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Optimization and validation of a high-performance liquid chromatographic method with UV detection for the determination of ketoconazole in canine plasma☆

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Abstract

An isocratic high-performance liquid chromatographic method with detection at 240 nm was developed, optimized and validated for the determination of ketoconazole in canine plasma. 9-Acetylanthracene was used as internal standard. A Hypersil BDS RP-C_{18} column (250 mm × 4.6 mm, 5 μm particle size), was equilibrated with a mobile phase composed of methanol, water and diethylamine 74:26:0.1 (v/v/v). Its flow rate was 1 ml/min. The elution time for ketoconazole and 9-acetylanthracene was approximately 9 and 8 min, respectively. Calibration curves of ketoconazole in plasma were linear in the concentration range of 0.015–10 μg/ml. Limits of detection and quantification in plasma were 5 and 15 ng/ml, respectively. Recovery was greater than 95%. Intra- and inter-day relative standard deviation for ketoconazole in plasma was less than 3.1 and 4.7%, respectively. This method was applied to the determination of ketoconazole plasma levels after administration of a commercially available tablet to dogs.

Keywords: Ketoconazole; RP-HPLC with UV detection; Canine plasma; Pharmacokinetic profile

1. Introduction

Ketoconazole, cis-1-Acetyl-4-[4-[[2-(2,4-dichlorophenyl)-2-(1H-imidazol-1-ylmethyl)-1,3-dioxolan-4-yl]methoxy]phenyl] piperazine, shown in Fig. 1A, is a synthetic imidazole type of oral broad-spectrum antifungal agent. It is effective in the treatment of superficial and systematic infections [1,2] and has been widely used in immunocompromized patients and advanced prostatic carcinoma [3]. Compared with other similar drugs, it has a broad-spectrum activity and few unwanted side effects.

Several analytical methods have been developed for the determination of ketoconazole in biological samples. Among them microbiological assays [4,5] lack specificity because they are based on measurement of antifungal activity, which may not only be due to ketoconazole but to other bioactive plasma components or metabolites as well. HPLC methods with UV [6–12], fluorescence [13,14], electrochemical [15] and MS–MS [16] detection have been reported in plasma samples. Chen et al. [16] have summarized the limitations of all the above HPLC methods. More specifically, main drawbacks of the existing HPLC methods with UV detections are either high detection limits (>50 ng/ml) [8–12], or complicated and time consuming sample preparation procedures including liquid-liquid extraction, evaporation and reconstitution in order to lower LODs [6–8,12] or long total time elution time (>20 min) with the internal standard eluted after ketoconazole [7–10] or lack of internal standard [6,11,12].

The aim of the present work was to develop and validate a simple, fast and reliable isocratic RP-HPLC method with UV detection for the determination of ketoconazole in plasma samples. The important features and novelty of the proposed method include simple sample treatment with acetonitrile precipitation, centrifugation and direct injection of the clear supernatant to the HPLC system; short elution time (less than 10 min) with internal standard eluted prior to ketoconazole; short analysis time (less than 20 min); low limit of detection 5 ng/ml; good precision (less

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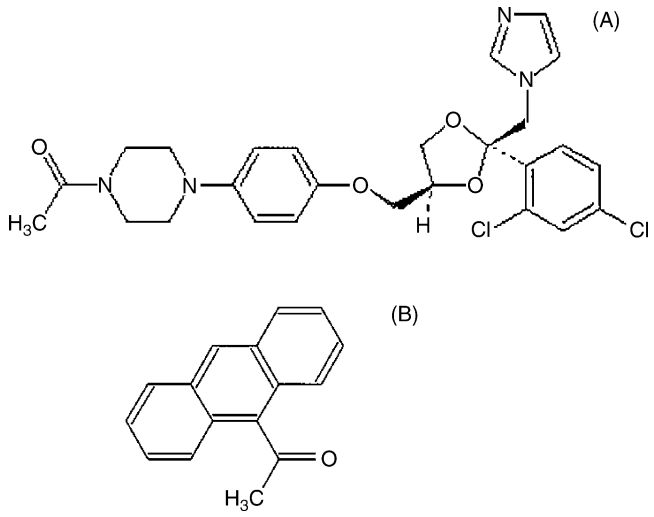

Fig. 1. Structures of ketoconazole (A) and 9-acetylanthracene (B).

than 5%) and high recovery (greater than 95%). Confirmation of the applicability of the developed method to pharmacokinetic studies of ketoconazole was also performed in dogs after single administration of a commercially available tablet (Fungoral®).

