3-Methoxytyrosine as an indicator of dopaminergic manipulation in equine plasma

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Abstract

The use of catechol-*O*-methyltransferase inhibitors may mask doping agents, primarily levodopa, administered to racehorses and prolong the stimulating effects of dopaminergic compounds such as dopamine. It is known that 3-methoxytyramine is a metabolite of dopamine and 3-methoxytyrosine is a metabolite of levodopa thus these compounds are proposed to be potential biomarkers of interest. Previous research established a urinary threshold of 4,000 ng/mL for 3-methoxytyramine to monitor misuse of dopaminergic agents. However, there is no equivalent biomarker in plasma. To address this deficiency a rapid protein precipitation method was developed and validated to isolate target compounds from 100 µL equine plasma. A liquid chromatography-high resolution accurate mass (LC-HRAM) method using an IMTAKT Intrada amino acid column provided quantitative analysis of 3-methoxytyrosine (3-MTyr) with lower limit of quantification of 5 ng/mL. Reference population profiling (n=1129) investigated the expected basal concentrations for raceday samples from equine athletes and showed a right-skewed distribution (skewness= 2.39, kurtosis= 10.65) which resulted from large variation (RSD=71%) within the data. Logarithmic transformation of the data provided a normal distribution (skewness = 0.26, kurtosis = 3.23) resulting in the proposal of a conservative threshold for plasma 3-MTyr of 1,000 ng/mL at a 99.995% confidence level. A 12-horse administration study of *Stalevo*® (800 mg L-DOPA, 200 mg carbidopa, 1600 mg entacapone) revealed elevated 3-MTyr concentrations for 24-hours post-administration.

Keywords: 3-methoxytyrosine, anti-doping, threshold, equine, plasma

1. Introduction

Bicker *et al.* comprehensively reviewed the chemistry of catecholamines, sample preparation requirements and chromatographic methodology for analysis of catecholamine compounds[1]. Dopamine is a catecholamine and the most widely known neurotransmitter and therefore, has been extensively studied in relation to human conditions, such as Parkinson's disease[2]. Levodopa (L-DOPA) is commonly used for treatment of diseases related to a deficiency of dopamine[3, 4]. The improvement of L-DOPA therapy can be

achieved through the addition of catechol-*O*-methyltransferase (COMT) inhibitors which limit the metabolism of dopamine to the inactive 3-methoxytyramine (3-MT)[5] (Figure 1). Carbidopa was first used as a COMT inhibitor with early Parkinson's therapies using L-DOPA[6]. Entacapone was found to increase the half-life of L-DOPA, without any negative effects on the established L-DOPA/carbidopa combination therapy, and is known to be rapidly absorbed following oral administration[3, 7]. The use of COMT inhibitor therapies in clinical settings is well known for disease management and treatment [1], however, the potential misuse of these compounds in the horseracing community is not known. While L-DOPA therapies require a prescription from a doctor, they are easier to access than other drugs, such as opioids, thereby potentially providing a performance enhancing effect to the horse through modulation of locomotor behaviour[8]. There is still limited knowledge of these compounds in relation to different equine matrices. Further, the direct detection of COMT inhibitors is difficult due to the instability of these catecholamine compounds under physiological conditions [1].

The aim of racing governing bodies, such as the International Federation of Horseracing Authorities (IFHA) and Fédération Equestre Internationale (FEI), is to detect any substance that may cause performance altering effects [9, 10]. The misuse of COMT inhibitors is of concern as it may prolong the stimulating effects of dopamine and can potentially avoid breaching the 3-MT threshold (4,000 ng/mL) thus enabling potential masking of dopaminergic manipulation[5, 7]. Wynne *et al.* determined the average basal concentration of 3-MT in equine urine to be 214 ng/mL[11]. This work contributed to an equine urinary threshold for 3-MT of 4 µg/mL being established to combat misuse of dopaminergic compounds[5, 9]. Following an administration of *Sinemet*® (800 mg L-DOPA, 200 mg carbidopa), the urinary threshold was breached 4 hours post-administration[12]. McKinney *et al.* also investigated the use of this threshold for another COMT therapy, *Stalevo*®, and found this use of peripheral COMT inhibitors to pose a threat to racing integrity[7]. Elbourne *et al.* expanded on this work by investigating the use of an endogenous reference compound; tyramine, which was found to provide further statistical power resulting in better intelligence for misuse of dopaminergic agents, such as L-DOPA[13]. The absence of an equine plasma threshold limits the transferability and expanded applicability of this research into routine testing. It is known that L-DOPA also metabolises to 3-methoxytyrosine (3-MTyr) (Figure 1), but the suitability of 3-MTyr as biomarker for L-DOPA misuse remains to be investigated[1].

