

# Detection of *myo*-inositol trispyrophosphate in equine urine and plasma by hydrophilic interaction chromatography-tandem mass spectrometry

April S.Y. Wong,\* Emmie N. M. Ho and Terence S. M. Wan\*

*Myo*-inositol trispyrophosphate (ITPP) is a new drug capable of increasing the amount of oxygen in hypoxic tissues. Studies have shown that administration of ITPP increases the maximal exercise capacity in normal mice as well as mice with severe heart failure. The properties of ITPP make it an ideal candidate as a doping agent to enhance performance in racehorses. While there have been speculations in the horseracing industry that the covert use of ITPP is already widespread, no reported method exists for the detection of ITPP in equine biological samples. ITPP is a difficult-to-detect drug due to its hydrophilic nature; the complexity of equine biological matrices also adds to the problem. This paper describes for the first time a method for the detection and confirmation of ITPP in equine urine and plasma. ITPP was isolated from the sample matrices by solid-phase extraction and the extract was analyzed by hydrophilic interaction chromatography-tandem mass spectrometry. ITPP could be detected at low ppb levels in both fortified equine plasma and urine with good precision, fast instrumental turnaround time, and negligible matrix interferences. To our knowledge, this is the first report of a validated method for the detection and unequivocal confirmation of low levels of ITPP in any biological fluid. Copyright © 2012 John Wiley & Sons, Ltd.

**Keywords:** *Myo*-inositol trispyrophosphate (ITPP); horse; plasma; urine; hydrophilic interaction chromatography (HILIC); mass spectrometry

## Introduction

Red blood cells (erythrocytes) transport and deliver oxygen to body tissues by means of the iron-containing oxygen-transport protein haemoglobin in cytoplasm. The binding of oxygen to haemoglobin and its release to body tissues are mainly governed by the allosteric properties of haemoglobin.<sup>[1]</sup> Oxygen delivery is regulated by the action of the endogenous allosteric effector 2,3-bisphosphoglycerate. When the latter binds to haemoglobin, the oxygen-binding affinity of haemoglobin decreases, allowing the release of oxygen to the tissues. Numerous illnesses (such as Alzheimer's disease, cardiovascular disease, and cancer) involve hypoxia, the reduced availability of oxygen.<sup>[2,3]</sup> For this reason, the discovery and synthesis of allosteric effectors of haemoglobin have significant therapeutic value. *Myo*-inositol trispyrophosphate (ITPP) was recently discovered to have the ability to penetrate red blood cells to modulate their oxygen supply ability,<sup>[4,5]</sup> and was characterized as a candidate as an anti-angiogenic and anti-cancer agent to counteract the effects of hypoxia.<sup>[6,7]</sup> ITPP has been shown to enhance maximal exercise capacity in normal mice as well as mice with severe heart failure.<sup>[8,9]</sup> As such, it is a potential doping agent in sports, and there are speculations that it is already being abused in the horseracing industry.<sup>[10–12]</sup>

The development of an analytical method for the screening of low levels of ITPP in complex biological matrices is challenging, due to its intrinsic properties. ITPP is very hydrophilic and ionizes extensively in aqueous medium, thus making liquid/liquid extraction ineffective. In addition, it is poorly retained on reversed phase chromatographic columns. Duarte *et al.* reported the quantification

of ITPP in human red blood cells by high performance ion-chromatography with a detection limit of  $< 0.001 \times 10^{-3} \text{ mol/L}^{-1}$  (or around 0.6  $\mu\text{g/ml}$ ), but detailed analysis procedures and method validation have not been described.<sup>[4]</sup> Apart from this recent publication, no other analytical method has been reported for the detection of ITPP. This paper describes a liquid chromatography-mass spectrometry method which is capable of screening and confirming the presence of ITPP in equine urine and plasma at low ppb levels. As the documented dose of ITPP to produce an effect is rather high (0.5 to 3 g/kg in mice<sup>[8]</sup>), the method's sensitivity should be adequate for monitoring its possible abuse in racehorses. To the best of our knowledge, this is the first report of a validated method for identifying ITPP in any biological matrix.

