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Short communication

Development and validation of HPLC method for the determination of Cyclosporin A and its impurities in Neoral® capsules and its generic versions

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ABSTRACT

Cyclosporin A (CyA) is a cornerstone immunosuppressant for the prophylaxis against allograft rejection after organ transplantation. The most widely prescribed CyA formulation is Neoral® soft gelatine capsules (Novartis Pharmaceuticals, Basel, Switzerland). After Novartis patent expiration, several generic formulations have been developed.

In this paper, a simple and reliable HPLC method was developed and validated for the evaluation of four CyA degradation products (ID-005-95, CyH, IsoCyH and IsoCyA) and two related compounds (CyB and CyG) aimed for the quality control of Neoral® capsules and its generic formulations. In a second step, the validated method was then compared to the USP assay method for capsules, where some of the mentioned impurities were not adequately resolved from the CyA peak.

Isocratic elution at a flow rate of 1.0 mL min^{-1} was employed on a Lichrospher RP-18 (4 mm × 250 mm; 5 μm) analytical column maintained at 75 °C with a tetrahydrofuran:phosphoric acid (0.05 M) (44:56, v/v) as mobile phase. The chromatograms were recorded using a Hewlett Packard 1100 chromatographic system. The UV detection wavelength was performed at 220 nm and 10 μL of sample was injected.

The developed method was validated in terms of selectivity, linearity, precision, accuracy, limit of detection and limit of quantitation. The validate method was successfully applied to commercial capsules, Neoral® and generic versions. Therefore, the proposed method is suitable for the simultaneous determination of CyA as well as its major impurities.

1. Introduction

The immunosuppressive properties of Cyclosporin A (CyA) have been widely explored clinically, predominantly in the prophylaxis against allograft rejection after organ transplantation and in the treatment of certain auto-immune diseases [1,2]. In addition to this predominant use in transplantation, CyA has also been indicated for a number of new clinical applications, like the reversal of multi-drug resistance, anti-malarial, herpes virus infection, rheumatoid arthritis, type I diabetes, and also as a potent anti-human immunodeficiency virus 1 (HIV-1) agent [3,4].

CyA is a cyclic undecapeptide produced by submerged culture fermentation with a number of congeners differing by one to three amino acids [5]. CyA are typically available from multiple sources, and each source may have a different manufacturing process. The result of a fermentation process is generally a mixture of products having closely related structural and/or physical properties, and the purification of this mixture is the most important stage to acquire a product in a substantially purified form. Therefore, it is essential that the dosage-form manufacturer evaluate each supplier's drug substance impurity profiles.

According to the current rules (e.g., US Food and Drugs Administration and European Pharmacopoeia), CyA is produced with a quality >98.5% [6,7]. In this respect, we should expect that the sum of all impurities do not exceed 1.5%, and that this amount might be respected no matter who produced the CyA. This is particularly interesting in view of some results indicating that industrial fungal strains also produced cyclosporins which were previously detected among human trace metabolites ([Leu^4]Cs = AM4N, [Leu^9]CS = AM9N) [8–10].

