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Research Article

Development and Validation of a HPLC Method for Dissolution and Stability Assay of Liquid-Filled Cyclosporine Capsule Drug Products

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Abstract. To assay the dissolution samples of a drug product from several sources, a simple but broadly applicable analytical method is always desired. For the liquid-filled cyclosporine capsules, while analyzing the dissolution samples, the current compendial and literature HPLC methods have been found to be inadequate to provide satisfactory separation of the drug and the excipient peaks. Accordingly, a suitable isocratic reverse-phase HPLC method was developed for the analysis of dissolution samples of liquid-filled cyclosporine capsules. The method successfully separated the cyclosporine peak from the interfering chromatographic peaks of the excipients. The method was validated according to the ICH and FDA guidelines. Specificity, selectivity, linearity, accuracy, precision, and robustness were established over 3 days as part of method validation. Additionally, the degradation kinetics of cyclosporine in dissolution media was determined. Cyclosporine degradation followed a zero-order kinetics in the dissolution media with the respective rate constants of −3.5, −1.5, and −0.3%/h at 37°C, 25°C, and 10°C.

KEY WORDS: cyclosporine; degradation; dissolution; HPLC; liquid-filled capsules.

INTRODUCTION

Cyclosporine (also known as cyclosporine A) is an immunosuppressant that has been a drug of choice for the prevention of graft rejection in organ transplant patients since 1980s (1). It reduces the activity of the immune system by interfering with the activity and growth of T cells (2). Currently, cyclosporine is marketed as liquid-filled capsules and solution for oral administration, solution for injection, and emulsion for ophthalmic administration (3). Despite its high immunosuppressive efficacy, cyclosporine has a low oral bioavailability attributed to its dissolution-limited absorption (due to the low water solubility (6.6 μg/mL in water at 37°C (4))), first-pass metabolism, intestinal degradation, *etc.* (5,6). For example, it has been reported that factors such as gastric emptying, gastrointestinal motility, pre-systemic metabolism, and intestine bile salt concentration all greatly influenced cyclosporine oral bioavailability (7–9). To alleviate the problem, the drug has been formulated into various dosage forms, including suspension, emulsion and microemulsion formulations to enhance its dissolution rate and solubility in order to increase the bioavailability (10–13).

The most challenging aspect of HPLC analysis for cyclosporine is the broadening of the cyclosporine peak (largely attributed to the presence of several conformational isomers of cyclosporine (14)), which leads to poor resolution of the drug peak from the other co-eluting peaks (5). This became an issue for several cyclosporine liquid-filled soft capsules dissolved in dissolution media, where presence of surfactants, ethanol, and oil in the formulation as well as the surfactant in the dissolution media resulted in co-eluting peaks. Using the current compendial HPLC methods, these co-eluting peaks cannot be separated from the drug peak of several liquid-filled cyclosporine capsule drug products (Fig. 1). Previously reported methods (15) also failed to resolve cyclosporine and excipient peaks. In addition to the separation efficiency, it is preferred that the method be simple and isocratic rather than a gradient method (16), which would reduce the analysis time.. Accordingly, a suitable isocratic reverse-phase HPLC method was developed to analyze the dissolution samples of liquid-filled cyclosporine capsules.

To maintain appropriate sink condition throughout the dissolution process and to mimic the gastric environment, 0.1 N hydrochloric acid solution containing 0.5% *w*/*v* SDS was used as the dissolution media. Under the acidic environment, cyclosporine may degrade (5). Without the prior knowledge of cyclosporine degradation, in the dissolution media, sample loss due to degradation remains unaccounted. This may result in an underestimation of the released drug amount. For this reason, the degradation kinetics study of cyclosporine in the presence of dissolution media was also performed, at three relevant temperatures (37°C for during dissolution, 25°C for during room temperature storage, and 10°C for during HPLC analysis).

