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Validation of a method for quantitation of the clopidogrel active metabolite, clopidogrel, clopidogrel carboxylic acid, and 2-oxo-clopidogrel in feline plasma

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Abstract

Objectives—To validate a new method for stabilization and quantitation of the clopidogrel active metabolite (CAM), clopidogrel, and inactive clopidogrel carboxylic acid and 2-oxo-clopidogrel.

Animals—Feline plasma was used for assay validation. A pilot pharmacokinetic study was conducted with 2 healthy cats.

Methods—CAM stabilization was achieved by adding 2-bromo-3'-methoxyacetophenone to blood tubes to form a derivatized CAM (CAM-D). CAM-D was quantified using high performance liquid chromatography and tandem mass spectrometry. Validation of the methodology included evaluation of calibration curve linearity, accuracy and precision, and stability using quality control samples spiked with CAM-D, clopidogrel, clopidogrel carboxylic acid, and 2-oxo-clopidogrel. *In vivo* utility of this assay was evaluated by conducting a pharmacokinetic study in cats receiving a single oral dose of 18.75mg clopidogrel.

Results—The 2-oxo-clopidogrel metabolite was unstable. CAM-D, clopidogrel, and clopidogrel carboxylic acid appear stable for 1 week at room temperature and 9 months at -80°C . Standard curves showed linearity for CAM-D, clopidogrel, and clopidogrel carboxylic acid ($r>0.99$). Between assay accuracy and precision was 2.6% and 2.8% for CAM-D and 17.9% and 11.3% for clopidogrel and clopidogrel carboxylic acid. Within assay precision for all three compounds was 7%. All three compounds were detected in plasma from healthy cats receiving clopidogrel.

Conclusions—This methodology is accurate and precise for simultaneous quantitation of CAM-D, clopidogrel, and clopidogrel carboxylic acid in feline plasma but unsuitable for 2-oxo-clopidogrel. Validation of this assay is the first step to more fully understanding the use of clopidogrel in cats in both a population and individual setting.

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CONFLICTS OF INTEREST

The authors do not have any conflicts of interest to disclose.

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Keywords

Plavix; cat; thromboprophylaxis; antithrombotic; antiplatelet

INTRODUCTION

Clopidogrel is an antiplatelet agent commonly used in cats and is superior to aspirin in the prevention of feline cardiogenic thromboembolism (1). Prevention of thromboembolic disease is paramount since survival rates post-embolism are low (less than 40%) with an approximate 17–75% recurrence rate (2, 3). In humans, individual response to clopidogrel has been demonstrated with some individuals being resistant to clopidogrel (4–6). Variable response to clopidogrel has also been observed in cats (7–9). Clopidogrel resistance in humans has been documented to be associated with increased risk for the development of major adverse cardiac events (4–6).

Clopidogrel is a prodrug and requires hepatic metabolic transformation for antiplatelet activity. In the human liver, metabolism of clopidogrel occurs through two main pathways. The clinically important pathway is the conversion of inactive parent drug clopidogrel by cytochrome P450 enzyme to an intermediate product 2-oxo-clopidogrel, which is then subsequently hydrolyzed to the clopidogrel active metabolite (CAM). The conversion of 2-oxo-clopidogrel can result in four diastereoisomers of CAM named H1, H2, H3, and H4. Only H4 and H2 have antiplatelet effects, with H4 exhibiting about twice the activity of H2 (10). In the human body, only H3 and H4 isomers can be detected (11, 12). As a result, the H4 isomer is the only active circulating form of CAM in humans (10). The H4 isomer selectively and irreversibly blocks adenosine diphosphate (ADP) binding to the P2Y₁₂ receptor thereby inhibiting ADP-induced platelet aggregation. In humans, about 15% of the parent clopidogrel undergoes bioactivation to both the H3 and H4 CAM isomers (11). The remaining 85% is metabolized in the liver to an inactive clopidogrel carboxylic acid (Fig. 1).

CAM is very unstable and has been challenging to quantitate thereby making complete pharmacokinetic studies in any species difficult. No pharmacokinetic studies of clopidogrel and/or its metabolites in cats have been reported. In humans and dogs, pharmacokinetic studies have focused instead on measuring plasma concentrations of clopidogrel or the clopidogrel carboxylic acid metabolite (13–15). Only recently has a method to stabilize CAM in human plasma been developed by adding 2-bromo-3'-methoxyacetophenone (BMAP) to blood tubes (12, 16). Once stabilized, the derivatized CAM (CAM-D, Fig. 1) can be readily quantitated using high-performance liquid chromatography with tandem mass spectrometry (HPLC-MS/MS). Validation of this assay has now allowed complete pharmacokinetic studies, pharmacodynamic-pharmacokinetic correlations, medication interactions, and mechanisms of clopidogrel resistance to be more fully evaluated in humans (12, 17, 18). In order to accomplish the same in cats, validation of this assay for use in cats is a critical first step.

