Paper spray high-resolution accurate mass spectrometry for quantitation of voriconazole in equine tears

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Abstract

Paper spray high-resolution accurate mass spectrometry is a fast and versatile analysis method. This ambient ionization technique enables the quantitation of xenobiotics in complex biological matrices without chromatography or conventional sample extraction. The simplicity, rapidity, and affordability of the paper spray mass spectrometry (PS-MS) method make the technique especially attractive for clinical investigations where fast and affordable sample analysis is crucial. A new PS-MS method for the quantitation of voriconazole in equine tears was developed and validated. For a concentration range of 10 to 1000 ng/mL, good linearity ($R^2 > 0.99$), inter- and intra-run precision (coefficient of variation (CV) max. 11.9%), accuracy (bias of the nominal concentration ± 13.9%), and selectivity (signal areas of the double blanks represent 0.13 ± 0.05% of the lower limit of quantitation (LLOQ) signal in equine tears) were observed. The quantitation of voriconazole was based on three product ions and calculated relative to the isotope-labeled internal standard, voriconazole-d$_3$, which had a final concentration of 250 ng/mL in the standards and samples. The matrix effect of the method showed an ionization suppression by reduction of the voriconazole response to 63.6%, 70.2%, and 81.9% for 30 ng/mL, 450 ng/mL, and 900 ng/mL in equine tears compared with voriconazole in solvent (methanol:water, 50:50, v:v). The method was used to analyze 126 study samples collected for a pharmacokinetic study investigating a novel approach for treatment of fungal keratitis in horses. Therefore, the integrity of the sample dilution ($n = 6$, CV 6.90%, and bias of nominal concentration + 8.40%) and the carryover effect (increase from 0.33 ± 0.21% to 1.33 ± 0.89% of the signal of the LLOQ) was further investigated. To our knowledge, this method is the first application of PS-MS for quantitation of drug concentrations in tears from any species.

Keywords Paper spray high-resolution accurate mass spectrometry · Equine tears · Antifungal drug · Voriconazole · Quantitation

Introduction

Paper spray mass spectrometry (PS-MS) is a fast and versatile analysis method. This ambient ionization technique enables the quantitation of xenobiotics in complex biological samples without chromatography and conventional sample preparation. In a typical PS-MS workflow, a few microliters of sample (e.g., 12 μL of whole blood [1]) are spotted onto a paper substrate shaped in a triangle and dried. Afterwards, the sharp tip is placed in front of the inlet of the mass spectrometer (e.g., in 5-mm distance) [2]. To extract the analyte, solvent is added. The solvent-sample interactions provide the extraction of the analyte from the sample, but simultaneously, the paper substrate retains unwanted sample components such as proteins, which results in a real-time purification of the sample [3, 4]. The solvent, in combination with the porous structure of the paper substrate, leads to the migration of the analyte from the spotting site to the paper tip driven by capillary action [5, 6].
With application of a direct current (DC), high-voltage (e.g., 4.5 kV) electrospray ionization is initiated [2]. The underlying process of paper spray ionization shows similarities to electrospray ionization (ESI) and is also considered a soft ionization technique [5].

PS-MS typically provides detection limits in the low nanograms per milliliter range. Measurement times of approximately 1 min make this ambient ionization technique exceptionally fast, and minimal sample (< 15 μL) and solvent volumes (< 120 μL per sample) are highly economical. Different paper substrates (e.g., filter paper or print paper) are easily available and low in costs [7]. The simplicity, rapidity, and affordability of PS-MS make the technique especially attractive for clinical investigations, where fast and cheap sample analysis is crucial.

