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Determination of voriconazole in aqueous humor by liquid chromatography–electrospray ionization-mass spectrometry ☆

Lei Zhou[a], Randolph D. Glickman[b,*], Nancy Chen[b], William E. Sponsel[b], John R. Graybill[c], Kwok-Wai Lam[a,d]

[a]*Singapore Eye Research Institute, Singapore National Eye Center, Singapore 168751, Singapore*
[b]*Department of Ophthalmology, The University of Texas Health Science Center, 7703 Floyd Curl Drive, San Antonio, TX 78229-0339, USA*
[c]*Department of Medicine, The University of Texas Health Science Center, 7703 Floyd Curl Drive, San Antonio, TX 78229-0339, USA*
[d]*Wilmer Ophthalmological Institute, Johns Hopkins University and Hospital, Baltimore, MD, USA*

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Abstract

A novel method based on liquid chromatography–mass spectrometry with electrospray ionization (LC–MS) has been developed for analysis of voriconazole in aqueous humor. The separation was achieved on a reversed-phase C_{18} column eluted by 70% acetonitrile–30% water–0.01% TFA. The correlation between the concentration of voriconazole to peak area was linear ($r^2=0.9990$) between 0.04 and 60 ng, with a coefficient of variance of less than 3%. Limit of quantitation (LOQ) was estimated to be 5 ng/ml voriconazole with an injection volume of 2 μl of aqueous humor. Both intra-day and inter-day imprecision were less than 3% over the whole analytical range. Parallel analyses of voriconazole samples by LC–MS and by high-performance liquid chromatography (HPLC)–UV showed that the two methods were highly correlated ($r^2=0.9985$). LC–MS was used to the determine voriconazole levels achieved in the aqueous humor of the rabbit eye, following topical application of 5 or 10 μg voriconazole in the form of eyedrops for 11 days b.i.d. The lower dosage produced an aqueous humor concentration of 7.29±5.84 μg/ml, while the higher dosage produced a concentration of 14.56±12.90 μg/ml.

Keywords: Voriconazole; Antifungal; Aqueous humor

1. Introduction

Voriconazole, (2*R*,3*S*)-2-(2,4-difluorophenyl)-3-(5-fluoro-4-pyrimidinyl)-1-(1H-1, 2, 4-triazol-1-yl)-2-butan-2-ol (chemical structure in Fig. 1), has been used as a novel broad spectrum antifungal agent [1,2]. It is especially effective against *Aspergillus* species, but is also active against molds such as *Fusarium* [3], and yeasts such as *Candida krusei* typically resistant to fluconazole and other conventional antifungal drugs [4]. Although clinical reports of voriconazole applications are starting to appear, e.g. for treatment of candidiasis [5] and aspergillosis [6], most of the data relating to the activity of

☆The LC–MS analysis of voriconazole in rabbit aqueous humor was performed in the Singapore Eye Research Institute.

*Corresponding author. Tel.: +1-210-567-8420; fax: +1-210-567-8413.
E-mail address: glickman@uthscsa.edu (R.D. Glickman).

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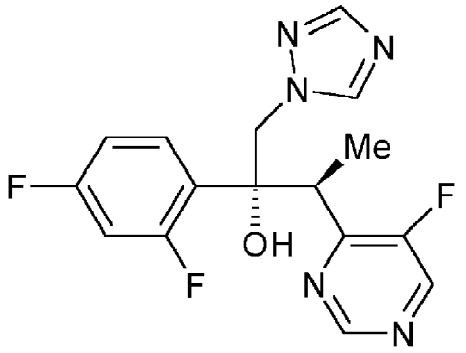

Fig. 1. Chemical structure of voriconazole molecule.

voriconzole is currently still derived from in vitro studies. In order to use this agent in the clinical setting, a better understanding of its in vivo pharmacokinetics is required, which in turn will require an analytical method with maximal sensitivity. Assay methods based on high-performance liquid chromatography with UV detection (HPLC–UV) have been developed to determine the concentration of voriconazole in human plasma [7–9]. Recently, liquid chromatography coupled to mass spectrometry (LC–MS) has emerged as a powerful tool for the analysis of drug samples in biological media because of its superior sensitivity and selectivity [10,11]. LC–MS has been successfully used to determine other antifungal agents in plasma [12].