Reversed-phase (RP) chromatography is suitable for hydrophobic analytes, whilst a more polar approach, such as hydrophilic interaction liquid chromatography (HILIC), is recommended for hydrophilic compounds which experience poor retention using RP chromatography[14-16]. Grecco *et al.* developed an automated in-tube solid-phase microextraction (SPME)-HILIC-tandem mass spectrometry (MS/MS) method which targeted 3-MTyr and other dopamine related compounds in human plasma[17]. The use of HILIC columns has been scrutinised due to their susceptibility to pH changes, mobile phase variation and long re-equilibration time[18]. Amino acid columns, such as the IMTAKT Intrada amino acid column, have proven to be successful in the analysis of small polar compounds without the need for prior derivatisation[19, 20].

The aim of this work was to develop and validate a LC-HRAM method using an amino acid column for the analysis of 3-MTyr. A reference population study was investigated to determine basal concentrations of 3-MTyr in equine plasma and propose a suitable threshold. The *Stalevo*® (800 mg L-DOPA, 200 mg carbidopa, 1600 mg entacapone) administration study was used to determine the suitability of the proposed threshold. Initial investigation also included 3-MT, L-DOPA, dopamine, carbidopa and entacapone but they were not included in further investigations due to a lack of detection in authentic plasma matrix and when screened for in the administration study.

2. Methods

2.1. Chemicals and reagents

Acetonitrile (ACN), ammonium formate ($HCOONH_4$), methanol (MeOH), formic acid, and trichloroacetic acid (TCA) of LC-MS grade were purchased from Merck (Darmstadt, Germany). Ultrapure grade (18.2 $M\Omega.cm^{-1}$) water was obtained from a ThermoFisher Scientific Barnstead Smart2Pure system (ThermoScientific; Langenselbold, Hungary). Racemic 3-MTyr was manufactured by Toronto Research Chemicals (Toronto, Canada) and supplied by PM Separations (Capalaba, Queensland, Australia). d_4-3-MT manufactured by

CDN Isotopes (Quebec, Canada) was used as internal standard (IS). 3-MT, dopamine and L-DOPA were manufactured and supplied by Sigma-Aldrich (St Louis, MO, USA). Stock and working solutions of the target compounds and IS were prepared in MeOH at 1 mg/mL and 10 μg/mL, respectively.

Bovine serum albumin (BSA) was obtained from Sigma-Aldrich (St Louis, MO, USA). A mixture of 70 mg of BSA was made up in 2 mL phosphate buffered saline (PBS) solution consisting of 137 mM NaCl, 10 mM Na_2HPO_4 and 1.8 mM KH_2PO_4. This gave a BSA protein content of 35% (w/v) which has previously been reported as optimal[21].

2.2. Sample preparation

Plasma (100 μL) was transferred into a 650-μL Costar microcentrifuge tube (Cat. No. 3208; Salt Lake City, UT, USA). A 1:10 dilution using MeOH of a diluted working solution (1,000 ng/mL) for the IS was made and 10 μL of this solution was spiked into the sample. The final concentration of the IS in the spiked sample was 100 ng/mL. Cold ACN (150 μL) and TCA (150 μL, 10% v/v prepared in ultrapure water) were added to precipitate the proteins in the plasma sample. The sample was centrifuged using a Beckman Coulter Microfuge 20 at 17968 xg for 5 minutes. The supernatant was collected and dried under a gentle flow of nitrogen gas at 50 °C. The dried sample was reconstituted with 100 μL of a 20 mM $HCOONH_4$ in 90% ACN solution, and transferred to a vial for LC-HRAM analysis. Samples were stored at 4 °C until analysis.

Calibrator and QC samples were prepared in PBS with BSA. Calibrators were prepared over a concentration range of 5-4,000 (ng/mL). QC samples were prepared at 20, 200 and 2,000 (ng/mL) concentrations.

