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Determination of Allopurinol and Oxypurinol in Dogs Plasma by High-Performance Liquid Chromatography with an Ultraviolet Detector: Application for Pharmacokinetic Studies

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Abstract

High performance liquid chromatography with ultraviolet detection (HPLC-UV) was developed and validated for quantification of allopurinol and its active metabolite oxypurinol; in dog plasma with naturally acquired zoonotic visceral leishmaniasis. Allopurinol is a potent inhibitor of the xanthine oxidase enzyme; an enzyme that catalyzes the conversion of hypoxanthine to xanthine and from xanthine to uric acid. In veterinary medicine allopurinol is indicated in the treatment of canine visceral leishmaniasis. Allopurinol is utilized to inhibit the synthesis of Leishmania RNA. The conditions defined for development of the chromatographic analysis of dog plasma samples by utilizing the mobile phase of the HPLC-UV consist of a mixture of 0.1% water, 88% formic acid and 0.25% acetonitrile. Allopurinol and oxypurinol were separated on a LiChroCART® 125-4 LiChrospher® 100 RP-8 (5 µm) column utilizing a flow rate of 0.7 mL min^{-1} and detector operation was at a wavelength of 254 nm. Acyclovir was utilized as an internal standard. The validation of the HPLC-UV method was determined by limits of detection and quantification, linearity, reproducibility and repeatability. The method had a lower quantification limit of 0.1 µg.mL^{-1} for both allopurinol and oxypurinol. The analysis was linear at concentrations of 0.1-20.0 µg.mL^{-1} for both allopurinol and its oxypurinol. The precision value intracurrent and intercurrent for all concentrations presented had a coefficient variation lower than 15%. The confidence limits of HPLC-UV for analysis of allopurinol and oxypurinol in dog plasma indicates that the method is applicable to the multiple dose pharmacokinetic study of allopurinol in dogs. It is efficient, accurate and sensitive. This study of Pharmacokinetic research of allopurinol and oxypurinol in dogs with Stage I and II visceral leishmaniasis resulted in similar research outcomes that correlated with the healthy dog investigation.

Keywords: HPLC-UV; Allopurinol; Oxypurinol; Pharmacokinetic; Canine visceral leishmaniasis

Introduction

Allopurinol (1H-pyrazolo (3,4-d) pyrimidin-4-ol) is an oxypurine base. It was first synthesized in the 1950s by George Hitchings and Gertrude Elion in New York with the aim to increase the efficacy of antineoplastic drugs [1,2]. However, it was later discovered that allopurinol was a strong inhibitor of the enzyme xanthine oxidase [1]. Currently allopurinol is utilized in the treatment of humans diagnosed with hyperuricemia [2,3].

In veterinary medicine, this drug has been used to dissolve uroliths formed by ammonia uric acid calcifications in dogs. Dalmatian genetics elicit a predisposition to developing high concentrations of uric acid [4,5]. Its use also extends to the treatment of canine visceral leishmaniasis (LV) therapy. In leishmaniostatic treatment is utilized either as monotherapy [6,7] or associated with leishmanicidal drugs [8-10].

Allopurinol is an analog of hypoxanthine and is converted by xanthine oxidase into its active metabolite oxypurinol (alloxanthin) (Figure 1). Both allopurinol and oxypurinol bind competitively to xanthine oxidase promoting its inhibition. They inhibit xanthine oxidase which is the enzyme that catalyzes the conversion of hypoxanthine to xanthine and xanthine into uric acid in both humans and dogs [3,11,12]. Peak plasma concentrations of allopurinol are obtained within 30 minutes to 1 hour after ingestion and about 60-70% of the drug is converted rapidly by xanthine oxidase to the active metabolite oxypurinol [3,13].

