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Doping control analysis of insulin and its analogues in equine urine by liquid chromatography–tandem mass spectrometry

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ABSTRACT

Insulin and its analogues have been banned in both human and equine sports owing to their potential for misuse. Insulin administration can increase muscle glycogen by utilising hyperinsulinaemic clamps prior to sports events or during the recovery phases, and increase muscle size by its chalonic action to inhibit protein breakdown. In order to control insulin abuse in equine sports, a method to effectively detect the use of insulins in horses is required. Besides the readily available human insulin and its synthetic analogues, structurally similar insulins from other species can also be used as doping agents. The author's laboratory has previously reported a method for the detection of bovine, porcine and human insulins, as well as the synthetic analogues Humalog (Lispro) and Novolog (Aspart) in equine plasma. This study describes a complementary method for the simultaneous detection of five exogenous insulins and their possible metabolites in equine urine. Insulins and their possible metabolites were isolated from equine urine by immunoaffinity purification, and analysed by nano liquid chromatography–tandem mass spectrometry (LC/MS/MS). Insulin and its analogues were detected and confirmed by comparing their retention times and major product ions. All five insulins (human insulin, Humalog, Novolog, bovine insulin and porcine insulin), which are exogenous in horse, could be detected and confirmed at 0.05 ng/mL. This method was successfully applied to confirm the presence of human insulin in urine collected from horses up to 4 h after having been administered a single low dose of recombinant human insulin (Humulin R, Eli Lilly). To our knowledge, this is the first identification of exogenous insulin in post-administration horse urine samples.

1. Introduction

Insulin is a polypeptide hormone consisting of two peptide chains (A- and B-chains) that are crossed-linked by two disulfide bonds. It is produced in the pancreas. Apart from being the primary agent in carbohydrate homeostasis, it exerts a variety of effects on fat metabolism, the liver's activity in the storage and release of glucose, and in the processing of blood lipids [1,2]. Exogenous sources of insulins are used in the treatment of insulin-dependent diabetes mellitus. However, insulin has been abused for building muscles and improving endurance in athletes [1,3,4]. Insulin administration can result in an increase in muscle glycogen prior to sports events or during the recovery phases by utilising hyperinsulinaemic clamps, and increase muscle size by its chalonic action to inhibit protein breakdown [1,5–7]. Since 1999, the use of insulins has been banned by the International Olympic Committee and the World Anti-Doping Agency for human athletes who are demonstrably not suffering from diabetes mellitus [8,9]. Similarly, insulins are prohibited in equine sports as implicit in Article 6 of the International Agreement on Breeding, Racing and Wagering (published by the International Federation of Horseracing Authorities) [10] and as stated in the Equine Prohibited List of the Veterinary Regulations of the Federation Equestre Internationale [11].

In order to control insulin abuse in equine sports, a method to effectively detect the use of insulins in horses is required. Up to now, *bona fide* products for equine use are unavailable. However, there are various forms of insulins for human use in the market. Short or rapidly acting human insulin preparations, like Humulin R, have been used in doping as they act fast and excrete from the body rapidly [12]. Therefore, the two most commonly used fast-acting human insulin analogues, Humalog (Lispro) and Novolog (Aspart), were included in this study in addition to human insulin. Apart from the readily available human insulin and its synthetic variants, insulins from other species can also be used as doping agents. Insulins from pigs and cows are thought to be pharmacologically effective in horses since they are structurally similar to equine insulin, with only one and three amino acid differences from porcine insulin and bovine insulin respectively (see Fig. 1). Indeed,

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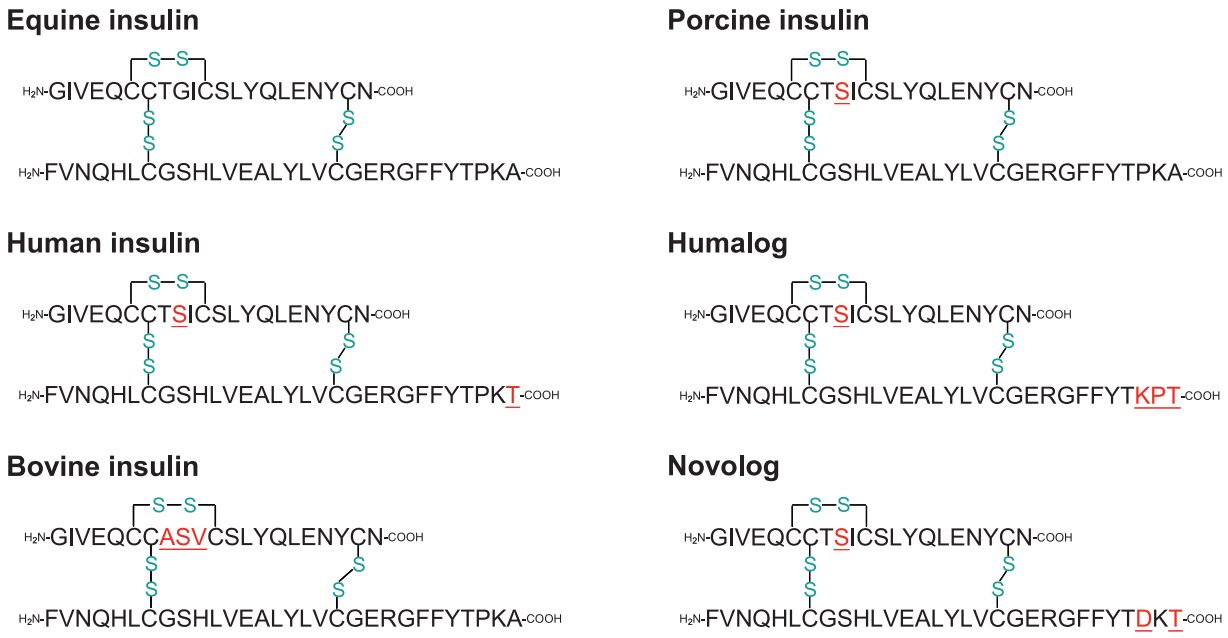

