Validated UHPLC–MS-MS Method for Rapid Analysis of Capsaicin and Dihydrocapsaicin in Equine Plasma for Doping Control

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A method involving ultra high-performance liquid chromatography–tandem mass spectrometry was developed and validated for the analysis of capsaicin and dihydrocapsaicin in equine plasma. The analytes were recovered from plasma by liquid–liquid extraction using methyl tert-butyl ether and separated on a sub-2 micron column. The mobile phase was composed of 2 mM ammonium formate and methanol. A triple quadrupole mass spectrometer was used to detect the analytes in positive electrospray ionization mode with selected reaction monitoring. The limits of detection, quantification and confirmation for both analytes were 0.5, 1.0 and 2.5 pg/mL, respectively. The linear dynamic range of quantification was 1.0–1,000 pg/mL. During storage, both analytes in equine plasma were unstable at room temperature but stable at –20 and –70°C. The retention time and product ion ratios were employed as the criteria for confirmation of the presence of the analytes in plasma. The total analysis time was 2 min. The method is fast, selectively sensitive, reproducible, reliable and fully validated.

Introduction

Capsaicin (8-methyl-N-vanillyl-trans-6-nonenamide) (Figure 1A) is the major chemical component in hot chili peppers (1–2). Exposure to capsaicin causes a burning sensation of the skin and mouth, lacrimation, disorientation, gagging and coughing (3). It is used in repellent spray products for self-defense and crowd management. Capsaicin is a highly selective agonist for vanilloid subtype I, and as a substance-P depletor, it produces significant desensitization and analgesia (4–9). Thus, it is used in topical ointments and creams as an analgesic agent to treat various pain-related conditions such as back pain, nerve pain, headaches and other painful conditions that the use of conventional analgesics does not help. In veterinary medical practice, capsaicin has been used to treat foot pain, laminar ischemia and arthritis (10). In addition to pain management, red pepper powder is also used to prevent horses from chewing on leg bandages.

Capsaicin is considered to be a potential substance for abuse in racehorses because of its pain-relieving and desensitizing effects. The degree of desensitization caused by capsaicin treatment helps a horse in pain to compete sooner than untreated pain would allow, consequently, the safety of other horses, drivers or jockeys in the race is compromised. In a reversible lameness model, the lameness score and other parameters indicating pain were markedly decreased in capsaicin-treated horses between 40 and 240 min after treatment (10). This time frame for the duration of the beneficial effect of capsaicin in horses is longer than the time it takes for horses to complete a race. It is for these compelling reasons that the use of capsaicin in racehorses is banned by the State of Pennsylvania (PA) Racing Commissions. Capsaicin is also a prohibited substance by the Federation Equestrian International (FEI). In the 2008 Olympic competition in Beijing, four out of 15 samples obtained from jumping-show horses tested positive for capsaicin and were disqualified from competition. Thus, to regulate the use of capsaicin in racehorses in PA, a fully validated method for detection, quantification and confirmation of the presence of capsaicin in post-race plasma samples was needed.

Methods for the analysis of capsaicin had been developed in the past two decades using various techniques such as colorimetry/spectrophotometry (11), complexation chromatography (12), supercritical fluid chromatography (13), liquid chromatography (LC) (14–15), enzyme immunoassays (16), gas chromatography (17) and LC–mass spectrometry (18–22). Direct injection of sample in screening for capsaicin in equine urine was achieved with a detection level of 10 ng/mL (23). Compared to urine, plasma is a more appropriate matrix for the detection of capsaicin, because only 0.095% of unchanged compounds is eliminated in the urine (24). In 2006, a more sensitive method for the screening of capsaicin in equine plasma resulted in a limit of detection of 20 pg/mL (25). Because the topical route of administration is the most commonly used, the concentration of capsaicin in equine plasma is very low. Thus, any method that is used for the detection of capsaicin in plasma must be highly sensitive and selective. Moreover, the ultimate goal of doping control analysis is to confirm the presence of any analyte that is suspected to be present in the test sample. Currently, there is no reported method to achieve the confirmation of low picogram concentrations of capsaicin in equine plasma. Thus, the impetus for the current study was to develop a sensitive method for the screening, quantification and confirmation of very low concentrations of capsaicin in equine plasma.