In humans, allopurinol and oxypurinol are excreted primarily via the kidneys. A dose of 100 mg allopurinol orally generates 90 mg of oxypurinol [11]. Allopurinol is excreted in the urine in its unchanged form at a rate of 10%; in the feces at a rate of 20%. The metabolite oxypurinol is eliminated in the urine at a concentration of 70% [12-14]. Limited research has been conducted on the pharmacokinetics of allopurinol in dogs [5,15,16]. The pharmacokinetics of allopurinol has been demonstrated in healthy Dalmatians where maximum concentration (C_{max}) was reached at 6.43 ± 0.18 µg.mL^{-1} at a time of (T_{max}) 1.9 hours and its apparent volume of distribution was (Vd / F) 1.17 ± 0.07 L.kg^{-1}. This illustrates it was well distributed within the tissues [5].
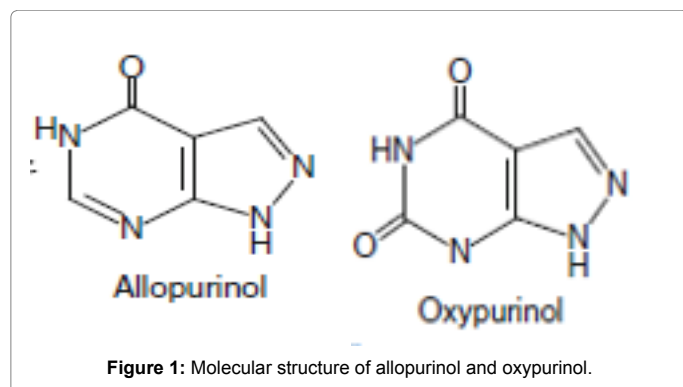
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Figure 1: Molecular structure of allopurinol and oxypurinol.

The allopurinol pharmacokinetic research by Ling et al. in healthy Dalmatians was also performed utilizing an HPLC-UV operating at 254 nm wavelength and had a limit of detection of 2.0 µg.mL^{-1} [5].

In human analysis, allopurinol and oxypurinol were evaluated utilizing high performance liquid chromatography (HPLC) with UV-detection. The percentage recovery and intra-assay coefficient of variation (CV%) for allopurinol was 97.4-101.3% and 0.66-5.13% respectively in the concentration range of 0.5 to 5.0 µg.mL^{-1}. For oxypurinol the percentage recovery and the intra-assay coefficient of variation (CV%) was 93.2-98.1% and 0.88-5.62% respectively in the concentration range of 0.4 to 20.0 µg.mL^{-1} [17].

Reinders et al. also evaluated allopurinol and oxypurinol in human plasma. Validation results for allopurinol lower and upper limits of quantification were 0.5 and 10.0 mg.L^{-1} and for oxypurinol 1.0 and 40.0 mg.L^{-1}. The assay was linear over the concentration range of 0.5-10.0 mg.L^{-1} (allopurinol) and 1.0 to 40.0 mg.L^{-1} (oxypurinol). Intra- and inter-day precision showed coefficients of variation <15% over the complete concentration range. Accuracy was within 5% for allopurinol and oxypurinol. Endogenous purine-like compounds were separated from allopurinol, oxypurinol and acyclovir with a factor of >1.5% [18].

In the present study, both allopurinol and oxypurinol in dog plasma were tested utilizing HPLC-UV. The development of an accurate, sensitive and reproducible method of plasma analysis is a fundamental foundation in the research of the kinetic arrangement and metabolism of allopurinol and oxypurinol utilizing multiple dose administration in dogs with visceral leishmaniasis.

Materials and Methods

Chemicals and reagents

Standard research concentrates (European Pharmacopoeia Reference Standard) of allopurinol 99.8% and oxypurinol 98.4% was obtained from Sigma (St. Louis, MO). Acyclovir was donated by Cristália, Produtos Químicos e Farmacêuticos Ltda. (Brazil). Standard stock solutions of allopurinol and oxypurinol were prepared in NaOH (1M) at a concentration of 400 µg/mL.

Chromatography grade acetonitrile and formic acid were purchased from Merck (Darmstadt, Germany). The analytical grade reagent sodium hydroxide was purchased from Mallinckrodt Baker (Paris, Kentucky). Water was purified using a Milli-Q Plus System.

Chromatography

Plasma sample analysis: High performance liquid chromatography with ultraviolet detection (HPLC-UV) was the method used for the quantitative analysis of allopurinol and oxypurinol in dog plasma.

This system consisted of a LC20AD high-pressure pump and a CTO-10ASvp column-conditioning oven from Shimadzu (Tokyo, Japan).

Allopurinol and oxypurinol were separated on a LiChroCART® 125-4 LiChrospher® 100 RP-8 (5 µm) column (Merck, Darmstadt, Germany) and using a pre-column LiChroCART® 4-4 LiChrospher® 100 RP-8 (5 Mm) (Merck, Darmstadt, Germany). The oven temperature was 25°C and the auto sampler was 12°C. The mobile phase consisted of a mixture of purified water, 0.1% formic acid (88%) and 0.25% acetonitrile. The HPLC-UV system operated at a flow rate of 0.7 mL.min^{-1}. Ultraviolet detection was performed at a wavelength of 254 nm.