Fig. 1. Amino acid sequence of the targeted insulins.

early sources of insulin for clinical use in human had been isolated from cows, horses or pigs. A method for the detection of bovine, porcine and human insulins, as well as the synthetic analogues Humalog and Novolog in equine plasma has recently been published by the authors' laboratory [13]. This study describes a complementary method for the simultaneous detection of five exogenous insulins and their possible metabolites in equine urine, which is the most common sample matrix for doping control in equine sports, since sample volumes are usually large and the concentrations of drugs and its metabolites are usually higher than blood.

A few LC/MS methods have been reported for the detection of insulins and their synthetic counterparts in human plasma or urine [14–21] and equine urine [22]. Targeted insulins were isolated from plasma [14,18–20] or urine [15,16,21,22] samples by means of solid-phase extraction [18,20] and/or immunoaffinity purification [14–16,19–22]. Mutliply charged intact insulins [14–16,18–22] and their B-chains [14–16,21,22] were analysed by LC/MS. However, there has been only one other report of the detection of insulins in biological samples obtained from either human or animal after surreptitious administration of insulins; reported in a very recent case study on the detection of Humalog in a blood sample from an 8-week-old infant [23]. The objective of this study was to develop a sensitive mass-spectrometry based method for the detection of insulins and their possible metabolites in post-administration horse urine samples. The possible metabolites of insulins were first identified from *in vitro* studies. Insulins and their metabolites were isolated from equine urine by immunoaffinity purification, and analysed by nano liquid chromatography–tandem mass spectrometry (LC/MS/MS). In order to reduce analysis turnaround time and to avoid possible reduction in recovery, neither enzyme digestion nor reduction of disulfide bonds was performed in this method. This top-down sequencing-based assay enabled a fast determination of insulins in equine urine samples as the retention times and diagnostic SRM transitions from intact insulins could provide sufficient information for unequivocal identification. All five exogenous insulins (human insulin, Humalog, Novolog, bovine insulin and porcine insulin) could be detected and confirmed at 0.05 ng/mL. In order to demonstrate the applicability of this method in doping control, post-administration urine samples collected from horses administered with a single low-dose short-acting recombinant human insulin preparation, Humulin R, were analysed by this method.

2. Experimental

2.1. Materials

Human, porcine and bovine insulin were obtained from Sigma (St. Louis, MO, USA). Humalog (Lispro) was purchased from Lilly (Fegersheim, France) and Novolog (Aspart) was obtained from Novo Nordisk (Bagsværd, Denmark). Stock solutions (1 mg/mL) of each insulin analogue were prepared with 2% acetic acid in deionised water. Stock solutions were stored in aliquots of 200 μL at −70 °C. Working standard solutions of individual insulin were prepared by consecutive dilution of the respective stock solutions. Recombinant human insulin Humulin R was obtained from Eli Lilly (Giessen, Germany). Polyclonal anti-human insulin antibodies (P/N No. 20-IP30) were purchased from Fitzgerald Industries International (Concord, MA, USA). Magnetic beads, Dynabeads M-280 tosylactivated, and magnetic particle concentrator (MPC) were obtained from Invitrogen (Carlsbad, CA, USA). Igepal CA-630 (biochemical grade) was obtained from Fluka (Buchs, Switzerland). Polyethylene glycol 6000 was purchased from BDH (Poole, UK). Sodium hydroxide (pellets, analytical grade) and boric acid (analytical grade) were purchased from Riedel-de Haën (Seelze, Germany). Acetic acid (100%, Suprapur) hydrochloric acid (30%, Suprapur) and formic acid (>98%, Suprapur) were obtained from Merck (Darmstadt, Germany). HPLC grade deionised water was obtained from a Millipore water purification system (Milli-Q, Molsheim, France). Acetonitrile (ACN) (Chromasolv; gradient grade), phosphate-buffered saline (PBS), EDTA, sodium azide, β-nicotine adenine dinucleotide (β-NAD), glucose-6-phosphate, magnesium chloride ($MgCl_2$), glucose-6-phosphate dehydrogenase and Trizma base were purchased from Sigma (St. Louis, MO, USA). Buffers used for linking the anti-insulin antibody to the magnetic beads and subsequent immunoaffinity purification of insulins were prepared

Table 1
Molecular weights, precursor ions, characteristic product ions, *in vitro* metabolites identified for the targeted insulins.

