Ultra High Performance Liquid Chromatography with Tandem Mass Spectrometry (UPLC-MS/MS) analysis of Gentamicin, Penicillin G, Flunixin, and 5-hydroxyflunixin in Horse Plasma

Samples were analyzed at the VA-MD College of Veterinary Medicine Analytical Chemistry Research Laboratory. Analyte concentrations in plasma samples were determined by UPLC-MS/MS. Special precautions were taken to avoid adsorption of the analytes to glass surfaces. All standards were made up in polypropylene (PP) containers, all pipette tips used were virgin PP, and dilute heptafluorobutyric acid (HFBA) was used to help reduce/remove any other potential adsorption or precipitation effects.

Reference standards of gentamicin sulfate (G) and isotopically labeled gentamicin C1-dx, pentaacetate salt (GIS) were obtained from Sigma-Aldrich, Inc. (St. Louis, MO, USA) and Toronto Research Chemicals (North York, ON, CAN), respectively. Penicillin G potassium salt (P), penicillin G-d7 (Pd7), flunixin meglumine (F), flunixin-d3 (Fd3), and 5-hydroxyflunixin (5OH) were obtained from Cayman Chemical (Ann Arbor, MI, USA). 5-hydroxyflunixin-d3 (5d3) was also obtained from Toronto Research Chemicals. Stock solutions of gentamicin and penicillin compounds were initially made up with 50% acetonitrile (ACN) in water while the flunixin compounds were made up in DMSO. Each of the individual stock solutions were then diluted in methanol (MeOH) + 1% HFBA to their final standard concentrations.

Plasma samples were prepared by combining 100 µL of plasma with 300 µL of the internal standard addition solution (626 ng/mL GIS + 500ppb Pd7 + 125ppb Fd3, 5d3 in MeOH + 1% HFBA) in 2 mL polypropylene (PP) microcentrifuge tubes. The protein precipitated samples were briefly shaken and then placed on a vortex table to extract for 5 minutes before being centrifuged (Eppendorf Microcentrifuge Model 5415R, Eppendorf North America, Enfield, CT, USA) at 16,100 g for 5 minutes. 50 µL of the resulting supernatant solutions were then combined with 100 µL of deionized water in a 2 mL amber autosampler vials with PP low volume inserts. These were then briefly vortexed to homogenize before being placed in the refrigerated autosampler of the UPLC-MS/MS for analysis.

Sample extracts were subjected to chromatographic separation performed on a Waters H-Class UPLC system with an HSS T3 reverse phase column (Acquity UPLC HSS T3, Waters Corporation, Milford, MA, USA, 100 mm length x 2.1 mm ID x 1.8 µm) and matching guard column (Acquity UPLC HSS T3 VanGuard Pre-Column, Waters Corporation, Milford, MA, USA, 5 mm length x 2.1 mm ID x 1.8 µm) maintained at 40°C. Nine microliters of sample was injected onto the column using a refrigerated autosampler maintained at 5 °C. Mobile phase A consisted of 0.5% HFBA in water (H_2O), mobile phase B consisted of 0.75% HFBA in MeOH. The mobile phase was delivered to the UPLC column at a flow rate of 0.4 mL per min. The gradient elution program is shown below in Table 1.

To reduce MS contamination, the divert valve was used to transfer the column effluent to the MS from 0.5 to 2.00 minutes. From 0 to 0.49 and 2.01 to 4.25 minutes, all the column effluent was transferred to waste. Simultaneous and efficient separation of penicillin G, flunixin, 5-hydroxyflunixin and the individual gentamicin components of interest was achieved using this ion pairing methodology with penicillin G, flunixin, and 5-hydroxyflunixin eluting at

approximately 0.92, 1.56, and 1.63 minutes, respectively. The retention times of the gentamicin components C1, C1a, and C2+C2a eluted at approximately 1.91, 1.87, 1.89, and 1.93 minutes, respectively. Baseline separation of C2 and C2a was not achieved, but given the similarity in MRM transitions and the reference standard's certificate of analysis providing a purity for C2+C2a combined, baseline separation was unnecessary. The UPLC column effluent was pumped directly without any split into a triple-quadrupole mass spectrometer (Xevo TQD, Waters Corporation, Milford, MA, USA) equipped with a Zspray ionization source which was operated in positive-ion electrospray mode (ESI+) using multiple reaction monitoring (MRM). The parent and product ion transitions for the compounds of interest are shown in Table 2.

Commercial software (MassLynx, Waters Corporation, Milford, MA, USA) was used to analyze the data. Tuning was performed on each analyte by direct infusion of standard solution (5 ng/µL) at a rate of 20 µL per min. Mass spectrometer parameters used for the detection of gentamicin are shown in Table 3.

A five-point calibration curve made up in blank plasma was prepared in the same manner as the samples but was spiked with a range of approximately 0.09 to 84.4 µg/mL plasma for total gentamicin as well as penicillin G. These same standards ranged from 0.018 to 16 µg/mL plasma for flunixin, and 0.06 to 5.63 µg/mL plasma for 5-hydroxyflunixin. Using these standards, calibration curves were constructed for each of the individual gentamicin components using the MassLynx software to determine analyte concentration in samples based on the sample / IS ratio.