Fungal keratitis is a common, challenging, and potentially vision-threatening ophthalmic issue in horses [8–10]. Due to the exposed position of the horse eyes, ocular injuries occur frequently and can be a point of entry for fungal infection. Numerous species of fungal organisms have been implicated in equine keratomycosis, with Aspergillus spp. and Fusarium spp. most frequently identified [11, 12]. Successful management of fungal infection requires early and aggressive treatment with topical antifungals, though surgery may be necessary in select cases. Because of the poor pharmacokinetic profiles of most topical antifungals, conventional dosing regimens typically consist of frequent ocular administration (every 2–6 h depending on clinical preference) [13]. This frequency of treatment is not always feasible for horse owners nor tolerated by the equine patients. Furthermore, fungal resistance or potential toxicity of several antifungal drugs constrains the number of possible drug candidates. The triazole voriconazole (Fig. 1) is not affected by either of these limitations and is therefore one of the most promising drugs for successful treatment of fungal keratitis [14]. Voriconazole is a derivative of fluconazole [15] and has demonstrated good effectiveness against Fusarium [16] and Aspergillus species [17]. Its mode of action consists in the inhibition of the ergosterol synthesis [18].

With the frustrating nature of equine fungal keratitis, challenges of treatment, and high costs of surgical intervention, strategies to improve treatment compliance and keratomycosis clinical outcomes are desirable. A possible approach for improved treatment is the optimization of the administration and formulation of voriconazole.

Poloxamer gels are thermoreversible carriers that exist as a fluid when refrigerated and change into a solid gel at body temperature [19]. This novel method of ocular drug delivery was recently utilized in rescued sea lions to manage corneal ulceration using antibiotic-impregnated poloxamer gel with positive clinical results [20]. An in vitro investigation demonstrated the successful release of voriconazole from a thermogel for over 21 days [21]. Based on these reports, the administration and formulation of voriconazole for treatment of fungal keratitis by subconjunctival injection of concentrated voriconazole-thermogel were investigated in healthy horse eyes for possible clinical applications. While there is pharmacokinetic (PK) data of voriconazole in several body fluids from horses available, for example plasma, urine, or aqueous humor [22, 23], only very little knowledge of the PK of voriconazole in tears exists [14, 21]. A typically used analytical technique for the quantitation of voriconazole in biofluids is the high-performance liquid chromatography (HPLC) equipped with a UV/Vis detector or coupled with mass spectrometry [24–26]. The observed retention times vary between 4 and 10 min. Independent from the detection technique applied, all methodologies require extensive sample preparation like solid-phase or liquid-liquid extraction. The additional working steps with the considerably longer analysis times make the procedures more time consuming and more complex. In this work, a simple quantitative PS-MS method was developed and validated to measure the release of voriconazole in equine tears without prior extraction steps.

**Experimental section**

**Materials**

All solvents used were purchased from Fisher Chemical (NJ, USA) at highest purity (LC-MS grade). To prepare calibration standards and quality control (QC) samples, two different lot

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*Fig. 1* Structural formula of voriconazole

Molecular formula: C₁₆H₁₄F₃N₉O
numbers of voriconazole Vetranal™ analytical standard were acquired from Sigma-Aldrich (MO, USA) and two independent sources of the internal standard, voriconazole-d₃, were obtained from Cerilliant Sigma-Aldrich (TX, USA). The Velox Sample Cartridges were provided from Prosolia, Inc. (IN, USA).

**Instrumentation**

The paper spray system Velox 360 (Prosolia, Inc.) coupled with a Q Exactive™ Focus Hybrid Quadrupole-Orbitrap™ mass spectrometer (Thermo Fisher Scientific, CA, USA) was used. The Velox 360 system is an ionization source, enabling the automated analysis of dried biofluids by PS-MS. The system uses commercially available, disposable cartridges consisting of a plastic body holding the paper substrate, a solvent chamber allowing the wetting of the paper, and a metal ball providing electrical contact for generation of the ESI directly from the paper. The autosampler magazine of the device holds up to 40 Velox Sample Cartridges.