Insulin	Molecular weight (Da)	Precursor ion $[M+5H]^{5+}$ (m/z)	Product ion monitored (m/z)	*In vitro* metabolites identified
Equine insulin	5747.6	1150.8	110.1, 120.1, 129.1, 136.1, 143.1, 219.1, 226.2, 315.2	N/A[a]
Human insulin	5807.7	1162.5	110.1, 120.1, 129.1, 136.1, 143.2, 219.1, 226.2, 345.1	DesB24-30, DesB25-30, DesB26-30, DesB27-30 and DesB30 human insulin
Bovine insulin	5733.6	1147.7	110.1, 120.1, 129.1, 136.1, 143.2, 219.2, 226.3	DesB24-30, DesB26-30 and DesB30 bovine insulin
Porcine insulin	5777.6	1157.2	110.1, 120.1, 129.1, 136.1, 219.2, 226.2, 315.1	DesB24-30, DesB25-30, DesB26-30, DesB27-30 and DesB30 porcine insulin
Humalog	5807.7	1162.5	110.1, 120.1, 129.2, 136.1, 143.2, 217.2, 345.2	DesB24-30, DesB25-30, DesB26-30 Humalog
Novolog	5825.6	1166.0	110.1, 120.1, 129.2, 136.1, 143.1, 219.2, 226.2	DesB24-30, DesB26-30, DesB27-30 and DesB30 Novolog

[a] *In vitro* studies for equine insulin could not be performed owing to the unavailability of the reference standard.

according to the Invitrogen's manual and another reference [13]. These included PBS, pH 7.4; borate buffer, 0.1 M, pH 9.5 (*buffer* B in the Invitrogen's manual); PBS (pH 7.4), plus 0.1% (w/v) bovine serum albumin (BSA), 2 mM EDTA, and 0.02% (w/v) sodium azide (*buffer* C); Tris buffer (0.2 M, pH 8.5) plus 0.1% (w/v) BSA (*buffer* D); washing buffer, 1% (w/v) Igepal CA-630 in PBS (pH 7.4); and elution buffer, 0.01% PEG 6000 in PBS (adjusted to pH 2.0 with concentrated HCl). All the buffers were stored at 4 °C.

2.2. Procedures for linking anti-human insulin antibody to magnetic beads

Magnetic beads (in 2 mL suspension) were gently shaken and immediately transferred to a 5-mL plastic tube. The tube was placed in a magnetic particle concentrator (MPC) to separate the magnetic beads from the solution. The supernatant was then discarded. The tube was removed from the MPC and the beads were washed with 2 mL *buffer* B (0.1 M borate buffer, pH 9.5). The supernatant was removed and the beads were re-suspended in 2 mL of *buffer* B. Anti-human insulin antibody (1 mL) was added to the magnetic beads in 2 mL of *buffer* B. The mixture was incubated at 37 °C overnight with shaking. It was then placed in the MPC to remove the supernatant. The coated beads were then washed twice with 2 mL *buffer* C (PBS (pH 7.4) plus 0.1% BSA, 2 mM EDTA and 0.02% sodium azide) for 5 min each at ambient temperature. The coated beads were then incubated with 2 mL of *buffer* D (0.2 M Tris buffer (pH 8.5) plus 0.1% BSA) for 4 h at 37 °C in order to block the residual tosyl groups. The supernatant was removed using the MPC. The coated beads were washed with 2 mL of *buffer* C for 5 min at ambient temperature and re-suspended in 2 mL of *buffer* C. The suspension was kept at 4 °C until use.

2.3. Immunoaffinity purification of insulins from equine urine

The coated magnetic beads in *buffer* C were re-suspended by gently shaking. An aliquot of 0.4 mL of the mixture was immediately transferred to a 5-mL plastic tube. The tube was placed in the MPC to separate the coated magnetic beads from the solution. The supernatant was then discarded. The beads were washed with 2 mL of *buffer* C for 5 min. The supernatant was removed and the beads were re-suspended in 5 mL of *buffer* C. Equine urine sample (5 mL) was added to the coated magnetic beads. The mixture was incubated at 37 °C overnight with shaking. After incubation, the mixture was then placed in the MPC to remove the supernatant. The magnetic beads were washed four times with 2 mL washing buffer (1% (w/v) Igepal CA-630 in PBS (pH 7.4)) for 5 min at ambient temperature. After discarding the supernatant, 240 μL of elution buffer containing 0.01% PEG 6000 in PBS (pH 2.0) was added. The mixture was incubated at ambient temperature for 30 min with shaking. The supernatant was transferred to a micro-centrifuge vial using the MPC. The magnetic beads were washed three times with 1 mL of elution buffer for 5 min at ambient temperature and stored in 2 mL of *buffer* C at 4 °C for future use. Fifteen microlitres of Angiotensin II solution (1 mg/mL) and 60 μL of ACN were added to the supernatant. The mixture was then centrifuged at 13,000 × g for 5 min. The supernatant obtained was ready for LC/MS/MS analysis. Unless otherwise stated, all the apparatus used were plasticware.

2.4. Instrumentation

LC/MS/MS analyses were performed on an Applied Biosystems 4000 QTrap mass spectrometer (Applied Biosystems, Foster City, CA, USA) with a nanospray ionisation source interfaced with a Tempo nano multi-dimensional liquid chromatograph along with a Tempo nano-LC autosampler (Applied Biosystems).

2.5. LC conditions for the detection of insulins

The analyses of insulins were performed using a nano-LC/MS/MS system. A peptide cap-trap (P/N No. TR1/25109/32; Michrom Biosources, Auburn, CA, USA) and a reversed-phase wide-pore ZC-15-C18SBWX-150 column (15 cm L × 150 μm I.D., 3 μm, 300 Å; MicroTech, Vista, CA, USA) was used for the analyses. Both channel 1 (binary pump system) and 2 (nano-flow pump system) used the same mobile phase solvent. The mobile phase was composed of 0.1% formic acid in a mixture of H_2O:ACN (98:2, v/v) as solvent A and 0.1% formic acid in a mixture of H_2O:ACN (2:98, v/v) as solvent B. The injected sample extract in the sample loop was delivered to the peptide cap-trap at 20 μL/min using 100% of solvent A from channel 1 (binary pump system) and the solute was concentrated on the peptide cap-trap for 8 min. Following removal of salts and other matrix components, the targeted compounds enriched on the peptide cap-trap were transferred to the analytical column by switching the 10-port valve under gradient elution mode at a flow rate of 1 μL/min. The linear gradient was then run from channel 2 (nano-flow pump system), with 85% solvent A at the start

