

The effect of ketoconazole on whole blood and skin ciclosporin concentrations in dogs

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Background – Ciclosporin (CSA) is approved for the treatment of canine atopic dermatitis. Ciclosporin is metabolized by liver cytochrome P450 enzymes, a process inhibited by ketoconazole (KTZ).

Hypothesis/Objectives – The aims of this study were to determine skin and blood CSA concentrations when CSA was administered alone at 5.0 (Treatment 1) or 2.5 mg/kg (Treatment 2) and when CSA was administered at 2.5 mg/kg concurrently with KTZ at 5 (Treatment 3) or 2.5 mg/kg (Treatment 4). We hypothesized that skin and blood CSA concentrations in Treatment 1 would not differ from those obtained with T3 or T4.

Animals – In a randomized cross-over study, six healthy research dogs received each of the treatments (Treatment 1, 2, 3 and 4) once daily for 7 days.

Methods – After the first, fourth and seventh dose for each treatment, a peak and trough skin punch biopsy sample and whole blood sample were collected and analysed with high-performance liquid chromatography–tandem mass spectrometry. Data were analysed using a repeated measures approach with PROC MIXED in SAS. Pairwise comparisons were performed with least squares means and Tukey–Kramer adjustment for multiple comparisons.

Results – Mean blood CSA concentrations in Treatment 1 were not different from those in Treatment 2 or 4, but were less than in Treatment 3. Mean skin CSA concentrations in Treatment 1 were greater than in Treatment 2, not different from those in Treatment 4, and less than those in Treatment 3.

Conclusions and clinical importance – Administration of CSA and KTZ concurrently at 2.5 mg/kg each may be as effective as CSA alone at 5.0 mg/kg for treatment of canine atopic dermatitis.

Introduction

Ciclosporin (CSA) is an immune-modulating drug currently labelled in the USA as Atopica® (Novartis Animal Health, Greensboro, NC, USA) for use in the treatment of canine atopic dermatitis (CAD).¹ As a calcineurin inhibitor, the primary mechanism of action of CSA is prevention of transcriptional activation of genes responsible for interleukin-2 production, a necessary step for full activation of the T-helper cell pathway.^{2,3} The absence of interleukin-2 synthesis prevents the activation and proliferation of T cells, in addition to the secondary synthesis of cytokines involved in CAD, such as interleukin-4, interleukin-5, interleukin-8 and interferon- γ .² A systematic review and meta-analysis of 10 controlled clinical trials, enrolling approximately 800 dogs, provided strong evidence for the efficacy of CSA in the treatment of CAD and concluded that the efficacy of CSA was comparable to that of oral

corticosteroids.⁴ The clinically effective dose of CSA for the treatment of CAD has been determined to be 5 mg/kg orally once daily.^{5,6} Treatment with CSA is typically maintained lifelong (as with all treatments for CAD), and 40–50% of dogs have continued control of their disease with every other day dosing at 5 mg/kg after 4–8 weeks of induction daily dosing.⁴

The cost of therapeutic agents as well as potential adverse effects are important factors for consideration, particularly when coupled with the fact that CAD commonly manifests at a young age and requires lifelong treatment. Long-term administration of Atopica® on a daily or every other day basis, particularly in medium-sized to large dogs, can be cost prohibitive. The cost of Atopica® was listed as a reason for discontinuing treatment in 10% (five of 51) of dogs with CAD being treated for at least 6 months in a study of long-term use of CSA.⁷ The authors are not aware of any published studies assessing the effect of drug cost on owners' initial choice of therapy for their dog with CAD.

As the cost of Atopica® may be a limiting factor for its use, methods to reduce the dose of CSA without loss of clinical efficacy have been evaluated. Ciclosporin is primarily metabolized in the liver by cytochrome P450

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enzymes 2B11 and 3A12/26.^{8,9} Consequently, research thus far has focused on pharmacotherapeutic manipulation of this enzyme system by co-administration of drugs known to be P450-enzyme inhibitors. Recent studies indicate that neither metoclopramide nor cimetidine, both P450 inhibitors, have any effect on the pharmacokinetic profile of concurrently administered CSA.^{10,11} Grapefruit juice and powdered whole grapefruit may variably affect the pharmacokinetic parameters of CSA in dogs.^{11,12}

Ketoconazole (KTZ) is a potent inhibitor of numerous cytochrome P450 enzymes, including CYP 2B11 and 3A12/26.^{8,9} Numerous *in vitro* and *in vivo* studies have demonstrated that co-administration of KTZ with CSA results in increased whole blood CSA concentrations in dogs.^{13–18} The inhibitory effect of KTZ on CSA clearance from blood was determined to be dose dependent, with the critical KTZ dosage range identified as 2.5–10 mg/kg daily.¹⁸ A study on normal research beagles investigating the dose of KTZ necessary to maintain whole blood CSA trough levels between 400 and 600 mg/mL showed that KTZ at 13.6 mg/kg daily enabled a 75% reduction of CSA dose, with estimated monetary savings of 57.8% at that time, and KTZ at 4.7 mg/kg daily reduced CSA dose by 38% and thus reduced cost by 23.8%.¹⁴ These results have been applied clinically in studies on the efficacy of combination KTZ and CSA for the treatment of canine perianal fistulas, with excellent short-term results and a significant decrease in the dose of CSA required for clinical efficacy. One study involving 12 dogs with perianal fistulas showed that doses of KTZ between 5 and 11 mg/kg daily allowed a 50–75% dose reduction of CSA while maintaining target CSA trough levels, and all 12 dogs had at least short-term resolution of clinical signs.¹⁶ This reduced dose equalled a monetary saving of 35–71% when compared with the cost of treatment with CSA alone. Further studies in dogs with perianal fistulas had concurring results; a dose of KTZ of 10 mg/kg daily allowed for an 80% reduction of CSA administration, and doses of KTZ of 5.3–8.9 mg/kg twice daily enabled a reduction of CSA dose equalling a monetary saving of 70% compared with CSA alone.^{15,17}

As CSA is a lipophilic drug, it distributes widely in tissue and has been reported in the skin at concentrations up to 10 times higher than blood concentrations in humans.^{19,20} An abstract of an unpublished study in dogs dosed with CSA at 3.8 mg/kg once daily for 14 days found that skin levels of CSA were 2.5–6.4 times higher than the whole blood CSA levels, and that depletion of CSA is slower from the skin than from the blood.²¹ Studies examining tissue levels of CSA are sparse and are primarily toxicological or post-mortem studies.

