Analyst

Cite this: *Analyst*, 2012, **137**, 2445

www.rsc.org/analyst

**PAPER**

# A new analytical method based on anti-EPO monolith column and LC-FAIMS-MS/MS for the detection of rHuEPOs in horse plasma and urine samples

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***Received 29th July 2011, Accepted 1st March 2012***
**DOI: 10.1039/c2an15662h**

Recombinant human erythropoietin (rHuEPO) is a 30–34 kDa glycoprotein banned by the racing authorities. For some years this molecule has been detected in race horses in USA and in Europe, and even in racing camels. Although direct methods to differentiate horse endogenous EPO and rHuEPO have been developed either by LC-MS/MS or by isoelectric focusing (IEF) with double-blotting, the short confirmation time of such prohibited hormone in plasma remains a problem for horseracing doping control laboratories. In order to improve the rHuEPOs confirmation process in horse plasma or urine in terms of reliability and delay, a small anti-EPO monolith membrane contained in a disposable column (anti-EPO monolith column) has been successfully used and validated ($n = 10$). This new sample preparation, combined with LC-FAIMS-MS/MS, has been performed on plasma and urine samples collected from one horse which received an Eprex® treatment during six consecutive days and a second one with a single injection of Aranesp®. This inventive technology allowed the possibility to confirm the presence of rHuEPO within one day with a limit of detection validated for both urine and plasma at 250 pg $mL^{-1}$ by means of a disposable, ready to use immunoaffinity column. The lower limit of detection (LLOD) obtained for each matrix was 100 pg $mL^{-1}$. These results provide an important improvement for rHuEPO doping control in horseracing especially the possibility to confirm these banned molecules in both matrices, urine and plasma, with a confidence of two specific target peptides.



## 1 Introduction

For some years, recombinant human erythropoietins (rHuEPOs) which are glycoprotein hormones[1–3] have been found to be used as a doping agent in horseracing industry in USA and in Europe to enhance the oxygen transport and aerobic power of race horses. Erythropoietin (EPO) is produced predominantly in the kidneys, with an inverse relation to oxygen availability, which ensures a fine production rate delivery of red blood cells in the body.[4–6] Recombinant human molecules of EPO were released on the market in the middle of 1990s in order to help patients with severe anaemia. Since this period and due to the wide use of genetic engineering techniques, the large-scale production of rHuEPO in a suitable mammalian cell line (Chinese hamster ovary, CHO) has been developed, thus allowing the possibility to acquire easily this molecule on the black market.[7]

Equine EPO (eEPO) and rHuEPO have 84% identity of amino acids composition[8] which is extremely similar; consequently it provides the opportunity to use such substances for horse doping. Nevertheless, the risk of side effects is high due to possible production of horse anti-EPO antibodies which was previously reported for human therapy[9–11] and such protein based drug antigenicity was also observed with recombinant equine growth hormone doping practices.[12]

For horses, the confirmation of rHuEPO was obtained either by the IEF double-blotting method, developed primarily for human urine samples,[13,14] or by liquid chromatography linked to mass spectrometry applied exclusively to equine plasma.[15–19] Unfortunately, these techniques are often extensive and need a high background in biochemistry to prepare antibodies for the immunoaffinity column. These columns are often reused because of their expensive cost and the potential carryover cannot be ignored.

In the field of human doping control, a novel kit for rHuEPO affinity purification has been tested for enrichment and purification of EPO from human urine and plasma samples. This kit showed a high recovery of rHuEPO, capturing 76% of the recombinant human EPO, while 99.7% of the non-relevant proteins were removed.[20] It was found to be a reliable tool for

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human urine and plasma sample preparation and significantly improved the sensitivity and specificity for IEF double-blotting EPO doping tests.[21] However, the human recommended procedure for this affinity purification tool was not suitable for use prior to mass spectrometry, and with equine plasma and/or urine samples, and required thus further improvements.

Therefore, this study focuses on two innovative aspects. The first one is the sample preparation modifications of the kit reagents which allows us to work with an anti-EPO monolith membrane contained in a disposable column[20] (anti-EPO monolith column) in order to fit to mass spectrometry analysis. This system was evaluated to extract efficiently recombinant human EPO (rHuEPO) from horse plasma and urine samples. The sample preparation was improved to allow the detection of rHuEPOs target peptides by mass spectrometry. The second significant improvement was obtained by using the continuous gas-phase ion separation and atmospheric pressure focusing properties of the high-Field Asymmetric waveform Ion Mobility Spectrometry (FAIMS) for an improved selectivity of the rHuEPO target peptides before their introduction into the mass spectrometer. The FAIMS technology has been well described for tryptic peptides analysis[22–24] especially the improvement of the signal-to-background ratio which is one of the main issues when a drug needs to be confirmed at trace level. This system has also been described for peptides quantification in plasma[25] or serum[26] samples and to characterize macromolecular conformations.[27] So far, the use of such technology in doping control was only reported by Guddat *et al.*[28] for the detection of anabolic steroids in sport drug testing.

In this article, the new analytical method based on anti-EPO monolith column and LC-FAIMS-MS/MS has been validated in spiked horse plasma and urine samples and was successfully applied to horse Eprex® and Aranesp® post-administration samples in order to confirm the presence of these banned molecules in plasma and urine.