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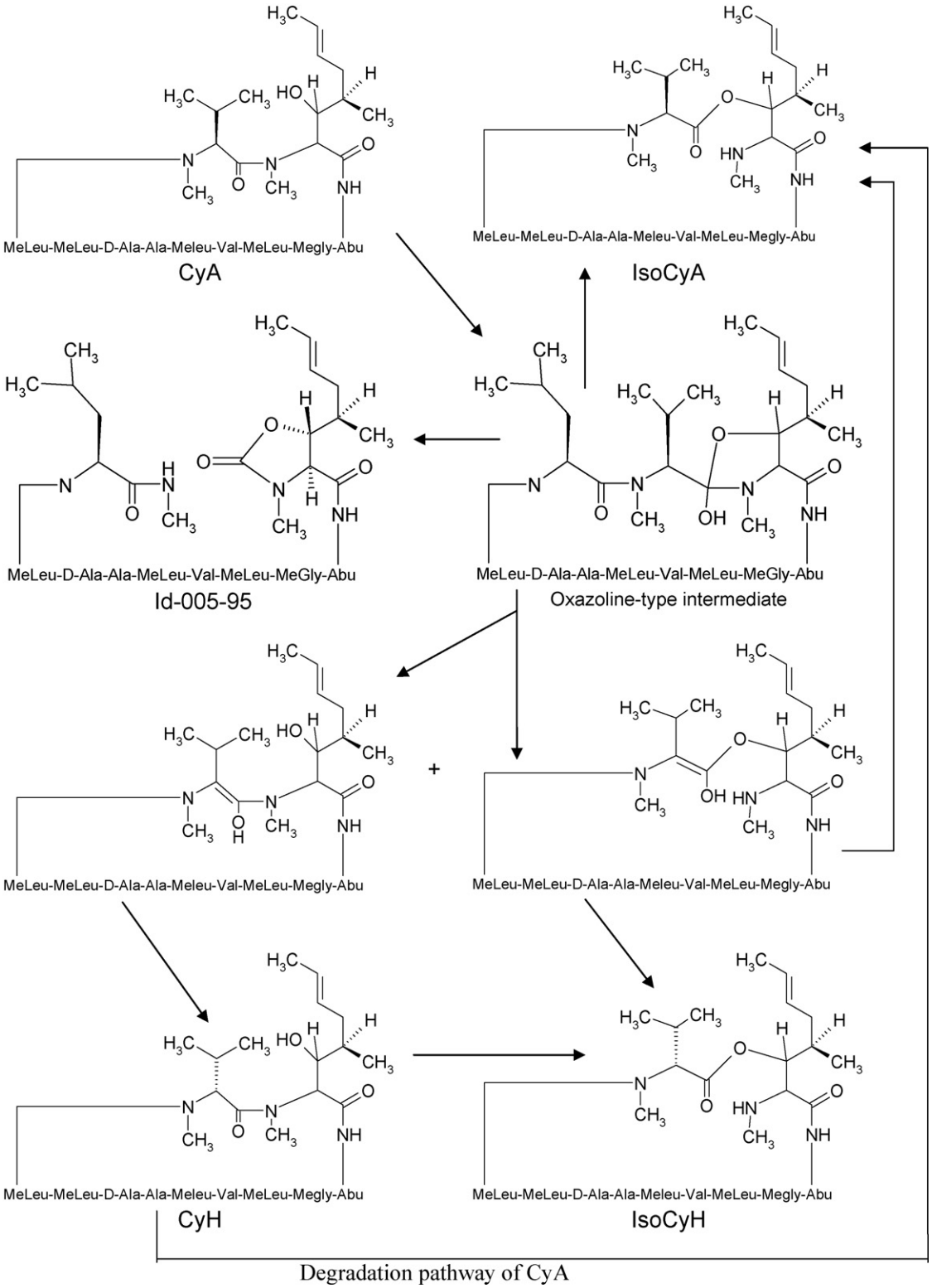

Fig. 1. Degradation pathway of CyA.

Due to its cyclic structure and lipophilic character, CyA is a very stable molecule. However, its stability is not unlimited and several degradation pathways have been reported: racemisation of [MeVal[11]] leading to Cyclosporin H (CyH) [11], N–O peptidyl shift providing IsoCyclosporine A (IsoCyA) [12–15]; photo-oxidation by singlet oxygen [16]; formation of open chain oligopeptides while avoiding complete degradation of peptide chain [17], dehydratation and even the loss of the [MeBmt[1]] side chain [18] (Fig. 1).

The most widely prescribed CyA formulation is Neoral® (Novartis Pharmaceuticals, Basel, Switzerland). In 1995, Novartis composition of matter patent on CyA expired. Since then, other companies are free to manufacture generic products. The fabrication processes, as well as the excipients of these generic versions

are likely to be different from the one used by the original manufacturer.

The safety of a drug product is dependent not only on the toxicological properties of the active drug substance itself, but also, on the impurities that it contains. Simultaneous identification of active drug substance and its impurities usually represents analytical challenge because of their structural similarity. A thorough literature search has revealed that only a few analytical methods are available for the determination of CyA in bulk drug, pharmaceuticals, and blood samples. Different columns and conditions were evaluated for the HPLC analysis of CyA, its congeners and degradation products and some modifications of the pharmacopoeial HPLC method were proposed by Husek [19]. A stability indicating HPLC assay method was developed for the analysis of stressed samples of CyA oral solutions [20]. A d-optimal design was applied to evaluate the importance of selected parameters in the chromatographic determination of CyA and its main metabolites [21]. In biological fluids, immunoassays [22–24] as well as liquid-chromatography coupled or not with mass spectrometry methods were used for the determination of CyA and/or its metabolites [23–26].

