

# Development and validation of a stability-indicating analytical method for simultaneous determination of drugs employed in canine leishmaniasis treatment

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We developed and validated a stability-indicating method for the simultaneous determination of allopurinol and ketoconazole in the pharmaceutical form of capsules. A Dionex® liquid chromatograph equipped with a diode array detector (DAD) and InertSustain® C18 column (4.6 x 100 mm x 3 µm) was used and InertSustain® C18 column (4.6 x 100 mm x 3 µm). The chromatographic separation occurred in the isocratic mode with a flow rate of 0.45 mL min<sup>-1</sup> and mobile phase composed of acetonitrile: water (52:48 v/v) with pH adjusted to 3.0. The wavelengths used were 250 nm for allopurinol and 225 nm for ketoconazole. The method was validated by evaluating the parameters of linearity, limits of detection and quantification, precision, accuracy, robustness, and selectivity. The method presented linearity in the concentration ranges of 2.0 to 16.0 µg mL<sup>-1</sup> for ALLO and 1.0 to 15.0 µg mL<sup>-1</sup> for KETO. It was precise, with coefficients of variation lower than 2.0%, and accurate, with recovery close to 100.00%. Robustness was indicated by the Plackett-Burman model, and the method was not significantly influenced by any of the variations. The selectivity was proven by the purity of the peaks of the drugs close to 1000, in the presence of excipients as degradation of drugs. Therefore the proposed method is simple and fast, with separation for both drugs less than four minutes.

**Keywords:** allopurinol, ketoconazole, canine visceral leishmaniasis, analytical validation, liquid chromatography.

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## Introduction

Visceral Leishmaniasis (LV) is a chronic disease with high morbidity and mortality rates caused by protozoa of the genus *Leishmania*. The first case of LV in humans in Brazil was reported in 1934 when the parasite was found after liver necropsy [1-3]. Currently, LV has a worldwide distribution, being endemic in countries of the Americas with 96% of these notifications in Brazil [4].

The dogs present in the urban transmission cycle as hosts of the parasite establishing fundamental importance in the biological cycle of the disease, act as a natural reservoir and become a source of infection for the sandflies during their repast, helping in the transmission of the disease to humans and other susceptible animals [5,6].

The great interest in the dog is given by the ever-closer relationship with man, both in rural areas and in urban areas, making these animals members of the family, and also by changing the epidemiological profile of the vector. These factors not only provide the essential care already carried out by the owners of these animals, but also generate a greater concern with their health.

The disease control measures indicated are euthanasia of infected animals, vector control, vaccination of animals, and the use of collars and repellents impregnated with deltamethrin [7]. Pharmacological therapy is complex because of the greater resistance of canine disease, high toxicity to the animal, and side effects, generating many relapses with little chance of cure. The most commonly used drugs are pentavalent antimonials, amphotericin B, allopurinol, azoles, and some antibiotics. These drugs are

used alone or in combination, which makes the treatment more efficient [8].

The combination of allopurinol (ALLO) and ketoconazole (KETO) (Figure 1) has shown therapeutic success.

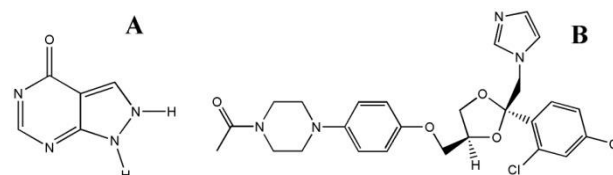


Figure 1. Molecular structure of allopurinol (A) and ketoconazole (B).

ALLO is a drug used to treat gout and acts by blocking the enzyme xanthine oxidase inhibiting the formation of uric acid [9,10]. In the parasite, this drug is incorporated into its RNA by blocking the synthesis of proteins essential for its development [11, 12].

