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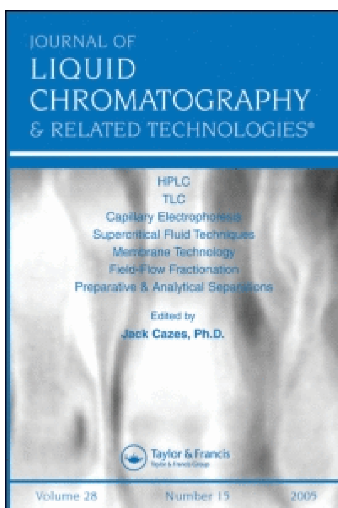

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF CGA-184699 (LUFENURON) IN DOG AND CAT BLOOD

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ABSTRACT

A method for quantitation of lufenuron (CGA-184699) in blood was developed for the purpose of determining lufenuron pharmacokinetics in cats and dogs. Lufenuron is an investigational insect development inhibitor used in the control of fleas on cats and dogs. An extract of lufenuron from blood is passed through a solid-phase extraction column before injection onto a C-18 column and elution with a mobile phase of acetonitrile and tetrahydrofuran in water. The eluate is monitored with a UV detector at 254 nm. Extraction efficiencies of lufenuron and the internal standard (CGA-112913) were 96% and 94%, respectively. The minimum detectable amount was 0.1 ng; day-to-day variation showed a CV% of less than 5%. Application of the method to a pharmacokinetic study in which one cat and dog received a single intravenous 10 mg/kg dose showed half-lives of approximately 60 days in both species.

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INTRODUCTION

Lufenuron (N-[2,5-dichloro-4-(1,1,2,3,3,3-hexafluoropropoxy)-phenylaminocarbonyl]-2,6-difluorobenzamide; CGA-184699) is used for the control of fleas on cats and dogs in various countries and is in the process of being registered in the United States. This insect development inhibitor has many characteristics that make it ideal for systemic use. It can be administered orally, it has long lasting effects after single doses, and was shown to have no detrimental effects in cats, dogs.(1) Lufenuron has no effect on adult fleas, but inhibits development of flea progeny when the adult flea ingests blood from a host animal that has been treated with lufenuron.(2) Adequate blood concentrations in the host animal must therefore be assured.

The reversed-phase high-performance liquid chromatographic (HPLC) method reported here was designed for the quantitation of lufenuron in cat and dog blood. The application of this method to pharmacokinetic studies of lufenuron in a mixed-breed cat and beagle dog is demonstrated.

MATERIALS AND METHODS

Apparatus

A model 2010 isocratic HPLC pump fitted with a Rheodyne sample injection valve (model 7125, Varian Associates, Sunnyvale, CA) was used with a variable wavelength ultraviolet detector (Varian model 2050) set at 254 nm. Detector output was recorded on a strip chart recorder (model 1200, Linear Instruments, Reno NV). A MicroPak-SP-18 HPLC column (5 µm particle diameter, 15 x

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0.40 cm) was purchased from Varian Associates (Sunnyvale, CA). Solid-phase extraction columns (1-mL volume, Bakerbond spe^R) were purchased from J.T. Baker, Inc., Phillipsburg, NJ. Eight-mL polypropylene test tubes and 12 x 100 mm glass culture tubes were purchased from KEW Scientific, Columbus, OH.

Chemicals, Reagents and Stock Solutions

Lufenuron and the internal standard (CGA-112913) were obtained from Ciba Geigy, Agricultural Division, Greensboro, NC. HPLC-grade acetonitrile, methanol, and tetrahydrofuran were purchased from Fisher Scientific, Fairlawn, NJ. Reagent-grade methyl-T-butyl ether was purchased from Baxter, Burdick and Jackson Division, Muskegon, MI. Water used for the mobile phase and solid-phase extraction procedure was double-distilled and stored in polypropylene containers.

Stock solutions (200 µg/L) of lufenuron and the internal standard were prepared in isopropyl alcohol. A working internal standard solution was prepared by diluting the stock solution with isopropyl alcohol to a final concentration of 4 µg/mL. Spiking solutions of lufenuron were prepared by serial dilution of the stock solution to concentrations ranging from 3.9 ng/25 µL to 500 ng/25 µL. A chromatography reference mixture containing 0.4 µg/mL of lufenuron and 0.4 µg/mL of internal standard was prepared in 50% methanol in water. All solutions were stored at -20°C when not in use.

Extraction Procedure

For calibration standards, 25 µL of each lufenuron spiking solution was added to an empty polypropylene tube and the contents dried at 50°C under filtered air. Drug-

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free human blood (0.5 mL) was added to create final blood lufenuron concentrations ranging from 7.8 µg/L to 1000 µg/L. For dog and cat samples, 0.5 mL of blood was transferred to an empty tube. Internal standard (25 µL or 100 ng) was added to standards and samples, followed by addition of 100 µL methanol; the mixture was vortex-mixed for 10 sec. Four mL of methyl-T-butyl ether were then added and the mixture was rocked for 20 min on a blood mixer. After centrifuging at 1500 g for 10 min, a 2-mL aliquot of the organic layer was transferred to a 12 x 100 mm glass culture tube and evaporated to dryness under filtered air using a heating block set at 50°C. The residue was dissolved in 1.0 mL of 50% methanol in water.

