# Bioanalytical method development and validation of natamycin in rabbit tears and its application to ocular pharmacokinetic studies☆

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## ABSTRACT

A new selective and sensitive high-performance liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS) method was developed for the quantification of natamycin in rabbit tears using amphotericin B as internal standard (IS). Chromatographic separation was achieved on a Luna Cyano column (100 mm × 2 mm, 3 μm) using ammonium acetate buffer (pH 4; 3.5 mM): methanol (10:90, v/v) as the mobile phase. The run time was 5 min. Detection was performed by negative ion electrospray ionization in multiple reaction monitoring (MRM) mode. The calibration curve was linear over the concentration range from 25 to 800 ng/ml, and lower limit of detection of 12.5 ng/ml. The accuracy and precision of the method were within the acceptable limit of ±20% at the lower limit of quantitation and ±15% at other concentrations. Natamycin was stable during the battery of stability studies viz., benchtop, auto-sampler, freeze/thaw cycles and 30 days storage in a freezer at −70 ± 10 °C. The method was successfully applied to the ocular pharmacokinetic studies of natamycin eye drops in New Zealand rabbit tears.

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## 1. Introduction

According to the World Health Organization, fungal keratitis has a worldwide prevalence and is recognized as leading cause of ocular morbidity, second only to cataract [1–3]. Natamycin (Fig. 1a) is considered as the mainstay of treatment for fungal keratitis and is the only available approved antifungal medication for this indication by the U.S. Food and Drug Administration [4–6].

The standard therapy consists of topical administration of natamycin (5%, w/v) eye suspension and its dosage regimen in fungal keratitis is based on clinical/physician's experience. However, rational pharmacokinetic-based dosage regimen has not been reported. For treatment of fungal keratitis natamycin concentration in tear fluid following topical administration must be higher than the minimum inhibitory concentration (MIC) for long enough to achieve clinical improvement or cure, reduce resistance and prevent re-occurrence.

In order to measure tear fluid concentration and optimize dosage regimen, a highly sensitive assay techniques is required since only few microliter of tear sample (~10–20 μl) can be collected.

LC–MS/MS is often used as a sensitive and fast analytical tool and requires low sample volume. The bioanalytical methods for quantitation of natamycin using LC–MS/MS has not been reported, however in few LC–MS/MS method natamycin was used as internal standard [7,8]. Therefore, in our present work, we have developed and validated a sensitive, selective and rapid method for estimation of natamycin in rabbit tears using LC–MS/MS. The method was used for ocular pharmacokinetic studies in NZ rabbit.

## 2. Materials and methods

### 2.1. Chemicals and materials

Natamycin and amphotercin B (Internal standard; IS) of pharmaceutical grade were gifted by Cipla Ltd. (Mumbai, India). HPLC grade methanol was purchased from Sisco Research Laboratories (SRL) Pvt. Ltd. (Mumbai, India). Ammonium acetate, glacial acetic acid AR, ammonia solution (25%) and dimethyl sulphoxide (DMSO) AR were purchased from E Merck Limited (Mumbai, India). Oasis HLB 3 cc, 60 mg solid phase extraction cartridges was procured from Waters (India) Pvt. Ltd. Ultrapure water was obtained from a Milli-Q PLUS PF water purification system. Natamycin ophthalmic suspension USP (Natamet®) was purchased from local pharmacy store. The artificial tear fluid (ATF) was used as a surrogate matrix and was prepared as reported by Arnold et al. [9]. Calibrated glass capillaries (microcaps) of 10 μl were purchased from Dummond
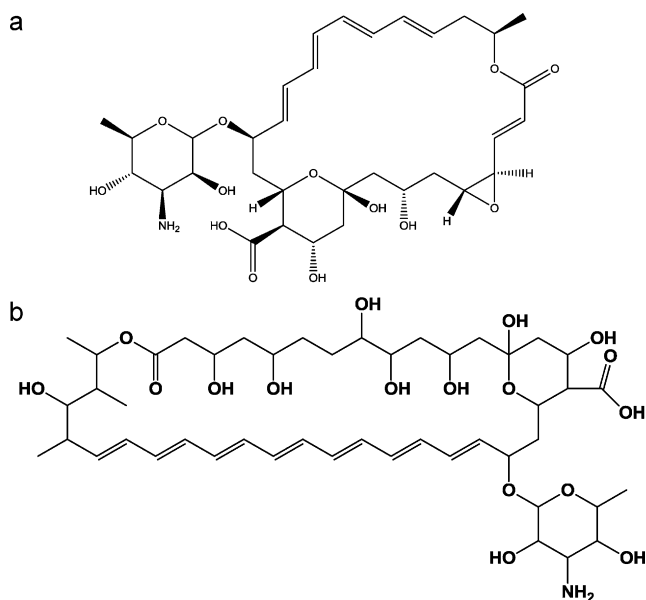
a

