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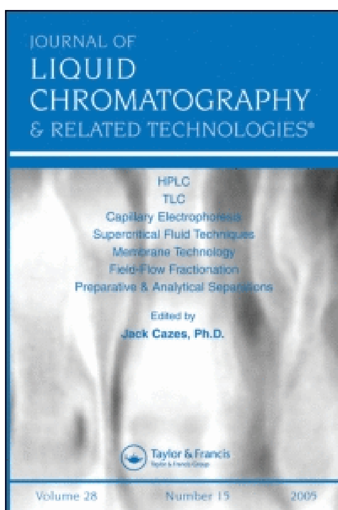

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Determination of L-(5-(3,4-Dimethoxyphenyl)-Pyrazol-3-Yl-Oxypropyl)-3-[N-methyl-N- 2-(3,4-dimethoxyphenyl)ethyl-amino] Propane Hydrochloride and Its Two Metabolites in Dog Plasma by High Performance Liquid Chromatography with Fluorescence Detection

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DETERMINATION OF 1-(5-(3,4-DIMETHOXYPHENYL)-PYRAZOL-3-YL-OXYPROPYL)-3-[N-METHYL-N- 2-(3,4-DIMETHOXYPHENYL)ETHYL-AMINO]PROPANE HYDROCHLORIDE AND ITS TWO METABOLITES IN DOG PLASMA BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORESCENCE DETECTION

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ABSTRACT

A simple, specific and sensitive high-performance liquid chromatographic (HPLC) method was developed for the determination of 1-(5-(3,4-dimetoxyphenyl)-pyrazol-3-yl-oxypropyl)-3-[n-methyl-n-[2-(3,4-dimethoxyphenyl)ethyl]amino] propane hydrochloride (KC11346) and two of its metabolites (KC12795, KC12816) in dog plasma. KC11346, KC12795, KC12816 and KC11294 (internal standard) are extracted from dog plasma by methyl t-butyl ether (MTBE) following an alkalization of plasma. MTBE is removed from the extract with

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a gentle stream of nitrogen at 40°C and reconstituted in 200 μL of mobile phase. Separation of the reconstituted extract is achieved by HPLC on a Zorbax SB-CN column with a mobile phase composed of (56:44) 50 mM sodium acetate/acetonitrile. The analytes were detected by fluorescence detection with a Corion UG-11 cut off filter and an excitation wavelength of 265 nm. The mean retention times of KC12816, KC12795, KC11346, and the internal standard were 4.8, 8.1, 9.0, and 14.4 minutes, respectively. The assay is linear over the concentration range 3 to 350 ng/mL. The analysis of pooled quality control samples (8, 30, and 300 ng/mL) demonstrates excellent precision with relative standard deviations (RSD) ($n = 18$) $\leq$ 7.5% for all three compounds. The method is accurate with all intraday ($n = 6$) and overall mean values $\leq$ 15.7% from theoretical at all control concentrations for all compounds.

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INTRODUCTION

1-(5- (3,4- Dimethoxyphenyl)- pyrazol- 3-yl- oxypropyl)- 3-[n- methyl- n-[2-(3,4-dimethoxyphenyl)ethyl] amino]propane hydrochloride (KC11346) is a new cardiovascular drug at the preclinical stage developed as bradycardic antianginal agent for treatment of ischemic heart disease and congestive heart failure as a potential second indication. A sensitive and selective method is needed to support preclinical toxicokinetic studies.

HPLC is a very viable separation technique that is widely used in analysis of pharmaceuticals.[1-6] Detection methods such as fluorescence,[1] ultra-violet,[2] electrochemical,[3] mass spectrometry[4-5] and refractive index[6] have been used with HPLC analysis. KC11346, and two of its metabolites (KC12795, KC12816) can be detected by fluorescence without any derivatization, therefore, a simple, sensitive and specific method with fluorescence detection was developed and is described in this manuscript.