The aim of this study was to validate this new method of BMAP addition and HPLC-MS/MS for CAM stabilization and quantitation in feline plasma. A secondary aim was to also validate this assay for quantitation of clopidogrel, clopidogrel carboxylic acid, and 2-

oxo-clopidogrel. *In vivo* utility of this assay was evaluated by conducting a preliminary pharmacokinetic study in 2 healthy cats that received a single dose of 18.75mg clopidogrel by mouth.

ANIMALS, MATERIALS AND METHODS

Validation of the HPLC-MS/MS methodology and sample analysis were all preformed through the Washington State University, Program in Individualized Medicine in the laboratory of one of the authors (MHC).

Chemicals and reagents

Mass spectrometry compatible reagents included acetonitrile (Fisher Scientific, Pittsburgh, PA, USA), formic acid (J.T. Baker, Avantor Performance Materials, Center Valley, PA, USA), and deionized ultrafiltered water (Milli-Q Advantage, EMD-Millipore, Billerica, MA, USA). Racemic (E)-2-bromo-3'-methoxyacetophenone (BMAP) was from Sigma-Aldrich (St. Louis, MO, USA). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) or Thermofisher (Waltham, MA, USA) unless otherwise indicated.

Assay standards and internal standards were purchased from Toronto Research Chemicals (Ontario, Canada). These included S-(+)-clopidogrel hydrogen sulfate (cat# C587250), 2-oxo-clopidogrel hydrochloride (cat# O869990), trans-clopidogrel-BMAP derivative (mixture of H1, H2, H3, H4 diastereomers derivatized with BMAP; CAM-D; cat# C587255), and clopidogrel carboxylic acid (cat# C587240). The corresponding stable isotope labelled compounds were used as internal standards including rac-clopidogrel-d4 hydrogen sulfate (cat# C587252), 2-oxoclopidogrel-d3 (cat# O869994), trans-clopidogrel-BMAP-¹³C-d3 derivative (mixture of H1, H2, H3, H4 diastereomers derivatized with BMAP-D-¹³C-d3; CAM-D-¹³C-d3; cat# C587257), and clopidogrel-d4 carboxylic acid (cat# C587242). Standard and internal standard solutions were prepared by dissolving the powdered compounds in acetonitrile and then stored in brown glass vials at -20°C.

Citrate treated feline plasma from healthy cats used as the blank matrix for standard curves and quality control samples were obtained from the small animal blood bank at Washington State University, College of Veterinary Medicine, Veterinary Teaching Hospital. Ethylene diamine tetra acetic acid (EDTA) treated feline plasma samples were obtained from the Washington State University, College of Veterinary Medicine, Veterinary Teaching Hospital, Clinical Pathology Laboratory.

Preparation of plasma samples for HPLC-MS/MS assay

Plasma samples were prepared for analysis by HPLC-MS/MS using a protein precipitation method as follows. Two milliliter polypropylene tubes were prepared containing 100 µL of BMAP-stabilized plasma (or quality control plasma samples), 300 µL of acetonitrile, and 100 µL of the internal standards (1 ng clopidogrel-d4, 100 ng clopidogrel-d4 carboxylic acid, 1 ng 2-oxoclopidogrel-d3, 1 ng CAM-D-¹³C-d3) dissolved in acetonitrile. Quality control plasma samples analyzed with each run were previously prepared by spiking blank cat plasma with known amounts of clopidogrel, 2-oxo-clopidogrel, CAM-D, and clopidogrel carboxylic acid and stored at -80°C. Standard curve samples were prepared with each run

by adding 100 μ L of blank cat plasma to 100 μ L of known amounts of standards dissolved in acetonitrile, 200 μ L of acetonitrile, and 100 μ L of the internal standard mixture. All samples were then vigorously mixed with a homogenizer (Bullet Blender, Next Advance Inc., Averill Park, NY, USA) for one minute and then centrifuged at 15,000 g for 10 min. For measuring clopidogrel, 2-oxo-clopidogrel, and CAM-D concentrations, 500 μ L of the supernatant was transferred to a 96-well polypropylene plate containing 500 μ L of 0.1% formic acid in water, mixed, and 5 μ L (for clopidogrel and 2-oxo-clopidogrel) or 20 μ L (for CAM-D) were analyzed by HPLC with mass spectrometry detection. For measurement of clopidogrel carboxylic acid concentrations, the samples from the first plate were further diluted by transferring 50 μ L to another 96-well plate containing 950 μ L of water containing 0.1% formic acid and 25% acetonitrile, mixing, and analyzing 5 μ L by HPLC with mass spectrometry detection.

