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Note

Simplified liquid chromatographic analysis for cyclosporin A in blood and plasma with use of rapid extraction

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Cyclosporin A is a new fungal cyclic undecapeptide with a potent immunosuppressive activity. It is notably effective in the prevention of graft-versus-host disease in allogenic bone marrow transplantation [1, 2] and in the prevention of graft rejection after kidney [3, 4], heart [5] and liver transplants [6]. The clinical success of cyclosporin and the utility of measuring blood or plasma concentrations of the drug have been well documented [4, 7].

Several methods have been developed for the analysis of cyclosporin A in plasma and/or in blood; these are generally time-consuming or need a high technology [7—15] (Table I).

TABLE I

PUBLISHED METHODS OF CYCLOSPORIN A ANALYSIS

Authors	Method	Sample	Extraction
Donatsch et al. [8]	RIA	Blood/plasma	—
Niederberger et al. [9]	HPLC, gradient	Plasma	Liquid—liquid
Lawrence and Allwood [10]	HPLC, isocratic	Serum	Column
Sawchuk and Cartier [16]	HPLC, isocratic	Blood/plasma	Liquid—liquid
Nussbaumer et al. [11]	HPLC, column switching	Blood/plasma	Protein precipitation
Leyland-Jones et al. [12]	HPLC, isocratic	Plasma	Column
Kahan et al. [7]	HPLC, isocratic	Blood/plasma	Liquid—liquid
Yee et al. [13]	HPLC, gradient	Serum	Column
Carruthers et al. [17]	HPLC, isocratic	Plasma	Liquid—liquid
Smith and Robinson [14]	HPLC, column switching	Blood/plasma	—

The following study reports the development of a rapid and selective high-performance liquid chromatographic (HPLC) method for the routine analysis of cyclosporin A in blood and plasma. Cyclosporin D was used as the internal standard. This method is suitable for pharmacokinetic studies.

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MATERIALS AND METHODS

Reagents

Chemicals used were cyclosporin (cyclosporin A and D) (Sandoz, Rueil Malmaison, France), diethyl ether Spectrosol (S.D.S., Peypin, France), *n*-hexane Spectrosol (S.D.S.), acetonitrile for chromatography LiChrosolv (E. Merck, Darmstadt, F.R.G.), trifluoroacetic acid (TFA) Uvasol (Merck), methanol Normapur (Prolabo, Paris, France), hydrochloric acid Normapur (Prolabo), buffer pH 10 (Merck).

Water was demineralized and filtered on a Millipore filter Type HA 0.4 μm.

A stock solution of cyclosporin was prepared by dissolving 10 mg of cyclosporin A (or D) in 100 ml of methanol. Further dilutions of this stock solution were made with methanol prior to preparing plasma and blood standards.

Chromatographic conditions

A Waters 6000A pump including a U6K injector was used for the chromatographic analysis (Waters Assoc., Milford, MA, U.S.A.). The analytical column was a RP-18 Hibar LiChrocart 4 μm (25 × 4.6 cm) (Merck). The ultraviolet (UV) detector was a UVIKON 722 LC. A 4 × 0.4 cm precolumn dry-packed with μBondapak C_{18} reversed-phase packing served to protect the analytical column from the adsorption of sample constituents.

The precolumn and analytical column were maintained at 75°C with a column heater block (built by Y. Clavel, Biophysics Department, Nice). This temperature was reached from room temperature in 30 min in order to minimize damages.

To separate cyclosporin A and D from other co-extracted serum or blood constituents, the following mobile phase was used: acetonitrile—1 ml/l trifluoroacetic acid in water (70:30) with a flow-rate of 1.2 ml/min and column pressure of 143 bar, with the detector set at 210 nm. The mobile phase was continuously degassed by helium flow. Under these conditions, the retention times were 6 min for cyclosporin A and 7.2 min for cyclosporin D (see Figs. 1 and 2).