b

**Fig. 1.** Structure of (a) natamycin and (b) amphotericin B.

Scientific Co, (Broomall, USA). All other reagents were of analytical grade and were obtained from standard commercial suppliers. The animal studies were carried out as per the approval and guidelines of the local ethical committee on animal experimentation.

### 2.2. Chromatographic and mass spectrometric conditions

The HPLC system consisted of Series 200 pumps and auto sampler with temperature controlled Peltier-tray (Perkin–Elmer instruments, Norwalk, CT, USA). Chromatographic separation was carried on a Phenomenex, Luna 3μ CN column (3 μm, 100 mm × 2 mm) at ambient temperature. The mobile phase consisting of a mixture of ammonium acetate buffer (3.5 mM, pH 4): methanol (10:90, v/v), was delivered at a flow rate of 0.3 ml/min. The samples (15 μl) were injected through auto-sampler on to the LC–MS/MS system.

Mass spectrometric detection was performed on an API 4000 mass spectrometer (Applied Biosystems, MDS Sciex Toronto, Canada) equipped with an API electrospray ionization (ESI) source. The ion spray voltage was set at −4500 V. The instrument parameters viz., nebulizer gas, curtain gas, auxiliary gas and collision gas were set at 30, 15, 25 and 3 arbitrary units respectively. Compound parameters viz., declustering potential (DP), collision energy (CE), entrance potential (EP) and collision exit potential (CXP) were −150, −35, −10, −10 V and −110, −35, −10, −10 V for natamycin and IS, respectively. Zero air was used as source gas while nitrogen was used as both curtain and collision gas. The detection and quantification of analytes were performed using the multiple reaction monitoring (MRM) mode using ion precursor → product ion combinations of $664.5 \rightarrow 137.2\,m/z$ for natamycin and $923.5 \rightarrow 183.4\,m/z$ for the IS. Data acquisition and quantitation were performed using Analyst software version 1.4.1 (Applied Biosystems, MDS Sciex Toronto, Canada).

### 2.3. Preparation of stock and standard solutions

The stock solution (1 mg/ml) of natamycin and IS were prepared in methanol and methanol:DMSO (1:1) respectively. The stock solutions were diluted with methanol to make working standard solutions which were further diluted to prepare the calibration standards and quality control samples (QC). Calibration standards were prepared by spiking working standard solution into ATF to obtain a concentration range of 25, 50, 100, 200, 400 and 800 ng/ml. QC samples (25, 100 and 800 ng/ml) were prepared in a similar manner with appropriate working stock solution. All standards and QCs were protected from light.

### 2.4. Sample preparation

Calibration standards, QC and tear samples were extracted using solid phase extraction method. Tear sample mixture was prepared by mixing 10 μl of tear test sample or standards with 10 μl internal standard (500 ng/ml) and 80 μl of methanol, followed by 5 min vortex. The Oasis HLB (3 cc, 60 mg) solid phase extraction (SPE) cartridge was preconditioned with 2.0 ml methanol followed by 2.0 ml of 0.1% aqueous ammonia solution. Tear sample mixture and 250 μl of 0.1% aqueous ammonia solution were simultaneously loaded to the SPE cartridge. The SPE cartridge was washed with 3.0 ml of 4% (v/v) aqueous methanol. Finally natamycin was eluted from SPE cartridge with 2.0 ml methanol. The eluate was collected and dried under vacuum in speed-vac concentrator (Savant Instrument, Farmingdale, USA). The residue was reconstituted in 35 μl of methanol and 15 μl was injected into LC–MS/MS.

### 2.5. Method validation

The method was validated to meet the acceptance criteria of industrial guidance for the bioanalytical method validation [10].