## Experimental

### Materials

*Myo*-inositol trispyrophosphate was obtained from Frontier Biopharm (Richmond, KY, USA). Acetic acid (100 %, Suprapur),

\* Correspondence to: Dr April S. Y. Wong, Racing Laboratory, The Hong Kong Jockey Club, Sha Tin Racecourse, Sha Tin, N.T., Hong Kong, China. E-mail: april.sy.wong-rl@hkjc.org.hk

Dr Terence S. M. Wan, Racing Laboratory, The Hong Kong Jockey Club, Sha Tin Racecourse, Sha Tin, N.T., Hong Kong, China. E-mail: terence.sm.wan@hkjc.org.hk

Racing Laboratory, The Hong Kong Jockey Club, Sha Tin Racecourse, Sha Tin, NT, Hong Kong, China

ammonia solution (25%) and formic acid (> 98 %, RDH) were purchased from Merck (Darmstadt, Germany). Methanol (AR grade) and anhydrous sodium acetate (AR grade) were obtained from Sigma-Aldrich (St Louis, MO, USA). Oasis<sup>®</sup> HLB cartridge (60 mg, 3 ml) and Oasis<sup>®</sup> WAX cartridge (60 mg, 3 ml) were purchased from Waters Corporation (Milford, Massachusetts, USA). Deionized water was generated from an in-house water purification system (Milli-Q, Molsheim, France). Sodium acetate buffer (1 M, pH 4) was prepared by dissolving 41 g of anhydrous sodium acetate in 300 ml deionized water, adjusted to pH 4 with hydrochloric acid, and the solution was made up to 500 ml with deionized water.

## Sample preparation and extraction procedures

Urine and blood samples were centrifuged at 3000 rpm for 10 min. The plasma fraction or urine (0.5 ml) was diluted to 3 ml with deionized water. Sodium acetate (1 M, 0.4 ml) was added and the mixture was adjusted to pH 4 with hydrochloric acid and filtered through an Oasis<sup>®</sup> HLB cartridge that had been pre-conditioned with methanol (3 ml) and deionized water (3 ml). Eluent was not used, and the filtrate was loaded onto an Oasis<sup>®</sup> WAX cartridge that had been pre-conditioned with methanol (2 ml) and formic acid (0.0005% in deionized water, pH 4; 2 ml). The cartridge was then washed with formic acid (0.0005% in deionized water, pH 4; 2 ml) and methanol (2 ml), and eluted with ammonia solution (10% in methanol; 3 ml). The eluate was evaporated to dryness under nitrogen at 60 °C, reconstituted with a mixture of ammonium acetate in deionized water (25 mM), methanol, and acetonitrile (10/45/45; v/v/v; 50 µl), and transferred to a conical insert in a Chrompack autosampler vial for liquid chromatography-mass spectrometry (LC-MS) analysis.

## Instrumentation

Solid-phase extraction (SPE) was carried out using a RapidTrace<sup>®</sup> SPE workstation (Zymark Corporation, Hopkinton, MA, USA). LC-MS analyses were performed on an Applied Biosystems 4000 QTrap mass spectrometer (Applied Biosystems, Foster City, CA, USA) equipped with an Agilent 1100 series high performance liquid chromatography (HPLC) system consisting of a binary gradient pump (Agilent Technologies, Palo Alto, CA, USA).

## LC conditions

A hydrophilic interaction chromatography column Atlantis<sup>®</sup> HILIC Silica (5 cm x 2.1 mm ID; 3 µm particle size, Waters) was used. The mobile phase was composed of ammonium acetate in deionized water (25 mM, pH 6.7) as solvent A and acetonitrile as solvent B. A linear gradient was run at a flow rate of 400 µl/min, with 0 % of

solvent A initially (t=0 min), increased to 100 % of solvent A from t=1 min to t=5 min, and held for 1 min (until t=6 min). The gradient was then returned to 0 % of solvent A from t=6 min to t=6.1 min, and stabilized until t=10 min before the next injection. The overall run time for each analysis was 10 min, and the injection volume was 10 µl.

## MS conditions

Detection of the ITPP was performed using negative electrospray ionization in the SRM mode. The SRM transitions, collision energies, and other transition-dependent parameters for ITPP were optimized to yield the best sensitivity automatically with the instrument software by infusing ITPP reference standard into the 4000 QTrap. The dwell time for each transition was 50 msec with a 5 msec pause time. The source was at 300 °C, with the nebulizer gas and heater gas set at 50 and 20 psi, respectively. Ionspray voltage was -4500 V, curtain gas at 10 psi, and collision gas was set to medium. The resolution for selecting respectively precursor ions in Q1 and product ions in Q3 was both set to low and unit mass. Data processing was performed using the Analyst (version 1.5) software.

## Estimation of ITPP concentration

For each batch of urine or plasma samples, a calibrator containing ITPP spiked in negative equine matrix was processed in parallel. The spiked concentration of ITPP in the calibrator was five times higher than the quality control (QC) level as shown in Table 1. A one-point calibration graph was prepared using the Analyst (version 1.5) software. The estimated concentration of ITPP in the urine or plasma samples was calculated automatically by the software.

