Kwan S H, Bahrudin F, Ismail M N

Comparison of purification techniques for erythropoietin anti-doping analysis

Doping Control Centre, Universiti Sains Malaysia, Penang, Malaysia

Abstract

The anti-doping testing among athletes has been a challenge since more than two decades ago to ensure a fair and justice competition can be held. An effective separation of recombinant human erythropoietin (rhEPO) from endogenous urinary erythropoietin (uEPO) is essential for the detection of EPO abuse. The separation of rhEPO and uEPO can be done by SDS-PAGE which is based on their respective molecular weight. A purification step is important prior to SDS-PAGE for a better and cleaner separation. In this project, we would like to compare the different methods of EPO purification methods commonly applied in anti-doping labs. In this work, three methods i.e. conventional ultrafiltration (UF), MAIIA purification with and Stemcell with UF purification, have been tested on the purification of urine samples. Based on the results we found that the application of MAIIA purification has a higher sensitivity and higher throughput when compared to the others. Conventional ultrafiltration is not competent enough to remove all the impurities and Stemcell with UF purification method has a lower recovery of EPO compared to MAIIA purification. MAIIA purification was efficient in removing foreign proteins except Bovine Serum Albumin (BSA) which we suspected it was contributed by part of the component applied in the MAIIA purification kit. The research shows MAIIA purification recovered higher amount of erythropoietin compared to other methods. The use of MAIIA purification prior to SDS-PAGE contributes to a high quality analytical result to the EPO analysis.

Introduction

Erythropoietin (EPO) is an acidic glycoprotein hormone that is responsible for erythropoiesis, the process that produces red blood cell [1,2]. The molecular weight of EPO is estimated to be around 30.4 kDa [3]. Recombinant human EPO (rhEPO) has been produced successfully using DNA recombinant technology since 1985 [4]. rhEPO is one of the commonly abused drugs by athletes to improve their performance [5]. Recently, SDS-PAGE [6] was approved to be used as a complement to isoelectric focusing (IEF) [7] to differentiatie between EPO and rhEPO [8]. SDS-PAGE can separate rhEPO from urinary EPO (uEPO) based on different their molecular weights. A purification step is required prior to SDS-PAGE to remove other impurities [9]. A proper purification method can enhance the detection of EPO in the urine samples.

Experimental

All urine samples, except blank urines, were pre-spiked with uEPO standard (NIBSC, Hertfordshire, UK) to ensure visible EPO bands on the gel. The ultrafiltration (UF) technique is based on the existing method in the lab. 20 µL of protease inhibitor cocktail and 2 mL of 3.75 M Tris buffer pH 7.4 were added to 20 mL urine. Urine samples were then applied to Amicon Ultra-15 MWCO 30kDa devices and centrifuged for 10 min. The retentate was washed with 50 mM Tris pH 7.4 buffer and re-centrifuged. Then the rentate was further concentrated to 50 µL with Amicon Ultra-0.5 MWCO 30 kDa devices.
The 96 microwell plate containing anti-EPO antibody was supplied by Stemcell Technologies (Vancouver, Canada). The protocols were independently developed. 20 mL samples were concentrated to 50 µL using the UF technique mentioned above. The samples were then transferred into the microwell plate and left for overnight in 4°C with gentle shaking. The wells were washed with PBS and tapped dry. Sample was eluted with 4.4% of CHAPS and incubated at 80-85°C for 25 minutes.