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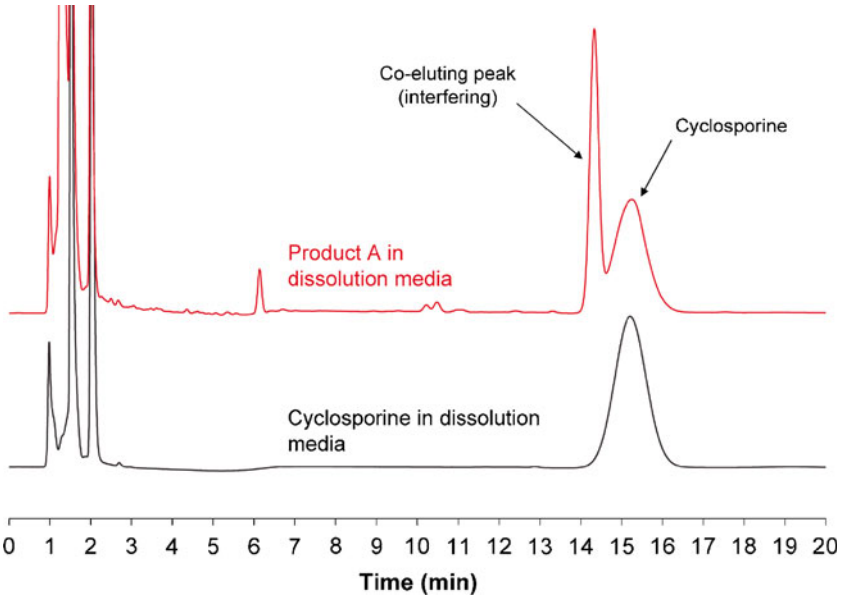

Fig. 1. Chromatograms of Cyclosporine and Product A Capsule Dissolved in Dissolution Media (0.1 N HCl+0.5%*w/v* SDS) using the USP Method (Isocratic Elution with Acetonitrile, Water, Methanol and Phosphoric Acid (900:450:50:0.5, *v/v/v/v*))

MATERIAL AND METHODS

Materials

Cyclosporine reference standard was purchased from the United States Pharmacopeia (Rockville, MD, USA). Four liquid-filled cyclosporine capsule drug products, referred to as Products A to D, respectively, were purchased from a local pharmacy. Nylon syringe filters were purchased from Millipore Corp. (Bedford, MA, USA). ACS grade phosphoric acid, ACS grade hydrochloric acid (HCl), HPLC-grade acetonitrile and HPLC-grade methanol were purchased from Fisher Scientific (Fairlawn, NJ, USA). HPLC-ready DI water was obtained, in-house, from a Milli-Q Gradient A-10 water purification system, Millipore Corp. (Bedford, MA, USA).

Chromatographic Conditions

The HPLC system consisted of a Hewlett Packard 1100 series (Agilent Technologies, Wilmington, DE, US) equipped with a quaternary pump, online degasser, column heater, autosampler and UV/Vis detector. Data collection and analysis were performed using ChemStation (Agilent Technologies). Separation was achieved on a Luna C8(2) 3 μm, 4.6× 150 mm column protected using a Phenomenex security guard cartridge (Phenomenex, Torrance, CA, US). The elution was isocratic at 1.0 mL/min with a mobile phase of acetonitrile–water–phosphoric acid (750:250:1, *v/v/v*). The column temperature was maintained at 60°C in a column oven, and autosampler was maintained at 10°C. The injection volume was 20 μL and detection was by UV at 210 nm.

Preparation of Cyclosporine Standards

Cyclosporine stock solution I of 200 μg/mL was prepared in mobile phase using the USP cyclosporine reference standard. Calibration standard solutions at seven levels were prepared daily by diluting the stock solution I to concentrations of 2, 5, 15, 30, 60, 100, 140 μg/mL.

Preparation of Cyclosporine Quality Control Standards

Cyclosporine stock solution II of 200 μg/mL was prepared in mobile phase using the USP cyclosporine reference standard. Quality control (QC) standard solutions were prepared by diluting the stock solution II to concentrations of 5, 60, and 100 μg/mL.

Preparation of Cyclosporine System Suitability Standards

System suitability standard solution which contained 100 μg/mL cyclosporine was prepared by diluting cyclosporine stock solution I with mobile phase.

Preparation of Cyclosporine Dissolution Standard

Cyclosporine stock solution III of 100 μg/mL was prepared in dissolution media containing 0.1 N HCl and 0.5% w/v sodium dodecyl sulfate (SDS) using the cyclosporine drug substance.

Method Validation

Validation was carried out according to ICH and FDA guidelines for the chromatographic method. The following validation

characteristics were established: linearity, range, accuracy, precision, specificity, limit of quantitation, and robustness.

Method Validation: System Suitability

System suitability was determined from ten replicate injections of the system suitability standard before sample analysis. The acceptance criteria were less than 2% relative standard deviation (%RSD) for peak area, greater than 700 column plates and USP tailing factor of less than 2.0.