## 2 Materials and methods

### 2.1 Chemicals and reagents

The EPO Purification Kit, containing the anti-EPO monolith membranes, was from MAIIA Diagnostics (Uppsala, Sweden). The rHuEPO used for the LC-MS/MS development was Aranesp® (Amgen S.A.S., Neuilly sur Seine, France) and Eprex® (Janssen-Cilag, Issy-les-Moulineaux, France). Synthetic tryptic peptides with amino acid sequences as follows, T5: $^{21}$EAENITTGCAEHCSLNENITVPDTK$^{45}$, T6: $^{46}$VNFYAWK$^{52}$, T9: $^{77}$GQALLVNSSQVNETLQLHVDK$^{97}$ and T17: $^{144}$VYSNFLR$^{150}$, were purchased from Millegen (Labège, France). Bovine serum albumin was purchased from Jackson ImmunoResearch (Suffolk, UK). Trypsin (sequencing grade) was purchased from Promega (Charbonnières-les-Bains, France). HPLC-grade acetonitrile was purchased from Carlo Erba (Val de Reuil, France). Formic acid (FA), hydrochloric acid, ammonium bicarbonate, octyl β-D-glucopyranoside (OGP), tris(2-carboxyethyl)phosphine hydrochloride (TCEP), Tris, NaCl, $NaN_3$ and EDTA were obtained from Sigma-Aldrich (St Louis, MO, USA). All buffers and solutions were exclusively prepared with high purity water with a resistivity of >18 MΩ cm, and filtered online through a 0.22 μm membrane (Millipore, Bedford, MA, USA).

Standard solutions of rHuEPOs were prepared daily in BSA buffer (50 mM Tris pH 7.4, 1% BSA) at a final concentration of 100 pg $\mu L^{-1}$. The concentrate commercial solution was aliquoted and stored at −20 °C. Standard solutions of synthetic peptides were prepared in water/acetonitrile (70 : 30 v/v, 0.2% FA) at a concentration of 1 mg $mL^{-1}$ and stored at 4 °C. Working standard solutions were prepared by consecutive 1 : 10 dilutions and used within one month with storage at 4 °C.

### 2.2 Horse administration and sample collection

The study was led in agreement with animal welfare rules at the administration and sampling centre of the Fédération Nationale des Courses Françaises (FNCF). Two thoroughbred horses were engaged in this experiment. The first one received 40 IU $kg^{-1}$ per day for six days of erythropoietin α (Eprex, Janssen-Cilag, Issy-Les-Moulineaux, France) and the second received a single dose (0.39 μg $kg^{-1}$) of darbepoetin α (Aranesp®, Amgen, Neuilly sur Seine, France). The two drugs were administered subcutaneously in the neck of each horse. Plasma, obtained from blood collected in 9 mL lithium heparinate tubes (Greiner Bio-One SAS, Courtaboeuf, France), and urine samples were collected before, during and after the end of administrations. All samples were stored at −20 °C until their analysis. Individual negative blank urine and plasma samples used for method validation were from routine analysis (post-race and post-training).

### 2.3 Vacuum manifold

A vacuum manifold for processing 1–24 columns, QIAvac 24 Plus, and the connecting system (Qiagen, Les Ulis, France) was used together with a vacuum pump for passing the liquids through the columns.

### 2.4 MS and FAIMS compensation voltage (CV) optimization

MS optimization was performed from the compound optimization workspace of Thermo TSQ Tune Master software (version 2.1.0) on T5, T6, T9 and T17 synthetic peptides in water/acetonitrile (70 : 30 v/v, 0.2% FA) at 1 ng $\mu L^{-1}$ in order to optimize their doubly or triply charged state. The standard solutions were injected at a flow rate of 3 μL $min^{-1}$ coupled to a HPLC system (400 μL $min^{-1}$) with a T tubing in order to obtain enough flow parameters for suitable optimization. The FAIMS parameters were adjusted as follows: the electrode dispersion voltage was −5000 V, the total gas flow was 1.2 L $min^{-1}$ with a composition of 50 : 50 helium/nitrogen, and inner and outer electrode temperatures were 70 °C and 90 °C, respectively. The compensation voltage of each peptide was then obtained by means of a compensation voltage scan from −45 V to −15 V (Table 1).

The collision-induced dissociation (CID) of $[M + 2H]^{2+}$ and $[M + 3H]^{3+}$ precursor ions was performed in selected reaction monitoring (SRM) scan type and the parameters were as follows: Q1 and Q3 peak widths were 1.20 *m/z*; the Q3 scan width was set at 0.05 *m/z* to improve detection selectivity; and collision energy (eV) optimised for each selected transition is shown in Table 1.

 

**Table 1** High-Field Asymmetric waveform Ion Mobility Spectrometry (FAIMS) compensation voltage and Selected Reaction Monitoring (SRM) parameters for the rHuEPO tryptic peptides detection used with a triple quadrupole mass spectrometer. S, signal followed during the method validation

| Peptides | Charge state | Precursor ion (*m*/*z*) | Compensation voltage (V) | Collision energy (eV) | Fragment ions (*m*/*z*) |
|---|---|---|---|---|---|
| T5 | $[M + 3H]^{3+}$ | 897.0 | −27.3 | 40 | 248.1 |
| | | | | 31 | 460.3 |
| | | | | 22 | 660.2 |
| | | | | 25 | 1067.4 |
| T6 | $[M + 2H]^{2+}$ | 464.3 | −33.0 | 14 | 214.0 (S1) |
| | | | | 17 | 479.2 (S2) |
| | | | | 15 | 567.3 (S3) |
| | | | | 13 | 714.5 (S4) |
| T9 | $[M + 3H]^{3+}$ | 765.4 | −30.3 | 35 | 186.0 |
| | | | | 28 | 257.0 |
| | | | | 18 | 906.1 |
| | | | | 17 | 962.7 |
| T17 | $[M + 2H]^{2+}$ | 450.0 | −33.0 | 26 | 135.8 (S1) |
| | | | | 15 | 235.2 (S2) |
| | | | | 13 | 262.9 (S3) |
| | | | | 16 | 799.5 (S4) |

### 2.5 rHuEPO plasma and urine purification before LC-MS/MS

**Plasma preparation.** To 4 mL of plasma were added 36 mL of pH neutral buffer (20 mM Tris, 0.1 M NaCl, 0.02% $NaN_3$), which was previously adjusted at pH 7.5–8.0 with hydrochloric acid (37%). The sample was then gently mixed with the buffer and was filtered through a syringe 0.22 μm filter unit before microcolumn extraction.

