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3-Methoxytyramine as an indicator of dopaminergic manipulation in the equine athlete

Paul M. Wynne[a,*], John H. Vine[a], R. Gary Amiet[b]

[a] *Racing Analytical Services Limited, 400 Epsom Road, Flemington, Vic. 3031, Australia*
[b] *Department of Applied Chemistry, RMIT University, G.P.O. Box 2476V, Melbourne, Vic. 3001, Australia*

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Abstract

The influence of sampling variables on the concentration of the dopamine metabolites 3-methoxytyramine (3MT), dihydroxyphenylacetic acid (DOPAC) and homovanilic acid (HVA) was examined in equine urine. A logarithmic transformation of the data for all horses gave distribution which approximated the normal distributions for each metabolite. The mean urinary concentration of 3MT in horses was 214 ng/mL and the application of a threshold with a probability of 1 in 10,000 gave an actionable level of 4 μg/mL. Environmental variables were not forensically significant in determining the population distribution. HVA was not found to be a reliable indicator of dopamine or levodopa administration.
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1. Introduction

Biogenic amine is a generic term used to describe a group of simple organic bases that have a significant role as mediators of many physiological actions and responses. Some of these compounds are available as pharmaceutical preparations (e.g. dopamine, adrenaline or levodopa) and their use may contravene the rules of many sporting bodies. Although the compounds are endogenous, some jurisdictions also dictate that if they are detected at abnormal concentrations in a biological sample then the finding is prima facie evidence of the use of a prohibited practice. Such cases are normally enforced where the substance concerned may be tied to the alteration of performance.

It is possible to influence the continuous up-regulation of an amine's biosynthesis by oversupplying its precursor amino acid. The technique, known as precursor loading, has been used effectively in conventional medicine by administering levodopa for the treatment of Parkinsonism. In addition to the symptomatic relief of disease or psychological disturbances, the manipulation of amine levels in the human may be attempted to alter athletic or mental performance or for "recreational" or behaviour altering purposes.

The catecholamines dopamine, adrenaline and noradrenaline are all derived in vivo from the amino acid levodopa and have profound effects on a variety of physiological systems. In this regard, each compound may be considered as performance enhancing. Before attempting to derive chemico-legal methods for the regulation of their use, it is important that the compounds that are likely to be abused and the routes by which they are likely to be administered to the animal be appreciated.

The abuse of dopamine, adrenaline and noradrenaline is not considered particularly advantageous in performance sports because of the relatively short duration of their activity following administration. These compounds require intravenous administration to be effective because they are rapidly deactivated following oral administration [1]. The inability to sustain their actions for extended periods after administration also limits their usefulness in improving performance. Of more interest to those attempting to influence performance

* Corresponding author. Present address: SGE International Pty. Ltd., 7 Argent Place, Ringwood, Vic. 3134, Australia. Tel.: +61 3 9837 4200; fax: +61 3 9874 5672.
E-mail address: pwynne@sge.com (P.M. Wynne).

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is any practice that results in a slow release of dopamine, adrenaline or noradrenaline such that any stimulatory effect remains significant after several hours. Further, the success of such a practice requires that peak levels of the amines not be so high as to result in the onset of post-administration fatigue prior to competition.

In an otherwise healthy equine athlete, where self-administration is unlikely, manipulation of the biogenic amines is likely to be attempted to modify performance. This paper discusses the manipulation of endogenous dopamine in the horse and considers a method to allow its regulation in racing animals.

2. Experimental

2.1. Terminology and standards

Throughout the text, the symbols 3MT, DOPAC and HVA have been used to represent the urinary concentrations of free and bound 3-methoxytyramine, 3,4-dihydroxyphenylacetic acid and 3-methoxy-4-hydroxyphenylacetic acid, respectively. Standards of 3-methoxytyramine, 3,4-dihydroxyphenylacetic acid and 3-methoxy-4-hydroxyphenylacetic acid, 3-chloro-4-hydroxyphenylacetic acid, fenfluramine and β-glucuronidase (*Helix pomatia* E.C. 3.2.1.31) were purchased from Sigma Chemical Company (St. Louis, Missouri, USA). An alternative standard of 3-methoxytyramine hydrochloride, for use in the preparation of spiked control samples for method validation, was purchased from Fluka Chemie AG (Buchs, Switzerland).

2.2. Collection of survey samples

Horse urine samples ($n = 247$) for the first survey of the normal levels of 3MT and HVA were drawn from post-event urine samples collected from standardbred horses. All the samples had been stored at −20 °C for up to 7 months prior to analysis.

Horse urine samples for the second survey were drawn from post-event urine samples ($n = 5280$) from standardbred, thoroughbred and other horses. Samples were stored at 4 °C prior to analysis and analysed within one week of collection.

