

Development and Validation of a HPLC Method for the Determination of Cyclosporine A in New Bioadhesive Nanoparticles for Oral Administration

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Pecchio, *et al.*: HPLC Method to Quantify Cyclosporine A in Nanoparticles

A simple and reliable high performance liquid chromatography method was developed and validated for the rapid determination of cyclosporine A in new pharmaceutical dosage forms based on the use of poly (methylvinylether-co-maleic anhydride) nanoparticles. The chromatographic separation was achieved using Ultrabase C₁₈ column (250×4.6 mm, 5 μm), which was kept at 75°. The gradient mobile phase consisted of acetonitrile and water with a flow rate of 1 ml/min. The effluent was monitored at 205 nm using diode array detector. The method exhibited linearity over the assayed concentration range (22-250 μg/ml) and demonstrated good intraday and interday precision and accuracy (relative standard deviations were less than 6.5% and the deviation from theoretical values is below 5.5%). The detection limit was 1.36 μg/ml. This method was also applied for quantitative analysis of cyclosporine A released from poly (methylvinylether-co-maleic anhydride) nanoparticles.

Key words: Cyclosporine A, HPLC-UV, nanoparticles, poly (methylvinylether-co-maleic anhydride), oral administration

Cyclosporine A (CYA) is a cyclic undecapeptide (fig. 1) with a potent immunosuppressive activity that is widely used to prevent transplanted organ rejection^[1]. CYA is also employed for the treatment of various autoimmune diseases^[2]. However, its oral absorption remains limited due to its high lipophilicity, low intestinal permeability, P-glycoprotein efflux from the enterocytes^[3] and extensive presystemic metabolism in the gut wall and liver^[4-6].

Colloidal drug carriers such as nanoparticles have been developed to improve oral bioavailability of drugs that are either poorly absorbed or are susceptible to gastrointestinal degradation^[7,8]. Hence, the formulation of CYA as nanoparticles has received much attention in recent years as a suitable alternative system for drug delivery^[9-17]. In this context, we have developed poly(methylvinylether-co-maleic anhydride) (PVM/MA) nanoparticles loaded with

CYA, in order to evaluate their ability as oral carriers. This synthetic copolymer (fig. 2) is widely used for pharmaceutical purposes as a thickening and suspending agent, denture adhesive and adjuvant for transdermal patches^[18]. PVM/MA nanoparticles have revealed an exceptional ability to establish bioadhesive interactions within the gut^[19].

Since the current research involves the development and evaluation of new pharmaceutical dosage forms for oral administration of CYA based on the use of the PVM/MA nanoparticles, the first important step in this project was to have a suitable analytical method to quantify the drug in these nanoparticulate systems. Several assays have been described for the measurement of this drug; some of them are based on radioimmunoassay (RIA)^[20,21]. This technique does not represent a viable analytical method to quantify the drug in routine analysis of quality control (QC) of CYA in pharmaceutical forms. Others analytical methods are based on high performance liquid chromatography (HPLC) coupled with mass-spectrometry^[22-25], however, it is a very

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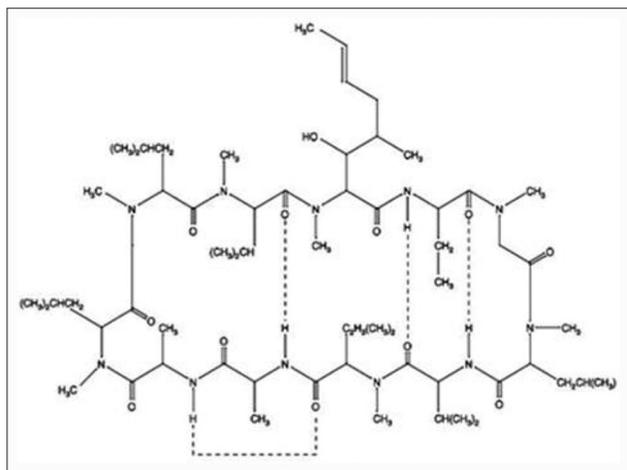


Fig. 1: Chemical structure of CYA.
CYA is cyclosporine A.

expensive equipment. Many methods reported in literature for assaying CYA are based on the HPLC with ultraviolet detection (UV)^[13,26-33]. Some of them have been successfully applied to assay CYA in pharmacokinetics studies and others to analysis of the drug in different pharmaceutical preparations. However, the chromatographic conditions of these methods were not suitable to quantify unequivocally CYA loaded in PVM/MA nanoparticles; the polymer interfered with the peak of the drug so these methods lacked selectivity to quantify CYA in the samples of this study. The difficulty with all HPLC-UV methods is related to CYA's lack of chromophores that imply the use of short-wavelength ultraviolet light detection (e.g. 205, 210 nm), and many molecular species absorb energy at this wavelength.

