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Intelligence benefit of the 3-methoxytyramine to tyramine ratio in equine urine

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Abstract
Equine urine analysis has evolved over time to detect thousands of urinary compounds for doping control in the horse racing industry. The longitudinal assessment of 3-methoxytyramine to tyramine ratio (3-MT/T) values in equine urine by GC-MS profiling was investigated to support the Racing NSW Equine Biological Passport (EBP) for detection of dopaminergic manipulation in racehorses. This involved comparison of routine urine samples to administration studies of *Sinemet*, a common Parkinson's disease medication containing levodopa. Using an endogenous reference compound (ERC) in a urinary ratio enabled greater confidence to provide intelligence of pharmaceutical manipulation as distinct from physiological variation. Population Reference Limits (PRLs) of 776 ng/mL for urinary 3-MT and 5.3 for 3-MT/T, together with the use of Individual Reference Limits (IRLs) are proposed.

Introduction
Levodopa (L-DOPA; *L*-3,4-dihydroxyphenylalanine) is used to treat the effects of Parkinson's disease (PD); a progressive, degenerative neurological condition that affects the control of body movements[1,2]. In suitable doses, L-DOPA reduces the symptoms of PD as it can cross the blood-brain barrier to produce more dopamine where it is needed[1]. Since discovery of the benefits of L-DOPA on human PD patients, it's misuse has been suspected in equine sports to increase locomotor activity[2,3]. To control the misuse of L-DOPA containing products, the L-DOPA and dopamine metabolite 3-methoxytyramine (3-MT) is monitored in equine urine samples for compliance below an international threshold of 4 µg/mL (4000 ng/mL)[3,4]. This threshold, however, is deliberately conservative and so may be insensitive to some doping practices[5,6]. To mitigate the risk of manipulating endogenous levels, the development of an Equine Biological Passport (EBP) can enable the longitudinal monitoring of biomarkers to improve the detection of doping[5].

p-Tyramine (T) is a biologically active amine from dietary plants, bacteria and fungi formed by enzymatic decarboxylation of the precursor amino acid *L*-tyrosine[7]. T occurs at substantial concentrations in many fermented and probiotic foods[8]. It is suspected that dietary variation, possibly resulting from a change in season and accessible vegetation (e.g. different pasture grasses) can alter the concentration of T excreted from horses[9]. This variation can be exploited to improve the assessment of biomarker measurements by incorporating an endogenous reference compound (ERC).

The aim of this work was to assess sub-threshold levels of 3-MT for intelligence purposes following dopaminergic manipulation with comparison to a reference population of raceday equine urine samples. Furthermore, the potential use of T as an ERC to provide 3-MT/T values was investigated to improve intelligence of dopaminergic manipulation and assess the

suitability of this biomarker for longitudinal monitoring as part of an EBP.

Materials and Methods

Reference population samples:

Equine urine samples were collected by veterinarians and swab officials employed by Racing NSW or Harness Racing NSW. Samples were transported to the Australian Racing Forensic Laboratory (ARFL) and stored at 4 °C until analysis. It is assumed but cannot be guaranteed that the donors of these samples have not been subject to L-DOPA containing products. Animal Ethics approval was provided by the Racing NSW Animal Care and Ethics Committee (ARA75).

Pre- (within 3 h prior) and post-race (up to 2 h after) samples were collected as routine doping controls between June and October of 2020 (Table 1).

Administration studies:

The administration of *Sinemet* (800 mg levodopa and 200 mg carbidopa, by oral (i.e., nasogastric tube) to Horse 1 was completed by the Singapore Turf Club with animal ethics approval. Samples were collected at t=0 h (pre-administration), 4 h, 6 h, 8 h and 24 h post-administration. The administration of *Sinemet* (same dose and route as Horse 1) to Horse 2 was completed by Racing NSW with animal ethics approval (RNSW ARA 51). Samples were collected daily for 7 days pre-administration, at t=0 h, 2 h, 4 h, 6 h, 8 h, 24 h, then daily (at approximately 8 am) for a further 6 days post-administration.