2.3. Sample extraction optimisation

A comparison between ACN and ACN:TCA in a 1:1 ratio was investigated. ACN has been commonly used as a protein precipitating agent[22-24], however, the combination of ACN and TCA was suspected to improve protein precipitation. Blank plasma and plasma spiked with 100 ng/mL of 3-MTyr was compared using seven replicates of each.

2.4. Analytical method

LC-HRAM analysis was undertaken with a 1290 Infinity II liquid chromatography (LC) system coupled to a 6545 quadrupole time-of-flight (QTOF) mass spectrometer from Agilent Technologies (Santa Clara, CA, USA). The LC system was equipped with an IMTAKT (Kyoto, Japan) Intrada Amino Acid column (2 mm x 100 mm, 3 μm) and a Phenomenex (Torrance, CA, USA) C18 SecurityGuard (2 mm x 4 mm) operated at 40 °C and using a gradient elution. Mobile phase A was 100 mM $HCOONH_4$ in ultrapure water and mobile phase B was 0.3% formic acid in ACN. The gradient used was as follows: 0-6 min 75-70% B, 6-9 min 70-0% B with a 2-minute post run equilibration time. The flow rate was constant at 0.5 mL/min. All injection volumes were 1 μL.

Positive electrospray (ESI) data was acquired using full scan MS mode from 0 to 9 minutes at a resolution of 17,500 (FWHM). The *m/z* range was 50 to 1000. The scan rate was 5 spectra/sec. The gas temperature was 275 °C with a gas flow of 12 L/min. The nebuliser was set to 35 psi. The sheath gas temperature was 350 °C with a gas flow of 11 L/min. The VCap was set to 3000 with 0 V applied to the nozzle. The fragmentor was set to 100 V with the skimmer at 65 V and the Octopole RF Peak at 750 V. Internal mass calibration was conducted using Agilent Technologies' reference mass solution that was prepared according to Agilent Technologies procedures. Positive reference ions of *m/z* 121.050873, *m/z* 149.02332, *m/z* 322.048121, *m/z* 922.009798, *m/z* 1221.990637 and *m/z* 1521.971475 were selected. External mass calibration was performed using an Agilent Technologies calibration mass solution and prepared according to the instructions provided with this solution. The mass solution was injected during

the mass tuning and calibration in positive and negative ionisation modes using the default settings set by Agilent Technologies for the 6545 QTOF mass spectrometer.

All data were processed using Agilent MassHunter Quantitative and Qualitative software (version B.09.00 and B.07.00, respectively). Further data analysis was performed using Excel (version 16.37) and Matlab software (R2018a) from Mathworks (Natick, MA, USA).

2.5. Method validation

The method was validated for selectivity, specificity, sensitivity, linearity, accuracy, precision, matrix effects, recovery, stability and column robustness. Validation criteria has been presented by Peters *et al* [25] except for column robustness which has been completed in accordance with Zhang and Watson [26]. Detailed explanation of the method validation can be found in the Supporting Information (SI).

2.6. Reference population study

Equine plasma samples were collected by veterinarians and swab officials employed by Racing NSW. Samples were transported to the Australian Racing Forensic Laboratory (ARFL) and stored at 4 °C until aliquoting. Plasma samples (n=1129) for the survey of basal levels of 3-MTyr were drawn from pre-race plasma samples collected on raceday from thoroughbred horses in the Autumn 2022 racing carnival season in New South Wales, Australia. All samples were aliquoted and frozen at -20 °C until extracted for analysis. Statistical analysis was performed using Microsoft Excel and Matlab software. The data was assessed for skewness and kurtosis. For the parametric approach, a coverage factor of 3.72 was used to establish an initial threshold approximating a 99.99% confidence level. Non parametric statistics was used in a complementary manner to identify outliers and far outliers using (Q3 + (1.5xIQR)) and (Q3 + (3xIQR)), respectively.

The use of raceday samples for reference population studies was approved by the Racing NSW Animal Care and Ethics Committee (ARA 80). It was assumed but could not be guaranteed that these horses were not exposed to L-DOPA containing products.