However, none of these methods address to the problem of simultaneous separation and determination of four principal degradation products (ID-005-95, Cyclosporin H, IsoCyclosporin H, and IsoCyclosporin A) and two related compounds (Cyclosporin B and Cyclosporin G) in capsules, which are most likely to be present in the finished products of cyclosporine.

The aim of the present work was to develop and validate a simple and reliable isocratic RP-HPLC method for the simultaneous quantitative analysis of CyA and six impurities CyB, CyH, CyG, IsoCyA, IsoCyH, and ID-005-95 in Neoral® and in generic capsules.

2. Experimental

2.1. Chemicals and reagents

All the reagents used in the experimental work were of analytical grade. HPLC grade water was prepared by Milli-Q reverse osmosis (Millipore, Bedford, USA) and meets European Pharmacopoeia requirements. Tetrahydrofuran and *ortho*-phosphoric acid (Sigma–Aldrich, Saint–Quentin–Fallavier, France) were used for preparing the mobile phase. Ethanol (Merck, Darmstadt, Germany) was used as solvent.

Working standards of CyA as well as four degradation products ID-005-95, CyH, IsoCyH and IsoCyA, and two related compounds CyB and CyG were kindly provided by Novartis (Basel, Switzerland). CyA was checked by comparison with European Pharmacopoeia CRS standards.

Generic CyA capsules were obtained from 6 international sources, representing 5 countries and 6 different manufacturers (Table 1) and further compared to the brand leader.

2.2. Chromatographic conditions (instrumentation and analytical conditions)

An Agilent 1100 Series (Agilent Technologies, Palo Alto, USA) chromatographic system was used, equipped with an HP 1100 binary pump, an HP 1100 diode array detector, HP 1100 column, auto sampler thermostat and HP 1100 degasser. Chromatographic software HP ChemStation was used for data collection and processing.

Separations were performed using Lichrospher 100 RP-18 analytical column, 4 mm × 250 mm (Merck, Darmstadt, Germany) packed with 5 μm particle size. A 1 m long steel capillary with 0.25 mm internal diameter, as described in CyA European Pharmacopoeia monograph [7], was inserted between the injection system and the entrance of the column, and adjusted thermostatically to 75 °C. Injection volume was 10 μL. Separations and simultaneous determination of CyA and its impurities ID-005-95, CyB, CyH, CyG, IsoCyA and IsoCyH were performed using the mixture of tetrahydrofuran:phosphoric acid (0.05 M) (44:56, v/v) as a mobile phase. Mobile phase was filtered through a 0.45 μm Millipore filter. The flow rate was 1.0 mL min^{-1} and the UV detection was performed at 220 nm.

2.3. Preparation of solutions

2.3.1. Stock and calibration standard solutions of CyA

Stock standard solution of 2 mg mL^{-1} of CyA was prepared by accurately weighing approximately 50 mg of CyA into a 25 mL volumetric flask and making up to volume with ethanol. The stock standard solution was diluted with ethanol to obtain five calibration standards in the concentration range of 0.80, 0.90, 1.00, 1.10 and 1.20 mg mL^{-1} of CyA, covering 80–120% of the intended test concentration of 100 mg mL^{-1} for the pharmaceutical formulation before dilution which corresponds to 1 mg mL^{-1} after dilution (see Section 2.4).

2.3.2. Stock and calibration standard solutions of impurities

Stock standard solutions of 500 μg mL^{-1} of each impurity (ID-005-95, CyB, CyH, CyG, IsoCyH, and IsoCyA) were prepared by accurately weighing approximately 5 mg of each impurity into a 10 mL volumetric flask and making up to volume with ethanol. For the calibration curve of each impurity, seven calibration standards containing 100 μg mL^{-1} of CyA were freshly prepared in the concentration range of 2, 5, 10, 20, 25, 40 and 50 μg mL^{-1} by diluting the stock standard solutions of each impurity with ethanol.

2.4. Assay sample preparation

20 capsules of Neoral® and generic versions were carefully cut open with a sharp blade, and with the aid of ethanol the contents of the capsules were transferred into a suitable volumetric flask. The blades and shells capsules were washed with ethanol and the washings were transferred to the volumetric flask. The volume of the volumetric flask was completed with the appropriated solvent and mixed. An accurately measured volume of this solution was quantitatively diluted with ethanol in order to obtain a solution having a concentration of 1 mg mL^{-1}.