Standards and reagents:

Two sources of 3-MT HCl were purchased for method validation and sample analysis. The calibration standard was manufactured by HPC Standards (Cunnersdorf, Germany), while the quality control standard was purchased from Sigma-Aldrich (Castle Hill, Australia). The internal standard (IS) 3-MT-d_4 was manufactured by CDN Isotopes (Quebec, Canada).

Pyridine (99.9%), acetic anhydride (99.5%), isopropanol, ethyl acetate (EtOAc) and methanol (MeOH), of liquid chromatography grade were purchased from Merck (Darmstadt, Germany) for routine extraction of the basic fraction from equine urine. ß-glucuronidase (from *Helix pomatia*) was purchased from BBI solutions (Crumlin, UK). Water used was ultrapure grade obtained from a Thermo Smart2Pure system.

Preparation of calibration and quality control (QC) samples:

The concentrations of 3-MT and T were determined for equine urine samples relative to calibration samples (50 to 800 ng/mL) prepared using non-hydrolysed blank equine urine, which contained no detectable presence of the target analytes. Dilutions (1:3 or 1:6) were performed where applicable for valid quantifications. QC samples were prepared at 50, 200 and 350 ng/mL.

Sample Preparation

Equine urine samples (3 mL) were adjusted to between pH 4.9 to 5.5 prior to addition of β-glucuronidase enzyme solution (150 µL), d_4-3-MT internal standard (200 ng/mL, 150 µL) and phosphate buffer (pH 5, 0.1 M, 3 mL). Samples were incubated at 37 °C overnight for

approximately 12 hours before being centrifuged at 3000 rpm (2093 x g) for 10 min.

Solid-phase extraction (SPE) used UCT (Bristol, PA, USA) Xtrackt cartridges pre-conditioned with methanol (2 mL), potassium phosphate buffer (0.1 M, 2 mL), and then loaded with the urine samples. This was followed by methanol (3 mL) and acetic acid (0.1 M, 3 mL) wash steps, before target compounds were eluted with a solution (3 mL) containing 3% ammonia and 0.5% methanol in ethyl acetate.

One drop (approx. 20 µL) of 0.1 M methanolic HCl was added to the eluates before being evaporated under a gentle stream of nitrogen at 60 °C. Acetic anhydride (25 µL) and pyridine (25 µL) were added to the dried residue and heated for 60 min at 60 °C. Extracts were left to cool, then dried again under a gentle stream of nitrogen at 60 °C. Extracts were stored at -20 °C before reconstitution with EtOAc (100 µL) for GC-MS analysis.

Gas Chromatography – Mass Spectrometry

Gas Chromatography–Mass Spectrometry (GC-MS) analysis was undertaken using a Shimadzu (Kyoto, Japan) QP2020 NX instrument. The GC was equipped with a Shimadzu SH-Rxi-5Sil MS (30 m x 0.25 mm internal diameter, 0.25 µm film thickness) column. GC parameters involved an injection volume of 2 µL (EtOAc) at 220 °C (splitless mode). The initial oven temperature at 45 °C (1.5 min hold) was increased to 300 °C at 30 °C/min (5 min hold). Column flow rate was 1.2 mL/min with helium as the carrier gas. The transfer line temperature was set to 320 °C.

MS acquisition involved simultaneous full scan (*m/z* 50 to 500) and Selected Ion Monitoring (SIM) modes (Table 2) with an ion source temperature of 200 °C. Data was acquired with Electron Impact Ionisation (70 eV) following a solvent delay of 2.5 min. LabSolutions software (Shimadzu, version 4.50 SP1) was used for data processing.

Refer to Supporting Information for information on method validation.

Data Analysis

Reference Population

GC-MS data was analysed using *Insight* (Shimadzu, version 3.7) and the basic statistical functions of Microsoft Excel (version 16.42). Additional statistical analysis was completed using *MATLAB* (version R2018a, MathWorks Inc, Natick, MA, USA) to produce frequency distributions, boxplots, and normal-probability plots. Non-parametric analysis was performed to estimate an outlier (Q3+1.5IQR) and intelligence (Q3+3IQR) levels. Population reference limits (PRLs) were estimated using a coverage factor of 3.72 to approximate a 99.99% confidence level.