Therefore, the aim of the present study was to develop a simple and reliable HPLC-UV method to quantify the CYA incorporated into PVM/MA nanoparticles. Additionally, the analytical method was applied to assay the drug released from these nanoparticulate systems.

MATERIALS AND METHODS

CYA (ref. L-0602070 AN0617424) was provided by Roig Parma S.A. (Barcelona, Spain). PVM/MA (Gantrez[®] AN 119; MW 200 000) was kindly gifted by ISP (Barcelona, Spain). Acetone and ethanol (analytical grade) were purchased from VWR Prolabo (Fontenay Sous Bois, France), acetonitrile (ACN) and ethanol (HPLC grade) were supplied by Merck (Darmstadt, Germany). Deionised reagent water (18.2 MΩ cm resistivity) was prepared

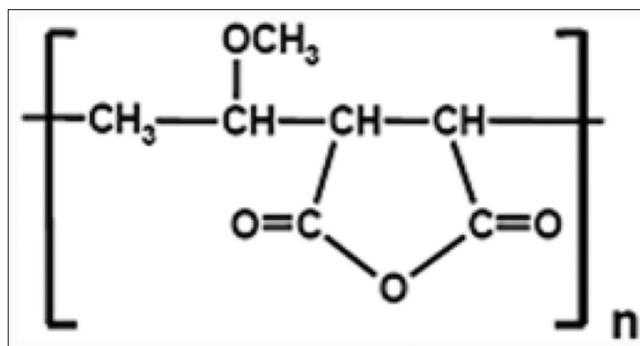


Fig. 2: Chemical structure of PVM/MA.
PVM/MA is poly(methylvinylether-co-maleic anhydride).

in our laboratory using a water purification system (Wasserlab, Pamplona, Spain).

Stock solutions of CYA (0.50 and 0.35 mg/ml) were prepared by dissolving 5.0 and 3.5 mg of the drug in 10 ml of ethanol:water (1:1, v/v), containing 100 and 40 mg of PVM/MA, respectively. The matrix to prepare standard curve and QC samples was the same as the nanoparticle samples. The stock solutions were stored at 4°. The standard calibration samples required for constructing calibration curve and QC samples were prepared by dilution from stock solutions with appropriate volumes of the ethanol:water (1:1, v/v). Although it was found that these solutions were stable for long periods, we preferred preparing them freshly each day.

Chromatographic system:

HPLC analysis was performed using a Hewlett Packard 1050 (Waldbronn, Germany) system equipped with a quaternary pump, an autosampler, a heated column compartment and a diode-array detector. System management and data acquisition were performed by the HP ChemStation 3D software used with a HP personal computer.

Chromatographic separation of the CYA from potentially interfering substances was achieved at 75° on an Ultrabase-C₁₈ column (250×4.6 mm, 5 μm particle size) (Scharlau, Barcelona, Spain), preceded by an ODS guard column (Teknokroma, Barcelona, Spain). The mobile phase consisted of ACN and deionised water (W) and was used as a gradient mode as follows: 80% ACN to 100% ACN over a period of 1 min and 100% ACN isocratic during 8 min. Then the gradient was returned to 80% ACN to prepare for the next run. The mobile phase flow rate was 1 ml/min and the effluent was monitored at 205 nm.

Method validation:

The method was validated according to the guidelines based on the International Conference on Harmonisation for the validation of analytical procedures^[34]. The parameters, which were used to validate the analytical method, were selectivity, limit of detection (LOD), limit of quantitation (LOQ), linearity, precision and accuracy.

The selectivity of the assay was determined by the individual analysis of blank samples. The retention time of the polymer used in the preparation process of those particulate systems was compared with that of CYA. Under the chromatographic conditions used, no peaks with retention times similar to that of CYA must be observed. LOD and LOQ were estimated from the signal-to-noise ratios. The detection limit was defined as the lowest concentration level resulting in a peak area of three times the baseline noise. The quantitation limit was defined as the lowest concentration level that provided a peak area with a signal-to-noise ratio higher than 10, with a precision and accuracy less than 10%.

