Cross Validation of HS-GC/MS to Quantify Total Carbon Dioxide in Horse Plasma

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Abstract

The use of alkinizing agents by trainers to counteract the accumulation of lactic acid in racehorses has been well documented. A by-product of this administration is elevated total carbon dioxide (tCO₂) concentrations. Most regulatory authorities have set the threshold for tCO₂ in plasma at 37 mM. The quantification of tCO₂ often presents a challenge to laboratories due to the instrumentation required, which can be expensive to use and maintain. The Beckman DxC 600 (Brea, CA) is currently used in our laboratory for tCO₂ quantification. The goal of this research was to determine if another analytical method could be used for this procedure. We report the use of headspace gas chromatography–mass spectrometry (HS-GC/MS) as an acceptable alternative to the use of the Beckman DxC 600. A six-point calibration curve ranging from 10 to 60 mM was analyzed along with controls at 15, 25 and 45 mM. Imprecision was found to be <3% at all concentrations. Inaccuracy was measured at <4% at all concentrations. Measurement of uncertainty was determined using the Simplified GUM approach and was found to be 3% at 99.7% confidence level with eight measurements at a tCO₂ concentration of 45 mM. Furthermore, the HS-GC/MS was cross-validated according to international protocols with all parameters being met. During cross validation, a standard at a known concentration was analyzed by both instruments. The average difference using 25 replicates in calculated concentrations was <0.1 mM. Also, plasma samples from four untreated horses were analyzed by both instruments. The difference in calculated concentrations between the two instruments was <0.6 mM for all horses. The results show that the use of HS-GC/MS is an acceptable alternative to the use of the Beckman DxC 600 for the quantification of tCO₂ in horse plasma.

Introduction

In horses, during prolonged exercising at high intensity, lactic acid will accumulate in the muscles, which lowers the pH of the blood (1). In an effort to allow a horse to run in an anaerobic state for a longer period, some trainers will administer a mixture of alkalinizing agents, usually containing sodium bicarbonate in a procedure sometimes referred to as “milkshaking” due to the appearance of the aqueous slurry of sodium bicarbonate in water (2, 3). It is thought that the high doses of bicarbonate will provide a buffer in the blood and muscle tissue, enabling the horse to run longer and faster. The efficacy of milkshaking in horses has shown mixed results (4). However, because this is an attempt to manipulate the outcome of a race, most horse racing authorities have banned its use. The primary means of detecting milkshaking is through the quantification of total carbon dioxide (tCO₂) in plasma. Under normal post-race conditions, a horse is expected to have an average tCO₂ concentration of between 29 and 31 mM (5, 6). In the USA, most racing authorities have set the regulatory threshold at 37 mM, which is more than 2 standard deviations (SDs) above the mean concentration (6).

Until 2010, the instrument used world-wide for the quantification of tCO₂ in horse plasma was the Beckman EL-ISE (6, 7). However, this instrument has been discontinued by the developer and is no longer in service in most racing laboratories. In order to...
maintain harmonization between laboratories, the Association of Racing Chemists (AORC) developed a protocol to cross-validate an instrument against the EL-ISE (8). One instrument that has been successfully cross-validated using this protocol is the Beckman DxC 600 (9). It is the Beckman DxC 600 that is currently used by our laboratory for tCO₂ quantification (5).

For equine regulatory laboratories, testing for tCO₂ using the Beckman DxC 600 or other similar instrument can present a challenge. Clinical chemistry analyzer instruments can only be used for the quantification of tCO₂ and electrolytes, with no other practical screening or confirmatory capabilities for other drugs. In addition, the costs of purchasing a clinical chemistry analyzer are relatively expensive and are further increased by the added costs for maintenance, unique consumables and training for the operation of chemistry analyzers. Gas chromatography–mass spectrometry with headspace injection (HS-GC/MS) is a technology that is already used by forensic toxicology laboratories for analyses such as the quantitation of volatile compounds in liquid samples (10). One of the most common uses in the industry is for the quantification of various compounds such as ethanol and other flammables (11).