Dihydrocapsaicin (Figure 1B) (N-[4-hydroxy-3-methoxyphenyl] methyl-8-methyl-nonanamide) is another major capsaicinoid in hot chili peppers and a major by-product in the pharmaceutical production of capsaicin ointments and creams. It coexists in medical capsaicin products. Confirmation of the presence of dihydrocapsaicin in equine plasma provides unequivocal evidence for the illegal use of a capsaicin-containing product on the horse. As an analog of capsaicin with similar chemical structure, dihydrocapsaicin is also pharmacologically active with pain-relieving and desensitizing properties (2). The purpose of this study was to develop a fast, sensitive and reliable method for the simultaneous screening, quantification and confirmation of capsaicin and dihydrocapsaicin in equine plasma.

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plasma to regulate the use of these drugs in racehorses competing in PA.

**Experimental**

**Chemicals and reagents**

Capsaicin (≥95% purity) and dihydrocapsaicin (~90% purity) were obtained from Sigma Chemical Corp. (St. Louis, MO). Capsaicin-d₅ (98% purity), as internal standard (IS), was purchased from Medical Isotopes, Inc. (Pelham, NH). Methyl tert-butyl ether (MTBE), formic acid and ammonium hydroxide were obtained from EMD Chemical, Inc. (Gibbstown, NJ). Water (Optima) was purchased from Honeywell Burdick & Jackson (Muskegon, MI), whereas methanol (LC grade) was purchased from Fisher Scientific (Fair Lawn, NJ).

**Preparation of standards and reagents**

Capsaicin, dihydrocapsaicin and IS (1.0 mg/mL) were individually prepared in methanol using the respective dry powder and stored at −70°C. A mixture of capsaicin and dihydrocapsaicin (10 μg/mL) was prepared by adding 100 μL of each stock solution (1.0 mg/mL) to 9,800 μL of methanol, for a total of 10 mL. Working standard solutions of 0.1, 0.25, 0.5, 1.0, 2.5, 5.0, 10, 25, 50, 75 and 100 ng/mL were prepared by serial dilution of 10 μg/mL of the solution mixture using methanol. All stock and working standard solutions were stored at −70°C to prevent degradation. Capsaicin-d₅ working solution (2.5 ng/mL) was prepared by dilution of the relevant stock solution (1.0 mg/mL) using methanol and stored at −70°C.

A stock solution of ammonium formate buffer composed of 1.0 mol/L ammonium formate and 1.0 mol/L formic acid was prepared by adding 15.4 mL of formic acid and 13.5 mL of ammonium hydroxide to 171 mL of water (unadjusted pH: 3.4 ~ 3.5). Ammonium formate buffer (2 mmol/L; pH 3.4 ~ 3.5) was prepared by diluting 2 mL of 1.0 mol/L of stock ammonium formate buffer into 1,000 mL of water.

**Preparation of calibration samples**

Blank equine plasma was collected from healthy but retired female or gelded racehorses now resident at the PA Racing Commission Equine Facility at the University of Pennsylvania, School of Veterinary Medicine. An aliquot (10 μL) of each working standard solution of 0.1, 0.25, 0.5, 1.0, 2.5, 5.0, 10, 25, 50, 75 and 100 ng/mL was spiked to 1.0 mL blank plasma to prepare calibration samples of 1.0, 2.5, 5.0, 10, 25, 50, 100, 250, 500 and 1,000 pg/mL. Calibration samples were processed in exactly the same manner as test samples.

**Sample preparation**

Equine plasma (1.0 mL) was added into 16 × 125 mm screw-cap culture tubes. First, 10 μL of 2.5 ng/mL IS were added to 1.0 mL of plasma and mixed prior to adding 5 mL of MTBE to the mixture. The tube was capped and mixed on a roto-rack shaker for 10 min before centrifugation at 1,610 × g for 10 min. The resulting organic layer (top) was transferred to a prelabeled culture tube and the content was evaporated to dryness at 50°C (Techni Dri-Block DB-3, Duxford, Cambridge, UK) under a steady stream of air or nitrogen. The dried extract was reconstituted in 100 μL of a 2 mM ammonium formate–methanol (40:60; v/v) mixture and transferred into a 200 μL insert (Target PP Polyspring, National Scientific Company, Rockwood, TN). An aliquot (20 μL) of the reconstituted extract was used for LC–MS analyses.