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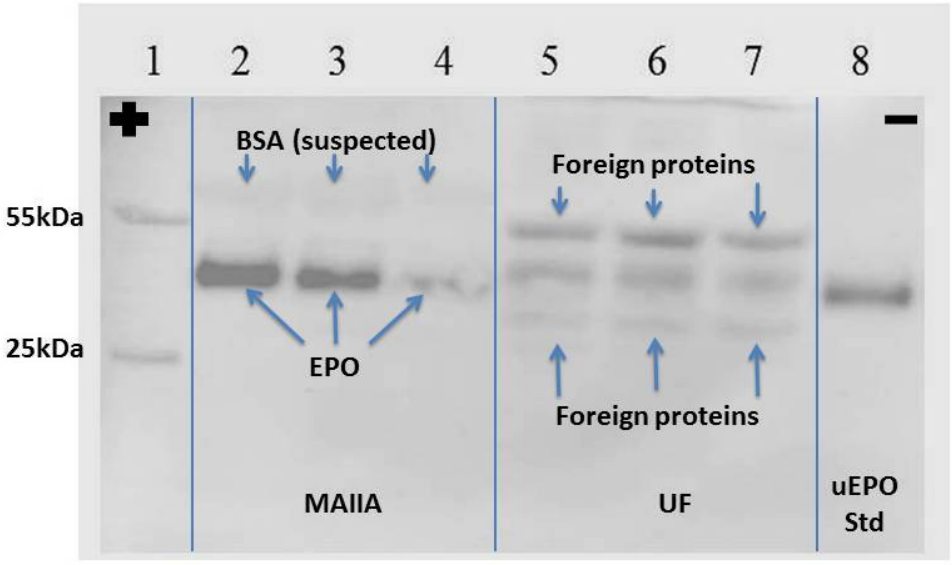
The procedure was carried out as described in the instructions in the EPO Purification Kit from MAIIA diagnostics (Uppsala, Sweeden). Briefly, 20 mL of urine sample was mixed with urine precipitate dissolvation (UPD) buffer. The UPD–urine mixture was heated to about 82-85°C. The pretreated filtered sample mixture was passed through the anti-EPO column, and later washed with washing buffer. EPO was eluted into a new vial containing pH-adjustment buffer supplemented with BSA and detergent, adding the desorption buffer to the column and spinning for 1 min at 1000×*g*.
Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed on handcast Tris-HCl gels (10% T, 1.0 mm, 10 wells, Tris/Glycine/SDS running buffer) according to Laemmli [6]. The Immulite 1000 Immunoassay system was used to determine the EPO concentration in each sample.

Results and Discussion

Figure 1 shows an in-depth comparison of UF and MAIIA purification. The UF method showed many foreign bands in the image regardless of the concentration of uEPO spiked into the sample. MAIIA purification has given promising result by producing higher intensity EPO bands. As compared to the UF method, the MAIIA purification has a higher recovery on purifying the urine samples. The bands intensity reflected that the MAIIA columns captured more EPO as compared to UF. At a low concentration of 0.25 mIU, both methods were able to purify EPO from the urine sample. However, it was easier to identify the EPO bands from MAIIA as it was specific and able to remove other foreign bands effectively. Unfortunately, a foreign band was also non-specifically detected at a molecular weight higher than 55 kDa. It is suspected to originate from bovine serum albumin (BSA) which is a component in one of the buffer.



Lane	2	3	4	5	6	7
EPO Concentration (mIU/50µl)	1.27	0.85	0.72	0.49	0.54	0.34

Figure 1: Image of EPO bands after ultrafiltration and MAIIA purification. Lane 1: marker; Lanes 2-4: pre-spiked urine samples with 1mIU/50µL, 0.5mIU/50µL and 0.25mIU/50µL respectively of uEPO then purified with MAIIA purification method; Lanes 5-7: pre-spiked urine samples with 1mIU/50µL, 0.5mIU/50µL and 0.25mIU/50µL respectively of uEPO then purified with conventional ultrafiltration method; Lane 8: urinary EPO standard. The quantitative analysis of EPO recovered using different purification respectively is shown at the bottom.

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Figure 2 shows a comparison between Stemcell with UF and MAIIA purification. Blank urine samples were tested with both methods as control. The image indicated that the blank urine already contained some endogenous EPO. The results for MAIIA purification were similar to the previous experiment where it has shown a high intensity band of EPO and the suspected BSA band was also detected. Stemcell with UF purification has also been able to provide an intense band as well. However, the disadvantage of Stemcell with UF purification is it demonstrated lower recovery of EPO compared to MAIIA. As shown in Figure 2, erythropoietin in 0.25 mIU of sample is hardly detected by using Stemcell purification. The MAIIA purification proved otherwise as the band appeared is quite intense and visible. This shows the recovery of MAIIA purification has a higher recovery compared to Stemcell purification with UF.

