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Control of the misuse of bromide in horses

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Bromide is a sedative hypnotic. Due to its potential use as a sedative or calmative agent in competition horses, a method to control bromide is needed. Colorimetric method had been employed in the authors' laboratory from 2003 for the semi-quantification of bromide in equine plasma samples. However, the method was found to be highly susceptible to matrix interference, and was replaced in 2008 with a more reliable inductively coupled plasma-mass spectrometry (ICP/MS) method. Equine plasma was protein-precipitated using trichloroacetic acid, diluted with nitric acid, and then submitted directly to ICP/MS analysis.

Since bromide is naturally occurring in equine plasma, a threshold is necessary to control its misuse in horses. Based on population studies (n = 325), a threshold of 90 μg/mL was proposed (with a risk factor of less than 1 in 10 000). Using the ICP/MS screening method, equine plasma samples with bromide greater than 85 μg/mL would be further quantified using the more accurate ICP/MS standard addition method. Confirmation of bromide was achieved by gas chromatography-mass spectrometry (GC-MS), with the bromide detected as its pentafluorobenzyl derivative. A sample is considered positive if its plasma bromide concentration exceeds the threshold (90 μg/mL) plus the measurement uncertainty of the quantification method (8 μg/mL at 99% 1-tailed confidence level) and its presence is confirmed using the GC-MS method.

Following oral administration of potassium bromide (60 g each) to two geldings, plasma bromide levels peaked after approximately 2 hours at about 300 μg/mL, and then remained above the threshold for 8 and 13 days respectively.

Keywords: bromide; inductively coupled plasma-mass spectrometry; plasma; horse; threshold; doping control

Introduction

Bromide was the first agent to be specifically introduced as a sedative and soon thereafter as a hypnotic.[1] The principal pharmacological effect of bromide is to produce drowsiness and promote sleep. The mechanism of its action is uncertain, but is thought to be due to its depressant effects on neuronal excitability and activity. Bromide is believed to compete with chloride for access to the brain, lowering the electrical activity in the central nervous system, thus making initiation of seizure more difficult. Potassium bromide (KBr) was used to treat human epilepsy as early as 1857 and remains a recommended treatment for refractory (difficult to control) canine epilepsy.[2–6] KBr has almost complete bioavailability, and its half-life in human serum is about 9–12 days, unless special effort is made to increase the bromide excretion, for example, by increasing chloride intake. Due to its potential use as a sedative or calmative agent in competition horses, a method to control bromide is needed.

Colorimetry has been used to measure bromide in human or equine plasma.[7,8] This involves the formation of an orange-coloured gold complex by adding gold (III) chloride to a protein-free plasma sample. The absorbance of the reaction mixture at 450 nm can be correlated with bromide concentration using a multi-point linear regression calibration curve. This colorimetric method had been employed in the authors' laboratory from 2003 for the semi-quantitative determination of bromide in equine plasma samples. However, the method was found to be highly susceptible to matrix interference, and was replaced in 2008 with a more reliable inductively coupled plasma-mass spectrometry (ICP/MS) method.

ICP/MS is by far the best technique for quantifying elements other than C, H, O, F and the inert gases in biological samples. Besides its high sensitivity and fast turnaround time, another major advantage of ICP/MS is its selectivity, which allows blood (as well as urine) to be analyzed directly only after a simple dilution step with acid.[9] This research article describes two ICP/MS methods for the screening and quantification of bromide in equine plasma. Equine plasma was first protein-precipitated. An aliquot of the supernatant was then diluted with nitric acid and submitted directly to ICP/MS analysis. The approximate bromide concentration was determined from a multi-point linear regression calibration curve using Germanium (Ge) as the internal standard. A more accurate ICP/MS quantitative determination of bromide could be achieved by means of standard addition. A set of calibrators was prepared by adding known amounts of bromide to aliquots of a test sample. The bromide concentration in the test sample was determined by extrapolation from the standard addition calibration curve.