Administration studies:

Quantified results for the administration studies are provided as the mean of six results from triplicate analysis of duplicate extractions.

Individual Reference Limits

Longitudinal monitoring was performed using the method proposed by McIntosh *et al.* to estimate individual reference limits (IRLs) from the parametric empirical Bayes (PEB) method, which provides posterior predictions based on prior observations (Equations 1 and 2).[10,11]

Equations 1 and 2 use μ (population mean), $\bar{x}$ (individual mean), B_n (intra-class correlator), z_α (coverage factor), V (total variance), σ^2 (population variance), s^2 (individual variance) and n is the number of samples. The coverage factor used was 3.09 to approximate a 99.9% confidence level.

Results and Discussion

Reference Population

Results for 3-MT and T are provided in the Supplementary Information (Figures S1 to S7). Summary statistics for urinary 3-MT, T and 3-MT/T are provided in Table 3.

Non-parametric review of the reference population data showed that there was no significant difference between the assessed variables; pre- and post-race, gender, breed and initial pH levels (Supplementary Information Figure S8 to S10). Variation in the data can be explained by sample size. The distribution of 3-MT levels (Figure S2) demonstrates the conservative nature of the international threshold set at 4000 ng/mL and the need for sub-threshold intelligence limits to be derived for effective anti-doping. It is important to note that the collection of samples in this study is approximately half that of in the work performed by Wynne *et al.*[3]. This could be considered a reason for the difference in frequency distribution plots.

From the reference samples, an outlier (Q3+1.5IQR) and intelligence (Q3+3IQR) limit of 539 ng/mL and 776 ng/mL were proposed for 3-MT, respectively. The latter could be used as an action level to notify the appropriate authority of a screening abnormality. The reference population data showed 99.8% of 3-MT levels to be less than the intelligence limit of 776 ng/mL. These limits were used for comparison to the administration studies.

Logarithmic transformation of 3-MT levels resulted in only 1768 samples (71%) displaying parametric behaviour, therefore 3-MT/T values were investigated. Transformed 3-MT/T results comprised a total of 84% of collected data. While variance was not reduced in comparison to the original 3-MT data, 3-MT/T values allowed for a higher percentage of parametric data to be included in the reference population for proposal of a PRL and application of IRLs.

The distribution of urinary 3-MT/T values (Figure 1) is skewed (skewness = 2.79, kurtosis = 16.85), reflected by a mean of 0.55 and median of 0.44 providing a relative difference of 25%.

The log-transformed distribution of 3-MT/T values (Figure 2) appears to exhibit bi-modal qualities most likely due to samples with high T levels.

There is potential for a parametric distribution to exist for log 3-MT/T values greater than -0.8, which equates to an untransformed value of 0.16. This inference is supported by the

Normal probability plot shown in Figure 3.

Reviewing the frequency distribution for log 3-MT/T values between 0.16 and 3.98 (Figure 4) provides a reasonable approximation of parametric behaviour for 3-MT/T values for a one-tailed approach to higher values. This provided a correlation coefficient of 0.9936 for the selected 2137 samples, which represented 86% of the population, suitable to apply parametric statistics to estimate a PRL for 3-MT/T values. Using the logarithmic mean (-0.2939) and standard deviation (0.2728), a putative PRL for equine urinary 3-MT/T can be estimated (mean+3.72*SD) at 5.3 with a 99.99% confidence level[12] (i.e. probability of exceedance at 1 in 10,000).

Administration Studies

Two administration studies involving *Sinemet* were analysed. Horse 1 displayed a maximum 3-MT concentration of 2866 ng/mL at 4 h. This did not breach the 4000 ng/mL threshold so would be declared negative. However, implementation of the 776 ng/mL intelligence limit could potentially identify an abnormal 3-MT result for approximately 22 hours post-administration (Figure S11). This is consistent with the estimated post-administration period using the PRL of 5.3 estimated for 3-MT/T values (Figure 5).