Horse urine samples for the third survey were drawn from post-event urine samples ($n = 875$) collected for routine drug screening in Australia and New Zealand. Australian samples were stored at 4 °C prior to analysis and analysed within one week of collection. New Zealand samples were stored at 4 °C for up to one week prior to shipment and remained in transit for 60–70 h in insulated containers but without refrigeration. On delivery, samples were stored at 4 °C and were analysed within 4 days of delivery.

Acid-preserved samples ($n = 50$) for the analysis of DOPAC were collected as an adjunct to the routine collection of post-event urine collection from thoroughbred and standardbred horses. The urine (40 mL) was diluted with 40 mL of aqueous hydrochloric acid (0.2 M) within 10 min of voiding. Treated urine samples were stored at 4 °C for up to 3 days while in transit and then at −20 °C until required for analysis. All samples were partial volumes of naturally voided urine and were drawn without favour from post-race urine samples collected for routine drug-testing purposes.

2.3. Extraction and analysis

2.3.1. 3-Methoxytyramine (3MT)

Aliquots of urine (3 mL), or blank surrogates spiked with 3-methoxytyramine to give concentrations of 0, 0.02, 0.20, 1.0, 5.0 and 20.0 μg/mL, were spiked with fenfluramine (internal standard) at a concentration of 1 μg/mL. Control samples were similarly prepared by spiking blank surrogates with an alternative source of 3-methoxytyramine at concentrations of 3.0, 4.0 and 5.0 μg/mL. All samples and spikes were diluted with 0.1 M potassium phosphate buffer (pH 6.0, 4.5 mL), adjusted to pH 6.2–6.3 and the diluted urine enzyme hydrolysed with β-glucuronidase (2500 IU) for 2 h at 50 °C. Extraction columns (Bond-Elut Certify™, 130 mg, 3 mL, Varian Inc., Harbor City, California, USA) were conditioned sequentially with methanol (2 mL), water (2 mL) and 0.1 M phosphate buffer (pH 6.0, 2 mL). Each sample was passed through a column followed by 0.1 M phosphate buffer (2 mL). The column was washed with 1 M acetic acid (2 mL) for pH adjustment and then dried with air at 200 mL/s for 3 min. The column was washed with methanol (2 mL) and again dried with air for 3 min. The base fraction was eluted with a 2 mL of a solvent mixture containing ethyl acetate/dichloromethane/2-propanol/concentrated aqueous ammonia ($\rho = 0.880$) in the ratios 5:4:1:0.02 (v/v), respectively.

The basic fraction was evaporated to dryness under a stream of nitrogen at a flow rate of 1 mL/min and a temperature of 20 °C and acetylated with pyridine–acetic anhydride (2:1, 100 μL) in a screw-capped culture tube at 80 °C for 20 min. The excess reagents were evaporated under a stream of nitrogen at 20 °C and the residues reconstituted in 100 μL of ethyl acetate prior to gas chromatography mass spectrometry (GCMS) analysis.

2.3.2. Homovanillic acid (HVA)

Aliquots of urine (1 mL) or blank surrogates spiked with 3-methoxy-4-hydroxyphenylacetic acid to give concentrations of 0, 1.0, 5.0, 20.0 and 100 μg/mL were spiked with 3-chloro-4-hydroxyphenylacetic acid (internal standard) to give a final urinary concentration of 10 μg/mL. The samples were made basic with the addition of sodium hydroxide solution (100 μL, 0.2 M) mixed and allowed to stand at room temperature for 30 min to effect hydrolysis. The samples were adjusted to pH 3 with the addition of aqueous hydrochloric acid (2 M) and extracted with hexane–dichloromethane–ethyl acetate (4:3:3, 1 mL). The organic phase was separated off, dried over anhydrous sodium sulphate and evaporated to dryness under nitrogen at 40 °C. The residues were treated with an ethereal solution of diazomethane containing 10%

by volume of methanol and the treated samples evaporated to dryness under a stream of nitrogen at 20 °C. The residues were reconstituted in 400 μL of ethyl acetate prior to GCMS analysis.

2.3.3. HVA and DOPAC in acid-preserved urine and DOPAC in unpreserved urine

Portions of urine (1 mL) or blank surrogates spiked with 3,4-dihydroxyphenylacetic acid at 0, 0.2, 2, 10 and 100 μg/mL or HVA at 0, 0.2, 2, 10 and 100 μg/mL were adjusted to pH 1. Samples and surrogates were spiked with 3-chloro-4-hydroxyphenylacetic acid to give a final urinary concentration of 10 μg/mL and then heated at 80 °C for 30 min in capped tubes. After hydrolysis, the samples were cooled and extracted with diethyl ether (2 mL) on a rotary mixer for 15 min at 60 rotations per minute. The ether was separated off, dried over anhydrous sodium sulphate and a 200 μL portion of the extract evaporated to dryness. The dried residues were treated with MSTFA (35 μL) and toluene (65 μL) in a sealed tube at 80 °C for 20 min and then the mixture was transferred to an autosampler vial for GCMS analysis.