2.7. *Stalevo* administration study

A 12-horse administration study was completed where 8 *Stalevo*® (800 mg L-DOPA, 200 mg carbidopa, 1600 mg entacapone) tablets were administered orally by nasogastric tube to each treated horse. Of the 12 horses, 6 were mares and 6 were geldings with 2 control horses in each gender group (Table 1). Blood samples were collected in 10-mL Vacutainer tubes at 24-hour intervals for 7 days prior to administration, immediately pre-administration (T=0), then 5 , 10 , 20 , 30 , 40 , 50 , 60 , 90 min and 2 , 3 , 4 , 6 , 8 , 12 , 24 hours, and then in 24-hour intervals for up to 7 days post-administration. Plasma was obtained immediately following blood collection by centrifugation at 1500 x g then stored at -20 °C until analysis. Analysis was completed in duplicates for each sample. Ethics approval for the administration study was provided by the Charles Sturt University Animal Care and Ethics Committee (A20277).

Table 1: Administration status of the 12 horses

Administration status	Mare	Gelding
Control	1, 6	1, 9
Treated	2, 3, 4, 9	2, 3, 4, 10

3. Results and discussion

3.1. Method optimisation

The supernatant from the ACN:TCA extracted sample was colourless while the ACN extracted sample was yellow (Figure S1). A noticeable difference in the colour of the precipitated protein was seen between the two with the ACN protein being orange and the ACN:TCA protein being yellow. The addition of the TCA also improved peak shape with the average response of only ACN being 1046 ± 182 and the combination of ACN and TCA being 7097 ± 1235 for 3-MTyr (Figure S2). It was proposed that the combination of ACN:TCA was able to release analyte that was protein bound in the plasma thus resulting in

a greater response for the analyte (Table S2). One of the main limitations of the introduction of the TCA was the increased drying time as the TCA was prepared in water.

3-MTyr displayed acceptable retention (>1 min) and excellent peak shape (Figure 2). However, 3-MT did not demonstrate sufficient abundance in equine plasma samples. Dopamine and L-DOPA also appear to be at a low concentration in plasma samples. Therefore, 3-MTyr was chosen as an investigative biomarker for quantitative analysis.

3.2. Method validation

The method was validated with respect to the quantification of 3-MTyr. BSA in PBS was used as the matrix for calibration and to estimate the limit of detection (LOD) and lower limit of quantification (LLOQ)[27, 28]. The endogenous content in plasma resulted in background interference that made estimation of the LLOQ problematic[27]. Therefore, a surrogate matrix of BSA in PBS was used for the calibration curves and to estimate LOD and LLOQ. Sensitivity was investigated with a LOD ($S/N \geq 3$) and LLOQ ($S/N \geq 10$) of 2 ng/mL and 5 ng/mL for 3-MTyr. The calibration was linear between LLOQ and 800 ng/mL for 3-MTyr with a R^2 value of 0.9986. Qualifier ions of 212.0917, 195.0641 and 166.0862 were used to assess the 3-MTyr selectivity. Quantitative accuracy and precision from the surrogate matrix were deemed to be acceptable based on relative error (RE) and relative standard deviation (RSD) being ± 20% RE and less than 20%RSD at LLOQ and ±15% RE and less than 15%RSD for other assessments, respectively. To ensure feasibility in matrix, accuracy and precision should be evaluated through further work with spiked samples in equine plasma. A standard addition approach would be considered appropriate for this further work. The recovery of 3-MTyr was deemed to be acceptable (100 ± 20%). Matrix effects were assessed to be 33% for 3-MTyr (>0 meaning ion enhancement, <0 meaning ion suppression and 0 meaning no matrix effect). A thorough stability study was unable to be completed due to time constraints but it would be recommended for future work. Mass accuracy was observed to be < 5 ppm.

3.3. Column robustness

Column robustness was assessed using a reference population study of 1129 samples collected from the Autumn racing carnival season, and an administration study of 696 samples where the same IMTAKT column was used for the duration. Precision was assessed for the retention time of 3-MTyr and relative retention to the d_4-3-MT IS. The column was determined to be robust for the reference population study (Table 2) as the %RSD for retention time and relative retention time was 1.35% and 2.81%, respectively. Analysis of the administration study revealed the column to be robust as well with the %RSD for retention time and relative retention time of 1.62% and 1.85%, respectively. Therefore, the column was determined to be suitable for long-term and routine analysis of small polar molecules in equine plasma using the simple protein precipitation method. It was possible that the use of a C18 column guard aided the filtering of samples prior to analysis on the column thus prolonging the column life.