Sample preparation solution: Plasma samples were obtained from a random sample of healthy dogs owned by the staff of the Teaching Hospital of the School of Veterinary Medicine and Zootechny, University Federal of Bahia (HOSPMEV-UFBA), Brazil. Aliquots of 100 µL of dog plasma were supplemented with 25 µL of IS solution (acyclovir). Allopurinol and oxypurinol were extracted from plasma samples with 200 µL of acetonitrile by shaking in a mechanical shaker (220 ± 10 cycles min^{-1}) for 1 minute. After 10 minutes at room temperature the samples were centrifuged at 21.500 × gravity for 10 minutes at 5°C to separate the organic phase. The organic phase was collected, evaporated and dried in an evaporator vacuum system set at 25°C (models RCT90 and RC10.22; Jouan AS, St. Herblain, France). The residues were dissolved in a 200 µL mixture of water plus 0.1% of formic acid and 0.25% of acetonitrile (mobile phase) and vortexed for 10 seconds. Then 60 µL was injected into the analytical column.

Method validation of plasma sample: The calibration curve was prepared from 100 µL of drug free plasma enriched with 25 µL of the solution for each concentration standard and the internal standard. The samples were prepared in triplicates resulting in concentrations of 0.1 to 20.0 µg.mL^{-1} of each plasma solution (allopurinol and oxypurinol).

Recovery of allopurinol and oxypurinol was evaluated five times at plasma concentrations of 0.25 µg.mL^{-1} and 16.0 µg.mL^{-1}. The internal standard acyclovir was present in the concentration at 40.0 µg.mL^{-1} (n=10). The peak concentration areas from the extracted samples were compared with the standard areas obtained in the analysis of standard solutions × 100.

The quantification limit was determined as the lowest quantified concentration of allopurinol and oxypurinol with precision and accuracy of less than 20%. Plasma samples were evaluated five separate times. Each plasma sample was enriched with allopurinol and oxypurinol at a concentration of 0.1 µg.mL^{-1}.

Linearity was verified at concentrations of 0.1 to 20.0 µg.mL^{-1}. Plasma samples for both allopurinol and oxypurinol were tested in triplicate at eight different concentrations.

Precision and accuracy were evaluated five times at concentrations of 0.1, 0.25, 10.0 and 16.0 µg.mL^{-1} for allopurinol and oxypurinol. In the analysis of the intra-crossover accuracy, four preparations of four different concentrations were evaluated. Five aliquots of each sample were used by means of a single calibration curve. The coefficient variation of the data achieved must be less than 15% to substantiate methodology accuracy. For precision analysis, the four independent concentration preparations were evaluated using five aliquots of each sample for three consecutive days. The precision evaluation was performed using the coefficient variation of the acquired values.

For the analysis of the intra and inter current accuracy the experimental results obtained in the precision research were utilized. The accuracy is expressed by the standard error at a rate of less than 15%. The long-term stability assessment (3 cycles for 24 hours) of five plasma samples of allopurinol and oxypurinol in plasma concentrations of 0.25 and 16.0 µg.mL^{-1} was evaluated. To evaluate the stability of freezing and thawing; the five samples were frozen at -20°C and after 24 hours were thawed and then frozen again for 12 hours. This cycle was repeated two more times and then the drug and metabolite tested.

The short-term stability assessment was also performed five times at the same concentrations of 0.25 and 16.0 µg.mL^{-1} of both allopurinol and oxypurinol in plasma samples. In this analysis, these samples were maintained at room temperature (25°C) for four hours.

To evaluate the post-processing stability assessment the samples were extracted and maintained at 12°C for twenty-four hours. The completed sample preparations were maintained until completion of analysis.

Experimental sampling

Animals and ethics: This investigation was conducted on male dogs consisting of various weights, ages and species. They were all carriers of zoonotic visceral leishmaniasis. These animals were patients at the HOSPMEV-UFBA in the Small Animal Clinic sector. The experiments were carried out in accordance with the rules and recommendations of The Ethics Committee Use of Animals, University Federal of Bahia.