**Urine preparation.** To 10 mL of urine was added 1 mL of urine precipitate dissolvation (UPD) buffer, provided in the EPO purification kit. The sample was then gently mixed and incubated at room temperature for 10 minutes. The sample was heated at 95 °C for 7 minutes in a simmering water bath and immediately cooled in a cold water bath for 5 minutes. To the cold sample was added 10.4 mL of dilution buffer (10 mL of high purity water supplemented with 200 μL of detergent aid and 200 μL of exposure aid included in the kit). The sample was then gently mixed with the buffer and was filtered through a syringe 0.22 μm filter unit before microcolumn extraction.

**Microcolumn extraction.** The EPO monolith membrane contained in the disposable column was conditioned with 1 mL of washing buffer (kit component) using a QIAvac vacuum manifold at a flow rate of 1 mL $min^{-1}$. The sample mixtures, 40 mL and 21.4 mL for plasma and urine, respectively, were then loaded at the same flow rate onto the microcolumn and allowed to pass completely through the column, followed by one washing with 1 mL of washing buffer. The microcolumn was then dried by centrifugation (1000 × *g*, 1 min). rHuEPO target molecules were eluted by centrifugation (1000 × *g*, 1 min) with 55 μL of a modified desorption buffer (desorption buffer component A (included in the kit) supplemented with 0.2% OGP and 0.016% TCEP) in a plastic tube containing 45 μL of denaturant buffer (100 mM ammonium bicarbonate, 2% OGP, 0.2% TCEP).

**Filtration step.** The eluate (100 μL) was completed with 400 μL of denaturant buffer before sample filtration on a 10 kDa MWCO (Millipore, Bedford, MA, USA) by centrifugation (5000 × *g*, 19 min).

**Denaturation step.** Following centrifugation, the retentate (45 μL) was kept and made up to 90 μL with TCEP (7 mM) in EPAB buffer (50 mM ammonium bicarbonate pH 7.9, 10 mM EDTA, 1 μM pepstatine A) before a denaturation step (95 °C, 15 min, alternating rotation set at 800 rpm) in Thermomixer Comfort (Eppendorf, Le Pecq, France) followed by a return to 37 °C for a minimum of 5 minutes.

**Trypsin digestion.** Trypsin (2 μg) was added to a final volume of 100 μL in EPAB buffer and gently mixed with repeated aspiration in a pipette tip. The protein solution was digested at 37 °C for 3 h 30 min with an alternating rotation of 800 rpm. Before LC-MS/MS analysis, the peptides obtained were dried under nitrogen at 48 °C and then dissolved in 30 μL water/acetonitrile (70 : 30 v/v, 0.2% FA).

### 2.6 LC-MS/MS

Peptide analyses were carried out on a triple quadrupole (TSQ Vantage, Thermo Fisher Scientific, San Jose, CA) with a heated electrospray ionization (HESI) source operated in the positive mode combined with a high-Field Asymmetric waveform Ion Mobility Spectrometry system (FAIMS, Thermo Fisher Scientific, San Jose, CA). The system was interfaced with an UFLC-XR binary pump (Shimadzu Corporation, Kyoto, Japan) equipped with a cooled autosampler maintained at 15 °C, a vacuum degasser and a column oven maintained at 30 °C. Separations were performed on a Hypersil Gold C18 column (175 Å, 150 mm × 2.1 mm, 5 μm) (Thermo Fisher Scientific) with a Hypersil Gold guard column (10 mm × 2.1 mm, 5 μm). The LC flow rate was 400 μL $min^{-1}$. The mobile phase comprised water/acetonitrile, 0.2% formic acid (FA). The liquid chromatography gradient was run with 95% water, 0.2% AF at the start ($t$ = 0 min), held for 2 min, decreased to 40% water, 0.2% AF at 12 min and to 100% acetonitrile, 0.2% AF at 14 min, held for 1 min and then returned to the initial mobile phase composition (95% water, 0.2% AF) at 16 min and stabilised for 3 min ($t$ = 19 min). The sample injected for analysis was 15 μL.

Parameters of the triple quadrupole mass spectrometer were 3 kV and 400 °C for spray voltage and vaporizer temperature of the probe, respectively. Sheath gas and auxiliary gas pressure applied to the HESI probe were 60 and 30 arbitrary units, respectively. The ion transfer capillary temperature and the source S-lens RF amplitude were 300 °C and 102 V, respectively. The argon collision gas pressure was 1.5 mTorr.

## 3 Results and discussion

### 3.1 MS/MS optimization

For enhancement of rHuEPO detection, sensitivity, and specificity, we focused on 4 specific peptides, T5, T6, T9 and T17, described previously for rHuEPO equine doping control.[15–17] T6 and T17 are short peptides (7 amino acids) which are not glycosylated, T5 (25 amino acids) and T9 (21 amino acids) are

glycosylated and need the PNGase F (peptidyl *N*-glycosidase F) treatment before sample analysis. Synthetic peptides were characterized by their doubly or triply charged state, as is common with peptides resulting from trypsin digestion.[29]

MS/MS experiments were performed on each synthetic peptide to optimize their SRM collision energy (Table 1). Our results are in good accordance with the results previously obtained from such peptides.[15,16] Only the four most abundant transitions of each molecule have been selected as targets.

### 3.2 Compensation voltage (CV)

Compensation voltage was optimized at *m/z* 450.0, *m/z* 464.3, *m/z* 765.4 and *m/z* 897.0 for peptides T17, T6, T9 and T5, respectively. The compensation voltage distribution of each target peptide was monitored and their maxima values corresponding to the highest degree of ion selectivity/mobility were selected. The compensation voltage value is related to the specific ion mobility between the FAIMS inner and outer electrode and depends also on the peptide conformation. In addition, the FAIMS interface placed between the electrospray probe and the mass spectrometer adds a complementary dimension of ion separation and thus increases the selectivity of the method by suppressing isobaric interferences.