HPLC-MS/MS method

Instrumentation including a gradient capable HPLC pump (Agilent 1100, Agilent Technologies, Santa Clara, CA, USA) with autosampler (CTC-PAL, CTC Analytics, Lake Elmo, MN, USA) and 2.0 x150 mm C18 column (Synergi Polar-RP, Phenomenex, Torrance, CA, USA). A triple quadrupole mass detector (API 4000, AB/Sciex, Framingham, MA, USA) was used to measure negative ion mass transitions including 504.4 $\rightarrow$ 155.2 (CAM-D), 508.2 $\rightarrow$ 155.2 (CAM-D- ^{13}C -d3), 322.2 $\rightarrow$ 212.1 (clopidogrel), 326.2 $\rightarrow$ 216.1 (clopidogrel-d4), 308.1 $\rightarrow$ 198.1 (clopidogrel carboxylic acid), 312.1 $\rightarrow$ 202.1 (clopidogrel-d4 carboxylic acid), 338.3 $\rightarrow$ 155 (2-oxoclopidogrel) and 341.3 $\rightarrow$ 158 (2-oxoclopidogrel-d3). The mobile phase was a mixture of 0.1% formic acid in water (mobile A) and acetonitrile (mobile B) run as a gradient at 400 μ L/min total flow rate. The mobile A:B mix (v/v) was 50:50 from the run start until 1.5 minutes, when it linearly changed to 0:100 at 1.6 minutes, remained there until 4 minutes, returned to 50:50 at 4.1 minutes, and remained there until the end of the run at 7 minutes. Typical retention times for clopidogrel carboxylic acid, clopidogrel-d4 carboxylic acid, clopidogrel, clopidogrel-d4, 2-oxoclopidogrel and 2-oxoclopidogrel-d3 were 1.1, 1.1, 2.95, 2.95, 3.7, and 3.7 minutes, respectively.

High performance liquid chromatography analysis of plasma samples spiked with CAM-D, which was provided by the manufacturer as a mixture of the diastereomers of the H1, H2, H3, and H4 metabolites derivatized with BMAP, showed four peaks with retention times of 2.75 (small peak), 3.25, 3.6, and 3.75 minutes (Fig. 2A). Unfortunately pure H1, H2, H3, and H4 or their BMAP-derivatives were not available from commercial or other sources to allow for positive identification of each of the observed peaks. However, plasma from both of the clopidogrel treated cats in this study showed a single (m/z 504.4 $\rightarrow$ 155.2) peak with an identical retention time (3.6 minutes) to one of the large peaks found in the CAM-D standard mixture (Fig. 2B and 2C). Consequently, the area of this CAM-D peak was used for calibration purposes after adjusting for the amount of this stereoisomer within the total CAM-D mixture (37% by mass based on relative peak areas). For the internal standard CAM-D- ^{13}C -d3, which also contained 4 peaks, the area of the largest peak found at 3.25 minutes retention time was used for calibrating standards and unknown samples.

The standard curves were made by back-calculating from the peak area ratios of clopidogrel or its metabolites compared to each internal standard and the intercept. Clopidogrel and metabolite concentrations in quality control and clopidogrel treated cat plasma samples were calculated from the slopes of standard curves of known analyte concentrations versus analyte to internal standard peak area ratios (Analyst ver. 1.6, AB/Sciex, Framingham, MA, USA). Concentrations are given as ng base mass equivalents per mL of plasma.

Method validation

Validation of the assay method included evaluation of calibration curve linearity, precision and accuracy, within and between assay precision and accuracy of measurements using quality control samples spiked with analytes (CAM-D, clopidogrel, and clopidogrel carboxylic acid) at three different concentrations spanning the calibration curve range. Analyte stability was also assessed by evaluating changes in analyte concentration in samples spiked with 100 ng/mL CAM-D, 38.3 ng/mL clopidogrel, and 500 ng/mL clopidogrel carboxylic acid at different storage temperatures (room temperature, 4°C, -20°C and -80°C) for up to 7 days. Additional samples spiked with 100 ng/mL CAM-D, 76.6 ng/mL clopidogrel, and 2,500 ng/mL clopidogrel carboxylic acid were also evaluated for analyte stability at -80°C for up to 9 months. Freeze thaw analyte stability was evaluated following three repeated freeze thaw cycles using samples spiked with 20 ng/mL CAM-D, 15.3 ng/mL clopidogrel, and 500 ng/mL clopidogrel carboxylic acid.