**UHPLC–MS-MS conditions**

The ultra high-performance liquid chromatography–tandem mass spectrometry (UHPLC–MS-MS) system used in this study consisted of a Thermo Accela UHPLC (Thermo Fisher Scientific, San Jose, CA) and a TSQ Quantum Ultra mass spectrometer with heated electrospray ionization (H-ESI) source (Thermo Fisher Scientific, San Jose, CA). A Hypsiber Gold column (50 × 2.1 mm i.d., 1.9 μm particle size; Thermo Fisher Scientific, Waltham, MA) was employed for analyte separation. The column oven chamber was maintained at 40°C. Mobile phase A was 2 mM ammonium formate, and mobile phase B was methanol. The LC gradient for analyte separation was programmed as: 0 min, 40/60 (A/B); 1.5 min, 10/90 (A/B); 1.51 min, 40/60 (A/B); and 2.00 min, 40/60 (A/B) with the flow at 500 μL/min. Total analysis time was 2 min.

H-ESI source parameters were optimized by syringe infusion of the analytes into a concurrent mobile phase flow mixture of 2 mM ammonium formate–methanol (40:60; v/v) at 500 μL/min. Data acquisition was conducted in selected-reaction monitoring (SRM) mode. The H-ESI source and mass spectrometric parameters were: spray voltage, 2,000 V; vaporizer temperature, 450°C; sheath gas, 50 arbitrary units (au); sweep gas, 20 au; auxiliary gas, 10 au; ion transfer capillary temperature, 300°C; peak width relating to resolution (FWHM), 0.1 μ for Q1 and 0.7 μ for Q3; collision gas pressure, 1.5 mTorr (1 Torr ≈ 133 pa);
The method was validated for specificity, extraction recovery, matrix effect, linearity, intra-day and inter-day accuracy/precision and analyte stability. Specificity was evaluated by comparing the chromatograms of six different batches of blank plasma, blank plasma spiked with IS and that spiked with 1.0 pg/mL [limit of quantification (LOQ)] of the analytes. Extraction recovery was determined at three concentrations (25, 250 and 750 pg/mL) by comparing the response of the extracted samples to those of the analytes added to blank plasma extracts. The difference in responses between these two processed samples is extraction recovery (26–28). The matrix effect was evaluated by comparing the chromatographic peak area of the analyte added to blank plasma extract \((A_{\text{extract}})\) with that of the analyte spiked in extraction solvent \((A_{\text{solvent}})\) at three different concentrations (25, 250 and 750 pg/mL), according to the following equation:

\[
\text{Matrix effect} (\%) = \left( \frac{A_{\text{extract}} - A_{\text{solvent}}}{A_{\text{solvent}}} \right) \times 100
\]

The linearity of the method was determined by plotting peak area ratios of the analytes to deuterated IS against concentrations of the analytes. The intra-day and inter-day precision in the method [relative standard deviation (RSD) calculated as percent of the standard deviation (SD) divided by the mean of observed concentrations] and accuracy (the agreement between the concentrations of analytes quantified and those spiked into blank plasma) were determined by assaying six replicate quality control (QC) samples at four different concentrations (1.0, 5.0, 25, 250 and 750 pg/mL). The stability of the analytes in equine plasma was evaluated for freeze-thaw cycles and at different storage conditions. In each freeze-thaw cycle, the samples were frozen at \(-20^\circ C\) for 24 h and thawed at ambient temperature for 1 h. Concentrations of the analytes in the freeze-thaw samples were determined using daily calibration curves. The stability of the analyte was evaluated by analyzing samples stored at room temperature, 4°C, \(-20^\circ C\) and \(-70^\circ C\) for different periods of time. The change in analyte concentration over the period of sample storage represents the stability of the analytes at a specified temperature condition and period of storage.