Previous publications have described the quantification of tCO₂ by GC–MS with routine liquid sample injections (12). However, procedures of this type use hazardous chemicals and take much longer to perform and are therefore not compatible with the expected sample volume that our laboratory would analyze. Other papers have described the use of HS-GC/MS to quantify biological gases such as carbon monoxide and carbon dioxide in post-mortem samples (13, 14). The goal of this project was to develop a quantitative method for tCO₂ in horse plasma samples and cross-validate that method with the Beckman DxC 600 using the AORC protocol.

Experimental

Standard curve and quality control for the Beckman DxC 600
A six-point standard curve kit was obtained from Verichem Laboratories (Providence, RI) and stored at 4°C prior to analysis on the Beckman DxC 600. These concentrations were 5, 10, 20, 30, 40 and 50 mM in water. The concentration of the calibrators was calculated by the Beckman instrument and was plotted against their nominal values, and a linear regression model was applied using no weighting.

The concentrations of tCO₂ in the control samples were calculated using the standard curve. Since control samples and calibrators prepared in horse plasma are not commercially available, human plasma quality control samples were used for analysis.

Reference compounds and reagents for the HS-GC/MS
Sodium carbonate, sodium bicarbonate and sulfuric acid were obtained from Fisher Scientific (Hanover Park, IL) and were all 99.9% pure or higher. About 99.9% purity 13C-sodium carbonate was obtained from Sigma Aldrich (St. Louis, MO). A 0.5 N sulfuric acid was prepared by adding 4 mL of concentrated sulfuric acid to 996 mL of deionized water. This solution was stored at room temperature and was stable for up to 6 months.

Standard curve and quality controls for the HS-GC/MS
Two stock solutions of carbonate were created at a concentration of 100 mM using equal parts sodium carbonate and sodium bicarbonate in water. To 20 mL of water, 1 mmol of sodium carbonate and 1 mmol of sodium bicarbonate were added and mixed until dissolved. Separate stock solutions were used for the calibrator curve and quality control samples.

For the HS-GC/MS, a six-point standard curve was made at the following concentrations in water: 10, 20, 30, 40, 50 and 60 mM. These curve points were analyzed before each batch of samples. All calibrators were stored at 4°C and stable for up to 3 months. To ensure the accuracy of these calibrators, during method development, these calibrators were analyzed for accuracy by running them on the DxC 600 instrument that had been calibrated according to previous publications (5). All curve points concentrations were found to be within 0.4% of expected concentration when analyzed with the DxC 600.

Quality control samples were prepared at the following concentrations: 25 and 45 mM in water. To ensure the accuracy of these quality control samples, during method development, these samples were also analyzed on the DxC 600 instrument that had been previously calibrated. Again, all quality control concentrations were found to be within 0.4% of expected concentrations when analyzed using the DxC 600. All controls were stored at 4°C and stable for up to 3 months. These control solutions were analyzed before each batch of samples.

For the internal standard, a solution of 13C-sodium carbonate at a concentration of 25 mM was prepared in water. This solution was stored at 4°C and was stable for up to 3 months.

Instrumentation

Beckman DxC 600 instrumentation
A Beckman Coulter UniCel DxC 600 (Brea, CA) instrument was used for cross validation and calibrated daily before samples were analyzed. Beckman Synchrotron® Calibrators were obtained from Beckman Instruments and stored at 4°C prior to analysis.

HS-GC/MS instrumentation conditions
An Agilent 7890 Gas Chromatograph with Headspace Injector model 7697 coupled with an Agilent 5977 Mass Spectrometer was used. For the headspace injection, the oven temperature was set to 50°C, the loop temperature to 110°C and the transfer line temperature to 115°C. The vial size was 10 mL. The contents of the vials were equilibrated in the oven for 4 min and were shaken at 50 shakes/min. For injection, the fill pressure was set at a constant flow until the pressure was 2 p.s.i. The injection duration was 0.02 min. The post-injection purge was 100 mL/min for 1 min.

