Nanoparticulate Mycophenolic Acid Eye Drops - Analytical Validation of a High Performance Liquid Chromatography Assay and Stability Studies

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Abstract

Background

Mycophenolic acid (MPA), an immunosuppressive agent, is used orally to reduce corneal graft rejection. However its oral use is associated with gastrointestinal side effects.

Objectives

To prepare MPA nanoparticle eye drops and a validated analytical method.

Methods

Aqueous MPA eye drops were prepared by nanoencapsulation of MPA using Nanomerics MET (N-palamitoyl-N-monomethyl-N,N-dimethyl-N,N,N-trimethyl-6-O-glycolchitosan) at a MET, MPA ratio of 7.5: 1 g g^{-1} in the presence of glycerol (2.75% w/w). A validated MPA in-formulation drug substance assay was then developed.

Results

MET-MPA formulations were prepared as well as a validated assay. Assay validation parameters for the analysis of MPA in the formulation were satisfactory [Plate count = 16458, Capacity Factor = 2.4, Tailing Factor = 1.02, linearity = 0.999 (0.016 – 0.5 mg mL^{-1}), limit of detection = 0.056 mg mL^{-1}, limit of quantification = 0.17 mg mL^{-1}, accuracy = 98%, intraday and interday relative standard deviation = 0.45% and 4% respectively]. The candidate formulation (z-average mean = 66 ± 0.4 nm, polydispersity index = 0.12 ± 0.012, drug content = 1.14 ± 0.003 mg mL^{-1}, zeta potential = +8.5 ± 1.4 mV, pH = 7.4 ± 0.02, osmolarity = 309 ± 1.5 mOSm L^{-1}, viscosity = 1.04 ± 0.001 mPa.s) was then found to be stable for 14 days with respect to drug content at refrigeration, room and accelerated (40°C)temperature and. All other formulation parameters were within the ocular comfort range.
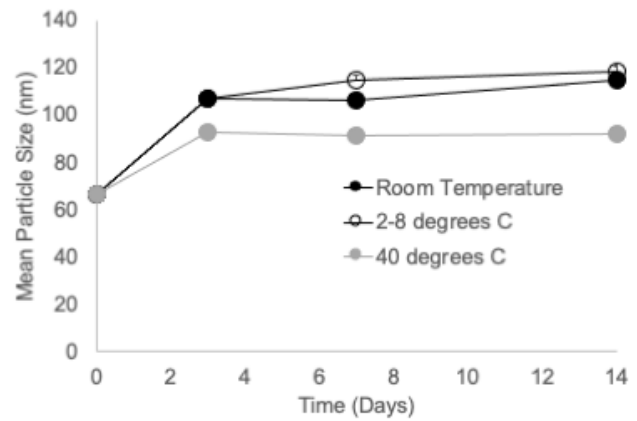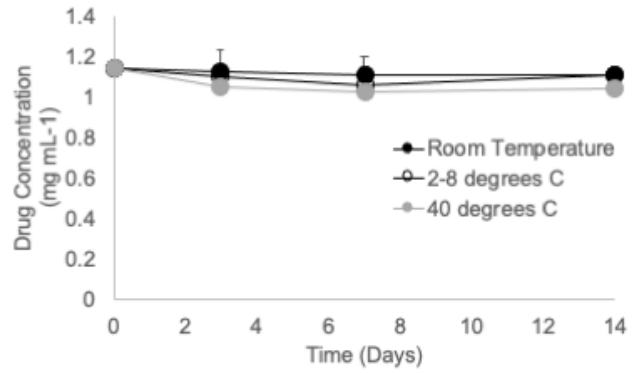
Conclusions

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A validated assay (ICH and US FDA guidelines) for new MPA nanoparticle eye drops has

been developed.

Graphical Abstract
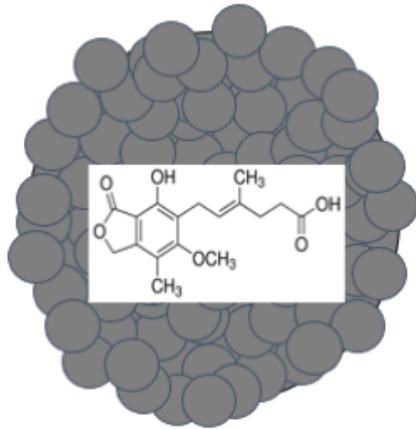
Polymeric nanoparticle
mycophenolic acid eye
drops

1. Introduction

MPA selectively and reversibly inhibits the enzyme inosine monophosphate dehydrogenase (IMPDH) [1]. IMPDH is the rate-limiting enzyme in the de novo synthesis of guanosine nucleotides, responsible for conversion of inosine monophosphate (IMP) to guanosine monophosphate (GMP), which is subsequently converted to guanosine diphosphate (GDP), and guanosine triphosphate (GTP) and both GTP and GDP are used in nucleic acid synthesis [2]. As both T and B lymphocytes are critically reliant on this de novo guanine nucleotide synthesis pathway, relative to cells that use the salvage pathway of nucleotide biosynthesis, MPA has a more potent cytostatic effect on lymphocytes than on other cells [2]. Therefore, MPA blocks proliferation and clonal expansion in T and B lymphocytes, inhibits antibody production and the generation of cytotoxic T cells, inevitably suppressing cell-mediated immune responses and exhibiting immunosuppressive activity [2]. MPA is available as the ester prodrug or sodium salt and is used orally to prevent transplant rejection in paediatric [3,4] and adult [5] renal patients. The drug is also used in patients undergoing corneal transplants to prevent corneal graft rejection [6]. The drug has also been used to treat a variety of ocular inflammations including uveitis (anterior, intermediate or posterior), scleritis and ocular mucous membrane pemphigoid [7,8]. MPA is associated with gastrointestinal side effects [4,7,8], such as diarrhoea as well as bone marrow suppression [9,10] motivating the development of a topical ocular formulation to treat ocular inflammation. The gastrointestinal side effects of MPA are linked to its mechanism of action as low intracellular guanosine levels are observed *in vitro* within gastrointestinal cells in culture when exposed to MPA [11]. Additionally, MPA causes a dysregulation of lipid metabolism which may affect the barrier function of the gastrointestinal epithelium and contribute to the ulceration and diarrhoea side effects observed [11]. Enteric coated formulations have been associated with reduced gastrointestinal side effects; albeit with a report of neutropenia [4]. The reduced gastrointestinal side effects

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demonstrate that reducing gastrointestinal exposure by utilising an eye drop formulation may lead to a safer immunosuppressive regimen with MPA.