The solid-phase extraction columns were conditioned with three 1.0 mL volumes of methanol, followed by two 1.0 mL volumes of water. Particular care was taken not to allow the columns to dry after the final water rinse. The reconstituted extracts were slowly pulled through the conditioned columns, and the columns were rinsed with two 1.0 mL volumes of water. The analytes were eluted from the columns into 12 x 100 mm glass culture tubes using 0.5 mL of methanol. The eluate was dried once again, and the final residue was dissolved in 150 µL of mobile phase by vortexing for 30 sec.

Chromatography and Quantitation

All chromatography was performed at room temperature. The mobile phase was a solution of acetonitrile:water:tetrahydrofuran (53:25:22 v/v/v) at a flow rate of 0.8 mL/min. Thirty to fifty microliters of each extract were injected at detector ranges of 0.005 to 0.04 a.u.f.s.. The recorder was set at 10 mV, with a speed of 30 cm/hr. Calibration curves were constructed relating peak height ratio (lufenuron:internal standard)

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to known concentrations of lufenuron in the calibration standards.

Analytical Variables

The extraction recoveries of lufenuron at two concentrations (65 µg/L and 375 µg/L) and internal standard (200 µg/L) were determined using fresh human blood anticoagulated with EDTA. The spiked samples were processed according to the above procedure except that additional internal standard was not added, and all transfers were carefully measured. Aliquots of the reconstituted extracts were injected and peak heights corresponding to lufenuron and the internal standard were measured. Absolute recovery was calculated by comparing the peak heights resulting from extracts (corrected for losses in transfers) with peak heights obtained by direct injection of pure reference standards.

Within-day precision of the method was evaluated by analysis on one day of ten blood samples spiked to either contain 65 µg/L or 375 µg/L lufenuron. Quality control samples prepared to contain 100 µg/L or 800 µg/L in blood were stored in aliquots at -20°C in polypropylene tubes and analyzed 15 times over a one-month period to assess day-to-day precision. Coefficients of variation (CV%) were determined for the corresponding mean concentrations.

The sensitivity limit was determined by injecting amounts of lufenuron ranging from 2 to 125 ng onto the column. Peak heights were plotted against the amount of lufenuron injected and a regression line was calculated. The regression line was used to determine the amount of lufenuron that corresponding to a peak height that is three-fold higher than baseline noise (minimum detectable amount). The optimal wavelength for detection was determined by multiple injections of a lufenuron stock

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solution at UV wavelengths ranging between 225 and 285 nm.

Application to a Pharmacokinetic Study

One cat and one dog received a single intravenous 10 mg/kg dose of lufenuron (5% solution) infused over 5 min. Approximately 30 3-mL blood samples were drawn over a 100-day period following the dose. All bloods were collected in evacuated glass tubes containing EDTA, and frozen at -20°C until analyzed. Areas under the blood concentration-time curve (AUC) were determined using the linear trapezoidal rule. (3) Areas were extrapolated to infinity using the terminal elimination rate constant (K). Elimination half-life was calculated by dividing 0.693 by K. Body clearance was determined by dividing the intravenous dose by its corresponding AUC. Steady-state volume of distribution corrected for duration of infusion was determined as described by Perrier and Mayersohn. (4)

RESULTS AND DISCUSSION

Representative chromatograms are shown in Figure 1 for analyte-free dog blood (A), an extract of a calibration standard containing 125 µg/L of lufenuron and 200 µg/L of internal standard (B), and an extract of blood from a dog that received 10 mg/kg of lufenuron intravenously. Retention times for lufenuron and the internal standard were 3.6 and 5.4 min, respectively. A small blood component was consistently observed to elute immediately before the lufenuron peak (Figures 1(A) and 1(B), but did not interfere with lufenuron quantitation. The solid-phase extraction step was found to be critical, as without it a large solvent obscured the peaks corresponding to these two analytes.

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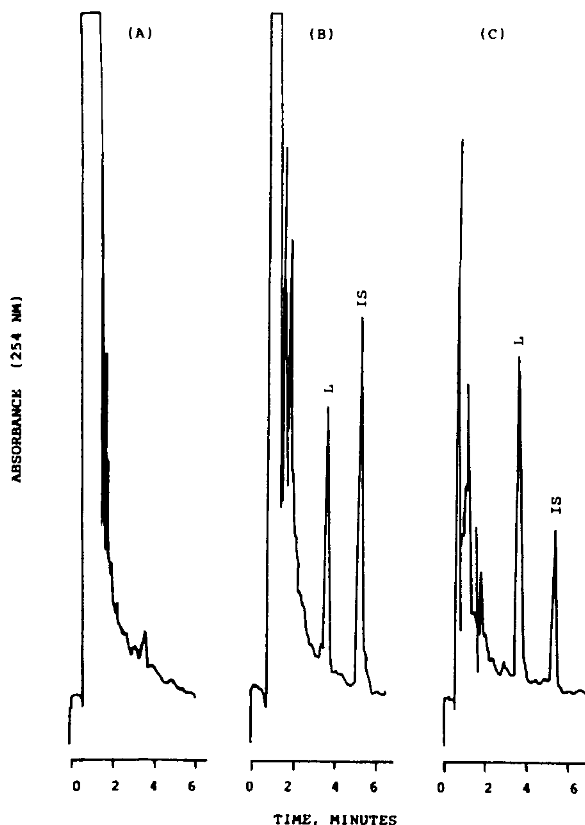

Figure 1(A). Chromatogram of extracted analyte-free pre-dose blood from dog. Range is 0.005 a.u.f.s..