EXPERIMENTAL

Test Materials

KC11346, KC12795, KC12816 and KC11294 (internal standard) were synthesised by Kali Chemie Pharma GmbH. Heparinized dog plasma was obtained from Rockland. Acetonitrile, HPLC grade were obtained from Fisher

(Fairlawn, NJ, USA). Methanol, hexane and methyl t-butyl ether, HPLC grade were obtained from Burdick & Jackson. Acetic acid and ammonium hydroxide, analytical reagent grade were obtained from EM Science (Gibbstown, NJ, USA). Sodium acetate anhydride, analytical reagent grade were obtained from Mallinckrodt. Deionized water was processed through a Milli-Q water purification system, Millipore Corporation.

Chromatographic Conditions

The HPLC system consisted of a Perkin-Elmer 200 LC pump (Norwick, CT, USA), a Waters 717 autoinjector (Milford, MA, USA), and ABI/Kratos Spectroflow 980 fluorescence detector (Foster City, CA, USA) with a Corion UG-11 cut off filter and 265 nm excitation wavelength. The analytical column was a Zorbax SB-CN, 250 mm x 4.6 mm, 5-µm particle size (Mac-Mod, Chadds Ford, PA, USA) protected by a Brownlee CN pre-column (15 mm x 3.2 mm, 7-µm particle size, ABI, San Jose, CA, USA). The column temperature was maintained at 35°C with an Eppendorf CH-30 column heater. The mobile phase was (56:44) 0.05M sodium acetate/acetonitrile with a flow rate of 1.2 mL/minute. Data collection and calculations were conducted using an HP1000, Model A990 computer with a 3350A Laboratory Automation System (Hewlett-Packard, Palo Alto, CA, USA)

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Preparation of Standard Solutions

A stock standard solution for KC11346 (100 µg/mL) was prepared by dissolving 2.75 mg of KC11346 salt in 25-mL water. The factor to convert the salt to the free base is 0.926. A stock standard solution for KC12795 (500 µg/mL) were prepared by dissolving 5 mg of KC12795 in 10-mL water. A stock standard solution for KC12816 Stock Standard Solution (7.79 mg/mL) was prepared by dissolving the entire material received in 25-mL of 50:50 methanol/water because the standard material received at CHW was stuck together and could not be weighed out in smaller portions. Combined standard spiking solutions (30 to 3500 ng/mL) for the standard curve were prepared in 1% acetic acid from the three stock solutions. A stock solution of internal standard solution (100 µg/mL) was prepared by dissolving 1 mg of KC11294 in 10-mL of methanol. The internal standard working solution (5 µg/mL) was prepared by diluting the stock solution with water. All stock and working solutions were prepared in glass volumetric flasks and vials, and were stored at approximately 5°C protected from light with aluminum foil. These solutions were stable for the duration of the validation.

Quality Control Samples

Pooled quality control samples (QC samples) were prepared to determine the precision and accuracy of the method, and to evaluate the stability of samples. Over-curve quality control samples were also prepared to evaluate the precision and accuracy when specimens required analysis at partial volume.

Plasma control pools (8, 30, and 300 ng/mL) were prepared by diluting 160 µL of 10 µg/mL, 600 µL of 10 µg/mL, and 1,200 µL of 50 µg/mL KC11346, KC12795, and KC12816, respectively, to a 200-mL volume using blank dog plasma. An over-curve control (3000 ng/mL) was prepared by diluting 12 mL of 50 µg/mL to a 200-mL volume with blank dog plasma.

All control pools were aliquoted into 6-mL polypropylene-vials and stored at approximately -70°C.

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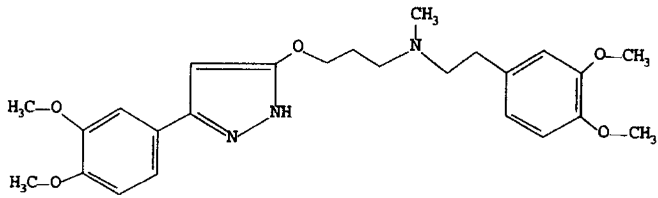
Sample Preparation

Calibration standards (3-350 ng/mL) were prepared by adding 50 µL of the appropriate KC11346, KC12795, and KC12816 combined spiking solutions (30-3500 ng/mL) to 0.5 mL of blank dog plasma. Clinical specimens and controls were prepared by aliquoting 0.5 mL into glass conical centrifuge tubes.