The specificity was investigated by analyzing processed blank tear fluid from six individuals rabbits and in ATF. Specificity was established by the lack of interfering peaks at the retention time for the natamycin and internal standard.

Linearity was tested at six different concentrations, covering a range of 25–800 ng/ml. The calibration curves were established by plotting the peak area ratio (peak area analyte/peak area IS) versus concentration. The regression parameters of slope, intercept and correlation coefficient were calculated by fitted to the $y = mx + c$ using weighing factor $(1/x^2)$.

The lower limit of quantitation (LLOQ) of the assay was assessed as the lowest concentration on the calibration curve that can be quantitatively determined within ±20% accuracy and precision. The LLOQ was established based on six replicates on three consecutive days.

The absolute extraction recoveries of natamycin were determined by comparing the mean peak area of extracted samples spiked with the mean peak area of analytical standards at corresponding concentration. These experiments were performed in five replicate at three concentration levels (low, medium and high QC) for natamycin, where as recovery of IS was determined at single concentration of 250 ng/ml.

The matrix effect was evaluated by comparing the corresponding peak areas of the post extraction spiked samples to those of the standard solutions evaporated directly and reconstituted in mobile phase. Matrix effect was evaluated in tear fluid and ATF. Matrix effect was determined at same concentrations of analyte and IS as in recovery experiment. If the ratio of peak area of post extracted spiked to standard solution is less than 85% or more than 115%, the matrix effect is implied [11].

The accuracy (% bias) and precision (% relative standard deviation; % RSD) of this analytical method were determined using QC samples in five replicates of 25, 100 and 800 ng/ml of natamycin in ATF. The accuracy of each sample preparation was determined by injection of calibration samples and three QC samples in five replicates for five days.

The criteria for acceptability of the data included accuracy and precision within ±15% from nominal values, except for LLOQ, where it should not exceed ±20% of accuracy and as well as precision. The
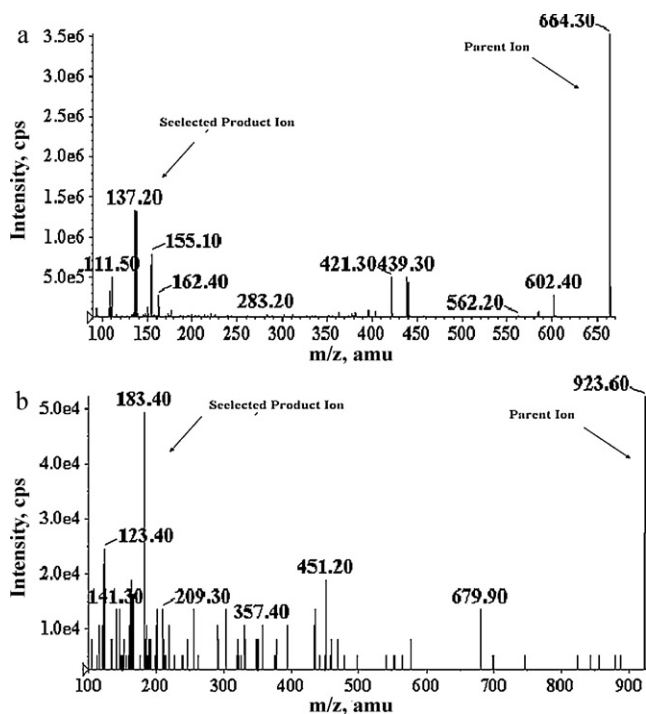
**Fig. 2.** MS/MS spectra of (a) natamycin and (b) amphotericin B (IS) in showing prominent precursor to product ion transitions.

accuracy was expressed as % bias:

$$\% \ \text{bias} = \frac{(\text{observed conc.} - \text{nominal conc.}) \times 100}{\text{nominal conc.}}$$

All stability studies were carried out at 25 ng/ml and 800 ng/ml. Stability of natamycin and amphotericin B in ATF was evaluated at 4 °C for 48 h. Other stability studies (bench-top, freeze–thaw and long-term stability) were carried out in pooled rabbit tear fluid in three replicates due to low availability of bio-matrix. The bench-top stability was evaluated at ambient temperature for 24 h. The long-term storage stability was carried out at −70 ± 10 °C for 30 days. The freeze–thaw stability was determined after three freeze–thaw cycles (room temperature to −70 ± 10 °C).