2. Experimental

2.1. Instrumentation

The chromatographic system consisted of a Spectra System P1000 pump, a Spectra System UV 2000 absorbance detector extended to the visible region and an autosampler AS 3000. The above system was controlled by a Specta System Controller SN 4000 and a software package Chromquest (Thermoquest Inc., San Jose, USA). A Hettich centrifuge Universal 32R (Tuttlingen, Germany) was utilized to centrifuge plasma samples.

2.2. Chemicals and reagents

Methanol (MeOH) and acetonitrile (ACN) of HPLC grade was purchased from E. Merck (Darmstadt, Germany). Diethylamine was of analytical purity grade (Riedel). Ketoconazole was of pharmaceutical purity grade and was donated by Recordati Espana S.L., Beniel, Spain. 9-Acetylanthracene (Fig. 1B) was purchased by Sigma Co. (St. Louis, MO, USA). Water purified with Labconco water pro ps system (Kansas City, Missouri, USA) was used in all procedures. Pooled canine blank plasma was obtained from dogs hosted at an animal facility that operates in our laboratory according to European Union regulations for the maintenance and experimentation on animals and which has been approved by the Veterinary Directorate of the Municipality of Athens. Aliquots of ketoconazole-free pooled canine plasma were used for preparation of spiked plasma standards.

2.3. Chromatographic conditions

A reversed-phase Hypersil BDS-C_{18} column (250 mm × 4.6 mm, 5 μm particle size) equipped with a precolumn Hypersil BDS-C_{18} (10 mm × 4 mm, 5 μm particle size) was used. The mobile phase was composed of methanol, water and diethylamine 74:26:0.1 (v/v/v) and its flow-rate was 1 ml/min. Injection volume was 50 μl. Experiments were performed at ambient temperature. Absorption was measured at 240 nm. The elution times for ketoconazole and 9-acetylanthracene were approximately 9 and 8 min, respectively.

2.4. Solution preparation

2.4.1. Stock solutions

Stock solutions of ketoconazole and 9-acetylanthracene (IS) (100 μg/ml) were prepared by dissolving 10 mg of each compound in 100 ml acetonitrile. These solutions were stored at 4 °C and were stable for at least two weeks.

2.4.2. Plasma samples

One hundred microlitres of sample were transferred to a microcentrifuge tube in which 40 μl of the working solution of IS (0.5 μg/ml) and 60 μl of acetonitrile were added. After vortexing for 30 s, the sample was centrifuged for 10 min in 11,000 rpm at 10 °C. Centrifugation at high speed and low temperature has been proved important in the sample preparation. The clear supernatant was then injected into the HPLC system.

2.4.3. Standard solutions

Usual ketoconazole calibration curves in plasma were constructed in the concentration range of 0.015–10 μg/ml as follows: in a microcentrifuge tube containing 100 μl of pooled blank canine plasma, 20 μl of ketoconazole working solutions (0.15–100 μg/ml in acetonitrile) were transferred. After vortexing, 40 μl of IS working solution (0.5 μg/ml) and 40 μl of acetonitrile were added. After vortexing for 30 s, the samples were centrifuged for 10 min in 11,000 rpm at 10 °C. The clear supernatant was then injected into the HPLC system.

For the establishment of the linearity range of the proposed method, calibration curves in plasma were prepared in the concentration range of 0.005–10 μg/ml. In addition, for the calculation of the limit of detection (LOD) and the limit of quantification (LOQ), similar curves in plasma were prepared in the range of 5–100 ng/ml. For recovery studies, similar calibration curves were constructed following the above procedure but replacing the amount of 100 μl of plasma with water.

Evaluation of precision and accuracy occurred in spiked plasma standards of low, medium and high concentration values (0.015, 0.025, 0.035, 0.05, 0.5, 1 and 3 μg/ml), prepared and measured five times each.