Unfortunately, due to the low electronic switching capacity of the FAIMS to apply different compensation voltages at the same time, it became difficult to analyze such a large number of molecules (T5, T6, T9 and T17) with their different optimized compensation voltages. Consequently, in regards of each compensation voltage curve pattern, it was decided to focus on T6 and T17 peptides with adjustments (Fig. 1). The compensation voltages obtained were −32 V and −38 V for T17 and T6, respectively. According to each peptide compensation voltage curve, it was decided to use a single compensation voltage for both T6 and T17 peptides, with a CV set at −33 V. The consequence in terms of sensitivity is low for T17 but there was a loss of sensitivity at about 35% for T6. In spite of this small compromise, the confirmation of T6 and T17 peptides has been possible either in spiked plasma/urine samples and rHuEPOs incurred plasma/urine samples.

### 3.3 Sample preparation

The analytical challenge was to develop a rapid method based on a single-use technology and consistent with mass spectrometry detection. To date, all the rHuEPO confirmation methods developed for horse doping control are based on coated beads immunoaffinity chromatography[15–19] which is often difficult to reproduce with the same quality and recovery. In this context, starting from the anti-EPO monolith membranes provided in the kit, some parameters have been tested and modified in order to improve the rHuEPO detection sensitivity by mass spectrometry.

In a first step of development, different sample volumes were tested. The best recovery with the lowest microcolumn membrane clogging was found for 4 mL and 10 mL of plasma and urine, respectively. Attempts to use a directly heated urine sample (95 °C, 7 min) without the use of the dedicated urine dilution buffer have been carried out but regularly without success. The viscosity of each different horse urine specimen was decreased very well by combination with the heating step and dilution with the mix of water, detergent aid and exposure aid. The provided elution buffer was modified in order to improve the link between the sample preparation, trypsinolysis and the mass spectrometry detection. The use of octyl β-D-glucopyranoside (OGP) as a detergent in each step of our method leads to improved repeatability and stability of the analyses. TCEP (tris-(2-carboxyethyl)phosphine hydrochloride) was preferred to DTT or β-mercaptoethanol for the disulfide bridge reduction because the molecule is more stable, odourless and more compatible with mass spectrometry than the other two. In addition, pH adjustment consecutive to the acidic elution was performed in 100 mM ammonium bicarbonate, which decreased highly the ion suppression effect. Prior to rHuEPO denaturation and trypsinolysis, the sample was washed and concentrated to 45 μL by means of a spin column with 10 kDa MWCO. Other molecular weight cut-off membranes have been evaluated (*i.e.* 100, 50, and 30 kDa) but the lowest limit of detection was obtained only with the 10 kDa MWCO. A denaturation step and trypsin digestion were specifically developed for rHuEPO confirmation procedures by LC-MS/MS.

### 3.4 Assay validation

The number of worldwide available rHuEPOs increases each year and must be directly correlated to the recent release on the market of numerous rHuEPO Chinese biosimilars. In the case of horse doping control all rHuEPOs are forbidden and the method

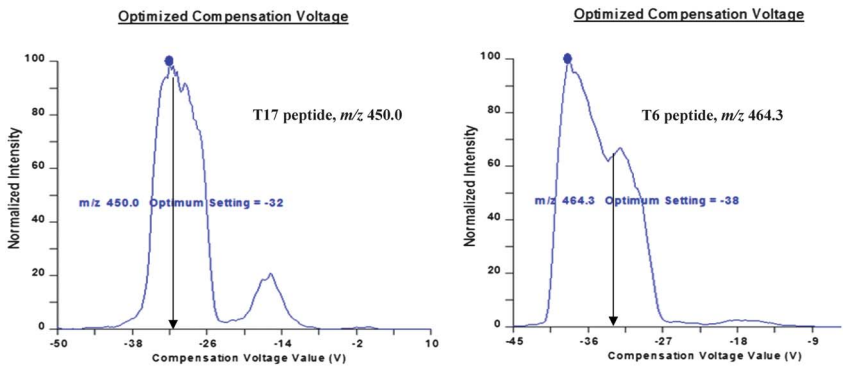


**Fig. 1** Representative compensation voltage (CV) pattern monitored for peptides T17 (*m/z* 450.0) and T6 (*m/z* 464.3). Optimal CVs obtained were −32 V and −38 V for T17 and T6, respectively. Black arrows show the use of a single compensation voltage for both T17 and T6 peptides set at −33 V and its consequence in terms of sensitivity.

 

**Table 2** Method validation performed from ten different plasma and urine samples spiked both at 250 pg mL$^{-1}$ with Aranesp®. The two rHuEPO target peptides monitored were T6 ([M + 2H]$^{2+}$ *m/z* 464.3) and T17 ([M + 2H]$^{2+}$ *m/z* 450.0). The standard deviation (SD) of their retention time (RT) expressed in percentage (%) was calculated for the ten plasma and urine replicates as well as their three characteristic fragment ion ratios (see Table 1 for transitions). The lower limit of detection (LLOD) was the lowest concentration measured at a signal-to-noise ratio greater or equal to three

| | Repeatability ($n$ = 10) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | T17 [M + 2H]$^{2+}$ *m/z* 450.0 | | | | T6 [M + 2H]$^{2+}$ *m/z* 464.3 | | | | | |
| | RT SD (%) | S2/S1 | S3/S1 | S4/S1 | RT SD (%) | S1/S3 | S2/S3 | S4/S3 | Validated performances (pg mL$^{-1}$) | LLOD (pg mL$^{-1}$) |
| Plasma | 1.1 | 10 | 11 | 9 | 1.0 | 17 | 9 | 16 | 250 | 100 |
| Urine | 1.1 | 15 | 15 | 12 | 0.1 | 14 | 12 | 18 | 250 | 100 |

presented in this paper is therefore dedicated to qualitative detection at the lowest validated concentration in plasma and in urine samples of all rHuEPOs without discrimination.