KETO is an antifungal that inhibits enzyme cytochrome P450 lanosterol-dependent 14-demethylase, which is responsible for transforming lanosterol into ergosterol, an essential component of cell membrane synthesis of microorganisms. In the absence of this component, there is a change in the permeability of this membrane with rupture of the phospholipid chains, consequently, important enzymatic processes for cell growth and replication are interrupted [13,14].

These drugs are administered orally, have low toxicity, low cost and studies report the cure of animals and humans [15-17]. For the treatment of dogs, the doses used

are: 10mg / kg, SID, orally, from KETO and 10mg / kg, BID, orally, from ALLO [15].

This association there is no commercial formulation available in drugstores, so it is obtained through manipulation in the form of capsules, which allows adjustment of the dose according to the weight of the animal.

In the literature, no studies are found regarding the quality control of this combination, and it is necessary to develop methods to ensure a safe and quality formulation and consequently an effective treatment.

Thus, the present work had as its purpose the development and validation of an analytical method indicative of stability by the HPLC-DAD technique for simultaneous and quantitative determination of ALLO and KETO drugs in compounded samples, as well as in industrialized samples as a mono drug.

## Experimental section

### Reference standards, samples, and reagents

The standards of allopurinol (purity 100.0%) and ketoconazole (purity 99.8%) were obtained from the Brazilian Pharmacopoeia through the Instituto Nacional de Controle de Qualidade e Saúde (INCQS). Compounded samples were obtained from local pharmacies containing ALLO 200 mg and KETO 100 mg. Commercial samples were purchased from local drugstores with ALLO 100 mg (batch: 17I153, 08/2019) and KETO 200 mg (batch: 17G766, 06/2019).

HPLC grade solvents were acetonitrile (ACN, Sigma-Aldrich<sup>®</sup>, JT Baker<sup>®</sup>, Tedia<sup>®</sup>), methanol (Vetec<sup>®</sup>), o-phosphoric acid (Synth<sup>®</sup>) and ultrapure water (Direct-Q R 3UV<sup>®</sup> equipment).

### Chromatographic system and conditions

The method was developed and validated on a Dionex<sup>®</sup> high-performance liquid chromatograph (Ultimate 3000 model) (Thermo Fisher Scientific, USA), equipped with the Ultimate 3000 RS Variable Wavelength diode array detector (DAD), Ultimate 3000 Pump, connected to a microcomputer with Chromeleon<sup>®</sup> 7.1 software - Chromatography Data System.

The chromatographic separation occurred in the isocratic mode with 0.45 mL min<sup>-1</sup> flow and injection volume was 20 µL using InertSustain<sup>®</sup> C18 column (4.6 x 100 mm and 3 µm particle size) from the manufacturer GL Sciences.

The mobile phase was composed of acetonitrile and acid water (52:48 v/v) with pH adjusted to 3.0 with o-phosphoric acid. The wavelengths used were 250 nm for ALLO and 225 nm for KETO.

### Development and optimization of the analytical method

Three chromatographic columns were tested: InertSustain<sup>®</sup> C18 GL Sciences (4.6 x 100 mm x 3 µm), Kinetex<sup>®</sup> C18 Phenomenex (4.6 x 100 mm x 2.6 µm) and Discovery<sup>®</sup> HS C18 Sigma-Aldrich (4.6 x 100 mm x 5

µm). The mobile phases tested were methanol and water without adjustment of pH, acetonitrile, and water without adjustment of pH and acetonitrile and water with pH 3, adjusted with o-phosphoric acid, in different proportions. The choice of the best chromatographic condition was made through the evaluation of the parameters: retention time, peak purity, number of theoretical plates, resolution factors and asymmetry, according to recommendations of the International Conference on Harmonisation [18].

### Validation of the analytical method

The method was validated based on the International Conference on Harmonization [18] and the Association of Official Analytical Chemists International [19] guidelines, following the parameters of linearity, detection limit, quantification limit, accuracy, robustness, and selectivity.