Bromide is ubiquitous in nature and present in all feedstuffs. Bowen[10] has reported that the average bromide content in soil is 5 ppm and that in land plants 15 ppm. Other dietary sources of bromide include salt prepared from brine. As bromide is naturally occurring in equine plasma, a threshold is necessary to control its misuse in horses. With a threshold set, any equine plasma sample is deemed to be positive for a prohibited substance if its bromide

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concentration determined accurately exceeds the plasma bromide threshold plus the measurement uncertainty of the quantification method, and if its presence can be confirmed using an unequivocal confirmation method. Numerous methods have been reported for the qualitative identification of bromide in biological samples using gas chromatography (GC),[11–13] liquid chromatography (LC),[14–16] capillary electrophoresis[17] and ICP/MS.[18] These methods, however, do not meet the requirements stipulated in the Association of Official Racing Chemists (AORC) *Guidelines for the Minimum Criteria for Identification by Chromatography and Mass Spectrometry*,[19] the criteria adopted by most racing laboratories. For this reason, a definitive GC-MS method based on the confirmation of bromide as its pentafluorobenzyl derivative[20–22] was adopted.

Materials and Methods

Materials

A reference standard solution of bromide (Br) was obtained from High Purity Standards (Charleston, SC, USA). The certified value of the bromide concentration was traceable to the Standard Reference Material (SRM) 3184 of the National Institute of Standards and Technology (NIST). Potassium bromide for administration trials, germanium (Ge) reference standard, and Triton-X (Ultra) were purchased from Sigma-Aldrich (St Louis, MO, USA). Suprapur grade of nitric acid (65%), trichloroacetic acid (pro analysi grade) and sodium chloride (NaCl) (pro analysi grade) were obtained from Merck (Darmstadt, Germany). High purity deionized water was obtained from a Milli-Q Element A10 water purification system (Milli-Q, Molsheim, France). Gold chloride was obtained from International Laboratory (San Bruno CA, USA). Pentafluorobenzyl *p*-toluenesulfonate (PFB-OTs) was purchased from Tokyo Kasei (Tokyo, Japan).

Working standard solutions

The working standard solutions of Br and Ge were prepared from the respective reference standard solutions from High Purity Standards and Sigma-Aldrich by dilution with 5% (*v*/*v*) nitric acid. Only plastic containers and labware were used for all ICP/MS analyses.

Sample preparation for ICP/MS analyses

Blood samples were centrifuged at 3000 rpm (~1650 g) for 10 min. Germanium standard solution (1 μg) was added as an internal standard to 100 μL of plasma, followed by addition of deionized water to make up to a total volume of 500 μL. The concentration of internal standard in a plasma sample was equivalent to 10 μg/mL. The mixture was deproteinated by the addition of trichloroacetic acid (1 mL; 10 g trichloroacetic acid and 120 mg NaCl in 100 mL deionized water). The deproteinated plasma was left standing at room temperature for 15 min and then centrifuged at 2000 rpm (~750 g) for 5 min. The supernatant (50 μL) was transferred to an ICP/MS autosampler tube (a 4-mL polypropylene tube) using a calibrated micropipette. The sample was then diluted with 5% nitric acid to give a total volume of 2 mL. The diluted sample was then infused *via* an autosampler to the ICP/MS.

Sample preparation for confirmation by GC-MS

Blood samples were centrifuged at 3000 rpm (~1650 g) for 10 min. An aliquot of plasma (50 μL) was added into a mixture of 100 μL phosphate buffer (0.5 M, pH 6.8) and 500 μL pentafluorobenzyl *p*-toluenesulfonate (PFB-OTs; 0.1 M in acetone). The solution of PFB-OTs was prepared by dissolving 1.76 g of PFB-OTs in 50 mL acetone. The mixture was vortexed for 1 min and incubated at 80 °C for 30 min. After cooling to room temperature, *n*-hexane (500 μL) was added. The mixture was then vortexed for 1 min and centrifuged at 3000 rpm (~1650 g) for 3 min. The supernatant (1 μL) was then injected into a GC-MS for analysis.

Sample preparation for colorimetric analyses

Blood samples were centrifuged at 3000 rpm (~1650 g) for 10 min. An aliquot of plasma (200 μL) was deproteinated by the addition of trichloroacetic acid (2 mL; 10 g trichloroacetic acid and 120 mg NaCl in 100 mL deionized water). The deproteinated plasma was left standing at room temperature for 15 min and then centrifuged at 2000 rpm (~750 g) for 5 min. The supernatant (1 mL) was then pipetted out and mixed with 0.25 mL of aqueous gold chloride solution (5 mg/mL). The absorbance of the resulted mixture was measured at 450 nm.