This paper reports a novel method based on LC–MS with electrospray ionization for analysis of voriconazole in samples of aqueous humor from the eye.

2. Experimental

2.1. Chemicals

Voriconazole (UK-109,496) was obtained from Pfizer Central Research (Sandwich, UK). The purity of this agent as supplied was 99.9%. For use in the animal experiments, it was dissolved in Noble agar at a concentration of 50 or 100 μg/ml. HPLC-grade acetonitrile was purchased from Fisher Scientific (USA). Trifluoroacetic acid (TFA) was purchased from Sigma (USA). The water used in the mobile phase was of Milli-Q grade (Millipore, MA, USA).

2.2. HPLC conditions

The HPLC system consisted of a Waters 2690 solvent delivery system including an auto-sampler and photodiode array detector. The Delta PAK C_{18} analytical column, (15-μm particle size, 300×3.9 mm, supplied by Waters Associates) was eluted by an acetonitrile–water–TFA mixture (70:29.99:0.01, v/v). The flow-rate was 0.5 ml/min.

2.3. Electrospray ionization-mass spectrometry conditions

The electrospray ionization-mass spectrometry system used in this study was the Micromass Platform LCZ (UK), coupled to the HPLC system. The optimized settings in the MS detector were as follows. The nitrogen gas flow was maintained at 350 l/h. The capillary and cone voltages were set to 3.5 kV and 10 V, respectively. The source temperature and desolvation temperature were set to 140 °C and 425 °C, respectively. All mass spectra were recorded under a full scan operation for positive ions, with a scan range from m/z 50 to 600. The quantification was carried out with the selected-ion monitoring (SIM) mode set to the protonated parent molecular ion ($m/z=350$).

2.4. Calibration curve

The determination of voriconazole was based on the external standard method. This was a suitable calibration method because sample pre-processing was not required, and fixed-volume injections (2 μl) were used throughout this study. For the preparation of calibration standards, a known amount of pure voriconazole was added to blank aqueous humor to obtain voriconazole concentrations of 0.02–30 μg/ml. Using 2 μl injection volumes, six-point calibration curves (triplicate injections) were created for the range from 0.04 to 60 ng by plotting the peak area of protonated voriconazole molecular ion (m/z 350) against the amount of voriconazole injected into the column.

2.5. Aqueous humor specimens and sample preparation

To study the penetration of voriconazole into the eye, albino rabbits each weighing ca. 2-kg were treated with topical eye drops of voriconazole at a low dose (50 μg/ml) or at a high dose (100 μg/ml) twice a day for 11 days. In each session, the drug was delivered in a volume of 0.1 ml to the eye, so that in the low-dose regimen, the animal received 5 μg per treatment, and in the high-dose regimen the animal received 10 μg per treatment. Thus, the animals in the low-dose group ($n=4$) received a total cumulative dose of 0.11 mg, and the high-dose animals ($n=7$) received a total cumulative dose of 0.22 mg. Samples of blank aqueous humor were obtained from five eyes of untreated rabbits. Aqueous humor samples were obtained from the anterior chamber of the eye by entering the limbus with a 30-ga needle fitted to a 1-ml syringe, and stored in the dark at −25 °C until analysis to minimize degradation. Under these conditions, voriconazole is stable for at least several months. The samples were obtained within 75 min following the last voriconazole treatment. The aqueous humor is a transparent liquid that fills the anterior chamber between the cornea and lens. It contains very little protein. Therefore, the aqueous humor samples required no further preparation, and 2 μl aliquots were directly injected into the column for analysis.