Method Validation: Linearity and Range

Seven calibration samples were prepared over a concentration range of 2–140 μg/mL for cyclosporine. The peak areas *versus* drug concentration were subjected to least square linear regression analysis. The obtained standard curves were evaluated for intra- and inter-day linearity. The range was the interval between the highest and lowest concentration of analyte where acceptable linearity, accuracy and precision were obtained.

Method Validation: Accuracy and Precision

Accuracy and precision of the method were determined by analyzing quality control standards at three cyclosporine concentrations (5, 60, 100 μg/mL). The method precision was established by injecting six standard QC samples at each concentration level for the intra-day precision and on 3 days for the intermediate precision. Precision was expressed as the %RSD of the analyte concentration. Accuracy was established by evaluating the amount determined from the quality control standards and comparing to the respective nominal value expressed as percent recovery.

Method Validation: Limit of Quantitation

The limit of quantitation for cyclosporine is the lowest concentration where acceptable accuracy and precision were obtained. As per ICH guideline (17), limit of quantitation (LoQ) was determined based on the standard deviation of the response and the slope. The quantitation limit is expressed as:

$$\mathrm{LoQ} = \frac{10.\sigma}{S}$$

where σ is the residual standard deviation of the regression line, and S is the slope of the calibration curve of the analyte.

Method Validation: Robustness

The robustness of the method was evaluated by analyzing the system suitability standard and evaluating system suitability parameter data after varying, individually, the HPLC pump flow rate (±10%), autosampler injector volume (±50%) and column compartment temperature (±2°C).

Method Validation: Specificity

Specificity of the method was determined by analyzing samples containing the drug products and cyclosporine drug substance. All chromatograms were examined to determine if cyclosporine co-eluted with any excipient peak.

Cyclosporine Degradation Kinetics in Dissolution Media

The degradation kinetics of cyclosporine in dissolution media was determined at three different temperatures (10°C, 25°C, and 37°C) by analyzing the concentration change of cyclosporine over a period of time using the newly developed and validated HPLC method. For samples to be tested at 10°C and 25°C, various HPLC vials each containing 1 mL of cyclosporine stock solution III were loaded onto HPLC autosampler, and temperature was controlled using the autosampler thermostats. The injection intervals (run time) were adjusted accordingly so that at predetermined time samples were injected directly for cyclosporine concentration analysis. For 37°C stability samples, seven HPLC vials each containing 1 mL of cyclosporine stock solution III were put inside an oven chamber maintained at 37±0.5°C. Every 15 min, one HPLC vial was taken and loaded onto the HPLC autosampler for analysis (10 min equilibration at 10°C before each injection). The degradation rate constants were determined after normalization (against the initial concentration) and were reported as percentage decrease per hour.

Dissolution Test

A calibrated dissolution apparatus (USP I) was utilized with basket at 150 rpm and 37±1°C. One thousand milliliters of freshly prepared and degassed 0.1 N hydrochloric acid solution containing 0.5% *w/v* SDS was used as the dissolution media. Six capsules were evaluated and 1 mL samples were collected at 5, 10, 20, 30, 45, 60, 90, and 120 min. One sample of the dissolution medium was also tested as the blank sample representing the sample at 0 min time point. Samples were removed from the vessels with the autosampler and filtered through a nylon filter (0.45 μm, 25 mm) into labeled glass vials for direct analysis using the newly developed and validated HPLC method. Calibration curves were generated by plotting the concentration of the calibration standard (0.1 mg/mL cyclosporine dissolved in mobile phase) *versus* its chromatographic peak area which gave the following the equation (one point calibration):

$$\text{Peak area (for standard)} = \text{Slope} \times \text{Concentration of standard}\left(\mathrm{mg}/\mathrm{mL}\right)$$

The amount of cyclosporine in the test samples was calculated, as % released, from the measured cyclosporine peak area using the following equation:

$$\%\text{Released} = \frac{100}{\text{Drug load(mg)}} \times \frac{\text{Peak area(for sample)}}{\text{Slope}} \times 1000(\text{ml})$$

where *Slope* value was derived from the calibration curve and *Drug Load* was 100.