2.3.4. Gas chromatography mass spectrometry analysis

Electron impact (EI)-GCMS was performed on a Hewlett Packard 6890 GC-5973 MSD equipped with a 7683 Autoinjector (Palo Alto, California, USA). The GC was equipped with a BPX5 column (12 m × 0.2 mm × 0.33 μm, SGE, Ringwood, Victoria, Australia) and used helium as the carrier gas with a constant flow of 1.0 mL/min. Injections of 2 μL were pulsed splitless with a nominal head pressure of 64 psi pulsed to 180 psi for 1 min after injection. The oven temperature was held at 75 °C for 2 min then heated at 30 °C/min to 300 °C with a final holding time of 9.0 min. The detector temperature was 280 °C and the injector temperature 250 °C. For the analysis of the acetylated basic fraction, a scan range of 50–650 Da at 2.48 scan/s was used. For the analysis of HVA as its methyl ester, the ions *m/z* 214, 200, 196, 155, 141 and 137 were scanned in selected-ion monitoring mode at a scan rate of 1.44 scan/s with a dwell time of 100 ms. For the analysis of DOPAC and HVA following silylation, the ions *m/z* 267, 326, 330 and 384 were scanned in selected-ion monitoring mode at a scan rate of 2.15 scan/s with a dwell time of 100 ms.

2.3.5. Data analysis

For the quantification of 3MT, the ions *m/z* 72 (fenfluramine base peak) and *m/z* 150 (3MT base peak) were extracted from the total ion chromatogram and the ratio of peak areas of 3MT to fenfluramine was calculated. The concentration of 3-methoxytyramine was interpolated from the standard curve ($r > 0.9990$). Linearity between 0.02 and 20 μg/mL 3MT was established by the extraction and analysis of independently spiked blank surrogate samples at the concentration mid-points. The limit of detection was determined following analysis of spiked blank surrogates to be 5 ng/mL. Precision was ±5% ($n = 10$ replicate extractions) and instrument precision was ±3% ($n = 10$ replicate injections). Degradation on storage at −20 °C for control specimens was determined to be <5% after 12 months. Enzyme efficiency was demonstrated by the analysis of control specimens that had been diluted with potassium phosphate buffer (pH 6.3, 0.1 M) to 40, 50, 60, 70, 80, 90 and 100% of their original concentration, that were incubated at 65 °C for 2 h or that had been dosed with 5000 or 10,000 IU of enzyme. Recovery of 3MT remained within ±5% regardless of the sample pretreatment. Control specimens were prepared from urine samples collected from animals that had been administered levodopa [2] and which had subsequently been diluted with blank urine to give a 3MT concentration of 4 μg/mL.

In each analysis, the identity of the target analyte was confirmed by matching its full scan mass spectrum with that of an acetylated standard of 3-methoxytyramine.

For the quantification of HVA as the methyl ester, the ions *m/z* 141 (methyl 3-chloro-4-hydroxyphenylacetate base peak) and *m/z* 137 (HVA methyl ester base peak), were extracted and the ratio of peak areas of *m/z* 137–141 was calculated. A typical example of the regression line equation was $y = 0.003907x - 0.00004907$ ($r = 0.9995$), where y is the peak area ratio of HVA to internal standard and x is HVA in μg/mL. The limit of detection was less than 100 ng/mL.

For the quantification of DOPAC and HVA as the TMS derivative, the ions *m/z* 330 (3-chloro-4-hydroxyphenylacetate base peak), *m/z* 384 (DOPAC) and *m/z* 326 (HVA) were extracted and the ratio of peak areas was calculated. The regression line equations were $y = 0.8331x - 0.001901$ ($r = 0.9986$) where y is the peak area ratio and x is the concentration of DOPAC in μg/mL and $y = 0.4501x - 0.00012$ ($r = 0.9987$), where y is the peak area ratio of HVA to internal standard and x is HVA in μg/mL. The method was linear between 0 and 100 μg/mL for both analytes with detection limits of <100 ng/mL for DOPAC and <50 ng/mL for HVA determined following the extraction and analysis of spiked surrogate samples.

Correlation coefficients ($\rho_{x,y}$) between populations were calculated using the correlation analysis function in Excel 97 (Microsoft Corporation, USA). The standard variances are given by $\sigma_x^2 = (1/n)\ \Sigma\ (x_i - \mu_x)^2$ and $\sigma_y^2 = (1/n)\ \Sigma\ (y_i - \mu_y)^2$ and the correlation coefficient is given by $\rho_{x,y}$ = covariance $(x,y)/\sigma_x\sigma_y$. Significant correlation between populations is indicated for values of $|\rho| \approx 1$.