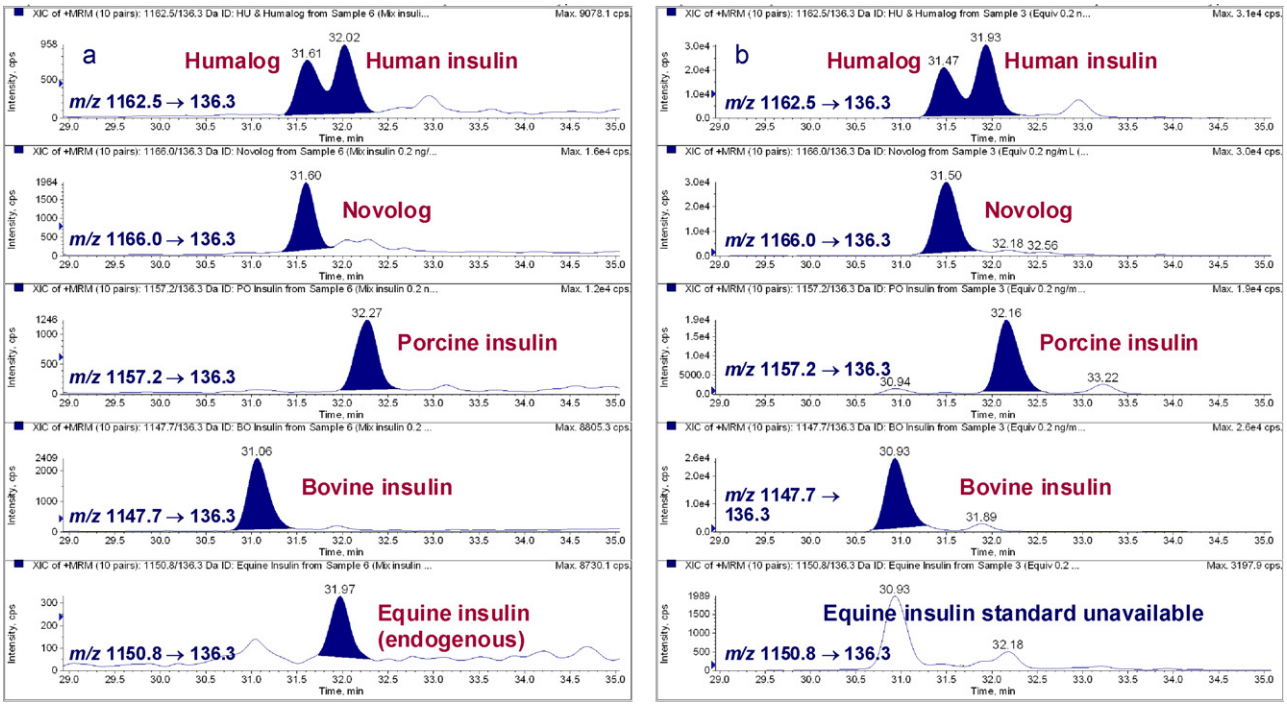

Fig. 2. Product-ion chromatograms of the targeted insulins obtained from the analysis of (a) a urine sample spiked with five exogenous insulins at 0.2 ng/mL each and (b) a mixed standard of exogenous insulins (18 pg each injected). A reference standard of equine insulin was unavailable for study.

(t = 0 min), held for 1 min, decreased to 65% solvent A at t = 44 min, and to 40% solvent A at t = 46 min and held there for 6 min (until t = 52 min). The gradient was then decreased to 0% solvent A at t = 55 min and held for 5 min (until t = 60 min). The gradient was returned to the initial mobile phase composition (85% solvent A) at t = 63 min, and stabilised until t = 65 min before starting the next injection. Injection volume was 100 μL each.

2.6. MS conditions for the detection of insulins

Detection of the five exogenous insulins together with the endogenous equine insulin was performed in positive nanospray ionisation using SRM mode in a single time segment. The non-coated nanospray PicoTip® emitter (20 μm tubing I.D.) used was from New Objective (Woburn, MA, USA). The SRM transitions, declustering potential, collision energies and the collision cell exit potential for each targeted insulin were optimised by infusion of the corresponding reference material into the 4000 QTrap. The dwell time for each transition was 150 ms and the collision energy was 129 eV for all targeted insulins. The selected precursor ions are listed in Table 1. The source was operated in positive nanospray mode with the nebuliser gas (ion source gas 1) set to 35 psi. The IonSpray voltage was set to 3600 V, the entrance potential for all targeted insulins was set at 10 V, declustering potential was set at 150 V, curtain gas was set to 20 psi, and nitrogen was used as collision gas and was set to high. The precursor ions in Q1 and the product ions in Q3 were isolated at low resolution to enhance the sensitivity. Data processing was performed using the Analyst (Version 1.4.1) software.