For the gas chromatograph, helium was used as the carrier gas and the front inlet was set on split mode with a split ratio of 10:1. The split flow was 30 mL/min. An Agilent DB-ALC 2 30 m x 320 μm x 1.8 μm was used. The column flow was 5 mL/min. The oven was set to 50°C and held for 2.25 min. The transfer line was set at 280°C. The total run time was 2.25 min.

For the mass spectrometer (MS), the acquisition mode was set to collect selected ion monitoring (SIM) and full scan data simultaneously. The MS Source and Quad were set at 230 and 150°C, respectively. The solvent delay was 1.85 min. The SIM ions collected were 44.1 and 45.1 m/z with dwell times for both ions being 10 ms. The full scan collected ions between 10 and 100 m/z. The delta EM volts was set to −100 mV.

MassHunter Acquisition and Quantitative Analysis version 7.0 was used for the analysis of all samples.
Sample preparation

Beckman DxC 600 sample preparation

For tCO2 screening, the instrument was calibrated, and standard curve and quality controls were analyzed according to previous publications (5). After calibration, one portion of 500 µL of sample was aliquoted into a 2 mL cup and delivered to the flow cell of the Beckman instrument.

HS-GC/MS sample preparation

After calibrators, controls and test samples were brought to room temperature, 100 µL of internal standard solution was aliquoted into each 10 mL crimp top headspace vial. Fifty microliters of sample was aliquoted into each vial followed by 1 mL of 0.5 N sulfuric acid. The vials were immediately capped after addition of the 0.5 N sulfuric acid and placed onto the headspace tray for analysis.

Method validation

Stability testing for tCO2 analysis has been studied elsewhere (16). In brief, for tCO2 analysis, changes in tCO2 concentration are not seen when unopened sample tubes are stored at 4°C for up to 72 h prior to analysis. Because of these previous studies, stability testing was only investigated for conditions unique to the HS-GC/MS method. Therefore, in addition to carryover, stability testing consisted of processed stability testing and repeat injection testing.

Carryover

Carryover was evaluated by injecting a water sample followed by a 100 mM calibrator aliquot followed by another water sample. This was performed in triplicate. The abundances of the peak of interest and internal standard for the pre- and post-calibrator water injections were measured and averaged.

Processed stability testing

Stability testing for processed samples was conducted by preparing six replicates of the 45 mM quality control sample for injection and separating them into two groups. Group 1 sample replicates were injected immediately. Group 2 sample replicates were injected 24 h after being placed on the autosampler. The area abundances of CO2 for each group of samples were averaged. The stability was calculated by dividing the average area abundance of Group 2 by that of Group 1 and multiplying by 100.

Repeat injection testing

Repeat injection testing was performed on three replicates of the 25 mM quality control sample. The samples were repeatedly injected up to six times, and the area abundances of the CO2 and 13CO2 were measured along with the calculated concentrations of CO2 in the sample.

Accuracy and precision

Accuracy and precision for the analytical method were determined by analyzing six samples at concentrations of 15, 25 and 45 mM. These samples were analyzed on five separate days. Accuracy was calculated by subtracting the mean calculated concentration from the theoretical concentration and dividing by 100. This result was then multiplied by 100 to show the accuracy as a percentage. The inter- and intra-day precisions were calculated using one-way ANOVA tables. These results are reported in Table I.