Estimations of normality were carried out using the Minitab 9 (Minitab Inc., Pennsylvania, USA) Ryan-Joiner correlation test for normality.

Significant differences between means for populations with estimable but unequal variances were determined for the $\mu_x = \mu_y$ hypothesis using the *t*-statistic according to the probability condition that $P(|T| < t_\alpha)$ where $T = (X_x - X_y)/\sqrt{((S_x^2/n_x) + (S_y^2/n_y))}$. The number of degrees of freedom was calculated from $v = ((S_x^2/n_x) + (S_y^2/n_y))^2/((S_x^2/n_x)^2/(n_x - 1) + (S_y^2/n_y)^2/(n_y - 1))$ using the method described by Walpole and Myers [3]. One-way analysis of variance calculations used Excel 97.

3. Results and discussion

Levodopa was rumoured to have been the subject of abuse in horse racing. In response, Canadian researchers attempted to detect levodopa, its catechol metabolites and carbidopa in horse plasma and urine samples following the administration of levodopa [4,5]. Subsequent studies [2,6] have also found that 3MT, HVA and DOPAC are elevated in the urine of horses that have been administered levodopa and dopamine. While the administration trials demonstrated that urine is an appropriate sample in which to detect levodopa metabolites they do not provide a means of regulating the use of levodopa (or dopamine) in horses without addressing issues of endogenous metabolite concentrations.

3.1. Urinary 3-methoxytyramine distribution in the horse

To investigate the possibility that 3MT or HVA could be used as threshold substances in equine urine, a preliminary survey of 247 standardbred horses was undertaken. The metabolite concentrations were determined in the samples and it was found that a logarithmic transformation of both gave approximately normal distributions. The means and standard deviations for the population are given in Table 1 and, on the basis of these statistical parameters, both compounds were considered as potentially useful threshold substances.

The preliminary study drew on only a small number of individuals and did not address the influence of possible variables including long-term sample storage, geographic range and season. Accordingly, a more extensive survey was undertaken of over 5000 urine samples collected from standardbred, thoroughbred and other horses in several states. Analyses were undertaken when the samples were fresh and therefore subject to the minimum of bacterial or thermal degradation. Again, the 3MT concentrations were determined and a logarithmic transformation of the data gave an approximately normal distribution (Fig. 1). The division of this population into various sub-groups based on breed and state of origin also produced distributions which were normal ($p < 0.05$) or very nearly so.

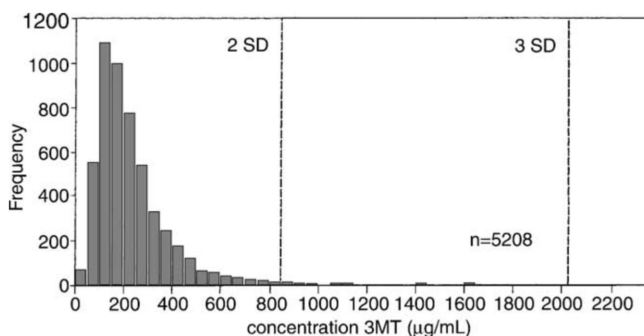

Fig. 1. The distribution of urinary 3-methoxytyramine in Australian horses sampled during the second survey.

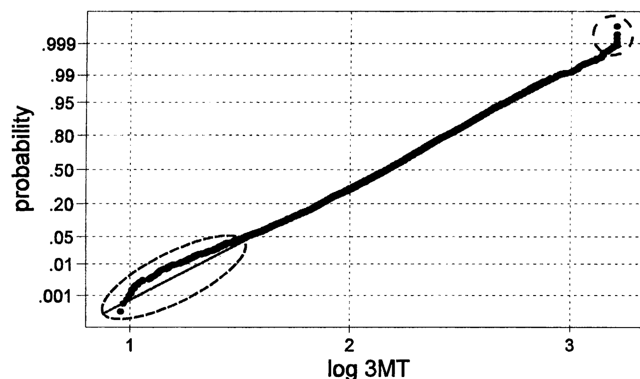

Fig. 2. Normal probability plot and Ryan-Joiner test for 3MT in horses sampled during the second survey. Areas enclosed by the dashed lines indicate deviations from the normal distribution.

The larger data set gave an untransformed mean approximately 40 ng/mL lower than that determined in the first study although other population parameters were comparable. In all cases, the differences between the various sub-groups were relatively small (means differ by <50 ng/mL) and of apparently little practical or forensic significance.

