Rapid Measurement of Cyclosporin A Plasma Levels by HPLC in Allograft Transplant Recipients

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Summary

An HPLC-method for the measurement of blood Cyclosporin A levels (CyA) of renal allograft transplanted patients within 9min is described. After a simple protein precipitation of the blood the supernatant is transferred to an HPLC-system. The short time of analysis is obtained by a step gradient elution technique and a precolumn separation of the fractions of interest followed by a backflush regeneration step of the precolumn. The analysis of the fraction of interest takes place on a column with high resolution power as long as the precolumn is regenerated. CyA is monitored by UV-absorption at 206nm. Detection of 20 to 2000ng/ml CyA allows the use of the method for patient monitoring and for research purposes.

Introduction

Peptides can be elegantly separated since 1976 by reversed-phase chromatography (RPC) [1]. Cyclosporin A, an 11 amino acid containing cyclic fungal peptide, is a new pharmaceutical agent. It receives increasing attention as the immunsuppressive drug of choice in organ transplantation (kidney, heart, liver, pancreas, bone marrow). Several groups measured plasma levels of CyA using modern HPLC techniques [2–10]. Manual sample preparation, however, is relatively long coupled with extended analysis time [2–9]. Smith and Robinson used an automated method, however, with an extensive experimental setup. Their sample handling coupled with expensive phase separators complicated routine application of their otherwise excellent technique [10].

Plasma concentrations by HPLC give lower values than by radio immuno assay (RIA) [8, 9]. There is no clear relationsship between the results of both methods, as the RIA cross-reacts with unknown metabolites or other plasma components. Therefore the specificity of RIA is lower than expected and CyA cannot be determined in a single plasma sample. The routine clinical analysis of CyA has to be carried out with high reliability, since the success of transplantations is highly depending on the proper administration of the drug and on an exact knowledge of its post-transplantation concentration. Measurement of the plasma sample should take a short time for immediate clinical monitoring. The metabolism of CyA in man is not yet clearly elucidated. For rapid and specific determination of CyA plasma levels a special HPLC column-switching technique had to be developed.

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Experimental Part

Materials

Cyclosporin A (Sandoz, Basle, Switzerland)

Acetonitrile LiChrosolv (Merck, Darmstadt, FRG)

Water HPLC-grade, obtained by a filter unit containing, charcoal + ion-exchanger + absorber for non-polar contaminants (Millipore, Milford, MA, USA)

Blood, to prepare standard mixtures, was received from a population of healthy adults.

Standard containing CyA 1000ng/10µl was made by dissolving 10mg of CyA in 100ml of (acetonitrile/water) (90/10, v/v).

Blood standard: 30ml of blood was incubated with 600µl of standard at 37°C for 30min (CyA 2µg/ml). Samples of lower concentrations were obtained by dilution with native blood down to 20ng/ml.

Columns: Precolumn (PC): LiChrosorb RP-8, 10µm, (30 × 4mm) (Merck, Darmstadt, FRG) connected to an electric high-pressure switching valve (V4) as a loop.

Analytical column (AC): Molnar-C-18, 5µm, 250 × 4.6mm (Molnar, Berlin-W) connected to a high-pressure switching valve (V5) as a loop.

Eluents Contrary to ref. [5] only three different mixtures of acetonitrile with water (v/v) were needed:

E1: 50/50 for the elution of the precolumn;
E2: 75/25 for the elution of the both columns;
E3: 90/10 for protein precipitation.

The flow rate was 1.5ml/min with both pumps, in all experiments.

A detailed description of all operations is given in Table I.

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Table I. Complete analysis run with manual sample injection and monitoring* AC–Eluent

	Time [sec]	P_1 V_1	P_1 V_2	P_1 Δp*	P_2	P_2 Δp*	$V4^{**}$	$V5^{**}$	$V6^{**}$	Note***
1	0	E_1	–	1.7	E_2	8.0	A	A	A	Injection of 200μl sample.
2	70	–	E_2	1.2	E_2	8.0	A	A	A	Increase of eluent strength on the PC.
3	90	–	E_2	9.7	E_2	–	A	B	A	Sample washed from the PC to the AC.
4	150	–	E_2	1.2	E2	8.0	B	A	A	Fraction containing CyA is developed on the AC. Late eluting components from the PC go to waste in backflush mode.
5	420	E_1	–	1.7	E_2	8.0	A	A	A	PC forward wash. Equilibration with E_1.
6	540	E_1	–	1.7	E_2	8.0	A	A	A	End of program. Ready for next injection.
				Position A: ———						
				Position B:						