## Results and discussion

### Hydrophilic interaction chromatography

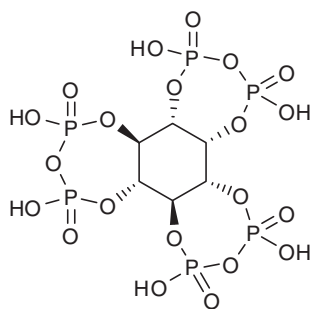
ITPP (Figure 1) is a hydrophilic compound that is poorly retained on reversed phase LC columns. During the initial stage of method development, it was observed that ITPP eluted with the solvent front on reversed phase columns even in the presence of a high percentage of aqueous mobile phase. Duarte *et al.* reported a quantification method for ITPP by ion-chromatography.<sup>[4]</sup> However, ion-chromatography is not preferred for coupling with mass spectrometry (MS), as ion-pairing agents could cause ion suppression leading to low and variable MS signals. In addition, ion-pairing agents are often non-volatile acids that could contaminate the ion source. A mode of liquid chromatography

**Table 1.** Method validation results for ITPP spiked in equine plasma and urine.

Sample Matrix	Estimated Limit of Detection (ng/ml)	QC Level (ng/ml)	Recovery* (%)	Intra-day Precision*		Inter-day Precision*	
				(% RSD; n = 6)		(% RSD; n = 6 + 6 + 6)	
				PA <sup>#</sup>	RT	PA <sup>#</sup>	RT
Equine plasma	< 2	50	75	10	0.08	16	0.19
Equine urine	< 5	50	55	16	0.21	19	0.23

\* Based on the SRM transition  $m/z$  301.9<sup>-</sup>  $m/z$  445

<sup>#</sup> PA = Peak Area



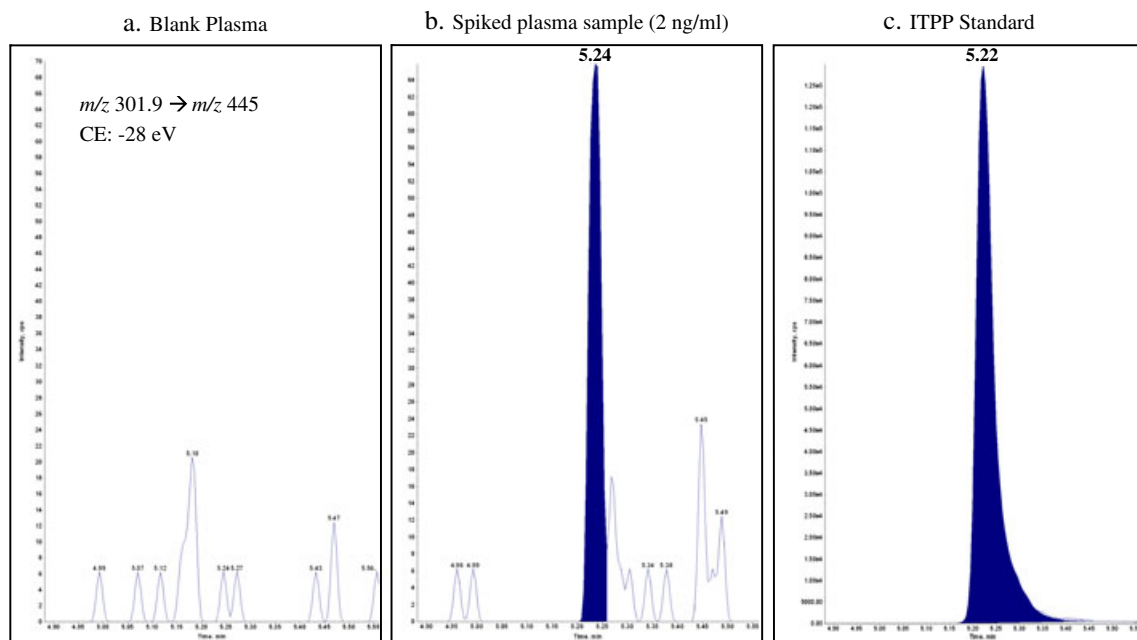
**Figure 1.** The chemical structure of *myo*-inositol trispyrophosphate (ITPP, *cis*-1,2,3,5-*trans*-4,6-cyclohexanehexol 1,6:2,3:4,5-tris(P,P'-dihydrogen diphosphate).