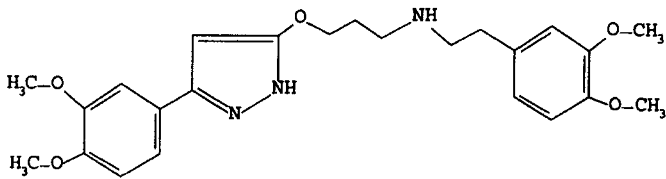
Calibration standards, clinical specimens and controls were processed by adding 50 µL of internal standard working solution into each tube except the reagent blank and blank plasma. Fifty microliters of 1% acetic acid was added to all QC samples, blanks, and reagent blanks to compensate for spiking solution volume added to calibration standards.

Four hundred microliters of 1:3 ammonium hydroxide and 6 mL of methyl t-butyl ether were added to each tube. The tubes were shaken for 10 minutes in a horizontal shaker at 72 cpm and centrifuged at approximately 980 g for 5 minutes.

The aqueous layer was frozen in an acetone/dry ice bath, the organic layer was transferred to a clean tube. The methyl t-butyl ether was dried down under nitrogen in Turbo Vap at 40°C and the specimen reconstituted in 200 µL of mobile phase. The resulting solution was washed with 1 mL of hexane. Forty microliters aliquots were injected onto the HPLC system.



KC11346



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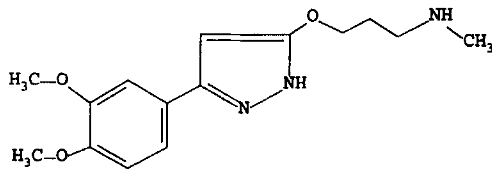
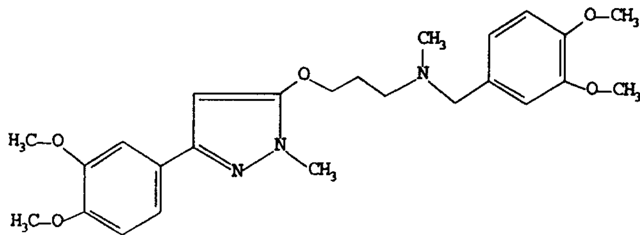


KC12816



KC11294

Figure 1. Molecular structures.

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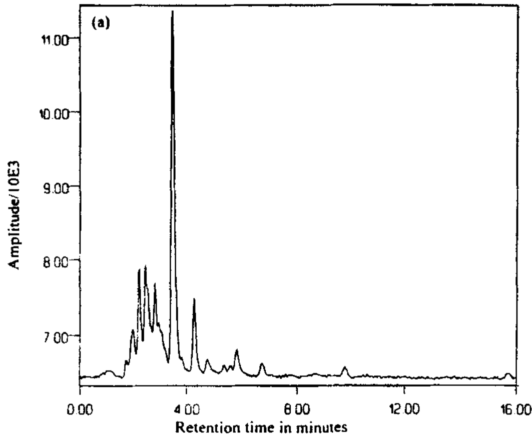