* Pressures (Δp) in MegaPascal (MPa)

** Valve positioning (V_{4-6}) in Fig. 1.

*** See Figs. 1 and 2.

Methods

Protein precipitation procedure from blood. 0.5ml of blood (sample or blood standard) and 1ml of (acetonitrile/water) (90/10, v/v) were given into a 5ml glass vial, shaked (Whirlmix) and centrifuged for 5min at 3000x *g*. The supernatant was injected manually using a Rheodyne 7125 loop injector with a 200μl loop (s. valve (V3) in Fig. 1). The advantage of this method is the high recovery of CyA contrary to other methods where protein precipitation takes place in aqueous systems (e.g. Cl_3CCOOH), and part of CyA is coprecipitated with the proteins. Here, in acetonitrile/water (90/10, v/v), CyA remains in solution, as hydrophobic interactions in this medium are almost eliminated. The recovery of CyA is complete.

HPLC-column-switching. Cyclosporin A–Analyzer (Fig. 1) was designed in cooperation with ACT (Dortmund, FRG). The dedicated HPLC system was built of commercially available components.

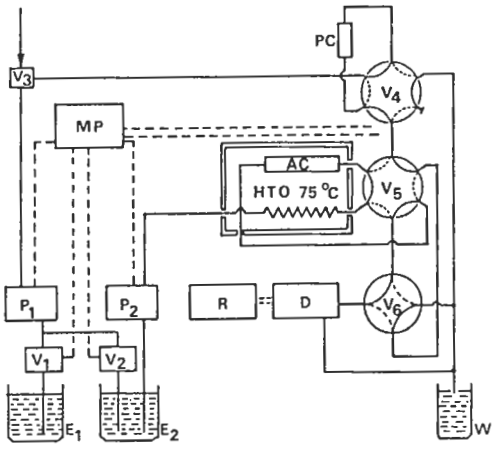

Fig. 1

HPLC-Analyzer for rapid measurement of Cyclosporin-A and of its metabolites in human plasma.

D variable wavelength UV-detector; **MP** HPLC Microprocessor-Programmer; **R** 2-channel chart recorder; **W** Waste. For further explanation and description see Experimental part.

The experimental setup consists of a precolumn (PC) and of an analytical column (AC). Two low-dead-volume high-pressure pumps (P1 and P2) are needed as the function of both columns is timewise different. Sample introduction into PC takes place with a Rheodyne 7125 valve (V3) using eluent E1.

Eluting the sample from PC with E2 alone, there is often a certain amount of other constituents in the fraction of interest as the elution is very short and the fractionation is unprecise. Consecutively, the regeneration of AC is not completed after a period of 15min.

In order to separate substances on the PC which have similar hydrophobicities as CyA, we found an optimal elution strength of 50:50 (v/v) acetonitrile-water. However, due to the already described chromatographic behaviour of CyA [4], we found a very broad peak of CyA (1.5 to 4.5min).

The elution of CyA from the PC needs a step gradient which is realized by switching two low-pressure, low-volume magnetic valves (V1, V2) in front of pump 1. As the dead volume of the pumps is about 100μl, eluent change takes a time of only 4sec at a flow rate of 1.5ml/min.

Both column systems are connected for a given time to transfer CyA from the PC to the AC by switching the high-pressure electric valve (V5) into position B (Table I, Fig. 1). Elution of CyA from the AC is done isocratically washing it with E2.

The technique needs a precise timing of the electric high-pressure switching valves (V4, V5, Fig. 1). We used for this purpose a time-programmable microprocessor (MP). The exact position of the fraction of interest requires constant flow through the precolumn. To constancy monitor the flow rate on the PC we used a two-channel recorder (R). As long as the first channel registered the detector output, the second channel monitored the pressure of pump 1. Post-transferring the fraction of interest to the AC, no CyA was missing if the pressure of pump 1 was constant. After changing eluent E1 to E2 by switching V1–V2, the pressure on pump 1 decreased from 1.7 to 1.2 MPa due to the lower viscosity of E2.

