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A Novel Validated Method for the Determination of Cyclosporine in Rabbit Ocular Tissues by LC-MS/MS

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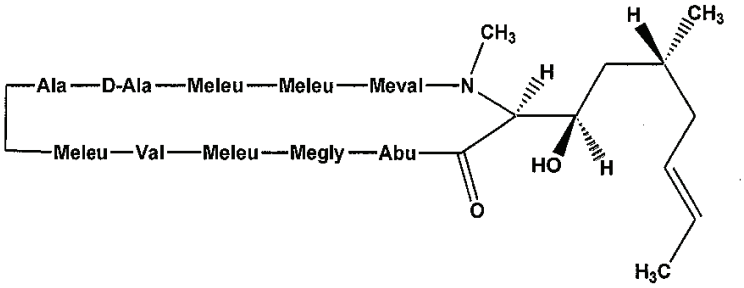
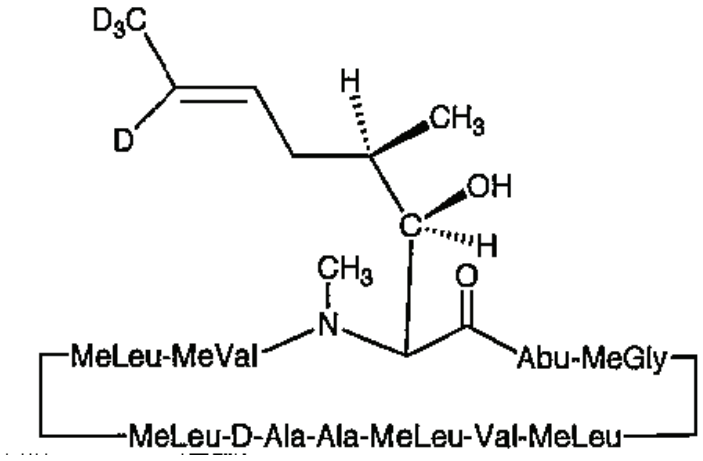
Introduction

Ocular tissues commonly selected for analysis during pre-clinical pharmacokinetic studies for ophthalmic drugs range from very small, soft perfused tissues (e.g., ICB or retina/choroid) to much larger, tougher collagenous tissues (e.g., sclera and eyelid). It is essential that the quality of the homogenate and subsequent extraction be designed properly to ensure optimal extraction of the drug from the tissues. Key to achieving this goal is the use of appropriate equipment such as a powerful bead homogenizer that efficiently and effectively prepares tissue homogenates in a high throughput manner. The methodology discussed here using the powerful OMNI Bead Ruptor Elite allowed for the preparation of suitable homogenates for the extraction of Cyclosporine from eight different ocular tissues with a simple, user friendly stepwise procedure. Due to the different phospholipid profiles between the tissues there were varying amounts of matrix suppression encountered during method development. While the stable labeled internal standard compensated quite well in most cases a Waters OstroTM phospholipid removal plate was needed to reduce the effect on analyte response. The higher range method used for analysis of cornea, conjunctiva, iris-ciliary body, sclera and eyelid used the OstroTM plate in the traditional manner of passing the extracted sample through free of phospholipids that are retained on the plate. The lower range method used for analysis of lens, lacrimal gland and retina/choroid tissues employed pre-dilution of



Cyclosporine

Bead Ruptor Elite



d4-Cyclosporine

7ml Tube

2.8 MM Ceramic Bulk Beads

OMNI International
The Homogenizer Company®

Method

A Rabbit ocular tissues were processed by bead-homogenization in 0.8 mL of buffer (20:80 acetonitrile: 50 mM ammonium acetate, pH 3.5) on a Bead Ruptor Elite (OMNI International Inc.) equipped with a 7 mL tube carriage and finger plate allowing for fast, simultaneous processing of 12 tissue samples. Each tissue was fully homogenized with 2.8 mm zirconium oxide beads in two 60 second cycles at 5.5 m/sec. followed by protein precipitation extraction using 2.0 mL of ACN with 0.1% formic acid and sonication. Samples were centrifuged and the ACN supernatants processed further on a phospholipid removal plate.