Instrumentation

ICP/MS analyses were performed on an Agilent 7500ce inductively coupled plasma mass spectrometer equipped with a G3160A integrated autosampler and a MicroMist nebulizer (Santa Clara Agilent Technologies, CA, USA). GC-MS analyses were performed on an Agilent 6890N Network GC system coupled to an Agilent 5973 Network Mass Selective Detector (Santa Clara Agilent Technologies, CA, USA). The absorbance was measured using microplate reader from Tecan (Männedorf, Switzerland).

ICP/MS conditions

An RF power of 1400 W was employed. The argon carrier gas flow rate was set at 1.05 L/min. The spray chamber temperature was set at 2 °C. Helium (4.0 mL/min) was used as the collision gas. The peristaltic pump speed was set at 0.2 revolution per sec (rps) during analysis. The sample uptake rate was about 0.8 mL/min, and the sample uptake time was set at 30 sec. The isotopes to be monitored for Br and Ge were *m/z* 79 and *m/z* 72 respectively. All data acquisitions were performed in 'spectrum analysis' mode with triplicate measurements. 'Peak area integration' mode was used, and the integration time per mass was 3 s for Br, and 1 s for Ge. The total acquisition time per sample was about 4 min. After each injection, the autosampler probe was rinsed with deionized water for 20 s in the rinse port and 40 s in the rinse vial, followed by intelligent rinse with 0.07% Triton-X for a maximum of 100 s to minimize carry over. The autosampler probe was finally rinsed with deionized water for 50 s before the next infusion.

GC-MS conditions

GC separation was performed on an EC-WAX column (30 m × 0.25 mm, 0.25 μm film thickness) with a constant helium flow of 1.2 mL/min. The oven temperature was set initially at 50 °C for 1.0 min, increased to 260 °C at 20 °C/min and held there for 10 min. Samples (1 μL) were injected at 220 °C in the split mode with a split ratio of 20 : 1. All GC-MS analyses were performed in the EI mode with full-scan acquisition.

Calibrators and quality control samples for ICP/MS analyses

Screening of bromide

A calibration curve was established by analysing a set of bromide calibrators at concentrations of 0, 40, 80, 120, 160 and 200 μg/mL in deionized water. Quality control (QC) samples at 90 μg/mL were prepared in duplicate by spiking bromide standard to a blank plasma sample. The calibrators, QC samples, and its corresponding blank plasma were analyzed alongside each batch of plasma samples using identical procedures. The calibrators and QC samples were prepared using Br working standard solutions prepared separately. The peak area ratios of bromide to the internal standard (Ge) versus the spiked concentrations of the calibrators were fitted using linear regression to obtain the calibration curve. Concentrations of bromide in the test samples were interpolated from the calibration curve using standard ChemStation quantification software. For the QC samples, the recovered concentration of bromide used for comparison with the spiked concentration was derived by subtracting the concentration of the corresponding blank plasma from the total concentration determined.

Quantification of bromide

The suspicious plasma sample was quantified in duplicate using standard addition. A QC sample was prepared by spiking bromide standard to deionized water to give a final concentration equivalent to 90 μg/mL of bromide. Portions of the quality control sample were transferred to capped plastic tubes and kept in a refrigerator at 4 °C. One portion was to be run in parallel with each batch of samples. Bromide standards were added to six aliquots each of a test sample and QC sample, in duplicate, to give equivalent bromide spiked concentrations of 0, 40, 80, 120, 160, 200 μg/mL. Each sample aliquot was analyzed according to the procedures stated. The working Br standard solutions for preparing the QC sample and for adding to sample aliquots were prepared separately. The peak area ratios of bromide to the internal standard (Ge) versus the spiked Br concentrations were fitted using linear regression to obtain the calibration curve. The concentrations of bromide in the test samples and QC samples were extrapolated from their corresponding standard addition calibration curves using standard ChemStation quantification software.

Statistical analysis

Statistical analysis was performed with Minitab computer software version 13.32 (2000) (State College Minitab Inc., PA, USA). The Kolmogorov-Smirnov normality test was used to compare the observed frequencies with the calculated distribution.