has gained immense interest recently and proved to be promising for the separation of polar analytes.<sup>[13,14]</sup> Albert *et al.* introduced the term HILIC (hydrophilic interaction chromatography) as a suitable acronym for this variant mode of normal phase chromatography.<sup>[15]</sup> The elution order of solutes in hydrophilic columns is in increasing order of their polarity. The retention mechanism of HILIC is yet to be fully established, but it is well-accepted that the complex retention process in a HILIC column is governed by partitioning of the polar analyte between the mobile phase and the water-enriched layer at the surface of the stationary phase through hydrogen bonding, dipole/dipole interactions and electrostatic forces.<sup>[16]</sup> In our studies, chromatographic conditions were optimized to attain adequate retention and favourable peak shape on the HILIC column. The predominant factor affecting retention in HILIC is the type and amount of organic modifier in the mobile phase.<sup>[17]</sup> Acetonitrile and methanol were tested as possible organic modifiers. Acetonitrile was preferred over methanol due to better retention, likely attributed to its lower polarity. The type and concentration of buffer salt in the mobile phase would also contribute to retention and selectivity. It was observed that sharper and

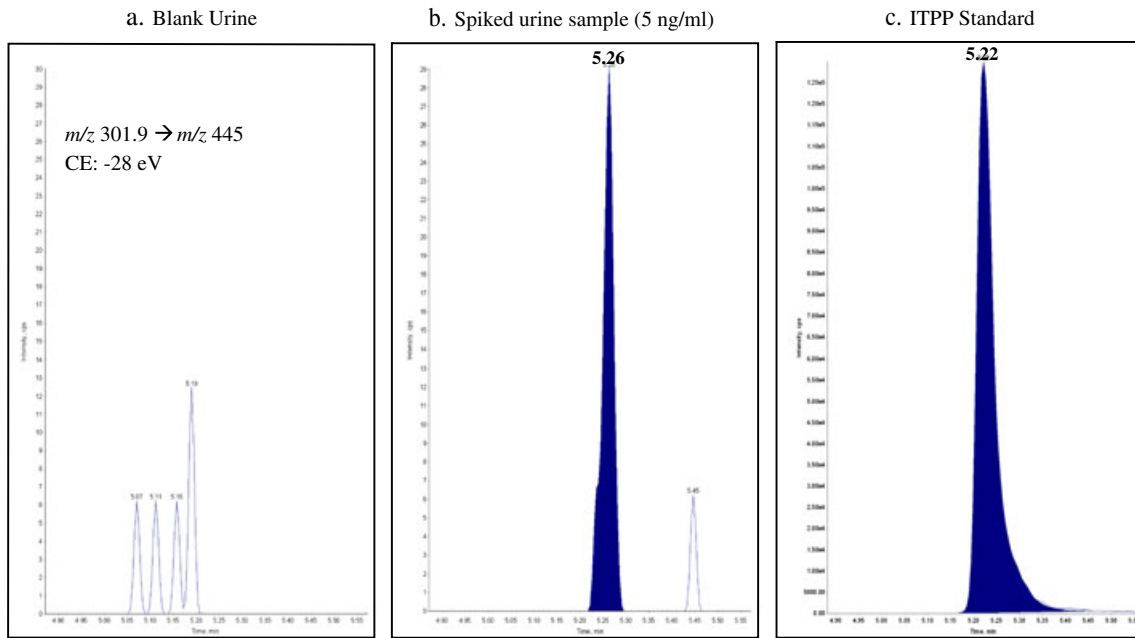
more symmetrical peaks with more reproducible retention times could be obtained by increasing the concentration of volatile ammonium acetate buffer from 5 mM to 25 mM. Wang *et al.* suggested that the increased salt concentration would promote the formation of ion-pairs for the charged solutes, which had better solubility in the mobile phase, resulting in slightly shorter retention times and better peak shapes.<sup>[18]</sup> Similar to reversed-phase chromatography, the sample reconstitution solvent can strongly influence solubility and peak shape. Ideally, the composition of the sample reconstitution solvent should be as close to the initial mobile phase as possible to avoid deterioration in chromatography; however, due to the low solubility of ITPP in organic solvent, a slightly polar solvent was used to allow effective reconstitution. Our studies have shown that a mixture of 25 mM ammonium acetate in deionized water (pH 6.7), methanol and acetonitrile (10/45/45, v/v/v) would provide the best response and peak shape for ITPP.

