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Development and validation of a stability indicating HPLC assay method for cyclosporine in cyclosporine oral solution USP

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Abstract

The suitability of the United States Pharmacopeia (USP) assay method for analysing stressed samples of cyclosporine oral solution USP was evaluated for stability samples by analyzing cyclosporine oral solution after acid, alkali, hydrogen peroxide, heat and light treatment. Some of the degradants generated during stress testing, as well as dihydrocyclosporine A, which is a known degradant of cyclosporine A, were not adequately resolved from the cyclosporine peak and mobile phase adjustments did not improve the resolution. In addition, isocyclosporine A, another known degradant of cyclosporine, could not be quantitated as it was eluting too early with the system peaks. Therefore, a binary gradient, reverse phase, stability indicating, HPLC method for the assay of cyclosporine in cyclosporine oral solution USP has been developed and validated. Analysis of degraded samples showed that the cyclosporine A eluted as a spectrally pure peak resolved from its degradation products.

Keywords: Cyclosporine; Oral solution; Stability indicating; Stress testing

1. Introduction

Cyclosporine A (CyA) is a cyclic undecapeptide (Fig. 1) produced by some imperfect fungi together with a number of cogeners differing by one–three amino acids [1]. Nowadays, it is widely used to prevent transplanted organ rejection and for the treatment of various autoimmune diseases [2].

Due to its cyclic structure and lipophilic character, CyA is a very stable molecule. However, its stability is not unlimited and recently several degradation pathways have been reported: dehydration and the loss of the [MeBmt[1]] side chain [3]; photo-oxidation by singlet oxygen [4]; formation of open chain oligopeptides while avoiding complete degradation of peptide chain [5]; and N–O peptidyl shift providing isocyclosporine A (isoCyA) [6–8]. There are a few reports in the literature covering the stability of CyA in its oral dosage form [9–11]. However, there is no report

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of stress testing of the cyclosporine oral dosage form to develop a stability indicating method of analysis of CyA.

The present work was conducted in order to develop and validate a stability indicating HPLC assay method that allowed resolution, detection and quantitation of the CyA in cyclosporine oral solution (COS).

2. Experimental

2.1. Materials

Acetonitrile and water of HPLC grade and *ortho*-phosphoric acid of reagent grade (Merck) were used to prepare the mobile phase. HPLC grade propan-2-ol (Merck) was used as sample diluent. Cyclosporine A reference standard was obtained from USPC, Inc., Rockville, MD, USA. Standards of process impurities/degradation products were obtained from Galena, a.s., Czech Republic. Panimun Bioral (modified cyclosporine oral solution USP 100 mg/ml) batch no. 139 manufactured by Panacea Biotec Ltd., India was used for stress testing purpose.

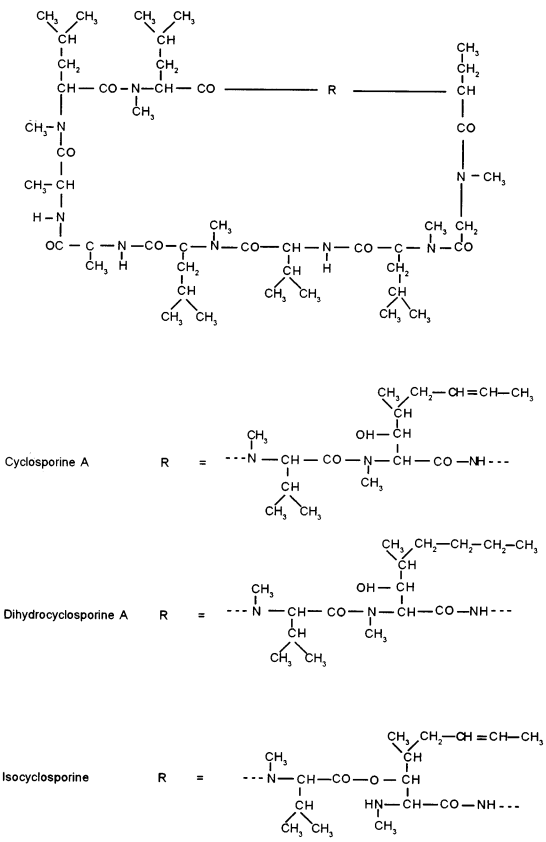

Fig. 1. Structure of cyclosporine A and its degradation products.