A major limitation of this administration study involving horse 1 was that only five samples were collected and so no data for the basal levels is available for the days pre- and post-administrations. A further limitation was that no samples were collected between the 8 h and 24 h time points. This limits interpretation of the post-administration detection period using the intelligence limits for 3-MT of 776 ng/mL and 3-MT/T of 5.3. Notwithstanding this, the estimated post-administration period of 22 h is advantageous for control of levodopa misuse on raceday.

Horse 2 displayed an estimated maximum 3-MT level of 6314 ng/mL at 4 hours post-administration. This exceeded the 4000 ng/mL threshold so would have been declared positive in routine analysis. A maximum 3-MT/T value of 290.5 was also observed (Figure 6), considerably higher in comparison to the maximum value for Horse 1 of 32.0. The results from horse 1 and 2 need to be considered with the limitation of only having two horses administered with levodopa for comparison to the reference population.

For analysis of the administration studies, the proposed outlier limit of 539 ng/mL and intelligence limit of 776 ng/mL for 3-MT, and 3-MT/T limit of 5.3 proved useful in the "detection" of a doped horse. Furthermore, T displayed consistent down-regulation when 3-MT was elevated to support its use as an ERC (Figures S12 and S13). The proposed limits provided comparable post-administration periods of approximately 22 hours to enable control of levodopa misuse on raceday.

Individual Reference Limits (IRLs)

The estimated IRL in Figure 7 commences at 4.1, equivalent to a PRL at 99.9% confidence. The PEB model then requires a minimum of 3 samples to account for the individual's mean

and variance, which is observed at -120 h.

This example using the Horse 2 administration results simulates consistent 24 h sampling. The results show that after 24 h, the administration of *Sinemet* is detectable using this method, where the predicted IRL of 2.1 was exceeded by the 3-MT/T value of 3.7. This provides the opportunity to further extend the detection period for levodopa administration in this specific horse beyond the 22 hours attributed to using the PRL of 5.3.

Conclusion

Population derived reference limits for 3-MT (776 ng/mL) and 3-MT/T values (5.3) are proposed to provide intelligence of dopaminergic manipulation of racehorses, without exceedance of the 4 μg/mL threshold. In addition, longitudinal assessment of 3-MT/T values in equine urine by GC-MS profiling was developed to support the Racing NSW EBP and improve the detection of levodopa misuse for individual horses. Additional longitudinal analysis of routine raceday samples and comparison to administration studies will be necessary to further validate the proposed use of IRLs.

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Tables

Table 1: Reference population samples collected on raceday.

Sample Type	Breed	Sex	Number
Pre-race	Thoroughbred	Male	4
		Gelding	58
		Female	89
Postrace	Thoroughbred	Male	146
		Gelding	814
		Female	579
	Standardbred	Male	38
		Gelding	481
		Female	287
Total			2496

Table 2: GC-MS monitoring of target compounds.

Compound	**Retention Time** *(min)*	**Relative Retention***	**Quantifier Ion** *(m/z)*	**Qualifier Ions** *(m/z)*
3-MT	8.52	1.001	150	209, 137
d4-3-MT*	8.51	1.000	153	213, 139
Tyramine	8.03	0.943	120	107, 162

* Internal standard

Equation 1: Estimation of IRL

$$IRL = \mu + (\bar{x} - \mu)B_n + z_\alpha\sqrt{1 - B_1 B_n}\sqrt{V}$$

Equation 2: Intra-class Correlator

$$B_n = \frac{\sigma^2}{s^2/n + \sigma^2}$$

Table 3: Summary statistics for urinary 3-MT, T and 3-MT/T values.

n = 2496	3-MT (ng/mL)	T (ng/mL)	3-MT/T
Mean	231	663	0.55
Standard deviation	122	547	0.46
Minimum	10	8	0.01
Maximum	939*	7265*	3.98
Q1	144	311	0.23
Median (Q2)	217	526	0.44
Q3	302	845	0.72
Interquartile range (IQR)	158	534	0.49
1%	32	80	0.02
2.5%	45	111	0.04
5%	58	144	0.07
10%	82	200	0.12
90%	387	1242*	1.07
95%	446	1591*	1.41
97.5%	503	1946*	1.79
99%	617	2788*	2.34

* Estimated level greater than the ULOQ of 800 ng/mL without dilution.