In vivo application and pilot pharmacokinetic study

To evaluate the utility of the assay, a pilot pharmacokinetic study was conducted in two healthy cats orally administered 18.75 mg of clopidogrel.^a Cats were determined to be healthy based on normal physical exam, complete blood count, chemistry panel, urinalysis, FIV/FelV testing, and echocardiogram. All blood work and urinalyses were performed by the Washington State University, Clinical Pathology Laboratory. All echocardiograms were performed by a single investigator (PML). Conventional 2D, M-mode, and Doppler examinations were performed using an echocardiographic system and transducers ranging from 4–10MHz.^b The use of these animals was approved by the Institutional Animal Care and Use Committee at Washington State University (IACUC protocol #04399). To obtain the 18.75 mg dose, a standard 75 mg clopidogrel tablet was weighed, crushed, and then divided by weight into gelatin capsules. Plasma samples were obtained before clopidogrel administration and at 20 min, 40 min, 60 min, 90 min, 2 h, 4 h, 6 h, 8 h, 12 h, and 24 h after administration.

CAM in whole blood collected from these cats were stabilized through derivatization with BMAP using the following approach. For each 1 mL whole blood sample, a 2 mL polypropylene tube was prepared containing 10 µL of a 50 mM solution of BMAP in acetonitrile and 20 µL of a 500 mM solution of EDTA in water. Tubes were stored at 4°C until use within 24 hours. For blood collection, 1 mL of whole blood was added to each tube. Immediately after addition, the tubes were capped, mixed gently by inversion 8–10

^aPlavix, Bristol-Myers Squibb/Sanofi Pharmaceuticals Partnership, Bridgewater, NJ

^bMyLab 70, Esaote North America, Inc., Indianapolis, Indiana, USA

times, and then centrifuged at 2,500 g for 5 min at room temperature. The upper plasma layer was transferred to a new 1.5 mL polypropylene tube and stored at -80°C until analysis by HPLC-MS/MS.

Pharmacokinetic parameters including the maximum observed concentration (C_{max}), the time at which C_{max} occurred (T_{max}), elimination half-life ($t_{1/2}$), area under the plasma concentration-time curve (AUC) until the last measured time point (AUC_{last}), and AUC extrapolated to infinity ($\text{AUC}_{\text{total}}$) were estimated by a non-compartmental approach using linear regression of log transformed concentration data (elimination half-life) and the trapezoidal method (AUC) with Microsoft Excel as we have recently described in detail (19).

RESULTS

The 2-oxo-clopidogrel metabolite was unstable ($>25\%$ decrease from nominal concentration within 6 hours) at all storage temperatures tested in both EDTA treated and citrate treated plasma (data not shown). As a result, selectivity, linearity, precision, and accuracy for 2-oxo-clopidogrel were not evaluated further.

Calibration curve linearity precision and accuracy

Seven to eight point standard curves were used to assess linearity for CAM-D, clopidogrel, and clopidogrel carboxylic acid. Standard curves estimated for CAM-D, clopidogrel, and clopidogrel carboxylic acid were linear at concentrations of 1–100 ng/mL for CAM-D, 0.8–76.6 ng/mL for clopidogrel, and 200–10,000 ng/mL for clopidogrel carboxylic acid. The mean, standard deviation from nominal, accuracy, and precision for each analyte are described in Table 1. The correlation coefficients obtained over 4 to 6 separate trials for the CAM-D, clopidogrel, and clopidogrel carboxylic acid standard curves indicated consistent linearity with $r > 0.99$. The lower limits of quantitation (lowest concentrations on the calibration curves with 20% or less precision and 20% or less deviation from nominal) were 1 ng/mL, 0.8 ng/mL, and 200 ng/mL for CAM-D, clopidogrel, and clopidogrel carboxylic acid, respectively. The lower limits of detection (defined as three times the baseline signal) were 0.5 ng/mL, 0.05 ng/mL, and 4 ng/mL for CAM-D, clopidogrel, and clopidogrel carboxylic acid, respectively.