Drug administration experiments

Potassium bromide (60 g each) was administered to two thoroughbred geldings by stomach tubing. Blood samples were collected before administration and at 0.5, 1, 2, 4, 6, 8, 10, 12 and 24 h post administration on Day 1, then at least once on Day 2, 3, 4, 5, 7, 9, 11, 14, 18, 27, 41 and 43. Blood samples were centrifuged upon receipt and the corresponding plasma samples were kept at below −60 °C until analysis.

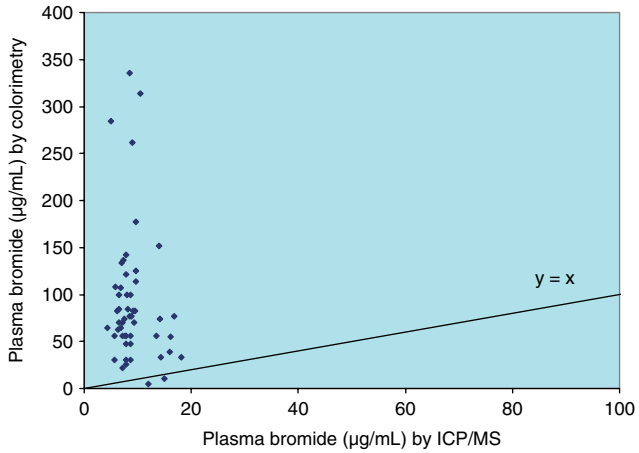

Figure 1. Comparison of plasma bromide (n=52) estimated by colorimetry and ICP/MS.

Results and Discussion

Screening of bromide in equine plasma by ICP/MS

In the authors' laboratory, the screening of bromide in equine plasma had been performed since 2003 by means of colorimetry. It involved the formation of an orange-coloured gold complex by adding gold (III) chloride to a protein-free plasma sample. The bromide concentration was determined from the absorbance of the reaction mixture at 450 nm using a multipoint linear regression calibration curve. However, the colorimetric method was not sensitive enough to endogenous levels of bromide and highly susceptible to matrix interference, and was replaced in 2008 with a more reliable ICP/MS screening method. Figure 1 shows the correlation of endogenous bromide in equine plasma (n = 52) as determined by the colorimetric and ICP/MS screening methods. The results show that the colorimetric method can have a high positive bias for plasma bromide.

The sample preparation procedures for the ICP/MS screening method were similar to those of the colorimetric method. Equine plasma was first deproteinated with trichloroacetic acid and the supernatant was then analyzed by ICP/MS after dilution with nitric acid. Ge was chosen as the internal standard for the ICP/MS method because there was essentially no interference with bromide. In addition, Ge (72 Da) is not normally present in horse blood, and has a mass close to that of Br thus minimizing possible mass-dependent gain of the detector. The bromide concentration in a plasma sample was interpolated directly from the multipoint linear regression calibration curve constructed using water spiked with bromide at 0, 40, 80, 120, 160 and 200 μg/mL. The correlation coefficients of the calibration curves were greater than 0.99 on all occasions (n = 50). The bromide threshold in equine plasma was established from a population study of 325 samples, comprising 229 post-race horse samples (local = 223; overseas = 6) and 96 samples from horses in training. Figure 2A shows the distribution of plasma bromide in the 325 equine plasma samples. The mean ± SD was determined to be 12.2 ± 8.3 μg/mL, which was higher than the average Br concentration reported in human blood of 5.3 ± 1.4 μg/mL.[23] This set of data has a skewed distribution and cannot be used directly to establish a threshold. A normal distribution was obtained after a one-fortieth power transformation (Figure 2B).