For analysis of cornea, conjunctiva, iris-ciliary body, sclera and eyelid on the high range method the surrogate matrix (rabbit plasma supernatant) was spiked with Cyclosporine at a range of 0.0500 – 100 ng (approximately 0.300 – 600 ng in the original ocular tissue sample when an aliquot factor is applied). A 0.5 mL aliquot of the supernatant is removed, mixed with stable labeled IS (d4-Cyclosporine) and run through a Waters OstroTM phospholipid removal plate alongside surrogate matrix calibration standards/analytical QC samples. Extracts were diluted further for subsequent analysis by LC-MS/MS. Run times (time between injections) are approximately 5.5 minutes. The total amount of Cyclosporine (ng) in the tissue supernatant (approximately 0.300 – 600 ng for rabbit cornea, conjunctiva, iris-ciliary body, sclera and eyelid) was back-calculated by applying an aliquot factor based on aliquot to supernatant volume ratio as follows.

$$\text{Correction (Aliquot) Factor} = \frac{\text{supernatant aliquot volume (500 µL)}}{\text{buffer volume (0.8 mL) + solvent volume (2.0 mL) + Cyclosporine spiking solution volume (25 µL)}} = \mathbf{0.1770}$$

For analysis of lens, retina-choroid, and lacrimal gland on the low range method the surrogate matrix was spiked with Cyclosporine at a range of 0.0100 – 20.0 ng (approximately 0.0500 – 100 ng in the original ocular tissue sample when an aliquot factor is applied). A 0.6 mL aliquot of the supernatant was removed, mixed with stable labeled IS (d4-Cyclosporine) and then diluted with 0.2 mL of 5 mM NH4OAc to reduce the concentration of organic solvent. Diluted extracts were then loaded onto a Waters OstroTM phospholipid removal plate alongside surrogate matrix calibration standards/analytical QC samples. Extracts were retained by the plate and then washed off free of phospholipids with 0.4 mL ACN with 0.1% formic acid for evaporation and reconstitution for subsequent analysis by LC-MS/MS. Run times were approximately 5.5 minutes. The total amount of Cyclosporine (ng) in the tissue supernatant (approximately 0.0500 – 100 ng for rabbit lens, retina-choroid and lacrimal gland) was back-calculated by applying an aliquot factor based on aliquot to supernatant volume ratio as follows:

$$\text{Correction (Aliquot) Factor} = \frac{\text{supernatant aliquot volume (600 µL)}}{\text{buffer volume (0.8 mL) + solvent volume (2.0 mL) + Cyclosporine spiking solution volume (25 µL)}} = \mathbf{0.2124}$$

Analytical Parameters

- **HPLC Pumps:** Shimadzu LC-10AD, LC-20AD or LC-20ADXR
- **Controller:** Shimadzu SCL-10A or CBM-20A
- **Autosampler:** Leap HTS-PAL or HTC-PAL
- **Eluent:** 5 minute linear gradient
- **Mobile Phase A:** 20 mM NH4COOH with 0.1% formic acid
- **Mobile Phase B:** 90:7.5:2.5 ACN:MeOH:0.4 M NH4COOH, pH 3.5
- **Mass Spectrometer:** AB Sciex API 4000 triple quadrupole mass spectrometer
- **Ionization:** Positive Ion Electrospray

LC-MS-MS MRM Parameters:

Compound	MRM transition (m/z)	DP	CE	CXP	ISV	Temp. (°C)
Cyclosporine	1220.0 – 1203.0	67	19	40	5000	350
d4-Cyclosporine	1224.0 – 1207.0	67	19	40	5000	350