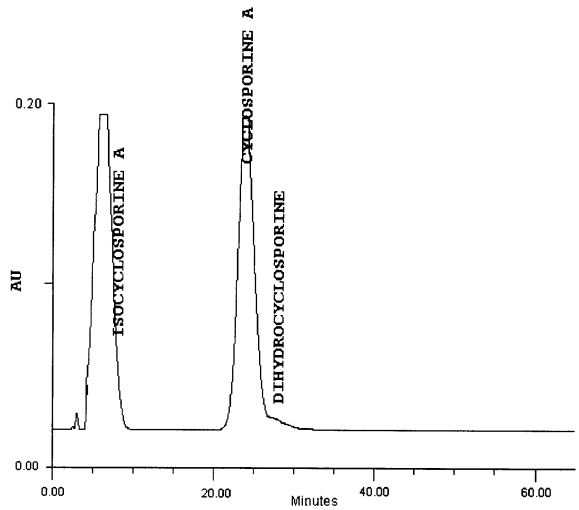

Fig. 2. Chromatogram of a cyclosporine A sample spiked with degraded products (USP method).

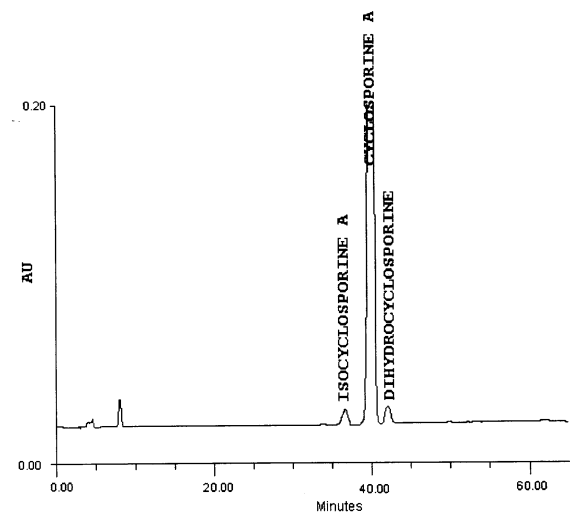

Fig. 3. Chromatogram of a cyclosporine A sample spiked wih degraded products (proposed method).

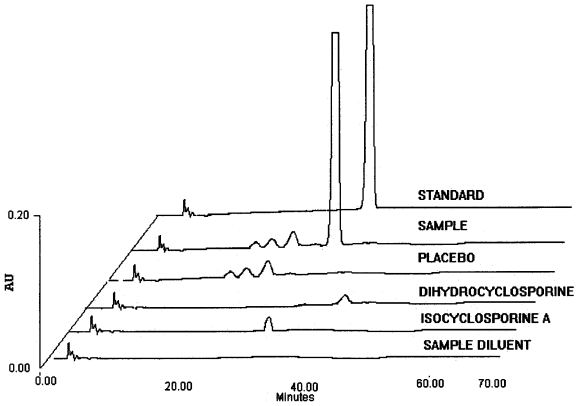

Fig. 4. Overlay of chromatograms of diluent, placebo, standard solution, sample solution, isocyclosporine A and dihydrocyclosporine A.

Table 1
Assay (%) of degraded cyclosporine oral solution

Conditions	USP method	Proposed method
1.Acid 1N HCl, 80°C[a]	34.1	27.3
2.Base, 1N NaOH, 80°C	100.8	100.3
3.15%v/v, hydrogen Peroxide 80°C	100.5	100.8
4.Heat, 80°C	102.9	92.7
5.Light (1000 ft-c)	100.1	101.0

[a] Degradation product was identified as isocyclosporine A.

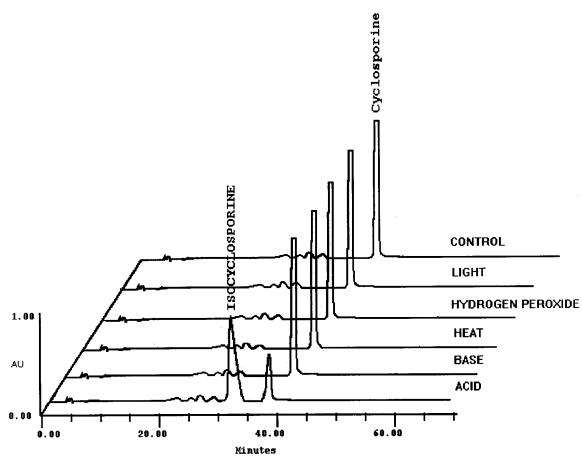

Fig. 5. Overlay of chromatograms from cyclosporine oral solution degradation study.

2.2. Equipment

A HPLC system consisting of a pair of Waters 510 pumps, a Rheodine 7725i injector, Millennium 2010 chromatography data software with a Pump Controller and a Waters 996 PDA detector was utilized.

The second instrument used for determination of intermediate precision was a HPLC system with Waters 515 pumps, a Rheodine 7725i injector, Millennium 32 chromatography data software with a Pump Controller and a Waters 2487 UV/VIS tunable detector.