#### Selectivity

This parameter was assessed by analyzing the placebo solution, prepared with excipients used in manipulated samples (stearate magnesium, aerosil, sodium lauryl sulfate, premium talc, and corn starch). [20].

The degradation study was carried out with solutions prepared with 20 mg of the standard powder of each medication in acetonitrile:water (52:48 v/v), which were placed in direct contact with the degrading agent. For comparison, ALLO and KETO control solutions were prepared without the addition of a degrading agent.

For the percentage of degradation, the range of 10 to 30% of the content loss of the main peak was considered in relation to the control [21]. The results were evaluated by the retention time, peak purity, and spectral profile in the UV region.

The acid condition was performed using a 1 M hydrochloric acid solution with 30 minutes exposure in a water bath at 60 °C.

The basic condition was performed differently for the two drugs. For ALLO a solution of 1 M sodium hydroxide with 30 minutes exposure in a water bath 60 °C was used, and for KETO a solution of 0.01 M sodium hydroxide for 10 minutes in a water bath 60 °C was used.

The oxidative condition was performed with a 0.3 % hydrogen peroxide solution with 30 minutes exposure in a water bath 60 °C.

The photolytic and thermal conditions were performed with 24 h exposure in a UVB chamber and a stove at 80 °C, respectively.

After the exposure time, aliquots of the solutions were removed and diluted and injected under the conditions in the proposed method.

#### Linearity

This parameter was evaluated through the construction of analytical curves.

Solutions of the mobile phase diluted standards were prepared considering six concentrations for ALLO (2.0,

4.0, 6.0, 10.0, 12.0, and 16.0  $\mu\text{g mL}^{-1}$ ) and six for KETO (1.0, 3.0, 6.0, 9.0, 12.0, and 15.0  $\mu\text{g mL}^{-1}$ ). The determinations were performed in triplicate and the results were submitted to linear regression analysis and statistical analysis by analysis of variance (ANOVA).

#### Precision

Precision was assessed using repeatability and intermediate precision using commercial sample solutions. Repeatability was confirmed by analyzing six determinations of solutions at concentrations of 5.0 and 10.0  $\mu\text{g mL}^{-1}$  for KETO and ALLO, respectively. The injections were performed on the same day, under the same chromatographic conditions and by the same analyst. Intermediate precision was assessed by two different analysts in six times, on three different days, at the concentrations mentioned above. From the results obtained, standard deviation (SD) and relative standard deviation (RSD) were calculated.

#### Accuracy

Accuracy was evaluated through standard recovery assays at three concentration levels. Solutions of the compounded sample were added with known amounts of the standard solution of the drugs, totalizing concentrations of 3.0, 5.0, and 7.0  $\mu\text{g mL}^{-1}$  for KETO and 6.0, 10.0, and 14.0  $\mu\text{g mL}^{-1}$  for ALLO. All determinations were performed in triplicate and percentages of recoveries of each drug were calculated according to AOAC [19].

#### Limit of detection (LOD) and limit of quantification (LOQ)

The limits were determined by the mathematical relationship between the standard deviation of the intercept with the Y-axis (SD), and the slope of the calibration curve (a), being  $3.3 \times (\text{SD} / a)$  and  $10 \times (\text{SD}/a)$ , respectively.

#### Robustness

The robustness was demonstrated by the Plackett-Burman factorial model for 15 experiments and 7 factors that vary between normal (0), major (1), and minor (-1) conditions [22] (Table 1). In order to determine the influence of each factor, solutions of the standards were prepared in the concentrations of 10  $\mu\text{g mL}^{-1}$  of ALLO and 5  $\mu\text{g mL}^{-1}$  of KETO and the determinations made in triplicate. The effect produced by each factor was calculated by the difference of the results obtained with the normal condition in relation to the variant conditions [23]. For the method to be considered robust the effect produced must be less than the critical value.

Table 1. Variable factors and conditions for the robustness test.