Figure 1(B). Chromatogram of extracted calibration standard containing 125 µg/L lufenuron (L) and 200 µg/L CGA-112913 (internal standard, IS) in human blood. Range is 0.01 a.u.f.s..

Figure 1(C). Chromatogram of extracted dog blood obtained 24 hours after a single 10 mg/kg intravenous dose of lufenuron. The concentration of lufenuron is 356.5 µg/L. Range is 0.02 a.u.f.s..

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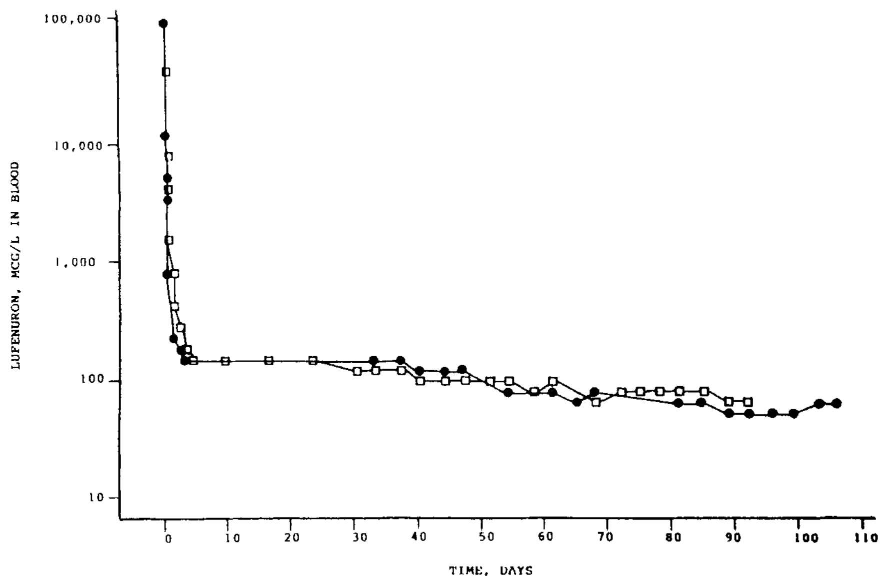

Figure 2. Semi-logarithmic plots of lufenuron blood concentration versus time in a healthy cat (□) and dog (●) following intravenous infusion of 10 mg/kg over 5 min.

Extraction recoveries of lufenuron (at both concentrations) and the internal standard were 96.0% and 93.5%, respectively. There was no dependence on concentration over the range studied. The mean concentrations and corresponding CV% values (in parentheses) for within-day determinations were 63.5 µg/L (2.7%) and 377.1 µg/L (1.6%), respectively. The corresponding values for day-to-day precision determinations were 99.1 µg/L (4.4%) and 805 µg/L (1.4%). The minimum detectable amount of lufenuron was 0.1 ng. The optimal wavelength for sensitivity was 260 nm, thus justifying the use of 254 nm.

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Semi-logarithmic plots of lufenuron blood concentration versus time are shown in Figure 2 with the cat and dog that received 10 mg/kg of lufenuron by intravenous infusion. The profiles are very similar to one another, showing a rapid initial decline followed by a slower decline. The highest measured concentrations, one hour after infusion of the dose were 104,000 and 41,000 μg/L in the dog and cat respectively. The terminal portions of the curves were linear between nine days after the dose and the time of the last sample. The body clearances (cat: 0.56 L/day per kg; dog: 0.54 L/day per kg) and steady-state volumes of distribution (cat: 44.8 L/kg; dog: 48.3 L/kg) were very similar between the animals. Hence, elimination half-lives were also similar between the cat (60.7 days) and dog (65.2 days). The large distribution volume is not surprising in light of the extremely lipophilic nature of lufenuron (log partition coefficient of n-octanol/water of 4.5)[5]. Most of the lufenuron body load resides in extravascular tisse and is slowly cleared with time. The slow blood clearance of lufenuron in comparison to normal hepatic blood flows[6] in the cat and dog suggest very weak extraction by the liver and hence negligable first-pass potential. Thus, an observation of less than 100% bioavailability following oral administration would be predicted to result only from incomplete absorption, not from a first-pass phenomenon.

The slow clearances and large distribution volumes both contribute to the prolonged elimination half-lives of approximately 60 days in these animals, and explain the prolonged effect of this systemic insect development inhibitor, which requires only monthly dosing.

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