2.6. Recovery, precision and accuracy

The recovery was determined by comparing the peak area of a blank aqueous humor sample premixed with a known amount of voriconazole with a sample containing the same concentration in pure water. These comparisons were justified because of the similar water-based matrix for both the biological samples and standard solutions. For determination of intra-assay precision, four parallel sets of aqueous humor samples spiked with voriconazole were prepared, such that every set contained one sample each of the following voriconazole concentrations (60, 200, 800 and 10 000 ng/ml). For the inter-assay precision, the above samples were analyzed on 3 subsequent days. Accuracy was measured using aqueous humor spiked with voriconazole at six different concentrations (60, 200, 500, 800, 1000 and 10 000 ng/ml), and calculated as the deviation from the theoretical values.

3. Results and discussion

The mobile phase used in this study was modified from the one described by Gage et al. [7]. In order to avoid using a non-evaporating buffer system, such as phosphate salt, in the LC–MS system, we used a mobile phase consisting of 70% acetonitrile–30% water–0.01% TFA.

With an injection of 15 ng of pure voriconazole, the typical HPLC–UV chromatogram at a detection wavelength of 255 nm is shown in Fig. 2A, while the SIM chromatogram of the same sample is shown in

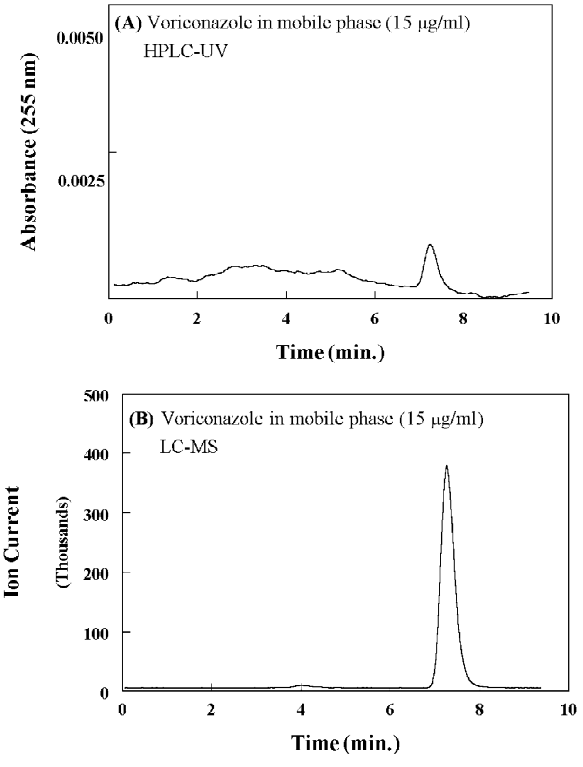

Fig. 2. (A) HPLC–UV chromatogram (detection at 255 nm) of an injection of 15 ng of voriconazole standard in acetonitrile (1 μl injection of a 15 μg/ml solution). The retention time of the voriconazole peak is 7.2 min. (B) The corresponding LC–MS SIM chromatogram ($m/z=350$).

Fig. 2B. The retention time (RT) for the voriconazole peak is 7.2 min. The corresponding mass spectra of a voriconazole standard as well as a sample in rabbit aqueous are shown in Fig. 3. The mass spectrum of a voriconazole standard is shown in Fig. 3A. The protonated ion ($[M+H]^+$, $m/z = 350$) and its acetonitrile adduct ($[M+ACN]^+$, $m/z = 391$) were observed in this mass spectrum. In addition, a minority fraction of daughter fragments, produced by loss of the triazole moiety from the parent molecule, was observed at $m/z = 281$. As stated above, rabbit aqueous could be analyzed by LC–MS without pre-processing. A total ion current chromatogram of a 2 μl aliquot of aqueous from an eye treated with voriconazole is shown in Fig. 3B. Early-eluting components of the aqueous form the largest peaks in this chromatogram, however, a voriconazole peak with the expected retention time of 7.2 min was clearly present. The mass spectrum of this peak (Fig. 3C) is similar to that of the voriconazole standard, i.e. the major peaks present are the parent peak at $m/z = 350$, the daughter peak at $m/z = 281$, and the acetonitrile adduct at $m/z = 391$. In this study, the electrospray ionization parameters were chosen to minimize fragmentation, and the quantification of voriconazole was based only on the dominant mass peak ($[M+H]^+$, $m/z = 350$).