Concurrent use of KTZ and CSA for treatment of CAD has been suggested by veterinary dermatologists at continuing education meetings in North America.^{22–26} However, we are not aware of any published studies that have evaluated the effects of KTZ on CSA skin levels in dogs, or any published clinical trials of the efficacy of this combination of drugs for the treatment of CAD. The specific aims of this study were to determine skin and whole blood CSA concentrations when CSA was administered

alone at a recommended (5.0 mg/kg/day) and a subtherapeutic dose (2.5 mg/kg/day) and when administered at a subtherapeutic dose (2.5 mg/kg/day) concurrently with KTZ at two different doses (2.5 or 5.0 mg/kg/day). We hypothesized that when CSA was administered alone at the recommended dose (5.0 mg/kg/day), the skin and whole blood CSA concentrations would not differ significantly from those obtained with subtherapeutic CSA dosing (2.5 mg/kg/day) concurrently with either dose (2.5 or 5.0 mg/kg/day) of KTZ.

Materials and methods

The experimental protocol was approved by the Institutional Animal Use and Care Committee (IACUC) of The Ohio State University.

Animals

Six clinically normal adult laboratory dogs (foxhounds) 1–4 years of age were used in this study. All dogs had complete blood counts and serum biochemical profiles prior to study enrolment. Dogs were housed in the laboratory research facility at the veterinary college and were under the care of the University Laboratory Animal Resources (ULAR) staff. The animals were housed indoors, in individual concrete runs, in a temperature- and humidity-controlled environment. They were maintained on a diet of Iams Mini Chunks (The Iams Company, Cincinnati, OH, USA) or Teklad 25% Lab Dog Diet (Harlan Laboratories, Indianapolis, IN, USA), fed once a day, with occasional dog treats, and water *ad libitum*. During the study periods, food was available from 14.00 to 07.30 h.

All treatments (CSA and CSA + KTZ) were administered at 09.30 h to comply with the Atopica® label recommendation that the drug should be administered at least 2 h before or after feeding because the bioavailability is better in fasted animals.²⁷ Vomiting for more than two consecutive dosing periods (48 h) was deemed a criterion for withdrawal from the study, as were signs of systemic illness (lethargy, fever, changes in complete blood counts or chemistry values above or below the reference range). Anti-emetics were not permitted because most of these agents are also metabolized by (or affect) the cytochrome P450 enzyme system.¹¹ The dogs were visually monitored daily, and any adverse events (including vomiting or diarrhoea/soft stool, as well as erythema, swelling or discharge from biopsy sites) were recorded. Biopsy sites that became infected were to be treated topically with chlorhexidine gluconate solution 2% (Phoenix™ Pharmaceutical Inc., St Joseph, MO, USA) and Triple antibiotic ointment® (E. Fougera and Co., Melville, NY, USA) twice daily until completion of the study period, and then with 5.0–10.0 mg/kg generic cefpodoxime (Proxetil Putney Inc., Portland, ME, USA) once daily until resolution of the infection. Antibiotic administration was not permitted during the study period to avoid increasing the likelihood of vomiting or diarrhoea.

Sample collection

Whole blood samples for CSA analysis (1.0 mL) were collected via cephalic, lateral saphenous or jugular venipuncture with a 22-gauge needle. The blood was placed into a lavender-topped tube (EDTA tube) and gently mixed, then transferred to Eppendorf tubes (VWR International, Radnor, PA, USA) and stored frozen at –80°C until analysis.

Skin samples were collected from the dorsal or dorsolateral neck. These sites were chosen for ease of access and maximal skin thickness for sample analysis. Local anaesthesia was performed by injecting 0.5 mL of 2% lidocaine (Butler Schein™ Animal Health, Dublin, OH, USA) subcutaneously with a 25-gauge needle. After 5 min, the skin sample was collected with an 8 mm biopsy punch (Medichoice® Tru Punch Disposable Biopsy Punch; Owens & Minor, Mechanicsville, VA, USA), and the site was closed with a single cruciate suture using 3–0 absorbable

monofilament (3–0 PDS, Ethicon®; Novartis Animal Health, Greensboro, NC, USA). All subcutaneous tissue was trimmed from the skin sample, and the skin was placed in an Eppendorf tube and stored frozen at -80°C until analysis.

Study design

Prior to commencement of the study, whole blood and skin samples were taken for CSA analysis to ensure that each dog was beginning with CSA levels below the limit of detection. Completion of each of the four treatment periods detailed below was followed by a 14-day washout, at which time whole blood and skin samples were collected for CSA analysis to ensure that all CSA concentrations were below the level of detection prior to entering the next treatment period. Complete blood counts and serum biochemical profiles were performed on all dogs within 90 days prior to study enrollment, as well as prior to (day 0) and immediately following completion (day 8) of Treatments 3 and 4.

The six dogs were randomly assigned via a computer-generated list (using the dog's research number) into two groups of three dogs each. One group was administered CSA at 5 mg/kg orally once daily for 7 days (Treatment 1), and the other group was administered CSA at 2.5 mg/kg orally once daily for 7 days (Treatment 2). Ciclosporin was administered as Atopica®, which is available as 10, 25, 50 and 100 mg capsules. Combinations of these capsule sizes were used to dose the dogs as closely as possible to 2.5 and 5.0 mg/kg/day. After the first, fourth and seventh dose of CSA, a skin sample was collected at 4 h (estimated peak skin concentration) and at 24 h (estimated trough skin concentration) to determine skin CSA levels.²¹ After the first, fourth and seventh dose of CSA, a whole blood sample was collected at 1.4 h (peak whole blood concentration) and at 24 h (trough whole blood concentration) to determine whole blood CSA levels.²⁸ Peak concentration samples were thus collected on days 1, 4 and 7, while trough concentration samples were collected on days 2, 5, and 8.