### 2.6. Dilution integrity

Dilution of biological matrix is required if some study sample concentrations are expected to be higher than the upper limit of quantitation. Dilution integrity experiments were carried out at six replicates by 250-fold dilution of sample to 100 ng/ml with blank ATF. The back calculated accuracy and precision are acceptable if it is within the limits described in Section 2.5.

### 2.7. Application of the method

The LC–MS/MS method was successfully applied in the pharmacokinetic studies of natamycin ophthalmic suspension USP (Natamet®) in three NZ rabbits. The *in vivo* pre-corneal drainage of suspension was determined after an instillation of 20 μl suspension onto the left cornea. After instillation, the eyelids were kept closed for 5 s to prevent the loss of the instilled solution. Food and water intake were free during the study. A small 10 μl calibrated glass capillary (Dummond Scientific Co, USA) was placed near the eye of the rabbit for tear collection without touching and irritating cornea. Tear samples (10 μl) were collected immediately before dosing and at 15, 30, 45, 60, 90, 120, 150, 180, 210 and 240 min post dose. They were stored in micro-centrifuge tubes at −20 °C until
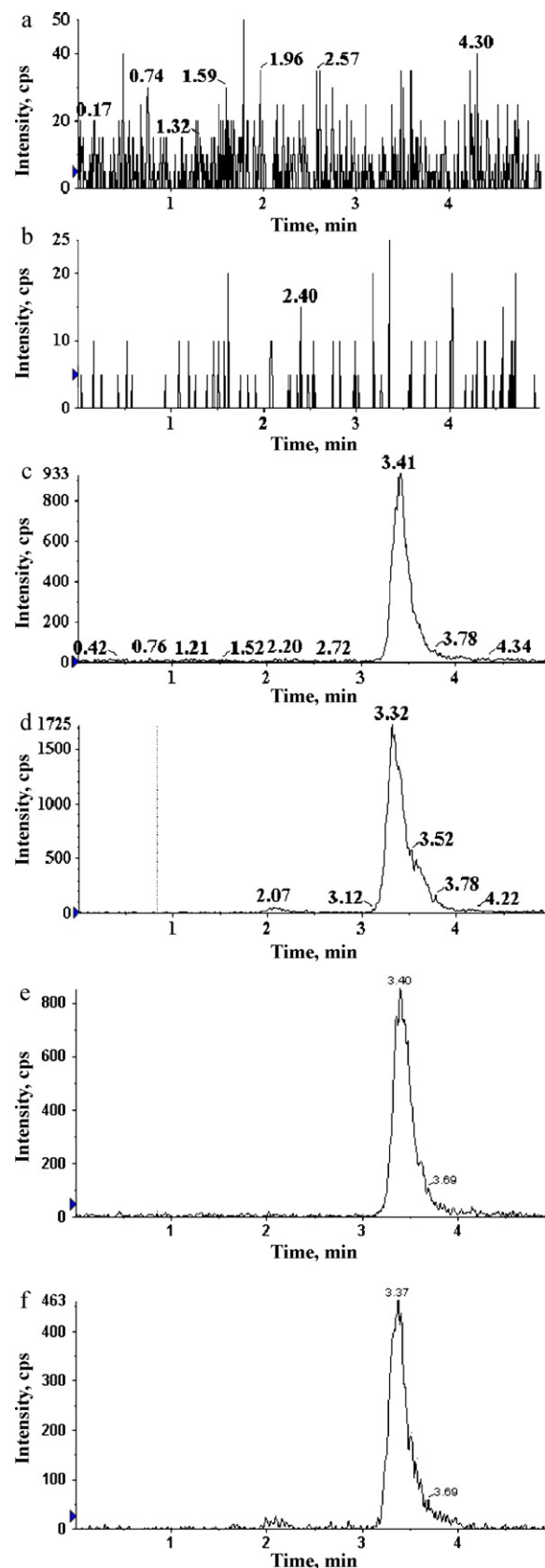


**Fig. 3.** Typical MRM chromatograms of (a) blank tear fluid at (MRM) for 664.5/137 natamycin, (b) blank tear fluid at (MRM) 923.5/183.4 for IS, (c) natamycin spiked in tear fluid, (d) IS spiked in tear fluid. (e) Pharmacokinetic sample at 30 min of natamycin and (f) pharmacokinetic sample at 30 min of IS.