Dosing and sample collection: The standard method was applied for pharmacokinetic studies of allopurinol and oxypurinol in dogs with naturally acquired visceral leishmaniasis. After 8 h fasting a blood serum sample was obtained from the dogs at a zero time; being in a steady state ($5 \times t_{1/2}$). An oral dose of 10 mg.kg^{-1} of allopurinol was then administered. Blood samples were obtained utilizing standard medical procedures at time intervals of: 15 minutes, 30 minutes, 1 h, 1.5 h, 2 h, 2.5 h, 3 h, 4 h, 5 h, 7 h, 10 h and 12 h. Blood samples were centrifuged and the plasma separated and stored at -20°C until analysis on high performance liquid chromatography (HPLC-UV).

Pharmacokinetics: The pharmacokinetic parameters were calculated on the basis of the plasma concentration *versus* time curves using WinNonlin software version 4.0 (Pharsight Corp, Mountain View, CA). No compartmental model was used in this study.

Statistical analysis: The median, mean and relative standard error with 95% confidence interval were calculated using Excel 2016.

Results and Discussion

Several preliminary experiments were evaluated to discriminate the allopurinol and oxypurinol analysis in plasma samples. The present study reports for the first time the simultaneous analysis of allopurinol and oxypurinol by HPLC-UV in dogs. This pharmacokinetic veterinary application was than applied to this study in male dogs with visceral leishmaniasis receiving multiple oral doses of allopurinol (10 mg.Kg^{-1}).

The optimal chromatographic separation of allopurinol and oxypurinol (Figure 2) was conducted with the LiChroCART® 125-4 LiChrospher® 100 RP-8 (5 µm) The mobile phase utilized a mixture of water plus 0.1% of formic acid and 0.25% of acetonitrile. Figure 2 show the chromatographic analysis of plasma blank (drug-free), plasma blank (drug-free) enriched with solutions of allopurinol, oxypurinol, acyclovir (internal standard - IS) and the plasma from one dog with

leishmaniasis treated with allopurinol (10 mg.Kg^{-1}). It was observed that the retention times of allopurinol, oxypurinol and acyclovir were 6.0 min; 7.8 min and 9.9 min, respectively.

The calibration curve constructed for the analysis of allopurinol and oxypurinol (0.1-20 µg.mL^{-1} plasma) showed a coefficient correlation higher than 0.99 (Table 1).

The methodology was validated utilizing assessments of recovery, limits of quantification, linearity, precision, accuracy and stability. Coefficients of variation and relative errors of less than 15% are within acceptable range. The limit of quantification values was extended to 20%.

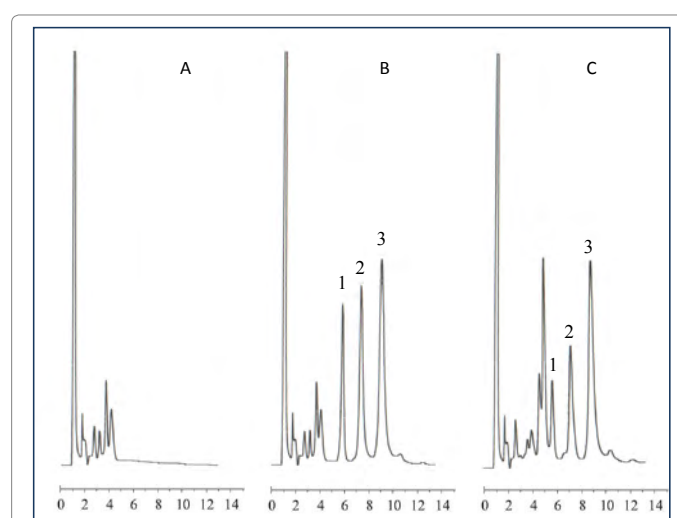Recovery rates obtained were ~90% for allopurinol and oxypurinol

Figure 2: Chromatograms for analysis of allopurinol and oxypurinol in canine plasma: A) White plasma; B) White plasma+enriched with standard solution of 5 µg/mL plasma; C) Plasma sample collected within 2 h after oral administration of 10 mg/kg allopurinol. 1=Oxypurinol; 2=Allopurinol; 3=Aciclovir (Internal Standard).
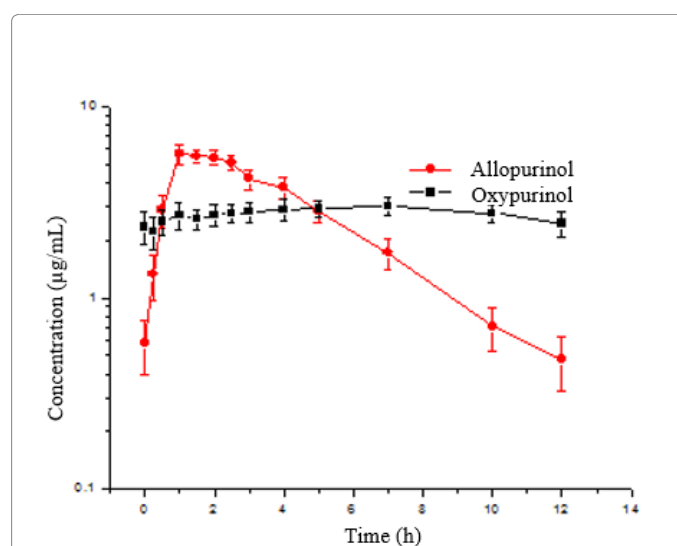