### Method sensitivity and specificity

The method's limit of detection (LoD) was estimated by analyzing ITPP in spiked plasma and urine samples at different concentrations. The estimated LoD represents the lowest spiked concentration amongst those evaluated that gave a signal-to-noise ratio of greater than 3 in the selected product-ion chromatogram. As shown in Figures 2 and 3, ITPP could be detected at less than 2 ng/ml in equine plasma and less than 5 ng/ml in equine urine. The precursor ion  $m/z$  301.9 was the doubly-charged deprotonated ITPP. Both singly- and doubly-charged deprotonated ITPP, corresponding to  $m/z$  604.9 and  $m/z$  301.9, were detected in the full-scan mass spectrum of ITPP. The doubly-charged deprotonated ITPP was chosen as the precursor ion as it was more abundant. Figure 4 shows the mass spectrum of the product ion scan of  $m/z$  301.9, showing product ions  $m/z$  159,  $m/z$  239,  $m/z$  329,  $m/z$  445, and  $m/z$  525. The product ions were mainly attributed to cleavage of various phosphate moieties from ITPP. For example,  $m/z$  159 and  $m/z$  239 should be pyrophosphate



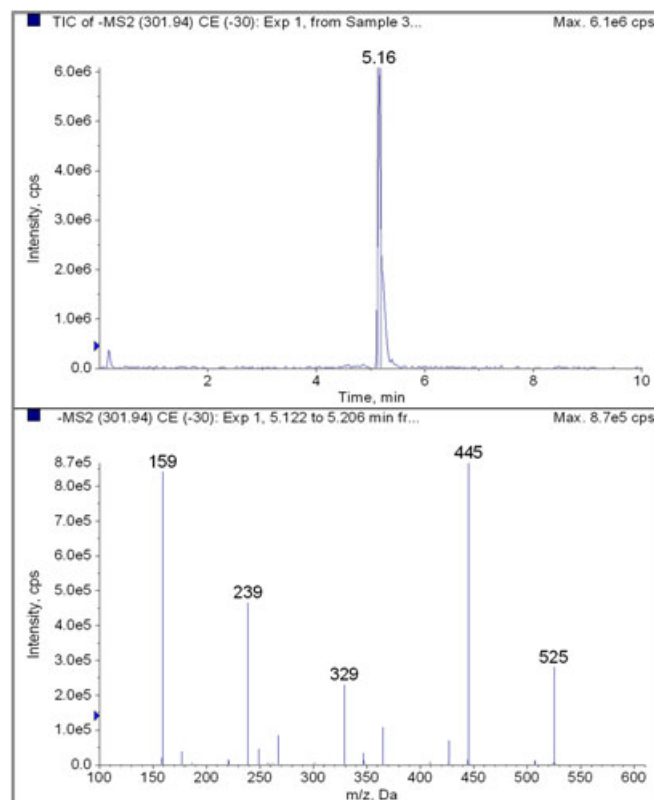
**Figure 2.** Estimated limit of detection in equine plasma: product ion chromatograms of ITPP obtained from the analysis of (a) blank plasma sample, and (b) plasma sample spiked with ITPP at 2 ng/ml, and (c) ITPP standard.



**Figure 3.** Estimated limit of detection in equine urine: product ion chromatograms of ITPP obtained from the analysis of (a) blank urine sample, and (b) urine sample spiked with ITPP at 5 ng/ml, and (c) ITPP standard.

ion ( $\text{HP}_2\text{O}_6^-$ ) and cyclic trimetaphosphate ion ( $\text{H}_2\text{P}_3\text{O}_6^-$ ) respectively;  $m/z$  525 and  $m/z$  445 would result from a loss of metaphosphate ion ( $m/z$  79;  $\text{PO}_3^-$ ) and pyrophosphate ion ( $m/z$  159;  $\text{HP}_2\text{O}_6^-$ ) from the doubly-charged deprotonated ITPP respectively. An unequivocal confirmation of the presence of ITPP in equine plasma and urine

was achieved by monitoring five SRM transitions with  $m/z$  301.9 as the precursor ion. This exceeded the three SRM transitions required in the *AORC Guidelines for the Minimum Criteria for Identification by Chromatography and Mass Spectrometry* published by the Association of Official Racing Chemists.<sup>[21]</sup> ITPP could be confirmed