**PS-MS conditions**

To control the hardware functions of the Velox 360, the Velox Control Software (Prosolia, Inc.) was used. The extraction of the analyte from the cartridge was performed using acetonitrile:water:acetic acid (90:10:0.1, v:v:v) as extraction solvent. In order to precipitate organic compounds (e.g., proteins) and dissolve the analyte, a total volume of 12 μL of the solvent was dispensed directly on the dried sample in four portions with 3 μL each. Additionally, 10 times 10 μL were applied in the solvent chamber located in the back of the cartridge to wet the rest of the paper substrate. Every solvent dispense was separated by 2.2 s. The total measurement time was 1 min with an applied ESI potential of 5 kV (from 0.1–0.8 min). Parameters typically used for the heated electrospray ionization (HESI) source like the sheath, sweep, and auxiliary gas flow rate were set to 0. The ion transfer capillary temperature was 250 °C and the S-lens RF level 60. The MS data was collected in positive ion parallel reaction monitoring (PRM) mode (collision energy of 15 eV) with selection of voriconazole ([M+H]⁺ 350.12232 m/z) and voriconazole-d₃ ([M+H]⁺ 353.14115 m/z) as precursor ions. The mass resolution was set to 35,000 with a precursor ion isolation window of ±0.5 m/z and a scan range of 50–375 m/z for voriconazole and 50–380 m/z for voriconazole-d₃.

**Methods**

**Preparation of calibration standards and QC samples**

The stock solutions of the reference substance, voriconazole, were prepared in methanol (100%) with a concentration of 1 mg/mL. The internal standard voriconazole-d₃ was purchased as premade solution (1 mg/mL in methanol 100%). For the preparation of working dilutions and spiking solutions, a serial dilution of the stock solutions was performed in methanol:water (50:50, v:v). The stock solutions, working dilutions, and spiking solutions were stored at -20 °C.

For preparation of the calibration standards and QC samples and to dilute the study samples, blank equine tears were collected from seven healthy, untreated horses at the Iowa State University’s Lloyd Veterinary Medical Center using polyvinyl acetal ophthalmic sponges [27]. Following the procedure described in [27], the sponges were centrifuged (5974×g, 10 min) to remove the tears from the ophthalmic sponges. The tears were stored in Eppendorf tubes at -80 °C, separated by the donating horses. To simplify the handling of the matrix, the blank equine tears from the single horses were additionally centrifuged (5974×g, 10 min) to remove the gross debris (e.g., mucus). The supernatants were pooled and vortex mixed (5 min). The combined tears were separated into aliquots (1 mL) and stored in the freezer (-80 °C). The calibration standards and QC samples used were prepared by spiking the reference compound voriconazole and the stable isotope-labeled analog voriconazole-d₃ into the prepared blank equine tears or solvent.

**Precision and accuracy**

For method validation, three independent sets of calibration standards and QC samples were prepared. The sets covered a concentration range from 10 ng/mL (lower limit of quantitation, LLOQ) to 1000 ng/mL (upper limit of quantitation, ULOQ) of voriconazole including eight different concentrations (10 ng/mL, 25 ng/mL, 50 ng/mL, 100 ng/mL, 250 ng/mL, 375 ng/mL, 500 ng/mL, and 1000 ng/mL) for the calibration standards and four different concentrations (10 ng/mL, 30 ng/mL, 450 ng/mL, and 900 ng/mL) for the QC samples. All standards contained a constant voriconazole-d₃ concentration of 250 ng/mL.

The sets were analyzed on three different days. Every analytical run included two replicates of each calibration standard and six replicates of each QC sample. Two independent stock solutions of voriconazole and voriconazole-d₃ were used for the preparation of the calibration sets.

**Selectivity testing**

To assess the selectivity of the analytical method, the signal area of voriconazole in blank equine tears was compared with the signal area at 10 ng/mL voriconazole (LLOQ). The interference in tears from seven different horses (H1–H7) was investigated. For the preparation of the samples, a working dilution of voriconazole in methanol:water (50:50, v:v) with a concentration of 200 ng/mL was prepared and spiked into
the unpooled equine tears from H1 to H7 until a concentration of 10 ng/mL was reached. The unpooled tears from H1–H7 were used as double blank. Both the samples at the LLOQ and the double blanks were analyzed with three replicates each by PS-MS.

Matrix effect

The matrix effect was determined at three concentrations (30 ng/mL, 450 ng/mL, and 900 ng/mL) by comparison of the signal areas of QC samples in solvent and in equine tears. The PS-MS analysis was performed with three replicates of each concentration.

Carryover effect

For further confirmation of the analytical performance of the method, the carryover effect was investigated. Pooled, double blank equine tears before and after the ULOQ (1000 ng/mL, n = 12) were analyzed and the signal areas for voriconazole compared.