All separations were achieved on a Nucleosil RP-2 column. All the samples and standard solutions were chromatographed at 80°C using the mobile phase in gradient mode with detection at 210nm and an injection volume of 20 μl. Peak area responses were used for quantitation of the cyclosporine A.

2.3. Preparation of mobile phase

Component A of the mobile phase was 0.8 ml *ortho*-phosphoric acid in 1000 ml of water, while component B was prepared by adding 0.8 ml *ortho*-phosphoric acid in 1000 ml of acetonitrile. Both components were filtered separately through a 0.22 μm nylon membrane filters and degassed, before use. Elution was carried out with a mixture of component A and component B in the form of a linear gradient from 65:35 to 40:60 over a period of 55 min.

2.4. Preparation of standard solution

An accurate amount (approx. 100.0 mg) of CyA USP Reference standard was dissolved in 100 ml of propan-2-ol to provide a concentration of approx. 1000 ppm.

2.5. Preparation of solution for LOD evaluation

An aliquot (1.0 ml) of the CyA standard solution was diluted to 100 ml with propan-2-ol. A sample of the resulting solution (5 ml) was further diluted to 50 ml with the same solvent.

2.6. Preparation of the samples

Degraded samples were prepared by subjecting 5.0 ml of COS to heat (80°C), acid (1N HCl; 80°C), base (1N NaOH; 80°C), hydrogen peroxide (15% v/v; 80°C) and light (1000 ft-c), for 72 h each. After the degradation treatments were complete, all the samples were allowed to cool to room temperature, neutralized with acid/base (if needed) and diluted with propan-2-ol to obtain a concentration of about 1000ppm of CyA. Placebo samples were also prepared in a similar way for each of the stress conditions.

Standard solutions for the evaluation of CyA linearity were prepared by diluting the CyA stock solution, of approx. 5000 ppm concentration, to 100, 500, 1000, 1500 and 2000 ppm in propan-2-ol.

To evaluate the linearity of isoCyA and dihydroCyA, stock solutions of about 100 ppm were diluted separately to 4, 8, 16, 20 and 24 ppm in propan-2-ol.

The precision of the method was checked by dissolving COS in propan-2-ol to give a known concentration of about 100, 1000 and 2000 ppm of CyA in propan-2-ol.

Samples to evaluate recovery of CyA, isoCyA and dihydroCyA were prepared by simultaneously spiking known amounts of standards in the placebo at three concentration levels. CyA was added at concentrations of approx. 500, 1000 and 1500 ppm. Standards of degradation products were added at concentration levels of approx. 4, 8 and 16 ppm.

3. Results and discussion

3.1. Optimization of chromatographic separation

The assay method for COS described in USP [12] uses isocratic elution (acetonitrile, water, methanol and *ortho*-phosphoric acid: 550: 400: 50: 0.5), a reverse phase column (RP-2) maintained at 50°C and chloroform/methanol (1:4) as the sample diluent. The stability indicating properties of the USP method for the assay of CyA in COS were evaluated by analysing CyA in the drug product after acid, alkali, hydrogen peroxide, heat and light treatments. Some of the degradation products from the acidic treatment, as well as dihydrocyclosporine A (dihydroCy A), a known degradation product of CyA, were not adequately resolved from the active peak (Fig. 2). The results were confirmed by spiking a COS with a known amount of the same impurity and analyzing the sample using a photo-diode array detector. Changes to the chromatographic conditions did not efficiently resolve the two peaks. Furthermore, one of the degradation products from the acidic treatment (as well as process impurity), isoCyA, was merging with the system peaks (Fig. 2). Therefore, the USP method did not meet the requirement for a stability indicating assay and a new method needed to be developed.

Various combinations of isocratic mobile phases and stationary phases ranging from reverse phase to normal phase failed to yield a reasonable separation between degradants and the cyclosporine A. One of the significant features of all the HPLC methods developed for CyA analysis is its peak broadening [13]. The characteristic peak broadening was the major reason for the poor resolution of the cyclosporine A from the degradants. It was found that the gradient system improved the peak shape of the cyclosporine A substantially. Normally, with the USP method 800–1000 theoretical plates are achieved, while in the present chromatographic conditions, more than 10 000 theoretical plates can be achieved with the same column. Elution of isoCyA was found to be very much dependent on the concentration of acetonitrile as well as the *ortho*-phosphoric acid in the mobile phase. A concentration of 0.08% *ortho*-phosphoric acid was found to be optimal to keep isoCyA resolved from CyA, as well as from the peaks due to placebo.

The sample diluent described in the USP method [12], i.e. chloroform and methanol, gave a high response for a long time. Therefore, it was not considered suitable, as some of the degradation products may get masked. Panimun Bioral solution, being a modified solution, has a different solubility profile compared to its drug substance, thus making the choice of sample diluent difficult. Propan-2-ol was found to be most suitable sample

diluent after trying several other alternatives including components of the mobile phase.