Correction of the Dissolution Data Using Degradation Constant

To account for degradation-related cyclosporine loss during dissolution, Percentage of drug release was corrected using

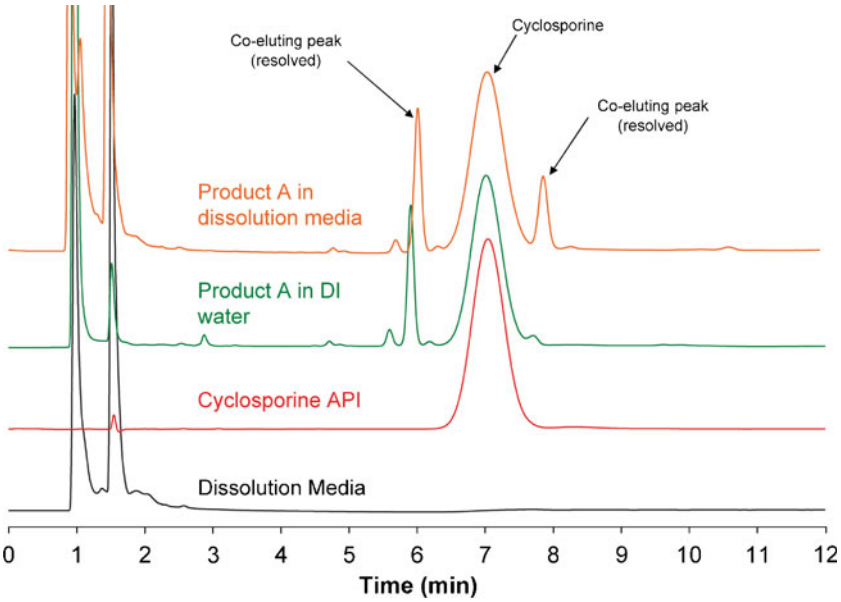

Fig. 2. Chromatograms of Dissolution Media, Cyclosporine (in Mobile Phase), and Product A Capsule Dissolved in DI Water and Dissolution Media, using the Newly Developed Method

the Correction Coefficient:

$$\%\text{Released(corrected)} = \%\text{Released} \times \text{Corr.Coeff.}$$

The Correction Coefficient was calculated for each dissolution sample based on the time and temperature the samples were stored at, using the following equation:

$$\text{Corr.Coeff.} = \frac{100}{(100 - t_{10} \times k_{10})} \times \frac{100}{(100 - t_{25} \times k_{25})} \times \frac{100}{(100 - t_{37} \times k_{37})}$$

Where, k_{10}, k_{25}, k_{37} were degradation rate constants (in per minute) for cyclosporine in dissolution media at 10, 25, and

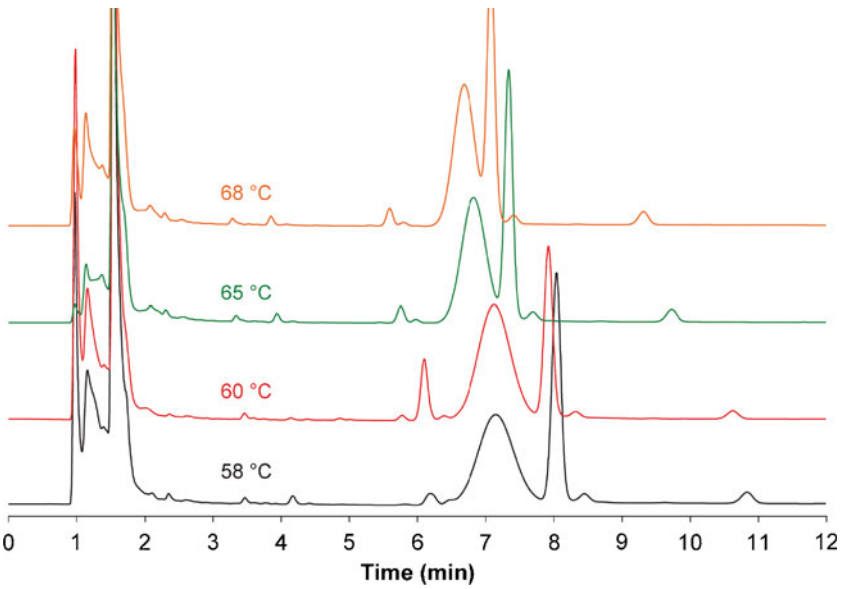

Fig. 3. Effect of Column Temperature on Separation of the Peaks for Product A Product Dissolved in Dissolution Media

