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Simple determination of cyclosporine in human whole blood by high-performance liquid chromatography

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Abstract

A simple and reproducible high-performance liquid chromatography (HPLC) method was developed for determination of cyclosporine (CyA, also known as cyclosporin A) in human whole blood. The method entailed direct injection of the blood samples after deproteination using acetonitrile. Chromatography was carried out using an ODS column under isocratic elution with acetonitrile–5 mM disodium hydrogen phosphate (75:25, v/v), pH 5.1 at 70 °C and a detector set at 210 nm. The mean absolute recovery of cyclosporine from blood was 97%, and the linearity was assessed in the range of 100–3000 ng/ml blood, with a correlation coefficient of greater than 0.999. The limit of quantification and detection of the present method were 100 and 50 ng/ml, respectively. This method has been used to analyze several hundred human blood samples for bioavailability studies.

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Keyword: Cyclosporine

1. Introduction

Cyclosporine (CyA) (Fig. 1), is a cyclic peptide consisting of 11 amino acids. It is an important immunosuppressive drug used commonly to reduce tissue rejection after organ transplantation [1,2]. Methodological approaches to measuring CyA include high-performance liquid chromatography (HPLC) and a variety of immunoassay-based methods. Although immunoassay-based methods are simple to use, the cross-reactivity of the antibody with the metabolites leads to overestimation of the parent CyA concentrations in blood by these methods [1,2].

Many laboratories prefer to measure whole blood concentration of CyA rather than plasma or serum, mainly due to the variation in the distribution of the drug in the plasma that depends on the sample temperature and hematocrit. However, the discussion is still open [3].

During the past two decades, many attempts have been made to improve HPLC method for CyA analysis in blood, resulting in numerous published methods [3–12]. The difficulty with all chromatographic methods is related to CyA's lack of chromophores that necessitates the use of short-wavelength (e.g. 210 nm) ultraviolet light detection. Because, so many

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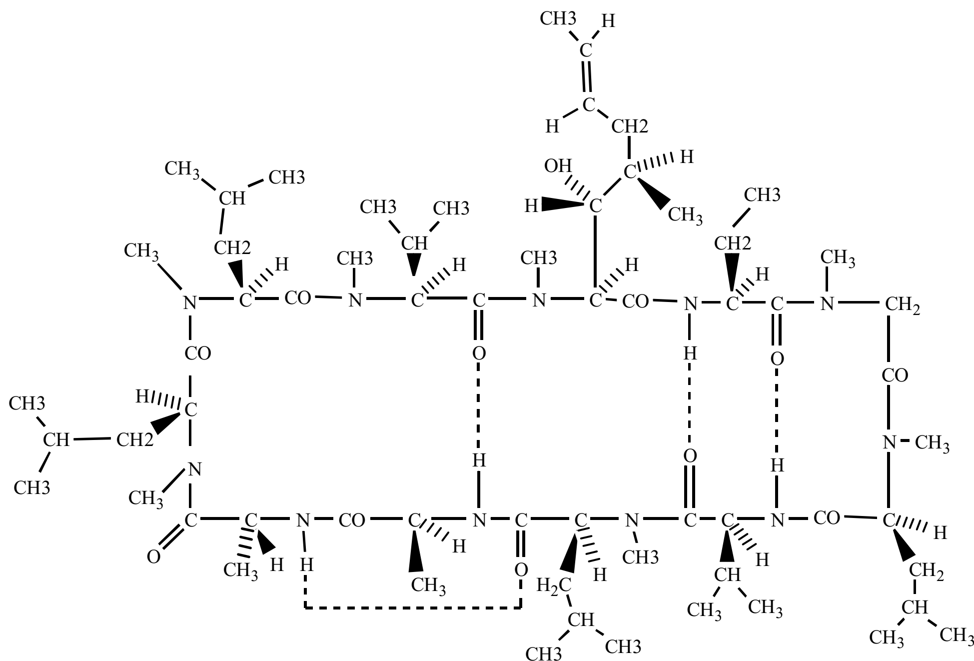

Fig. 1. Structure of cyclosporine.

molecular species absorb energy in this wavelength, sample preparation procedures are usually complex and involve vigorously removing potentially interfering compounds. Most of the published methods involve the use of solid-phase extraction (SPE) procedure following rapid protein precipitation with acetonitrile, which takes so long and is time-consuming [3–10].