Factors	Unit	Limit	Variable condition (-1)	Variable condition (0)	Variable condition (+1)
A - mobile phase pH	---	$\pm 0,2$	2,8	3,0	3,2
B - proportion of mobile phase	%	$\pm 2$	50:50	52:48	54:46
C - wavelength	nm	$\pm 2$	223/248	225/250	227/252
D - sonication time	min	$\pm 2$	8	10	12
E - room temperature	$^{\circ}\text{C}$	$\pm 2$	23	25	27
F- acetonitrile brand	---	---	Tedia	Sigma-Aldrich	JT Baker
G - mobile phaseflow	mL min <sup>-1</sup>	$\pm 0,0$ 2	0,43	0,45	0,47

#### Application of the analytical method

The developed method was used to quantify ALLO and KETO in compounded samples of the drugs associated with 200 mg of ALLO and 100 mg of KETO and in commercial samples in tablet of the drugs in monodrugs being ALLO 100 mg and KETO 200 mg.

## Results and Discussion

#### Method optimization

In the mobile phases composed of acetonitrile:water and methanol:water, both without pH adjustment, the separation of the chromatographic peaks in the tested columns was not obtained.

The mobile phase of acetonitrile and water at pH 3.0 showed good separation by increasing the concentration of acetonitrile, reaching the optimum ratio of 52:48 v/v.

The Sigma-Aldrich column presented parameters of resolution and asymmetry out of the recommended and thus discarded.

The Phenomenex and GL Sciences columns were those that showed separation of the compounds and parameters within the recommended, however, the GL Sciences column obtained superior performance with lower run time, better separation of the chromatographic peaks, and higher resolution parameters, plates and peak purity (Figure 2).

Thus the chromatographic conditions chosen were InertSustain® C18 column from the manufacturer GL Sciences (4.6 x 100 mm x 3  $\mu\text{m}$ ); mobile phase consisting of acetonitrile and acidic water with pH adjusted to 3.0 using o-phosphoric acid (52:48 v/v); flow rate of 0.45 mL min<sup>-1</sup>.

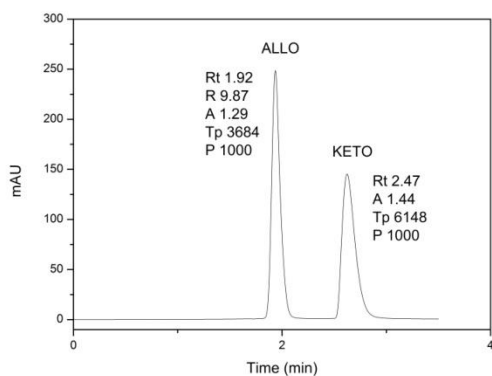


Figure 2. Chromatogram of ALLO (allopurinol) and KETO (ketoconazole) obtained with the InertSustain® C18 GL Sciences (4.6 x 100 mm x 3 µm) column using a mobile phase composed of acetonitrile:water (52:48 v/v) pH 3.0 adjusted with o-phosphoric acid and 0.45 mL min<sup>-1</sup> flow rate.

### Validation of the analytical method

#### Selectivity

In the placebo analysis, no peak was observed in drug retention times, indicating the selectivity of the method. The forced degradation study was evaluated through peak purity (> 950) and percent degradation (10-30%). The control solution presented levels of 99.30 ± 0.25% for ALLO and 101.61 ± 0.12% for KETO, peak purities were 1000 for both drugs. The acid condition presented degradation of 13.15% for ALLO and 27.11% for KETO, the peak purity index higher than 950. In the degradation of KETO (Figure 3) a peak in retention time and spectral profile different from that of the drug indicating to be a degradation product.