Table 2: Column robustness data

	Reference population (n=1129)		Administration Study (n=696)	
	Retention time (min)	Relative retention	Retention time (min)	Relative retention
Average	1.32	2.57	1.29	2.53
Standard deviation	0.018	0.072	0.021	0.047
Relative standard deviation (%RSD)	1.35	2.81	1.62	1.85

Note: The retention time was for 3-MTyr and the relative retention time was the ratio of 3-MTyr to the IS (d_4-3-MT)

3.4. Reference population study

The reference population data consisted of 1129 samples analysed during the Autumn 2022 racing carnival period. Within the sample population 430 were female, 507 were geldings and 192 were entire males. The number of entire male samples was expected to be smaller due to the lower prevalence within the male racehorse population.

Quantifiable results were obtained for 3-MTyr in all samples analysed (Table 3). The results were variable with a mean of 119 ng/mL and a SD of 85 ng/mL resulting in a RSD of 71%.

Minimum and maximum 3-MTyr concentrations of 16 and 665 ng/mL, respectively, were obtained.

Table 3: Summary statistics for plasma 3-MTyr (ng/mL)

n = 1129	3-MTyr (ng/mL)
Mean	119
Standard deviation	85
Minimum	16
Maximum	665
Q1	67
Median (Q2)	95
Q3	142
Interquartile range (IQR)	75
1%	23
2.5%	33
5%	40
95%	285
97.5%	367
99%	468

Non parametric review showed 3-MTyr values greater than 255 ng/mL (Q3+(1.5xIQR)) to be considered outliers (Figure 3). This assessment would classify 77 (6.8%) samples as outliers. Far outliers (Q3+3*IQR) were considered to be greater than 367 ng/mL which was represented in the 97.5 percentile. The distribution of plasma 3-MTyr concentrations (Figure 3) was right-skewed (skewness = 2.39, kurtosis = 10.65) with a mean of 119 ng/mL and a median of 95 ng/mL. The difference in the mean and median (Table 4) also gives an indication to the skewness of the data implying a lack of normal distribution. The lack of symmetry within the boxplot furthers what was seen in the frequency plot with majority of values occurring within a lower 3-MTyr concentration. This highlights the large kurtosis value meaning there were a considerable number (n=77, 6.8%) of outliers present within the dataset.

Generally, if raw data does not follow normal distribution, then the data will be transformed to fit a normal distribution [29]. Log transformation of the entire reference population dataset was attempted to fit a normal distribution (Figure 4) by reducing the skewness and kurtosis by 89% and 70% to 0.26 and 3.23, respectively. The resulting boxplot (Figure 4) displayed a symmetric range of data relative to the untransformed median (95 ng/mL) with a lower number (n=17, 1.5%) of high log plasma 3-MTyr outliers. Review of the normal probability plot (Figure 4) showed evidence of a lognormal trend between 32 ng/mL and 200 ng/mL, deviation from normal at concentrations higher than 200 ng/mL, before a return to normal at the maximum concentration of 665 ng/mL.

A proposed parametric-based threshold for equine plasma 3-MTyr was estimated at the 99.99% confidence level (i.e. mean+3.72SD) to provide an untransformed 3-MTyr concentration of 908 ng/mL. It would be recommended that this be rounded up to propose a conservative threshold at 1000 ng/mL, which represents a 1:20,000 probability of natural exceedance.

To verify the suitability for application of the parametric approach, the Anderson-Darling test was applied to the entire data set, however this was not achieved ($p<0.05$). Therefore, the boxplot results in Figure 3 were used to exclude outliers less than 21 ng/mL (n=2) and greater than 440 ng/mL (n=17) to provide a data set of 1110 samples (98.3%) to again attempt an Anderson-Darling test (Figure 5) that returned $p=0.114$. Using this sample sub-set an untransformed 3-MTyr concentration of 780 ng/mL was obtained thus showing how conservative the proposed threshold of 1000 ng/mL is. The normal probability plot for this distribution (Figure 5) was reviewed to demonstrate its suitability.