Following a 14-day washout period, the same two groups of three dogs were then randomly assigned to receive either CSA at 2.5 mg/kg and KTZ at 5.0 mg/kg orally once daily for 7 days (Treatment 3) or CSA at 2.5 mg/kg and KTZ at 2.5 mg/kg orally once daily for 7 days (Treatment 4). Ketoconazole (Teva, Sellersville, PA, USA) was administered in the generic form of 200 mg tablets (or part thereof) or as a compounded solution of the tablets per Trissel's formulary (prepared by the facility's pharmacy) in order to ensure dosing as close to 2.5 or 5.0 mg/kg as possible.²⁹ The compounded solution was not used for the 5.0 mg/kg KTZ dose owing to the large volume required. The CSA and the KTZ were administered concurrently. After the first, fourth and seventh dose of CSA and KTZ, a skin sample was collected at 4 h (estimated peak skin concentration) and at 24 h (estimated trough skin concentration) to determine skin tissue CSA levels. After the first, fourth and seventh dose of CSA and KTZ a whole blood sample was collected at 1.4 h (peak whole blood concentration) and at 24 h (trough whole blood concentration) to determine whole blood CSA levels. Peak concentration samples were thus collected on days 1, 4 and 7, while trough concentration samples were collected on days 2, 5 and 8.

Cross-over

After a 14-day washout period, the two groups of dogs then followed a full cross-over study design. Thus, at completion of the study each of the six dogs had received each of the four treatments in random order.

Ciclosporin analysis

Skin and whole blood samples were shipped overnight on dry ice to iC42 Bioanalytics (UC Denver, Denver, CO, USA) for analysis. Samples were shipped and analysed in two batches; the first sent half-way through the study and the second at study completion.

Ciclosporin in EDTA whole blood and tissue was quantified using high-performance liquid chromatography–tandem mass spectrometry (HPLC-MS/MS) following the procedures as previously described.³⁰ All tissues were weighed. Tissues were pulverized

under liquid nitrogen, and 100 mg of the frozen tissue powder was measured into 1 mL of the 0.5 mol/L potassium phosphate buffer (pH 7.4) and homogenized. Homogenates were prepared as 200 μL aliquots for extraction.

For protein precipitation, 800 μL of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (17.28 g/L) in 30:70 (v/v) HPLC grade water/methanol containing the deuterated internal standard ciclosporin-D4 (50 ng/mL) was added to 200 μL aliquots of EDTA whole blood and tissue homogenates. Samples were vortexed for 2.5 min and then centrifuged at 13,000g for 10 min at 4°C . The supernatant was transferred into glass HPLC vials for analysis.

The extracts were analysed using an LC/LC-MS/MS system (HPLC Agilent 1100 Series, Applied Biosystems and Sciex API 4000 triple quadrupole mass spectrometer, Carlsbad, CA, USA). For on-line sample clean-up, 20 μL supernatant was loaded and cleaned on a 4.6 mm $\times$ 12.5 mm, 5 μm , Eclipse XDB-C8 column (Agilent, Santa Clara, CA, USA) using a mobile phase of 20% methanol and 80% 0.1% formic acid at a flow rate of 5 mL/min for 1 min. Then the column switching valve (Rheodyne, Cotati, CA, USA) was activated and the analytes were back-flushed onto the analytical column (4.6 mm $\times$ 150 mm, 5 μm , Eclipse Zorbax XDB-C8, Agilent), which was kept at 65°C . A gradient was used from 87% methanol and 13% 0.1% formic acid to 100% methanol in 2.0 min at a flow rate of 1 mL/min and held at 100% for 1.5 min. The mass spectrometer was run in the positive MRM (multiple reaction monitoring) mode. The declustering potential (DP) was set to 131 V. Detection of the ions was performed by monitoring the transitions of m/z 1224.6 $\rightarrow$ 1112.4 for ciclosporin $[\text{M}+\text{Na}^+]$ and m/z 1228.6 $\rightarrow$ 1112.4 for the deuterated internal standard ciclosporin-D4 $[\text{M}+\text{Na}^+]$. The collision energy (CE) was 85 eV.

The lower limit of quantification for ciclosporin in EDTA whole blood was 5.0 ng/mL, and for tissues 25 ng/g (0.025 ng/mg). The assay was linear over three orders of magnitude. The interassay accuracy was between 85 and 115%, and total imprecision was $\leq 17\%$. There were no matrix interferences, carry-over or ion suppression. Both batches of samples were reported with assay specific quality control data and calculated interassay accuracy.

Sample size estimation

A pilot study with two dogs was performed prior to the study. Results from the pilot study were used for a power calculation, using a power of 80% and significance level of 0.05, which indicated that six dogs would be needed in order to detect a significant difference in CSA concentrations in skin using the trough value from day 8 between treatment groups 1 and 2. The preliminary data suggested that differences when comparing the additional treatment groups were greater, and would require less than six dogs to achieve statistical significance (Stata 10; Stata Corp., College Station, TX, USA).

Statistical analysis

Daily CSA blood and skin concentrations were considered the outcomes in the data analyses using a repeated measures approach with PROC MIXED in SAS (version 9.2; SAS Institute Inc., Cary, NC, USA). Compound symmetry covariance structure was used to account for the nonindependence of the repeated observations from individual dogs. The effects of day, treatment and order of treatments on skin and blood CSA concentrations were initially assessed and then treatment–day interaction was also tested. If the treatment–day interaction was significant, analyses were further stratified by treatment; and blood and skin CSA concentrations between the days were compared within each treatment, separately for days with presumed peak (days 1, 4 and 7) and trough values (days 2, 5 and 8). Pairwise comparisons between days and between treatments were performed by obtaining least squares means and using Tukey–Kramer adjustment to account for multiple comparisons. The correlation between skin CSA and blood CSA values was assessed using Spearman correlation coefficients. For all analyses, values of $P \leq 0.05$ were considered statistically significant.

Results

All treatments were administered as intended (Table 1) except for one dog (no. 38), that received medications in ~15 g (1 tablespoon) of canned food because the dog was resistant to manual pilling. The interassay accuracy of the CSA HPLC-MS/MS according to comparison with internal quality controls was reported for the two batches of samples, with each batch containing samples of skin and whole blood from all four treatment groups. The first batch analysis showed that blood CSA detection accuracy ranged from 98.3 to 104%, and skin accuracy ranged from 95.7 to 115%. The second batch showed that blood CSA detection accuracy ranged from 86.7 to 102%, and skin accuracy ranged from 93.6 to 117%. None of the skin and whole blood samples collected prior to entry into the study or prior to each treatment (following the 14-day washout period) had detectable levels of CSA.