2.5. Peak capacity

The peak capacity of a column is the number of hypothetical peaks which could be separated with resolution $R = 1$ within a certain run time [27]. If a few or even only two analytes within a sample need to be separated it is best to look for a chromatographic system with high selectivity. This approach will lead to a short analysis time. If, on the contrary, the task is to separate the components within a complex sample with numerous peaks of interest, then peak capacity is more important than selectivity. Based on Meyer's results peak capacity (n) was calculated with the following formula for isocratic separation

$$n = 1 + \delta \frac{Lc}{\sqrt{\mathrm{Tr}_{\max}}} \quad (1)$$

Peak capacity has a great importance because it governs the chance for peak resolution. The probability P for the separation (with resolution $R = 1$ or better) of a certain analyte is given by Davis and Giddings [28] as

$$P = e - 2m/n \quad (2)$$

Table 1
Description of CyA capsules: manufacturer, shelf-life and batch number.

Proprietary name	Manufacturer	Date of		Batch number
		Manufacture	Shelf-life	
Abrammune	Amoun Pharmaceutical (Cairo, Egypt)	04/2001	04/2004	807
Arpimune	RPG Life Science (Mumbai, India)	12/2000	11/2002	S24A0001
Equoral	Ivax Pharmaceuticals (Opava, Czech Republic)	Unknown	05/2007	4T5050001
Gengraf	Abbott Laboratories (North Chicago, USA)	Unknown	11/2002	69919AF21
Ciclosporina Germed	Germed Farmacêutica/Group EMS Sigma Pharma (Portugal/Brazil)	03/2006	03/2009	001570
Imusporin	Cipla Ltd. (Mumbai, India)	02/2000	02/02	Y00517
Neoral®	Novartis (Bazel, Switzerland)	10/2005	09/2008	SO169

where m is the total number of analyte peaks present in the chromatogram (although not all of them may be visible due to peak overlap) and n is the peak capacity. However, the probability P' for the resolution of all analytes is much lower as shown by El Fallah and Martin [29]:

$$P' = \left(1 - \frac{m-1}{n-1}\right)^{m-2} \quad (3)$$

2.6. Validation procedure

Chromatographic separation was optimized in the aim to obtain a resolution above 1.5 between all components, with the respect of stationary and mobile phase compositions, flow rate, sample volume, detection wavelength and temperature.

The method was validated for linearity, range, precision (repeatability and intermediated precision), specificity, limit of quantitation, limit of detection in accordance with standard procedure [30].

2.6.1. Linearity and range

Standard calibration curves were prepared with five calibrators over a concentration range of 0.80–1.20 mg mL^{-1} for CyA and with seven calibrators over 2–50 μg mL^{-1} for each impurity. The data of peak area versus drug concentration were treated by linear least square regression analysis. The standard curves were evaluated for intra-day and inter-day linearity.

2.6.2. Precision

The precision of the assay was studied with respect to both repeatability and intermediated precision. Repeatability was calculated from six replicate injections of freshly prepared solution in the same equipment on the same day. Repeatability for CyA was realized with a 1000 μg mL^{-1} solution. Impurities were tested at a concentration of 20 μg mL^{-1} of each impurity in a solution containing also 100 μg mL^{-1} of CyA. The experiment was repeated by assaying freshly prepared solution at the same concentration on 2 additionally consecutive days to determine intermediate precision. Precision was expressed by the % of the relative standard deviation (R.S.D.) of the analyte peaks.

2.6.3. Specificity

Specificity of a method can be defined as absence of any interference at retention times of peaks of interest, and was evaluated by observing the chromatograms of blank samples and samples spiked with CyA and all six impurities. The variable number of excipient used in generic versions of CyA, as well as the lack of information in the composition of some generic formulations makes it difficult to assess selectivity by traditional analysis comparison with a placebo solution.

2.6.4. Limits of detection and quantitation

Limits of detection (LOD) and limits of quantitation (LOQ) were provided for four degradation products (ID-005-95, CyH, IsoCyH, and IsoCyA) and two related compounds (CyB and CyG), and calculation was made with the following equations:

$$\mathrm{LOD} = \frac{3.3\sigma}{S}; \qquad \mathrm{LOQ} = \frac{10\sigma}{S}$$

where σ was the standard deviation of the response (estimated from the standard deviation of y-intercepts of regression lines) and S was the slope of the standard curve [30].