Sample preparation

Blood samples were drawn into heparinized tubes from patients having received oral or intravenous cyclosporin A. If the determinations are performed in the whole blood, the sample may be immediately extracted, whereas when the determinations are performed on plasma, the samples must remain for 2 h at room temperature before centrifugation in order to stabilize exchanges between blood cells and plasma.

Conical glass centrifuge tubes (25 ml) were cleaned with diethyl ether and then dried. Different volumes of the standard solution of cyclosporin A were added to standard tubes. The same volume of the internal standard solution was added to all standard curve tubes and to each sample tube. All tubes were dried with a nitrogen flow. Then 0.5—1 ml of drug-free human blood or plasma was added to each standard tube and 0.5—1 ml of patient's blood or plasma to the appropriate sample tubes. The blood samples were lysed by rapid deep freezing and then thawed.

Hereafter all tubes were treated identically; 0.5—1 ml of pH 10 buffer was added to each tube, mixed for 5 sec with a vortex mixer and then 7 of ether were dispensed with an automatic pipette. The tubes were shaken mechanically for 5 min on a horizontal shaker and centrifuged for 5 min at 800 *g*. A 6-ml aliquot of the separated diethyl ether layer was then transferred to another conical centrifuge tube, which had been previously rinsed with diethyl ether. Another extraction was performed with 7 ml of diethyl ether and then the two ether extracts were mixed and evaporated to dryness under vacuum and nitrogen flow for 10 min at 40°C.

The plasma or blood residue concentrated at the bottom of the conical tube and was then dissolved in 200 μl of methanol—0.1 *M* hydrochloric acid (1:2); 1 ml of *n*-hexane was added and then mixed for 20 sec with the vortex mixer.

An aliquot of 30 μl was injected into the chromatograph.

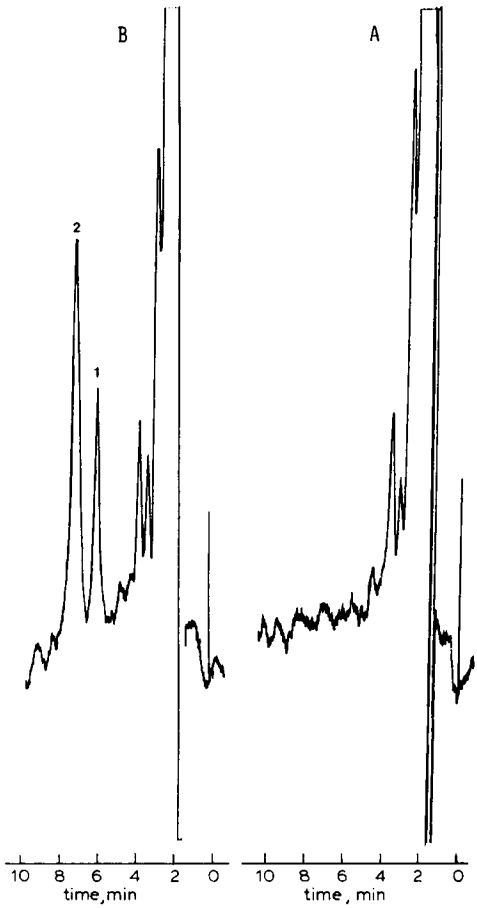

Fig. 1. (A) Chromatogram obtained from analysis of an extract of cyclosporin-A-free human plasma without addition of internal standard. (B) Chromatogram obtained from analysis of an extract of a plasma sample containing 250 μg/l cyclosporin A (1) and internal standard (cyclosporin D) (2).

RESULTS

Chromatograms

No interfering peak was detected in the plasma or blood used for blank standards or from subjects who had received cyclosporin A.

The chromatograms obtained in the analysis of 1 ml of blank plasma or blood and 1 ml of patient's plasma or blood sample containing 250 μg/l (plasma sample) and 150 μg/l (blood sample) are shown in Figs. 1 and 2.

Concentrations of cyclosporin A in plasma or blood were determined from calibration curves of peak height ratios of cyclosporin A to cyclosporin D. The use of the internal standard cyclosporin D was essential for a good precision and linearity of the method.