<table>
<thead>
<tr>
<th>Table I. Precision and accuracy of quality control samples</th>
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<tr>
<td></td>
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<tr>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>Intra-day precision</td>
</tr>
<tr>
<td>Inter-day precision</td>
</tr>
<tr>
<td>Total precision</td>
</tr>
<tr>
<td>Accuracy</td>
</tr>
</tbody>
</table>

Cross-validation procedures

To cross-validate the HS-GC/MS to the Beckman DxC 600, the AORC Protocol for Cross-Validating tCO2-Measuring Instruments against the Beckman Synchron EL-ISE was used (15). This procedure consisted of three main steps:

Step 1 was to compare the results of a prepared sample at a concentration of 35 mM. The sample was prepared by taking 350 µL of the quality control stock solution and diluting it with 650 µL of water. Five replicates of this sample were analyzed by both instruments on five different days with the data captured in quadruplicate. This was completed and the results are reported in Table II.

Step 2 is to investigate bias from matrix effects by analyzing plasma samples from untreated horses. For this step, venous blood samples were collected from the jugular veins of four untreated horses in lithium-heparin tubes, then centrifuged and then stored at 4°C until analysis, which occurred within 24 h. This study was completed and the results are reported in Table III.

Step 3 was to investigate bias from matrix effects at concentrations above the regulatory threshold. This was accomplished by supplementing plasma from four untreated horses with an appropriate volume of sodium bicarbonate in water solution to give an estimated concentration of 45 mM. Four samples from each horse were measured in quadruplicate. This study was completed and the results are reported in Table IV.

For Steps 2 and 3, blood samples were collected by a veterinarian. Venous blood samples were collected from each horse in two 3 mL plasma tubes which were inverted gently several times before being centrifuged at 3000 r.p.m. for 15 min and then stored at 4°C prior to analysis. The time between blood collection and analysis was <30 h. This time frame is in agreement with previously published data on plasma tCO2 stability (16).

Results

Samples analyzed using the HS-GC/MS method proved stable when submitted to normal analytical conditions. When testing for injection carryover, no observable difference in the mean between the
control, the intra- and inter-day precisions were measured at 1.92 and 0.9, respectively and the bias was calculated at 0.53%. These results can be seen in Table I.

For Step 1 of the AORC Cross-validation protocol, the prescribed calculations were performed and are reported in Table II. Measurements of the prepared reference standard on both instruments were within 1% of the theoretical concentration with the mean for the DxC 600 at 35.19 mM and that for the HS-GC/MS at 35.36 mM. Within-run precision for the DxC 600 and HS-GC/MS were calculated at 0.35 and 0.42, respectively, using one-way ANOVA tables. The data were subjected to Bland-Altman analysis and showed random scattering with 97% of sample measurement differences falling between ±2 SD of the mean difference. Results can be seen graphically in Supplemental Figure 1.

For Step 2, we attempted to demonstrate that the analysis does not suffer from matrix effects. For each of the samples from the four horses, the average tCO2 concentration, SD and relative standard deviation (RSD%) were calculated and the results are reported in Table III. For three of the four horses, the average tCO2 concentration was within 0.5 mM between the two instruments. For the fourth horse, the difference between the average tCO2 values was 0.6 mM. Results on both instruments showed SDs of <0.6 mM with calculated RSD% <2.0 on both instruments for samples from all horses. The data were subjected to Bland-Altman analysis and showed random scattering with 96.3% of sample measurement differences falling between ±2 SD of the mean difference. Results can be seen graphically in Supplemental Figure 2.

For Step 3, we attempted to demonstrate that the analysis does not suffer from bias from matrix effects above the regulatory threshold. For each of the four horses, the average tCO2 concentration, SD and RSD% were calculated and the results are reported in Table IV. For all the horses, the difference between the average calculated tCO2 concentrations was <0.5 mM. The SDs for both instruments were <0.9 with calculated RSD% <2.0. The data were subjected to Bland-Altman analysis and showed random scattering with 96.8% of sample measurement differences falling between ±2 SD of the mean difference. Results can be seen graphically in Supplemental Figure 3.