Dilution integrity of study samples

For study samples with concentrations above the ULOQ, the integrity of the dilution of these samples needed to be proven. To do so, a sample with a voriconazole concentration of 5000 ng/mL was prepared in equine tears to simulate the samples above the ULOQ. This sample was diluted (1:10) with blank equine tears and spiked with the stable isotope-labeled standard voriconazole-d₃ to obtain six dilutions. To reach a concentration of 250 ng/mL of voriconazole-d₃ and a concentration of 500 ng/mL of voriconazole within the six dilutions, 2 μL of a spiking solution with 25 μg/mL of voriconazole-d₃ was added to 178 μL of blank pooled equine tears and 20 μL from the simulated sample above the ULOQ for every dilution integrity sample.

Analysis of study samples

The validated analytical method was used for quantitation of voriconazole in 126 tear samples collected from six healthy horses as part of an in vivo proof-of-concept pharmacokinetic study [28]. This clinical study aimed to determine if a sustained release of voriconazole can be achieved in tear film by use of a thermosensitive poloxamer gel (Thermafixx®, Med Specialties Pharmacy, CA, USA). For the treatment of the horses, chilled Thermafixx® was mixed with 5% voriconazole solution (1:1) to result in a 2.5% voriconazole-thermogel combination and kept on ice until use. A volume of 0.3 mL of the voriconazole-thermogel combination was administered to both eyes of one horse and one randomly selected eye of five horses (n = 7 treated eyes) by dorsal bulbar subconjunctival injection. The contralateral eyes of five horses (n = 5 control eyes) received 0.3 mL of thermosensitive poloxamer gel without voriconazole. Tear samples were collected from all treated, as well as control eyes, immediately prior to the subconjunctival injections and at multiple time points following drug administration (72 h) to monitor the release rate of voriconazole in equine tears. The samples were stored at −80 °C. For the quantitation, the study samples were thawed slowly at room temperature and vortexed to provide proper homogenization. Afterwards, 38 μL of the samples was spiked with 2 μL of the internal standard voriconazole-d₃ (spiking solution 5 μg/mL in methanol:water, 50:50, v:v). In case the concentration of the sample was above the ULOQ or the sample volume was below 38 μL, the sample was diluted with blank pooled equine tears.

The analysis of the study samples was performed with the same eight levels of calibration standards used for the method validation and with three levels of QC samples (30 ng/mL, 450 ng/mL, and 800 ng/mL). All standards contained a concentration of 250 ng/mL of voriconazole-d₃.

Analytical method

Eight microliters of the calibration standards, study samples, or QC samples was applied onto Velox Sample Cartridges, dried in an Isotemp incubator (Fisher Scientific, NJ, USA) for 30 min at 37 °C, and analyzed by PS-MS. The overall workflow of the analytical procedure is summarized in Fig. 2.

Data analysis

For data processing, the QualBrowser tool from the Xcalibur 4.0 software (Thermo) and Excel (2016, Microsoft) were used.

The quantitation of voriconazole was performed by summing up the signal areas of three product ions for voriconazole ([281.08962 m/z, 224.06299 m/z, and 127.06660 m/z] and voriconazole-d₃ (284.10845 m/z, 224.06299 m/z, and 130.08543 m/z). The areas analyzed contained a narrow mass range of approximately ± 50 ppm up to ± 70 ppm around the accurate mass of each product ion. The calibration curve (area ratio of voriconazole/voriconazole-d₃ plotted over concentration of voriconazole) was obtained with a weighing factor of 1/x² at least square linear regression fit. The resulting concentrations of the QC samples, calibration standards, LLOQ, and the regression coefficient (R²) were evaluated by typical guidance criteria for method validation [29]. The accuracy of the method and the analytical runs was accepted with a bias of the nominal concentration within ± 15% (for LLOQ ± 20%). The precision was sufficient with values for the coefficient of variation (CV) within + 15% and the linearity was adequate with $R^2 > 0.96$. 

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Results and discussion

Method development

PS-MS for quantitation of voriconazole in equine tears

For investigation of the ionization of voriconazole from Velox Samples Cartridges, calibration standards and QC samples in equine tears were prepared and analyzed as described (“Methods” section). A constant concentration of 250 ng/mL of the stable isotope-labeled internal standard, voriconazole-d₃, was included.