Linearity

The relationship between the peak height ratio of cyclosporin A to the internal standard (cyclosporin D) and the cyclosporin A concentration in blood

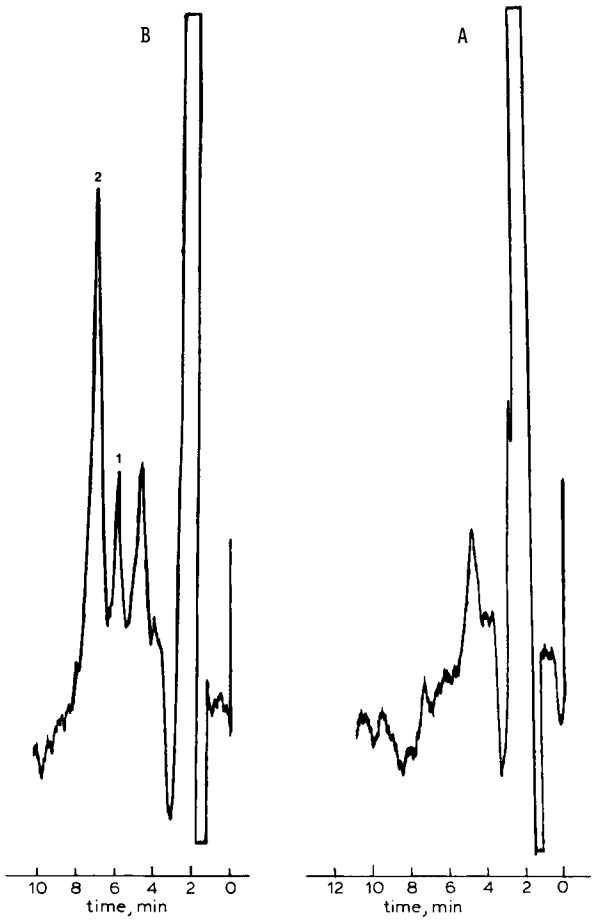

Fig. 2. (A) Chromatogram obtained from analysis of an extract of cyclosporin-A-free human blood without addition of internal standard. (B) Chromatogram obtained from analysis of an extract of a blood sample containing 150 μg/l cyclosporin A (1) and internal standard (2).

or plasma was linear over the range 0—1000 μg/l (plasma, r = 0.980) and 0—2000 μg/l (blood, r = 0.988).

Analytical recovery, sensitivity and reproducibility

Recovery of cyclosporin A was determined by comparing the peak height ratios after extraction of plasma and blood samples containing known amounts of cyclosporin A with those of unextracted samples supplemented with the same amounts of cyclosporin A. For the purpose of this calculation, the internal standard was added to the samples just before injection into the chromatograph.

Cyclosporin A recoveries of 85% and 74% from plasma and blood, respectively, were calculated at a concentration of 300 μg/l.

The sensitivity of this method was evaluated by analysing plasma and blood samples after addition of cyclosporin A in concentrations approaching the sensitivity limit. Method parameters remained consistent to 25 μg/l for plasma and 35 μg/l for blood.

The reproducibility was evaluated after extraction of several plasma and blood samples with given concentrations of cyclosporin (inter-assay) or after repeated injections of the same extract (intra-assay).

The results are given in Table II.

TABLE II

INTRA- AND INTER-ASSAY COEFFICIENTS OF VARIATION (C.V.) FOR CYCLOSPORIN A IN PLASMA AND BLOOD

	Concentration (μg/l)	n	C.V. (%)
Intra-assay			
Plasma	150	8	5
Blood	300	8	4.5
Inter-assay			
Plasma	100	5	7
	400	5	5
Blood	150	5	4
	1000	5	7

APPLICATION OF THE METHOD

Approximatively 600 plasma and blood samples from ten patients have been analysed by this method, with dosage just before treatment and sometimes 2 or 4 h after to assess the serum or blood peak. Fig. 3 shows a typical blood concentration curve after a single dose of cyclosporin A in a treated patient (2 × 200 mg per 24 h per os).