**Table 1**
Intra-day and inter-day assay precision and accuracy for natamycin ($n = 5$).

| Concentration (ng/ml) | Accuracy (% bias) | | Precision (% RSD) | |
|---|---|---|---|---|
| | Intra-day | Inter-day | Intra-day | Inter-day |
| 25 | −2.94 | −3.04 | 3.19 | 6.69 |
| 100 | −0.82 | −1.46 | 2.93 | 3.71 |
| 800 | −0.24 | 3.05 | 3.68 | 5.78 |

analysis. Along with the tear samples, QC samples were distributed among calibrators and unknown samples in the analytical run. Ocular pharmacokinetic parameters of natamycin in tear was derived from tear concentration-time profile of natamycin using WinNonlin software Ver 5.1 (Pharsight Corporation, Mountain Veiw, CA).

# 3. Results and discussion

## 3.1. Method development

Liquid chromatography method development began with the optimizing mobile phase composition and column type. The feasibility of several mixtures of solvent such as acetonitrile and methanol using different buffers such as ammonium acetate, ammonium formate, acetic acid and formic acid with variable pH range 3–6 was tested for complete chromatographic resolution of natamycin and IS from interfering tear matrix. The versatility, suitability and robustness of the method were checked with several C18 and Cyano (CN) columns. The Phenomenex, Luna Cyano column ($100 \times 2$ mm, 3 μm) provided very good selectivity, sensitivity and peak shape for natamycin and IS (Fig. 3) as compared to C-18 column. The mobile phase consisting of methanol: 3.5 mM ammonium acetate solution pH 4 (90:10, v/v) with flow rate of 0.3 ml/min was found to be suitable during LC optimization. Then total run time was 5 min. Under the employed chromatographic conditions, the retention time for natamycin and IS were 3.4 and 3.3 min, respectively.

Natamycin and amphotericin B exhibits fragmentation in both negative and positive mode. However negative mode exhibits low noise and good sensitivity as compared to positive mode. During direct infusion experiment in negative mode, the mass spectra for natamycin and IS revealed molecular ion peaks at $m/z$ 664.5 and 923.5, respectively as molecular ions $[M-H]^-$. The product ion mass spectrum for natamycin shows the formation of characteristic product ions at $m/z$ 111.3, 137.2, 155.1, 421.2 and 439.4 (Fig. 2). The selected MRM transition for natamycin and IS was 664.5/137.2 and 923.5.5/183.4 respectively. Nebulizer and evaporator gases were optimized with flow injection analysis (FIA) mode. Amphotericin was selected as internal standard due to its structural similarities with natamycin, elutes at same retention time and reproducibly extracted by a common extraction procedure.

## 3.2. Validation procedures

### 3.2.1. Specificity and selectivity

Chromatograms of five batches of control drug-free tear fluid and ATF contained no co-eluting peaks of analyte and IS at their respective retention time. Representative chromatograms of blank tear, blank tear fortified with natamycin and IS are shown in Fig. 3. The retention times of all the analyte and IS showed less variability with a % RSD well within the acceptable limit of ±5%.

### 3.2.2. Calibration curve

The peak area ratio of analyte to IS was linear over a concentration range of 25–800 ng/ml for natamycin. The average regression ($n = 5$) was found to be $\geq 0.998 \pm 0.0019$. The lowest concentration with RSD < ±20% was taken as LLOQ and was found to be 25 ng/ml.

### 3.2.3. Matrix effect

The adverse consequences of matrix effects on the results of quantitative LC–MS/MS analyses have been fully recognized and its assessment is an integral part of method development and validation [12]. The matrix effect for natamycin at 25, 100 and 800 ng/ml concentration levels in tear fluid and ATF was less than ±15%. Thus no significant matrix effect was observed.

### 3.2.4. Recovery

The extraction recoveries of natamycin were determined at low (25 ng/ml), medium (100 ng/ml) and high (800 ng/ml) concentrations by comparing the responses from tear samples spiked before extraction with those of the corresponding standard solutions without extraction. The absolute mean recovery of natamycin was 75.1% and the absolute mean recovery of the IS was 57.51%.