A normal probability plot for the population of all horses, using the Ryan-Joiner method, is shown in Fig. 2. Slight deviations from the normal distribution were observed for samples with a 3MT concentration of less than 25 ng/mL or greater than approximately 1 μg/mL. While the numbers of samples within these groups was very small relative to the total population (less than 5% of all values), they were observed across all sub-populations.

Of those samples with 3MT concentrations less than 25 ng/mL, many were also low in other endogenous urinary bases. Such an observation may be attributed to two possible causes. Either the enzyme system, which is normally sufficient, had failed to hydrolyse conjugate species or, the urine was excessively diluted. As the low concentration samples were randomly distributed through a population tested over a period of 12 months, it is likely that any failure of the enzyme system was non-systematic.

The 3MT concentration showed a small trend to higher values with increasing urinary pH, although this effect was not statistically significant (Table 1). Further, the mean urinary pH for samples with a 3MT concentration below 25 ng/mL was not significantly different to that of samples of greater than 25 ng/mL (see Table 2). On this basis, it appears reasonable to conclude that urinary alkalinisers do not play a significant role in yielding unexpectedly low 3MT values.

The resistance of certain urine samples to enzyme hydrolysis and the propensity for others to auto-oxidation has been described before [7] and may account for many of these low values. These samples, comprising less than 3% of the total population, may therefore be regarded as a separate population in which the 3MT concentration is influenced by the administration of some other substance (resulting in increased diuresis) or incomplete hydrolysis prior to extraction. The remaining samples in this group having a 3MT concentration of

Table 1
Urinary 3MT and HVA concentrations in Australian horses on the basis of breed, state of origin, urinary pH and gender

Population	3MT				HVA			
	n	log 3MT mean	log S.D.	Untransformed mean	*n*	log HVA mean	log S.D.	Untransformed mean
First survey								
	247	2.2527	0.3694	179	247	0.6403	0.4612	4.4
Second survey								
Breed								
Standardbred	2086	2.1101	0.3840	129[a]	921	0.7863	0.3711	6.1[a]
Thoroughbred	2818	2.1687	0.3887	149[a]	1274	0.8311	0.3687	6.8[a]
Pleasure horse	376	2.0913	0.4065	123[a]	113	0.9941	0.4096	9.8[a]
State of origin								
Victoria	3501	2.1534	0.3681	142[a]	1503	0.8172	0.3796	6.6
South Australia	1168	2.0552	0.5191	113[a]	510	0.8303	0.3706	6.8
Tasmania	408	2.0868	0.4313	122[a]	185	0.8205	0.3214	6.6
Unspecified	131							
pH								
3	1	1.6854		49	0			
4	24	2.0607	0.3784	115	10	0.6262	0.5379	4.2
5	170	2.1197	0.4621	132	82	0.7336	0.3740	5.4
6	564	2.1448	0.3998	140	245	0.7829	0.3777	6.1
7	1285	2.1252	0.3976	133	591	0.8250	0.3510	6.7
8	2034	2.1621	0.3593	145[a]	864	0.8346	0.3760	6.8
9	915	2.1474	0.3585	140	385	0.8353	0.3899	6.8
10	88	2.1791	0.4038	148	51	0.7739	0.2641	5.9
11	7	2.2348	0.1597	172	4	0.8437	0.2854	7.0
Not measured	120				76			
Greater than 6	4893	2.1238	0.4476	133	2140	0.8195	0.3703	6.6
Gender								
Female	1484	2.1412	0.3854	138	617	0.8383	0.3764	6.9
Gelding	3054	2.1443	0.3881	139	1367	0.8087	0.3721	6.4
Male	537	2.1668	0.3736	147	252	0.8269	0.3571	6.7
Unspecified	205				72			
All horses	5280	2.1401	0.3893	138	2308	0.8212	0.3743	6.6

Untransformed means refer to the logarithm transform mean transposed back to a concentration.
[a] Denotes groups which differ significantly within categories ($p < 0.05$).

less than 25 ng/mL gave a normal basic urinary profile and are therefore likely to be true outliers in the normal population.

At the opposite end of the distribution, the number of samples in the range 1–2 μg/mL is slightly in excess of that predicted for a normal distribution. Often, these samples also showed increased levels of other compounds derived from pasture species or dietary supplements. It is therefore likely, that a small sub-population of samples is also observed in which 3MT is elevated, albeit slightly, by the addition of some exogenous source of dopamine or one of its precursors. Whether these components were administered deliberately as ergogenic aids or found their way into individual feeds as random contaminants remains unknown.