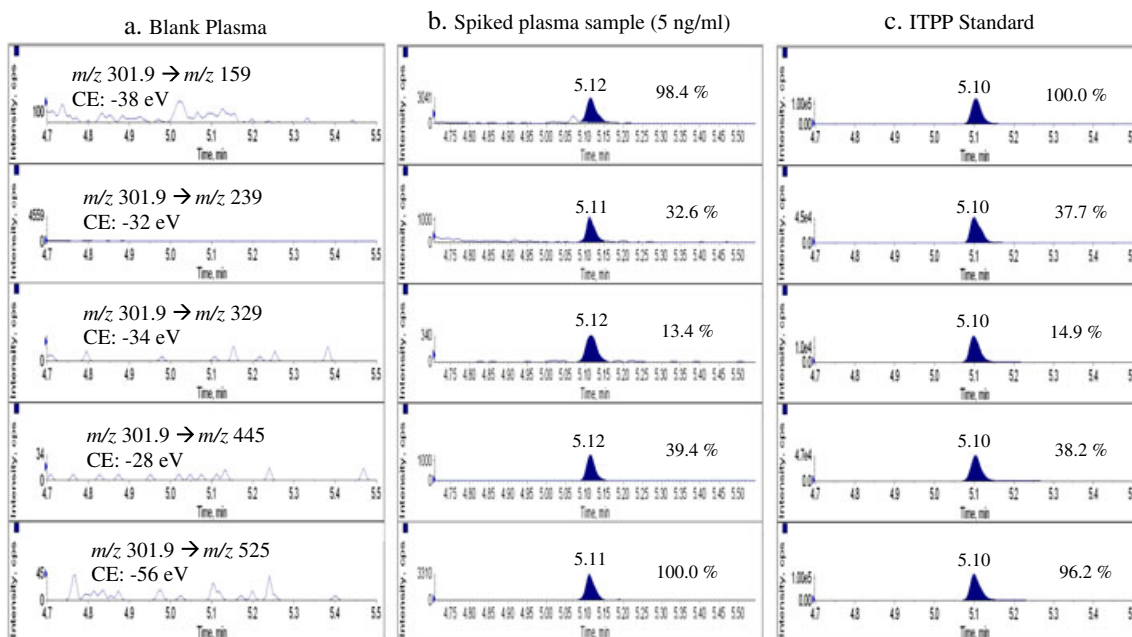
**Figure 4.** Product ion scan mass spectrum of  $m/z$  301.9, the doubly-charged deprotonated ITPP.

reliably at 5 ng/ml in plasma (Figure 5) and 10 ng/ml in urine (Figure 6). Both the retention times and relative abundances of the five transitions in the spiked samples matched well with those of the authentic standard within the limits stipulated in the above criteria.

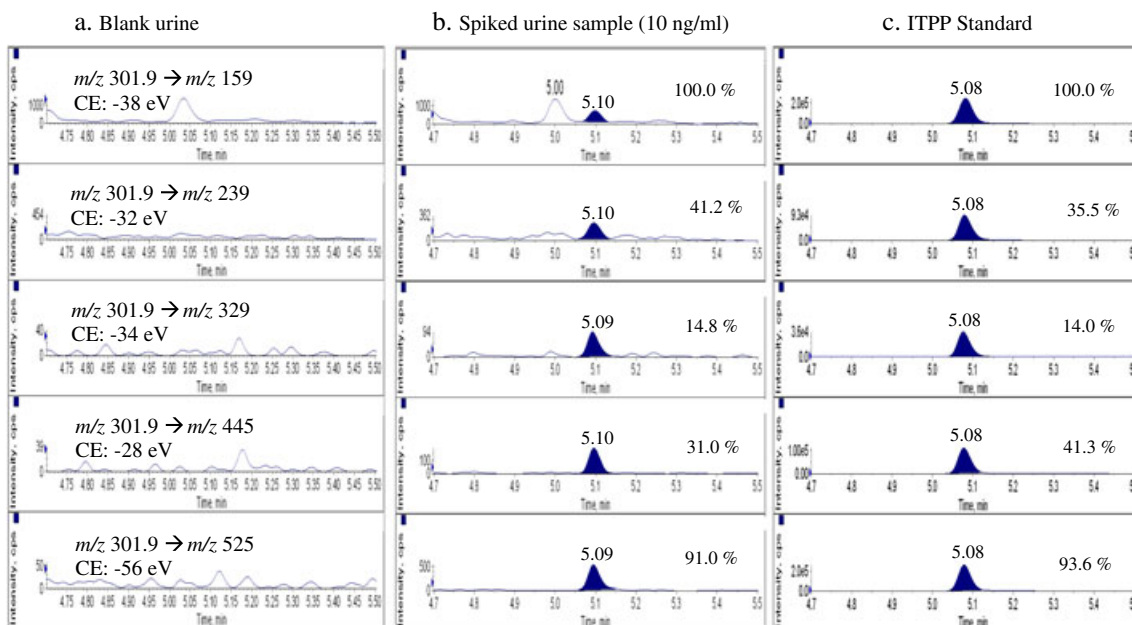
The method specificity was assessed by analyzing different equine urine ( $n = 25$ ) and plasma ( $n = 25$ ) samples. No significant interference from the blank matrices was observed at the expected retention times of the target transitions.

### Method precision

The precision of the method, based on peak area (PA) and retention time (RT) of ITPP, was evaluated by replicate analyses of a spiked urine sample or a spiked plasma sample at their respective QC levels on three different days. The results (Table 1) showed this method had reasonably good precision for PA (RSD < 20 %) and RT (RSD < 0.25 %) to be used regularly for the qualitative identification of ITPP in equine plasma and urine.