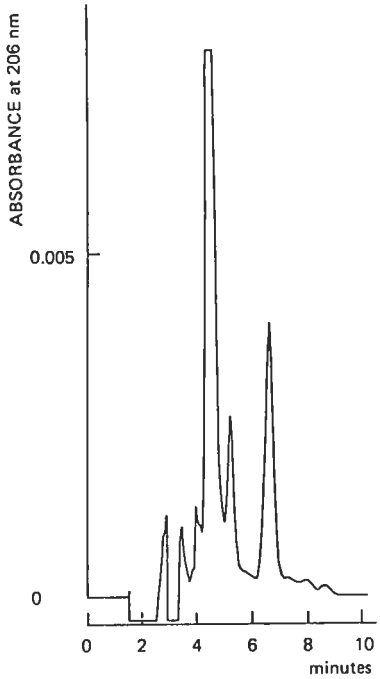

Fig. 2
Chromatogram of CyA from human plasma. For conditions see Table I.

Functions of V1, V2, V4, V5 and of P1 and P2 were controlled by the microprocessor (MP) (Fig. 1, Table I).

Optimization of elution conditions. Visualization of the separation processes on each column can be done individually and independently by switching the manual valve V6 into position A (for AC) or position B (for PC) respectively (Fig. 1). Improved peak shape and better resolution of CyA from other components on the AC at a working temperature of 75 °C were shown by Sawchuk and Cartier [4]. Therefore, we used a high-temperature oven for heating the AC and prewarming the eluent. The PC was kept at ambient temperature (25 °C).

Detection. A variable wavelength detector (D, Fig. 1) was used at 206 nm as there is an absorbance maximum of CyA. No major components were seen at the retention time of CyA when measuring a native blood sample containing no CyA. In order to reach an adequate peak height at low amounts of CyA in the sample, a sensitivity of E = 0.01 AUFS was chosen. At this sensitivity the baseline was constant after about 1 hour. During this time both columns were cleaned by a washing program with E2.

Results and Discussion

In clinical HPLC the complete analysis of a plasma sample on an analytical column is only possible by gradient elution, which is, due to development and reequilibration time, rather tedious.

Another disadvantage is the long clean-up step of a 25 cm column. Contrary to this, a precolumn offers short regeneration and clean-up time and allows a pre-separation step, by which a gradient elution becomes obsolete. In this pre-separation step we can divide the eluted sample components in *three groups* according to their hydrophobicity:

the first fraction is containing hydrophilic substances;
the second fraction containes compounds of medium hydrophobicity, including CyA;
the third fraction contains strongly hydrophobic and non-soluble molecules which would only elute after a long time or would not elute at all.

By a back-flushing step the precolumn can be easily and thoroughly regenerated.

Instead of using a linear gradient to elute the CyA-fraction from the PC, the analysis time on the PC can be shortened by changing the eluent after 70 sec from E1 to E2. Due to the liquid volume of the precolumn of 0.37 ml, the CyA-