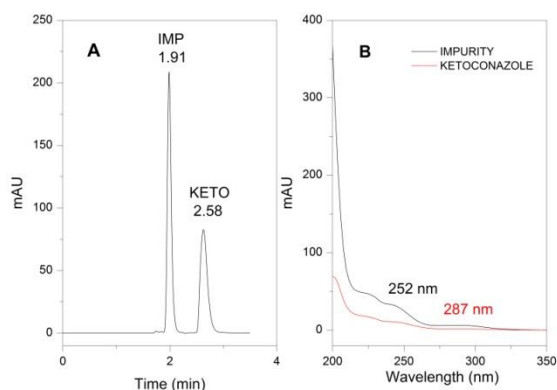


Figure 3. Chromatogram of KETO (ketoconazole) after acid stress (A). Spectrum in the ultraviolet region of impurity (IMP) 1.91 min and KETO 2.58 min, resulting from acid stress (B).

In the basic condition of ALLO, a degradation of 10.84% with a purity greater than 950 was obtained. In the condition used for KETO, the degradation value was 64.88%, indicating a great sensitivity of the drug for this condition.

In the oxidative condition, the degradation was 29.60% for ALLO and 18.93% for KETO, peak purities were

greater than 950. A peak in time 1.99 was observed in the KETO chromatogram not corresponding to the drug and can be observed in the images of its spectra, featuring drug degradation product (Figure 4).

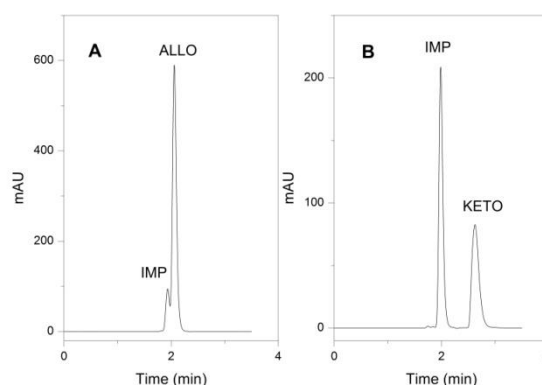


Figure 4. Chromatogram of ALLO (allopurinol) (A) and KETO (ketoconazole) (B) after oxidative stress.

In the thermal and photolytic conditions, there was no degradation of the drugs, indicating greater stability for these media, the peak purities were greater than 950. Singh and Gadhwala (2013) performed a study of ALLO degradation observing greater sensitivity in the oxidative medium with the formation of drug degradation product. Similarly, the researcher verified the greater stability of the drug in the photolytic and thermal medium [24].

Sajan *et al.* (2014) observed significant degradation of ALLO in acidic, basic, and photolytic medium with the formation of drug degradation products [25].

Tian *et al.* (2016) observed higher sensitivity of KETO in the oxidative medium [26]. However, the study of Mhaske and Sahasrabudhe (2011) showed great degradation of KETO in the acidic, basic, and oxidative medium with formation of degradation products of the drug, and greater stability in photolytic and thermal media [27].

Studies related to the photostability of KETO in formulations demonstrate its instability against this medium with the formation of drug degradation products. These studies work with semisolid formulations of the drug, usually shampoos and emulsions, providing an environment more conducive to its degradation against UV light and other degradation media, differently in this study using the solid-state drug itself (powder) in direct contact with the light [28-31].

#### Linearity, limit of detection and limit of quantification

The linearity of the method was demonstrated with correlation coefficients of 0.9995 for ALLO and 0.9999 for KETO, the regression equations obtained were  $y = 2.9421x + 0.2436$  and  $y = 4.0947x + 0.0930$ , respectively. The ANOVA test showed that linear regression has significance, where calculated F was greater than F critical for ALLO.

The inverse occurred for KETO indicating that another model could better explain the data, but does not mean

that the model in question has no significance because by the value of  $r^2$  it is possible to state that 99.987% of the variance is explained by the mathematical model.

The correlation between the analyte concentration and the equipment signal was also demonstrated by the Regression Residue analysis. Residual is defined as the difference between the values observed and the values estimated by the regression. In Figure 5 it is possible to visualize that the residues are distributed heterogeneously and randomly around the axis, indicating that there is no tendency.