Discussion

As can been seen in Table I, the developed method was found to be both accurate and precise over the range of concentrations analyzed. The quality control sample concentrations were selected because they represent the general lower and upper limits that are expected to be seen in horse plasma. Using the quality control data for the 45 mM level, the measurement of uncertainty for this method was 3% at 99.7% confidence level, which is the same measurement of uncertainty calculated using the DxC 600.

The use of 13C-sodium carbonate as an internal standard proved essential for reliable quantitation of tCO2 in plasma samples. The stability testing showed that both the compound of interest and its internal standard were stable for up to 24 h after processing and repeated injections. This is an advantage over testing using the DxC 600. When the sample plasma tubes are opened, CO2 immediately begins to be released from the sample. Therefore, if a sample needs to be reinjected due to power failure or some other fault occurs, the results will be substantially lower. However, with the HS-GC/MS, once samples are placed and capped in the autosampler vials, they are stable with no discernable change in calculated tCO2 concentration over a period of 24 h.

Table III. Comparison of analysis of untreated equine plasma

<table>
<thead>
<tr>
<th>Horse</th>
<th>Average tCO2 value (mM)</th>
<th>Beckman DxC 600</th>
<th>Agilent HS-GC/MS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Horse 1</td>
<td>28.55</td>
<td>29.14</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SD (mM)</td>
<td>0.28</td>
<td>0.41</td>
</tr>
<tr>
<td></td>
<td>RSD%</td>
<td>0.97</td>
<td>1.41</td>
</tr>
<tr>
<td>Horse 2</td>
<td>29.89</td>
<td>29.49</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SD (mM)</td>
<td>0.26</td>
<td>0.45</td>
</tr>
<tr>
<td></td>
<td>RSD%</td>
<td>0.88</td>
<td>1.54</td>
</tr>
<tr>
<td>Horse 3</td>
<td>30.09</td>
<td>29.79</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SD (mM)</td>
<td>0.32</td>
<td>0.56</td>
</tr>
<tr>
<td></td>
<td>RSD%</td>
<td>1.07</td>
<td>1.89</td>
</tr>
<tr>
<td>Horse 4</td>
<td>31.55</td>
<td>30.99</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SD (mM)</td>
<td>0.55</td>
<td>0.31</td>
</tr>
<tr>
<td></td>
<td>RSD%</td>
<td>1.74</td>
<td>1.01</td>
</tr>
</tbody>
</table>

Table IV. Comparison of analysis of fortified equine plasma

<table>
<thead>
<tr>
<th>Horse</th>
<th>Average tCO2 value (mM)</th>
<th>Beckman DxC 600</th>
<th>Agilent HS-GC/MS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Horse 1</td>
<td>42.50</td>
<td>42.84</td>
<td></td>
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<tr>
<td></td>
<td>SD (mM)</td>
<td>0.32</td>
<td>0.80</td>
</tr>
<tr>
<td></td>
<td>RSD%</td>
<td>1.23</td>
<td>1.87</td>
</tr>
<tr>
<td>Horse 2</td>
<td>44.06</td>
<td>44.40</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SD (mM)</td>
<td>0.33</td>
<td>0.89</td>
</tr>
<tr>
<td></td>
<td>RSD%</td>
<td>0.75</td>
<td>2.00</td>
</tr>
<tr>
<td>Horse 3</td>
<td>44.44</td>
<td>44.97</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SD (mM)</td>
<td>0.36</td>
<td>0.58</td>
</tr>
<tr>
<td></td>
<td>RSD%</td>
<td>0.81</td>
<td>1.28</td>
</tr>
<tr>
<td>Horse 4</td>
<td>46.23</td>
<td>46.67</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SD (mM)</td>
<td>0.39</td>
<td>0.32</td>
</tr>
<tr>
<td></td>
<td>RSD%</td>
<td>0.85</td>
<td>0.69</td>
</tr>
</tbody>
</table>
For cross-validation purposes, the results obtained with the Agilent HS-GC/MS satisfied all the required criteria set forth in the AORC protocol. When measuring the prepared standard at 35 mM, the HS-GC/MS showed an average difference of 0.14 mM from the theoretical concentration, nearly identical to the Beckman DxC 600 calculation, which showed a mean difference of 0.19 mM. The cross-validation protocol indicated that the procedure suffered no matrix effects above or below regulatory thresholds. For the untreated samples, the concentrations calculated by the two different instruments differed by <0.5 mM in three of the four horses and 0.6 mM in one horse. The treated sample calculated concentrations all differed by <0.5 mM between the two instruments. The AORC protocol was confined to the use of instruments that used the same basic technology as the EL-ISE (10). However, this was due more to the fact that instrumentation that used different technology to quantitate tCO₂ was not in use at the regulatory laboratories at the time of publication as opposed to any inherent advantage of the blood gas analyzer over other technology.