**Method Validation**

**Administration of capsaicin to research horses**

The study protocol was approved by the University of Pennsylvania Institutional Animal Care and Use Committee. Eight Thoroughbred horses (5–12 years old weighing 553.8 ± 39.2 kg) were used in the study. The horses were no longer actively racing. However, based on direct physical examination and routine clinical chemistry, inspection, palpation and routine examination for lameness, all horses were in good health. The administration of capsaicin was on a randomly assigned basis. Each horse in the group was administered capsaicin by topical (TOP) application. All horses were fed grass hay and had water *ad libitum*. Grain supplement was based on activity and weight of the horse.

Before capsaicin administration, a 14-F catheter (Angiocath, Becton Dickinson, Sandy, UT) was inserted into the jugular vein under sterile conditions for collection of blood samples.

The anterior-posterior surface of the front knee was used for the TOP application of 15 g of paste containing 0.025% capsaicin (Equi-Block, MiracleCorp, Dayton, OH). Latex gloves were worn during the application of the capsaicin paste to the joint. A total of \(\sim 5\) min was required to apply and rub the material onto the surrounding skin surface of the joint and adjacent tendons.

All studies started at 8:00 AM. Blood samples were collected shortly before drug administration and at various time intervals up to 24 h post-administration. After collection, blood samples were stored in crushed ice for 10 min before centrifugation (2,500 × g for 15 min) to harvest plasma. Aliquots of 2 mL of plasma were immediately frozen at \(-20^\circ C\), and within 24 h they were stored at \(-70^\circ C\) until analysis. Each 2 mL aliquot was used once to eliminate any effect of freeze-thaw cycles on the concentration of capsaicin and dihydrocapsaicin in the sample.

**Data analysis**

Data acquisition and analysis were accomplished by Xcaliber software (version 2.0.7; Thermo Fisher Scientific). The quantification of both analytes was performed via construction of calibration plots of analyte/IS peak area ratios versus analyte concentration by using linear regression with \(1/x\) weighting. The concentrations in test samples were determined from the line of best fit. Regression analysis was automatically performed using the instrument’s software (Xcaliber, v 2.0.7).

**Results**

**Development of the analytical method**

During method development, different mobile phase combinations and LC gradients were investigated to achieve sharp peaks with well-resolved separation of capsaicin and dihydrocapsaicin in a short analysis time. The LC gradient chosen for the study generated sharp and asymmetrical peaks for both analytes, with complete separation achieved within 2 min (Figure 2). The retention times were 1.16 and 1.35 min for capsaicin and dihydrocapsaicin, respectively, and 1.16 min for IS (Figure 2). The method is efficient for the rapid analysis of capsaicin and dihydrocapsaicin in equine plasma.

Full-scan mass spectra of capsaicin and dihydrocapsaicin were acquired for selecting precursor ions for SRM analysis. In the positive ESI mode, capsaicin, dihydrocapsaicin and IS generated protonated molecular ions, \([M + H]^+\), at \(m/z\) 306, 308 and 309, respectively, as the most abundant ions. Adduct ions of \([M + Na]^+\) were also observed at \(m/z\) 328, 330 and 331 for capsaicin, dihydrocapsaicin and IS, respectively. Because \([M + H]^+\) ions were the most abundant, they were selected for further collision-induced dissociation (CID) study and product ion spectra were obtained by varying collision energy to optimize the fragmentation of \([M + H]^+\) ions for SRM analysis. For each analyte, three SRM transitions were selected for screening, quantification and confirmation study (Table I). Among the SRM transitions and LC gradients were investigated to achieve sharp peaks with well-resolved separation of capsaicin and dihydrocapsaicin in a short analysis time.
transitions obtained, the most intense product ions at $m/z$ 137, 137, and 140 were used for quantitative analyses. For confirmation of the presence of capsaicin and dihydrocapsaicin in a test sample, $m/z$ 137, 122, and 66 ions were used for capsaicin, and $m/z$ 137, 122, and 94 were employed for dihydrocapsaicin. The fragmentation of capsaicin in ESI$^+$ mode has previously been discussed (29–30).