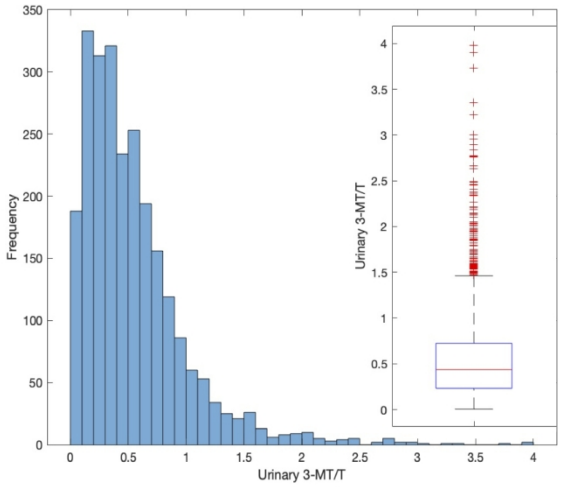

Figure 1: Frequency distribution and boxplot (inset) for urinary 3-MT/T values (n = 2496).

677x381mm (72 x 72 DPI)

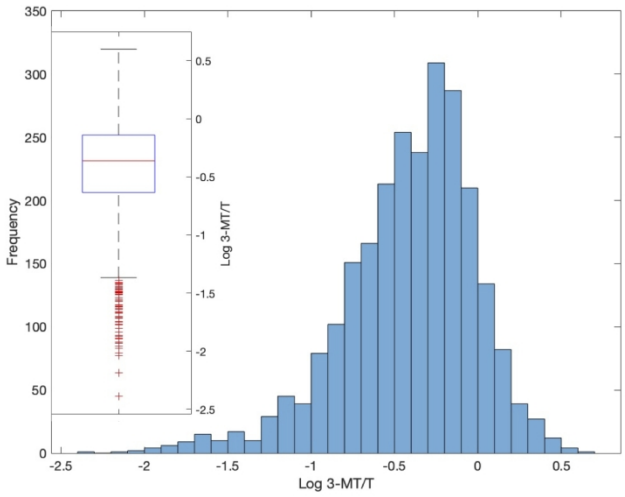

Figure 2: Frequency distribution and boxplot (inset) of log-transformed 3-MT/T values (n = 2496).

677x381mm (72 x 72 DPI)