The calibration curve of CYA was established from eight standard calibration samples over the 22-250 µg/ml concentration range. Calibration curve was done in triplicate. Peak areas of CYA versus the corresponding drug concentrations were plotted. Calibration curves were determined by least square linear regression analysis. Moreover, the coefficient of variation of the factors of response (linearity test) was calculated in order to confirm the linearity. This value must be less than 10%.

The intraday and interday precision were determined by analysing six replicates of QC samples at concentrations of 31.25, 62.5, 125 and 250 µg/ml on the same day and on six different days, respectively. The variability was expressed as the percentage of the relative standard deviation (%RSD) of replicate measurements. To be acceptable, the values should be less than 10% at all concentrations. Accuracy of the assay method was defined as the percentage of the systematic error, which was calculated as the agreement between the measured value and the true value as follows, % accuracy = $(C_{\text{obs}} - C_{\text{theor}} / C_{\text{theor}}) \times 100$, where C_{obs} is the determined concentration of a QC and C_{theor} is the theoretical concentration. To be acceptable, the values should be less than 10% at all concentrations. Accuracy was calculated from the same samples as those used for precision studies.

Preparation of CYA-loaded PVM/MA nanoparticles:

PVM/MA nanoparticles were prepared by the solvent displacement method previously described by Arbos *et al.*^[18] with a few modifications. Briefly, the required amounts of PVM/MA and CYA were dissolved in acetone and incubated for 60 min with constant stirring. Later, an appropriate volume of ethanol:water (10:3, v/v) was poured into the mixture. After incubating for 20 min at 300 rpm, the organic solvents were eliminated under reduced pressure and a suitable volume of ethanol was added to the aqueous suspension. The cyclosporine nonencapsulated was present in the supernatant. The resulting nanoparticles were purified twice by ultracentrifugation at $27\,141 \times g$ for 20 min and dried by lyophilisation. In the same way, empty nanoparticles were also prepared. The particle size and the zeta potential of nanoparticles were determined by photon correlation spectroscopy (PCS) and electrophoretic laser Doppler anemometry, respectively, using a Zetamaster analyser system (Malvern Instruments, UK).

Application of the method:

This HPLC-UV method was applied to the determination of CYA content in PVM/MA nanoparticles as well as to *in vitro* drug release studies. For analysis, nanoparticles were solubilised with ACN (1:4, v/v). The sample was filtrated on 0.45 µm filters and an aliquot (100 µl) was injected into the HPLC. The analysis was done in triplicate and the results were expressed as the drug loading, calculated as follows, drug loading = amount of CYA in nanoparticles (µg)/PVM/MA nanoparticles (mg). The amount of PVM/MA transformed into nanoparticles was calculated by gravimetry^[18].

Moreover, the encapsulation efficiency (%EE) of CYA in the nanoparticles was calculated as $(Q_{\text{associated}} / Q_{\text{initial}}) \times 100$; where $Q_{\text{associated}}$ is the amount of entrapped CYA into the particles and Q_{initial} is the initial amount of CYA added to the formulation.

The *in vitro* release of CYA from PVM/MA nanoparticles was carried out under sink conditions in two different media: gastric (SGF) and intestinal (SIF) simulated fluids (pH 1.7 and 6.8, respectively). These studies were conducted independently. Each study was performed at $37 \pm 1^\circ$ under agitation in a thermostated water bath (Unitronic-Orbital, J. P. Selecta, Barcelona, Spain) after the dispersion of CYA-nanoparticle

formulation with the appropriate medium (SGF or SIF). Under these experimental conditions, an exact amount of nanoparticles, sufficient for establishing sink conditions, was resuspended in 15 ml of the assayed medium. Eight samples were prepared and incubated for 4 h. Each study was performed in triplicate. At different time intervals (5, 30, 60, 90, 150, 180, 210 and 240 min) samples were centrifuged ($27\ 141\times g$, 20 min, 4°) and CYA in the supernatant was determined by proposed HPLC method. The release rate profiles were drawn as the percentage drug released from the nanoparticles versus time.