**Method validation**

**Specificity**

A method with high specificity in racehorse doping control analysis is essential for reducing false positive findings and improving method performance and reliability. Figure 3 indicates that no interfering peaks from endogenous compounds were observed at the retention times of capsaicin and dihydrocapsaicin, suggesting that the extraction method resulted in clean extracts of the analytes and that the LC gradient used is adequate for separating endogenous interferences from the analytes. Thus, the method is highly specific for the analysis of capsaicin and dihydrocapsaicin in equine plasma.

**Extraction recovery**

Concentrations of capsaicin and dihydrocapsaicin in equine plasma were in the pg/mL range due to the route (TOP) of administration. To recover such a low drug concentration from plasma, a highly efficient extraction method is required. Study
results (Table II) indicated that extraction recoveries of capsaicin and dihydrocapsaicin from equine plasma were >85%, suggesting that MTBE is an efficient extraction solvent for the recovery of the analytes and that liquid–liquid extraction provides high extraction recovery of the analytes from equine plasma.

Matrix effect
Table II lists the matrix effects on the analysis of capsaicin and dihydrocapsaicin in equine plasma. Negative values represent plasma-induced ion suppression on the analysis of capsaicin and dihydrocapsaicin, suggesting that the background impurity decreased the signal intensity of the analytes under ESI\(^+\) mode. The contribution of ion suppression by equine plasma was between 25 and 35% for both analytes.

Linearity and sensitivity
The linear dynamic range was 1.0–1,000 pg/mL, with correlation coefficient \((r) > 0.995\). The limit of detection (LOD) [signal-to-noise ratio (S/N) > 3] was 0.5 pg/mL for both analytes. The lower LOQ was defined as the lowest concentration in a calibration curve that yielded precision with coefficient of
variation <20% and accuracy of 80–120%. The lower LOQ for capsaicin and dihydrocapsaicin in the present study was 1.0 pg/mL.

**Inter-day and intra-day accuracy and precision**

Table III shows that intra-day precision and accuracy were 1.9–18.0% and 91.4–104.3%, respectively whereas inter-day accuracy and precision were 4.6–18.1% and 92.6–105.0%, respectively. These results suggested that the method is highly precise and accurate.

**Stability**

The results of freeze-thaw cycle evaluations indicated that after the fifth freeze-thaw cycle, the concentrations of the analytes were within 90–110%, suggesting that both drugs were stable after five freeze-thaw cycles (Table IV).

The results of evaluations of analyte stability at different temperature conditions (Table V) indicated that at room temperature, capsaicin and dihydrocapsaicin were stable for only 6 h. After 24 h, concentrations of capsaicin and dihydrocapsaicin decreased from 100 to ~70 and ~35%, respectively. At 4°C, capsaicin concentration was ~80% of the initial concentration after four days of storage, indicating that capsaicin was relatively stable for up to four days. However, after seven days of storage at 4°C, capsaicin concentration was 60–70% of the original concentration. Dihydrocapsaicin was less stable than capsaicin at 4°C. After one day of storage at 4°C, the concentration of dihydrocapsaicin decreased to ~70% of the initial concentration. After seven days, only ~30% of the initial concentration in plasma was present. Capsaicin and dihydrocapsaicin were stable at −20 and −70°C. The concentration of capsaicin was >80% for up to 90 days of storage at −20 and −70°C. The concentration of dihydrocapsaicin was >60% after 90 days of storage at −20 and −70°C.

**Confirmation of the presence of capsaicin and dihydrocapsaicin in equine plasma**

The ultimate purpose of this method was to confirm the presence of capsaicin and dihydrocapsaicin in equine plasma. The confirmation of the presence of a compound in a test samples is to demonstrate that the chemical fingerprints of the compound are the same as those of an authentic drug standard. Chromatographic retention time (tR) and mass spectra using LC–MS-MS serve as chemical fingerprints of the analyte for confirmation analysis, therefore, a match of the chemical fingerprints between the sample and reference standards must be established for retention time and mass spectrum. For a triple stage quadrupole mass spectrometer, at least three product ions are required to calculate ion ratios for analyte confirmation (31). Table I lists the product ions chosen for confirmation of each analyte. Ion ratios were calculated using the three product ions based on peak height, and the similarity in ion ratios between an unknown sample and calibration/QC samples served as one of the criteria for analyte confirmation (26, 27). The presence of capsaicin and dihydrocapsaicin in equine plasma were confirmed using two criteria: (i) the similarity in ion intensity ratios between an unknown sample and those of the authentic reference drug standards must be within 80–120% and (ii) the retention times of capsaicin and dihydrocapsaicin in the unknown samples must be within ±0.2 min of those of the authentic reference drug standards.