Descriptive statistics for the measured (unadjusted) whole blood and skin CSA concentrations for all treatment groups by day are presented in Table 2. Using the repeated measures model and evaluating for fixed effects, the treatment (Treatment 1, 2, 3 or 4), the day (day 1, 2, 4, 5, 7, 8) and the treatment–day interaction each had a significant effect on skin and whole blood CSA concentrations (F -test, $P < 0.0001$). However, the order in which treatments were received did not have a significant effect on whole blood or skin CSA concentrations (F -test, $P = 0.854$ and F -test, $P = 0.756$, respectively).

The adjusted mean whole blood CSA concentration across all days for Treatment 1 (307.5 ng/mL) was not significantly different from the adjusted mean whole blood CSA concentration for Treatment 2 (169.41 ng/mL, Tukey–Kramer, $P = 0.14$) or Treatment 4 (417.74 ng/mL, Tukey–Kramer, $P = 0.136$), when evaluated with a mixed-model approach considering treatment, day and treatment–day interaction. However, the adjusted mean whole blood CSA concentration for Treatment 1 was significantly lower than the adjusted mean whole blood CSA concentration for Treatment 3 (644.83 ng/mL, Tukey–Kramer, $P = 0.0002$). The adjusted mean whole blood CSA concentration for Treatment 3 was significantly higher than the adjusted mean whole blood CSA concentration for Treatment 4 (Tukey–Kramer, $P = 0.0081$), and the adjusted mean whole blood CSA concentration for Treatment 2 was significantly lower than that for Treatments 3 and 4 (Tukey–Kramer, $P < 0.0001$ and $P = 0.0040$, respectively).

Utilizing least squares means obtained from the mixed models, the daily mean peak whole blood CSA concentrations did not differ significantly from each other within any treatment group (Table 3). Likewise, the daily mean trough whole blood CSA concentrations were not significantly different within Treatment 3; however, they were lower on day 2 compared with day 5 and on day 2 when compared with day 8 in Treatments 1, 2 and 4 (Table 3).

Using the mixed-model approach considering treatment, day and treatment–day interaction, the adjusted mean skin CSA concentration for Treatment 1 (0.61 ng/mg) was significantly higher than the adjusted mean skin CSA concentrations for Treatment 2 (0.262 ng/mg, Tukey–Kramer, $P = 0.05$), not significantly different from the adjusted mean skin CSA concentration for Treatment 4 (0.697 ng/mg, Tukey–Kramer, $P = 0.895$), but was significantly lower than the adjusted mean skin CSA concentration for Treatment 3 (1.236 ng/mg, Tukey–Kramer, $P = 0.0006$). The adjusted mean skin CSA concentration for Treatment 3 was also significantly higher than the adjusted mean skin CSA concentration for Treatment 4 (Tukey–Kramer, $P = 0.0024$), and the adjusted mean skin CSA concentration for Treatment 2 was significantly lower than that of Treatments 3 and 4 (Tukey–Kramer, $P < 0.0001$ and $P = 0.0129$, respectively).

Utilizing least squares means obtained from the mixed models, the mean daily peak skin CSA concentrations on day 4 were significantly higher than day 1 mean daily peak CSA concentrations in Treatments 1, 2 and 3, while day 7 mean daily peak CSA concentrations were significantly higher than those of day 1 within all four treatments (Table 4). The mean trough CSA concentrations on day 5 were significantly higher than those on day 2 for Treatments 1 and 4; day 8 mean trough CSA concentrations were significantly higher than day 2 mean trough CSA concentrations for Treatments 1, 2 and 4; and day 8 mean trough CSA concentrations were significantly higher than those of day 5 only for Treatment 4 (Table 4).

The correlation between whole blood and skin CSA concentrations combining values from all treatments was moderate (Spearman correlation coefficient, $r^2 = 0.6689$). The correlation of whole blood and skin CSA values within each treatment group were moderate for Treatments 1 and 2 (Spearman correlation coefficient of 0.786 and 0.6881, respectively) and for Treatments 3 and 4

Table 1. Target and measured dosage range of ciclosporin administered alone or in combination with ketoconazole to six healthy dogs

	Treatment 1 (mg/kg)	Treatment 2 (mg/kg)	Treatment 3 (mg/kg)	Treatment 4 (mg/kg)
Ciclosporin				
Target dosage	5.0	2.5	2.5	2.5
Administered dosage (range)	5.0–5.3	2.5–2.9	2.5–2.9	2.5–2.9
Ketoconazole				
Target dosage	—	—	5.0	2.5
Measured dosage (range)			5.0–5.3	2.5

Treatment 1, 5.0 mg/kg of ciclosporin (CSA) once daily; Treatment 2, 2.5 mg/kg of CSA once daily; Treatment 3, 2.5 mg/kg of CSA and 5.0 mg/kg of ketoconazole (KTZ) once daily; Treatment 4, 2.5 mg/kg of CSA and 2.5 mg/kg of KTZ once daily.

Table 2. Descriptive statistics for ciclosporin concentrations in whole blood and skin

	Day 1	Day 2	Day 4	Day 5	Day 7	Day 8
Treatment 1						
Blood	544 (0–895)	34.5 (0–73.2)	682.3 (447–798)	87.0 (44.4–199)	710 (191–160)	94.8 (47.9–182)
Skin	0.27 (0–0.53)	0.27 (0.54)	1.21 (0.78–1.96)	0.69 (0.33–1.03)	1.06 (0.84–1.32)	0.78 (0.39–1.19)
Treatment 2						
Blood	338.5 (144–422)	18.7 (10.3–33.5)	412 (309–553)	31.6 (14.6–57.5)	347 (53.1–586)	380 (112–814)
Skin	0.16 (0.12–0.25)	0.19 (0.08–0.61)	0.44 (0.26–0.66)	0.24 (0.12–0.41)	0.43 (0.25–0.59)	0.36 (0.16–0.75)
Treatment 3						
Blood	738.7 (371–1420)	699.9 (423–1470)	981.4 (22.2–2130)	249.9 (95.1–522)	1451.3 (848–1950)	392.7 (112–814)
Skin	0.48 (0.19–0.94)	0.37 (0.17–0.60)	1.68 (0.98–2.91)	1.16 (0.96–1.57)	2.37 (1.25–4.57)	2.59 (1.26–8.11)
Treatment 4						
Blood	821 (574–1010)	42.6 (21.7–65.9)	813.3 (151–1190)	102 (66.5–179)	1013.7 (710–1270)	131.6 (68.8–206)
Skin	0.36 (0.20–0.49)	0.30 (0.20–0.40)	0.84 (0.51–1.05)	0.82 (0.52–1.31)	1.37 (0.97–2.44)	1.18 (0.78–1.42)

Values are expressed as means (range). Blood concentrations are reported in nanograms per millilitre, whereas skin concentrations are reported in nanograms per milligram. Days 1, 4 and 7 represent peaks, while days 2, 5 and 8 represent troughs.