Figure 3: Plasma concentration curves vs time of allopurinol and oxypurinol after oral administration of 10 mg/kg of allopurinol. Mean ± standard error of the mean.

extracted from plasma with acetonitrile (Table 1). Reinders et al. utilized dichloromethane as an extractor solvent and these recovery rates were 65% for allopurinol and 75% for oxypurinol [18]. Reddy and Reddy utilized an extractor solvent of ethyl acetate for their plasma samples and elicited recovery rates of less than 50% [19].

Quantification limits of 0.1 µg.mL^{-1} dog plasma were obtained for both allopurinol and oxypurinol, using 100 µL plasma sample and the HPLC-UV system. Ling et al. reported in his investigation quantification limits of 2 µg.L^{-1} for allopurinol when testing dog plasma samples [5]. Quantification limit research performed by Reinders et al. utilized 100 µL of human plasma samples [18]. It elicited quantification limits of 0.5 mg.L^{-1} and 10 mg.L^{-1} for allopurinol and oxypurinol, respectively.

Analysis of precision and accuracy showed coefficients of variation and percent of error of less than 15%. This indicates that the method is precise, duplicable and accurate (Table 1). The evaluation of stability after 3 days of freeze-thaw cycles and after storage at room temperature for 4 h showed deviations of less than 15% for both allopurinol and oxypurinol. This guarantees the stability of samples stored at -20°C.

This research study of kinetic disposition of allopurinol and oxypurinol as multiple doses of 10 mg.kg^{-1} to male dogs with naturally acquired visceral leishmaniasis was validated by recovery, limits of quantification, linearity, precision, accuracy and stability. In Figure 3 it is illustrated the curve of plasma concentrations versus the time of

allopurinol and oxypurinol concentrations in a steady state.

Canine visceral leishmaniasis is classified in four stages: Stage I (mild diseases), Stage II (moderate disease), Stage III (severe disease) and Stage IV (very severe disease); diagnosis is based upon clinical signs, laboratory findings, serological status, and types of therapy and prognosis for each patient [20]. In this investigation, the studied dogs were in Stage I and II.

The present pharmacokinetic study of allopurinol and oxypurinol in dogs with Stage I and II of visceral leishmaniasis resulted in similar conclusions as the study of Ling et al. when they studied these drugs in healthy Dalmatians [5]. The following parameters of drug kinetic disposition found in our study were equivalent to the study of Ling et al.: distribution volume (Vd.F^{-1}.Kg^{-1}), time to reach the maximum concentration (T_{max}), maximum concentration (C_{max}), half-life ($t_{1/2}$) and constant of elimination (Kel), as illustrated in Table 2. Even though we have studied infected dogs, the results we have found using HPLC-UV method for the pharmacokinetic analyses of allopurinol and oxipurinol in canine plasma were comparable to the results in a study of the same parameters in healthy dogs reported in the literature [5].

Conclusion

In the present study, it has been shown that allopurinol and oxypurinol can be accurately and precisely measured in dog plasma