Only limited pharmacokinetic and pharmacodynamics data for the application of voriconazole for eye treatment exists [14, 22] and no standardization for minimal inhibitory concentrations (MIC) is available for veterinary medicine [30, 31]. Furthermore, the variety of possible fungal species related to fungal keratitis made the estimation of a concentration range for effective treatment challenging. The concentration range from 10 to 1000 ng/mL covers a possible lower MIC, related for example to Candida species (MIC₉₀ 160 ng/mL) [31], and was therefore chosen as concentration range. A possibly lower concentration range was additionally interesting for the application of the PS-MS since the absence of sample preparation steps could result in limitations of the sensitivity. However, a calibration curve with 10 ng/mL, 25 ng/mL, 50 ng/mL, 100 ng/mL, 250 ng/mL, 375 ng/mL, 500 ng/mL, and 1000 ng/mL provided sufficient linearity ($R^2 > 0.99$) and good accuracy ($±5.00\%$ bias of the nominal concentration) with three QC samples (37.5 ng/mL, 300 ng/mL, and 800 ng/mL) within $±15\%$ bias of the nominal concentration. For the quantitation, three product ions were chosen (see Electronic Supplementary Material (ESM) Fig. S1). These observed results confirmed the suitability of voriconazole for direct analysis using PS-MS.

Analytical performance

Precision and accuracy

Determination of precision and accuracy was performed by calculation of the mean concentrations, bias, and CV by the use of the QC sample results for every single run and for the entire data (set MV1-3). Comparison of the data within one analytical run provided the intra-run accuracy and precision,
and a comparison of the three sets resulted in the inter-run accuracy and precision (Table 1).

For the intra-run accuracy, set MV1 showed mean concentrations within +13.9% bias of the nominal concentration, set MV2 within +13.1%, and set MV3 within +9.29%. The intra-run precision of all sets showed a maximum CV of 9.60%, which is within the acceptance limit of 15%. In the case of the inter-run analysis, the accuracy was within +8.73% bias of the nominal concentration and the precision within 11.9% CV.

Selectivity testing

The interference in blank tears from seven different horses was investigated. Both the double blanks and the samples at the LLOQ level were analyzed with three replicates by PSM-MS. The mean signal areas of voriconazole \( (n = 21) \) were calculated. The response of the double blanks was compared with that of the mean signal areas at the LLOQ, which were defined as 100%.

The interference of the blank equine tears at the LLOQ of voriconazole was between 0.13 ± 0.05%. According to bioanalytical guidance criteria [29], an interference of up to 20% would have been acceptable; therefore, an excellent selectivity of the method was provided.

Matrix effect

The evaluation of the matrix effect is essential for ambient ionization techniques since there is no purification or enrichment of the analyte prior to the MS analysis. All matrix components are included and can affect the ionization of the analyte.

The matrix effect was calculated with Eq. (1), which is based on the method of Wick [32] neglecting the signal area contributed by the unlabeled residue of voriconazole present in the isotope-labeled internal standard voriconazole-d3. This was possible because the mean signal area of this unlabeled voriconazole was < 1% and was determined by the analysis of single blanks \( (n = 3) \) containing only the stable labeled voriconazole-d3 (250 ng/mL). With the matrix effect (ME) value, the identification of ionization suppression (ME < 100) or enhancement (ME > 100) was possible [33].

\[
ME (\%) = \left( \frac{A_{\text{Spike}}}{A_{\text{SS}}} \right) \times 100
\]

\( A_{\text{Spike}} \) is the signal area of QC samples in equine tears, \( A_{\text{SS}} \) is the signal area of QC samples in solvent, and ME is the matrix effect value.

The matrix effect from equine tears of the ionization of voriconazole was determined at three different concentrations (30 ng/mL, 450 ng/mL, 900 ng/mL with 250 ng/mL of voriconazole-d3). To conserve equine tears, the QC samples from calibration set MV1 were used and analyzed with three replicates. The QC samples in solvent were prepared on the same day as the QC samples in tears. The mean signal area and ME values are calculated (Table 2).