Table 3. Least squares means obtained from the mixed models for daily peak and trough whole blood ciclosporin (CSA) concentrations for six healthy dogs

	Peak blood CSA			Trough blood CSA		
	Day 1	Day 4	Day 7	Day 2	Day 5	Day 8
Treatment 1	544.00	710.00	682.33	34.47 ^{a,b}	87.02 ^a	94.83 ^b
Treatment 2	338.50	412.00	347.02	18.70 ^{c,d}	31.63 ^c	38.02 ^d
Treatment 3	738.67	981.37	1451.33	699.93	249.85	392.67
Treatment 4	821.00	813.33	1013.67	42.53 ^{e,f}	101.98 ^e	131.63 ^f

Blood concentrations are reported in nanograms per millilitre. The same superscripts on the daily peak or trough values within a treatment indicate a significant difference between the days, with the Tukey–Kramer adjusted *P*-values given, as follows: ^a*P* = 0.003, ^b*P* = 0.012, ^c*P* = 0.011, ^d*P* = 0.007, ^e*P* = 0.0008 and ^f*P* < 0.0001.

Table 4. Least squares means obtained from the mixed models for daily peak and trough skin ciclosporin (CSA) concentrations for six healthy dogs

	Peak skin CSA			Trough skin CSA		
	Day 1	Day 4	Day 7	Day 2	Day 5	Day 8
Treatment 1	0.27 ^{a,b}	1.21 ^a	1.06 ^b	0.27 ^{i,j}	0.68 ^j	0.78 ^j
Treatment 2	0.16 ^{c,d}	0.44 ^c	0.43 ^d	0.19 ^k	0.24	0.36 ^k
Treatment 3	0.48 ^{e,f}	1.68 ^e	2.37 ^f	0.36	1.16	2.58
Treatment 4	0.36 ^g	0.84 ^h	1.37 ^{g,h}	0.30 ^{l,m}	0.82 ^{l,n}	1.19 ^{m,n}

Skin concentrations are reported in nanograms per milligram. The same superscripts on the daily peak or trough values within a treatment indicate a significant difference between the days, with the Tukey–Kramer adjusted *P*-values given, as follows: ^a*P* < 0.0001, ^b*P* = 0.0001, ^c*P* = 0.0008, ^d*P* = 0.0011, ^e*P* = 0.023, ^f*P* = 0.0013, ^g*P* = 0.0006, ^h*P* = 0.035, ⁱ*P* = 0.0010, ^j*P* = 0.0002, ^k*P* = 0.0083, ^l*P* = 0.0002, ^m*P* < 0.0001 and ⁿ*P* = 0.0030.

(Spearman correlation coefficient of 0.5623 and 0.5766, respectively).

One dog (no. 38) exhibited higher skin CSA concentrations than other animals, especially for the day-7 peak and day-8 trough of Treatment 3. Figures 1 and 2 show the whole blood CSA (Figure 1) and skin CSA concentrations (Figure 2) for dog no. 38 (red), as well as the concentrations for the other five dogs obtained during Treatment 3. Statistical significance was not affected by these values.

Adverse events

Chemistry profile values and complete blood counts for all dogs were within the normal reference range for the laboratory prior to study commencement and remained within the reference ranges following completion of Treatments 3 and 4. Gastrointestinal upset was the most commonly noted adverse event and

was generally sporadic. Four of six dogs vomited during the study period; two dogs vomited once, one dog vomited three times, and one dog vomited four times. Only one dog (no. 97) vomited for two consecutive dosing periods (48 h) during Treatment 1, but did not continue vomiting and thus was not withdrawn from the study. Six of the nine vomiting episodes occurred during Treatment 1. Soft stool was reported from one dog on two occasions during Treatment 1, and two dogs experienced diarrhoea on four and five total separate occasions during Treatments 1, 3 and 4.

Discussion

In this study, we have established the skin concentrations of CSA that are achieved in normal research dogs when administered CSA at 5.0 and 2.5 mg/kg orally once daily,

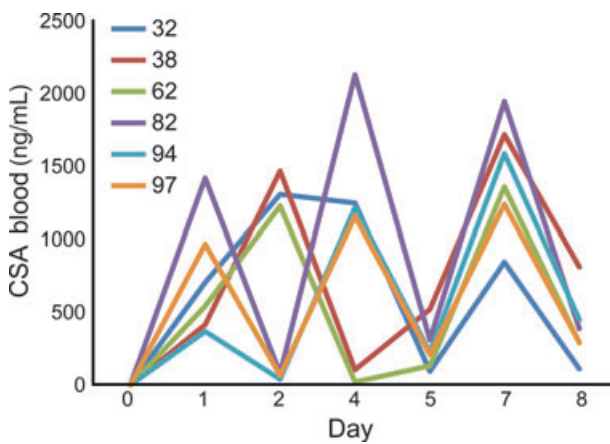


Figure 1. Ciclosporin (CSA) concentrations in whole blood for six healthy dogs (nos 32, 38, 62, 82, 94 and 97) treated with 2.5 mg/kg of CSA and 5.0 mg/kg of ketoconazole once daily (Treatment 3). Days 1, 4 and 7 represent peaks, while days 2, 5 and 8 represent troughs.