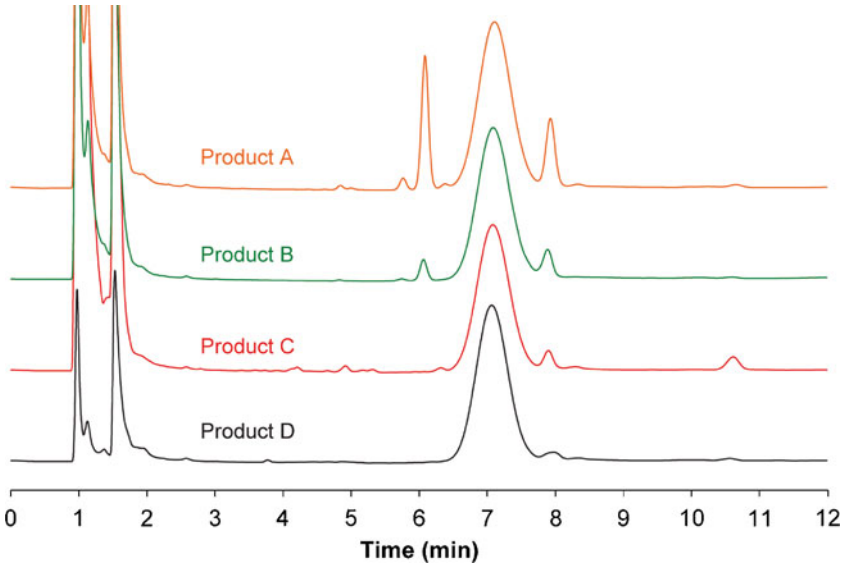

Fig. 4. Chromatograms of Various Cyclosporine Products Dissolved in Dissolution Media using the Newly Developed Method

37°C, respectively. t_{10}, t_{25}, t_{37} were time (in minute) for dissolution samples stored on the HPLC tray (10°C), at room temperature (25°C), and in the dissolution media (37°C), respectively.

RESULTS AND DISCUSSION

Selection and Optimization of Analytical Method

Cyclosporine is a hydrophobic peptide with Log*P* of 2.9 (18). One significant feature of all of the HPLC methods, reported for the cyclosporine analysis, is the broadening of the cyclosporine peak. This leads to poor resolution of the drug peak from the other co-eluting peaks. These co-eluting peaks were absent in the pure cyclosporine samples, dissolved in dissolution media and in mobile phase.

Various combinations of chromatographic conditions were evaluated, including varying the mobile phase organic to aqueous ratio, mobile phase organic modifier composition, column temperature, flow rate, *etc.* to resolve the interfering peaks from the cyclosporine peak. The separation was found

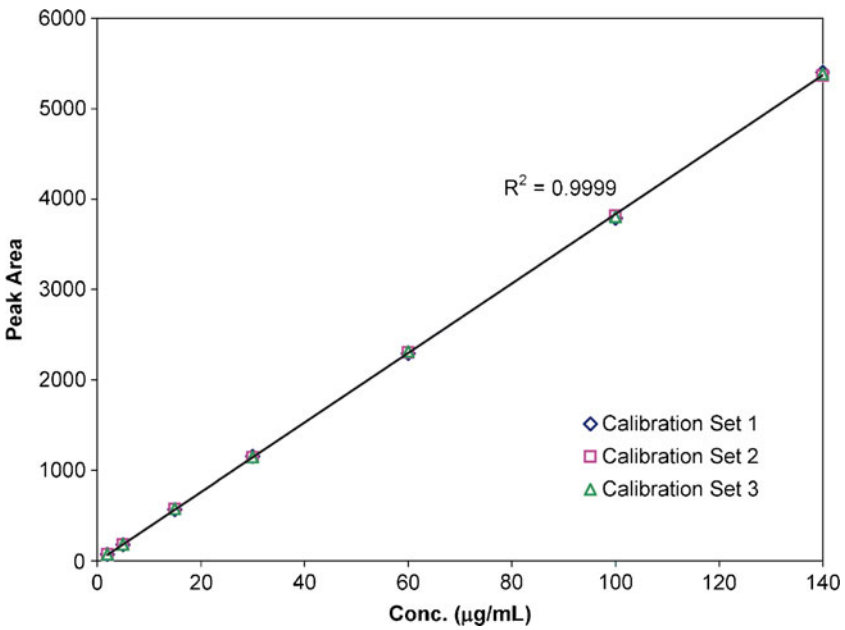

Fig. 5. Linearity Range of Cyclosporine (2–140 µg/ml)