The data were also analysed to determine if there were any significant gender differences. Comparison of genders using a boxplot of the raw data revealed similar profiles in terms of median concentrations for each gender and a similar outlier pattern, taking into account the smaller number of males (Figure S3). The raw gender data were not normally distributed and the same statistical data treatment as the entire population data set presented above were followed. The log transformed data were normally distributed and thus the gender difference test was performed on the log transformed data. A summary of the reference population log transformation data (Table 4) revealed similar mean (2.07 ± 0.01) and median (1.98 ± 0.02) values between genders. A one-way ANOVA was performed to determine any difference

within the genders with a null hypothesis of no significant differences between genders. The ANOVA resulted in a p-value of 0.35 meaning there were no significant differences and the proposed threshold may be applied to the administration study.

Table 4: Summary of the different genders' log 3-MTyr concentration within the reference population

	Gelding	**Female**	**Entire Male**
Count	507	430	192
Mean	2.07	2.08	2.08
SD	1.90	1.98	1.85
Median	1.99	1.96	1.98
Minimum	1.32	1.20	1.49
Maximum	2.79	2.82	2.67

To convert the proposed threshold to an accepted threshold by racing authorities, such as the IFHA and FEI, further studies will be required including sample exchange between international laboratories. During the sample exchange, incurred sample reanalysis is required to overcome the lack of accuracy and precision validation data and ensure feasibility of the plasma matrix. To verify the applicability of this proposed threshold, pre-race and post-race samples should be investigated along with seasonal variation.

3.5. Plasma 3-MTyr profiles from the *Stalevo* administration study

The minimum, mean and maximum concentrations from the reference population study were used for comparison to the administration profiles, including the control horses. The 3-MTyr concentration profiles for the mares are shown in Figure 6. The average 3-MTyr concentration for control mares 1 and 6 were 214 ng/mL (RSD 13%) and 394 ng/mL (RSD 16%), respectively. Importantly, the control horses displayed 3-MTyr levels within the reference population range confirming that they were comparative to raceday samples. However, some of the endogenous concentrations recorded for mare 6 may be treated as outliers when compared to the reference population study thus highlighting the importance of including further testing from other racing laboratories to assess the proposed threshold.

The 4 treated mares; 2, 3, 4 and 9 all showed elevated plasma 3-MTyr levels between 0.5- and 24-hours post-administration in comparison to their basal concentrations. The peak

plasma 3-MTyr concentrations (689 to 2111 ng/mL) occurred over the time range between 1.5- and 8-hours post-administration. Mare 2 displayed an increase (410%) of 3-MTyr for the longest period (~96 hours). Mare 4 displayed a similar profile to mare 2 with high plasma 3-MTyr (1938 ng/mL) at 4 hours post-administration, representing a 466% increase from pre-administration levels. Mare 3 and 9 had similar profiles but did not show as large of a 3-MTyr concentration increase (307% and 362%, respectively). One reason for this may be the lower pre-administration concentrations (mean 263 and 190 ng/mL, respectively) for these two horses, compared to a mean concentration of 515 and 416 ng/mL for mare 2 and 4, respectively. Notwithstanding this, mare 3 and 9 still showed an increase when compared to the control mares; 1 and 6.

Out of the treated mares, mare 2 and 4 would surpass the 1000 ng/mL proposed threshold between 0.67 and 8 to 12 hours post-administration. This timeframe of detection is suitable for control of L-DOPA misuse since this would most likely occur on raceday. The lack of detection for mare 3 and 9 highlights the need for more sensitive strategies, such as longitudinal profiling to provide an individualised approach for improved doping detection efforts. Future work should focus on further development of the equine biological passport (EBP) to include measurement of 3-MTyr in plasma.

The plasma 3-MTyr profiles for the geldings are shown in Figure 7 with comparative minimum, mean and maximum concentrations from the reference population study. Control geldings 1 and 9 displayed average basal concentrations of 391 ng/mL (RSD 23%) and 569 ng/mL (RSD 13%), respectively. Gelding 9 also displayed some higher 3-MTyr levels than the maximum basal concentration, particularly in the pre-administration sampling period. Therefore, the majority of the control gelding samples profiled within the reference population study demonstrated that 3-MTyr concentrations were representative of raceday samples.