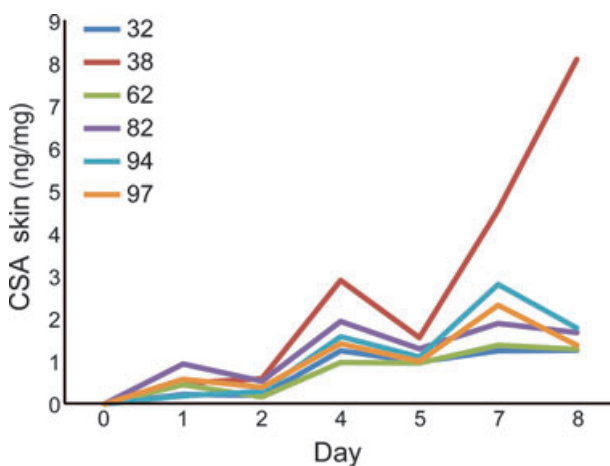


Figure 2. Ciclosporin concentrations in skin for six healthy dogs (nos 32, 38, 62, 82, 94 and 97) treated with 2.5 mg/kg of CSA and 5.0 mg/kg of ketoconazole once daily (Treatment 3). Days 1, 4 and 7 represent peaks, while days 2, 5 and 8 represent troughs.

as well as skin CSA concentrations achieved when CSA is administered at 2.5 mg/kg orally once daily with concurrent KTZ at either 2.5 or 5.0 mg/kg. The results from this study provide a baseline for comparison of CSA levels in skin and blood at two clinically relevant CSA dosages (5.0 and 2.5 mg/kg). They also provide evidence that concurrent administration of a CYP 450 inhibitor (KTZ) significantly elevates CSA levels in skin as well as blood.

Whole blood peak and trough concentrations of CSA when administered at 5.0 mg/kg in this study were within the range of those documented in previous studies.^{10,28} Although 2 h blood CSA concentrations in humans are predictive of acute rejection and clinical outcome in solid organ transplantation, a study of 97 dogs with AD treated with CSA at a mean dose of 4.6 mg/kg orally once daily for 28 days found no significant correlation between clinical improvement and whole blood CSA concentrations.^{27,28,31,32} As skin is likely to be the target organ for treatment of atopic dermatitis, the concentration of CSA achieved in the skin may be important in

determining the clinical response to CSA therapy. This is further exemplified by the moderate correlation between skin and whole blood CSA concentrations in our study, which indicates that whole blood concentrations do not accurately predict CSA concentrations in the skin. Additionally, skin concentrations in Treatment 2, a dosage of CSA known to be ineffective for the control of CAD, were significantly lower than those found in Treatment 1.⁶ The lack of significant difference in whole blood concentrations between Treatment groups 1 and 2 provides further support that skin concentrations may be more representative of the concentration of CSA required for clinical efficacy. Skin concentrations achieved in Treatment 4 were not different from those obtained in Treatment 1. It would be a fair expectation that clinically, treatment of CAD with either treatment regimen would yield similar results. Treatment 4 would offer a considerable (50%) reduction in cost of CSA administration. However, as it is not yet known whether skin CSA concentrations are correlated with clinical efficacy, identical treatment outcomes cannot be assumed and should be verified in atopic dogs. While Treatment group 1 establishes anticipated skin CSA concentrations, CSA concentrations in dogs clinically affected with CAD may differ from those found in research (normal) dogs, and may differ in clinically affected versus nonaffected skin. The skin levels established in this study were those found in the dorsal neck, which may differ from those sites typically affected by CAD, such as the inguinal and axillary regions, face, ears and ventral neck.

The highest CSA skin concentrations were obtained during Treatment 3, and were significantly higher than those found in both Treatment 1 and Treatment 4. If skin concentrations are in fact correlated with clinical efficacy, the higher CSA skin concentrations found with Treatment 3 may provide additional clinical effect for the control of CAD. However, it is possible that the degree of elevation of CSA paired with 5.0 mg/kg of KTZ may provide higher concentrations of CSA than necessary for control of CAD. Although the margin of safety for CSA administration in canines is much greater than that in humans, adverse effects from CSA have generally been observed to be dose dependent, supporting the importance of using the lowest effective dose. Although a recent retrospective analysis of the adverse effects of KTZ in dogs did not indicate that dose was a factor in the number or severity of adverse effects for KTZ, it has been shown that vomiting occurred more often with co-administration of CSA and KTZ.^{7,33} Vomiting in the present study, however, occurred more frequently during Treatment 1. Additionally, inhibition of the CYP 450 enzymes by KTZ has been shown to be dose dependent, which was supported in the present study, with blood and skin CSA concentrations obtained in Treatment 3 being higher than those in Treatment 4.¹⁸ As KTZ inhibition of CYP 450 can affect the metabolism of many other drugs and would be a lifelong therapeutic when paired with CSA for treatment of CAD, it may be important to use the lowest effective KTZ dose to limit the potential degree of interaction with other drugs.⁹ Furthermore, it should be noted that in the present study, CSA and KTZ were administered for only 7 days, and the long-term

effect of this drug combination on the whole blood and skin CSA concentrations is unknown.

The significant increases in mean skin CSA peaks on days 4 and 7 when compared with day 1, as well as significant increases in the mean skin CSA troughs on days 5 and 8 when compared with day 2, concur with previous studies indicating that CSA accumulates in skin after repeated administration.^{34,35} It appears that skin CSA concentrations may have been approaching a steady state in Treatments 1, 2 and 3 after 7 days of drug administration, because there were no significant differences in the skin CSA concentration between days 4 and 7, or between days 5 and 8. However, this does not appear to be the case in Treatment 4, where day 4 peak skin CSA concentrations were significantly less than those on day 7, and day 5 trough skin CSA concentrations were significantly less than those on day 8. Further studies on CSA skin concentration after continuous daily treatment for more than 7 days are necessary in order to establish accurately when true skin CSA steady state is achieved. The accumulation or storage of CSA in skin may be a factor explaining why every other day or even every 3 day administration of CSA can control signs of CAD, and may be associated with the time of drug administration required to observe a clinical effect for treatment of CAD. As CSA does accumulate in the skin with repeated administration and the skin is not a true compartment for drug distribution when considering pharmacokinetics, it is unlikely that skin CSA levels have a clearly defined peak or time of maximal concentration. The time of maximal concentration is likely to be variable, depending on the duration of treatment, dose, co-administration of drugs affecting CSA metabolism (such as KTZ) and individual patient factors. Four hours was chosen as an estimated time for peak skin CSA concentration in this study based on previous reports.^{21,35}