We could find no reports on the formulation of MPA eye drops and found one report on the formulation of mycophenolate mofetil (MPM) in which MPM is formulated with cyclodextrins or as a suspension [12]. We used Nanomerics' Molecular Envelope Technology (MET) [13,14] to formulate the hydrophobic MPA into a nanoparticle based eye drop formulation.

Analytical validation of a nanoparticle formulation is an area in which more data should be published in order to facilitate the translation of pharmaceutical nanotechnology formulations from the laboratory to the clinic. The current paper focuses on the validation of an assay for MPA in a nano-enabled eye drop formulation and refers to the guidelines published by the International Committee on Harmonisation (ICH) [15] and the US Food and Drug Administration (FDA) [16].

2. Materials and Methods

2.1 Materials

All reagents and solvents were obtained from Sigma Aldrich Chemical Company, UK unless otherwise stated. Nanomerics' Molecular Envelope Technology (MET, N-palmitoyl,N-monomethyl,N,N-dimethyl, N,N,N-trimethyl-6-O-glycolchitosan) was obtained from Nanomerics Ltd. Mycophenolic acid (MPA) was obtained from Millipore (LOT:2887139, Millipore, USA).

2.2 Methods

2.2.1 Formulation Manufacture

Procedure 1

Glycerol (2.7 mL) was added to deionised water (97.3 mL) to prepare a 2.7% w/v solution (100 mL). Nanomerics' MET (mole% palmitoylation = 22mol%, mole% quaternary ammonium groups = 11mole%) was dispersed at a concentration of 0.75% w/v in the glycerol solution. The polymer was allowed to disperse by gently shaking on an orbital shaker for at least 2 hours. This polymer dispersion was added to a weighed amount of MPA powder at two times the target amount. MPA was dispersed by initially vortexing the mixture and stirring for 12h and, subsequently, by processing for 15 cycles at 18000 psi using a high pressure homogeniser (Avestin Emulsiflex C5, Avestin, Germany). After high pressure homogenisation, the formulation was transferred to glass vials. The pH was adjusted to 7.4 using NaOH (1M), a magnetic stirrer and a pH meter (Jenway 3310, VWR, UK). The formulation was analysed using the unvalidated method given below for drug content, then diluted with an appropriate volume of the MET polymer dispersion containing 0.75% w/v of MT and 2.7% w/v of glycerol (previously adjusted to pH 7.4) to make up the formulation to the required strength. The solution was sterile filtered using a 10mL syringe (BD Plastipak,

LOT: 1503003) and a 0.22μm Millex GP filter unit (Millipore, LOT: R7PA99493) into 28mL glass vials, with each vial containing 16mL of the formulation.

Samples of MPA dissolved in acetonitrile (0.0625 – 2.0 mg mL^{-1}) were chromatographed over an Onyx™ Monolithic C18, LC Column (100 x 4.6 mm) using an Agilent 1200 series HPLC system and chromatograms analysed using Agilent ChemStation software (Agilent, USA) and a triethylamine, acetonitrile (35: 65) mobile phase. The flow rate was set at 1 mL min^{-1}, the injection volume at 10 μL, the column temperature at 60°C and the detection wavelength at 304 nm. The retention time was 1.92 minutes and the run time was 6 mins. For analysis of MPA in formulations, MPA was spiked within the MPA eye drop matrix (0.01563 – 0.5 mg mL^{-1}) and samples were chromatographed over the same system to produce a standard curve (0.00195 - 1mg mL^{-1}, Peak Area = 8.9395[MPA] + 8.755, r^2 = 0.99994). To quantify MPA in the formulation, an aliquot of the formulation (50 μL) was diluted with acetonitrile (200 μL) and chromatographed as described above. The formulation was then made up to the required strength by dilution with a volume of MET (0.75% w/v) dispersed in glycerol (2.7% w/v).

Procedure 2

Nanomerics MET (60 mg) was dispersed in glycerol (2.75%, 8 mL) at a concentration of 0.75% w/v. To this was added MPA (8mg) to give a GCPQ, MPA weight ratio of 7.5: 1 and the mixture was initially vortexed, stirred for 2h and subsequently processed for 15 cycles at 18,000 psi using a high pressure homogeniser (Avestin Emulsiflex C5, Avestin, Germany). After high pressure homogenisation, the formulation was transferred to glass vials. The pH was adjusted to pH = 7.4 using NaOH (1M), a magnetic stirrer and a pH meter (Jenway 3310, VWR, UK). The resulting solution was filtered using a 2mL syringe (BD Plastipak, LOT: 1503003) and 0.22μm Millex GP filter unit (Millipore, LOT: R7PA99493).

A blank formulation is prepared by carrying out the steps above but omitting the addition of MPA. Spiked formulations used in precision tests were prepared by spiking a portion of the blank formulation with an equal volume of MPA (of a required concentration) dissolved in acetonitrile.

2.2.2. HPLC Analytical Method Development and Validation

Samples were chromatographed over an Onyx™ Monolithic C18, LC Column (100 x 4.6 mm) using an Agilent 1200 series HPLC system and a triethylamine (0.1%v/v adjusted to pH = 6 with phosphoric acid), acetonitrile gradient (Table 1) mobile phase. The flow rate was set at 1.2 mL min^{-1}, the injection volume at 10 μL, the column temperature at 45°C and the detection wavelength at 304 nm. Chromatograms were analysed using Agilent ChemStation software (Agilent, USA). The retention time was 4.9 minutes and the run time was 8 mins.