Within and between assay precision and accuracy

The within and between assay precision ($\pm 7\%$ and $\pm 7.1\%$, respectively) and accuracy ($\pm 2.6\%$) for CAM-D were excellent at low, middle, and high quality control concentrations. There was an excellent within and between assay precision ($\pm 5.6\%$ and $\pm 3.7\%$, respectively) and good to acceptable accuracy ($\pm 16.2\%$) at low, middle, and high quality control concentrations for clopidogrel. There was good within and between assay precision ($\pm 7.0\%$ and $\pm 11.3\%$, respectively) and acceptable accuracy ($\pm 17.9\%$) at low, middle, and high quality control concentrations for clopidogrel carboxylic acid (20) (Table 2).

Analyte stability during storage and freeze-thaw

Stability of CAM-D, clopidogrel, and clopidogrel carboxylic acid were evaluated during storage over 1 week at -80°C , -20°C , 4°C , and room temperature in both citrate and EDTA treated plasma. Stability at -80°C was also evaluated for up to 9 months in citrate treated plasma. As shown in Table 3, all analytes were stable (20% or less decrease) for up to 1 week under all storage temperatures tested, and for up to 9 months at -80°C for citrate treated plasma. In addition, there were no differences in analyte stability associated with matrix (citrate versus EDTA treated feline plasma). Analyte stability in citrate treated plasma was also evaluated over three freeze-thaw cycles. As shown in Table 4, all analytes showed 13% or less decrease from nominal concentration over the three freeze-thaw cycles evaluated.

Pilot pharmacokinetic study

Plasma concentration-time profiles of CAM-D, clopidogrel, and clopidogrel carboxylic acid after a single oral dose of clopidogrel administered to two healthy cats are presented in Figure 3. Derived pharmacokinetic parameters for CAM-D, clopidogrel, and clopidogrel carboxylic acid for both cats are summarized in Table 5. The pharmacokinetic profiles and parameters for clopidogrel and clopidogrel carboxylic acid were quite similar for the 2 cats studied. However, the C_{max} and AUC values of CAM-D were more than 2-fold higher, and the CAM-D elimination half-life were nearly 2 times longer in Cat 1 compared with Cat 2. The C_{max} and AUC values were much higher (by over 100-fold) and elimination half-life longer (by about 2 times) for clopidogrel carboxylic acid compared with clopidogrel and CAM-D (in both cats). The AUC that was extrapolated was quite low for clopidogrel ($<2\%$), moderate for CAM-D ($\sim 12\%$) and more substantial for clopidogrel carboxylic acid (15–20%).

DISCUSSION

This study demonstrates the first quantitation of CAM-D in feline plasma. Based on national guidelines (20), this HPLC-MS/MS method is an accurate and precise method for the quantitation of CAM-D in feline plasma. This method also allows for simultaneous quantitation of parent clopidogrel and the clopidogrel carboxylic acid metabolite but is unsuitable for the quantitation of the 2-oxo-clopidogrel metabolite because of significant analyte instability.

The standard curves displayed linearity for CAM-D, clopidogrel, and clopidogrel carboxylic acid. The maximum plasma concentration of clopidogrel in both cats, however, exceeded the clopidogrel calibration range of 0.8–76.6 ng/mL. As a result, the values for both cats from the pharmacokinetic study were derived by extrapolation. This extrapolation is likely valid since further expansion of the calibration range has been conducted since this study that demonstrates linearity for clopidogrel concentrations up to 500 ng/mL (data not shown). The initial calibration range (0.8–76.6 ng/mL) was selected for validation based on the C_{max} of clopidogrel of 20 ng/mL reported for healthy humans (16). One possible explanation for the substantially higher clopidogrel C_{max} in cats is the significantly higher mg/kg dosage of clopidogrel given to cats compared to humans. The clinically used daily dose of clopidogrel

for cats and people are 18.75 mg/cat and 75 mg/person (21), respectively. As a result, if an average cat weighs 4.5 kg and an average human weighs 80 kg (22), then the dosage would translate to 4.2 mg/kg for a cat vs. 0.9 mg/kg for a person. For future evaluation of clopidogrel pharmacokinetics in cats, an expansion of the upper end of the standard curve is needed. The expansion of the lower end of the standard curve for CAM-D, clopidogrel, and clopidogrel carboxylic acid may also be feasible since the lower limits of detection is lower than the current lower limits of quantitation for each compound.

CAM-D, clopidogrel, and clopidogrel carboxylic acid appear to be stable in feline plasma for at least 1 week at room temperature in EDTA treated plasma and at least 9 months at -80°C in citrate treated plasma. This data is particularly noteworthy since it indicates that once blood is collected into BMAP-spiked anticoagulant tubes, the derived plasma samples can be easily shipped without risk of significant analyte degradation. The ability to ship samples could allow for more widespread use of this assay in both research and clinical settings.