The 4 treated geldings; 2, 3, 4 and 10 displayed similarly elevated plasma 3-MTyr levels between 0.5- and 24-hours post-administration, with peak concentrations (1425 to 2018ng/mL) recorded between 1.5- and 4-hours post-administration. Gelding 2 recorded the highest 3-MTyr plasma concentration of 2018 ng/mL, a 412% increase. Gelding 3 displayed the lowest mean pre-administration level (442 ng/mL) but largest increase of 425% to its peak concentration of 1880 ng/mL. Geldings 4 and 10 both displayed lower increases of

291% and 248%, respectively, which was attributed to higher pre-administration levels of 3-MTyr compared to geldings 2 and 3.

All 4 treated geldings surpassed the 1000 ng/mL proposed threshold between 0.67 and 24-hours post-administration. Elevation above the proposed threshold limit again occurred within the desired time period for raceday detection of the misuse of L-DOPA in equine plasma. Despite no significant differences found in the basal concentration of geldings and mares from the reference population study, this detection period, occurring above the proposed threshold limit, was larger for the geldings compared to the mares. This difference could potentially be attributed to differences in metabolic rates or physiological differences, which are influenced by neurotransmitters between the two genders. A previous study by Elbourne *et al.* also demonstrated differences when analysing the 3-MT threshold against the administration of COMT inhibitors in equine urine thus they proposed the use of a biomarker ratio to account for such differences [13]. Previous studies have shown significant differences in neurotransmitters of mares and geldings due to diet; where mares had significantly higher tryptophan levels compared to geldings on a high fibre diet[30, 31]. It is important to note that these horses were all given the same feed, husbandry and were all run together as one herd. The only difference was the day of administration with mares receiving the dose of *Stalevo*® one day prior to the geldings. Therefore, further metabolic influences, such as diet, environmental factors and physiological factors, should be investigated to determine significant confounding factors between the different genders.

The lack of significantly increased 3-MTyr plasma concentrations from the control horses confirmed that the observed increase was due to the administration of *Stalevo*® and not confounding factors, such as stress and environmental influence. The results from both mares and geldings indicate that 3-MTyr was a suitable biomarker for L-DOPA administration in equine plasma.

4. Conclusion

The misuse of L-DOPA containing agents is a concern for the racing industry due to the potential for improved performance from dopaminergic effects. A robust amino acid method was validated for the analysis of 3-MTyr in equine plasma, with a LLOQ of 5 ng/mL, enabling a reference population (n=1129) of raceday samples to assess variance in 3-MTyr. Log-transformation of the raw data resulted in a normal distribution which allowed a conservative proposed threshold of 1000 ng/mL to be proposed at a 99.995% confidence level. It is worth to note that despite log-transformation, there was still 1.5% outliers. It is possible that various confounding factors, such as diet and environmental influence, could result in outliers as seen in this population study that cannot be confirmed without further research. A *Stalevo*® administration study was performed to investigate the elimination profile and detection time of 3-MTyr in equine plasma. This revealed elevated plasma 3-MTyr levels for up to 24-hours post-administration. The majority (6 out of 8) of the horses administered with *Stalevo*® would be detected as being administered with L-DOPA. 3-MTyr was found to be a suitable biomarker for the misuse of L-DOPA administered together with COMT inhibitors; carbidopa and entacapone. Future work should involve international collaboration to assess the suitability of the proposed threshold including analysis of pre- and post-race samples with seasonal variation and investigate the potential of longitudinal profiling of plasma 3-MTyr levels as part of an EBP. Furthermore, the incorporation of endogenous reference compounds to further reduce inter-individual variance may provide greater confidence to discern dopaminergic manipulation from physiological variation.

5. Acknowledgements

The ARFL has maintained an interest in the issue of dopaminergic manipulation in equine athletes following the earlier work of Dr Andrew McKinney. The current work presented here followed a pilot (unpublished) study assisted by Dr Shawn Stanley. The authors thank PM separations (Capalaba, Queensland, Australia), particularly Michael Rennie, for the introduction to IMTAKT and their unique LC columns designed for amino acid analysis. Lauren Kennan at the ARFL is acknowledged for assisting the maintenance and operation of the LC-QTOF-MS. Funding for the *Stalevo*® administration study was provided by AgriFutures Australia as part of the Thoroughbred Horses Program.

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