Table 1: Mobile Phase Gradient

Time (minutes)	0.1%v/v triethylamine, pH = 6 (%)	Acetonitrile (%)
0	80	20
2.5	80	20
3.0	60	40
6.0	60	40
6.5	80	20
8.0	80	20

The system suitability was assessed using US FDA guidelines[1] and used to develop the above HPLC method and the other analytical parameters (specificity, linearity and limits, accuracy and precision) were validated against US FDA and ICH guidelines [1,2].

System Suitability

Samples (MPA in acetonitrile and MPA in the formulation) were injected 3 times and the peaks analysed in order to calculate the under noted peak characteristics.

Capacity Factor - This is a measure of the column's ability to retain the analyte. It is simply the ratio of the difference between solvent and analyte retention time to the solvent peak retention time (Equation 1)

Equation 1

$$k' = \frac{t_R - t_0}{t_0}$$

where:

k'= Capacity Factor

t_R = Retention time of the analyte peak

t_0 = Solvent peak retention time

The FDA recommends that this value does not fall below 2. [1]

Tailing Factor - This is a measure of the degree of tailing exhibited by the peak (Equation 2).

Equation 2

$$t = \frac{w_x}{2f}$$

where:

t = Tailing Factor

w_x = Width of the peak at 5% of the peak height

f = The difference between peak maximum and the peak front at 5% peak height

The tailing factor must not exceed 2, as recommended by the FDA. [1]

Theoretical Plate Number - The theoretical plate number (n) measures the column's efficiency. It indicates the theoretical maximum number of peaks that can be detected by a single run (Equation 3).

Equation 3

$$n = 16 \left(\frac{t_R}{t_w}\right)^2$$

where:

n = Plate Number

t_w = The width of the peak at the baseline

t_R = Retention time of the analyte peak

The US FDA recommends that theoretical plate number should generally be greater than 2000.[1]

Resolution - Resolution (R_s) refers to the separating power of the method and was determined from the two closest eluting peaks (Equation 4).

Equation 4

$$R_S = 2\frac{\left(t_{R_2} - t_{R_1}\right)}{\left(t_{w_1} + t_{\omega_2}\right)}$$

where:

R_s = Resolution

t_R = The retention times of the two closest eluting peaks. 1 refers to the first peak and 2 refers to the second peak.

t_w = The widths of the peaks at the baseline. 1 refers to the first peak and 2 refers to the second peak.

The FDA[1] recommends that resolution should be greater than 2 and this would have been the target resolution value when developing the method if more than 2 peaks were present.

Specificity

To evaluate method specificity, blank samples (formulation matrix alone) were run and the chromatogram was compared to that of matrix spiked with MPA. All peaks in the chromatogram were labelled and any overlap between peaks assessed.

Linearity and limits

To evaluate method linearity, 6 concentrations of MPA in the final formulation were tested. To produce these samples, a 1mg mL^{-1} solution of MPA in acetonitrile was prepared by adding 5 mg of MPA to 5 ml of ACN. This was diluted to produce separate 0.5, 0.25, 0.125, 0.0625, 0.0313 mg mL^{-1} solutions. 0.5ml of each solution was then be mixed with an equal

volume of the blank formulation matrix to give solutions of half the original concentrations
(0.0156 – 0.5 mg mL^{-1}). This was repeated 3 times using separately prepared replicate
1mg/ml MPA solutions for a total of 18 samples (3 replicates per concentration). Each
sample was run, and the peak area was recorded. This data was then used to evaluate linearity
by determining the regression coefficient. According to the US FDA guidelines, the
regression coefficient must be greater than or equal to 0.999 [1] to demonstrate acceptable
linearity. The data was also plotted to provide a standard curve that was utilised in the
accuracy investigation. The standard deviation of the residuals from the standard curve
produced was used to estimate the limits of detection (LOD, Equation 5) and quantification
(LOQ, Equation 6).

These limits were calculated using

Equation 5

$$LOD = \frac{3.3\sigma}{S}$$

And

Equation 6

$$LOD = \frac{10\sigma}{S}$$

Where S = Slope of the calibration curve

σ was calculated from the deviation of each point on the calibration curve from the regression
line (Equation 7):

Equation 7

$$\sigma = \sqrt{\frac{\Sigma(\text{residual})^2}{n-1}}$$

where:

σ = Residual Standard Deviation

Residual = The difference between each data point and the regression line.

n = number of data points (in this case, 18; 3 replicates x 6 concentrations)

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"Residual" is calculated by subtracting the signal detected from the theoretical value based on the calibration curve. This value is taken for each replicate at each concentration (18 values in total).

MPA in acetonitrile was evaluated for linearity and limits by dissolving 2mg of MPA in 1 mL of acetonitrile and diluting to give the following concentrations: 0.0625, 0.125, 0.25, 0.5, 1.0 and 2.0 mg mL^{-1}. These standard solutions were evaluated as described above.

Accuracy

To evaluate accuracy, solutions of MPA in acetonitrile were prepared: 0.5, 1.0 and 1.5 mg mL^{-1}. Furthermore, solutions of MPA in acetonitrile (1, 0.5 and 0.25 mg mL^{-1}) were then mixed with an equal volume of formulation matrix to produce 0.5, 0.25 and 0.125mg mL^{-1} MPA formulation samples. Each MPA solution and MPA formulation sample was prepared at least 3 times and chromatographed. The calibration curve constructed earlier was used to interpret the peak areas and give an "experimental concentration" for each sample analysed. These experimental values were then compared to the nominal concentrations to determine the % recovery (Equation 8)

Equation 8

$$Recovery \ (\%) = \ 100\frac{E}{A}$$

where:

E = The concentration measured in the experiment

A = The nominal concentration of the sample

The US FDA recommends that accuracy may be accepted when the Recovery ranges from 98% - 102% [1].

Precision

Precision was tested at 2 levels: repeatability and intermediate precision. Repeatability was assessed by injecting samples at 3 concentrations, 3 times each for a total of 9 determinations.