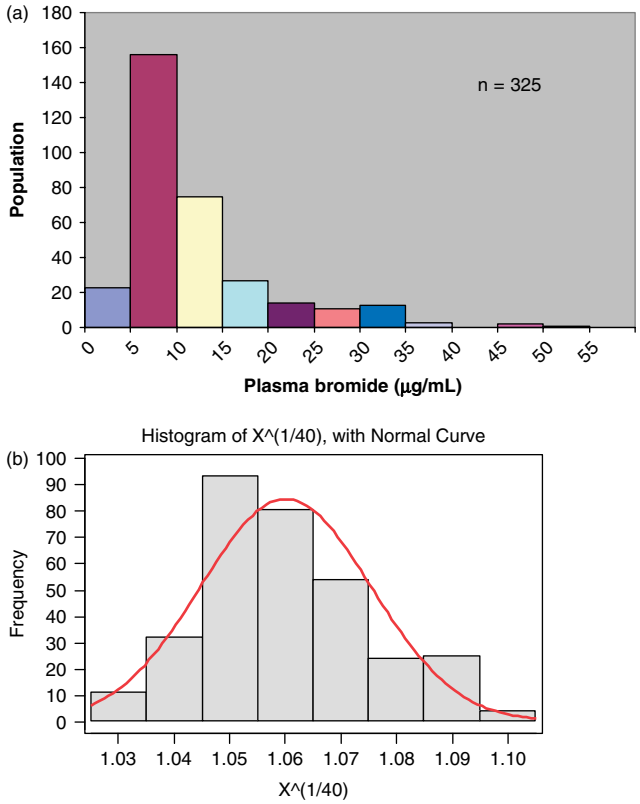

Figure 2. (A) Bromide concentration in equine plasma samples (n = 325); (B) bromide concentration in equine plasma samples after a power (1/40) transformation.

The transformed data were then subjected to the Kolmogorov-Smirnov normality test, resulting in an acceptable significance level of 0.053 ($P > 0.05$). A threshold could then be proposed at a level equal to the untransformed 'mean + 3.72 standard deviation (SD)' value of 82 µg/mL, representing a risk of 1 in 10 000 for a normal sample to exceed this threshold. Based on this approach, which is often used to establish internationally accepted thresholds in horseracing industry,[9,24,25] a 'rounded-up' threshold of 90 µg/mL of bromide in equine plasma was established. The risk associated with this threshold was about 1 in 21 000. This threshold could be useful as a benchmark to determine whether or not an observed bromide concentration is abnormal or suspiciously high. A QC sample prepared with plasma spiked with bromide at 90 µg/mL and its corresponding 'plasma blank' were analyzed in duplicate along with each batch of test samples. The recovered bromide concentration used for comparison with the spiked concentration was the difference between the mean readings from the QC samples and those from the corresponding blank samples. After replicate analyses (n = 15), the mean and SD of the recovered bromide concentration in QC samples prepared on separate occasions were found to be 94.1 µg/mL and 2.8 µg/mL respectively. Control limits for the QC samples could then be set at mean ±3 SD (85.7 µg/mL and 102.5 µg/mL). In order to minimize false negatives, a cut-off level for the screening of test samples was set at 85 µg/mL (rounded-down value of the lower QC limit). Test samples with a plasma bromide concentration greater than 85 µg/mL from this screening test were deemed suspicious and would be quantified using the more accurate ICP/MS standard addition method as described below.

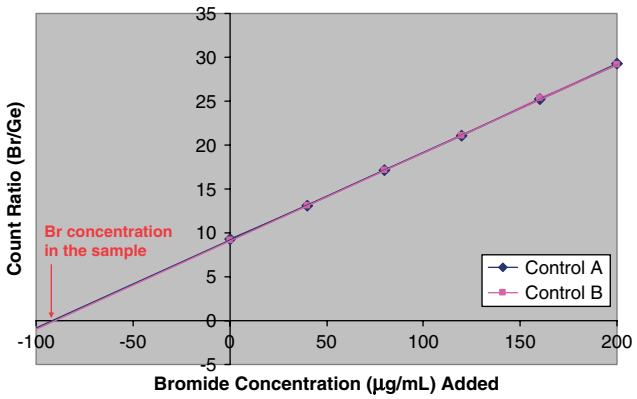

Figure 3. Calibration curve of a QC sample of bromide in deionized water at 90 µg/mL.