Evaluation of precision and accuracy was also obtained in canine samples of a pharmacokinetic study, prepared and measured five times each. More specifically, recovery data were achieved by the standard addition method applied in plasma samples of 30 min, 2.5 and 10 h after a single administration of a commercially available ketoconazole tablet. Each sample was prepared as described in Section 2.4.2. Then, an appropriate amount of ketoconazole standard was added and the sample was treated and run again. From the difference of these two signals, recovery values were extracted.

2.5. Pharmacokinetic application

A single dose oral administration of ketoconazole was performed in two female, 4-year-old mongrel dogs weighting about 30 kg. Before administration, each dog was fasted for 16 h from food but not water. Each dog was administered one tablet containing 200 mg of ketoconazole (Fungoral®, Janssen-Cilag AEBE, Greece) with 500 ml of milk (3.5% fat) via an orogastric tube. Blood samples were drawn by means of an indwelling catheter positioned in a suitable foreleg vein. After centrifugation, plasma was stored at −20 °C until assayed. This protocol had been approved by the Committee for Research of the University of Athens.

3. Results and discussion

3.1. Mobile phase

In the search of a proper and simple mobile phase, trying to avoid use of phosphate buffers, several solvent mixtures containing methanol (or acetonitrile) and water were examined. Triethylamine or diethylamine was necessary for minimizing peak asymmetry even though a BDS (Base Deactivated Silica)-C_{18} column was used. The finally accepted mobile phase was methanol-water-diethylamine 74:26:01 (v/v/v), where peaks of the drug and IS were clearly separated and not interfered with plasma constituents while the total elution time was reasonable (t∼10 min). Presence of acetonitrile did not improve the chromatographic performance. Moreover, changes in triethylamine content, as well as use of diethylamine instead, did not affect the separation features.

3.2. Choice of internal standard

Several substances were tested as internal standards. Among these, 9-acetylanthracene has been chosen as the most appropriate in the present analysis because it is stable and does not exist endogenously in plasma. Moreover, in the present study, it did not interfere with the matrix of plasma samples and it was well separated from ketoconazole. In addition, a significant advantage of this IS was its elution time, that was shorter than that of ketoconazole. The last feature was the goal of the performed internal standard optimization since in all relevant previously developed methods, the elution time of internal standard, whenever existed, was longer than that of ketoconazole resulting in considerable elongation of the total elution time [7–10].

3.3. Sample preparation

The proposed method involved a very easy and simple sample treatment (addition of acetonitrile for protein precipitation, centrifugation and injection to the HPLC system). Alkalinization of samples, proposed by several authors [7,9,14,16] did not improve the results, thus it was not adopted in this method. In addition, tedious and time consuming preparation procedures, such as sample evaporation and reconstitution aiming for lower limits of detection, were eliminated. However, the limit of detection of the developed method, is comparable with the lowest existing in the literature for HPLC methods [6] with UV [6–12], MS [16] and fluorimetric [13,14] detection.

Mixing acetonitrile with plasma in 1:1 v/v ratio resulted in effective plasma protein precipitation with simultaneous minimum sample dilution. In addition, centrifugation times ranging from 5 to 10 min did not affect the analysis. Moreover, the sample size was kept as low as possible (0.1 ml) to ensure the high quality of the analytical features of the developed method and minimal quantity of plasma sample needed for that.

3.4. Selectivity

Typical chromatograms of blank plasma, a spiked plasma standard and a real canine plasma sample, obtained under the optimized experimental conditions, are shown in Figs. 2 and 3, respectively. Good resolution for every peak and its nearest ones was confirmed by R_s values, which were greater than 2. Also, both ketoconazole and IS peaks were symmetrical (asymmetry factors, determined at 10% of the total peak height, were 1.05 and 1.10, respectively).