MPA samples at concentrations of 0.5, 0.25 and 0.125 mg mL^{-1} were prepared in the same way as the samples used to construct the standard curve. The % relative standard deviation (RSD) between peak areas was calculated for each concentration and the mean RSD is taken across all 9 determinations. The US FDA recommends that a relative standard deviation of 1% is an acceptable level of repeatability[1]. Intermediate precision was tested by running each of these concentrations once on 3 consecutive days. The RSD was calculated in the same manner as the repeatability study. The FDA does not specify a limit for repeatability and so the intermediate precision RSD is simply stated.

2.2.3 MPA Formulation Stability Study

MET - MPA Formulations (0.1% MPA and 0.75% MET) were prepared (Procedure 1) and stored at each at room temperature (18-23°C), refrigeration temperature (5-6°C) and at elevated temperature (40°C). These formulations were periodically analysed for drug content using the HPLC method detailed above and after dilution of an aliquot of the formulation (50 µL) with acetonitrile (200 µL). The particle size distribution and particle zeta potential of the formulations were determined on a Malvern ZetaSizer Nano SZ (Malvern Instruments, UK) by adding the MPA formulation (0.1% w/v, 700µL) to a disposable cuvette and particle size and zeta potential measured at 25°C. The osmolarity of the MPA formulation was measured using a Roebling Micro-osmometer Type5R (Camlab, UK) using the freezing point depression technique. Distilled water (0 mOsm) and the osmometer reference solution (300 mOsm) were used as standards for the instrument calibration. A sample (100 µL) of the formulation was pipetted into a 1.5 mL Eppendorf tube and the measurement conducted. The viscosity of the formulation was measured using an m-VROC viscometer (Rheosense, USA). Samples were inserted into the measuring cell using a 0.5 mL syringe. The apparent viscosity (mPa s) was measured at three different shear rates (10,700 s^{-1},

12,500 s^{-1} and 14,300 s^{-1}) and at 25°C. The formulations were treated as Newtonian liquids (fluids, where the viscosity is the same for all shear rates). The pH of the formulation was measured using a magnetic stirrer and a pH meter (Jenway 3310, VWR, UK). Before measurement, the pH was calibrated using reference standards at pH = 4 and pH = 10.

2.2.4 Statistical Analysis

Statistical analyses were performed via two-way ANOVA Test using GraphPad Prism software. Statistical significance was set as a $p < 0.05$ and all measurements were compared to formulation parameters at the start of the stability study (Day 0).

3. Results

The gradient HPLC method was then validated as described in the Methods section and the results appear below for MPA in acetonitrile and MPA spiked in the nanoparticulate eye drop formulation.
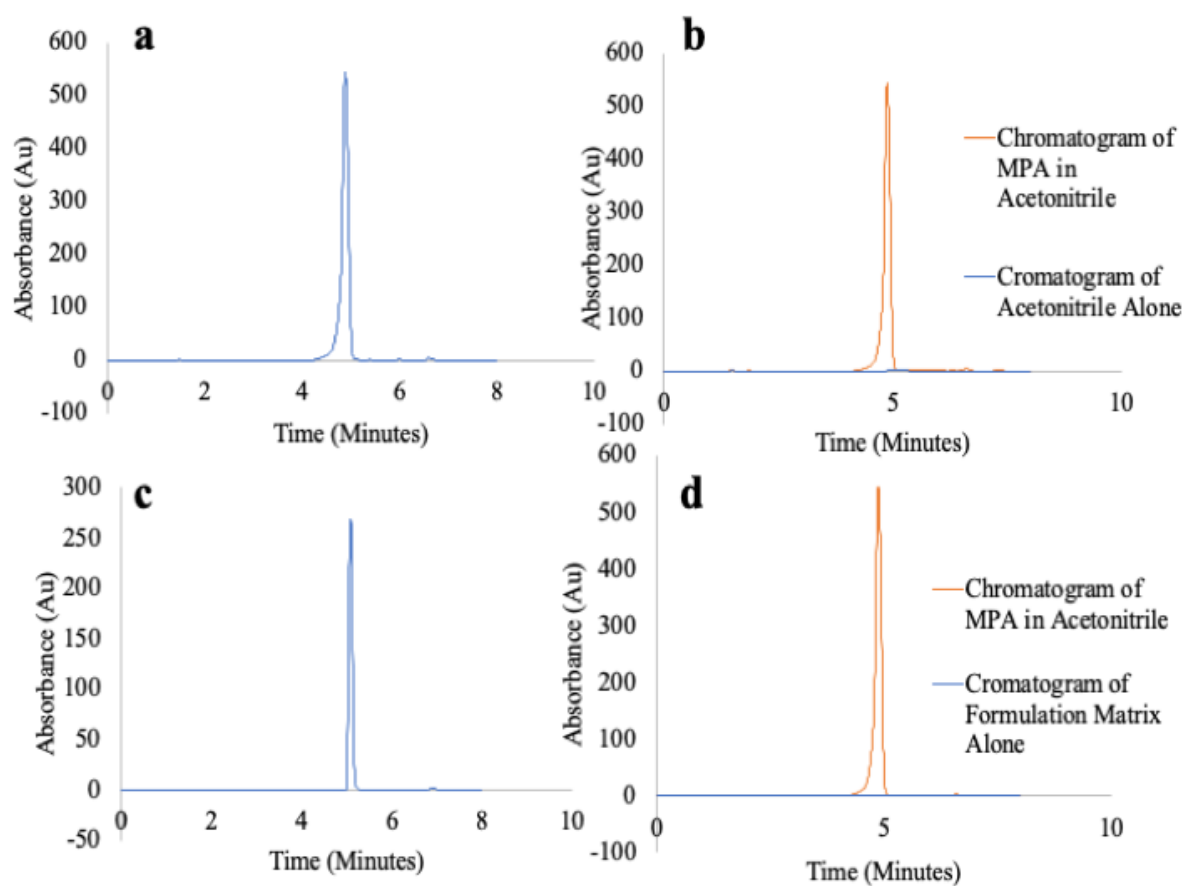
3.1 MPA in Acetonitrile

3.1.1 System Suitability

The HPLC method passed the system suitability criteria (Table 2). As the drug eluted as a single sharp peak (Figure 1a), the resolution test was not applicable here.