The degree of interindividual variability in whole blood CSA concentrations was comparable to those reported in previous studies.^{10,28} Interindividual variation in whole blood CSA concentrations is a well-recognized phenomenon in humans and is associated with a variety of factors, such as age, race and comorbidities.³⁶ The genetic expression of a functional polymorphism of the CYP 450 enzyme responsible for CSA metabolism as well as *ABCB1* genotypes have been the focus of recent research on the interindividual variation of CSA concentration in humans.^{37–39} *ABCB1* genotypes are important because CSA is a substrate of the P-glycoprotein system. *ABCB1* genetic polymorphisms have been identified in dogs, although not in the context of CSA administration.^{40,41} One dog (no. 38) did have very high skin CSA concentrations, particularly during Treatment 3, even though this dog's blood CSA concentrations taken at the same time point fell within the normal range. Interestingly, this is the dog that received medications in a small amount of food (owing to difficulty in administration of the oral medications), which has been shown to increase variability in CSA concentrations.²⁸ However, there is no obvious explanation for why only the skin CSA concentrations were so significantly elevated, with a normal blood CSA concentration. It is possible that certain dogs may accumulate CSA in their skin at higher than expected lev-

els, which could be correlated with the degree of clinical efficacy, the time taken to see clinical effect, the ability to reduce CSA dosing or adverse effects.

In conclusion, in the present study we have established skin CSA concentrations in normal research dogs when administered CSA at 5.0 mg/kg/day, the known therapeutic dose for treatment of CAD, and at 2.5 mg/kg/day, a known subtherapeutic dose for treatment of CAD. Additionally, skin and whole blood CSA concentrations when 2.5 mg/kg/day of CSA was administered with 2.5 or 5.0 mg/kg of KTZ were established. As there was no significant difference in mean blood or skin CSA concentrations when CSA was dosed at 5.0 mg/kg once daily (Treatment 1) when compared with CSA dosed at 2.5 mg/kg once daily with 2.5 mg/kg of KTZ once daily (Treatment 4), it is anticipated that administration of CSA and KTZ concurrently at 2.5 mg/kg/day may be as effective as CSA alone at 5.0 mg/kg/day for treatment of CAD. As the highest skin CSA concentrations were achieved with CSA dosed at 2.5 mg/kg concurrently with KTZ at 5 mg/kg, the possibility that this dosing regimen may translate into increased clinical efficacy in the treatment of CAD deserves further investigation. However, this dosing regimen may result in higher levels of CSA than needed for control of CAD, and caution is warranted because this combination of CSA and KTZ was also associated with significantly higher whole blood CSA concentrations, which may result in increased risk of adverse effects.

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Résumé

Contexte – La cyclosporine (CSA) est validée pour le traitement de la dermatite atopique canine. La cyclosporine est métabolisée par les enzymes cytochrome P450 hépatiques, processus inhibé par le kétoconazole (KTZ).

Hypothèses/Objectifs – Les objectifs de cette étude étaient de déterminer les concentrations cutanées et sanguines de CSA en cas d'administration de CSA seule à 5.0 (Traitement 1) ou 2.5 mg/kg (Traitement 2) et lorsque la CSA était administrée à 2.5 mg/kg et associée avec le KTZ à 5 (Traitement 3) ou 2.5 mg/kg (Traitement 4). Nous avons supposé que les concentrations cutanées et sanguines de CSA dans le Traitement 1 ne seraient pas modifiées de celles obtenues avec T3 ou T4.

Animaux – Six chiens sains de laboratoire ont reçu chaque traitement (Traitements 1, 2, 3 et 4) une fois par jour pendant 7 jours dans une étude croisée randomisée.

Méthodes – Après la première, la quatrième et la septième dose de chaque traitement, le haut et le bas d'échantillons de biopsies cutanées au trocart et les échantillons de sang total ont été recueillis et analysés par spectrométrie de masse en tandem avec une chromatographie liquide à haute performance. Les données ont été analysées à l'aide d'une approche de mesures répétées avec PROC MIXED de SAS. Des comparaisons par paire ont été réalisées par la méthode des moindres carrés et ajustées par le test de Tukey–Kramer pour les comparaisons multiples.

Résultats – Les concentrations moyennes de CSA dans le Traitement 1 n'étaient pas différentes de celles des Traitements 2 et 4 mais étaient plus basses que celles du Traitement 3. Les concentrations moyennes de CSA cutanées du Traitement 1 étaient plus élevées que dans le Traitement 2 mais identiques à celles du Traitement 4 et plus basses que celles du Traitement 3.

Conclusions et importance clinique – L'administration simultanée de CSA et de KTZ à 2.5 mg/kg chacun peut être aussi efficace que la CSA seule à 5.0 mg/kg pour le traitement de la dermatite atopique canine.

Resumen

Introducción – La ciclosporina A (CSA) está aprobada para el tratamiento de la dermatitis atópica canina. La CSA es metabolizada por las enzimas del citocromo P450 del hígado, un proceso inhibido por ketoconazol (KTZ).

Objetivos / hipótesis – el propósito de este estudio fue determinar las concentraciones en la piel y la sangre de CSA cuando se administraba por sí sola a una concentración de 5.0 mg/kg (tratamiento 1) o a 2.5 mg/kg (tratamiento 2) y cuando la CSA era administrada a 2.5 mg/kg junto con KTZ a dosis de 5 (tratamiento 3) o 2.5 mg/kg (tratamiento 4). Nuestra hipótesis era que las concentraciones en la piel y la sangre de CSA en animales con el tratamiento 1 no serían diferentes de las de obtenidas con los tratamientos T3 o T4.

Animales – en un estudio seleccionado al azar y cruzado, seis perros sanos de laboratorio recibieron cada uno de los tratamientos (tratamientos 1, 2, 3 y 4) una vez al día durante 7 días.

Métodos – tras la primera, cuarta y séptima dosis de cada tratamiento, se obtuvieron biopsias de piel y sangre para calcular el pico de concentración y la concentración mínima antes de la siguiente dosis de CSA. Las muestras fueron analizadas mediante cromatografía líquida de alta resolución y espectrofotometría total en tándem. Los datos fueron analizados usando un método de medidas repetidas con PROC MIXED en SAS. Se realizaron comparaciones pareadas realizadas con el cuadrado de las mínimas medias y ajuste de Tukey–Kramer para las comparaciones múltiples.

Resultados – las concentraciones medias en sangre de CSA en animales con el tratamiento 1 no fueron diferentes de las concentraciones en los animales con tratamientos 2 o 4, pero fueron menores que en animales con el tratamiento 3. Las concentraciones medias de CSA en la piel de animales con el tratamiento 1 fueron mayores que en la de los animales con el tratamiento 2, pero no diferentes de animales en el tratamiento 4, y menos que de los animales con el tratamiento 3.

