Corneal concentrations and preliminary toxicological evaluation of an itraconazole/dimethyl sulphoxide ophthalmic ointment

M. A. BALL*,†
W. C. REBHUN*
L. TREPANIER †
J. GAARDER* &
W. S. SCHWARK†

*Departments of *Clinical Science and †Pharmacology, College of Veterinary Medicine, Cornell University, Ithaca, NY 14853, USA*

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The objectives of this study were to determine the concentration of itraconazole achieved in corneal tissue and aqueous humour after topical application of a 1% itraconazole ointment; to determine the effect of including dimethyl sulphoxide (DMSO) in the ointment on achievable ocular tissue itraconazole concentrations; and to assess if any gross or histopathologic ocular toxicity results from the topical application of 1% itraconazole with or without the addition of DMSO.

The experimental trial consisted of 6 horses considered to have normal eyes. Each horse had one eye treated with 0.3 mL of 1% ultra-micronized itraconazole ointment and the fellow eye with 0.3 mL of 1% itraconazole/30% DMSO ointment. The ointment was applied every 6 h for a total of 28 treatments. Both ointments were well tolerated and no gross or histopathologic abnormalities developed during the trial.

Corneal tissue and aqueous humour concentrations of itraconazole were determined using high performance liquid chromatography. Corneal tissue concentration averaged 1.1 ($\pm$ 0.4) μg/g in horses treated with the 1% ultra-micronized itraconazole ointment and 7.9 ($\pm$ 3.3) μg/g for those treated with the 1% itraconazole/30% DMSO ointment; there was a statistically significant difference between ointments ($P = 0.005$) No itraconazole could be detected in the aqueous humour in either treatment group.

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M.A. Ball, Department of Clinical Science, College of Veterinary Medicine, Cornell University, Ithaca, 14853 NY, USA.

INTRODUCTION

Itraconazole (Janssen Pharmaceutica, Beerse, Belgium: R51211) is a new, highly lipophilic third generation triazole imidazole antifungal compound. As with other azoles, itraconazole appears to affect the demethylation of lanosterol and its analogues in the biosynthesis of cell-membrane ergosterol. Itraconazole is thought to have more activity than the other azole compounds (miconazole, ketoconazole, fluconazole) against *Aspergillus* species (Remmel *et al.*, 1988; Schmitt *et al.*, 1992). Itraconazole also was demonstrated to possess a broad spectrum of antifungal activity (Van Cutsem *et al.*, 1987).

Fungal keratitis was first described in humans in 1879 (Leber, 1879) and appears to have a higher incidence in the horse compared to other species (Peiffer, 1979; Kern *et al.*, 1983, Samuelson *et al.*, 1984; Barton, 1992). Horses are the only domestic animals that have a high incidence of spontaneous fungal keratitis and therefore provide the only natural animal model for comparative ophthalmic study. Due to a paucity of commercially formulated ophthalmic antifungal medications, most topical medications used for treatment of mycotic keratitis in humans and horses are custom formulated from parenteral antifungal medication. We hoped that if itraconazole could be formulated to achieve therapeutic corneal concentrations following topical application, it might become a treatment option for fungal keratitis in horses that would be economical and improve cure rates. This also may lead to a new viable treatment modality in people.

Among the fungal pathogens, the multinucleate septate filamentous are the most important group to cause ocular disease in both horses and people. These include *Aspergillus, Fusarium,* and *Penicillium* spp., with *Aspergillus* being most common in the horse (Moore *et al.*, 1983). However, less common fungi and yeast have been identified.

Itraconazole showed superior *in vitro* testing over amphotericin B and clotrimazole in one group of Aspergillosis organisms isolated from a recalcitrant case of keratomycosis in a human (Hahn *et al.*, 1993). Systemic treatment of keratomycosis with itraconazole also has been reported in humans (Thomas *et al.*, 1988). The minimum inhibitory concentration (MIC) of itra-

conazole for *Aspergillus* spp. has been shown to be in the range of 0.09–0.36 μg/mL (Dupont & Drouhet, 1987).

We hypothesized that higher corneal tissue and aqueous concentrations might be achieved with topical treatment and could potentially be enhanced by the addition of dimethyl sulphoxide (DMSO) to the preparation. This would be more economically feasible in the horse and could provide a higher cure rate.

The objectives of this study were: to determine the concentration of itraconazole achieved in corneal tissue and aqueous humour after topical application of a 1% itraconazole ointment; to determine the effect of including DMSO in the ointment on achievable tissue itraconazole concentrations; and to assess if any gross or histopathologic ocular toxicity results from the topical application of 1% itraconazole with or without the addition of DMSO.