The aim of the present work was to develop a suitable method for CyA assay in whole blood. In this paper, we report a simple and reproducible HPLC method using simple sample preparation procedure and demonstrate the pharmacokinetic applicability of the method.

2. Experimental

2.1. Reagents

All chemicals were reagent or HPLC grade. Acetonitrile, methanol, disodium hydrogen phosphate, sodium hydroxide and concentrated phosphoric acid were obtained from Merck, Germany. CyA from Sandoz (Switzerland) was provided by Zahravi Pharmaceutical Co. (Tabriz, Iran).

2.2. Instrumentation

The apparatus used for this work was an LC-6A solvent delivery pump equipped with an SPD-10A UV-Vis detector and a C-R4A integrator (all from Shimadzu, Kyoto, Japan). The detector was set to 210 nm. The samples were applied by a Rheodyne 7725 loop injector with an effective volume of 100 μl. A Shim-pack CLC-ODS (150 mm × 6 mm i.d.; 5 μm particle size) (from Shimadzu Co., Kyoto, Japan) was used for the chromatographic separation. The mobile phase comprised of acetonitrile–5 mM disodium hydrogen phosphate (75:25, v/v), adjusted to pH 5.1 with concentrated phosphoric acid and 1 M sodium hydroxide.

Analyses were run at flow rate of 1.5 ml/min at 70 °C for 12 min and then 10 min at flow rate of 2.5 ml/min. Before the next injection, the flow rate was decreased to 1.5 ml/min. The samples were quantified using CyA peak height.

2.3. Sample preparation

Five hundred microliters acetonitrile was added to a 250 μl of whole blood in a 1.5 ml polypropylene microcentrifuge tube. The tube was vortex-mixed for 30 s and centrifuged for 2 min at 11,000 × *g*. The supernatant was transferred to another clean tube, and a 50 μl volume was injected into the chromatograph.

2.4. Assay validation

Standard calibration curves were constructed by spiking drug-free blood with a known amount of CyA in the concentration of 100–3000 ng/ml. Known standards of CyA were used to determine the within-day and between-day precision and accuracy ($n = 3$). In addition, the absolute recovery ($n = 3$) was estimated by comparison with direct injection of methanolic drug solutions of corresponding concentrations.

2.5. Application

The assay was used for a comparative bioavailability study of a generic CyA preparation (100 mg soft gelatin capsule, Zahravi Pharmaceutical Co., Tabriz, Iran) versus reference product (100 mg soft gelatin capsule, Neoral, Novartis pharmaceutical Co., Switzerland).

Eighteen healthy male volunteers participated in the study. The study was conducted using a two-way crossover design, as a single dose, randomized, trial. The two preparations were administered on two treatment days, separated by a washout period of 7 days, to fasting subjects who received a single oral dose of 200 mg (i.e. two capsules) of one study medication of CyA. Food and drink were not allowed until 3 h after ingestion of the capsules. Multiple blood samples (3 ml) were collected before and 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 8 and 10 h post-dosing in heparinized tubes and stored at 4 °C until analysis.

3. Result and discussion

Although deproteination of the blood samples by acetonitrile is used in most of the previous CyA analysis methods, presence of many interferences in the chromatogram makes it difficult to inject supernatant directly. To overcome this problem, most of the HPLC methods [3–10] use extensive sample clean-up procedure following deproteination that complicates HPLC assay of CyA in the whole blood. This may explain why most of the laboratories prefer to use immunoassay methods. In the present study, it is demonstrated that CyA could be analyzed in whole blood by HPLC following direct injection of deproteinized blood samples.

We tested several commercially available HPLC columns and found great differences in their chromatographic behavior. The Shimpack CLC columns allowed more efficient separation of CyA peak from interferences. Shimpack CLC columns with different packing materials such as C18, C8, CN, and TMS were tested and all were found suitable. Columns like CN or TMS should be operated at 60 °C with the mobile phase of 50% acetonitrile, while 70 °C and 75% acetonitrile should be used for C18 or C8 columns.

While a guard column is usually used in HPLC methods, we observed that separation is completely lost when a guard column is used in the present work. Therefore, it is important to avoid a guard column between injector and column. However, without a guard column, the performance of the HPLC column did not changed at least after 500 injections. The exact reason of negative effect of guard column on separation is not clear, but it may be one of the reasons that direct injection of deproteinized whole blood was not tried by pervious investigators.

