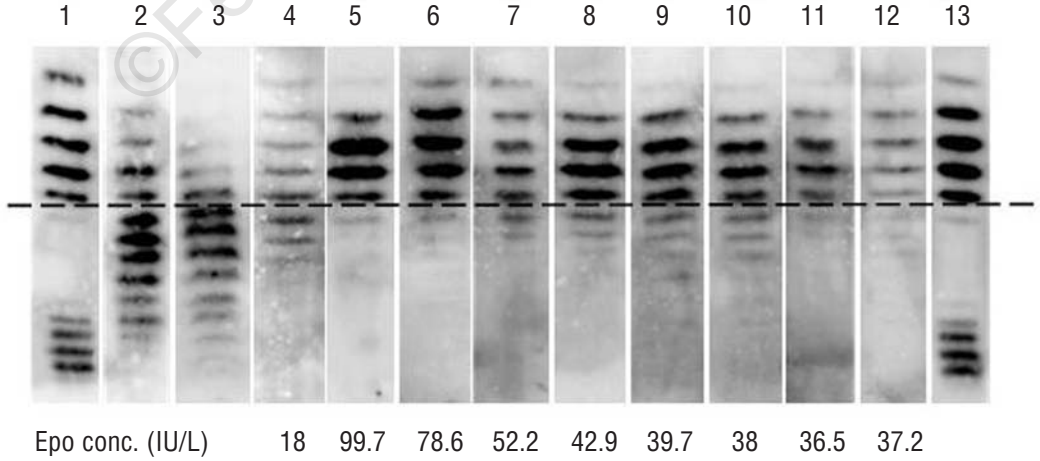

Figure 1. Analysis of erythropoietin (EPO) in seized blood plasma by isoelectric focusing. (1,13) Mixture of the Biological Reference Preparation (BRP) of recombinant (r) EPO (bands above dashed line) plus its analogue NESP; (2) Human urinary EPO preparation from the National Institute for Biological Standards and Control (NIBSC); (3) Urine from a subject known to contain endogenous EPO only; (4) Plasma from a subject known to contain endogenous EPO only; (5-12) Plasma samples seized as part of the Operation Puerto Spanish anti-doping investigation showing the presence of rEPO, characterized by predominant bands above the dashed line (cathode at the top). Concentrations of EPO measured by chemiluminescent immunoassay are indicated in the lower panel.

circulating EPO is shifted towards less acidic pH values, in comparison to the corresponding urinary EPO profile (Figure 1, lanes 2-4), a fact recently confirmed using serum and a similar affinity purification protocol.[8] Nevertheless, the profile is easily recognizable as endogenous.[9]

When the OP plasma samples with elevated EPO were immuno-purified and applied to IEF (Figure 1, lanes 5-12), they showed the presence of rEPO[9] with an intensity of the bands corresponding to the exogenous substance (Figure 1, above dashed line) far more intense than the depleted endogenous ones (Figure 1, below dashed line). Also, when WADA criteria[10] for the identification of rEPO were applied, the recombinant nature of the EPO was evident. This demonstrates the feasibility of using plasma to detect the administration of rEPO. As compared with the usual method for urine, the immunoaffinity step with a different anti-EPO antibody to that used in the IEF procedure strengthens the specificity, while using blood withdrawn by a phlebotomist prevents tampering by the concealed addition of proteases.

The finding of rEPO in samples connected to a blood transfusion doping network shows that to administer rEPO prior to blood withdrawal and storage appears to be current practice. Given the difficulties in detecting autologous transfusions, identifying rEPO in plasma could be a way to deter such practices.

Joaquim Mallorquí,[1] Jordi Segura,[1,3] Carme de Bolòs,[2] Ricardo Gutiérrez-Gallego,[1,3] and Jose A. Pascual[1,3]

[1]Pharmacology Research Unit, IMIM-Hospital del Mar, PRBB, Barcelona, Spain; [2]Cellular and Molecular Biology ResearchUnit, IMIM-Hospital del Mar, PRBB, Barcelona, Spain; [3]Dept. Experimental and Health Sciences, Pompeu Fabra University, PRBB, Barcelona, Spain

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Correspondence: Jordi Segura, IMIM-Hospital del Mar, PRBB, c/Doctor Aiguader, 88, 08003, Barcelona, Spain. Phone: international +34.933160470. Fax: international +34.933160467.E-mail: jsegura@imim.es

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