MATERIALS AND METHODS

Animals

Six healthy adult horses without keratomycosis were selected for this study. This study was approved by the Cornell Institutional Animal Care and use Committee (protocol # 94–119). Complete ophthalmic examination was performed by a board certified veterinary ophthalmologist (Rebhun) prior to the start of the study. Horses were excluded if any pre-existing ocular lesions would present difficulty in evaluating ocular toxicity.

Formulations

Itraconazole (Janssen Pharmaceutica, Beerse, Belgium) is extremely insoluble in aqueous media and most organic solvents. It can be dissolved in DMSO in concentrations up to 1.6% under normal conditions and it was determined that it had good stability in DMSO (Janssen Pharmaceutica, Beerse, Belgium, unpublished technical data).

The 1% itraconazole petroleum-based ointment was prepared by adding 1000 mg of ultra-micronized itraconazole to 100 mL of petrolatum that had been heated to 80 °C. The mixture was agitated for 5 min at that temperature and then allowed to cool with continued agitation until it solidified. The obtained ointment was of soft consistency and appeared to be homogeneous at room temperature.

The 1% itraconazole/30% DMSO petroleum-based ointment was prepared by dissolving 1000 mg of itraconazole in 33.3 mL of 90% DMSO heated to 80 °C under gentle agitation. The heated DMSO/itraconazole solution was then combined with 66.6 mL of petrolatum that had also been heated to 80 °C. The mixture was then cooled under constant agitation until it solidified. The obtained ointment was of soft consistency and appeared to be homogeneous at room temperature. Although a detailed stability study of the ophthalmic compounding has not been completed, one sample that had been stored at room temperature for 6 months was assayed for itraconazole and was determined to contain approximately 90% of the original quantity of drug.

As always, DMSO should be handled with caution by caregivers especially when formulated with another drug as it may enhance penetration of such substances through intact skin and increase exposure risks. (Schuh *et al.*, 1988).

Treatment

The treatments were assigned randomly as to which eye received the 1% ultra-micronized itraconazole ointment; each animal acted as its own control, with the fellow eye receiving the 1% itraconazole/30% DMSO ointment. A 0.3 mL dose of the assigned ointment was applied to the designated eye every 6 h for a total of 28 treatments. All treatments were performed by the same individual. The globe and surrounding ocular tissues were monitored for any tissue irritation or hypersensitivity reaction. The animals were observed for epiphora, chemosis, conjunctiva/scleral injection, blepharospasm, corneal edema/endothelial dysfunction, ulcer formation, uveitis, lenticular changes, or other notable abnormalities.

The last treatment was administered 6 h prior to the horse's euthanasia for an unrelated study. After euthanasia, 1 mL of aqueous humour was collected from all eyes and immediately frozen in liquid nitrogen. The eyes were removed and one half of the cornea was harvested and cut into $\approx 5 \times 5$ mm sections that were placed in reaction vials and snap-frozen in liquid nitrogen. The aqueous humour and tissue samples were stored at –20 °C until analysis was performed. The remaining ocular tissue was placed in Bouin's solution and fixed for histology. All histolopathology was performed by an ocular pathologist unaware of which eyes received which treatment.

Itraconazole assay

This technique was based on those described by Badcock & Davies (1990) with modifications. Stock solutions of itraconazole and hydroxy-itraconazole (internal standard) were prepared at 500 μM concentrations in HPLC grade methanol. These solutions have been shown to be stable at 4 °C for up to 3 months (Remmel *et al.*, 1988). Healthy corneal tissue for preparation of standard curves was harvested from horses that had been euthanized for non-ophthalmic reasons. The cornea was cut into $\approx 5 \times 5$ mm pieces, placed into plastic reaction vials, and snap-frozen in liquid nitrogen. The tissue was then stored at –20 °C until used.

Standard curves were created by placing a weighed tissue sample ($\approx$ 30 mg) into a reaction vial. To that, 100 μL of 4 M potassium hydroxide was added along with 300 μL of varied concentrations of itraconazole in methanol and 100 μL of 50 μM hydroxy-itraconazole. The final concentrations of itraconazole in the standard curve mixtures were 29 nM, 58 nM, 172 nM, 0.52 μM, 1.55 μM, 4.73 μM, and 14.2 μM. The samples were incubated at 80 °C for 15 min. After incubating, HPLC grade methanol was added to bring the total volume up to 700 μL, and the mixture was vortexed for 2 min. The mixture was then centrifuged at 2000 ***g*** for 5 min and the supernatant was harvested for HPLC analysis. A second set of reaction mixtures

was also prepared without tissue added to determine approximate percentage recovery.