The *in vivo* utility of this assay was demonstrated by our preliminary pharmacokinetic study following the oral administration of 18.75mg clopidogrel to two cats. Our preliminary pharmacokinetic data shows that, in cats, clopidogrel is rapidly absorbed through the gastrointestinal tract. Plasma concentrations of clopidogrel could be detected at the first sampling time point 20 minutes after drug administration. For Cat 1, plasma concentrations of parent clopidogrel at 90 minutes post administration were lower than 60 minutes and 120 minutes post administration. We suspect that this drop could represent an error in handling of that sample, although other causes cannot be excluded. Once absorbed, clopidogrel is rapidly converted to both the clopidogrel carboxylic acid and CAM. As in people, the majority of clopidogrel in cats is converted to the inactive clopidogrel carboxylic acid metabolite, and a smaller proportion of clopidogrel is converted to CAM. Both CAM-D and clopidogrel carboxylic acid plasma concentrations were detected at 40 minutes after drug administration in both cats and reached $C_{\max}$ values of 16–39 ng/mL and 2,900–3,300 ng/mL, respectively, approximately 1.5–2 hours post administration. In people, the CAM-D $C_{\max}$ appears to be similar with values around 18–30 ng/mL, and the $T_{\max}$ values occur around 1 hour post administration for both clopidogrel and CAM-D (16).

Estimates of the amount of AUC that was extrapolated beyond 24 hours for each analyte indicated that plasma sampling for up to 24 hours is likely sufficient to accurately estimate elimination parameters of clopidogrel and CAM-D. However, additional sampling time may be needed to accurately estimate these parameters for clopidogrel carboxylic acid.

Interestingly, although the $C_{\max}$, AUC, and elimination half-life values were quite similar between cats for clopidogrel and clopidogrel carboxylic acid, the CAM-D values were somewhat different between cats. Lower $C_{\max}$, AUC, and faster elimination half-life of CAM-D (by about 2-times) were found for Cat 2 compared with Cat 1, which is consistent with a slower rate of formation of CAM in Cat 2 compared with Cat 1. Variation between cats in the formation of CAM could impact the efficacy of clopidogrel therapy in preventing thromboembolism. Interindividual variability in platelet inhibition by clopidogrel has already been previously reported in cats (7–9). Additional pharmacokinetic studies with

more cats as well as studies correlating clopidogrel pharmacokinetics and pharmacodynamics are indicated to better describe the magnitude and causes of this variability.

Unlike people who demonstrate two distinct peaks on chromatograms of CAM (12), cats appear to demonstrate only one peak (Fig. 2C). In people, the two distinct peaks represent the formation of both the H3 (inactive) and H4 (active) diastereoisomers. We suspect that the single peak in cats either represents the H2 or H4 diastereoisomer because the H2 and H4 isomers are the only active isomers of CAM (10) and because platelet inhibition by clopidogrel in cats has been demonstrated (1, 7). Based on the retention time of the CAM-D peak in cats, the peak is would be most consistent with H4. However, this hypothesis will need to be confirmed once pure diastereomers become available. This interspecies difference may be the result of different cytochrome P450 isoenzymes present in the human compared to the feline liver since CAM is generated through the cytochrome P450 pathway and since the uniqueness of feline hepatic drug metabolism has been well established (23–26). Additionally, clopidogrel in humans undergoes metabolism through pathways other than the hepatic P450 pathway (27), such as the enteric P450 pathway (28) and serum paraoxonase-1 (29). Interspecies variations in these pathways may also influence clopidogrel metabolism in the cat.

CONCLUSIONS

Validation of this HPLC-MS/MS assay for quantitation of feline plasma concentrations of CAM-D is the first step to more fully understanding the use of clopidogrel in cats in both a population and individual setting. Complete clopidogrel pharmacokinetic studies, clopidogrel pharmacodynamic-pharmacokinetic correlations, and evaluation of alterations in clopidogrel metabolism in cats can now be explored using this accurate and precise assay.

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Abbreviations

BMAP	2-bromo-3' methoxyacetophenone
CAM	clopidogrel active metabolite
CAM-D	derivatized clopidogrel active metabolite
EDTA	ethylene diamine tetra acetic acid
HPLC-MS/MS	high-performance liquid chromatography with tandem mass spectrometry

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Fig. 1.

Metabolic pathway of clopidogrel. The parent clopidogrel undergoes metabolism by two different hepatic pathways. A proportion of clopidogrel is hydrolyzed by carboxyl esterase to form the inactive clopidogrel carboxylic acid metabolite. The remaining clopidogrel is converted by the cytochrome P450 enzyme system (CYP450) to an intermediate product 2-oxo-clopidogrel, which is subsequently hydrolyzed to form the unstable clopidogrel active metabolite (CAM). In the presence of 2-bromo-3' methoxyacetophenone (BMAP), the reactive thiol group of CAM binds with BMAP thereby forming a stable compound, the derivatized clopidogrel active metabolite (CAM-D).