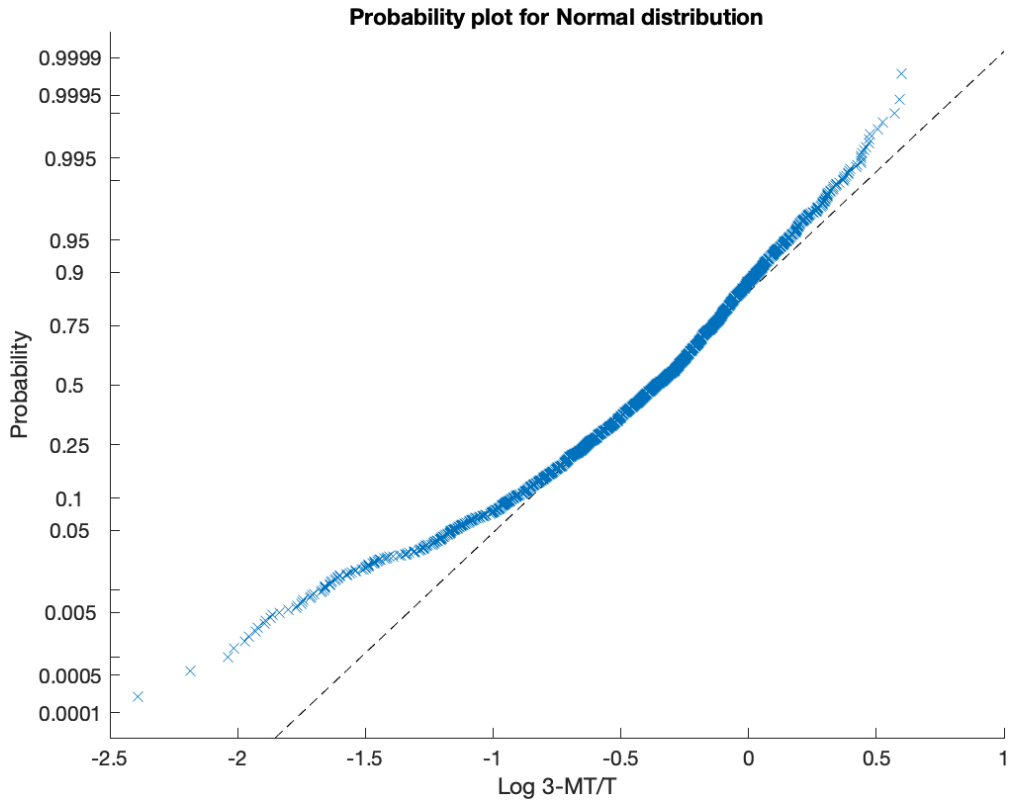

Figure 3: Probability plot for log 3-MT/T values. Dashed line provides parametric approximation (n = 2496).

435x346mm (59 x 59 DPI)

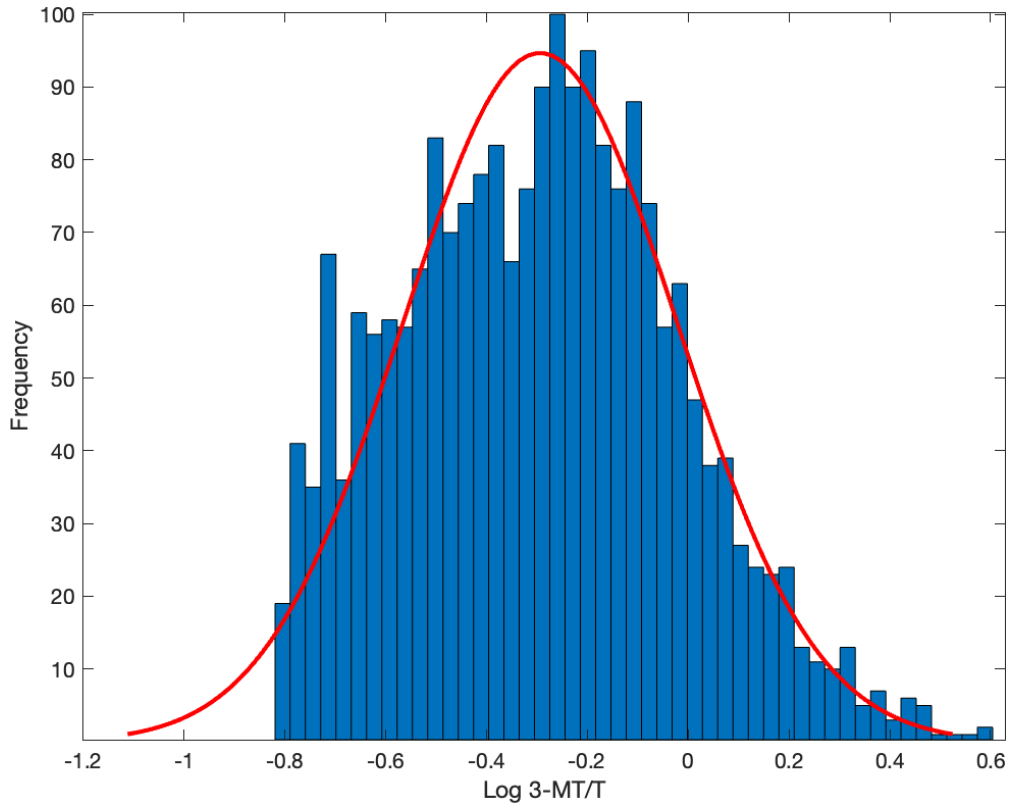

Figure 4: Frequency distribution of log-transformed 3-MT/Tyramine values between -0.8 and 0.6 (n = 2137). A normal distribution is approximated by the red line.