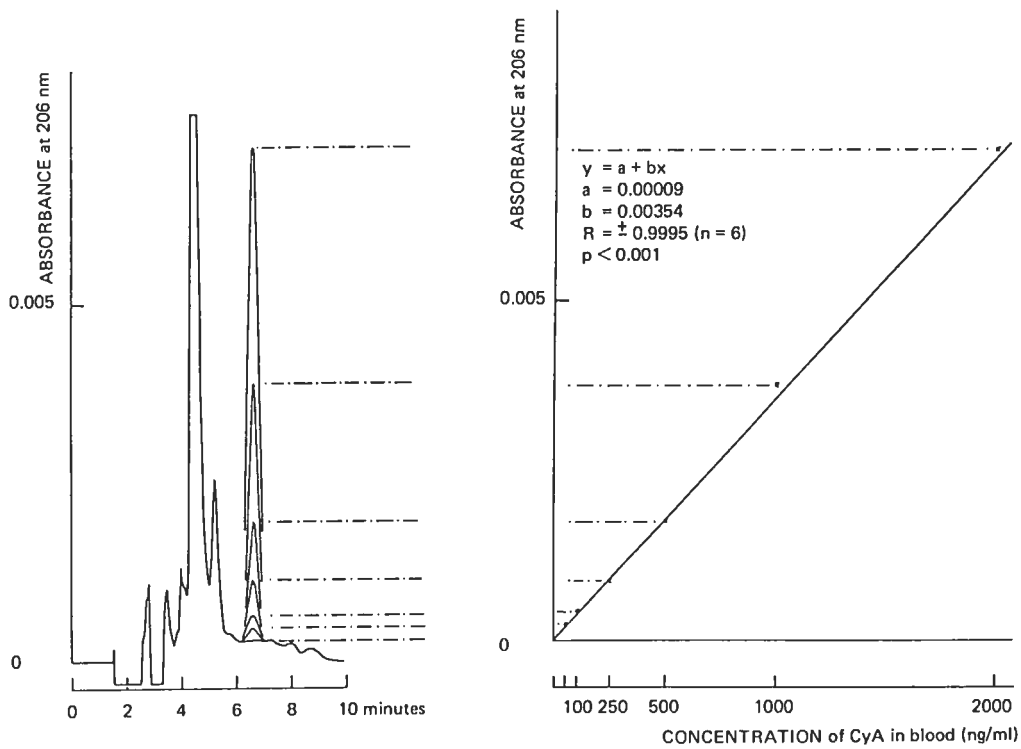

Fig. 3
Linearity test of CyA detection.

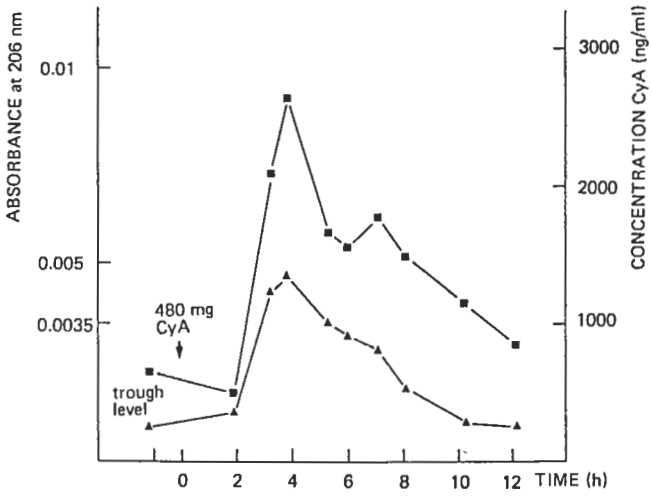

Fig. 4

Detection of CyA in plasma of a renal allograft recipient after a single oral dose. Comparison between RIA and HPLC.

peak is emerging at the time of 90sec. In this way the elution of the enriched CyA fraction from the PC is achieved without the specific spreading of CyA. The narrow band can completely be transferred to the AC which results in a shorter analysis time.

A complete analysis of a plasma sample containing 1000ng/ml CyA is shown in Fig. 2. The cycle-time is presently optimized to 9min including the cleaning and reequilibration of the PC which is carried out while the analysis proceeds on the AC.

Detection limit. Due to the almost quantitative recovery of CyA by the protein precipitation procedure used here, a concentration of 20ng CyA/ml blood can be detected by injecting only 200µl of the supernatant. The lowest detection limit (2x the noise) at a sensitivity of E = 0.01 AUFS corresponds to a quantity of only 2ng CyA.

Linearity test. Linearity was tested by the measurement of blood standards. The height of the CyA-peaks depend linearly on the concentration of CyA in the blood, in a range of 50 to 2000ng/ml ($r = 0.9995$; $n = 6$) (Fig. 3b).

Measurement of blood samples from a renal transplanted patient and comparison with values from RIA. A 45-year old man received renal transplantation because of chronic renal failure. Fig. 4 shows a plasma-concentration-curve from this patient after a single oral dose of 480mg Cyclosporin A (Sandimmun R) (14mg/kg weight). After the trough level from previous dosis, the concentration of CyA in blood increases, reaching a maximum after about 3.5 hours. Subsequently, it is decreasing to the next trough level after about 10 hours.

The kinetic is the same for HPLC and RIA although RIA gives higher values, by approximately a factor of two. The reason for this difference is not yet clear. Cross-reactions of RIA-antibodies with metabolites of CyA or other plasma constituents are conceivable.

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