Parameters for High Range method (Cornea, Conjunctiva, Iris Ciliary Body, Sclera, and Eyelid)		
Column: ACE 5 µm Phenyl, 50 x 2.1 mm		
Time (min)	Event	Parameter
Initial	%B	60
0.50	%B	60
1.80	%B	80
2.31	%B	100
2.32	Flow Rate (mL/min)	0.75
2.33	Flow Rate (mL/min)	1.00
3.60	%B	100
3.75	%B	60
3.90	Flow Ratw (mL/min)	1.00
4.00	Flow Rate (mL/min)	0.75
5.00	System Controller	Stop

Parameters for Low Range method (Lens, Lacrimal Gland, Retina/Choroid)		
Column: ACE 3 µm C18-AR, 100 x 2.1 mm		
Time (min)	Event	Parameter
Initial	%B	60
0.25	%B	60
2.50	%B	85
2.55	%B	98
2.60	Flow Rate (mL/min)	0.75
2.65	Flow Rate (mL/min)	0.85
3.75	%B	98
4.00	%B	60
4.70	Flow Ratw (mL/min)	0.85
4.75	Flow Rate (mL/min)	0.75
5.00	System Controller	Stop

Results

Ocular tissues commonly selected for analysis during preclinical pharmacokinetic studies for ophthalmic drugs range from very small, soft perfused tissues (e.g., ICB or retina/ choroid) to much larger, tougher collagenous tissues (e.g., sclera and eyelid). It is essential that the quality of the homogenate and subsequent extraction be designed properly to ensure optimal extraction of the drug from the tissues. Key to achieving this goal is the use of appropriate equipment such as a powerful bead homogenizer that efficiently and effectively prepares tissue homogenates in a high throughput manner. The methodology discussed here using the powerful OMNI Bead Ruptor Elite allowed for the preparation of suitable homogenates for the extraction of Cyclosporine from eight different ocular tissues with a simple, user friendly stepwise procedure. Due to the different phospholipid profiles between the tissues there were varying amounts of matrix suppression encountered during method development. While the stable labeled internal standard compensated quite well in most cases a Waters OstroTM phospholipid removal plate was needed to reduce the effect on analyte response. The higher range method used for analysis of cornea, conjunctiva, iris-ciliary body, sclera and eyelid used the OstroTM plate in the traditional manner of passing the extracted sample through free of phospholipids that are retained on the plate. The lower range method used for analysis of lens, lacrimal gland and retina/choroid tissues employed pre-dilution of samples to an acetonitrile concentration that bound the analyte like that of solid phase extraction (SPE). The analyte and IS were then eluted free of phospholipids in 100% organic solvent for evaporation and concentration of extracts necessary to achieve the desired lower sensitivity.

Cyclosporine notoriously is poorly ionized and difficult to fragment in the mass spectrometer. In order to overcome this and achieve the necessary detection limits an ammonium adduct was used for detection in Q1 which is then dissociated in the collision cell for detection of the molecular ion (M+H) in Q3 (Figures 2 and 3). Maintaining an appropriate concentration of NH_4OAc in the reconstitution solvent and mobile phases ensures consistent formation of the ammonium adduct for proper ionization with ~10x more sensitivity than using the 1203.0 – 425.0 fragment ion.

Quality control samples of each tissue as well as the surrogate matrix were evaluated at four concentrations to determine precision and accuracy.

These represent the total amount of drug in tissue near the lower limit of quantitation (LLOQ) and in the lower, middle and upper portions of the calibration curve. The precision and % deviation was within acceptance criteria (±15%) for the surrogate matrix and all tissues except for sclera and lens which were within ±20%. Cyclosporine was shown to be stable in all tissues for ≥22 hours at room temperature and when stored frozen at -70°C for ≥99 days. The Cyclosporine extracts were shown to be stable for ≥28 hours. Acceptable selectivity, matrix factor and dilution integrity were demonstrated for all tissues. Extraction efficiency was ~87% for the high range method and ~65% for the lower range method. In order to ensure stability of Cyclosporine for reanalysis the tissue homogenate supernatants were evaluated and showed at least 18 hours of room temperature stability and 13 days -70°C storage. Three freeze/thaw cycles were demonstrated for all tissues except retina/choroid and lacrimal gland which demonstrated only one cycle.