An ionization suppression of voriconazole was observed. Only 63.6% to 81.9% of the signal detected in solvent was present in equine tears. A decrease of the ionization suppression, with an increase of the concentration, is most likely caused by processes during the ionization. A higher concentration provides a higher amount of analyte molecules, competing for available charges, but the amount of interfering matrix components is approximately consistent; therefore, the ionization may be less suppressed.

Carryover effect

The mean signal areas detected for voriconazole in blank equine tear samples located before and after the ULOQ in an analysis sequence were compared \( (n = 12 \) each). Both signal areas observed were reported relative to the mean of the

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Precision and accuracy of the quantitation of voriconazole in equine tears</th>
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<tbody>
<tr>
<td></td>
<td>10 ng/mL</td>
</tr>
<tr>
<td>MV1 ( (n = 6) )</td>
<td>Intra-run mean [ng/mL]]</td>
</tr>
<tr>
<td></td>
<td>Intra-run bias [%]</td>
</tr>
<tr>
<td></td>
<td>Intra-run CV [%]</td>
</tr>
<tr>
<td>MV2 ( (n = 6) )</td>
<td>Intra-run mean [ng/mL]]</td>
</tr>
<tr>
<td></td>
<td>Intra-run bias [%]</td>
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<tr>
<td></td>
<td>Intra-run CV [%]</td>
</tr>
<tr>
<td>MV3 ( (n = 6) )</td>
<td>Intra-run mean [ng/mL]]</td>
</tr>
<tr>
<td></td>
<td>Intra-run bias [%]</td>
</tr>
<tr>
<td></td>
<td>Intra-run CV [%]</td>
</tr>
<tr>
<td>Total ( (n = 18) )</td>
<td>Inter-run mean [ng/mL]]</td>
</tr>
<tr>
<td></td>
<td>Inter-run bias [%]</td>
</tr>
<tr>
<td></td>
<td>Inter-run CV [%]</td>
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</table>
integrated signal area ($5.10 \times 10^7 \pm 3.70 \times 10^6$ representing $100 \pm 7.25\%$) of the LLOQ (10 ng/mL, $n=6$). In this way, the acceptance was easier to judge since the guidance criteria allow interferences from the matrix of up to 20%. A slight increase in the signal area of voriconazole, from $0.30 \pm 0.21\%$ to $1.33 \pm 0.89\%$ relative to the signal area of the LLOQ, was observed in the blank sample analyzed after the ULOQ standard. This indicated a low carryover effect, which was present but negligible.

Quantitation limit and linearity

For all calibration sets analyzed, the LLOQ of 10 ng/mL showed a reliable precision and accuracy for the intra- as well as inter-run data (bias $-9.29\%$ to $+13.9\%$, CV $2.13\%$–$11.9\%$); therefore, the quantitation limit of the method was confirmed. Additionally, an excellent linearity of $R^2 > 0.99$ was observed for all calibration curves, as exemplified in Fig. 3.

Dilution integrity of study samples

The six dilutions were quantitated ($n=3$) and the mean concentration ($n=18$), CV, and bias of the nominal concentration (500 ng/mL) were calculated. The mean concentration of $542 \pm 37.4$ ng/mL gave a bias of $8.40\%$ and a CV of $6.90\%$, both within the acceptance criteria of 15% [29].

Application for quantitation of voriconazole in study samples

The study samples were analyzed in seven analytical runs containing calibration standards, double blanks of equine tears (two replicates each), and QC samples (two replicates at each level). The analytical performance for the quantitation of the samples was accepted if at least 67% of the QC samples and 75% of the calibration standards were within $\pm 15\%$ (or for the LLOQ $\pm 20\%$) bias of the nominal concentration. Additionally, the linearity was claimed to be sufficient with a correlation coefficient over $R^2 > 0.96$. Comparison of the QC samples determined within the seven analytical runs confirmed the inter-run precision and accuracy of the developed method (Table 3). The inter-run accuracy (bias of the nominal concentration) was within $\pm 7.83\%$ and the inter-run precision (CV) within $4.77\%$. Both percentages are within the required range from 15%, whereby the analytical performance of the applied method was confirmed.