3.5. Calibration curves

Linear calibration curves for ketoconazole were obtained throughout the concentration range studied (0.005–10 μg/ml). Regression analysis was performed for the ratios of peak-area of ketoconazole to that of the IS (y) versus ketoconazole concentration (x). The results are presented in Table 1. In each case, the slope of the calibration curve obtained from standard solutions,

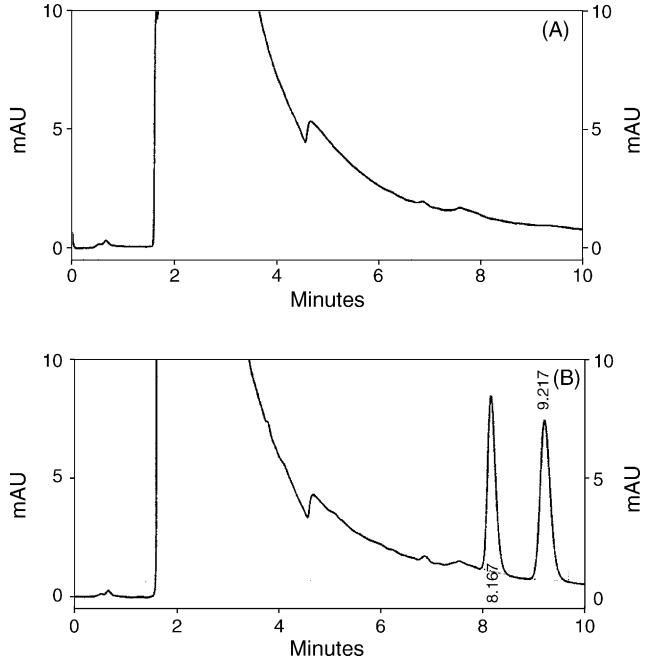

Fig. 2. Typical chromatograms of blank plasma (A) and a ketoconazole spiked plasma standard (0.25 μg/ml), containing 0.1 μg/ml of IS (B). Retention times for IS and ketoconazole were 8.2 and 9.2 min, respectively. The chromatographic conditions used were: BDS RP-C_{18} column (250 mm × 4.6 mm), mobile phase methanol, water and diethylamine 74:26:0.1 (v/v/v), flow rate 1 ml/min, detection wavelength 240 nm and room temperature.

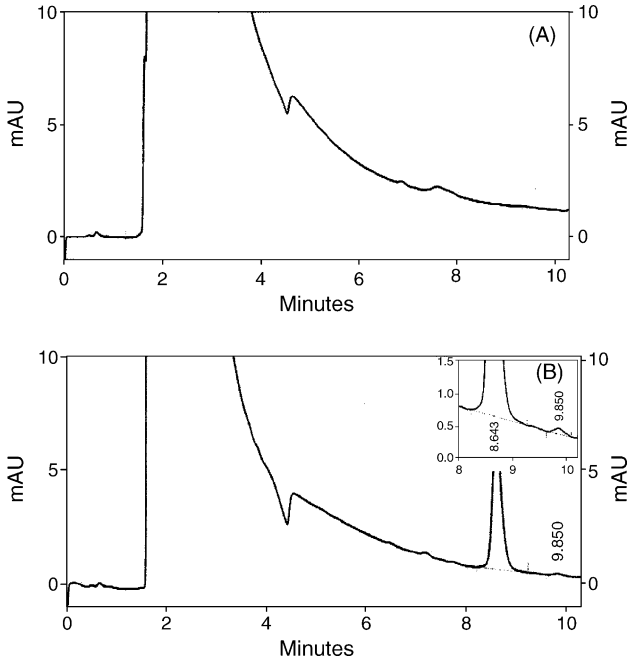

Fig. 3. Chromatograms of canine plasma prior to ketoconazole administration (A) and 15 min after a single administration of a tablet containing 200 mg of ketoconazole (Fungoral®) (B). Ketoconazole plasma concentration (10 ng/ml) was close to the LOQ of the method (15 ng/ml). Retention times for IS (0.1 μg/ml) and ketoconazole were 8.6 and 9.8 min, respectively.

prepared in plasma matrix, was not statistically different from that obtained in water (*t*-test, 95% confidence level).

3.6. Precision and accuracy

The precision of the proposed HPLC method was examined in standards and samples. After preparing and measuring plasma samples and standards of the same concentration of ketoconazole five times each, values of intra- and inter-day relative standard deviation (R.S.D.) were calculated. Results showed that intra-day relative standard deviation was less than 3.1%, while the corresponding inter-day value was less than 4.7%. However, at concentration levels close to the limit of quantification, R.S.D. values were 15–20%.