<table>
<thead>
<tr>
<th>Table II</th>
<th>Analyte Recovery* and Matrix Effects† of Plasma on the Analysis of Capsaicin and Dihydrocapsaicin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Analyte</td>
<td>Extraction recovery</td>
</tr>
<tr>
<td></td>
<td>25 pg/mL</td>
</tr>
<tr>
<td>Capsaicin</td>
<td>93.3</td>
</tr>
<tr>
<td>Dihydrocapsaicin</td>
<td>93.0</td>
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<td>Matrix effect</td>
<td>4.9</td>
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<tr>
<td>Capsaicin</td>
<td>−33.3</td>
</tr>
<tr>
<td>Dihydrocapsaicin</td>
<td>−31.7</td>
</tr>
</tbody>
</table>

*Extraction recovery (%) = Aprocessed/Astandard × 100, where Aprocessed is the peak area of an analyte spiked in blank equine plasma and processed and Astandard is the peak area of analytes fortified blank plasma extract.

†Matrix effect (ion suppression or enhancement, %) = (Vsample − Vstandard/Astandard) × 100, where Vstandard is the peak area of an analyte in extraction solvent and Astandard is the peak area of an analyte spiked in blank equine plasma extract.

<table>
<thead>
<tr>
<th>Table III</th>
<th>Intra-Day and Inter-Day Accuracy and Precision for the Quantification of Capsaicin and Dihydrocapsaicin in Equine Plasma (n = 6)</th>
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<tbody>
<tr>
<td>Concentration spiked (pg/mL)</td>
<td>Intra-day</td>
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<tr>
<td>C2</td>
<td>Concentration determined (pg/mL)</td>
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<tr>
<td>C2</td>
<td>Capsaicin</td>
</tr>
<tr>
<td>C2</td>
<td>Dihydrocapsaicin</td>
</tr>
</tbody>
</table>

*Precision (RSD, %) = SD of concentration determined/concentration determined × 100.

†Accuracy = concentration determined/concentration spiked × 100.
Stability of Capsaicin and Dihydrocapsaicin in Equine Plasma after One, Two and Five Freeze-Thaw Cycles Following Storage at –20°C

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Concentration spiked (pg/mL)</th>
<th>Stability*</th>
<th>One cycle</th>
<th>Two cycles</th>
<th>Five cycles</th>
</tr>
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<tbody>
<tr>
<td>Capsaicin</td>
<td>25</td>
<td>89.6%</td>
<td>94.4%</td>
<td>91.4%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>88.8%</td>
<td>94.0%</td>
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<tr>
<td></td>
<td>750</td>
<td>90.0%</td>
<td>97.7%</td>
<td>90.7%</td>
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</tr>
<tr>
<td>Dihydrocapsaicin</td>
<td>25</td>
<td>87.8%</td>
<td>98.1%</td>
<td>90.7%</td>
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<tr>
<td></td>
<td>250</td>
<td>92.6%</td>
<td>108.2%</td>
<td>110.8%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>750</td>
<td>105.2%</td>
<td>110.3%</td>
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</table>

*Stability = concentration determined/concentration spiked × 100%.