**Figure 5.** Confirmation of ITPP in equine plasma: product ion chromatograms of ITPP obtained from the analysis of (a) blank plasma sample, and (b) plasma sample spiked with ITPP at 5 ng/ml, and (c) ITPP standard.



**Figure 6.** Confirmation of ITPP in equine urine: product ion chromatograms of ITPP obtained from the analysis of (a) blank urine sample, and (b) urine sample spiked with ITPP at 10 ng/ml, and (c) ITPP standard.

Internal standards are often used in qualitative and quantitative analyses to compensate for variations in extraction recovery, sample manipulation loss, ionization efficiency, detector response, etc. A suitable internal standard should be one with similar physical and chemical properties to the analyte. The use of an appropriate internal standard would often improve the method performance. However, using an improper internal standard can often lead to major variations (easily up to as much as 500%) in the quantity measured. For the present investigation of mass spectral analysis of ITPP, since a stable-isotope labelled ITPP was not available commercially, other non-labelled internal standards have been evaluated. Less than satisfactory results were obtained with different bisphosphonates as internal standard, including alendronic acid, clodronic acid, ibandronic acid, residronic acid, and tiludronic acid. These bisphosphonates would elute as broad and tailing peaks under a range of chromatographic conditions that provided adequate retention and good peak shape for ITPP on the HILIC column. Since the primary purpose of this method was qualitative identification rather than quantitative determination of ITPP, and the method's precision without using an internal standard was already demonstrated to be satisfactory, a suitable internal standard was not pursued further. Nevertheless, the use of a stable-isotope labelled ITPP as internal standard will be examined and adopted whenever such material would become available.

### Extraction recovery

The extraction of hydrophilic substances such as ITPP from complex biological matrices was expected to be difficult. The SPE protocol used in the present study was originally developed by the authors' laboratory for the extraction of bisphosphonates – bone resorption inhibitors used for the management of skeletal disorder in humans as well as in horses.<sup>[19,20]</sup> As ITPP showed some similarity in its chemical structure with bisphosphonates, the same SPE protocol was used on ITPP. The extraction recovery of ITPP was investigated by analyzing equine plasma or urine samples in duplicate, which had been spiked with ITPP at 100 ng/ml either before or after sample extraction. The peak area ratios of ITPP obtained from samples spiked before extraction were compared with those obtained from blank extracts spiked with the same amount of ITPP after extraction. Adequate recoveries of ITPP, at 75% in equine plasma and 55% in equine urine, were observed.

### Method applicability

An ITPP administration study has yet to be performed in horses to determine its pharmacokinetic profile. As the very polar ITPP would unlikely undergo extensive metabolism before elimination, and the effective dose of ITPP which could cause an increase in maximal exercise capacity should be rather high (0.5 to 3 g/kg in mice),<sup>[8]</sup> the present method should have adequate sensitivity for monitoring the abuse of ITPP in racehorses.

### Conclusion

An LC-MS method involving simple SPE extraction has been developed and validated, for the first time, for the screening and unequivocal confirmation of ITPP in equine plasma and urine. ITPP is a hydrophilic substance that is difficult to extract from biological matrices and is poorly retained on reversed phase columns. The authors have overcome these problems by employing a mixed-mode weak anion exchange SPE, followed by hydrophilic

interaction chromatography – tandem mass spectrometry analysis. The developed method showed a fast instrumental turnaround time, adequate sensitivity, good precision and recovery, and negligible interference from sample matrices, which could be adopted as a regular qualitative screening and confirmation method for controlling the abuse of ITPP in equine sports.

### Acknowledgement

The technical assistance of Mr Stephen Cheung is gratefully acknowledged.