Quantification of bromide in equine plasma by ICP/MS

The sample preparation procedures for the ICP/MS quantification method were the same as those in the above ICP/MS screening method, except calibrators were prepared using aliquots of the test sample. Bromide standards were added to six aliquots of a test sample to give equivalent bromide spiked concentrations at 0, 40, 80, 120, 160, 200 µg/mL. Each sample aliquot was analyzed in duplicate. The bromide concentration of the test sample was determined by extrapolation from the standard addition calibration curve. In order to control the method's performance, a QC sample of bromide in deionized water at 90 µg/mL was analyzed along with each batch of the test samples. A calibration curve generated from duplicate analyses of a QC sample is shown in Figure 3. All calibration curves generated from the QC samples prepared on separate occasions (n=21) were linear with correlation coefficient greater than 0.99. The resulting mean and range chart and their control limits are shown in Figures 4A and 4B. The warning and rejection limits of the mean chart were marked at respectively ±2SD and ±3SD based on 20 data points, whilst the warning and rejection limits of the range chart were set at respectively 2.512 and 3.267 times of the average range obtained (n=20). The limit of quantification (LoQ) was derived from the SD of bromide standards at 10 µg/mL in deionized water (n=10). The LoQ was estimated at 10 µg/mL (SD × 10). The measurement uncertainty of this quantification method was estimated using the approach stipulated in the Eurachem/CITAC Guide.[26] The detailed calculations were similar to those reported by Leung *et al.*[27] The expanded uncertainty of measurement for quantifying bromide at 90 µg/mL was estimated to be 8 µg/mL at 99% 1-tailed confidence level. Therefore, a blood sample is deemed to be positive (having breached the threshold) if its plasma bromide level exceeds 98 µg/mL.

In order to regulate the misuse of bromide in equestrian sports, an accurate quantification of bromide in plasma is necessary. However, the ICP/MS standard addition method, although independent of matrix interference, requires the analysis of 12 aliquots from each test sample, it is not cost-effective for screening purposes. For regular testing, samples are tested initially using the ICP/MS screening (semi-quantification) method to identify any suspicious samples, which can then be further investigated by the more accurate ICP/MS standard addition method. Table 1 shows the plasma bromide concentrations of six horse-blood samples tested with the ICP/MS screening and standard addition methods. The results