Conclusiones e importancia clínica – la administración de CSA y KTZ de forma simultánea a 2.5 mg/kg de cada fármaco puede ser tan efectiva con la administración de CSA por sí sola a dosis de 5.0 mg/kg en el tratamiento de la dermatitis atópica canina.

Zusammenfassung

Hintergrund – Ciclosporin (CSA) ist eine zugelassene Behandlung für die canine atopische Dermatitis. Ciclosporin wird vom Cytochrom P450 Enzym der Leber metabolisiert; dabei handelt es sich um einen Prozess, der durch Ketokonazol (KTZ) beeinträchtigt wird.

Hypothese / Ziele – Die Ziele dieser Studie waren eine Bestimmung der CSA Konzentrationen in der Haut und im Blut nach einer alleinigen CSA Verabreichung bei einer Dosierung von 5.0 (Behandlung 1) oder 2.5 mg/kg (Behandlung 2) sowie nach Verabreichung von CSA bei einer Dosierung von 2.5 mg/kg gleichzeitig mit Ketokonazol bei einer Dosis von 5 (Behandlung 3) oder 2.5 mg/kg (Behandlung 4). Wir stellten die Hypothese auf, dass die CSA Konzentrationen in der Haut und im Blut bei Behandlung 1 sich nicht von denen bei T3 oder T4 unterscheiden würden.

Tiere – In einer randomisierten Cross-Over Studie erhielten sechs gesunde Versuchshunde einmal täglich 7 Tage lang eine jede der Behandlungen (Behandlung 1, 2, 3 und 4).

Methoden – Nach der ersten, vierten und siebten Dosis einer jeden Behandlung wurden eine 'Peak and Trough' Hautbiopsie und eine Blutprobe entnommen und mittels Hochleistungsflüssigkeitschromatographie–Tandem-Massenspektrometrie analysiert. Die Daten wurden mittels 'repeated measures approach' mit PROC MIXED in SAS analysiert. Paarweise Vergleiche wurden mittels 'least square means' durchgeführt, mit einer Anpassung nach Tukey–Kramer für multiple Vergleiche.

Ergebnisse – Durchschnittliche CSA Konzentrationen im Blut bei Behandlung 1 unterschieden sich von jenen bei Behandlung 2 oder 4 nicht, waren aber niedriger als bei Behandlung 3. Durchschnittliche CSA Konzentrationen bei Behandlung 1 waren höher als bei Behandlung 2, unterschieden sich von jenen bei Behandlung 4 nicht und waren niedriger als jene bei Behandlung 3.

Schlussfolgerung und klinische Bedeutung – Eine gleichzeitige Verabreichung von CSA und KTZ bei einer Dosierung von jeweils 2.5 mg/kg könnte für die Behandlung der caninen atopischen Dermatitis gleich wirksam sein, wie eine Verabreichung von CSA alleine bei einer Dosis von 5 mg/kg.

要約

背景-シクロスポリン（CSA）は犬アトピー性皮膚炎の治療薬として承認されている。シクロスポリンは肝チトクロームP450酵素で代謝され、そのプロセスはケトコナゾール（KTZ）により阻害される。

仮説/目的-この研究の目的は CSA を 5.0(処置 1)あるいは 2.5mg/kg（処置 2）の用量で単独投与した時と、2.5mg/kg の CSA と 5 mg/kg（処置 3）と 2.5mg/kg(処置 4)の KTZ を同時に投与した時の、皮膚と血中の CSA 濃度を測定することである。著者らは処置 1 の皮膚と血中 CSA 濃度は処置 3 または処置 4 で得られた結果と差がないと仮説を立てた。

供与動物-ランダム化したクロスオーバー試験を用い、6 頭の健康な実験犬にそれぞれの処置（処置 1, 2, 3 と 4）を 1 日 1 度、7 日間行った。

方法-それぞれの処置の 1 回, 4 回, 7 回目の投与の後に、最大濃度とトラフ濃度のときに皮膚の生検材料と全血を採取し、高性能液体クロマトグラフィー-タンデム質量分析を用いて解析した。データを SAS の PROC MIXED による繰り返し測定方法を用いて解析した。一対比較は最小自乗平均を行い、多重比較には Tukey-Kramer を用いた。

結果-処置 1 における平均血中 CSA 濃度は処置 2, 4 の濃度とかわらなかったが、処置 3 よりも低かった。処置 1 の平均皮膚 CSA 濃度は処置 2 より高かったが、処置 4 ではかわらず、処置 3 よりも低かった。

結論と臨床的な重要性-犬アトピー性皮膚炎の治療において、CSA と KTZ のそれぞれ 2.5mg/kg を同時に投与すると、CSA の 5.0mg/kg 単独投与と同様な効果が認められる可能性がある。

摘要

背景-环孢素（CSA）被许可用于治疗犬异位性皮炎。环孢素经肝细胞色素酶P450代谢，而酮康唑（KTZ）对其过程有抑制作用。

假设/目的-这个研究旨在确定在单独给予5.0（治疗1）或2.5mg/kg（治疗2）CSA，以及在给予2.5mg/kg CSA同时给予5（治疗3）或2.5mg/kg（治疗4）KTZ时，确定皮肤和血液CSA浓度。我们推测皮肤和血液CSA浓度在治疗1中与T3或T4没有区别。

动物-在随机交叉研究中，研究六只健康犬接受每天一次的治疗（治疗1、2、3和4）持续7天。

方法-每次治疗在第一次、第四和第七次剂量后，收集最高和最低值皮肤打孔活检样本和全血样本，用高性能液相色谱串联质谱分析法分析。数据分析在SAS中使用PROC MIXED方式重复测量。执行配对比较，并使用最小平方平均值和Tukey-Kramer调节进行多重比较。

结果-治疗1组的血CSA浓度平均值与治疗2或4组没有区别，但低于治疗3组。治疗1组皮肤CSA浓度平均值大于治疗2组，与治疗4组没有区别，并低于治疗3组。

结论和临床价值-同时给予CSA和KTZ各2.5mg/kg，可能比单独使用CSA5mg/kg治疗犬异位性皮炎更有效。