2.7. Microsomal incubation

For the *in vitro* metabolism study of insulins, a mixture of freshly prepared horse liver microsomes (30 μL), β-NAD (1.5 mM), glucose-6-phosphate (7.5 mM), magnesium chloride (4.4 mM), EDTA (1 mM) and glucose-6-phosphate dehydrogenase (1 U/mL) in phosphate buffer (pH 7.4, 5 mL) was incubated with each individual insulin at 37 °C overnight with shaking. The reaction was terminated by boiling at 100 °C for 10 min. The mixture was centrifuged at 3000 rpm for 10 min. The supernatant was extracted using the immunoaffinity purification procedures as described above for the urine samples. The residue was then analysed by LC/MS/MS. Control experiments in the absence of either (a) the insulin or (b) microsomes were performed in parallel.

2.8. Administration studies

A single dose of Humulin R (10 IU) was administered intramuscularly to each of two thoroughbred geldings. Blood samples were collected before administration, and 0.5, 1, 2, 4, 6, 8, 12, 24 h post administration. Urine samples were collected before administration, and as many samples as possible in the first two days post administration. The administration experiments had been approved in respect of the Animals (Control of Experiments) Ordinance by the Licensing Authority of the Department of Health, the Government of the Hong Kong Special Administrative Region.

3. Results and discussion

Blood has long been considered as the most suitable specimen for doping control testing of protein-based drugs, including insulins. Nevertheless, there were reports in detection of insulin and its analogues in human urine from athletes suffering diabetes [15,16,21] and endogenous equine insulin in horse urine [22]. Although no literature data so far is available proving the detection of exogenous insulins in human or animal urine samples after surreptitious administration, those studies implied the possibility of using urine samples (the most commonly used sample matrix) for doping control testing of insulins. This paper documents a complementary method for the simultaneous detection of bovine, porcine and human insulins, Humalog and Novolog, and their possible metabolites in equine urine.

Table 2
Detection levels, precision data (%RSD) and recovery of the targeted insulins in equine urine.

Insulin	Detection level in equine urine (pg/mL) [fmol/mL]	Urine spike at 0.5 ng/mL (%)[a]		Recovery (%) at 0.5 ng/mL
		Relative retention time (%RSD)	Peak area ratio (%RSD)	
Equine insulin	N/A[b]	N/A[b]	N/A[b]	N/A[b]
Human insulin	50 [8.6]	0.12	24.2	48
Bovine insulin	50 [8.7]	0.32	29.3	37
Porcine insulin	50 [8.7]	0.09	30.4	46
Humalog	50 [8.6]	0.14	31.0	39
Novolog	50 [8.6]	0.09	24.3	32

[a] The endogenous equine insulin in the urine blank sample was used as internal standard to estimate the %RSD on peak area ratio and relative retention time.
[b] Detection level, precision and recovery for equine insulin could not be estimated owing to the unavailability of the reference standard.

3.1. Method sensitivity and specificity

The amino acid sequences of the five targeted insulins as well as equine insulin are shown in Fig. 1 [22,24–26]. All five targeted insulins are different from equine insulin in no more than four amino acid residues. Differentiation among the insulins was achieved based on their differences in molecular weights and chromatographic retention times of the targeted ions. The precursor $[M+5H]^{5+}$ ions and the corresponding product ions chosen for the SRM experiments are shown in Table 1. Since the tyrosine immonium ion (m/z 136) was observed in the product-ion spectra of all five exogenous insulins as well as equine insulin, these transitions were used to screen for the presence of the six insulins. For the purpose of confirmation, at least three characteristic SRM transitions were used to provide unequivocal proof for the presence of a particular insulin in the sample.

Fig. 2a shows the selected product-ion chromatograms for the five exogenous insulins and endogenous equine insulin from a spiked horse urine sample, which is routinely run with each batch of unknown samples. The concentration of each exogenous insulin in the spiked sample was 0.2 ng/mL (equivalent to about 35 fmol/mL). For comparison purposes, the corresponding product-ion chromatograms from a mixed standard of the five exogenous insulins are shown in Fig. 2b. A standard for equine insulin was not available for this study and hence could not be included in the mix standard. However, its identity could be confirmed from the product-ion spectrum reported previously by other workers [22]. Human insulin and Humalog have identical molecular weight as they have the same amino acid composition with only proline and lysine residues at B_{28} and B_{29} positions switched (see Fig. 1). Thus they were monitored using the same transitions. Although there was only a slight difference between their retention times, their product-ion spectra showed significant differences, allowing for unequivocal identification. The characteristic product ions m/z 226 and m/z 217 for human insulin and Humalog respectively (see Table 1) were fragment ions from their corresponding C-termini of their B-chains [14,15].

An estimate of the method's limit of detection was performed by analysing insulins at different concentrations in the spiked urine samples. Fig. 3 shows the product-ion chromatograms of the five exogenous insulins obtained from a urine sample spiked at 0.05 ng/mL (equivalent to around 8.6 fmol/mL) each. At this level, good signal-to-noise ratio (S/N > 3, the general definition for limit of detection) could still be obtained for all five insulins, indicating that their limits of detection are well below 0.05 ng/mL. The detection level for equine insulin could not be determined owing to the lack of a reference material. The specificity of this LC/MS screening method was assessed by testing 10 different post-race horse urine samples. No interfering peak was observed at the expected retention times of the targeted insulins.