### References

- [1] F.B. Jensen. The dual roles of red blood cells in tissue oxygen delivery: oxygen carriers and regulators of local blood flow. *J. Exp. Biol.* **2009**, *212*, 3387.
- [2] R.S. Schwatz, H.K. Eltzching, P. Carmeliet. Hypoxia and Inflammation. *New. Engl. J. Med.* **2011**, *364*, 656.
- [3] M. Gassman. Hypoxia, from integrative biology to human disease. *High Alt. Med. Biol.* **2008**, *9*, 100.
- [4] C.D. Duarte, R. Greferath, C. Nicolau, J.M. Lehn. Myo-inositol trispyrophosphate: A novel allosteric effector of haemoglobin with high permeation selectivity across the red blood cell plasma membrane. *Chem. BioChem.* **2010**, *11*, 2543.
- [5] K.C. Fylaktakidou, J.M. Lehn, R. Greferath, C. Nicolau. Inositol tripyrophosphate: A new membrane permeant allosteric effector of haemoglobin. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 1605.
- [6] C. Kieda, R. Greferath, C.C. Da Silva, K.C. Fylaktakidou, J.M. Lehn, C. Nicolau. Suppression of hypoxia-induced HIF-1 $\alpha$  and of angiogenesis in endothelial cells by myo-inositol trispyrophosphate-treated erythrocytes. *P. Natl Acad. Sci. USA* **2006**, *103*, 15576.
- [7] G. Sihn, T. Walter, J.-C. Klein, I. Queguiner, H. Iwao, C. Nicolau, J.-M. Lehn, P. Corvol, J.-M. Gasc. Anti-angiogenic properties of myo-inositol trispyrophosphate in ovo and growth reduction of implanted glioma. *FEBS Lett.* **2007**, *581*, 962.
- [8] T. Watanabe, T. Takeda, S. Omiya, et al. Reduction in haemoglobin-oxygen affinity results in the improvement of exercise capacity in mice with chronic heart failure. *J. Am. Coll. Cardiol.* **2008**, *52*, 779.
- [9] A. Biolo, R. Grefereath, D.A. Siwik, et al. Enhanced exercise capacity in mice with severe heart failure treated with an allosteric effector of haemoglobin, myo-inositol trispyrophosphate. *P. Natl Acad. Sci. USA* **2009**, *106*, 1926.
- [10] ITPP – What is it? *Horseprerace*. 26 Nov **2010**. Available at: <http://www.horseprerace.com/blog/itpp-what-is-it/> [August 2011]
- [11] Doctors Discuss Undetectable Drugs. *Standardbred Canada*. 4 Nov **2010**. Available at: <http://standardbredcanada.ca/news/11-4-10/doctors-discuss-undetectable-drugs.html> [August 2011]
- [12] A. Cohen. New drugs are here – what can be done? *Harnesslink*. 17 Sept **2010**. Available at: <http://www.harnesslink.com/www.Article.cgi?ID=83740> [August 2011]
- [13] A.J. Albert. HILIC at 21: Reflections and perspectives. *J. Chromatogr. A* **2011**, *1218*, 5879.
- [14] N. Fontanals, R.M. Marce, F. Borrull. On-line solid-phase extraction coupled to hydrophilic interaction chromatography-mass spectrometry for the determination of polar drugs. *J. Chromatogr. A* **2011**, *1218*, 5975.
- [15] A.J. Albert. Hydrophilic-interaction chromatography for the separation of peptides, nucleic acids and other polar compounds. *J. Chromatogr.* **1990**, *499*, 177.
- [16] D.V. McCalley. Study of the selectivity, retention mechanisms and performance of alternative silica-based stationary phases for separation of ionized solutes in hydrophilic interaction chromatography. *J. Chromatogr. A* **2010**, *1217*, 3408.
- [17] Y. Kawachi, T. Ikegami, H. Takubo, Y. Ikegami, M. Miyamoto, N. Tanada. Chromatographic characterization of hydrophilic interaction liquid chromatography stationary phases: Hydrophilicity, charge effects, structural selectivity, and separation efficiently. *J. Chromatogr. A* **2011**, *1218*, 5903.
- [18] X. Wang, W. Li, H.T. Rasmussen. Orthogonal method development using hydrophilic interaction chromatography and reversed-phase high-performance liquid chromatography for the determination of pharmaceuticals and impurities. *J. Chromatogr. A* **2005**, *1083*, 58.

- [19] A.S.Y. Wong, E.N.M. Ho, T.S.M. Wan, C.H.F. Wong, K.K.H. Lam, B.D. Stewart. Liquid chromatography–mass spectrometry detection of bisphosphonates in equine urine and plasma and application to an administration study of tiludronic acid in horse. *Proceedings of the 56th ASMS Conference in Mass Spectrometry & Allied Topics*, Denver, USA, 1–5 June **2008**, TA319.
- [20] A.S.Y. Wong, E.N.M. Ho, T.S.M. Wan, C.H.F. Wong, K.K.H. Lam, B.D. Stewart. Liquid chromatography – mass spectrometry analysis of five bisphosphonates in equine urine and plasma. *Proceedings of the 18<sup>th</sup> International Conference of Racing Analysts and Veterinarians*, Queenstown, New Zealand, 7–13 March **2010** (in press).
- [21] The Association of Official Racing Chemists. AORC Guidelines for the Minimum Criteria for Identification by Chromatography and Mass Spectrometry (May 2011). Available at: <http://www.aorc-online.org/documents/aorc-ms-criteria-may-2011/> [August 2011]