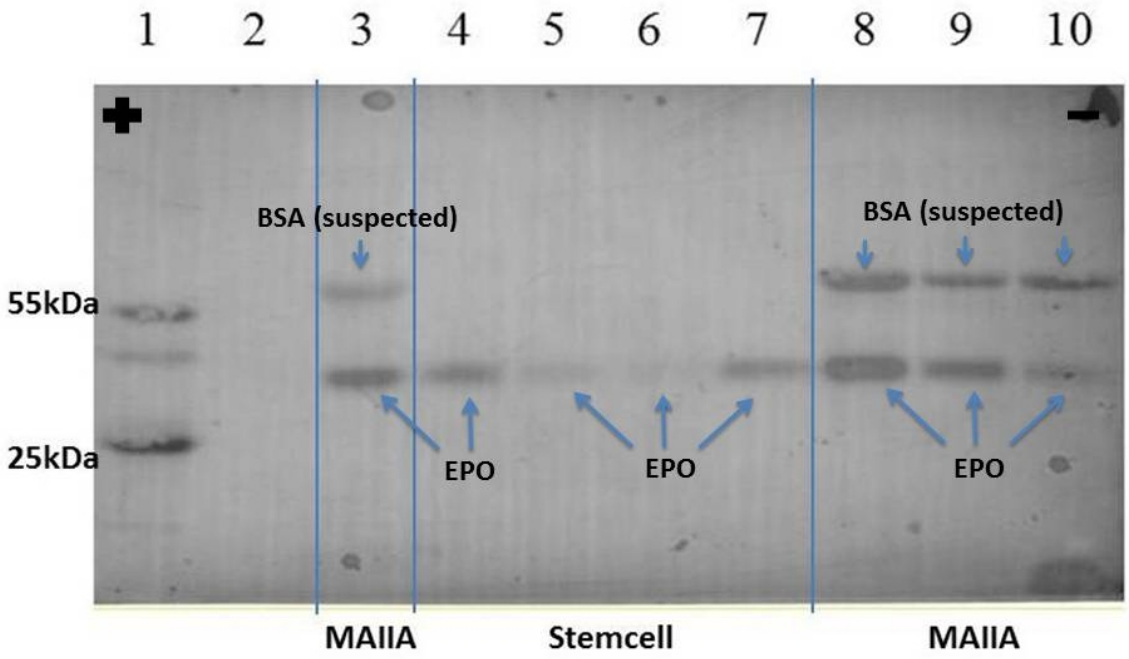

Figure 2: Image of EPO bands after Stemcell purification and MAIIA purification. Lane 1: marker; Lane 2: empty; Lane 3: blank Urine samples purified with MAIIA purification method; Lanes 4-6: pre-spiked urine samples with 1mIU/50µL, 0.5mIU/50µL and 0.25mIU/50µL respectively of uEPO then purified with Stemcell with UF purification method; Lane 7: blank Urine samples purified with Stemcell with UF purification method; Lanes 8-10: Pre-spiked urine samples with 1mIU/50µL, 0.5mIU/50µL and 0.25mIU/50µL respectively of uEPO then purified with MAIIA purification method.

Conclusions

An effective purification is required for an accurate and precise SDS-PAGE analysis of EPO in urine samples. MAIIA purification was proven to be the most efficient purification method compared to ultrafiltration and Stemcell purification. It was able to provide the highest recovery of EPO. This enhances the quality of anti-doping analysis and improves the quality of detection of EPO abuse among the athletes. MAIIA purification was efficient in removing foreign proteins except suspected to be Bovine Serum Albumin (BSA) which we suspected it was contributed by part of the component applied in the MAIIA purification kit. Nevertheless, it is not affecting the result due to its high mass is out from the molecular weight range of interest.

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