Table 2
The distribution of samples with low (<25 ng/mL) 3MT concentrations

3MT (ng/mL)	*n*	% Population	pH	S.D.
Thoroughbred[a]				
<25	217	8	7.14	1.17
>25	2697	92	7.39	1.13
All	2914		7.38	1.14
Standardbred[a]				
<25	159	8.1	7.92	1.01
>25	1957	91.9	7.91	0.94
All	2116		7.91	0.94

The low values include both those predicted by the normal population and the unexpected number of low values resulting from other causes.
[a] Breeds.

In retrospect, these sub-groups were entirely predictable but, importantly, they remain of little forensic significance in determining the population mean and had only a minor impact on the approximation to the normal distribution.

The division of the population into sub-populations on the basis of breed, state of origin, urinary pH and gender also produced logarithmically normal distributions. Statistical tests for differences between the various sub-groups showed that in some cases the differences were significant ($p < 0.05$) (see Table 1). Season variations, measured by plotting the movement in the weekly mean (see Fig. 3), also showed some differences when the values were subjected to an analysis of variance. However, for all the variables studied, these

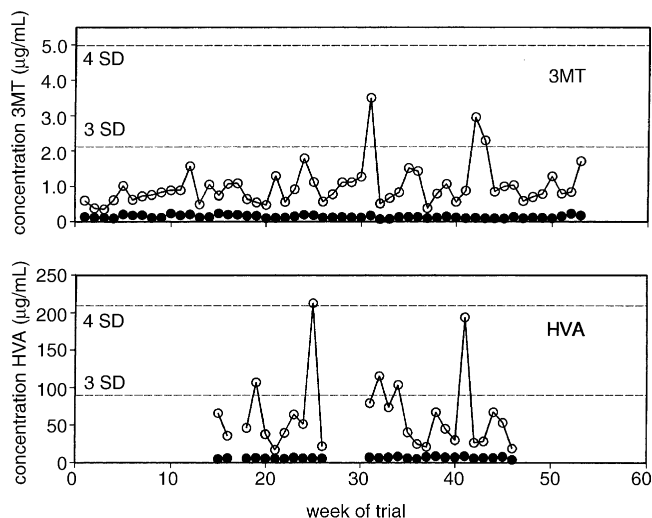

Fig. 3. Weekly mean (closed circles) and maximum (open circles) 3MT and HVA concentrations for Australian horses sampled during the second survey.

differences were generally small and of no practical or forensic significance with means differing by less than 50 ng/mL.

To address the possibility of regional bias, a third study was undertaken and the population was expanded to include horses competing in Australia (n = 620) and New Zealand (n = 261). The 3MT concentrations were measured and the population parameters determined following a logarithmic transformation of the data. Ryan-Joiner tests for normality indicated that both the Australian and New Zealand sub-populations were approximately normal with correlation coefficients of $\rho = 0.9865$ and 0.9855, respectively. The slight deviations from normality at the extremes of the distribution that had been observed in the earlier survey were also evident in both sub-populations. The population statistics for the New Zealand and Australian sub-populations are shown in Table 3.

Both the New Zealand sub-population and the first survey population show a slight bias towards higher concentrations but this effect is only apparent when comparing the transformed population means. Both groups were comprised of samples that had been subjected to degradative stress during transport or storage. The first group had been frozen for up to 7 months prior to analysis and the New Zealand samples were also subjected to thermal stress while in transit. It is therefore probable that both groups were degraded prior to analysis and it is noteworthy that many of the New Zealand samples typically exhibited a strong odour and showed the

Table 3
Urinary 3MT concentrations in Australian and New Zealand horses on the basis of breed and state of origin

Population	n	log 3MT mean	log S.D.	Untransformed mean	Urinary pH mean	Urinary pH S.D.
Third survey						
All horses						
All	875	2.1949	0.4180	157	7.9	1.3
Standardbred	361	2.1687	0.3887	148	8.1	1.2
Thoroughbred	514	2.1469	0.5103	140	7.7	1.4
Victoria						
All	432	2.1534	0.3681	143	7.8	1.2
Standardbred	152	2.0970	0.5626	125	8.1	1.1
Thoroughbred	280	2.2175	0.4288	165[a]	7.7	1.3
South Australia						
All	127	2.1391	0.3682	138	7.6	1.4
Standardbred	50	2.0883	0.4488	123	8.2	1.2
Thoroughbred	77	2.1721	0.3034	148	7.3	1.4
Tasmania						
All	61	2.1539	0.4529	143	7.3	1.1
Standardbred	46	2.1820	0.4452	152	7.4	1.1
Thoroughbred	15	2.1141	0.3624	130	7.1	1.1
Australia						
All	620	2.1560	0.4354	143	7.7	1.2
Standardbred	248	2.1002	0.5069	126	8.0	1.1
Thoroughbred	372	2.1788	0.3996	151	7.5	1.3
New Zealand						
All	255	2.2639	0.3716	183[a]	8.3	1.4
Standardbred	113	2.2525	0.3968	178[a]	8.3	1.4
Thoroughbred	142	2.2776	0.3546	189[a]	8.2	1.5
Other surveys						
First survey	247	2.2527	0.3694	179[a]	8.0	1.0
Second survey	5280	2.1401	0.3893	138	7.6	1.1

Untransformed means refer to the logarithm transform mean transposed back to a concentration.
[a] Denotes groups which differ significantly ($p < 0.025$).