The samples were analysed by high performance liquid chromatography using a Shimadzu LC10 controller, pump, auto-injector, dual wavelength UV detector, and EZChrome® integrated software (Shimadzu Scientific Instruments, Inc., Columbia, MD, U.S.A.). The mobile phase consisted of distilled water, HPLC grade acetonitrile, and HPLC grade diethylamine (30:70:0.05, v/v/v) that had been passed through a 0.2 μm filter. The samples were eluted isocratically at a constant 2 mL/min from a 250 × 4.6 mm (i.d.) C-18 Ultrasphere® reverse phase analytical column (Beckman Instruments, Inc., Fullerton, CA) with a 45 × 4.6 mm (i.d.) Ultrasphere® guard column, and detected at 261 nm with a dual wavelength UV detector. Peak heights were used to generate the standard curves and calculate itraconazole concentrations. The retention time for itraconazole eluted under the aforementioned conditions was 6.0 min.

Analysis of 7 standard calibration curves generated on different days over a 2 month period indicated a coefficient of variation of less than 10% for both within-assay and between-assay data at all concentrations evaluated (29 nM, 58 nM, 172 nM, 0.52 μM, 1.55 μM, 4.73 μM, and 14.2 μM). The mean percentage recovery was determined as the peak ratio of hydroxy-itraconazole in processed hydrolyzed corneal tissue samples compared to that in unprocessed reaction mixes without corneal tissue; based on 14 data sets, the mean percentage recovery was 98.5%. The limit of detection was determined to be ≈0.7 nmol/g of corneal tissue.

It was hypothesized that the extremely low concentration of albumin in aqueous humour would not lead to a significant degree of protein drug binding. To confirm this, samples of aqueous were dehydrated and then reconstituted in methanol and processed in the aforementioned method described for corneal tissue. Subsequent analysis and comparison of processed and non-processed aqueous humour did not indicate a difference. We therefore directly injected the aqueous without undergoing extraction procedures.

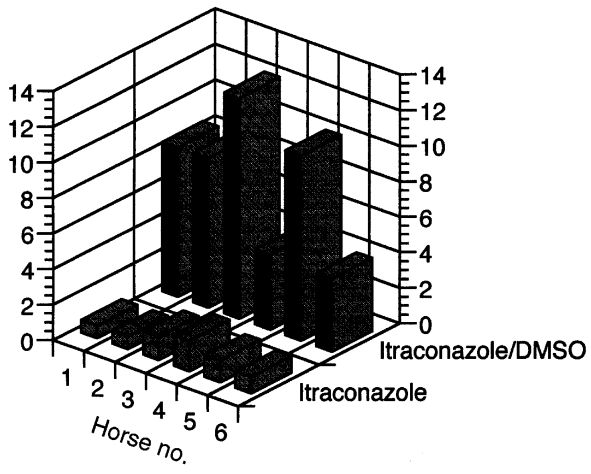

Fig. 1. Concentrations of itraconazole in corneal tissue in micrograms/gram of tissue.

RESULTS

Mean corneal tissue concentrations were significantly different between the two treatments. Mean corneal tissue concentrations were 1.1 ± 0.4 μg/g for those treated with the 1% ultra-micronized itraconazole ointment and was $7.9 \pm 3.$ μg/g in horses treated with the 1% itraconazole/30% DMSO ointment (Fig. 1). A paired Student's *t*-test (two tailed) was used to evaluate the difference, $P = 0.005$. No itraconazole could be detected in the aqueous humour in either treatment group.

During the treatment period, no gross ocular abnormalities were detected. In both the itraconazole and the itraconazole/DMSO treated eyes, there was mild blepharospasm and tearing that lasted ≈ 90 s following application of the drug. The ointments were easy to apply and appeared to have good contact time. At 60 min post-application, there was still a detectable film of either ointment on the corneal surface. Subsequent histologic evaluation of the conjunctiva, cornea, uveal tract, lens, and retina was normal for all eyes in either treatment group.

DISCUSSION

There are a limited number of topical anti-fungal drugs available with natamycin being the only one commercially formulated and approved for this use in people. Natamycin (Bistner & Riis, 1979; Kern *et al.*, 1983; Johns & O'Day, 1988; Barton, 1992); amphotericin B (Barton, 1992; Johns & O'Day, 1988; Peiffer, 1979; Bistner & Riis, 1979); ketoconazole (Torres *et al.*, 1985; Johns & O'Day, 1988; Barton, 1992); miconazole (Foster & Stefanyszyn, 1979; Johns & O'Day, 1988; Barton, 1992) and silver sulfadiazine (Mohan *et al.*, 1988) have been used extensively and evaluated for treatment of fungal keratitis in both humans and horses. Most are only thought to be fungistatic in the deeper layers of the cornea due to poor corneal penetration and many are quite expensive and severely irritating to the eye when used for extended periods of time. There has therefore been keen veterinary interest regarding other treatment options that might prove more economical and efficacious.