2.6.5. Sensitivity

The sensitivity (Δx) of an analytical method is defined by the minimum variation that requires to be applied to the magnitude measured in order to obtain a significant variation in the signal measured [31].

$\Delta X = (\left|t(1-\alpha/2) + t(1-\beta)\right| \mathrm{Se}\sqrt{2})/b$ where Se is the standard deviation estimated over 10 measurements of a 100% standard and b is the slope of the curve.

3. Results and discussion

3.1. Optimisation of the chromatographic conditions

The challenges involved in the commercial production of CyA generic version can lead to the incidence of non-chemically equivalent products, or products without the same quality presented by the reference formulation. This quality is closely related not only with the quality of active substance itself, but also the very small amounts of impurities in the preparation. Moreover, impurity levels of degradation products are a good indicator of active substance stability, and consequently the formulation stability.

It is very important to give great consideration to these detrimental degradation products. In addition to chemical stability, impurities in pharmaceutical products can cause problems associated with toxicity or pharmaceutical products performance.

Therefore, determination of related substances and degradation products in CyA capsules is necessary, due to a possible decrease of the effectiveness of CyA, which could cause a therapeutic failure.

HPLC with UV detection was chosen as a simple, reliable and effective separation method for determination of CyA and four degradation products (IsoCyA, IsoCyH, CyH and ID-005-95), as well as two related compounds (CyB and CyG). All tested compounds had a low detector response at wavelengths above 230 nm. The optimal absorption wavelength for detection of the compounds was chosen especially with regard to absorption spectra of degradation products IsoCyA and IsoCyH; both give higher detector response at 210 nm, but at this wavelength, a decrease in the signal to noise ratio was observed. Therefore, the final absorption wavelength for detection was chose at 220 nm in which no marked changes in signal to noise ratio, as well as in the shape peaks was observed.

As described in previous works [32–34], relatively high temperature is necessary for analysis of cyclosporins to ensure fast equilibration of individual conformers and therefore to achieve satisfactory peak shape. In addition, it has been suggested that CyA undergoes temperature-related conformational changes, possibly

Table 2
Linearity, limits of detection and quantification of standard curves.

Substance	Retention times (min)	Resolution	Calibration equation (y = area, x = μg mL^{-1})	Correlation coefficients (r)	LOD (μg mL^{-1})	LOQ (μg mL^{-1})
ID-005-95	7.3	–	$y = 8.18x + 0.995$	0.9998	0.14	0.42
CyB	10.4	6.48	$y = 11.05x + 4.40$	0.9996	0.17	0.51
CyA	11.7	2.42	$y = 10.15x + 362.8$	0.9995		
CyG	13.5	3.02	$y = 11.05x - 0.029$	0.9999	0.08	0.23
CyH	15.2	2.57	$y = 11.40x - 6.044$	0.9996	0.17	0.51
IsoCyH	30.9	15.62	$y = 6.38x - 2.04$	0.9954	0.72	2.15
IsoCyA	35.4	3.43	$y = 5.98x - 9.58$	0.9990	0.41	1.22

Table 3
Intra-day and inter-days precision and accuracy.

	Theoretical concentrations (μg mL^{-1})	R.S.D. (%)		Accuracy (%)	Sensitivity (μg mL^{-1})
		Intra-day precision (n = 10 (CyA) or 7 (impurities))	Inter-day precision (3 days)		
CyA	1000	0.258	0.270	97.5 ± 2.92	0.40
ID-005-95	20	1.216	1.292	99.1 ± 1.2	0.80
CyB	20	1.327	1.582	101.2 ± 1.4	0.65
CyG	20	1.209	1.276	100.5 ± 1.2	0.57
CyH	20	1.283	1.715	98.3 ± 1.5	0.63
IsoCyH	20	1.207	1.287	90.6 ± 1.1	1.01
IsoCyA	20	1.625	2.075	93.3 ± 1.7	1.53

related to intramolecular hydrogen-bonding, that could alter solubility and contribute to the narrow peak shape and high resolution observed with increasing temperatures [35].