Table V
Stability* of Capsaicin and Dihydrocapsaicin under Different Temperature Conditions

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Concentration spiked (pg/mL)</th>
<th>Room temperature</th>
<th>0 h</th>
<th>2 h</th>
<th>4 h</th>
<th>6 h</th>
<th>24 h</th>
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<tbody>
<tr>
<td>Capsaicin</td>
<td>25</td>
<td>–20°C</td>
<td>86.6</td>
<td>85.3</td>
<td>81.3</td>
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<td></td>
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<td>85.0</td>
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<td>86.9</td>
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<td>Capsaicin</td>
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<td>90.7</td>
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<td></td>
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<td>80.0</td>
<td>72.5</td>
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<td></td>
<td>750</td>
<td>75.9</td>
<td>64.8</td>
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<tr>
<td>Capsaicin</td>
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<td>0 days –30°C</td>
<td>99.1</td>
<td></td>
<td>90.4</td>
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<td></td>
<td>250</td>
<td>107.6</td>
<td>92.0</td>
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<tr>
<td></td>
<td>750</td>
<td>105.3</td>
<td>92.0</td>
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<tr>
<td>Dihydrocapsaicin</td>
<td>25</td>
<td>74.8</td>
<td>77.1</td>
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<td></td>
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<tr>
<td></td>
<td>250</td>
<td>83.2</td>
<td>99.3</td>
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<td></td>
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<td>77.2</td>
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*Stability = concentration determined / concentration spiked × 100%.

Method verification
The present method was successfully applied to the screening, quantification and confirmation of the presence of capsaicin and dihydrocapsaicin in equine plasma samples collected from racehorses post-competition in PA. Figure 4 shows that the retention times of capsaicin (1.13 ± 0.2 min) and dihydrocapsaicin (1.31 ± 0.2 min) in a post-race plasma sample matched those of the QC sample spiked with the standard drug. The concentrations of capsaicin and dihydrocapsaicin in the post-race sample were 345.3 ± 10.2 pg/mL and 84.8 ± 7.6 pg/mL, respectively. By comparing the product ion ratios between the unknown sample, standard calibration samples and QC samples (Table VI, Figure 5), the similarity in ion intensity ratios between the unknown sample and those of the standard calibration and QC samples was within 80–120%. Based on the match in tR and ion intensity ratios, the presence of capsaicin and dihydrocapsaicin in the post-race plasma sample was unequivocally confirmed.

The method was also successfully applied to the analysis of samples collected from research horses after TOP application of a capsaicin-containing paste. Following TOP application of 15 g of paste containing 0.025% of capsaicin to the left carpus (knee), capsaicin and dihydrocapsaicin peaked at 2 h in plasma with concentrations of 241.9 and 154.8 pg/mL, respectively (Figure 6). At 24 h post capsaicin treatment, the concentrations of capsaicin and dihydrocapsaicin decreased from 241.9 to 4.7 pg/mL and from 154.8 to 2.8 pg/mL, respectively (Figure 6). These results suggested that at 48 h post-TOP application of a capsaicin-containing paste (0.025% × 15 g) on a horse, there might be no quantifiable concentration of capsaicin and dihydrocapsaicin in the plasma.

Discussion
UHPLC provides the opportunity for fast separation/elution of analytes with sharp chromatographic peaks. However, due to the sub-micron particle size of the UHPLC column, high back-pressure in the LC system may cause concern. In the present study, a short column (50 mm) was used to reduce potential back-pressure, and column temperature was increased to 40°C to reduce the viscosity of the mobile phase, and therefore, decrease back-pressure. Compared to room temperature, a column temperature of 40°C reduced the LC backpressure from ~480 to ~360 bar at the initial gradient condition.

Ion suppression was observed for both capsaicin and dihydrocapsaicin (Table II). Because deuterated capsaicin was employed as the IS in this study, ion suppression was eliminated by using the peak area ratio of the analyte to IS for quantification. Thus, accurate quantification results were still achieved, although no further action was taken to reduce matrix effect in the present study.

Capsaicin and dihydrocapsaicin were not stable at room temperature and 4°C, with dihydrocapsaicin being less stable than capsaicin (Table V). Due to the instability of the analytes, adequate attention must be given to the storage conditions, especially when samples are not immediately transported to the laboratory following completion of races. Any positive samples should be immediately preserved at either −20 or −70°C for long-term storage in case reanalysis of the sample is needed.

Administration study results indicated that both capsaicin and dihydrocapsaicin concentrations in equine plasma peaked at 2 h post-application (Figure 6). Thus, the detection of any quantifiable concentration of capsaicin and/or dihydrocapsaicin in a post-race equine plasma sample is indicative of race day (within 24 h of race time) application of capsaicin, and this action constitutes a blatant disregard for the ban on race day administration of any unauthorized medication to any horse competing in an official race in PA.