RESULTS AND DISCUSSION

The HPLC method was developed to provide a specific procedure for the rapid determination of CYA in PVM/MA nanoparticles. During the preliminary investigations, a Zorbax Eclipse XBD- C_8 and Ultrabase C_{18} columns were tested for the selection of the most appropriate. In order to shorten the retention time of CYA and to improve peak symmetry, different mobile phases like ACN:water or ACN:water:isopropanol, different ratios, isocratic or gradient elutions, flow rates, water pHs, and column temperatures were studied. Taken into consideration the preliminary results, we decided to use the Ultrabase C_{18} column; its sensitivity and resolution was better when compared with the Zorbax Eclipse XBD- C_8 column. In this sense, optimal separation was obtained using ACN:water as mobile phase, the gradient elution previously described, a flow-rate of 1 ml/min and the column temperature set at 75° .

Under the assay conditions described, the retention time for CYA was around 6.5 min and the analysis time was less than 10 min, which make this method suitable for analysing a large number of samples in a short period of time. The method demonstrated excellent chromatographic selectivity; the PVM/MA present in experimental nanoparticles does not interfere with the drug peak. In addition, good peak symmetry and minimal band spreading were obtained. Representative chromatograms are shown in (fig. 3).

LOD and LOQ values of the drug with the developed HPLC method were 1.36 and $2.35\ \mu\text{g/ml}$, respectively. The quantitation limit was confirmed, in separate experiments, using five calibrators with nominal

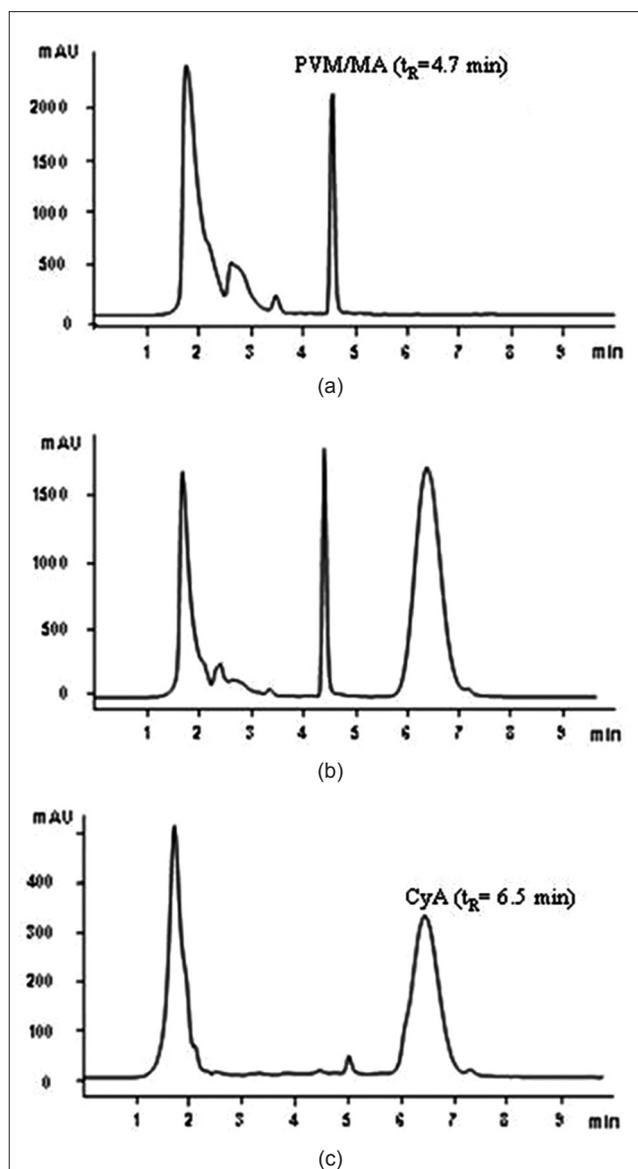


Fig. 3: Chromatograms of drug, blank and drug-loaded nanoparticles. Chromatograms obtained after processing a drug-free PVM/MA solution (a), a calibration standard containing $250\ \mu\text{g/ml}$ of CYA (b) and PVM/MA nanoparticles loading CYA (c). t_R is retention time, PVM/MA is poly(methylvinylether-co-maleic anhydride) with t_R of 4.7 min, CYA is cyclosporine A with t_R of 6.5 min.

concentrations of $2.35\ \mu\text{g/ml}$. The %RSD of replicate measurements and the accuracy value were below 7.5%.