The assay validation was performed according to the requirement of AORC guidelines[30] for minimum criteria for analyte identification by means of the selectivity and repeatability assessment of the retention time (RT SD $\leq$ 2%), the robustness of the characteristic fragment ions (ion ratio $\leq$ 20%) and the signal to noise ratio ($S/N \geq 3$). This was carried out using ten different blank plasma (4 mL) and urine (10 mL) samples to appraise the selectivity and repeatability followed by the same samples spiked at 250 pg mL$^{-1}$ with Aranesp® for robustness. In regards to the recent knowledge of such sample preparation to equine matrix,[20] it was noticed that the recoveries of Neorecormon®, Aranesp® and Mircera® added to equine plasma were 69%, 47% and 35%, respectively. During the validation step, it was decided to focus on the substance with the lowest reported recovery in order to be sure to catch the maximum of positive sample. The validation procedure was therefore performed with Aranesp® as rHuEPO reference molecule. The assay validation with Mircera® (third generation) is in progress (data not shown).

Table 2 summarizes the results obtained during the validation procedure for both urine and plasma samples. For these samples, no interferences were observed at the specific retention time of the fragment ions generated from the fragmentation of T6 and T17 at 9.9 min and 9.0 min, respectively. Furthermore for each peptide confirmed during the validation process in plasma and in urine the retention time standard deviation was below 2%. This demonstrated the specificity of the method used for the detection of T6 and T17 rHuEPO peptides. This result is a key point obtained by means of ion mobility with the FAIMS interface which improves, with specific compensation voltage, such

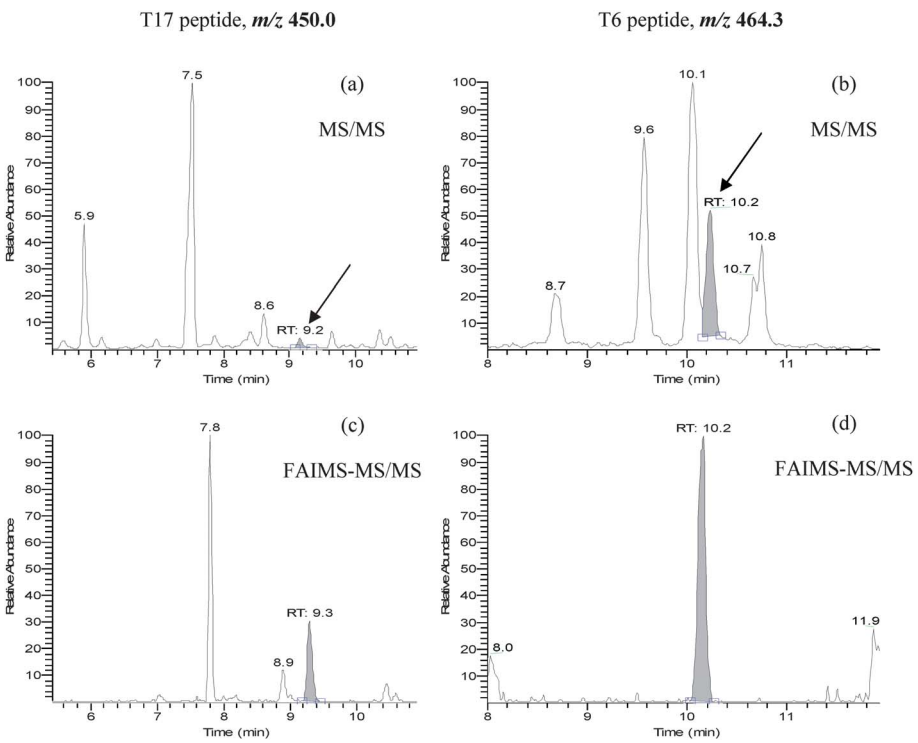


**Fig. 2** Comparison of a plasma spiked at 1 ng mL$^{-1}$ of Aranesp® analyzed without and with FAIMS ion mobility selectivity. (a) and (b) are obtained from LC-ESI-MS/MS of the precursor ions T17 (*m/z* 450.0) and T6 (*m/z* 464.3). (c) and (d) are obtained from LC-ESI-FAIMS-MS/MS of the precursor ions T17 (*m/z* 450.0) and T6 (*m/z* 464.3). Black arrows show the identified target peptides T17 and T6 in the overall signal.