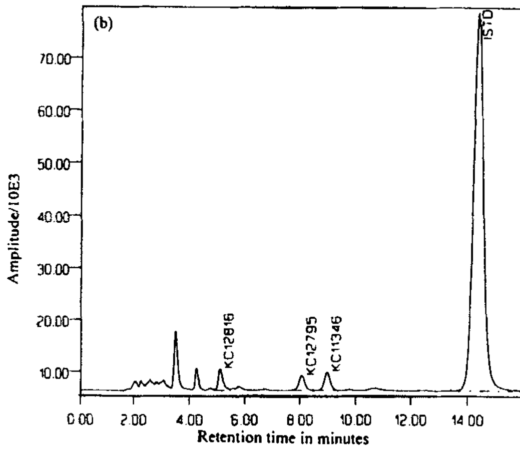

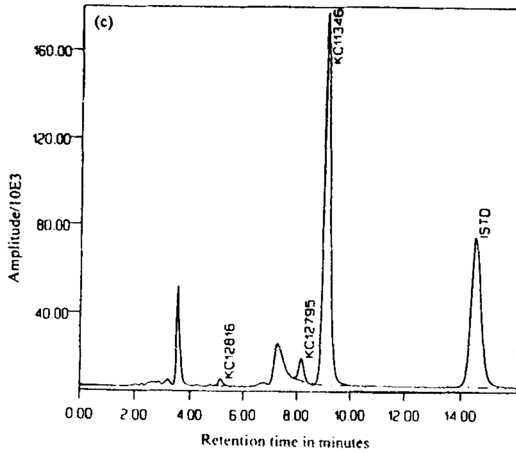

Validation Study

Duplicate calibration curves (3, 5, 10, 15, 50, 100, and 350 ng/mL) were analyzed on each of three days. One reagent blank (water substituted for plasma), blank plasma, control zero (blank plasma spiked with internal standard) and triplicate controls at each concentration (8, 30, 300 ng/mL of KC11346, KC12795 and KC12816 in plasma) were analyzed with each calibration curve. The calibration curves were obtained by weighted ($1/C^2$) least-squares linear regression analysis of the peak height ratios of KC11346, KC12795 or KC12816/internal standard vs the concentration of KC11346, KC12795 or KC12816. The equations of the calibration curves were then used to calculate the concentration of KC11346, KC12795, and KC12816 in the samples and controls from their peak height ratios.

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RESULTS AND DISCUSSION

Separation and Specificity

The molecular structures of KC11346, KC12795, KC12816 and KC11294 (internal standard) are shown in Figure 1. The four compounds were well separated from each other as shown in Figure 2. The mean retention times of KC12816, KC12795, KC11346, and the internal standard were approximately 4.8, 8.1, 9.0 and 14.4 minutes, respectively.

Blank dog plasma from ten pools was tested for endogenous interferences. All regions were free of endogenous interference peaks for seven of the lots. Three pools had minor interference peaks in the KC12816 and KC11346 regions and were not used in validation.

Linearity, Precision and Accuracy

Calibration curve parameters for KC12816, KC12795, and KC11346 are in Tables 1-3. Calibration curves for KC12816, KC12795, and KC11346 in plasma were linear in the concentration range from 3.00 to 350 ng/mL, with correlation coefficients greater than 0.9972 for all curves.

Figure 2. (left) Chromatograms of dog plasma from control animals (a), 3.00-ng/mL Calibration Standard (b), and plasma from a dog taken 2 hours after dosing with KC11346 (c).

Table 1

Calibration Curve Parameters for KC11346 in Dog Plasma

Day	Curve	Slope	Intercept	Correlation Coefficient
1	1	1.59E-2	-1.25E-3	0.9989
	2	1.70E-2	-1.67E-3	1.0000
2	3	1.33E-2	3.47E-3	0.9989
	4	1.30E-2	1.39E-2	0.9983
3	5	1.58E-2	-7.52E-3	0.9977
	6	1.74E-2	-4.92E-3	0.0076

Table 2

Calibration Curve Parameters for KC12795 in Dog Plasma

Day	Curve	Slope	Intercept	Correlation Coefficient
1	1	1.37E-2	3.45E-3	0.9991
	2	1.46E-2	3.22E-3	0.09992
2	3	1.12E-2	4.07E-3	0.9972
	4	1.13E-2	1.12E-3	0.9997
3	5	1.36E-2	-6.08E-3	0.9981
	6	1.49E-2	-4.05E-3	0.9981

Table 3

Calibration Curve Parameters for KC12816 in Dog Plasma

Day	Curve	Slope	Intercept	Correlation Coefficient
1	1	3.32E-2	-1.42E-4	0.9990
	2	2.59E-2	-9.29E-4	0.9997
2	3	1.64E-2	-1.52E-3	0.9990
	4	1.62E-2	-2.14E-4	0.9998
3	5	1.55E-2	-3.02E-3	0.9993
	6	1.61E-2	-1.65E-3	0.9990