Although the 1% ultra-micronized itraconazole ointment had corneal concentrations above the reported MIC (0.09–0.36 μg/mL) for most *Aspergillus* spp., the 1% itraconazole/30% DMSO ointment had levels that were 7.2 fold greater. The higher concentrations achieved with the addition of DMSO could greatly increase efficacy as some *Aspergillus* and *Fusarium* spp. have been shown to have an MIC as great as 6.3 μ g/mL (Ganer *et al.*, 1987).

In many clinical patients affected with fungal keratitis there is substantial ulcerative loss of corneal epithelium. It can be assumed that the concentrations of itraconazole and DMSO would be greater in the corneal stroma given the loss of corneal epithelium as a barrier to drug penetration. This has the potential to increase clinical efficacy and was not observed to have negative effects in 14 clinical cases (Ball *et al.*, 1996

Also, because DMSO has been shown to have fungicidal properties at concentration of 30% or greater (Basch & Gadebusch, 1968), it is possible that there could be a therapeutic

synergism when using itraconazole in combination with DMSO. In addition, there may be a therapeutic benefit from the anti-inflammatory free radical scavenging effects of DMSO.

Lenticular changes have been documented in dogs receiving daily oral doses of DMSO after 13 weeks of treatment (2.5 g/kg) and as early as 9 weeks after treatment (5–40 g/kg) (Rubin & Mattis, 1966; Rubin & Barnett, 1967). Dimethyl sulphoxide has also been reported to induce a refractile lenticular change in rabbits, dogs, and swine after an oral or dermal dose of a least 900 mg/kg/day for a minimum of 68 days (Jacob & Wood, 1971).

The direct topical ophthalmic application of 100 mg/kg/day of DMSO for prolonged periods of time (up to 6 months) in the eyes of rabbits produced no abnormalities in ophthalmoscopy, biomicroscopy, retinoscopy, or intraocular pressure (Wood *et al*., 1967). These rabbits had an average cumulative dose of 18 g of DMSO applied to the eye over the first 60 days of treatment. During a typical 60 day treatment course using 0.3 mL of 1% itraconazole/30% DMSO QID, the horse would receive a total cumulative dose of 21.6 g of DMSO. Given the average weight of a rabbit eye of 4.75 g and that of an equine eye of 52.5 g (Nicolas, 1914) there is a great difference in the total cumulative dose per g of ocular tissue. The rabbits in Wood's study received $\approx$3.8 g DMSO/g of ocular tissue. Horses treated under the aforementioned regime would receive 0.4 g DMSO/gram of ocular tissue, an amount considerably less that that which failed to produce toxicity in rabbit eyes. In addition, a clinical investigation in people noted that direct ocular application of 7.5–66% DMSO to 157 eyes for 1–5 months did not elucidate any deleterious ocular changes attributable to DMSO (Gordon, 1967). We observed no evidence of ocular pathology in the eyes being treated topically with 30% DMSO in this study.

A 1% itraconazole/30% DMSO was shown to be well tolerated by the horse eye when applied every 6 h. In addition, we also have noted that application every 4 h is well tolerated in 9 clinical cases of mycotic keratitis (10 eyes); there was excessive irritation with every 2 h frequency (Ball and Rebhun, unpublished observations, 1996).

In conclusion, it has been demonstrated that a 1% ultramicronized itraconazole ointment administered at a dose of 0.3 mL every 6 h produces corneal tissue levels that are up to 3-fold greater than the MIC for some species of Aspergillus. It has also been demonstrated that a 1% itraconazole/30% DMSO ointment achieved significantly increased corneal tissue levels up to 7-fold greater than the ointment not containing DMSO. The resultant increase in itraconazole concentration was up to 20-fold the MIC for some species of Aspergillus and within the therapeutic range for *Fusarium* spp.

The absence of detectable itraconazole in the aqueous humour in either treatment group is not unexpected due to itraconazole's aqueous insolubility (less than 0.0001 g/100 mL) and would preclude this therapeutic approach in a case of mycotic endophthalmitis. The ointments were easy to administer and well tolerated. There was no evidence of ocular toxicity associated with either preparation. In addition, this compounding of itraconazole/DMSO has been evaluated on a total of 9 clinical patients (10 eyes) with an average treatment duration of 33 days (16–53) at a dose frequency of every 4 h without signs of toxicity or impaired healing; several of these horses have been followed for over 1 year (Ball *et al*., 1996).

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