3.2. Method precision and recovery

The precision on peak area ratios and relative retention times of the five exogenous insulins were evaluated by replicate analyses ($n = 6$) of a spiked urine sample with each insulin at 0.5 ng/mL. The endogenous equine insulin in the urine blank sample was used as internal standard to estimate the %RSD on peak area ratio and relative retention time. The precision for peak area ratios were around 24–31%, and for the relative retention times were around 0.09–0.32%, see Table 2. These results indicate that the method has good enough precision to be used for qualitative identification. The recoveries of the five exogenous insulins were each determined by analysing different urine samples spiked with 0.5 ng/mL of each insulin and compared to analyses of corresponding blank extracts spiked with the same amount of insulins after sample extraction. The results are summarised in Table 2. Recoveries of 32–48% were obtained for the five exogenous insulins. Since only moderate recoveries were observed for five targeted insulins as compared to our reported method for plasma samples [13], larger

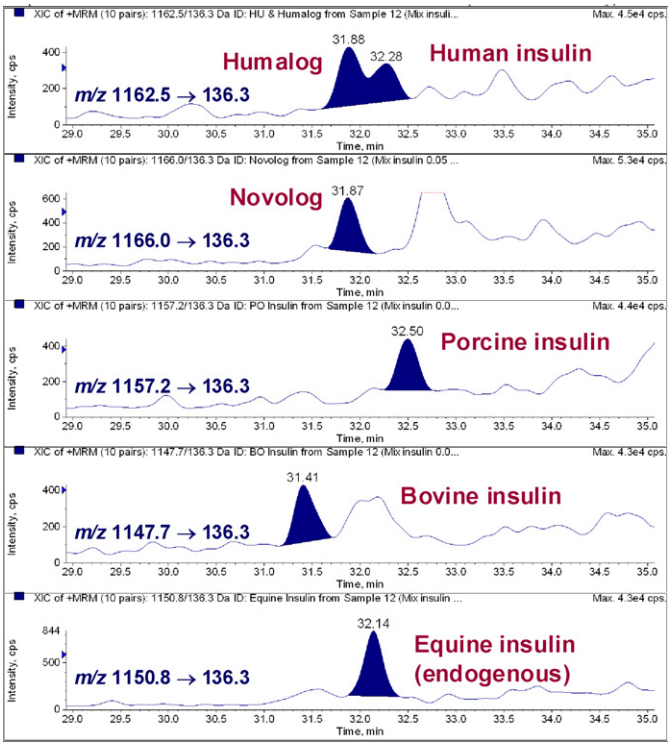

Fig. 3. Product-ion chromatograms of the targeted insulins obtained from the analysis of a urine sample spiked with five exogenous insulins at 0.05 ng/mL each.

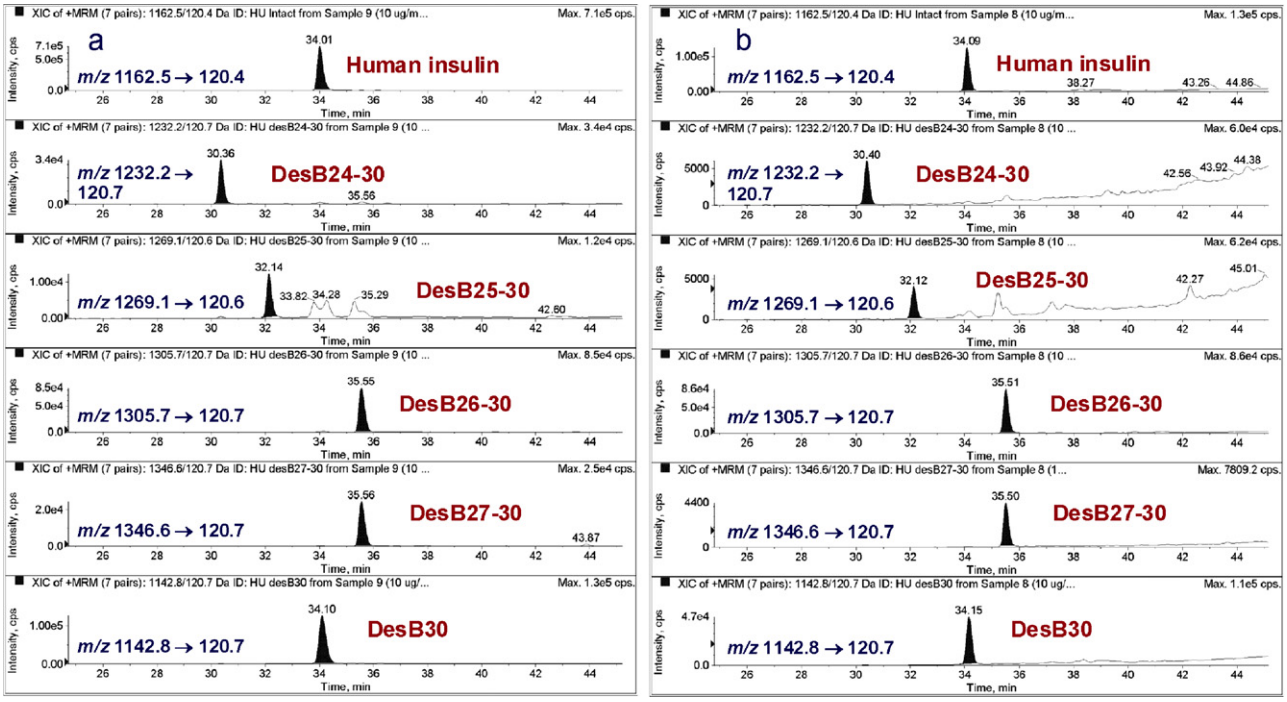

Fig. 4. Product-ion chromatograms of human insulin and its *in vitro* metabolites obtained from the analysis of (a) microsomal incubation mixture of human insulin (*without* immunoaffinity purification), and (b) microsomal incubation mixture of human insulin (*with* immunoaffinity purification).

sample volume and injection volume were used in order to achieve comparable detection sensitivity to plasma.