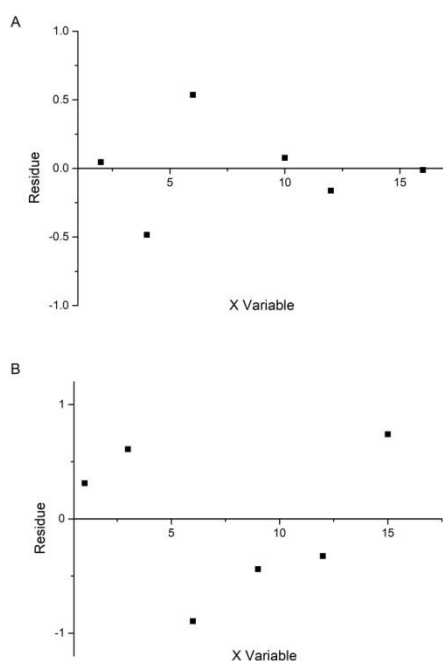


Figure 5. Analyzes of the residuals of the calibration analytical curves of allopurinol (A) and ketoconazole (B).

The LOD obtained were 0.0858 and 0.2599  $\mu\text{g mL}^{-1}$  for ALLO and KETO, respectively. The LOQ was 0.0331 and 0.1004  $\mu\text{g mL}^{-1}$  for ALLO and KETO, respectively.

#### Precision

The RSD values for ALLO and KETO were 0.21 and 0.24%, respectively, for the intermediate precision. For repeatability, 0.11 and 0.10% were obtained for ALLO and KETO, respectively. All values are within the recommended maximum limit of 2.0% [18].

#### Accuracy

The recovery values and the RSD obtained were  $100.77 \pm 0.93\%$  for ALLO and  $100.16 \pm 0.30\%$  for KETO. The results are in agreement with the acceptable limit range of 98.0 - 102.0% [19].

#### Robustness

The critical values calculated for ALLO and KETO were 1.8609 and 2.7350, respectively, for the major levels (+1),

and 3.67676 and 1.1819, respectively, for the minor levels (-1). The results of the effects produced (Table 2) indicate that small variations in the conditions tested do not significantly alter the proposed method.

Table 2. Results of the effects produced by each factor in the major and minor conditions in relation to the normal condition.

Factors	Allopurinol		Ketoconazole	
	(+1)	(-1)	(+1)	(-1)
A	-0,3203	0,3810	0,4817	0,3325
B	-1,4595	-1,3318	-1,2727	-0,3425
C	1,6002	-1,2911	0,0502	-0,6800
D	-0,0863	0,2071	-1,8559	0,8175
E	-0,2446	-0,1452	-1,1427	-0,2075
F	0,1746	0,5015	-1,0592	0,4475
G	1,1838	-1,0234	1,2168	0,3250

#### Application of the analytical method

Three manipulated samples of the association were analyzed, the average result of the content obtained in sample 1 was  $101.28 \pm 0.34\%$  for ALLO and  $102.35 \pm 0.25\%$  for KETO, in sample 2 it was  $103.06 \pm 0.13\%$  for ALLO and  $103.56 \pm 0.23\%$  for KETO, and in sample 3 it was  $104.67 \pm 0.42\%$  for ALLO and  $100.66 \pm 0.15\%$  for KETO. In the commercial samples available separately from the drugs, the contents of  $104.70 \pm 0.14\%$  for ALLO and  $104.02 \pm 0.11\%$  for KETO were obtained.

#### Conclusions

A stability-indicating HPLC-DAD method was developed and validated for the simultaneous determination of ALLO and KETO. This method proved to be selective, precise, accurate, linear, and robust. The method also showed good results in the quantification of drugs in other types of industrialized solid formulations, such as tablets. In addition to being a fast methodology and making use of low-cost solvents and can be inserted into the routine of drug quality control laboratories.

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#### Conflict of interest

The authors declare no conflicts of interest.

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