The tissue method discussed here along with validated methods for Cyclosporine in rabbit whole blood, aqueous humor, vitreous humor and tears were successfully used in support of a comparative topical ocular tissue distribution study in NZW rabbits comparing Restasis® to a proprietary formulation of Cyclosporine.

	High Range Assay (0.0500 – 100 ng)		Low Range Assay (0.0500 – 100 ng)	
	Mean Acc.(%Dev)	Precision (%CV	Mean Acc. (%Dev)	Precision (%CV)
Surrogate Matrix				
Intra-Assay	-2.44 to 0.417% (5.20% at LLOQ)	≤2.43% (5.36% at LLOQ)	-5.00 to 0.0417% (2.92% at LLOQ)	≤3.0% (14.8% at LLOQ)
Inter-Assay	0.917 to 2.45% (4.88% at LLOQ)	≤3.74% (7.04% at LLOQ)	-2.15 to 0.771% (2.36% at LLOQ)	≤7.25% (9.85% at LLOQ)
Process QC				
Cornea	2.94 to 4.86% (4.33% at LLOQ)	≤5.63% (7.92% at LLOQ)	—	—
Conjuctiva	5.69 to 8.48% (7.33% at LLOQ)	≤4.46% (5.90% at LLOQ)	—	—
ICB	0.333 to 8.82% (-1.22% at LLOQ)	≤3.26% (5.90% at LLOQ)	—	—
Sclera	-0.556 to 7.22% (-10.2% at LLOQ)	≤8.49% (4.93% at LLOQ)	—	—
Eyelid	-1.25 to 5.37% (-3.00% at LLOQ)	≤16.0% (18.4% at LLOQ)	—	—
Lens (Between- Batch)	—	—	-2.22 to 3.92% (4.91% at LLOQ)	≤16.3% (8.94% at LLOQ)
Retina-Choroid	—	—	-3.92 to 3.11% (14.2% at LLOQ)	7.18% (7.55% at LLOQ)
Lacrimal Gland	—	—	-4.38to 2.00% (6.80% at LLOQ)	≤4.11% (5.58% at LLOQ)