Voriconazole was present in tears of treated eyes for up to 3 h following subconjunctival injection. The apparent maximum concentration in tears was observed after 10 min of dosing (~0.17 h), with a time-dependent decrease until 3 h, at which point voriconazole tear levels were close to the lower limit of quantitation of the analytical method. Therefore, drug release of this new voriconazole formulation occurred for only 3 h. Additionally, large between-horse variability in drug exposure was observed, primarily at the early time points (Fig. 4). All samples from the control eyes and those collected prior to injection showed no detectable voriconazole concentrations (below limit of quantitation).
Still, the subconjunctival injection of poloxamer gel, with or without voriconazole, was well tolerated by horses with only transient conjunctival hyperemia noted as an adverse effect. A visible gel bleb was appreciable immediately following injection but was no longer visible at examination (12 h post-injection) or globe enucleation (72 h post-injection). Despite poloxamer gel typically forming a solid at body temperature, it is presumed that the gel returned to liquid form and dispersed prematurely in both vehicle and treated eyes. In fact, voriconazole was only measureable in the tears of treated eyes for up to 3 h following subconjunctival injection, a finding that is in contrast with the prolonged drug delivery which was expected from the poloxamer gel.

The observed differences noted in lacrimal voriconazole concentrations within and between horses could be partly explained by the method used for tear collection. The used PVA sponges yield large volumes of tears for analysis but may be less reliable than other collection methods such as Schirmer tear strips because the volume of tears collected during each sampling process can vary [34, 35].

Regardless, subconjunctival administration of 2.5% voriconazole/Thermafixx® did not appear clinically advantageous for treatment of fungal keratitis in horses given the lack of prolonged voriconazole delivery in tears.

**Conclusion**

A PS-MS method for the quantitation of voriconazole in equine tears was developed and validated following typical bioanalytical guidance criteria [29]. The calibration range of voriconazole from 10 to 1000 ng/mL in equine tears showed sufficient linearity ($R^2 > 0.99$). Good selectivity, precision, and accuracy were demonstrated. Matrix and carryover effects were evaluated and found to be acceptable as well. The method was applied for the quantitation of 126 study samples. For

<table>
<thead>
<tr>
<th>Concentrations</th>
<th>Inter-run mean</th>
<th>Inter-run bias [%]</th>
<th>Inter-run CV [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>QC samples n = 14</td>
<td>30 ng/mL</td>
<td>34.3 ng/mL</td>
<td>−4.01</td>
</tr>
<tr>
<td>450 ng/mL</td>
<td>432 ng/mL</td>
<td>5.34</td>
<td>3.07</td>
</tr>
<tr>
<td>800 ng/mL</td>
<td>863 ng/mL</td>
<td>7.83</td>
<td>3.92</td>
</tr>
</tbody>
</table>

![Fig. 4](image-url)  
**Fig. 4** Box plot of voriconazole concentrations in equine tears within the first 3 h of dosing. Box, 1st quartile, median, and 3rd quartile; whiskers, ± 1.5 interquartile range; open circles, outliers
this application, dilution integrity for samples above the ULOQ was confirmed.

The quantitation of voriconazole was performed without traditional sample preparation steps (e.g., solid-phase extraction) and with analysis times of approximately 2 min per sample, which emphasizes the simplicity and rapidity of the analytical approach. These facts together with its affordability and reliability prove the value of this PS-MS method for clinical investigations.

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Compliance with ethical standards

Ethical conduct of research The use of horses and all procedures in this study were approved by the Institutional Animal Care and Use Committee at Iowa State University (protocol no. 12-16-8403-E). Horses were obtained by client donation due to severe and/or chronic lameness and following completion of our study were humanely euthanized for reasons unrelated to ophthalmic research. Horses were housed individually in stalls and provided free choice hay and water. Ophthalmic examinations were performed, and all the eyes of all horses were deemed healthy with no confounding ocular conditions.

Informed consent All equine horse owners donated their horses to the Iowa State University College of Veterinary Medicine Lloyd Veterinary Medical Center. They did this by signing a form that officially transferred ownership of the horse and provided informed consent for the horses to be used for approved teaching and research procedures, both before and after euthanasia.

Conflict of interest The authors declare that they have no conflict of interest.

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