Table 2: System suitability for MPA

		MPA dissolved in acetonitrile	MPA in nanoparticle formulation
System Suitability Parameter	Requirement	Observed Value	Observed Value
Plate Count	No less than 2000	7684	16458
Capacity Factor	No less than 2	2.3	2.4
Tailing Factor	No more than 2	0.78	1.02

Figure 1: HPLC Chromatogram of a) MPA (0.5 mg mL-1) dissolved in acetonitrile, retention time = 4.9 minutes, the small peak at 1.5 minutes is the solvent peak; b) overlay of the acetonitrile chromatographic run against the MPA (0.5 mg mL^{-1}) in acetonitrile chromatographic run; c) of MPA (0.5 mg mL-1) in the nanoparticle eye drop formulation, retention time = 5.0 minutes; d) overlay of the formulation matrix chromatographic run against the MPA (0.5 mg mL^{-1}) in the formulation chromatographic run.

3.1.2 Specificity

The analytical method passes the specificity test (Figure 1b) as there is no interference from

the solvent in the relevant area of the chromatogram. The acetonitrile peak appears at 1.5

minutes (confirmatory data not shown).

3.1.3 Linearity and limits

Table 3: Linearity and limit data

Sample	MPA dissolved in acetonitrile	MPA in nanoparticle formulation
Equation of the straight line	Peak area = 6,739 [MPA] + 57.54	Peak area = 7,305 [MPA] + 2.64
r^2 value	0.99999	0.99986
Sum of Squared Residuals	48484	261459.20
Standard Deviation of the Residuals (σ)	53.4	124.02
Limit of Detection (mg mL^{-1})	0.0262	0.056
Limit of Quantification (mg mL^{-1})	0.0792	0.170

The standard curve constructed using MPA in acetonitrile shows excellent linearity (Table 3) and the regression coefficient passes the FDA[1] recommended value (0.999).

The limits of quantification and detection are quite conservative but well within the range of expected of the samples ~ 0.2 mg mL^{-1}.

3.1.4 Accuracy

For the accuracy study, three trials were run as Trial 1 revealed a possible weighing or pipetting error with Trial 1, Replicate 2 (

Table 4). Each replicate was separately prepared and diluted to the concentrations required. The peak area from each run was then recorded and used to calculate the experimental analyte concentration. This experimental value was then used to calculate % recovery, as outlined in the methods (

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Table 4).

Table 4: The percentage recovery from Trials 1,2 and 3 of the MPA in ACN accuracy study, overall mean% recovery from all trials = 99%

	Parameter	Replicate 1			Replicate 2			Replicate 3			Mean % recovery
Trial 1	**Concentration (mg mL^{-1})**	**0.5**	**1.0**	**1.5**	**0.5**	**1.0**	**1.5**	**0.5**	**1.0**	**1.5**	
	Peak Area (Au)	3560	6964	10161	4166	8256	12355	3651	6935	10235	
	Measured Concentration (mg mL^{-1})	0.520	1.025	1.499	0.610	1.217	1.825	0.533	1.021	1.510	
	Recovery (%)	104%	102%	100%	122%	122%	122%	107%	102%	101%	109%
Trial 2	**Concentration (mg mL^{-1})**	**0.5**	**1.0**	**1.5**	**0.5**	**1.0**	**1.5**	**0.5**	**1.0**	**1.5**	
	Peak Area (Au)	3432	6645	9726	2695	5235	7901	3932	7818	11662	
	Measured Concentration (mg mL^{-1})	0.501	0.978	1.435	0.391	0.768	1.164	0.574974	1.152	1.722	
	Recovery (%)	100%	98%	96%	78%	77%	78%	115%	115%	115%	97%
Trial 3	**Concentration (mg mL^{-1})**	**0.5**	**1.0**	**1.5**	**0.5**	**1.0**	**1.5**	**0.5**	**1.0**	**1.5**	
	Peak Area (Au)	2395	4858	9177	3829	7651	11661	2915	6041	8849	
	Measured Concentration (mg mL^{-1})	0.347	0.712	1.353	0.560	1.127	1.722	0.424	0.888	1.305	
	Recovery (%)	69%	71%	90%	112%	113%	115%	85%	89%	87%	92%

Pooling the data however produced an overall mean recovery that was within the range of 98 – 102% (Table 4). It was noted that multiple replicates would have to be produced when analysing MPA solutions in acetonitrile. However, we are focused on the analysis of MPA in nanoparticle formulations.

3.1.5 Precision

Table 5: Intraday and Interday precision data

	Experiment	Injection 1			Injection 2			Injection 3		
Intraday Precision on MPA in acetonitrile	**Concentration (mg mL^{-1})**	0.5	1.0	1.5	0.5	1.0	1.5	0.5	1.0	1.5
	Peak Area (Au)	3560	6964	10161	3556	6897	10182	3565	6889	10165
Intraday Precision on MPA in nanoparticle formulation	**Concentration (mg mL^{-1})**	0.125	0.25	0.5	0.125	0.25	0.5	0.125	0.25	0.5
	Peak Area (Au)	1014	2022	4008	1025	2043	4002	1019	2015	4001
Interday Precision on MPA in nanoparticle formulation		**Day 1**			**Day 2**			**Day 3**		
	Concentration (mg mL^{-1})	0.125	0.25	0.5	0.125	0.25	0.5	0.125	0.25	0.5
	Peak Area (Au)	1014	2022	4008	1058	2067	4133	1113	2177	4299

Table 6: Relative standard deviation on the intraday and interday precision samples

					Mean Relative Standard deviation (%)
Intraday precision on MPA in acetonitrile	**MPA Concentration (mg mL⁻¹)**	0.5	1.0	1.5	
	Relative Standard Deviation (%)	0.127	0.589	0.107	0.277
Intraday Precision on MPA in the nanoparticle formulation	**MPA Concentration (mg mL⁻¹)**	0.125	0.25	0.5	
	Relative Standard Deviation (%)	0.091	0.723	0.536	0.45
Interday Precision on MPA in the nanoparticle formulation	**MPA Concentration (mg mL⁻¹)**	0.125	0.25	0.5	
	Relative Standard Deviation (%)	3.53	3.81	4.67	4.00

This precision study was carried out on MPA in acetonitrile and the intra-day precision

evaluated by repeat injections from the same sample on the same day (

Table 6). The relative standard deviation was calculated and found to be well within the US

FDA[1] recommended limit of 1% (

Table 6). The method passes the intra-day precision requirement when analysing MPA dissolved in acetonitrile.