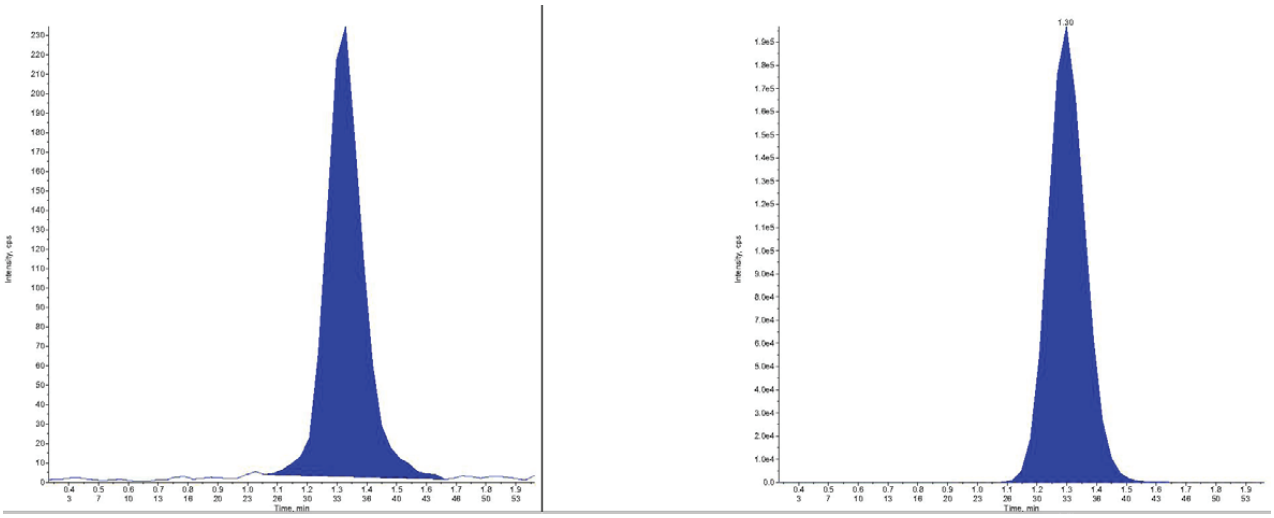


Figure 1. Figure 2 LC-MS-MS Chromatograms of Cyclosporine and its Internal Standard in Rabbit Cornea at the LLOQFigure

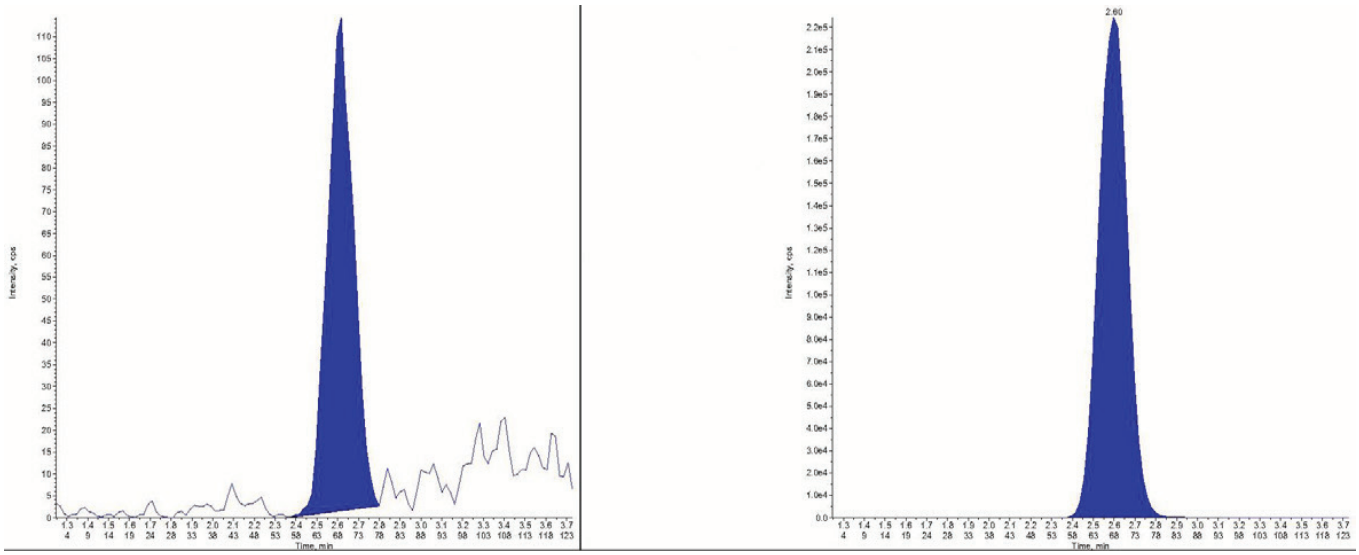


Figure 1. LLOQFigure 3 LC-MS-MS Chromatograms of Cyclosporine and its Internal Standard in Rabbit Lens at the LLOQ

Conclusion

• A robust, reproducible, efficient and sensitive assay was developed for the quantification of Cyclosporine in eight very different ocular tissues.

• Homogenization using the OMNI Bead Ruptor ensures rapid, high performance and complete homogenization of multiple tissue types

.• The use of a surrogate matrix lowers validation and sample analysis costs considerably and allows for multiple tissues to be analyzed in the same run

.• The methodology was flexible enough to allow for reanalysis of over range samples as well as lower Cyclosporine levels expected in posterior tissues.

Conclusion

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