Fig. 2. Chromatograms of (A) the derivatized clopidogrel active metabolite (CAM-D) following protein precipitation of a standard solution and plasma samples obtained from a healthy cat (B) before administration of clopidogrel and (C) 2 h after receiving a single oral dose of 18.75mg of clopidogrel.

Fig. 3. Plasma concentration versus time curves of (A) CAM-D, (B) parent clopidogrel, and (C) the clopidogrel carboxylic acid metabolite for two different cats that received a single oral dose of 18.75mg clopidogrel. Samples were collected before and also 20 min, 40 min, 1 h, 90 min, 2 h, 4 h, 6 h, 8 h, 12 h, and 24 h post administration.

Table 1

Linearity of the derivatized clopidogrel active metabolite (CAM-D), clopidogrel, and clopidogrel carboxylic acid calibration standard curves over a range of concentrations presented as mean $\pm$ standard deviation (SD) as well as accuracy (percent deviation from nominal) and precision (percent coefficient of variation, CV). The number of trials performed is indicated by n. Samples were run in duplicate for each trial.

Nominal (ng/mL)	Mean (ng/mL)	$\pm$ SD (ng/mL)	Accuracy (% deviation)	Precision (% CV)	n
<i>CAM-D</i>					
100	102.0	4.3	2.0	4.2	6
50	50.7	3.2	1.3	6.3	6
20	20.4	1.7	1.7	8.5	6
10	10.4	0.9	4.1	8.6	6
5	5.3	0.2	6.5	4.1	6
2	2.2	0.1	8.6	4.7	6
1	1.0	0.1	3.2	4.7	4
<i>Clopidogrel</i>					
76.6	78.6	7.4	2.6	9.4	6
38.3	41.2	3.1	7.5	7.4	6
15.3	16.8	1.8	9.6	10.8	6
7.7	8.4	0.8	9.9	9.4	6
3.8	4.3	0.4	11.2	9.7	6
1.5	1.6	0.2	6.7	8.8	6
0.8	0.8	0.1	9.3	10.6	6
<i>Clopidogrel carboxylic acid</i>					
10,000	10,010	350	0.1	3.4	6
4,000	4,170	190	4.3	4.5	6
2,000	2,020	70	0.9	3.5	5
1,000	1,080	80	8	7.8	6
400	410	40	2.5	8.6	6
200	210	30	4.2	13.4	6

Table 2

Within and between assay precision and accuracy for the derivatized clopidogrel active metabolite (CAM-D), clopidogrel, and clopidogrel carboxylic acid for low, middle, and high quality control concentrations represented as mean $\pm$ standard deviation (SD) as well as accuracy (percent deviation from nominal) and precision (percent coefficient of variation, CV). The number of trials performed is indicated by n. Samples were run in duplicate for each trial.

Nominal (ng/mL)	n	Within Assay		Between Assay	
		Mean Precision (% deviation) ± SD	Grand Mean ± SD	Accuracy (% deviation)	Precision (% CV)
CAM-D					
100	5	7.0 ± 3.6	101.0 ± 7.1	1.0	7.1
20	5	6.1 ± 0.9	20.47 ± 0.6	2.3	2.8
5	5	6.2 ± 2.0	5.1 ± 0.1	2.6	2.3
Clopidogrel					
76.6	5	5.6 ± 4.2	64.2 ± 1.9	16.2	3.0
15.3	5	2.7 ± 2.6	15.3 ± 3.1	9.4	2.1
3.8	5	4.9 ± 3.3	3.6 ± 0.8	14.8	3.7
Clopidogrel carboxylic acid					
2,500	5	6.4 ± 4.7	2,870 ± 240	14.8	8.3
500	5	4.9 ± 3.8	590 ± 70	17.9	11.3
125	5	7.0 ± 3.8	140 ± 10	14.7	7.7

Table 3

Stability of the derivatized clopidogrel active metabolite (CAM-D) was evaluated at a concentration 100 ng/mL for 1 week (wk) stability, 4 months (mo) stability, and 9 mo stability. Stability of clopidogrel was evaluated at concentrations of 38.3 ng/mL for 1 wk stability and 76.6 ng/mL for 4 and 9 mo stability. Stability of clopidogrel carboxylic acid were evaluated at concentrations of 500 ng/mL for 1 wk stability and 2,500 ng/mL for 4 and 9 mo stability. Short term stability was assessed for up to 1 wk at room temperature (RT), 4°C, -20°C, or -80°C in citrate or EDTA treated feline plasma and up to 9 months (mo) at -80°C in citrate treated feline plasma. Long term stability at -80°C was not determined (ND) for EDTA treated feline plasma. Data are presented as percent deviation from the nominal concentration.