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Table 4

Precision and Accuracy of KC11346 Standards

Calibration Standard Concentration (ng/mL)	Calculated Concentration (mean±S.D., n=6) (ng/mL)	RSD (%)	Deviation (%)
3	3.1±0.05	1.7	3.3
5	4.9±0.15	3.1	-2.6
10	9.6±0.32	3.4	-4.4
15	14.6±0.25	1.7	-2.9
50	50.5±1.75	3.5	1.0
100	101±1.4	1.4	1.0
350	364±11.8	3.2	4.0

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Table 5

Precision and Accuracy of KC-127595 Standards

Calibration Standard Concentration (ng/mL)	Calculated Concentration (mean±S.D., n=6) (ng/mL)	RSD (%)	Deviation (%)
3	3.1±0.08	2.6	3.0
5	4.9±0.23	4.8	-2.8
10	9.8±0.45	4.6	-2.5
15	14.7±0.21	1.4	-2.0
50	50.6±2.10	4.1	1.2
100	101±1.9	1.8	1.0
350	361±13.0	3.6	3.1

Precision and accuracy of KC12816, KC12795, and KC11346 standards are in Tables 4-6. The standards show low values in deviation (<4.6%) and relative standard deviation (<4.8%).

Table 6

Precision and Accuracy of KCl2816 Standards

Calibration Standard Concentration (ng/mL)	Calculated Concentration (mean±S.D., n=6) (ng/mL)	RSD (%)	Deviation (%)
3	3.0±0.067	2.2	1.0
5	5.0±0.14	2.8	-0.4
10	9.9±0.29	30	-1.5
15	14.8±0.28	1.9	-1.3
50	50.9±1.54	3.0	1.8
100	99.1±1.93	2.0	-0.9
350	356±12.0	3.4	1.7

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Data from the spiked quality control samples are shown in Tables 7-9. The within-day precision of the method as measured by the relative standard deviation (RSD) of the daily mean (n = 6) was less than 7.8% at the three control concentrations in dog plasma. The overall precision ranged from 4.3% to 7.5% RSD (n = 18) for the 8.00-, 30.0-, and 300-ng/mL KC12816, KC12795 and KC11346 controls.

The accuracy of the method was determined by comparing the means of the measured concentrations with the nominal (theoretical) concentrations of KC12816, KC12795 and KC11346 in the plasma QC samples. All of the daily mean (n = 6) and overall mean (n = 18) values for the QC samples were within 15.6% of their expected values.

Partial Volume Analysis Precision and Accuracy

A quality control samples containing 3000 ng/mL KC12816, KC12795 and KC11346 was prepared and analyzed with the high QC sample (300 ng/mL) at the partial volumes of 50 μL. These aliquots were diluted to a final volume of 500 μL with dog plasma from control animals. The mean (n = 6) values for all partial volumes were within 5.4% of their expected values. The precision was better than 6.1% RSD (n = 6) at all partial volumes.

Table 7

Precision and Accuracy of KC11346 Quality Controls

Calibration Concentration (ng/mL)	Calculated Concentration (Overall Mean±S.D., n=6) (ng/mL)	RSD (%)	Deviation (%)
8	8.1±0.61	7.5	2.0
30	29.2±1.55	5.3	-2.7
300	340±15.7	4.6	13.3

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Table 8

Precision and Accuracy of KCl2795 Quality Control

Calibration Concentration (ng/mL)	Calculated Concentration (Overall mean±S.D., n=6) (ng/mL)	RSD (%)	Deviation (%)
8	8.0±0.60	7.5	-0.1
30	29.8±1.61	5.4	-0.7
300	316±15.9	5.0	5.3

Table 9

Precision and Accuracy of KCl2816 Quality Control

Calibration Concentration (ng/mL)	Calculated Concentration (Overall mean±S.D., n=6) (ng/mL)	RSD (%)	Deviation (%)
8	8.3±0.52	6.3	4.3
30	29.3±1.26	4.3	-2.3
300	321±14.9	4.6	7.0

Limit of Quantitation

The lower limit of quantitation (LLOQ) was set at 3.00 ng/mL of KC12816, KC12795, and KC11346 in dog plasma. Six replicates of the lowest standards (3 ng/mL) were analyzed to evaluate the LLOQ. At the LLOQ, the RSD (n = 6) of the peak height ratios was within 7.1%, the RSD (n = 6) of the measured concentrations was within 6.9%, and the deviation of the mean of the measured concentrations from their nominal value was within 3.8% for all three compounds.