Table I. Parameters and linearity data of cyclosporine calibration curves

Standard curve	Analytical range (μg/mL)	Calibrators	Slope	*y*-intercept	Response standard deviation	r^2
Validation set 1	2–140	7	38.45	−11.94	14.31	0.9998
Validation set 2	2–140	7	38.34	−6.72	6.29	1.0000
Validation set 3	2–140	7	38.41	−6.90	9.74	0.9999

to be very sensitive to the composition of the mobile phase, particularly the composition of the organic modifier (*i.e.* acetonitrile or mixture of acetonitrile and methanol). Best separation was obtained in the absence of methanol and when 75% acetonitrile was used (Fig. 2). Column temperature was another major factor affecting cyclosporine peak. A narrower cyclosporine peak was observed with increasing column temperature. A corresponding increase in peak overlap was also observed (Fig. 3). A decrease in column temperature resulted in slightly better peak separation but was accompanied by a decrease in plate number, which at 58°C fell below 700, the USP minimum requirement for the theoretical plates. Accordingly, 60°C was determined to be the optimal temperature for the analysis of the cyclosporine dissolution samples.

The optimized HPLC method resulted in good separation between the cyclosporine and interfering peaks for all four liquid-filled cyclosporine capsule drug products (Fig. 4). The total run time was 12 min, and the retention time of cyclosporine was 7.2±0.1 min. The new method also allowed for direct analysis of cyclosporine dissolution samples without dilution, greatly reducing the sampling and analysis time.

Validation of the Method

The following method validation characteristics were addressed for cyclosporine: linearity, range, accuracy, precision, specificity, quantitation limit, and robustness. The method was validated for accuracy, precision, specificity and quantitation limit using sets of three quality control standards. Standard calibrators were used to establish linearity and range. Robustness was established using the system suitability standard. The validation characteristics met the acceptance criteria for USP Category I.

System Suitability

The system suitability assessment for the analytical HPLC method established instrument performance parameters such as peak area %RSD, total plate number, USP tailing factor for cyclosporine. The mean (n=10) peak area %RSD was 0.26%, the mean total plate number was 962, the mean peak width was 0.55, the mean capacity factor was 3.2, the resolution for cyclosporine and neighboring peak was 1.7±0.2, and the mean tailing factor was 1.04. All critical parameters tested met the acceptance criteria on all days.

Table II. Accuracy: drug substance, expressed as % (n=6)

Sample	5 μg/mL	60 μg/mL	100 μg/mL
Validation set 1	101.8±0.2	98.9±0.1	99.3±0.2
Validation set 2	98.2±0.2	100.0±0.5	100.3±0.5
Validation set 3	99.8±0.1	100.4±0.1	100.2±0.2

Linearity and Range

Linearity of the method was established over the analytical range of 2–140 μg/mL (Fig. 5). Excellent correlation between analyte peak area and concentration of the drug was obtained with $r^2 \geq 0.999$ for all standard curves (Table I). Precision and accuracy were established for drug substance from 5 to 100 μg/mL. This range represents 5–100% dissolution for 100 mg cyclosporine drug product.

Accuracy and Precision

Accuracy and precision were established across the analytical range for the cyclosporine drug substance. The accuracy and intra- and inter-day precision were calculated from the quality control samples for cyclosporine. Results for the intra-day accuracy are summarized in Table II, while the results for the intra- and inter-day precision are summarized in Table III. Ideally, the accuracy and precision studies should also be performed on the drug products in the dissolution media. However, the cyclosporine drug substance was found to degrade over time in the dissolution media making it difficult to accurately determine its concentration in these samples. For this reason, the degradation kinetics of cyclosporine in the dissolution media were determined to account for the degradation-related drug loss during the dissolution process. Furthermore, all drug products evaluated as part of this research were commercial formulations and it was not possible to prepare the respective placebo formulations. Hence, the recovery studies were not performed for these drug products.

Limit of Quantitation and Detection

The limit of quantitation for cyclosporine based on the lowest concentration where acceptable accuracy and precision were obtained is 2 μg/mL. An estimate of the limit of quantitation based on standard deviation of the response and the slope is 2.6 μg/mL. An estimate of the

Table III. Precision: drug substance, expressed as %RSD (n=6)

Sample	5 μg/mL	60 μg/mL	100 μg/mL
Validation set 1	0.22	0.09	0.15
Validation set 2	0.19	0.54	0.47
Validation set 3	0.14	0.03	0.23
Intermediate	0.18	0.22	0.28

limit of detection based on standard deviation of the response and the slope is 0.86 μg/mL for cyclosporine.

Robustness

To ensure the insensitivity of the HPLC method to minor changes in the experimental conditions, it is important to demonstrate robustness of the method. None of the alternations caused a significant change in the peak area %RSD, USP tailing factor, theoretical plates.