3.2 MPA in the Nanoparticle Eye Drop Formulation

3.2.1 System Suitability

The HPLC method passed the system suitability criteria (Table 2). As the drug eluted as a single sharp peak (Figure 1c), the resolution test was not applicable here. It should be noted that there is a slight change in the MPA retention time (0.1 minutes) when compared with the analysis of MPA in acetonitrile (Figure 1a).

3.2.2 Specificity

The analytical method passes the specificity test (Figure 1d) as there is no interference from the formulation matrix in the relevant area of the chromatogram. The acetonitrile peak appears at 1.5 minutes (confirmatory data not shown).

3.2.3 Linearity and limits

The standard curve constructed using MPA in acetonitrile shows excellent linearity (Table 3) and the regression coefficient passes the FDA[1] recommended value (0.999).

The limits of quantification and detection are quite conservative but within the range of expected of the samples ~ 0.2 mg mL^{-1} and are within 25% of the target formulation concentration (0.25 mg mL^{-1}). The limit of quantification just complies with the US FDA and ICH guidelines[1,2]. It should be noted that the limit of detection is far lower than this calculated value and quantifiable peaks were seen with the lowest concentration used (0.015 mg mL^{-1}).

3.2.4 Accuracy

Each replicate was separately prepared and diluted to the concentrations required. The peak area from each run was then recorded and used to calculate the experimental analyte

concentration. This experimental value was then used to calculate % recovery, as outlined in the methods (Table 7)

Table 7: The percentage recovery from the MPA nanoparticle formulation accuracy study

Experiment	Replicate 1			Replicate 2			Replicate 3		
Concentration (mg/mL)	0.125	0.25	0.5	0.125	0.25	0.5	0.5	0.25	0.5
Peak Area (Au)	924	1827	3693	908	1790	3463	887	1748	3412
Measured Concentration (mg/mL)	0.127	0.250	0.506	0.125	0.245	0.474	0.122	0.240	0.467
Recovery (%)	101%	100%	101%	100%	98%	95%	97%	96%	93%

The mean recovery for all runs in the study was 98% (Table 7). This falls just within the

FDA[1]-recommended limit of 98 - 102%. Unlike the accuracy study carried out on MPA

dissolved in acetonitrile, there were no large deviations from the mean in this study. A greater

mass of MPA was weighed out to produce the starting 1mg/mL solution for each replicate in

this study: 5mg of MPA was dissolved in 5mL of acetonitrile. Although the method passes

the accuracy criteria, it should be noted that Replicates 2 and 3 show a low % recovery at the

higher concentrations.

3.2.5 Precision

For the MPA nanoparticle formulation study, we studied both the intraday and interday

precision by injecting the same samples three times on a single day and injecting the same

samples over 3 days respectively (Table 5).

As with the investigation on MPA in mobile phase, the intraday precision test presented an

RSD (0.45%) that was within the (1%) limit set. The method passes the intra-day precision

requirement when analysing MPA in the nanoparticle eye drop formulation (

Table 6).

The FDA does not give a recommendation for the maximum allowed RSD in interday precision studies, however, it is recommended that this value is stated[1]. For this reason, the mean RSD from the interday precision study is simply stated (4%) and no pass requirement was applied to it. This value is significantly higher than the intraday RSD (0.45%) so it may be advisable to produce a fresh standard curve for each day of testing if greater precision is required from the method

3.3 Stability Studies

The formulation was manufactured with the characteristics outlined in Table 8.

Table 8: Characteristics of the MET – MPA formulation (mean ± s.d., n = 3)