Limit-of-detection and limit-of-quantification were not measured by this method because carbon dioxide is a normal component of room air. Because of this, blank sample injections yielded detectable amounts of carbon dioxide. This was not a cause of concern, however, since the lack of carbon dioxide will not occur with a tested horse plasma sample. This background concentration of carbon dioxide did not affect the quantification of tCO₂ in samples and quality controls.

Also, it is worth noting that during method development, it was found that routine settings for the headspace injector and GC front inlet resulted in over-saturation of the MS detector which resulted in plateauing of the calibration curve at concentrations >30 mM. Reductions in the injection time and an increase in the front inlet split ratio reduced the amount of sample put on the column. Also, a reduction in the electron multiplier voltage reduced signal intensity as well. With these adjustments, as can be seen in the example shown in Figure 1, the calibration curve was proven to be linear over the range of concentrations with r² values >0.9995 and slope values between 0.95 and 1.05 for all curves. Residual plot graphs showed all points along the calibration curve were within ±2 SDs, with 98% of residuals falling within ±1 SD. Results can be seen in Supplemental Figure 4. The use of an internally prepared calibration curve showed no significant difference in measurements between the two instruments. The use of this curve allowed us to prepare a calibration range where the expected plasma concentrations would be in the middle of the curve as well as reduce costs.

The simultaneous collection of SIM and full scan data was chosen because of the lack of clear qualifier ions available for carbon dioxide. This was due to the incomplete separation of the carbon dioxide peak from other components of the air peak. Other product ions available for carbon dioxide are 28 and 16 m/z, which are the same spectrum ions for nitrogen and oxygen gases. Because of the lack of qualifier ions, full scan data was used in order to detect if there were any co-eluting peaks with spectrum ions of 44 and 45 m/z. No interfering peaks were found in any injection. Future studies could look into the use of other columns or adjustments to the method that would allow full separation of carbon dioxide from components of ambient air.

Other advantages to this method include the reduction in the sample volume needed for testing. With only 50 µL of sample needed per vial, screening analysis and confirmatory quantification can be performed with <500 µL of total sample volume. This is helpful since plasma is being used more frequently for screening and confirmatory analyses of other drugs and prohibited compounds.

**Conclusion**

The use of HS-GC/MS was shown to be highly effective and accurate and precise for the quantification of tCO₂ in plasma at concentrations typically seen in pre- and post-race horse samples. The method run time is relatively short, which would allow the analysis of up to 20 samples per hour. Additionally, this method allows any GC–MS that can be fitted with a headspace autosampler to be used for quantification, offering potential cost savings to a laboratory since it would not need to purchase and maintain an instrument dedicated to tCO₂ determinations. Future studies should also investigate the use of other columns to resolve carbon dioxide from other components of ambient air and also into multi-laboratory cross validation.

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Example calibration curve for the quantification of tCO₂ in equine plasma.
**Supplementary data**

Supplementary data is available at *Journal of Analytical Toxicology* online.

**Acknowledgments**

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**References**