To quantify CYA in the different samples obtained in this work, a calibration curve over the $22\text{--}250\ \mu\text{g/ml}$ range was adequate although LOQ was $2.35\ \mu\text{g/ml}$. The method exhibited linearity between the chromatographic response (y) and the corresponding concentration of CYA (x) over this concentration range (typical Eqn.: $y=247.79x+0.072$). Furthermore, linear regression analysis showed

correlation coefficient higher than 0.999 and the coefficient of variation of the response factors was less than 5%.

Though the use of internal standards in HPLC analyses generally improve the accuracy and precision of the assay, in this case was not necessary. Accuracy values were within acceptable limits; the deviation from theoretical values was less than 5.5% at all concentrations levels studied (Table 1). The results for intraday and interday precision (Table 1) were also acceptable; RSDs were less than 6.5% for all samples over the concentration range assayed.

The reported method was successfully applied to the determination of CYA content in PVM/MA nanoparticles. After analysis of the samples, it was found that the drug loading was 3.3 ± 0.56 $\mu\text{g}/\text{mg}$ of PVM/MA nanoparticles and the %EE was $4.7 \pm 1.70\%$. In contrast, these nanoparticles displayed a size of about 200 nm, close to that measured for empty nanoparticles, and the particle size distribution was quite homogeneous showing low polydispersity.

Additionally, the developed method was applied to assay the drug released from PVM/MA nanoparticles. After incubation in SGF and SIF, the *in vitro* release of CYA from these nanoparticulate systems showed an initial soft burst effect (fig. 4). In the first 5 min, the drug released in SGF was about 30% and remained constant over 4 h. In parallel, drug released in SIF at 5 min was 20% and 4 h later this percentage was 42%.

Presently, this experimental formulation is being optimised in terms of drug loading by testing different excipients such as cyclodextrins and polyethylene glycol. Importantly, we found that under the chromatographic conditions described, these substances do not interfere with the drug peak. Therefore, the developed method could be applied to quantify CYA in these new nanoparticles.

In conclusion, the method described is sensitive enough for the quantitative determination of CYA loaded in PVM/MA nanoparticles as well as the released drug from them. Moreover, simplicity and efficacy of the assay as well as the relatively short retention time of the drug permit the analysis of a large number of samples in a short time, making it a fast and inexpensive method.

TABLE 1: INTRADAY PRECISION, ACCURACY AND INTERDAY PRECISION OF THE HPLC ASSAY FOR CYA

True value (CYA, $\mu\text{g}/\text{ml}$)	Intraday precision and accuracy			Interday precision	
	Measured value (mean \pm SD)	% RSD	% Accuracy	Measured value (mean \pm SD)	% RSD
250.00	256.0 \pm 11.6	4.5	102.4	254.0 \pm 14.2	5.6
125.00	129.0 \pm 6.3	4.8	103.2	133.0 \pm 7.9	6.0
62.50	65.8 \pm 2.9	4.4	105.3	67.1 \pm 4.2	6.2
31.25	32.0 \pm 1.8	5.8	102.4	33.6 \pm 1.6	4.7

CYA is cyclosporine A, SD is standard deviation for $n=6$ observations, RSD is relative standard deviation

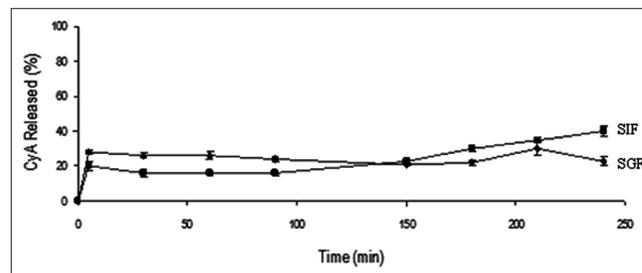


Fig. 4: CYA release profile from PVM/MA nanoparticles in SGF and SIF.

CYA release profile from PVM/MA nanoparticles after incubation in simulated gastric and intestinal fluids at 37°. Data represented as mean \pm SD ($n=3$). CYA=cyclosporine A, PVM/MA=poly(methylvinylether-co-maleic anhydride), SGF=simulated gastric fluid, SIF=simulated intestinal fluid, SD=standard deviation for $n=3$ observations.

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