In order to facilitate the measurement of voriconazole concentration in aqueous humor with high sensitivity, the electrospray ionization interface parameters were optimized for maximum intensity of the protonated voriconazole molecular ion ($m/z = 350$). An aliquot of 0.3 ng voriconazole was injected for each test, and the ion intensity was measured in the SIM mode. The highest ion intensity was achieved when the cone voltage was set to 10 V. When the cone voltage was increased higher than 15 V, the ion current signal decreased significantly because of increased fragmentation of the selected parent ion. The ion current intensity was enhanced with increasing desolvation temperature up to 425 °C. Further increases in desolvation temperature (from 425 to 475 °C), however, caused the depression of the ion current signal. The maximum ion intensity was achieved at 350 l/h for nebulizer nitrogen gas flow, and was not increased at a higher N_2 flow-rate.

Under optimized mass detector conditions, lineari-

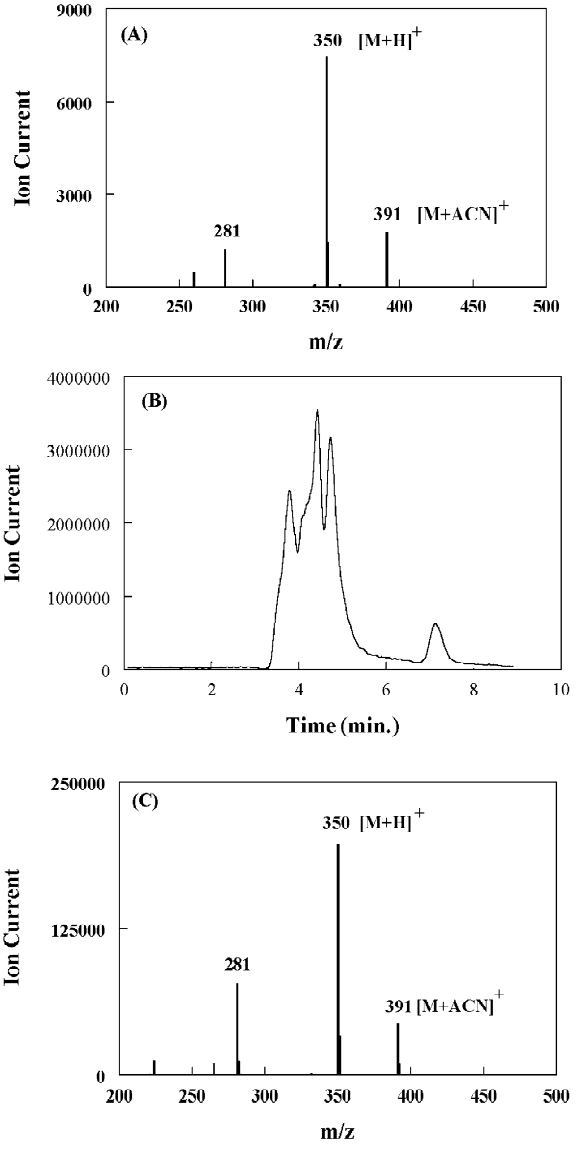

Fig. 3. (A) Mass spectrum of voriconazole standard in water. The spectrum shows the protonated voriconazole ion ($[M+H]^+$, $m/z = 350$), its acetonitrile adduct ($[M+ACN]^+$, $m/z = 391$), and a daughter fragment resulting from loss of the triazole ring at $m/z = 281$. (B) A total ion current chromatogram of an aqueous sample from a rabbit eye treated topically with voriconazole. The voriconazole peak with a retention time of 7.2 min is well separated from early-eluting, poorly resolved aqueous components. (C) Mass spectrum of the voriconazole peak with RT = 7.2 min from rabbit aqueous. The same major peaks are observed in the aqueous sample as in the voriconazole standard, i.e. at $m/z =$ 281, 350, and 391, and in comparable relative abundance.

ty was assessed over the range from 0.04 to 60 ng of injected voriconazole. A calibration curve was constructed with data obtained from injection of six different amounts of voriconzole over this range. A linear function was determined, with the following equation

$$\text{Peak area} = 64455 \times (\text{ng voriconazole injected}) + 5761.3 \quad (1)$$