Specificity

The dissolution media showed no peaks beyond the void volume while only one peak was observed for the drug substance samples (Fig. 2). However, co-eluting peaks were observed in the chromatograms of the drug products, likely from the excipients present in these drug products. Due to lack of availability of the different excipients present in these formulations, the excipients eluting at these peak positions could not be investigated. Since the cyclosporine peak for all four cyclosporine drug products showed good separation from these co-eluting peaks (Fig. 4), the method was determined to be specific for cyclosporine.

Cyclosporine Degradation in Dissolution Media

During dissolution, cyclosporine was found to degrade over time in the dissolution media. Hence, degradation studies were conducted on the drug substance using the same media and under the same conditions as used for the evaluation of the commercial drug products. Three temperature conditions (37°C, 25°C, and 10°C) were selected to mimic the temperature conditions encountered by the samples during the dissolution test, on the dissolution autosampler, and on the HPLC autosampler, respectively. The degradation of cyclosporine in dissolution media was found to be following zero-order kinetics, and the rate constant were −3.5, −1.5, and −0.3%/h at 37°C, 25°C, and 10°C, respectively (Fig. 6). Using Arrhenius equation, it was determined that the activation energy is 70 kJ and pre-exponential constant is 2.72e12. These values may be used to predict the degradation rate of cyclosporine in the dissolution media at any given temperature and time, which can be used to account for degradation-related sample loss during dissolution analysis. Additionally, no peak, other than cyclosporine peak, was observed in any of the samples from the degradation studies using cyclosporine drug substance. The only change that was observed was a decrease in the cyclosporine peak as a function of storage temperature and storage time, indicating a loss of cyclosporine from these samples.

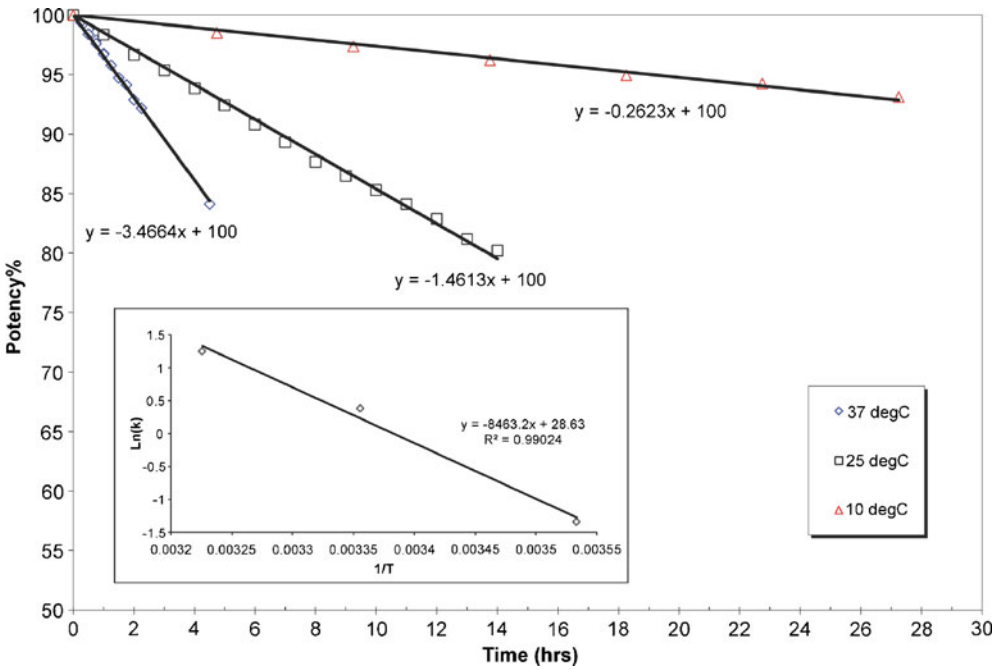

Fig. 6. Degradation of Cyclosporine (Zero Order) in Dissolution Media (0.1 N HCl and 0.5% *w*/*v* SDS) at Three Different Temperatures (10°C, 25°C, and 37°C). The *Insert* is the Arrhenius Plot