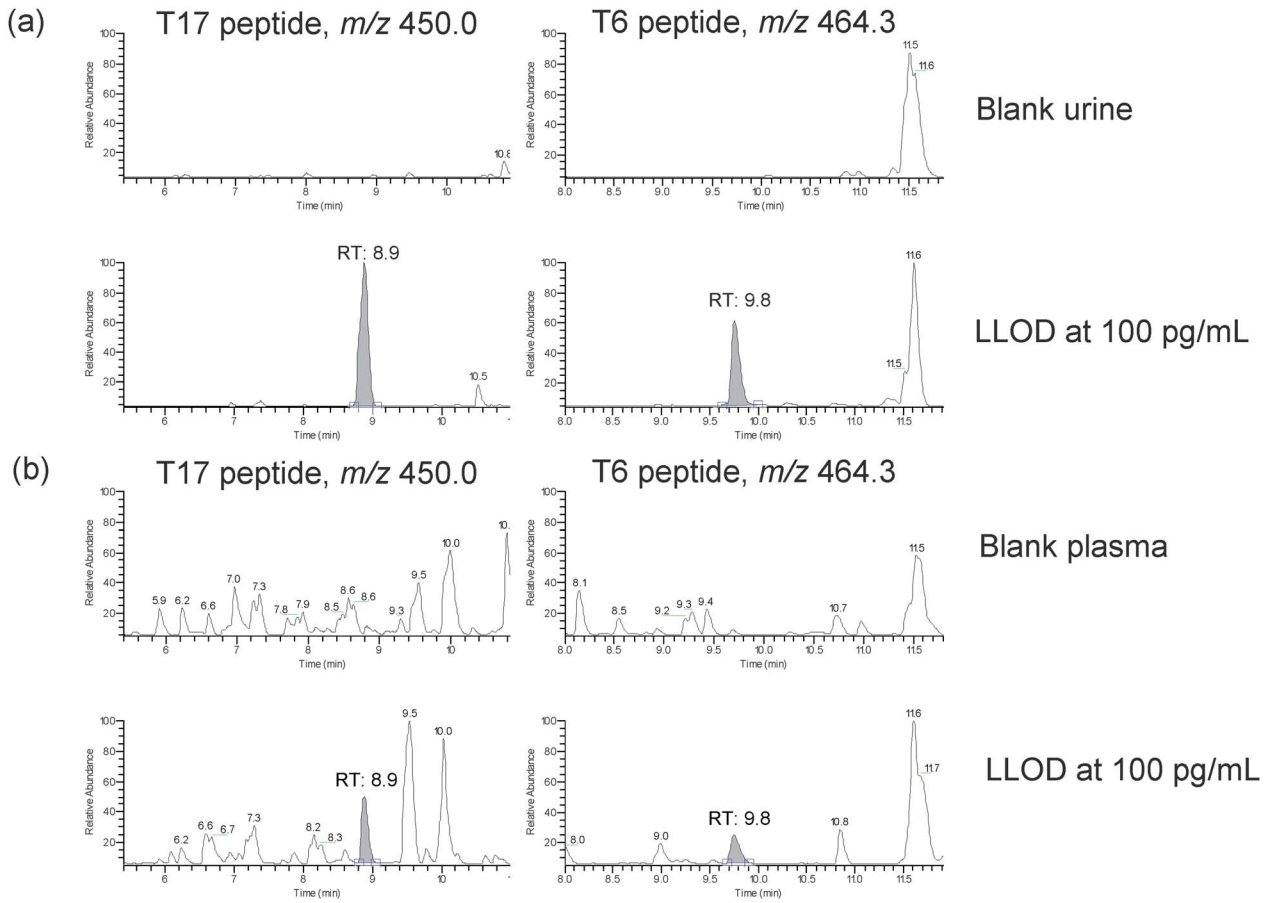


**Fig. 3** Lower limit of detection (LLOD) in both urine (a) and plasma (b) spiked at 100 pg $mL^{-1}$ obtained from LC-ESI-FAIMS-MS/MS of the precursor ions T17 (*m/z* 450.0) and T6 (*m/z* 464.3).

criteria. The analysis of the same sample performed without FAIMS has led to high number of interferences, especially with T6 (Fig. 2). Regarding the four transitions selected for T6 and T17 peptides analysis (Table 2), standard deviations of each signal intensity ratio were below 20%. The AORC criteria for small molecules were fulfilled for this method indicating that the method is capable of detecting and confirming the presence of rHuEPO in equine plasma and urine at 250 pg $mL^{-1}$. As part of the assay validation, Table 2 reports the LLOD which is defined as the lower limit of detection at which a substance may be measured at a signal-to-noise ratio greater than or equal to three. For each matrix, the LLOD is presented in Fig. 3 and was at 100 pg $mL^{-1}$.

### 3.5 Confirmation of Eprex® and Aranesp® in post-administration samples

The method was successfully applied to a plasma sample collected from a thoroughbred horse that was daily subcutaneously administered with Eprex® at a dose of 40 IU $kg^{-1}$ per day for six consecutive days. Fig. 4 shows the unambiguous detection of Eprex® in the plasma sample by means of the four fragments of peptide T6 and T17 at 9.9 min and 9.0 min, respectively. The retention time obtained is similar to the reference spiked plasma sample. Furthermore, the method was also successfully applied to a plasma sample collected from a thoroughbred horse that was subcutaneously administered with a single dose of Aranesp® (0.39 μg $kg^{-1}$). Fig. 4 shows the detection of Aranesp® at $D_{+4}$ with the four fragments of peptide T6 and T17 at 9.9 min and 9.0 min, respectively. The confirmation of Aranesp® was therefore possible 4 days after the single injection. For both post-administration samples, the signal to noise ratio was greater than three and very well correlated to the SRM transitions monitored for the rHuEPO spiked plasma.

In spite of difficulties associated with the use of horse urine in doping control, Fig. 5 shows four transitions well identified in the Eprex® urine post-administration sample at a retention time of 9.9 min and 9.1 min for T6 and T17, respectively. Surprisingly, the chemical background noise in the urine sample was very low but this is explained by the high selectivity provided by the use of the ion mobility technology in this confirmation method.

The present study compared to the recent method described by Yu *et al.*, 2010 decreases the time of sample preparation to one day compared to three days including the time to prepare the immunoaffinity column, uses only 4 mL of plasma sample compared to 5 mL which can be very sensitive in the case of low sample volume and is the first to provide the possibility to confirm the presence of rHuEPO in horse plasma or urine sample with a validated limit of detection of 250 pg $mL^{-1}$ for two target peptides according to AORC guidelines. Further investigations are in progress to improve this limit.