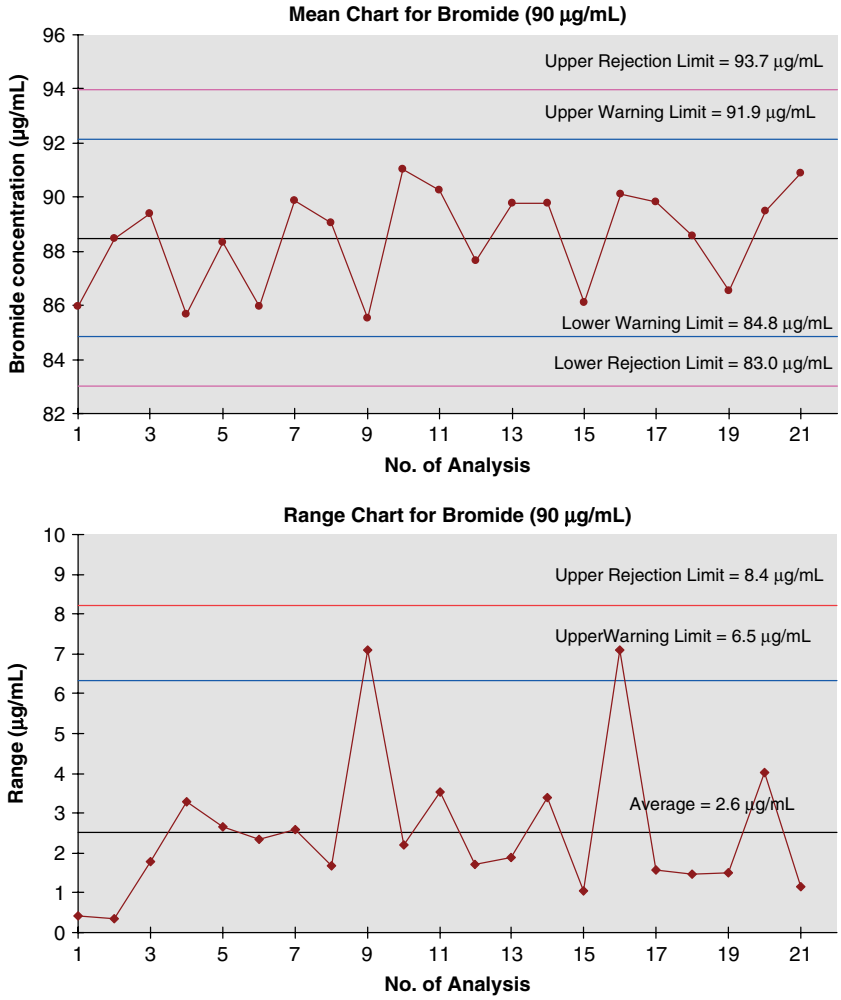

Figure 4. (A) Mean chart and (B) range chart for bromide QC samples at 90 µg/mL in deionized water.

Table 1. Horse blood samples analyzed by the screening method (using calibrators in water) and quantification method (by standard addition) methods by ICP/MS

Horse Blood Sample	Bromide concentration in plasma (µg/mL)	
	Screening method *(Using calibrators in water)*	Quantification method *(by standard addition)*
1	15.9	16.1
2	11.3	11.1
3	5.8	6.0
4	14.3	14.3
5	6.8	6.6
6	7.0	8.0

showed that there was good correlation between the two methods.

Confirmation of bromide in equine plasma by GC-MS

The presence of bromide in a positive regulatory sample should be established unequivocally using a confirmation method. The confirmation method adopted was based on the method reported by Kage *et al*.[20] and Funazo *et al*. [21,22] Our detection was performed on a GC-MS. Bromide in plasma was derivatized with pentafluorobenzyl *p*-toluenesulfonate (PFB-OTs) in a mixture of acetone and phosphate buffer. The resulting pentafluorobenzyl bromide was analyzed by GC-MS in EI mode. Bromide could be confirmed easily in a plasma sample collected 10.2 days post administration as shown in Figure 5A. Both the retention time and mass spectrum of pentafluorobenzyl bromide obtained from the post-administration sample matched well with those from the bromide standard (Figure 5B). The molecular ions at *m/z* 262 and *m/z* 260, corresponding to the isotopes of ^{81}Br and ^{79}Br respectively, were observed with an ion ratio of approximately 1 to 1. The base peak of $[M\text{-}Br]^+$ was observed at *m/z* 181. These GC-MS data met the criteria stipulated in the *AORC Guidelines for the Minimum Criteria for Identification by Chromatography and Mass Spectrometry*.[19] The bromide concentration in this post-administration sample was about 110 µg/mL, suggesting that this GC-MS method has adequate sensitivity to confirm the presence of bromide in horse plasma exceeding the threshold of 90 µg/mL.

Bromide administration trials

Pre- and post-administration samples from the administration of potassium bromide (60 g each) to two thoroughbred geldings were analyzed. The elimination profiles of bromide in two horses