Although the analgesic effect of capsaicin is beneficial, the risk associated with desensitization, caused by the blockade of C-fiber conduction that may result in long-lasting sensory deficits, outweighs any benefits of this drug to the horse during competition. Capsaicin-sensitive fibers are nociceptors that respond to sensory stimuli such as noxious pressure, heat and chemical irritants (32). Nociceptive neurons may release...
glutamate (excitatory amino acid) as a rapid central neurotransmitter and neuropeptides such as calcitonin gene-related peptide (CGRP), substance P, neurokinin A and somatostatin into the spinal cord under intense stimulation (33); thus, the central and peripheral nervous effects of capsaicin mediated through neurotransmitters, desensitization resulting from blockade of C-fiber conduction and the release of neuropeptides cannot be ignored when considering the risk in using capsaicin in a racehorse on the day of competition in PA. The results of studies conducted in cultured non-equine sensory neurons and isolated nerves have shown that capsaicin induces depolarization, resulting in membrane permeability to cations, specifically to calcium and sodium ions (34–37). The dorsal root ganglion (DRG) is an important therapeutic target in neuropathic pain disorders such as laminitis in the horse (37). Unlike in humans, capsaicin does not cause ion fluxes in cultured DRG from birds, which may explain why birds are insensitive to capsaicin (36–37) and thus, birds can eat hot chili peppers on farms, especially when they are ready for harvest. The membrane ion channel activated by capsaicin and its analogs is insensitive to calcium and sodium ion channel blockers such as tetrodotoxin and conotoxin, but is blocked by ruthenium red, which attenuates the effects induced by capsaicin (36).

Capsaicin also activates many biochemical processes and increases cellular concentrations of cyclic guanosine monophosphate (38) and nitric oxide (39) and stimulates inositol tris-phosphate turnover and arachidonic acid release (40). Thus, other secondary effects of capsaicin mediated through activation of biochemical systems may be of physiological benefit to the body. The existence of a specific membrane receptor for capsaicin was proposed based on a highly selective competitive capsaicin antagonist, capsazepine, which does not possess analgesic

Figure 4. UHPLC–ESI+–MS–SRM chromatograms of capsaicin (transition m/z 306 → 137) and dihydrocapsaicin (transition m/z 308 → 137) in racehorse plasma samples: blank plasma scan for capsaicin (A); racehorse plasma sample scan for capsaicin (B); QC sample of 250 pg/mL of capsaicin spiked in blank plasma (C); blank plasma scan for dihydrocapsaicin (D); racehorse plasma sample scan for dihydrocapsaicin (E); QC sample of 250 pg/mL dihydrocapsaicin spiked in blank plasma (F).
property, but it inhibits the excitatory effects of capsaicin in both in vivo and in vitro experiments (41). Capsaicin analogs such as resiniferatoxin, nuvanil and olvanil activate sensory neurons and nociceptors in a manner similar to that of capsaicin, and thus, they also need to be studied to prevent capsaicin abusers from shifting treatment of racehorses from capsaicin to its analogs, because the present method is limited to capsaicin and dihydrocapsaicin in equine plasma (42). Although capsaicin may be of therapeutic use to horses, its use on race day in PA does not absolve the trainer from sanctions that may be imposed by the PA Racing Commissions for violating the rules of racing.

**Conclusion**

A rapid and sensitive LC–MS-MS method was successfully developed and validated for the screening, quantification and
confirmation of capsaicin and dihydrocapsaicin in equine plasma. This method meets the requirements for doping control analysis for the presence of capsaicin and dihydrocapsaicin at a picogram concentration in equine plasma. Capsaicin and dihydrocapsaicin in equine plasma are unstable at room temperature, thus, adequate steps must be taken to preserve post-race samples to prevent degradation of these analytes. The method is shown to be sensitive, reproducible, and reliable and is adequate for regulating the use of capsaicin and dihydrocapsaicin in racehorses competing in PA.

References