In accordance to Li et al. [36] it was observed that the use of THF–water mobile phases (e.g. THF–phosphoric acid 0.05 M) in the chromatographic separation of CyA offers a different selectivity, and reduces the peak broadening effect. Different proportions of THF and phosphoric acid (0.05 M) were tested. The selectivity of ID-005-95, CyB, CyG, CyH and CyA can be altered by modifying the proportion of THF. Increasing the amount of THF reduces the retention time of mentioned compounds. On the other hand, the selectivity of IsoCyA and IsoCyH can be altered by modifying the proportion of phosphoric acid (0.05 M). The higher the concentration of phosphoric acid, the shorter are the retention time of IsoCyA and IsoCyH.

3.2. Peak capacity

In Eq. (1), the parameter δ, depends on mobile phase. Because of the low viscosity of our mobile phase at 75 °C, the chosen value for δ was 21 as described in Meyer's study [27].

In this case, the formula for Eq. (1) was: $n = 1 + 21(250/\sqrt{\mathrm{Tr}_{\max}})$.

The $\mathrm{Tr}_{\max}$ was for IsoCyA and n was estimated at 115.

Therefore, with m = 15, P and P' was calculated at 81 and 36%, respectively.

The same equations were applied in Husek's previous work [19]. P and P' were 74 and 22%, respectively, but with a major difference, the flow rate was 2 mL min^{-1} in this study. The proposed method shows an excellent peak capacity and can be able to separate the peaks of interest.

3.3. Validation of methods

3.3.1. Linearity

Areas of peak of each compound were measured and served for quantitation. Peak areas of the drug versus concentration were plotted and found to be linear within the concentration range except IsoCyH, which was found linear in the range 2.15–50 μg mL^{-1}. Standard curves were constructed on three consecutive days and the evaluation parameters like regression, slope, intercept, and correlation coefficient, were calculated and presented in Table 2.

3.3.2. Accuracy and precision

The percent recovery of method was found to be less than 100 ± 5% and with acceptable R.S.D. limits indicating that method could be considered as accurate and precise (Table 3).

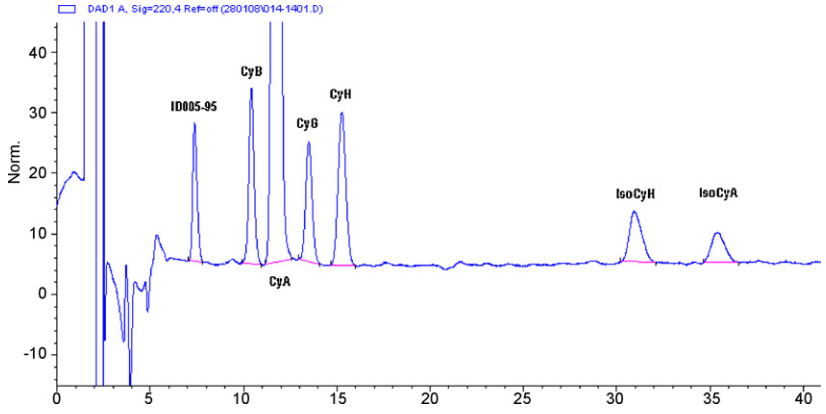

Fig. 2. Chromatogram of (ID-005-95, CyH, IsoCyH and IsoCyA) and two related compounds (CyB and CyG). Chromatographic condition: see text.

Table 4
Assay of active substance and impurities for 7 commercial capsules with proposed HPLC method.

Compounds	Neoral®	Germed	Equoral	Imusporin	Arpimune	Gengraf	Abrammune
CyA	97.67%	97.65%	100.30%	101.90%	94.04%	94.73%	67.97%
ID-005-95	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
CyB	0.50%	0.40%	<LOD	<LOD	<LOD	<LOD	2.19%
CyG	<LOD	<LOD	<LOD	<LOD	0.94%	<LOD	0.30%
CyH	<LOD	<LOD	<LOD	<LOD	<LOD	0.93%	25.26%
IsoCyH	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
IsoCyA	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Unknown 1	0.17%	0.05%		0.08%	0.05%		0.43%
Unknown 2							0.06%
Unknown 3				0.05%		0.05%	0.08%
Unknown 4							0.10%
Unknown 5							0.08%
Total unknown	0.17%	0.05%	0.00%	0.13%	0.05%	0.05%	0.76%
Total impurities	0.67%	0.46%	0.00%	0.13%	0.99%	0.98%	28.5%

3.3.3. Specificity

The elution peaks of analytes were presented in representative chromatograms shown in Fig. 2. The retention times of ID-005-95, CyB, CyA, CyH, CyG, IsoCyH and IsoCyA was presented in Table 2.