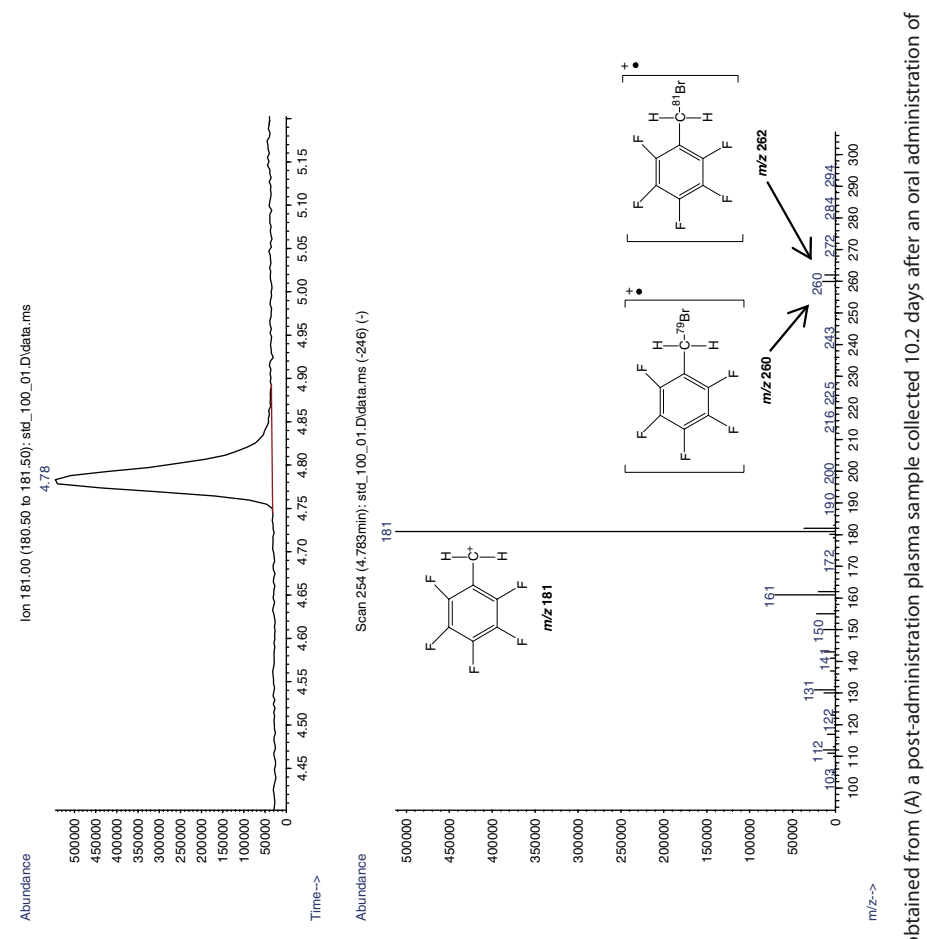

Figure 5. GC-MS extracted-ion chromatograms and EI mass spectra of pentafluorobenzyl bromide obtained from (A) a post-administration plasma sample collected 10.2 days after an oral administration of 60 g of potassium bromide to a horse, and (B) a standard solution of bromide in water.

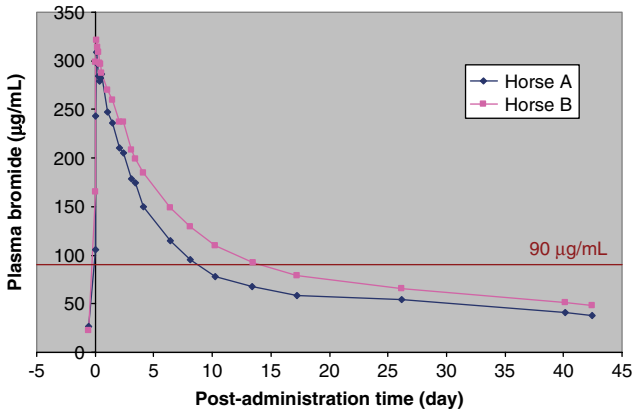

Figure 6. Plasma elimination of bromide following oral administration of 60 g each of potassium bromide to two horses.

are shown in Figure 6. Plasma bromide levels peaked after approximately 2 h at about 310–320 µg/mL, and then remained above the threshold for up to 8 and 13 days respectively. Plasma bromide did not return to the basal values even up to 43 days, which was when the last samples were collected. The initial half life for bromide in plasma was determined to be 3.6–4.6 days and the terminal phase half life was found to be 31.9–36.7 days.

Conclusion

ICP/MS screening and quantification methods for bromide in equine plasma samples were developed. Plasma samples were analyzed directly by ICP/MS after protein precipitation and simple dilution. Endogenous bromide levels in equine plasma samples (n=325) were determined with the aim of establishing a threshold to control the misuse of bromide in horses. After a one-fortieth power transformation, a normal distribution was obtained from this set of population data. A threshold of 90 µg/mL was proposed with a risk factor of less than 1 in 10 000 (actually 1 in 21 000). Any sample with plasma bromide greater than 85 µg/mL as determined by the ICP/MS screening method would be quantified using the more accurate ICP/MS standard addition method. A blood sample is considered positive if its plasma bromide exceeds the threshold plus the method's uncertainty of measurement and if the presence of bromide in the test sample can be established unequivocally using the GC-MS method reported in this study. The applicability of the threshold was evaluated by analyzing post-administration samples. Following oral administration of potassium bromide (60 g each) to two thoroughbred geldings, plasma bromide in post-administration samples remained above the proposed threshold for 8–13 days. This article provided an effective and complete approach for doping control analysis of bromide in horses.

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