Accuracy of the developed method was examined by recovery studies at low, medium and high concentration values. These results are summarized in Table 2. Recovery data were determined as ratios of integrated peak area of ketoconazole to internal standard in standard solutions prepared in plasma matrix compared to the working standards in water. An estimation of the recovery of the method can also be obtained comparing the slopes of calibration curves in water and in plasma matrix.

Similar recovery data (97–103%) were obtained by the standard addition method applied in canine plasma samples of the pharmacokinetic application conducted as described in Section 2.4.3.

Table 1
Analytical parameters of usual calibration curves of ketoconazole in water and canine plasma in the concentration range of 0.015–10 μg/ml

Matrix	Regression equation[a]		
	Intercept ($a \pm$ S.D.)	Slope ($b \pm$ S.D.)	Correlation coefficient[b], r
Water	−0.125 ± 0.092	1.113 ± 0.021	0.9997
Plasma	−0.0081 ± 0.0066	1.124 ± 0.012	0.9995

[a] Linear unweighted regression analysis, with a regression equation $y = a + bx$, where y was the peak-area ratio of ketoconazole to IS and x was ketoconazole concentration in μg/ml. S.D. is the standard deviation.

[b] The number of points in each calibration curve was 10 and each point was the mean of three experimental measurements.

Table 2
Recovery data for ketoconazole in spiked plasma standards

Concentration (μg/ml)		Ketoconazole recovery[a]	
Injected	Plasma	Mean relative recovery ± S.D. (%)	Mean absolute recovery ± S.D. (%)
0.015	0.03	105.3 ± 3.1	102.6 ± 3.0
0.025	0.05	97.3 ± 3.0	95.2 ± 2.9
0.035	0.07	99.2 ± 2.6	96.7 ± 2.5
0.05	0.1	100.2 ± 1.2	97.9 ± 1.3
0.5	1	96.1 ± 2.9	93.8 ± 3.0
1	2	101.3 ± 3.0	98.6 ± 3.1
3	6	99.9 ± 1.5	97.3 ± 1.3

Each standard was prepared and measured five times.

[a] Mean absolute recovery of internal standard was 97.3 ± 3.3.

3.7. Limits of detection (LOD) and quantification (LOQ)

The LOD was defined as the analyte concentration that gives a signal equal to $y_b + 3.3s_b$, where y_b is the signal of the blank and s_b is its standard deviation. Similarly, the LOQ was defined as $y_b + 10s_b$. In the unweighted least-squares method is quite suitable in practice to use $s_{y/x}$ [17] instead of s_b and the value of the calculated intercept a instead of y_b. Thus,

$$\text{LOD} = \frac{3.3s_{y/x}}{b} \quad \text{and} \quad \text{LOQ} = \frac{10s_{y/x}}{b}$$

where b is the slope of the regression line.

Based on the above equations and calibration curves in plasma matrix, ranging from 5 to 100 ng/ml, LOD and LOQ values for ketoconazole were found equal to 5 and 15 ng/ml of plasma sample, respectively.

3.8. Robustness

The robustness of the proposed method was assessed with respect to small alterations in mobile phase (composition and flow rate). Changes in mobile phase from methanol, water and diethylamine 74:26:0.1 (v/v/v) to 73:27:0.1 and 75:25:0.1 (v/v/v) are presented in Table 3. Changes in the flow rate of mobile phase from 1 ml/min to 0.9 and 1.1 ml/min are also shown in Table 3. The effect of the above changes to critical separation parameters (e.g. retention time and resolution) is also included in Table 3. Slightly altered mobile phase diethylamine content and sample centrifugation time affected the results by less than 2.4%.

