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SEMI-AUTOMATED HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF CYCLOSPORINE A IN WHOLE BLOOD USING ONE-STEP SAMPLE PURIFICATION AND COLUMN-SWITCHING

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SUMMARY

A highly sensitive and semi-automated high-performance liquid chromatographic method, utilizing acetonitrile protein precipitation and column-switching, is described for the determination of cyclosporine A in whole blood. Following a rapid manual acetonitrile treatment of the blood samples, the supernatant is loaded automatically onto a 5-μm high-speed protein separation column without any further clean-up operations. The fraction containing cyclosporine A is switched to a 3-μm C_{18} reversed-phase high-speed column by a microprocessor-controlled column-switching unit for final separation and detection by absorption at 214 nm.

Minimal sample handling and efficient separation resulted in a high recovery (75 ± 3%) of cyclosporine A from blood and a detection limit as low as 2 μg/l with a highly reproducible and linear response up to 2500 μg/l using 0.5 ml of sample. A separation cycle including regeneration of the first column is finished in 15 min, and this system was used continuously for ca. 1000 blood samples from heart, liver, kidney, pancreas and bone marrow recipients without change in separation parameters or material replacement.

The method described allows accurate and very fast daily routine monitoring of cyclosporine A in large numbers of blood samples from transplant recipients.

INTRODUCTION

Cyclosporine A (CyA, OL 27-400, Sandimmune®) is a hydrophobic cyclic undecapeptide of fungal origin [1], which is being used increasingly for immunosuppression in patients undergoing organ transplantation [2]. This compound is effective in preventing rejection of heart—lung, liver, kidney,

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pancreas and bone marrow grafts by interfering selectively with T-lymphocyte activation and proliferation [3].

Concentrations of CyA in blood that exceed therapeutic levels are associated with adverse effects, such as nephrotoxicity, hepatotoxicity, hirsutism and tremor [4, 5]. Since patients show large individual variations in CyA metabolism and absorption rates after oral application, in addition to drug interactions, methods should be available for the frequent and rapid estimation of the drug to provide adequate immunosuppression without a significant incidence of toxicity [6].

Since cyclosporine A is taken up by erythrocytes, and its distribution in blood depends on temperature, drug concentration, haematocrit and lipoprotein content, the drug should be measured in whole blood [7]. This may be done by a radioimmunoassay (RIA) method [8], but the one that was developed for CyA shows a cross-reactivity to certain metabolites of CyA [9]. The proportions of the unchanged drug and its metabolites seem to be affected by graft function in liver graft recipients and may also be altered by drug interactions.

Several high-performance liquid chromatographic (HPLC) methods have therefore been developed to measure native CyA in blood or plasma samples. A semi-automated method that excludes labour-intensive sample extraction and purification procedures has been published recently, but needs still an additional sample hexane wash system [10] for sample purification.

The aim of the present study was the development of a highly sensitive and reproducible HPLC analysis system for CyA measurement in blood samples which would involve minimal technical equipment with one-step sample purification omitting any further clean-up of the protein precipitation supernatant.

MATERIALS AND METHODS

Reagents

Cyclosporines A, D (Val-2-cyclosporine) and dihydro-C (Thr-2-cyclosporine) [11] were a gift from Biochemie (Vienna, Austria).

Water was purified with a Milli-Q system (Millipore), and fractions with a specific resistance higher than 18 MΩ/cm were used for HPLC analysis and sample preparation.

Acetonitrile (Art. 30, chromatography grade), methanol (Art. 6007) and trifluoroacetic acid (Art. 8262) were obtained from Merck (Darmstadt, F.R.G.).

Heparinized or EDTA blood for preparation of standards was drawn from healthy volunteers. For stock solutions, the cyclosporines were dissolved in methanol (2.5—250 μg/ml) and stored at 4°C.

Mobile phases

A and B were acetonitrile and water (purified as described above), respectively, and C was acetonitrile—water (71:29). Trifluoroacetic acid was added to mobile phases B (160 μl/l) and C (50 μl/l), and a continuous stream of helium was allowed to pass through the mobile phases.

Sample preparation

A 500-μl volume of whole blood (plasma or bile may also be used) was pipetted into a 15-ml glass test-tube, and cell lysis/protein precipitation was initiated by the addition of 890 μl of acetonitrile (≡ 68% acetonitrile). The stoppered tube was vortexed for 30 sec (Vortex Genie® mixer), incubated for 10 min at room temperature and then centrifuged at 1200 *g* for 10 min. The 1000 μl of the supernatant were transferred to a fresh tube and mixed with 615 μl of water (42% aqueous acetonitrile). This purified sample was filled into 2-ml glass automatic sampler vials (Model 125, Spark Holland), which were each capped with a septum. CyA blood standards were prepared the same way, except that the blood was incubated for 30 min at 37°C with different amounts of CyA stock solutions (less than 5% methanol) prior to treatment with acetonitrile.