 

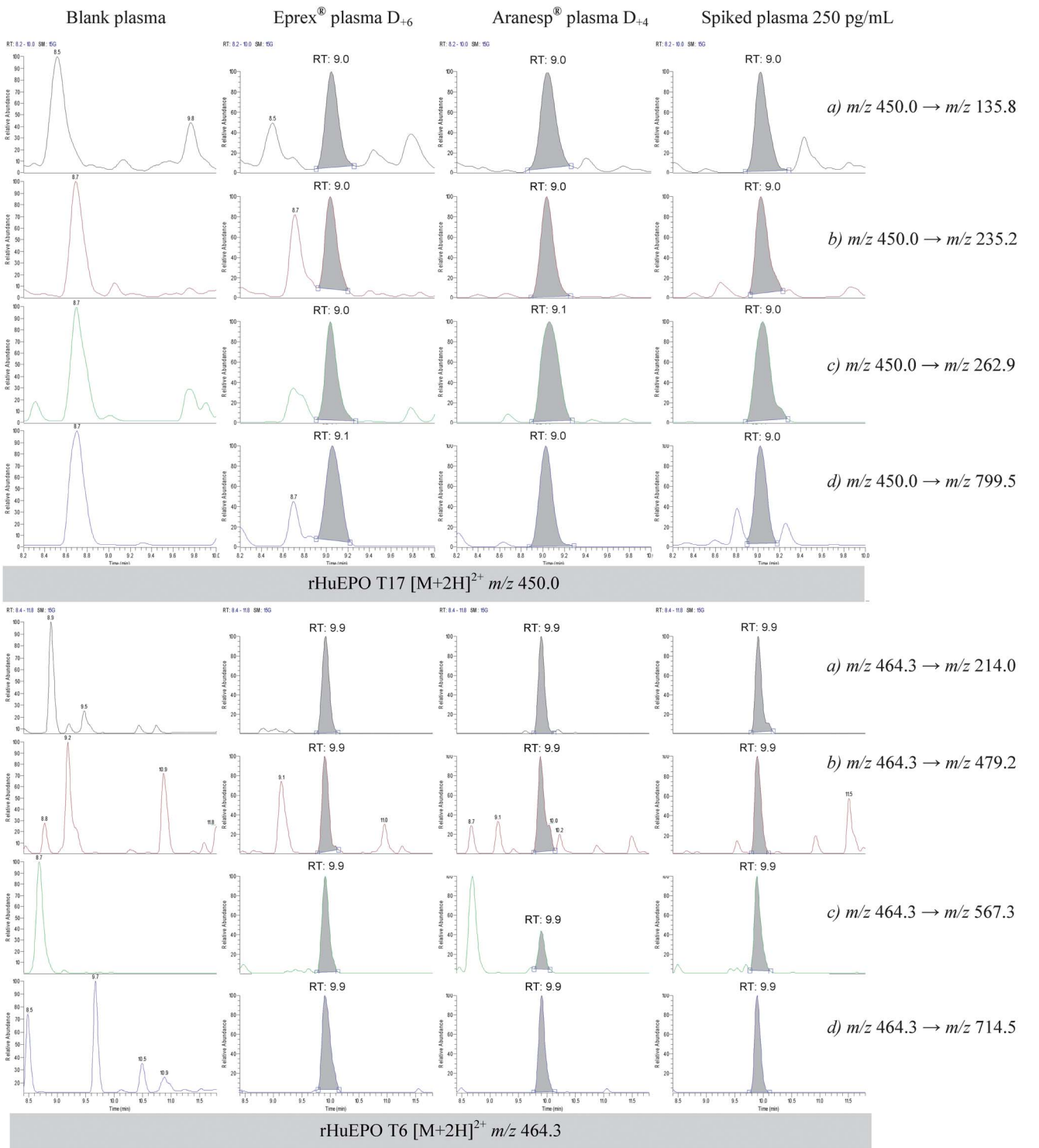


**Fig. 4** Ion chromatograms of blank plasma, Eprex® $D_{+6}$ post-administration, Aranesp® $D_{+4}$ post-administration and blank plasma spiked at 250 pg $mL^{-1}$ with Aranesp® of the four selected fragment ions of T17: VYSNFLR ($[M + 2H]^{2+}$, *m/z* 450.0, retention time of 9.0 min) and T6: VNFYAWK ($[M + 2H]^{2+}$, *m/z* 464.3, retention time of 9.9 min) obtained by LC-ESI-FAIMS-MS/MS (SRM scan type) analysis with Vantage triple quadrupole. The presence of Eprex® and Aranesp® was confirmed at $D_{+6}$ and $D_{+4}$, respectively according to the rHuEPO transitions as follows: T17 (a) *m/z* 450.0 → *m/z* 135.8, (b) *m/z* 450.0 → *m/z* 235.2, (c) *m/z* 450.0 → *m/z* 262.9, and (d) *m/z* 450.0 → *m/z* 799.5 and T16 (a) *m/z* 464.3 → *m/z* 214.0, (b) *m/z* 464.3 → *m/z* 479.2, (c) *m/z* 464.3 → *m/z* 567.2 and (d) *m/z* 464.3 → *m/z* 714.5.