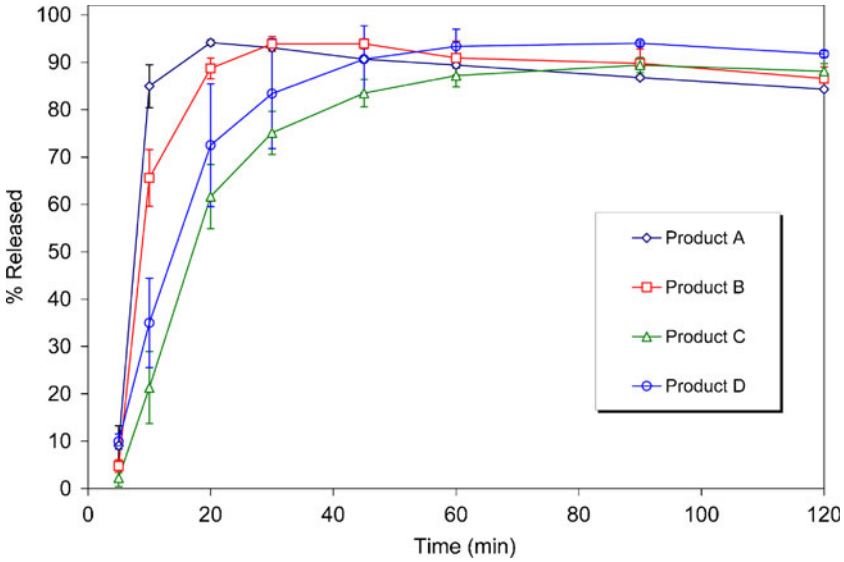

Fig. 7. Dissolution Profiles of Four Liquid-Filled Cyclosporine Capsule Drug Products without Correction. USP Apparatus I, 150 rpm, in 0.1 N HCl and 0.5% SDS, 37°C

Dissolution Test of the Marketed Products

In dissolution media (0.1 N HCl+0.5% SDS), all four liquid-filled cyclosporine capsule drug products met the USP requirement of $Q>80\%$ in 90 min (Fig. 7), but their release rates exhibited some differences (Product A>Product B> Product D>Product C in the order of time required to achieve >80% dissolution). Additionally, for all four drug products, a slight decrease in the concentration of cyclosporine in the dissolution media was observed after reaching the maximum value due to drug degradation. The difference (before and after correction) is demonstrated in Fig. 8. As can be seen, the degradation caused as much as 9% decrease in the released drug content (84.3% instead of 92.7% for Product A at the end of 2 h).

CONCLUSIONS

A new HPLC method for the analysis of the dissolution samples of liquid-filled cyclosporine drug products was developed and found to resolve the cyclosporine peak from the interfering peaks. The method was accurate, precise, and

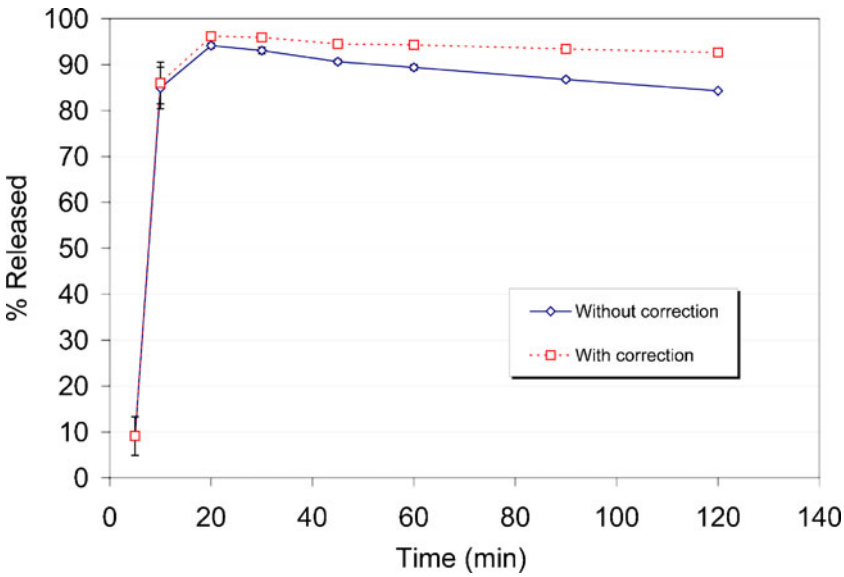

Fig. 8. Dissolution Profiles of Product A With and Without Correction. USP Apparatus I, 150 rpm, in 0.1 N HCl and 0.5% SDS, 37°C

linear across the analytical range and was specific for the determination and quantitation of cyclosporine in dissolution samples. Additionally, the degradation kinetics of cyclosporine in the presence of dissolution media was determined to follow zero-order kinetics and may be used to account for degradation-related drug loss during dissolution analysis.

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Disclaimer The findings and conclusions in this article have not been formally disseminated by the Food and Drug Administration and should not be construed to represent any Agency determination or policy.

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