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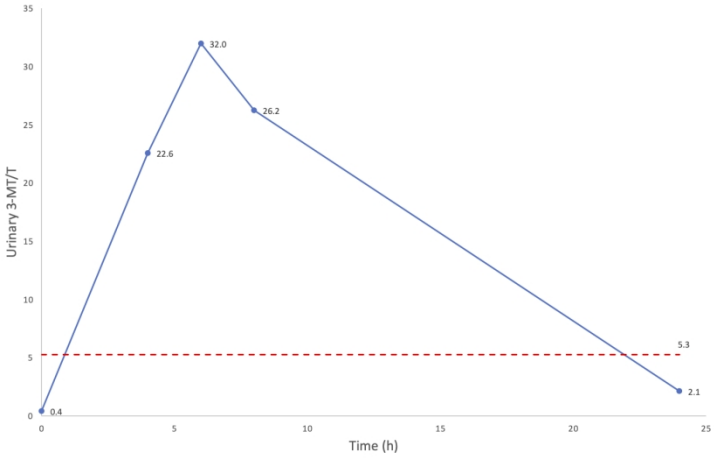

Figure 5: 3-MT/T values following Sinemet (800 mg levodopa, 200 mg carbidopa) administration to Horse 1. Red dotted line is the proposed PRL at 5.3.

677x381mm (72 x 72 DPI)

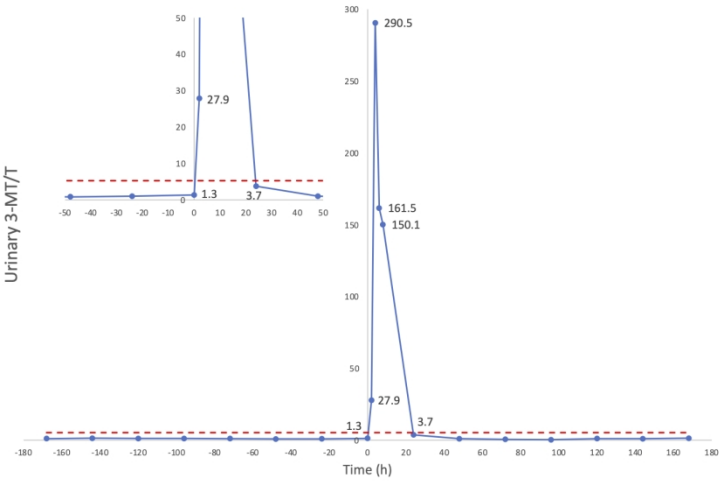

Figure 6: Urinary 3-MT/T values following Sinemet (800 mg levodopa, 200 mg carbidopa) administration to Horse 2. Red dotted line is the proposed PRL of 5.3.

677x381mm (72 x 72 DPI)

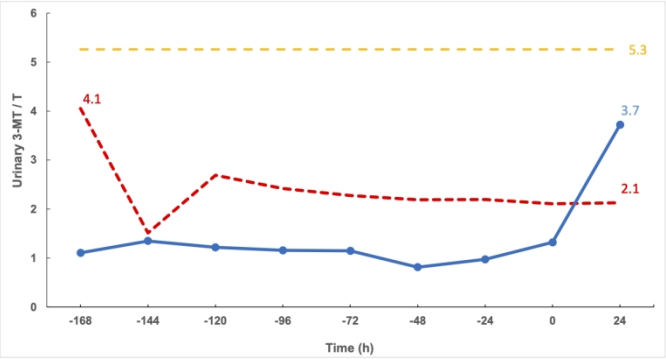

Figure 7: Urinary 3-MT/T values following Sinemet (800 mg levodopa, 200 mg carbidopa) administration to Horse 2 in relation to PRL (dotted yellow line) and IRL (dotted red line).

677x381mm (72 x 72 DPI)