3.3. *In vitro* biotransformation studies

In order to identify the possible urinary metabolites of insulins in horses, each individual insulin was incubated with horse liver microsomes. The much cleaner extracts from the *in vitro* studies would facilitate the identification of metabolites. After overnight incubation, insulins and their metabolites were analysed by LC/MS/MS. Various *in vitro* metabolites were identified for the five targeted exogenous insulins. All *in vitro* metabolites consisted of structures with a truncated B-chain and intact disulfide bonds. For example, the five identified *in vitro* metabolites (DesB24-30, DesB25-30, DesB26-30, DesB27-30 and DesB30) of human insulin (Table 1) are formed by the losses of one, or from four and up to seven amino acids from the C-terminus of the B chains. Similar metabolites were identified for the other four targeted exogenous insulins (Table 1). All these metabolites have not yet been reported for horses, and only DesB24-30, DesB25-30 and DesB30 of human insulin have been reported in human.

As the truncated B-chain may affect the extraction efficiency of the anti-bodies, attempts were made to check whether these metabolites could be extracted by the described immunoaffinity purification protocol. Fig. 4a shows the product-ion chromatograms of the five metabolites identified from the microsomal incubation mixture of human insulin. After extraction using the immunoaffinity purification protocol for urine samples, all five metabolites could still be detected (Fig. 4b). Similar experiments were also performed for the microsomal incubation mixtures of the other targeted insulins. All the identified *in vitro* metabolites could be extracted by the anti-bodies. If these metabolites are indeed

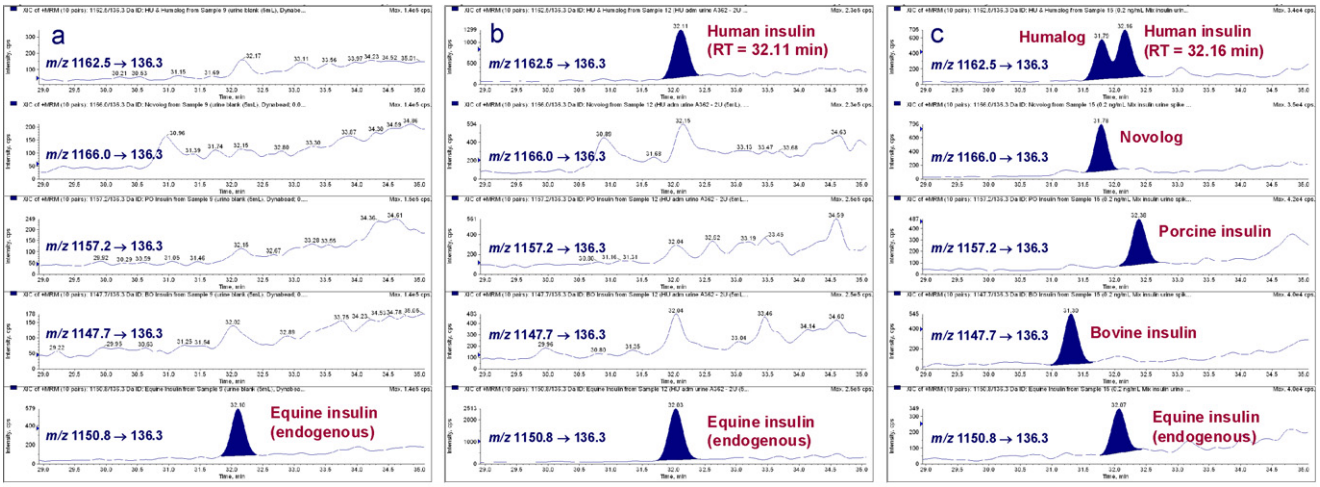

Fig. 5. Product-ion chromatograms of the targeted insulins obtained from the analysis of (a) a blank urine sample, (b) a post-administration urine sample collected from a horse 4 h after administration with a single dose of 10 IU of human insulin, and (c) a spiked urine sample with the five exogenous insulins at 0.2 ng/mL each.