Mycophenolic acid Concentration (mg mL^{-1})	Z-Average Mean Particle Size (nm)	Zeta Potential (mV)	pH	Osmolarity (mOsm L^{-1})	Viscosity (mPa.s)
1.141 ± 0.003	66 ± 0.4	+8.5 ± 1.4	7.40 ± 0.02	309 ± 1.5	1.035 ± 0.001
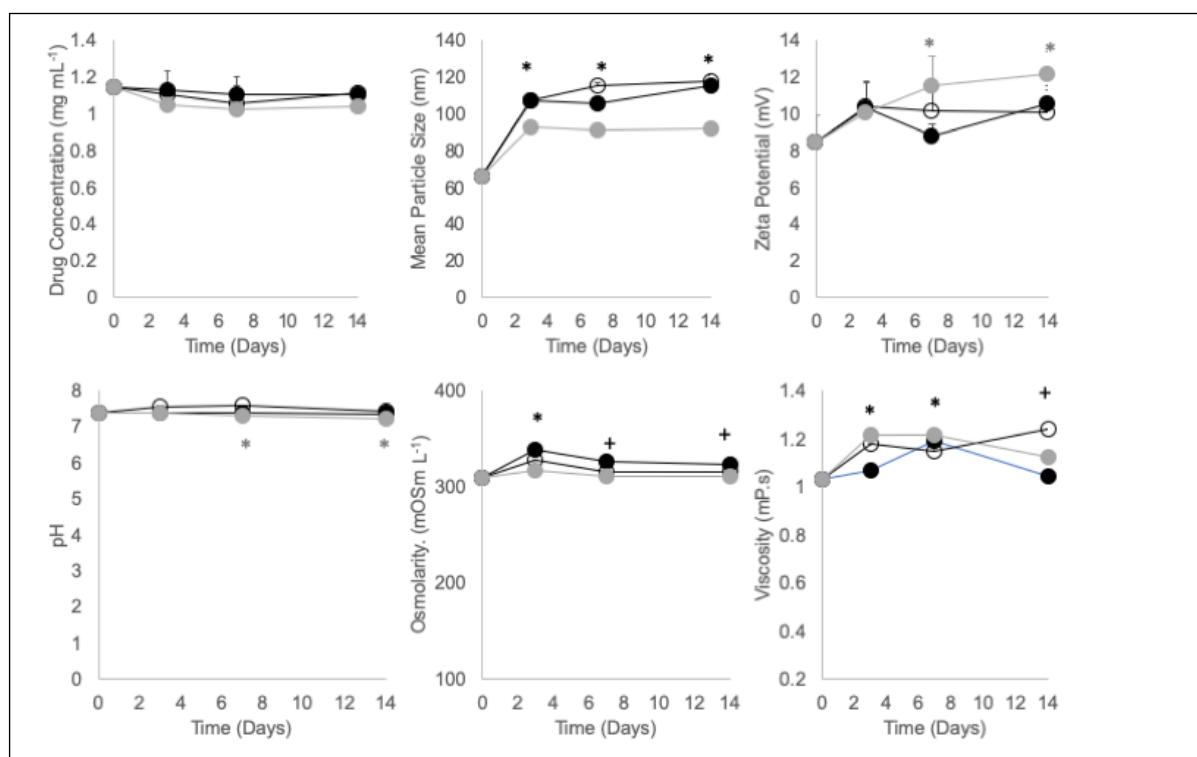
Figure 2: MET – MPA (0.1%) 14 day stability (mean ± s.d., n = 3) at: ○ = 2 – 8 ℃, ● = room temperature (16 - 25 ℃), ● = 40 ℃, * = all formulations statistically significantly different from formulation on Day = 0 (p < 0.05), * = 40 ℃ formulation statistically significantly different from formulation on Day = 0, + = 2 – 8 ℃ and room temperature formulations statistically significantly different from formulation on Day = 0.
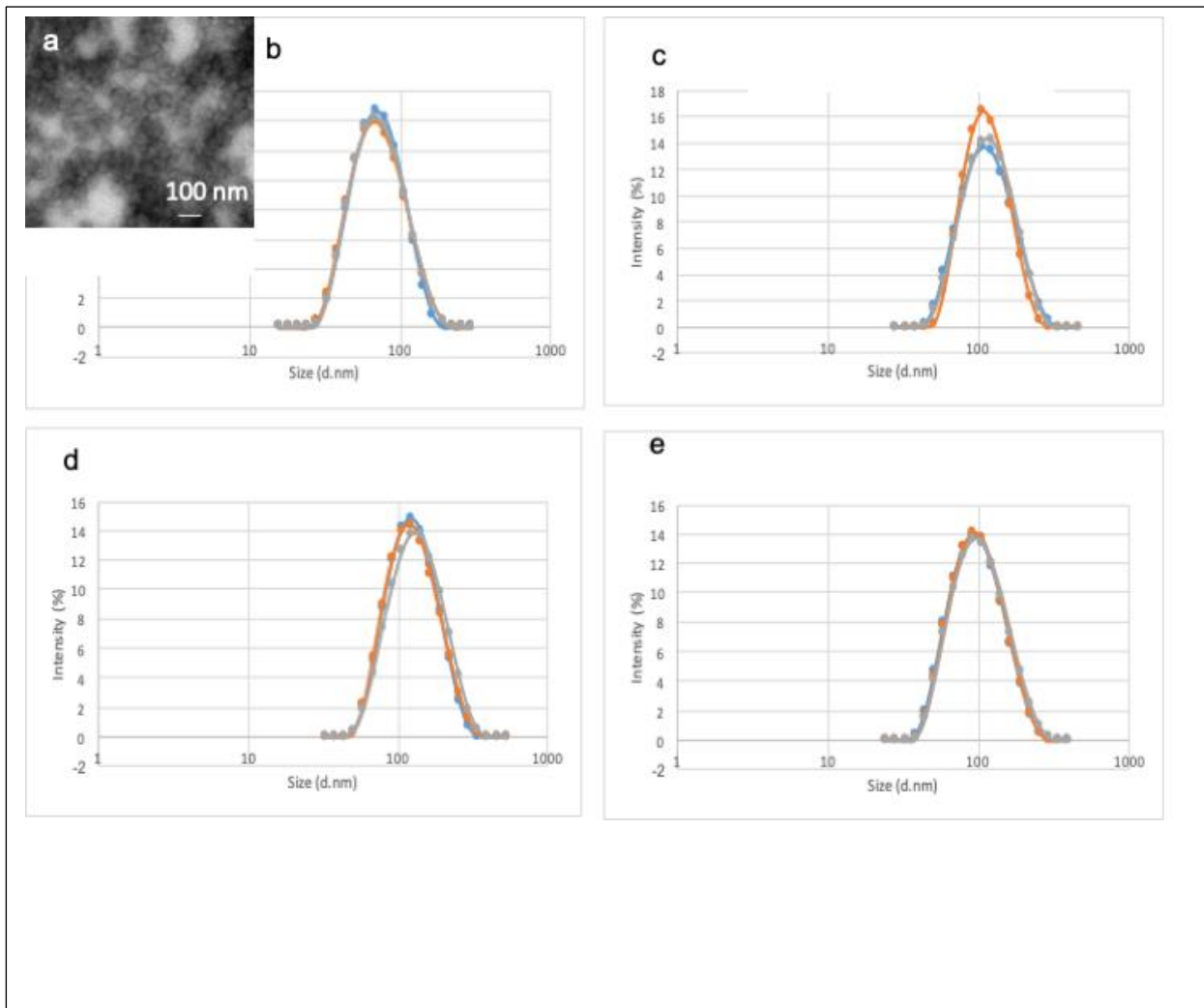
Figure 3: MET – MPA (0.1%) formulation stability: a) transmission electron micrograph with negative staining after storage for 7 days at 40 ℃, b) size distribution at Day = 0, c) size distribution on storage for 1 week at room temperature (18 – 25 ℃, d) size distribution on storage for 1 week at refrigeration temperature (2 – 8 ℃), e) size distribution on storage for 1 week at 40 ℃, n = 3 separate preparations.