### 3.2.5. Accuracy and precision

Accuracy and precision data for intra- and inter-day tear samples are presented in Table 1. The assay values on both the occasions (intra- and inter-day) were found to be within the accepted variable limits.

### 3.2.6. Stability

The predicted concentrations for natamycin at 25 and 800 ng/ml samples deviated within the nominal concentrations in a battery of stability tests, viz., bench top (6 h), repeated three freeze/thaw cycles and at $-70 \pm 10\,°C$ for at least for 30 days (Table 2). The results were found to be within the assay variability limits during the entire process.

## 3.3. Dilution study

The % accuracy and precision (% RSD) observed for the mean of back-calculated concentrations for diluted sample were 94–101.3% and 2.5–3.9% respectively. The results suggested that samples with concentration above upper limit of calibration curve could be reanalyzed by appropriate dilution.

**Table 2**
Stability of natamycin in artificial tear fluid (ATF) and pooled tear fluid ($n = 3$).

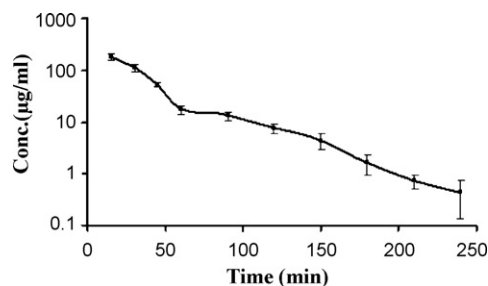| Nominal conc. | Stability | Mean | S.D. | Precision (% RSD) | Accuracy (% bias) |
|---|---|---|---|---|---|
| 25 ng/ml | Stability in ATF (4 °C, 48 h) | 25.20 | 0.650 | 2.57 | 0.80 |
| | Bench-top stability (6 h) | 24.30 | 0.90 | 3.71 | −2.80 |
| | Freeze–thaw stability | 24.50 | 0.80 | 3.56 | −2.00 |
| | Long-term stability (30 days, −70 °C) | 22.40 | 2.44 | 10.91 | −10.40 |
| 800 ng/ml | Stability in ATF (4 °C, 48 h) | 814.50 | 20.75 | 2.55 | 1.81 |
| | Bench-top stability | 787.00 | 15.79 | 2.01 | −1.63 |
| | Freeze–thaw stability | 797.83 | 10.70 | 1.34 | −0.27 |
| | Long-term stability | 786.33 | 63.04 | 8.02 | −1.71 |

**Fig. 4.** Tear fluid concentration–time profile of natamycin in rabbit tear fluid following topical administration of natamycin eye drop (5%, w/v).

### 3.4. Application of the method

The rabbit tear samples were analyzed by the newly developed and validated method along with QC samples. Initial time point samples usually have higher concentration than calibration range due to rapid removal of excess volume (eye drop) and high ocular clearance. These samples are reanalyzed after dilution along with the diluted QCs. The method was also sensitive enough to quantitate low concentration for calculation of appropriate terminal elimination rate constant and other pharmacokinetic parameters.

Time-concentration curve was smooth enough and well fitted to first order one compartment pharmacokinetic model (Fig. 4). Tear fluid concentration above the LOQ was observed for 240 min. Maximum natamycin concentration in tears was $C_{max}$ 360.24 ± 71.6 µg/ml while the AUC$_{(0-\infty)}$ was 8601.92 ± 379.29 min µg/ml. The half-life ($t_{1/2}$) of natamycin was 16.94 ± 3.08 min. The sensitivity and specificity of the assay were found to be sufficient for accurately characterizing the ocular pharmacokinetics of natamycin in rabbits. The pharmacological indices such as $C_{max}$/MIC and time above MIC were 182.67 and 2.6 h. Thus $C_{max}$/MIC were greater than 10, indicates optimal anti-fungal activity of the single instillation (Natmet®) for 2.6 h (time above MIC). Further the pharmacokinetic parameters will be evaluated to predict effectiveness of optimal dose and dosing frequency in disease condition.

### 4. Conclusion

The current validated LC–MS/MS method for natamycin offers significant advantages in terms of sensitivity and selectivity, sample preparation, short run time (5 min) and lower volume of sample requirements (∼10 µl). From the results of all the validation parameters and applicability of the assay, we can conclude that the present method can be useful for ocular pharmacokinetic studies of natamycin with desired precision and accuracy along with high-throughput.

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