	Allopurinol			Oxypurinol		
Linearity	0.1-20.0 µg/mL plasma			0.1-20.0 µg/mL plasma		
Equation of the line	y=0.15257x-0.02218			y=0.13523x-0.0166		
(r)	0.99962			0.99966		
(r²)	0.99924			0.99933		
* Absolute Recovery (%) (n=10)	87%			86%		
Aciclovir-PI (n=10)	89%					
Precision and Accuracy						
Intracorridas	**Mean**	**CV%**	**EPR%**	**Mean**	**CV%**	**EPR%**
LIQ (0.1 µg/mL, n=5)	0.10	5.64	-0.20	0.10	3.96	-4.40
CQB (0.25 µg/mL, n=5)	0.23	3.74	-7.68	0.22	3.53	-11.68
CQM (10.0 µg/mL, n=5)	10.53	1.84	5.32	10.12	1.87	1.18
CQA (16.0 µg/mL, n=5)	16.45	1.53	2.81	15.76	1.49	-1.49
Intercorridas	**Mean**	**CV%**	**EPR%**	**Mean**	**CV%**	**EPR%**
LIQ (0.1 µg/mL, n=5)	0.10	9.22	1.40	0.10	6.90	-3.60
CQB (0.25 µg/mL, n=5)	0.24	3.01	-3.68	0.24	4.55	-5.97
CQM (10.0 µg/mL, n=5)	10.19	4.81	1.90	10.01	4.82	0.07
CQA (16.0 µg/mL, n=5)	15.14	5.14	-5.40	14.79	5.03	-7.53
Stability						
Freezing and Defrosting	**Mean**	**CV%**	**EPR%**	**Mean**	**CV%**	**EPR%**
CQB (0.25 µg/mL, n=5)	0.24	3.24	-3.36	0.22	2.38	-12.00
CQA (16.0 µg/mL, n=5)	16.73	3.18	4.56	17.46	3.05	9.13
Post Processing (12°C-24 h)	**Mean**	**CV%**	**EPR%**	**Mean**	**CV%**	**EPR%**
CQB (0.25 µg/mL, n=5)	0.23	9.88	-8.16	0.22	9.96	-12.48
CQA (16.0 µg/mL, n=5)	14.22	1.38	-11.3	14.45	1.47	-9.70
Short term (25°C-4 h)	**Mean**	**CV%**	**EPR%**	**Mean**	**CV%**	**EPR%**
CQB (0.25 µg/mL, n=5)	0.26	0.52	3.04	15.65	3.68	-2.20
CQA (16 µg/mL, n=5)	0.27	5.38	9.78	16.40	3.44	2.49

r: Linear Correlation coefficient; r²: determination coefficient; CV: coefficient of variation, EPR: Relative standard error; LIQ: Lower Limit of Quantification; CQB: Low Concentration Quality Control; CQM: Quality Control of Medium Concentration; CQA: High Concentration Quality Control'; *Recovery=Mean determined by the ratio of analyte processed/analyte in solution × 100, Concentrations of allopurinol and oxypurinol of 0.25 µg/mL (CQB, n=5) e 16.0 µg/mL (CQA, n=5), and acyclovir de 40.0 µg/mL solution (n=10)

Table 1: Confidence limits of the analysis method of allopurinol and oxypurinol in dog plasma. Linearity, recovery, precision, accuracy, and stability values for allopurinol and oxypurinol.

Parameters	Allopurinol	Allopurinol
	Mean ± SD LV dogs	Men ± SD Healthy dogs
Cl/F/Kg (L/h/Kg)	0.35 ± 0.11	-
Vd/F/Kg (L/Kg)	1.37 ± 0.83	1.17 ± 0.07
Tmax (hr)	1.50 ± 0.67	1.9 ± 0.01
Cmax (µg/mL)	6.00 ± 1.78	6.43 ± 0.18
$t_{1/2}$ (hr)	2.81 ± 1.29	2.69 ± 0.14
Kel (1/hr)	0.31 ± 0.17	0.26 ± 0.01
AUC 0-12 h (hr* µg/mL)	29.47 ± 10.58	-

Cl_F: Apparent clearance; Vd: Volume of Distribution; T_{max}: Time to reach C_{max}; C_{max}: Maximum concentration; $t_{1/2}$: Elimination half-life; Kel: Elimination constant; AUC 0-12: Area under the curve in the dose interval (0-12 h)

Table 2: Mean values of the pharmacokinetic parameters corresponding to the allopurinol and oxypurinol analysis in plasma obtained after allopurinol administration in multiple dosage of 10 mg/kg every 12 hours in dogs with naturally acquired visceral leishmaniasis.

samples in less than 10 minutes using HPLC-UV. This method proved to be simple, accurate, reproducible and sensitive when applied for the pharmacokinetic analyses of those drugs using plasma from dogs with visceral leishmaniasis treated with multiple doses of 10 mg.kg^{-1} of allopurinol. Thus, HPLC-UV is a suitable method to evaluate the pharmacokinetics of allopurinol and oxypurinol in naturally infected dogs under treatment for visceral leishmaniasis.

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