Although buffer had no effect on the retention time of CyA, it changed the retention times of some interferences. We found that dilute buffer in the mobile phase helps to provide an empty bond in the retention time of CyA. Use of higher concentration of salt did not improve chromatographic separation and therefore was avoided to reduce the risk of salt precipitation in the system.

The storage condition for whole blood samples is also important for using our method. We found that freezing of blood samples increases interferences dramatically and should be avoided. It had been reported that CyA is stable in blood samples for 3 months when stored at 1–4 °C [11,12]. In our study, CyA had no stability problem at 4 °C, at least for 2 months. Although, the number and intensity of interfering peaks were gradually increased after long storage at 4 °C, the

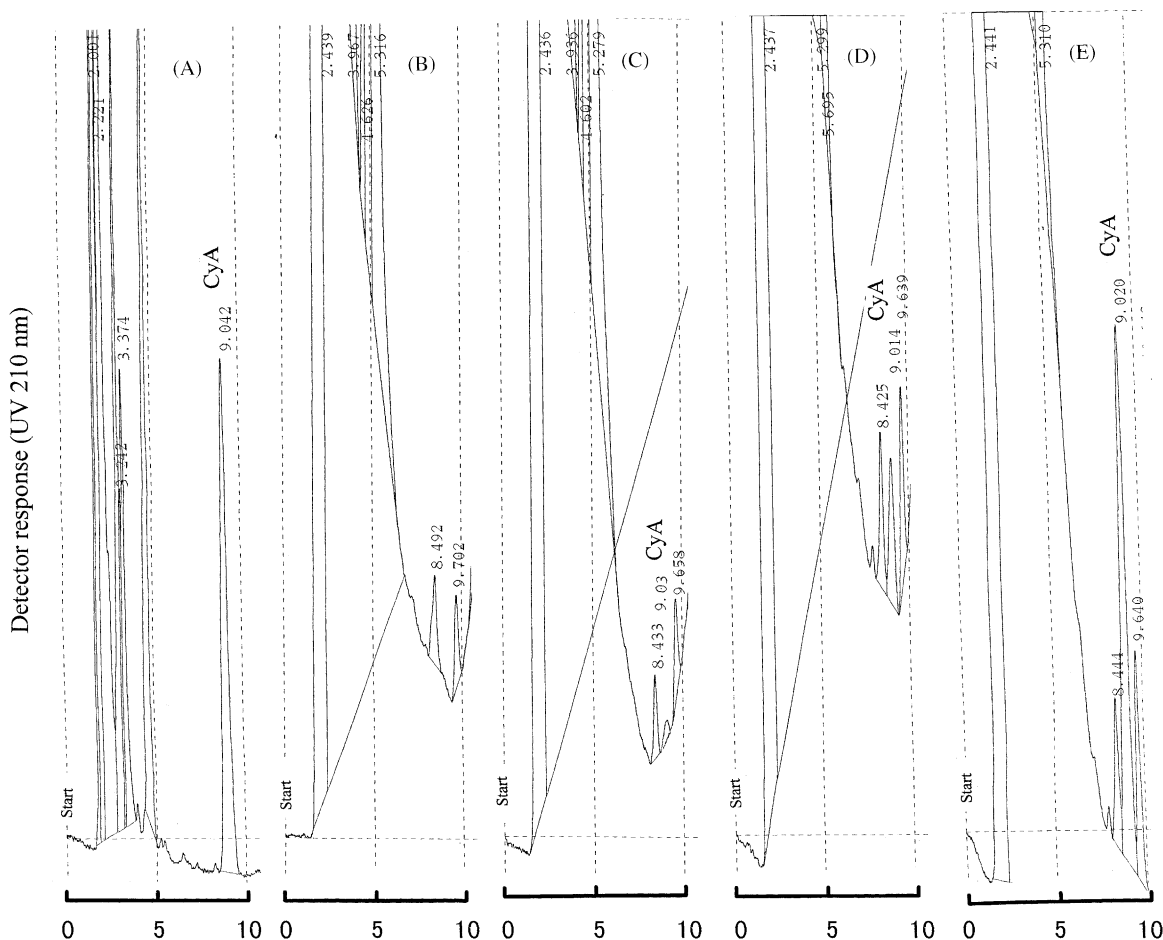

Fig. 2. Chromatograms from (A) 20 ng of cyclosporine (CyA) in 10 μl methanol, (B) blank blood, (C) CyA blood standard of 100 ng/ml, (D) CyA blood standard of 400 ng/ml, (E) a volunteer sample 2 h after taking 200 mg CyA (attenuation = 0, chart speed = 4 mm/min).