The coefficient of regression of this equation was 0.9990. Detailed accuracy data obtained by analysis of a set of voriconazole standard samples spiked in blank aqueous humor are listed in Table 1. The coefficients of variance (C.V.) were less than 3% over the whole analytical range, and the deviations were less than 5%. Table 2 illustrates the efficiency of voriconazole recovery (mean plus one standard deviation (SD)) at concentrations of 50, 500, 1250 and 10 000 ng/ml. The recovery of voriconazole from aqueous humor ranged from 92.30 to 98.67% for different concentrations (Table 2). The intra-day and inter-day imprecision of voriconazole determination for samples of 60, 200, 800 and 10 000 ng/ml was less than 3% (Table 3).

Typical HPLC–UV (255 nm) chromatograms obtained for blank aqueous humor and blank aqueous humor spiked with 300 ng/ml voriconazole are shown in Fig. 4A (blank) and 4B (spiked sample). There is no detectable voriconazole peak in Fig. 4B (note: 0.6 ng of voriconazole was injected into the column). The sensitivity of HPLC with UV detection (255 nm) is not high enough to detect the voriconazole in this case. According to Gage and Stropher [7], the limit of detection of voriconazole using HPLC with UV detection at 255 nm is about 5.0 ng (in their study, they injected 100-μl of a sample containing 50 ng/ml of voriconazole) [7]. The corresponding SIM chromatograms ($m/z = 350$) obtained with 2 μl injections of blank aqueous humor (Fig. 5A) or blank aqueous humor spiked with 300 ng/ml voriconazole (Fig. 5B) clearly demonstrate a voriconazole peak in the spiked sample (Fig. 5B, RT = 7.2 min, indicated by an arrow). Another peak, seen in both Fig. 5A and 5B with RT of 4.1 min, appeared in both blank aqueous humor and aqueous humor spiked with voriconazole; therefore, it is not a voriconazole peak, but apparently is a component in aqueous humor with the same

Table 2
Recovery (%) of voriconazole ($n = 4$) from aqueous humor

Concentration (ng/ml)	Recovery (%±SD)
50	98.67±1.87
500	96.38±2.12
1250	94.19±3.34
10000	92.30±3.78

Table 3
Imprecision of the LC–MS method

Theoretical concentration (ng/ml)	Imprecision (%)	
	Intra-day	Inter-day
60	1.44	2.87
200	0.48	1.76
800	0.16	1.37
10000	0.75	2.60

Imprecision was expressed in terms of coefficient of variation (four sets of four samples each).

Table 1
Accuracy of the LC–MS method

Theoretical concentration (ng/ml)	Mean*±SD Measured concentration (ng/ml)	C.V. (%)	Accuracy (%)	Deviation (%)
60	58.48±1.38	2.36	97.47	−2.53
200	208.26±3.09	1.48	104.13	4.13
500	518.54±12.45	2.40	103.71	3.71
800	789.05±9.65	1.22	98.63	−1.37
1000	960.48±6.08	0.63	96.05	−3.95
10000	9899.50±74.03	0.75	99.00	−1.00

$n = 4$; SD, standard deviation; C.V., coefficient of variance. Accuracy was expressed as a percentage of the mean measured concentration over the theoretical concentration.