DISCUSSION

The first reported method employed for cyclosporin A determination was radioimmunoassay (RIA) [8] which was initially developed for plasma and, as shown later, was also applicable to blood samples, Its main drawback has been

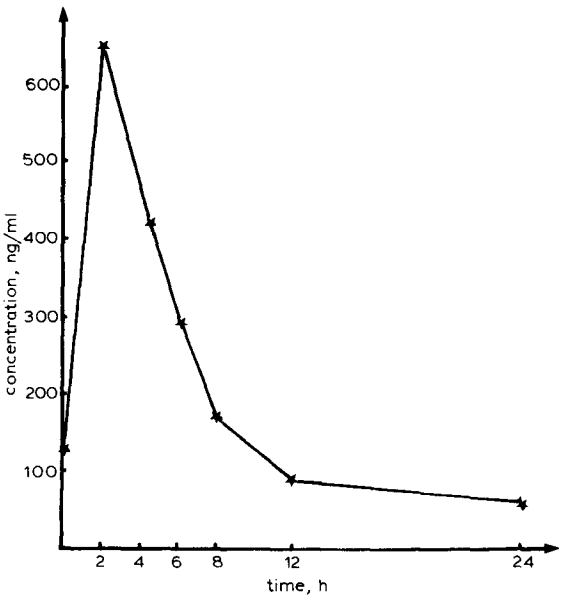

Fig. 3. Blood concentration curve of cyclosporin A from a patient who received an oral dose of 200 mg (chronic treatment).

the cross-reactivity of the antisera with some of the circulating metabolites of cyclosporin A.

The chemical nature of cyclosporin A has delayed the development of analytical techniques, but in the last three years high-performance liquid chromatography (HPLC) has proved to be a valuable technique for the analysis of this drug. Compared to RIA, HPLC methods have a greater specificity. But most of these HPLC methods have disadvantages, especially the long duration of sample preparation and/or chromatography times which make them unsuitable for routine determinations [16, 17]. Other methods have a shorter clean-up procedure, but require a higher technical complexity based on gradient elution or column switching [7, 9, 11, 13, 14].

We describe a method that is now routinely used in our laboratory to quantitate cyclosporin A in concentrations as low as 35 μg/l in whole blood or 25 μg/l in plasma. Compared to other methods, this method provides a combination of rapidity of extraction and simplicity of chromatographic analysis.

The use of an alkaline extraction allows us to reduce the number of steps in the clean-up procedure. Thus, the protein precipitation and the double extraction (acid then alkaline) described by Carruthers et al. [17] can be prevented. Moreover, the double wash with *n*-hexane introduced by Sawchuk and Cartier [16] is not useful; a similar result is obtained without delay by adding *n*-hexane just before injection, together with methanol—0.1 *M* hydrochloric acid. Furthermore, a good recovery is obtained with this extraction procedure.

The use of the internal standard (cyclosporin D) which differs only slightly from cyclosporin A in structure and chromatographic properties, confers to the analytical method a good precision and linearity.

The analytical column used, RP-18 4 μm, gives a good selectivity, sufficient

resolution and allows for isocratic conditions with the mobile phase 0.1% TFA—acetonitrile (30:70). We preferred 0.1% TFA to water, because its use results in sharper peaks.

The chromatograms of blanks from plasma and blood show fluctuations of the baseline, which cannot be considered as interfering peaks, and are due to the very high sensitivity of the detection (0.005 a.u.f.s.) and the short wavelength (210 nm).

The time required for a sample analysis (extraction and chromatographic analysis) is less than 45 min. The sensitivity limit appears adequate for the monitoring of plasma or blood cyclosporin A concentrations in patients receiving regular treatment with this drug.

The possibility of blood determination minimizes the problems of the kinetics of cyclosporin A exchange between red cells and plasma. Moreover, as the concentrations in whole blood are always significantly higher than in plasma, we need in many cases a lower sensitivity.

CONCLUSION

The method reported is rapid and simple, and suitable for routine analysis and therapeutic monitoring in most pharmacology laboratories.

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