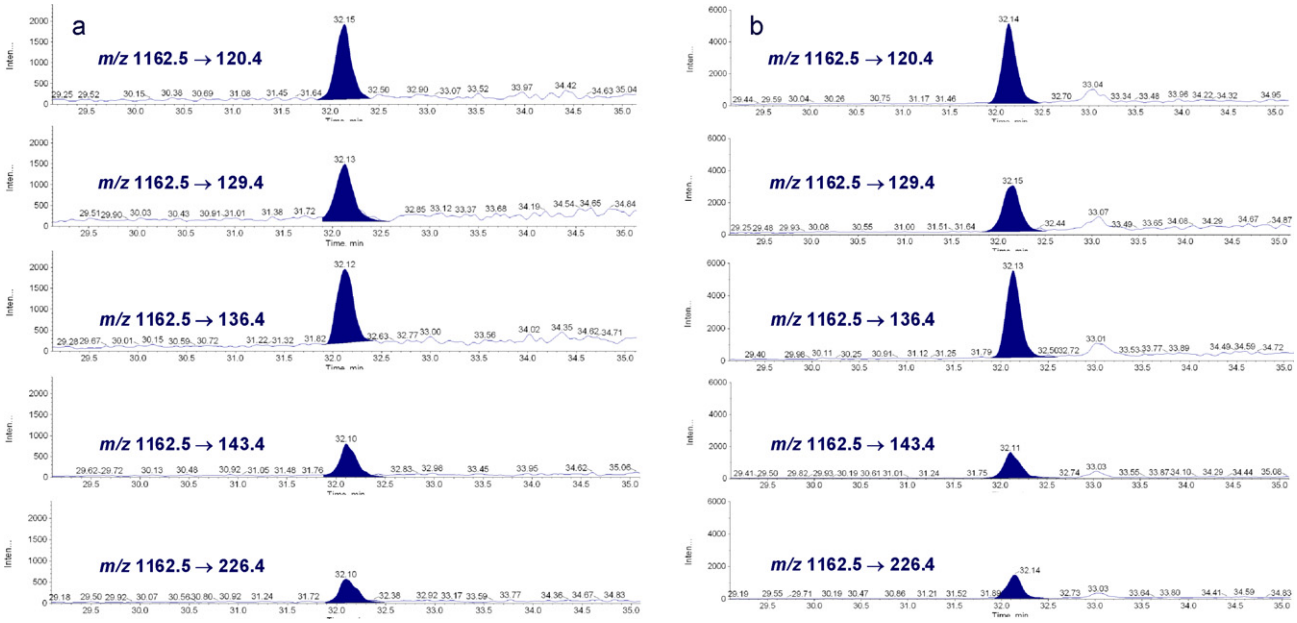

Fig. 6. Product-ion chromatograms of human insulin obtained from the analysis of (a) a post-administration urine sample collected from a horse 4 h after administration with a single dose of 10 IU of human insulin, (b) a spiked urine sample with human insulin at 0.2 ng/mL.

present in the urine samples, they should be able to be detected by the method.

3.4. Application of the method to administration samples

In order to demonstrate that the screening method is effective in detecting the use of insulins in horses, blood and urine samples collected from two horses having been administered a single low-dose of human insulin (10 IU per horse) were analysed. The results from the analysis of post-administration blood samples were reported elsewhere [13]. Human insulin in a short-acting formulation was used in these experiments, as this was more likely to be used in doping. The 10 IU applied was a common dosage for the abuse of insulin in human athletes. Since the body weight of a mature horse is about 7 times that of human (500 kg vs. 70 kg), the amount administered was in fact a very low dose for a horse. The LC/MS/MS results for a urine sample collected 4 h after administration is shown in Fig. 5b. Human insulin was clearly detected with a good signal-to-noise ratio and a well-matched retention time to that from the corresponding spiked sample at 0.2 ng/mL (Fig. 5c). The product-ion chromatogram from a blank urine processed in parallel with the other samples is shown in Fig. 5a, showing that only endogenous equine insulin was detected in the blank sample.

As mentioned above, the confirmation for the presence of suspected exogenous insulins that are detected by the screening method could be achieved by monitoring at least three characteristic SRM transitions of the analyte in question. Fig. 6a shows the confirmation results from the same 4 h post-administration urine sample. Five transitions characteristic of human insulin were used. All product-ion chromatograms were normalised to the most abundant product-ion at *m*/*z* 120.4. Both the retention times and relative ion ratios of the product-ions obtained from the post-administration sample matched well with those from the human insulin spiked urine sample (Fig. 6b). Our in-house match criteria for the confirmation of macromolecules [27] were established based on our experience of analysing macromolecules and are identical to the LC and full-scan MS/MS criteria published by the Association of Official Racing Chemists [28]. In our studies, human insulin could be detected for up to 4 h in the post-administration urine samples. However, none of the identified *in vitro* metabolites of human insulin was detected in the post-administration urine samples which is in contrast to the reported studies [15,16,21,22]. One of the possible reasons is that the reported insulin metabolites were detected in human urine from athletes suffering diabetes [15,16,21] and endogenous equine insulin in horse urine [22]. Parent insulins should have been present in the human or horse bodies for a prolonged period and in a relatively higher concentration. As only a single low-dose of human insulin was given to each horse in our study, the levels of insulin metabolites, if they were present in the post-administration samples, may not be sufficiently high to allow them to be detected by our method. Indeed, pharmacokinetics (i.e. absorption, distribution, metabolism and elimination) are dependent on many variables, including species (human vs. equine), form of substance given (insulin is available in many formulations, including different analogues in both long and short-acting ones), the particular dose, route and frequency of administration and the individual variability of the species. For the purpose of glycogen loading or muscle building, a larger dose of insulin (>10 IU) would normally be used on a horse, which should result in a longer detection time and/or detection of metabolites.

4. Conclusion

Pharmaceutical preparations of equine insulin are currently unavailable and unlikely to be available in the near future as the prevalence of equine diabetes mellitus is low [29,30]. In contrast to human sports testing, any abuse of insulins on horses will be based on insulins from other species and their analogues. In this paper, an immunoaffinity purification – LC/MS/MS method was presented for the identification of bovine, porcine and human insulins and the synthetic human insulin analogues Humalog and Novolog in equine urine. The method is also capable in detecting potential urinary insulin metabolites identified in the *in vitro* studies. The identification of the exogenous insulins and their potential metabolites together with endogenous equine insulin were based on retention times and SRM transitions. The detection of the five exogenous insulins could be achieved at less than 0.05 ng/mL (equivalent to less than 9 fmol/mL). The method gave adequate recoveries, precision and no interference from the urine matrix. Human insulin was successfully identified in urine samples collected from two horses

after a single low-dose administration of a short-acting formulation of human insulin (10 IU per horse). The presence of human insulin could be detected and confirmed in urine collected up to 4 h post administration, however, none of the identified *in vitro* metabolites was detected. To our knowledge, this is the first report on the identification of exogenous insulin in equine urine samples collected from horses after insulin administration. It is a direct proof of the applicability of urine for doping control testing of insulins in horses.

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