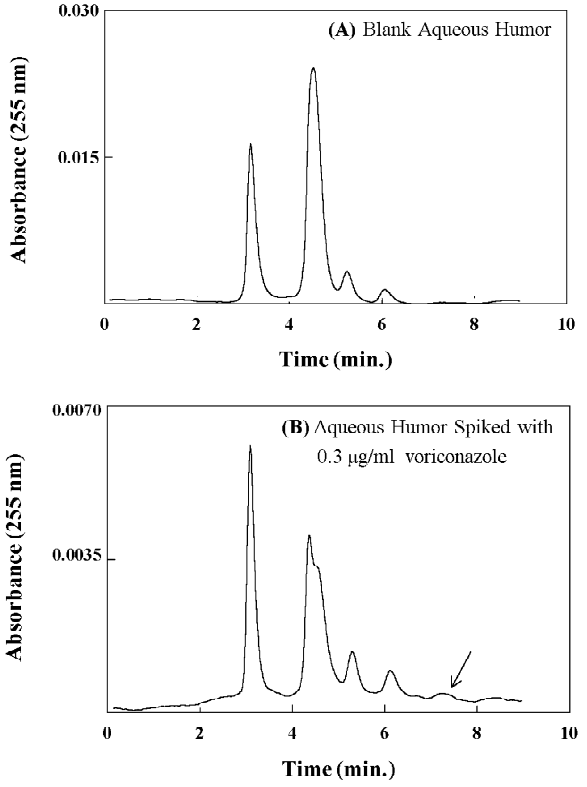

Fig. 4. HPLC–UV chromatogram (255 nm) of (A) blank aqueous humor, and (B) blank aqueous humor spiked with 0.3 μg/ml voriconazole. Arrow indicates the voriconzole peak.

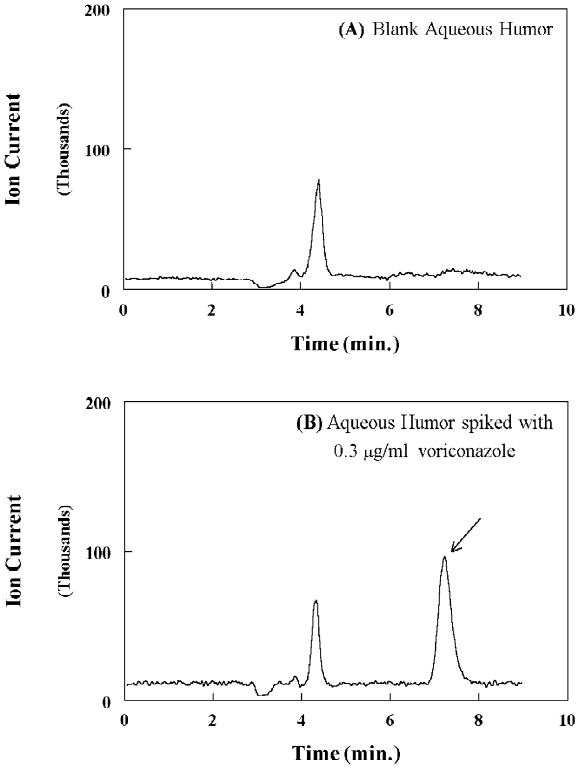

Fig. 5. SIM chromatogram ($m/z = 350$) of (A) blank aqueous humor, and (B) blank aqueous humor spiked with 0.3 μg/ml voriconazole. Arrow indicates the voriconazole peak.

ion size of $m/z = 350$. Because it separated well from the voriconazole peak (RT = 7.2), it did not interfere with the selectivity of the analysis. The limit of quantitation using the LC–MS method was 5 ng/ml using an injection volume of 2 μl, at a signal-to-noise ratio of ten based on the RMS deviation of the noise baseline. This corresponded to a column injection of 0.01 ng of voriconazole. In practice, therefore, LC–MS can be used to determine the penetration of voriconazole into aqueous humor at a concentration as low as 0.1 ng/ml, assuming an injection volume as large as 100 μl. Although this large an injection volume is probably impractical with the small analytical columns preferred for use in LC–MS, nevertheless the quantitation limit of LC–MS for voriconazole is potentially two orders of magnitude greater than that of HPLC with UV detection [7,9]. When voriconazole analyses by LC–MS and by HPLC–UV were compared, the two methods correlated very well. A regression analysis of LC–MS against HPLC–UV found the following relation

$$[\mu g/ml\ Voriconazole]_{LC-MS} = 0.9647 \times [\mu g/ml\ Voriconazole]_{HPLC-UV} - 0.0788 \quad (2)$$

The regression coefficient was 0.9985. This correlation is shown graphically in Fig. 6.