Table 3
Effect of small alterations in mobile phase on ketoconazole concentration in plasma samples and separation parameters

Chromatographic conditions		Ketoconazole concentration in plasma (μg/ml) (% difference)[a]				Separation parameters (% Difference)[a]	
Mobile Phase methanol-water-diethylamine (v/v/v)	Flow rate (ml/min)					t_R (min)	R_s
74-26-0.1	1	1.041	1.381	1.969	6.007	9.312	3.40
75-25-0.1	1	1.017 (2.4)	1.365 (1.2)	1.954 (0.79)	6.015 (0.13)	8.660 (7.0)	2.79 (18)
73-27-0.1	1	0.998 (4.1)	1.378 (0.27)	2.066 (4.9)	5.972 (0.59)	11.174 (20)	4.05 (19)
74-26-0.1	0.9	1.041 (0.004)	1.382 (0.065)	1.968 (0.049)	6.007 (0.003)	10.336 (11)	3.55 (4.4)
74-26-0.1	1.1	1.021 (2.0)	1.348 (2.4)	1.941 (1.4)	6.022 (0.24)	8.437 (9.4)	3.13 (7.8)

[a] Numbers in parentheses indicate the % difference in ketoconazole plasma concentration and separation parameters obtained under the deliberately altered chromatographic conditions [composition (v/v/v) or flow rate] vs. the optimized ones.

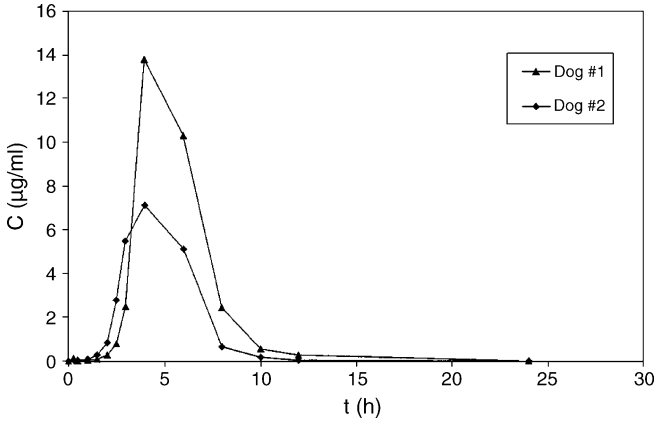

Fig. 4. Pharmacokinetic profiles of ketoconazole in canine plasma after a single administration of a commercially available tablet (Fungoral®, 200 mg).

3.9. Pharmacokinetic application

Fig. 4 shows two individual pharmacokinetic profiles of ketoconazole in plasma after single administration of a commercially available tablet (Fungoral®, 200 mg) with 500 ml of milk (3.5% fat) to two dogs. Table 4 shows the corresponding individual pharmacokinetic parameters. The values for the elimination rate constant are similar with previously reported values in dogs [18]. To the best of our knowledge there are no plasma levels of ketoconazole after single administration to fed dogs. However, after repeated administration of a ketoconazole tablet one hour after feeding (200 mg/tablet, every 12 h) to four dogs, the average plasma levels on day 30 ranged from 3.64 to 19.1 μg/ml [19]. Those data are in agreement with the increased interdog variability and fast elimination characteristics of ketoconazole from canine plasma that are implied by the data shown in Fig. 4. Also, both profiles in Fig. 4 show a lag phase prior to the onset of plasma levels that most likely relates to the reduced rate of emptying of canine gastric contents in the fed state [20] and makes the observed T_{max} values (Table 4) longer than those after administration in the fasting state [18,21]. A similar effect of food on absorption rates has been also observed in humans after administration of a single 200-mg tablet formulation of ketoconazole [22].

The slow absorption rates of ketoconazole in the fed state in both dogs and humans confirm the usefulness of the proposed method, to measure very low ketoconazole concentration in plasma, versus previously reported HPLC methods with much higher LOQ values [8–12].

Table 4
Individual values for the maximum concentration in plasma (C_{max}), the time at which C_{max} was observed (T_{max}), the estimated area under the concentration vs. time profile ($AUC_{0\text{-}12h}$), and the estimated elimination rate constant (Kel) after single administrations of a commercially available tablet (Fungoral®, 200 mg) with 500 ml of milk (3.5% fat) to two dogs

Pharmacokinetic parameters	Dog #1	Dog #2
C_{max} (μg/ml)	13.80	7.13
T_{max} (h)	4	4
$AUC_{0\text{-}12h}$ (μg ml^{-1} h)	49.23	27.35
Kel (h^{-1})	0.62	0.81

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