Table 4
Urinary 3MT concentrations in Australian and New Zealand horses on the basis of urinary pH

pH	n	log 3MT mean	log S.D.	3MT untransformed mean
New Zealand horses				
3	1	2.8334		681
4	1	2.5651		367
5	9	2.3200	0.3951	208
6	17	2.3700	0.2675	234
7	49	2.3700	0.3413	234
8	55	2.2900	0.4184	195
9	56	2.1910	0.4011	155[a]
10	72	2.1200	0.4963	131[a]
11	1	1.9224		84
Australian horses				
3	3	2.1524	0.3909	142
4	1	2.5166		329
5	39	2.0520	0.4243	113
6	43	2.2324	0.3464	171
7	144	2.1473	0.4726	140
8	206	2.1164	0.4478	131
9	166	2.1978	0.4166	158
10	18	2.2268	0.5095	169

Untransformed means refer to the logarithm transform mean transposed back to a concentration.
[a] Denotes groups which differ significantly from pH 7 mean ($p < 0.025$).

presence of a higher than normal level of putrefactive bases during routine analysis.

An increase in pH is one indicator of the bacterial degradation of equine urine. It is therefore not unexpected that the New Zealand samples had a mean urinary pH approximately half a pH unit higher than that observed for all local samples in the same group. A division of both populations on the basis of urinary pH showed that the New Zealand samples had a higher 3MT levels for samples below pH 8.5 and it is within this range that conditions for putrefactive processes would be more active (see Table 4). In contrast, Australian samples did not show a significant relationship between urinary pH and 3MT concentration. Again, while statistically anomalous, these differences were small and had little practical influence on the population distribution.

3.2. Urinary homovanillic acid distribution in the horse

The population distributions for HVA concentrations in the first and second survey groups are summarised in Table 1. As with the 3MT populations, logarithmic transformation of the HVA data gave approximately normal distributions that showed a slightly higher than expected number of outlying samples at both the upper and lower ends of the distribution (see Fig. 4). The outlier groups in the equine HVA population may also be readily attributed to limitations in hydrolysis of some samples, samples diluted by induced diuresis and by exogenous sources of HVA or its precursor DOPAC.

The sub-division of the population on the basis of breed, state of origin and gender also produced logarithmically normal distributions. While differences were generally small

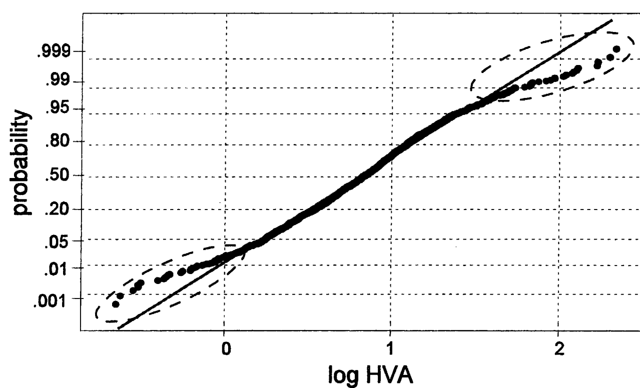

Fig. 4. Normal probability plot and Ryan-Joiner test for HVA in horses sampled during the second survey. Areas enclosed by the dashed lines indicate deviations from the normal distribution.

(see Table 1) they had minimal impact on the sub-group means or distributions.

Sub-populations created on the basis of pH were also normal. Some pH dependence was suggested by the lower mean for samples with a pH < 6. As 3MT appeared to be reduced over the same range (see Table 1), the effect is more indicative of increased urine volume rather than a function of reduced HVA solubility at low pH. The number of acidic samples made up less than 5% of the HVA population and had demonstrably little impact on the population.

The correlation coefficients calculated between the raw and logarithm transform data pairs for 3MT and HVA were $\rho = 0.049$ and 0.276, respectively. As has been observed in the human [8], no significant correlation was observed between the concentrations of the two metabolites.

As for 3MT, an analysis of variance for HVA showed some small seasonal variations (Fig. 3). It is also noteworthy that the mean concentration of HVA was approximately 2 μg/mL higher in the second survey than in the preliminary study. The reason for the differences is unclear but may be due to HVA degradation on storage of unpreserved samples.