3.3.4. Limit of detection and limit of quantitation

The LOD and LOQ were presented in Table 2. The guideline for validation of analytical procedures produced by the International Conference on Harmonization, recommends that when the LOD was determined by calculation, it may subsequently be validated by analysis of a suitable number of samples known to be near or prepared at the LOD [30].

3.3.5. Stability

The stability of CyA and its six impurities in standard solutions were checked by storing these solutions at room temperature. The solutions were injected in triplicate, during 3 days, and data were compared with freshly prepared standards. The results demonstrated that working standard solution is stable for at least 72 h. During this time, no additional peak appears and no changes in the chromatographic pattern were observed. Therefore, solution was stable for 72 h, which was considered as sufficient for assay.

3.3.6. Sensitivity

Sensitivity for all compounds was presented in Table 3. It is always low and in the same order than LOQ, by which we can conclude about the good sensitivity of the proposed method.

3.3.7. System suitability

The system suitability test ensures the validity of the analytical procedure as well as confirms the resolution between different peaks of interest. All critical parameters (resolution above 2 between CyB and CyA, not less than 3 between IsoCyH and IsoCyA, and asymmetry of each analyte comprise between 1.0 and 1.4) tested met the acceptance criteria on all days (see Table 2). Asymmetry was always between 1.08 and 1.30 corresponding to very symmetric peak.

The statistical evaluation of the proposed method revealed its good linearity, reproducibility and its validation for different parameters and led us to the conclusion that it could be used for the simple and reliable determination of CyA and its major impurities in Neoral® capsules and generic versions.

3.4. Assay of capsules

A comparison of proposed method and USP method for CyA capsule analysis were investigated. The assay method for CyA described in USP 30 [6] uses isocratic elution (acetonitrile, water, methanol and *ortho*-phosphoric acid: 550:400:50:0.5), a reversed phase column (L-16), maintained at 70 °C and dehydrated alcohol as sample diluent. The detection wavelength is lower at 210 nm. The standard sample containing CyA (1 mg mL^{-1}) and its six major impurities (50 μg mL^{-1}) was evaluated under those conditions. As described in a previous work [20], the USP method was not able to ensure the separation of the investigated impurities. With exception of two degradation products (IsoCyA and IsoCyH) that presented a relative retention time of 3.3 and 3.7, respectively, all the other compounds (ID-005-95, CyB, CyH, CyG and CyA) coeluted in 3 peaks. Therefore, USP method was not used for assay of capsules.

The proposed method was applied for the analysis of seven commercial capsules, claimed 100 mg of CyA. These included drug products from seven different manufacturers and six different countries (Table 1).

Except one (Abrammune), it appears that assay of CyA was conform for all products and was always between 94 and 102% (Table 4). USP states that cyclosporine capsules contain not less than 90% and not more than 110% of labelled amount of cyclosporine. ID-005-95, IsoCyH and IsoCyA were not found in all products. CyB was found only in two manufacturers at a level below 0.5%. CyH and CyG was found once in two manufacturers at levels below 1.0%. In all of theses six products, a total impurity was always below 1.0%.

The last product, manufacturer by Amoun Pharmaceutical (Cairo, Egypt) was totally out of specification. CyH was about 25%, CyB about 2.2% and the total of impurity was about 28.5%. In the same time, CyA represent only 68% of the labelled amount. However, shelf-life was exceeding 4 years without control of storage condition. We can conclude that the proposed method is sufficiently sensible to detect any variation on manufactured products and impurities are well detected.

4. Conclusion

An isocratic HPLC-UV method for the determination of four degradation products (ID-005-95, CyH, IsoCyH and IsoCyA) and two related compounds (CyB and CyG) in CyA capsules was developed and validated. The method was evaluated through the method validation and proved to be accurate, precise and specific, and limits of quantitation of CyA impurities were sufficiently low, in accordance with the requirements of current rules.

This study also demonstrates marked differences in chemical composition and formulation of current commercially available CyA products. Therefore, considering the worldwide development of generic versions of Neoral®, the proposed method could be useful for routine analysis and quality control of the tested compounds in CyA capsules formulations.

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