As a practical validation, LC–MS was used to determine the aqueous humor concentration of voriconazole in the rabbit eye after topical application twice daily of 5 μg or 10 μg of voriconazole for 11 days. The voriconazole concentration found in aqueous humor from animals receiving the lower dose was 7.29±5.84 μg/ml ($n = 4$), whereas in the animals receiving the higher dose, the concentration

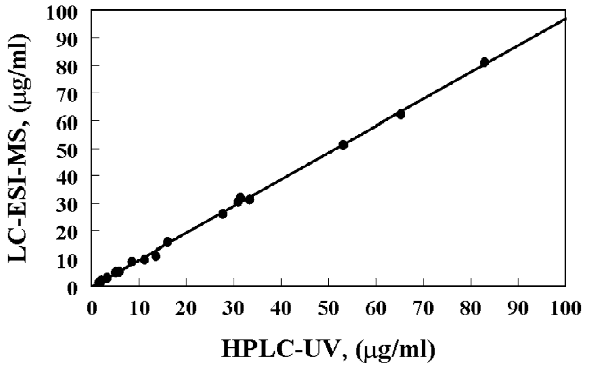

Fig. 6. Comparison of LC–MS with HPLC–UV using rabbit aqueous humor samples after topical application of voriconazole eye-drops. The solid circles indicate the experimental determination of voriconazole by HPLC–UV and LC–MS; the solid line is a plot of the regression equation of LC–MS on HPLC–UV

$$[\mu g/ml\ \text{Voriconazole}]_{LC-MS} = 0.9647 \times [\mu g/ml\ \text{Voriconazole}]_{HPLC-UV} - 0.0788).$$

was 14.56±12.90 μg/ml ($n=7$). The HPLC–UV and SIM chromatograms of the aqueous humor samples in treated eyes resembled those of the spiked aqueous samples shown in Figs. 4B and 5B. Although the mean values of voriconazole in the aqueous humor of rabbits, as determined in this study, were high enough to be measured by HPLC–UV alone, there was significant variability among individual animals, such that useable data were only obtained in some samples with the greater sensitivity of LC–MS. In addition, the LC–MS data indicate that voriconazole largely penetrates the normal eye without metabolic modification. This conclusion is supported by the similarity of the mass spectra of a voriconazole standard (see Fig. 3A) and that of an aqueous sample from a normal eye receiving topical voriconazole treatment (see Fig. 3C). In an eye with an active fungal infection, however, it is likely that the drug would be metabolized by fungal cytochrome P450 enzymes [13].

The utility of the LC–MS method for pharmacokinetic studies of voriconazole in animal or human investigations may be appreciated by comparing the sensitivity of this method to the reported minimum inhibitory concentration (MIC) of voriconazole against various fungal and related organisms. The *Aspergillus* family is considered to be especially suitable for treatment with voriconazole. For *A. fumigatus*, the MIC is approximately 0.2 μg/ml, and for other *Aspergillus* species the MIC ranges from 80 ng/ml to 0.8 μg/ml [14,15]. The MIC for *Candida* species has been reported to range from 1 ng/ml to 0.4 μg/ml [3,16]. In general, the MIC for yeasts and molds is on the order of 0.5–0.6 μg/ml [14,16]. Based on these in vitro observations, a clinically useful blood plasma concentration of 1.2–4.7 μg/ml has been recommended [9]. The effective dose for treatment of ocular infections has not yet been established but the present laboratory data, as well as emerging clinical experience, indicate that an effective therapeutic concentration of voriconazole in the eye can be achieved after topical application of the drug. While the clinically relevant *plasma* concentration of voriconazole can be monitored by HPLC–UV, *ocular* treatment levels may be below the practical sensitivity of routine UV detection, but are well within the working range of LC–MS. Therefore, LC–MS, because of its greater sensitivity, is better suited to measuring tissue penetration of the drug during pharmacokinetic investigations.

4. Conclusion

This paper describes a method based on LC–MS to determine voriconazole concentration in aqueous humor. The sensitivity and selectivity exceeded those of HPLC–UV methods, without significantly increasing the time required for the analysis. The analytical method developed in this paper is well suited to the study of voriconazole pharmacokinetics in animals and humans.

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