Absolute Recoveries

Absolute recoveries were determined by comparing the peak heights of extracted calibration standards with the peak heights of pure recovery standards at the same nominal concentrations. The mean recoveries for KC12816, KC12795, KC11346 and the internal standard were 83.4%, 98.7%, 93.3% and 76.4%, respectively.

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Stability

Working solutions of KC11346, KC12795 and KC12816 were stable in 1% acetic acid for at least 2 months when stored at 5°C and protected from light.

The stability of KC11346, KC12795 and KC12816 were determined by measuring the concentration changes in the control samples over time. The plasma controls stored in polypropylene at -70°C were stable for more than 2 months.

Stability was tested by subjecting the QC samples to three freeze/thaw cycles, and storage for 24 hours at room temperature. The thawing and refreezing of QC samples and the storage of QC samples at room temperature had little effect on the precision or accuracy of the results. The mean (n = 3) value was within 11.7% of the expected values.

Process stability was tested by extracting two sets of calibration standards with duplicate QC samples. One set was stored overnight at room temperature, and the other at 5°C before analyzing. The storage of extracted samples at room temperature or 5°C had little effect on the accuracy and precision of the results. The mean values of the controls was within 11.3% of the expected values.

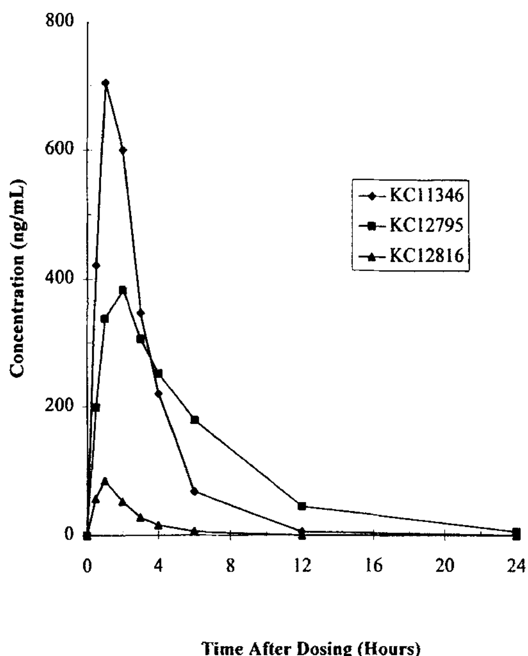

Figure 3. Mean pharmacokinetic profile of 8 animals (dosed with 25 mg/kg KC11346) from a toxicokinetic study.

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Application

The method presented here for the determination of KC12816, KC12795 and KC11346 in dog plasma shows acceptable linearity, precision, and accuracy down to a concentration of 3 ng/mL. The method is simple and rugged, with no observable matrix interferences. Figure 3 presents the mean pharmacokinetic profile of KC12816, KC12795 and KC11346 from 8 animals participating in a toxicokinetic study. Following dosing, KC11346 was rapidly absorbed and the plasma level reached its maximum (mean t_{max})within 1.19 hours. The mean maximum concentration (C_{max}) was 815±211.2 ng/mL. The metabolites KC12795 and KC12816 rapidly appeared in dog plasma after dosing and the plasma level reached its maximum, on average, within 1.75 hours and 0.94 hours, respectively. The C_{max} was 426±85.0 ng/mL and 108±30.3 ng/mL for KC12795 and KC12816, respectively. All three compounds were rapidly eliminated in dogs, with apparent $t_{1/2}$ 1.19, 3.58 and 1.35 hours for KC11346, KC12795 and KC12816, respectively.

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