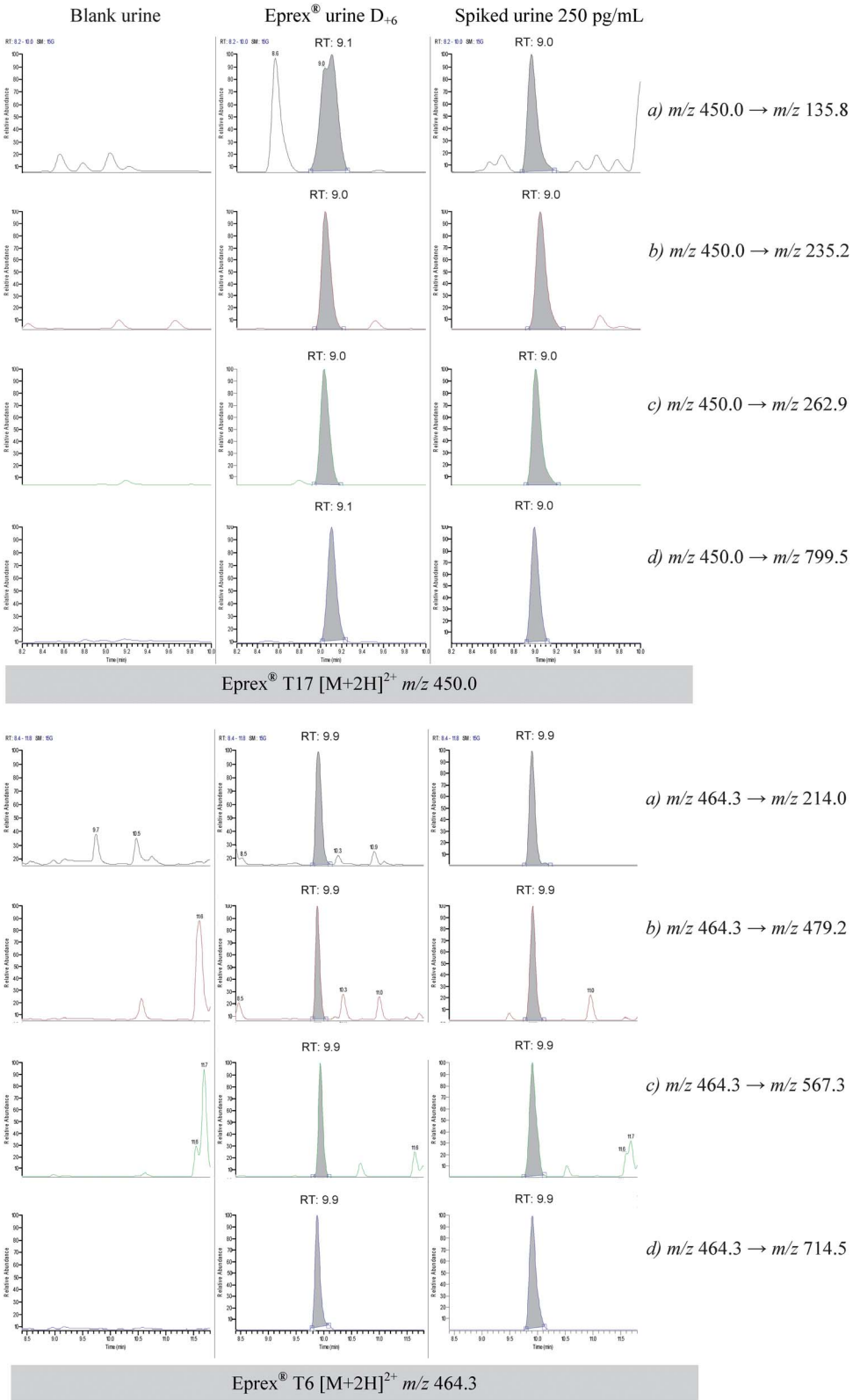


**Fig. 5** Ion chromatograms of blank urine, Eprex® $D_{+6}$ post-administration and blank urine spiked at 250 pg $mL^{-1}$ with Aranesp® of the four selected fragment ions of T17: VYSNFLR ($[M + 2H]^{2+}$, *m/z* 450.0, retention time of 9.0 min) and T6: VNFYAWK ($[M + 2H]^{2+}$, *m/z* 464.3, retention time of 9.9 min) obtained by LC-ESI-FAIMS-MS/MS (SRM scan type) analysis with Vantage triple quadrupole. The presence of Eprex® was confirmed in urine at $D_{+6}$ according to rHuEPO transitions as follows: T17 (a) *m/z* 450.0 → *m/z* 135.8, (b) *m/z* 450.0 → *m/z* 235.2, (c) *m/z* 450.0 → *m/z* 262.9, and (d) *m/z* 450.0 → *m/z* 799.5 and T6 (a) *m/z* 464.3 → *m/z* 214.0, (b) *m/z* 464.3 → *m/z* 479.2, (c) *m/z* 464.3 → *m/z* 567.2 and (d) *m/z* 464.3 → *m/z* 714.5.

 

## 4 Conclusion

The use of an anti-EPO monolith membrane contained in a disposable column as easy sample preparation associated with a sensitive LC-MS/MS method coupled to a selective high-Field Asymmetric waveform Ion Mobility Spectrometry (FAIMS) technology was sufficiently specific to directly confirm the presence of rHuEPO in a spiked plasma and urine sample at 250 pg $mL^{-1}$ with two specific target peptides. The method was therefore validated at this concentration for both plasma and urine matrices. The lower limit of detection obtained was 100 pg $mL^{-1}$ for both matrices. FAIMS coupled to the TSQ Vantage triple quadrupole mass spectrometer allowed a decreased chemical noise and thus an increased signal to noise ratio at low EPO concentrations. The FAIMS device is a mass spectrometer option which can be used for other analytical issues in doping control especially to improve the detection and selectivity of some other difficult banned molecules such as growth hormone or related anabolic molecules. The present method was sufficiently specific and sensitive to confirm the presence of Eprex® and Aranesp® in post-administration samples. In regards of doping control sensitivity the goal is to reach 50 pg $mL^{-1}$ in order to increase the detection time of the first generation of rHuEPO and biosimilars. Nevertheless, the proposed method is robust and sensitive for both plasma and urine samples with two target peptides and this new process could be useful for horseracing laboratories because the technology is rapid, easy-to-use, disposable and is expected to detect rHuEPO at low amount (100 pg $mL^{-1}$) in one day by LC-MS/MS.

## Acknowledgements

The authors are indebted to Dr Jean-Jacques Garin, veterinary surgeon at FNCF, to the horse farm manager in Coye la Forêt, and to the staff who participated in the drug administration, sampling, and horse care. The Swedish Foundation for Equine Research (SSH), Stockholm, funded the adaptation of the EPO Purification Kit for detection in equine samples. ThermoFisher Scientific (Les Ulis, France) is acknowledged for the loan of FAIMS unit. Finally authors thank Amgen laboratories for its contribution in giving Aranesp®.

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