Storage	Time	Deviation from nominal concentration (%)					
		Citrate treated plasma			EDTA treated plasma		
		CAM-D	Clopidogrel	Clopidogrel carboxylic acid	CAM-D	Clopidogrel	Clopidogrel carboxylic acid
		-10	10	4	-2	-4	-11
		-10	6	9	1	-2	3
RT	24 h	-5	7	14	5	-2	-2
RT	48 h	-17	7	20	-4	-5	-10
RT	72 h	-20	0	17	-3	-1	1
RT	1 wk	-20	2	25	-13	6	-1
4°C	6 h	3	6	8	-15	-7	-6
4°C	12 h	0	6	13	7	1	11
4°C	24 h	-2	7	11	7	-1	-1
4°C	48 h	6	6	17	1	1	-7
4°C	72 h	3	2	7	2	-3	-11
4°C	1 wk	7	6	19	-2	-10	-15
-20°C	6 h	-5	7	0	4	-6	-6
-20°C	12 h	-9	7	18	6	0	10
-20°C	24 h	-9	6	10	6	-2	-1
-20°C	48 h	-4	6	16	0	-2	-6
-20°C	72 h	-16	5	8	2	-5	-1
-20°C	1 wk	-13	7	11	3	-2	1
-80°C	6 h	6	10	5	1	-2	-7
-80°C	12 h	-1	8	22	2	-1	-4
-80°C	24 h	3	10	19	0	1	-3

Deviation from nominal concentration (%)									
Storage	Time	Citrate treated plasma			EDTA treated plasma			Clopidogrel	Clopidogrel carboxylic acid
		CAM-D	Clopidogrel	Clopidogrel carboxylic acid	CAM-D	Clopidogrel	Clopidogrel carboxylic acid		
-80°C	48 h	10	5	17	5	-3	-10		
-80°C	72 h	-8	1	7	7	1	-1		
-80°C	1 wk	1	2	15	-1	1	1		
-80°C	4 mo	12	8	15	ND	ND	ND		
-80°C	9 mo	-12	14	-11	ND	ND	ND		

Table 4

Stability of the derivatized clopidogrel active metabolite (CAM-D) at 20 ng/mL, clopidogrel at 15.3 ng/mL, and clopidogrel carboxylic acid at 500 ng/mL was evaluated after three freeze-thaw cycles (-80°C /room temperature) in citrate-treated plasma. Data are presented as percent deviation from the nominal concentration. Values are the average of duplicate determinations, which varied by less than 15% from the nominal concentration. The largest decrease was 13% of both clopidogrel and clopidogrel carboxylic acid and 8% decrease of CAM-D.

	Deviation from nominal concentration (%)		
	CAM-D	Clopidogrel	Clopidogrel carboxylic acid
1 Freeze Thaw Cycle	-7	1	-7
2 Freeze Thaw Cycles	1	15	-7
3 Freeze Thaw Cycles	-8	-13	-13

Table 5

Summary of pharmacokinetic parameters for the derivatized clopidogrel active metabolite (CAM-D), clopidogrel, and clopidogrel carboxylic acid metabolite from cats treated with a single oral dose of 18.75mg clopidogrel.

Parameter (units)	CAM-D		Clopidogrel		Clopidogrel carboxylic acid	
	Cat 1	Cat 2	Cat 1	Cat 2	Cat 1	Cat 2
C_{max} (ng/mL)	39.0	16	134	144	3,300	2,900
T_{max} (h)	1.5	2.0	2	0.7	2.0	1.5
$t_{1/2}$ (h)	4.9	2.8	5.4	5.2	9.2	10.4
AUC_{last} (ng.h/mL)	117	60	374	225	25,000	18,100
AUC_{total} (ng.h/mL)	134	68	377	229	31,400	21,200
Area extrapolated (%)	12.3	12	0.8	1.8	19.6	14.6

C_{max} , maximum concentration; T_{max} , time taken to reach maximum concentration; $t_{1/2}$, elimination half-life; AUC_{last} , area under the plasma concentration-time curve from 0 to 24hrs; AUC_{total} , area under the plasma concentration-time curve extrapolated to infinity.