CyA was still well separated in blood samples when stored up to 2 months at 4 °C.

Chromatograms obtained with CyA are shown in Fig. 2. The CyA peak, which had a retention time of 9.1 min, was well resolved and free of interference from endogenous compounds in the blood. Some late eluting peaks were present in the chromatogram (which are not shown in the chromatograms) that was eliminated by increasing the flow rate up to 2.5 ml/min for 10 min before the next injection. Alternatively, gradient elution is suggested to wash column between runs in future studies. The absolute recovery, within-day and between-day accuracy and precision values are presented in Table 1. The absolute recovery value was approximately 97%. The standard calibration curves ($n = 3$) were found to be linear over the concentration range of 100–3000 ng/ml, with correlation coefficient of greater than 0.999. The limits of quantification and detection were 100 and 50 ng/ml, respectively, which seem sufficient for pharmacokinetic studies. To increase the sensitivity, in the first steps of method development, we were evaporating the supernatant and then redissolving the pellet in 100 μl of 80% acetonitrile in water. However, using this method, the increased sensitivity is associated with increased intensity of interfering peaks.

The present method was applied to analyze blood samples obtained from volunteers who participated in

Table 1
Absolute recovery, within-day and between-day precision and accuracy ($n = 3$)

Concentration (ng/ml)	Recovery		Within-day		Between-day	
	Mean (%)	CV (%)	Precision (CV, %)	Accuracy (%)	Precision (CV, %)	Accuracy (%)
100	96.8	7.8	9.7	7.8	10.2	9.6
500	96.1	1.1	2.5	3.6	2.4	4.5
3000	98.4	2.4	0.5	1.1	0.5	1.3

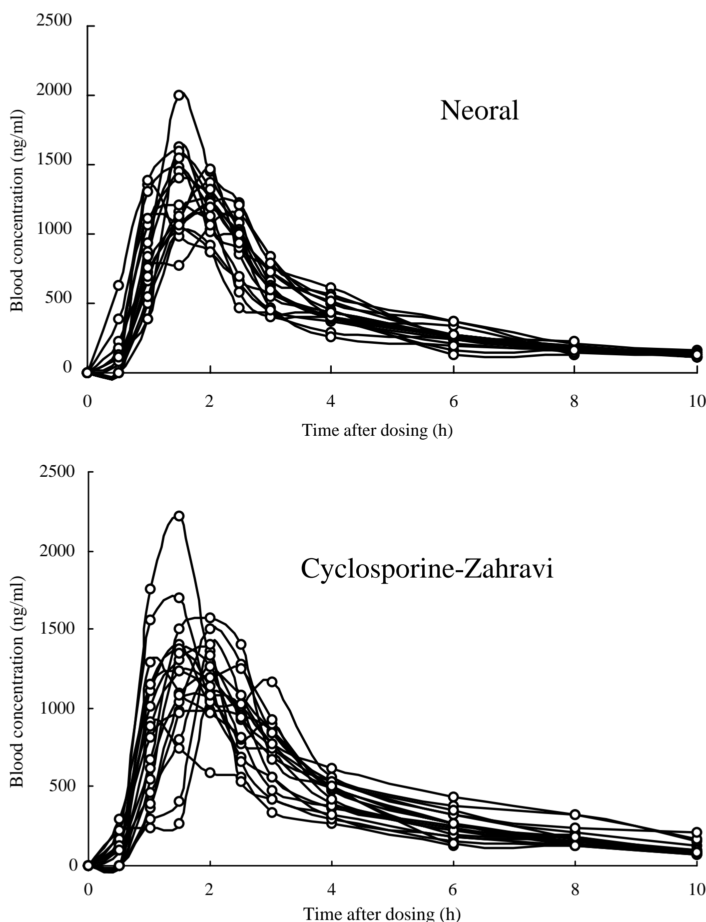

Fig. 3. Blood concentration–time profiles of cyclosporine in 18 healthy male volunteers following oral administration of 200 mg of Neoral (upper panel) and cyclosporine–Zahravi (lower panel) in a crossover study.

a comparative bioavailability of CyA. Fig. 3 shows the individual blood CyA concentration versus time profiles from the volunteers after oral dosing with CyA.

In conclusion, the HPLC method described here is simple, sensitive, reproducible and is applicable to pharmacokinetic studies of CyA.

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