3.3. Urinary DOPAC distribution in the horse

Like 3MT, DOPAC is a primary metabolite of dopamine and is likely to be influenced by dopaminergic manipulation. However, unlike the 3-*O*-methoxy metabolites, DOPAC is a catechol and may be lost through oxidative pathways from unpreserved urine. To generate a statistically sound data set to describe the DOPAC population, a separate trial was undertaken in which 50 acid-preserved urine samples were collected from standardbred ($n = 25$) and thoroughbred ($n = 25$) horses.

The HVA and DOPAC concentrations were determined in each sample and the population statistics are shown in Table 5. A strong correlation between the paired concentration data was found with a correlation coefficient of $\rho = 0.967$. Ryan-Joiner tests of the logarithm transformed data again showed a high correlation with a normal distribution for both DOPAC

Table 5
DOPAC and HVA concentrations in acid-preserved urine collected from Victorian horses determined within 3 months of sample collection after storage at −20 °C

Analyte	*n*	log mean	log S.D.	Untransformed mean (ug/mL)	Thresholds	
					Mean + 3S.D. (ug/mL)	Mean + 4S.D. (ug/mL)
DOPAC	50	0.8171	0.3565	6.6	77	175
HVA	50	0.8112	0.2766	6.5	45	83

and HVA (r = 0.9803 and 0.9564, respectively). As HVA is a primary metabolite of DOPAC and both compounds occur as endogenous urinary components, the strong correlation is not unexpected.

A value four standard deviations from the mean DOPAC concentration (6.9 μg/mL) gives a nominal threshold of 64 μg/mL and, as for 3MT, DOPAC in excess of this level may be attributed to some exogenous source of DOPAC or one of its metabolic precursors.

Unpreserved samples that had been stored at −20 °C for periods of up to 7 months showed little or no evidence of DOPAC. Given that no DOPAC free samples were found during the acid-preserved urine survey, the loss from stored samples indicated that it was destroyed on storage, even when the sample was frozen. Retesting of acid-preserved urine samples also showed that DOPAC was not protected from degradation for periods in excess of 6 months, even when the samples were stored at −20 °C.

Unlike 3MT and HVA, which are relatively stable to oxidation, oxidative loss of DOPAC is likely to limit its usefulness as a regulatory substance. Thus, while its detection in urine at abnormally high levels may be indicative of dopaminergic manipulation, its absence or detection at low levels cannot be considered to demonstrate the absence of manipulatory practices.

3.4. Theoretical limitations

Previous studies [2,6,7] have noted that when levodopa was administered to horses there was a simultaneous and dose dependant increase in 3MT and DOPAC excretion. In contrast, HVA first increased and then decreased to near pre-administration levels while the concentration of its precursors continued to rise.

The effect is readily explained in an environment where the systemic concentration of dopamine is significantly elevated by an exogenous precursor and it competitively inhibits both the 3-catechol-*O*-methyltransferase (3-COMT) and monoamine oxidase (MAO) pathways. That is, dopamine is capable of displacing DOPAC from 3-COMT sites and 3MT from MAO sites. In both cases, as the affinity of each compound for its particular deactivating enzyme is determined by the relative concentrations of all competing substrates, the effect is observed across a relatively wide range of DOPAC and 3MT concentrations.

Inhibition of this type has serious implications for the use of HVA as a threshold substance. As its formation from either DOPAC or 3MT is likely to be suppressed by high dopamine concentrations, HVA is less likely than 3MT or DOPAC to be significantly elevated following successful dopaminergic manipulation.

4. Conclusion

The population studies have shown that the post-race 3MT level in horses approximates a logarithmic normal distribution. On this basis, a mean urinary concentration of 138 ng/mL and a threshold of 4 μg/mL (which represent a probability level of approximately 1 in 10,000) was calculated for 3MT. Urinary concentrations in excess of the threshold are abnormal and influenced by exogenous sources of dopamine, levodopa or their precursors.

The net effect of outlier groups on the 3MT distribution was to increase the standard deviation and so shift the threshold to a higher value. While statistically anomalous, the only forensic significance of these groups was to increase the safety margin of the proposed threshold such that genuine dopaminergic manipulation could be detected but inadvertent effects arising from feed and pasture composition would remain unremarkable.

Only the acid metabolite DOPAC appears useful as an alternative threshold substance to 3MT but it is limited by its instability in urine.

The statistical parameters that have been described provide a basis for determining when an individual excretes 3MT at a concentration that is abnormal in comparison to the population. The ability to measure an abnormal state, in the absence of pathological abnormalities, is the primary requirement for establishing a threshold value that may be enforced in law. Having established the scientific basis for measuring indicators of dopaminergic manipulation, regulation of the practice can only be achieved with appropriate amendments to the laws governing racing sports.

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