The particulate formulation was prepared and the storage stability studied at three storage conditions (refrigeration temperature, room temperature and 40°C) over a two week period (Figure 2 and Figure 3). Over the two week period there was no change in the drug content under any of the storage conditions (Figure 2a). The particle size increased from about 65 nm to 100 nm (Figure 2b and Figure 3) but the polydispersity remained stable (0.1 – 0.15, data not shown). The formulations when first prepared were clear but become opalescent on storage. It is not clear why these kinetic aspects were evident. Spherical drug filled particles were seen in the electron micrograph (Figure 3a). MPA has a water solubility of 36 µg mL^{-1} hence a nanoparticle formulation containing 1mg mL^{-1} MPA and devoid of crystals (Figure 3a) or a sediment (Figure 3) comprises drug filled particles. There was very little change in the pH and osmolarity with any changes falling within 10% of the starting values, a ~ 20% change in the particle zeta potential when the formulation was stored at 40°C and a ± 15% fluctuation in the viscosity over the time period at all storage conditions (Figure 2).

4. Discussion

MPA is an immunosuppressive agent available in two oral formulations: as an ester prodrug or as a sodium salt[2-5]. Mycophenolate mofetil (MMF, CellCept) is the 2-morpholinoethyl ester prodrug of MPA that was initially formulated to improve its bioavailability, however more recently the enteric-coated mycophenolate sodium (EC-MPS, Myfortic) has been formulated. As the systemic use of MPA is associated with gastrointestinal and bone marrow suppression side effects [4,7-10] and MPA is useful in the treatment of ocular conditions [6-8], we decided to prepare a topical eye drop nanoparticle formulation and assessed the short term stability (14 days, Table 8, Figure 2 and Figure 3) of this formulation with a validated drug substance assay.

The formulation was a nanoparticle formulation containing 0.1%w/v MPA and prepared using Nanomerics' MET [13,14], in which the hydrophobic MPA (aqueous solubility = 36 µg

mL^{-1}) is essentially encapsulated in 60 - 100 nm polymeric nanoparticles [14] (Figure 2 and Figure 3) at a neutral pH [17] and with a suitable osmolarity for ocular use (~ 300 mOsm L^{-1}) [18]. This is the first report of a mycophenolic acid eye drop nanoparticle formulation. Nanomerics' MET was chosen as a formulation excipient as this technology is known to serve as a topical ocular penetration enhancer [14]. The assay of the drug substance MPA was validated according to ICH [15] and US FDA [16] guidelines. The assay procedure was derived from a published HPLC method used to quantify MPA in plasma samples [19]. The validated assay on the drug substance in solution and the drug substance within the formulation matrix passed the criteria outlined for: system suitability (a measure of the efficacy of the equipment in analysing the analyte, Table 2), specificity (a measure of the ability of the method to determine the analyte in the presence of other agents, Figure 1), linearity and limits of quantification/ detection (a measure of the linearity of the calibration standards and a definition of the analyte quantity boundary conditions of the assay, Table 3), accuracy (a measure of the relationship of the analytical result to the real data, Table 4 and Table 7) and precision (a measure of the relationship of the analytical result to the real data over time, Table 5 and

Table 6). Assay validation is usually carried out by adopting pre-defined criteria for the

robustness of the assay and then a qualification of the pre-defined parameters using the assay

results. These analytical procedures are critical components of the supporting

documentation submitted to regulators in new drug applications [1,2].

The storage stability was a preliminary study and did not follow ICH guidelines as humidity

was not controlled. There were no changes in drug content in the formulation over 14 days at

all storage temperatures (Table 9), but there were changes in the nanoparticle size at all

storage temperatures, the zeta potential and pH when stored at 40°C and both the viscosity

and osmolarity at all storage temperatures (Table 9).   Most formulation characteristics were

within the acceptable comfort range for ophthalmic formulations: pH = 6.6 – 7.8 [17],

osmolarity = 270 – 340 mOsm L^{-1} [18], viscosity < 25 mPa.s [20] over the storage period. The

changes to particle size occurred at the initial storage point with the particle size stabilising

thereafter at about 100 nm (Table 9). There are no acceptable particle size ranges for

nanoparticle eye drop formulations at present and the MET formulations do not cause eye

irritation [14]. This promising data make it clear that this MPA formulation warrants further

study. This was a preliminary stability study. In order to progress this formulation, longer

term (24 month) stability studies will have to be conducted to ICH standards.

Table 9: Effects of Storage on the MPA Formulation Parameters

Storage Temperature	18 - 23°C	5 - 6°C	40°C
Drug Content	no change	no change	no change
Particle Size	increased	increased	increased

Storage Temperature	18 - 23°C	5 - 6°C	40°C
Viscosity	no change	< 20% increase	< 20% increase
Zeta Potential	no change	no change	< 20% increase
pH	no change	< 0.2 pH points increase	< 0.2 pH points decrease
Osmolarity	< 10% increase	< 10% increase	no change

5. Conclusions

For the first time MPA eye drops have been formulated and found to be stable over a 2 week period on storage, with stability defined as the formulation characteristics not exceeding pre-defined ranges relevant to ocular comfort and product quality. The drug substance was analysed with a newly validated assay, which was validated according to ICH and US FDA guidelines. Based on the data produced we conclude that this formulation warrants further study.

5.1 Current and future developments

Our data show that the analytical validation of a nanomedicine eye drop formulation is fairly straight forward and may be carried out to ICH standards.

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