3.2. Validation of the method

3.2.1. System precision

System precision was determined by making five replicate injections of the standard solution. The relative standard deviation of the area of the CyA peak was found to be 0.3%.

3.2.2. Intermediate precision

The study was performed by assaying COS at three different concentration levels i.e. 10, 100 and 200% of the recommended CyA concentration. The coefficient of variance for these levels were found to be 0.45, 0.23, and 0.28, respectively.

3.2.3. Linearity

Linearity parameters of the curve for the CyA peak area response versus the CyA concentration were studied in the concentration range corresponding to about 10–200% of the nominal analytical concentration of 1000 ppm. The correlation coefficient was found to be 0.999933. Using the least square method, the regression equation between the peak area (Y) and the concentration (X) was found to be

$$Y = 2.56 \times 10^4 X + 2.38 \times 10^5$$

The linearity parameters of the curves for the degraded products/process impurities of CyA, i.e. isoCyA and dihydroCyA, were studied from 0.4 to 2.4% of the nominal CyA concentration of 1000 ppm. The correlation coefficients for isoCy A and dihydroCyA were 0.999851 and 0.999995, respectively. Using the least square method, the regression equation was calculated separately, for each of the impurities.

IsoCyA $\quad Y = 1.30 \times 10^4 X - 1.43 \times 10^3$

DihydroCyA $\quad Y = 2.58 \times 10^4 X - 7.35 \times 10^2$

3.2.4. Limits of detection (LOD) and quantitation (LOQ)

The limits of detection and quantitation were evaluated by serial dilutions of CyA, isoCyA and dihydroCyA stock solutions in order to obtain signal to noise ratios of $\approx 3{:}1$ for LOD and $\approx 10{:}1$ for LOQ. The LOD and LOQ values for CyA were found to be 1 (signal/noise = 3.1) and 3 ppm (signal/noise = 10.8), respectively. The LOD and LOQ values for isoCyA were found to be 2 (signal/noise = 3.0) and 4 ppm (signal/noise = 10.1), respectively. The LOD and LOQ values for dihydroCyA were found to be 1 (signal/noise = 2.9) and 4 ppm (signal/noise = 11.2), respectively.

As per the USP method, LOD and LOQ values for CyA were 2 (signal/noise = 3.5) and 5 ppm ppm (signal/noise = 10.1), respectively. It was not possible to determine LOD and LOQ values for isoCyA as the peak was merging with system peaks. LOD and LOQ values for dihydroCyA were 1 (signal/noise = 3.0) and 4 ppm (signal/noise = 9.7), respectively.

3.2.5. Relative response factors for impurities

The response factors of isoCyA and dihydroCyA were compared with that of CyA. Impurities were injected at a concentration of 10 ppm (1% of the CyA nominal concentration) while CyA was injected at the concentration of 1000 ppm (nominal concentration). The relative response factors were found to be 0.61 and 1.02 for isoCyA and dihydroCyA, respectively.

3.2.6. Accuracy/recovery studies

Recoveries of CyA, isoCyA and dihydroCyA were determined at three concentration levels. The mean recovery for CyA was 99.73% in the concentration range 500–1500 ppm. Mean recoveries for isoCyA and dihydroCyA were found to be 99.20 and 96.16%, respectively, in the concentration range 4–16 ppm.

3.2.7. Stability of analytical solution

The stability of the standard and sample solutions was checked by analyzing these solutions aged at room temperature, against freshly prepared standards. The results demonstrated that the working standard solution, as well as the sample solution is stable for at least 15 days. During the stability studies no additional peaks developed and no changes in the chromatographic pattern were observed in either of the solutions.

3.2.8. Selectivity/specificity

There was no interference due to placebo and sample diluent. Moreover, known degradants, i.e. isoCy A and dihydroCy A, were also well separated (Fig. 3 and Fig. 4).

Forced degradation studies were performed to evaluate the specificity and the percentage of the cyclosporine A recovered in each case is shown in Table 1. It is evident from Fig. 5 that the method has been able to separate the peaks due to the degraded products from that of CyA. This was further confirmed by peak purity analysis on a photo-diode array detector.

4. Conclusion

Degradation studies of the COS reveal that the solution is stable under the described conditions with the exception of acidic conditions, and the main degradation product generated, isoCyA, cannot be monitored by the USP method. Moreover, another known degradation product of CyA, dihydroA, is not separated from the CyA peak in the USP method. However, isoCyA, as well as dihydroCyA, can be analyzed by the proposed method. The validation data also shows that the proposed method is selective, precise, accurate and can be used to monitor the stability of CyA in COS in the range of 50–150% of the nominal CyA concentration.

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