Chromatography

A diagram of the HPLC system is shown in Fig. 1A and B. Sample volumes of 1000 μl are loaded automatically (Model 125, Spark Holland automatic sampler) onto an Ultrapore® RPSC protein separation column (5 μm mean particle diameter, 75 x 4.6 mm I.D.; Altex Berkeley, CA, U.S.A.) kept at 333°K by a thermostatted waterjacket (Braun, Thermomix). The autosampler

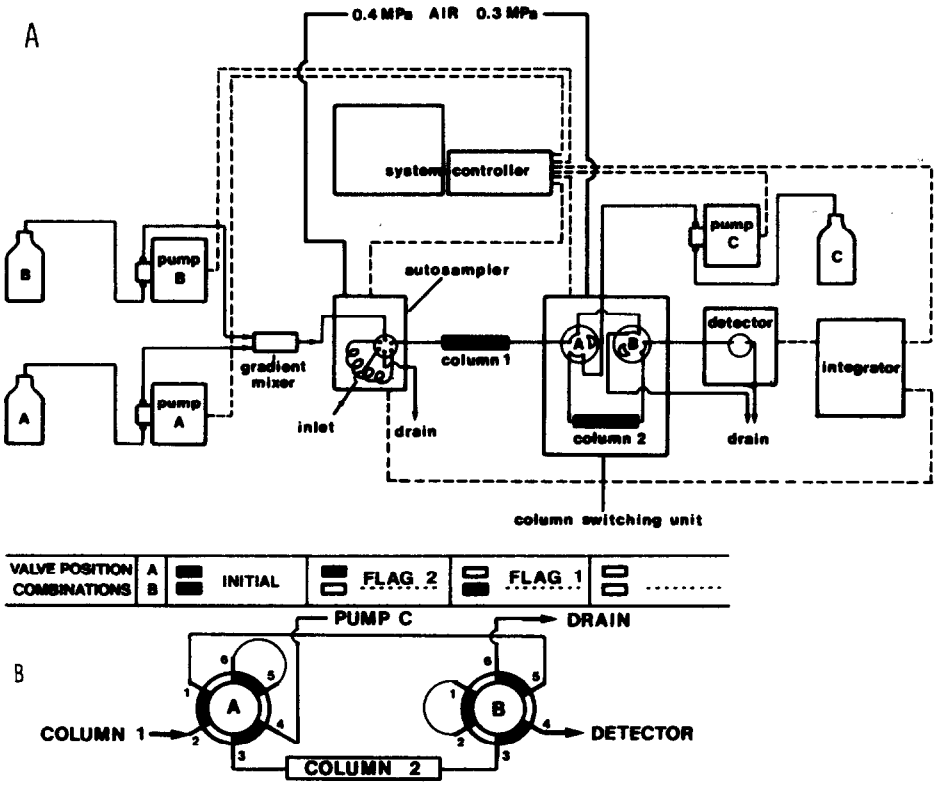

Fig. 1. (A) Scheme of the chromatography system; dashed lines represent microprocessor or autosampler control connections. (B) Scheme of the column switching device.

is started by a Beckman Model 421 CRT HPLC system controller, and itself activates data processing by a Chromatopac CR2AX Integrator (Shimadzu, Kyoto, Japan). The mobile phase consists of 42.4% B and 57.6% A, delivered at a flow-rate of 1 ml/min by a Model 110A and a Model 112 pump (Beckman), respectively (back-pressure ca. 4 MPa), and mixed in a gradient system (Beckman). At time 5.90 min after injection initiation ($t = 4.0$ min) flag 1 (column-switching unit, ventil 1) is activated for 1.80 min, and the fraction containing CyA is diverted automatically onto an Ultrasphere® ODS column (3 μm mean particle diameter, 75 x 4.6 mm I.D.; Altex), which was also kept at 333°K. The column-switching unit was Model 9210 (Spark Holland); its connections and valve positions are shown in Fig. 1A. CyA is eluted from this column with mobile phase C at a flow-rate of 1 ml/min using pump C (Model 112, Beckman). Cyclosporine is detected by its UV absorption at 214 nm using a fixed-wavelength detector (Model 160 absorbance detector, Beckman). Peak height, peak area calculation and baseline drawing are performed by the Chromatopac CR2AX. Concentrations of CyA in blood are calculated by relating peak area to standard curve area measurements run intermittent to unknown samples. In parallel with CyA detection on column II, the Ultrapore RPSC column is regenerated by washing with 90% mobile phase A. (After 11.0 min, the percentage of B is changed to 5% in 1 min, and after 13.0 min it is brought to the initial percentage of 42.4% in 1 min.) Under these conditions, column I can be used for at least 1000 sample determinations without significant increase in back-pressure and without changes in separation parameters, provided each sample is carefully freed from precipitated material as described above. Since a minimum of contaminating material is loaded onto column II in addition to CyA, no increase in back-pressure could be detected for ca. 1000 samples. The cycle finishes at time 3.30 min with activation of flag 4 (integrator stop) and starts again at 4.00 min with automatic injection of the next sample.

RESULTS

Sample treatment

Whole blood was drawn into heparinized or EDTA-coated tubes and prepared for CyA analysis. Coated tubes should be used since the method is sensitive enough to show sample dilution by addition of heparin solutions for inhibition of clot formation. Recovery of CyA from whole blood after acetonitrile treatment was 75 ± 3% for all CyA concentrations tested. Lysis of erythrocytes by addition of Zaponin® (Coulter Electronics, Luton, U.K.) prior to acetonitrile treatment showed no influence on cyclosporine recovery (data not shown). (The cyclosporine fraction that was not recovered seems to be occluded in the protein precipitate.)

Chromatograms

Separation of CyA from most of the contaminating sample components by protein separation column I could be monitored by activating flag 1 to divert the effluent directly to the UV detector. A typical result, using a whole blood standard containing 1000 ng/ml CyA is shown in Fig. 2A. Such determinations

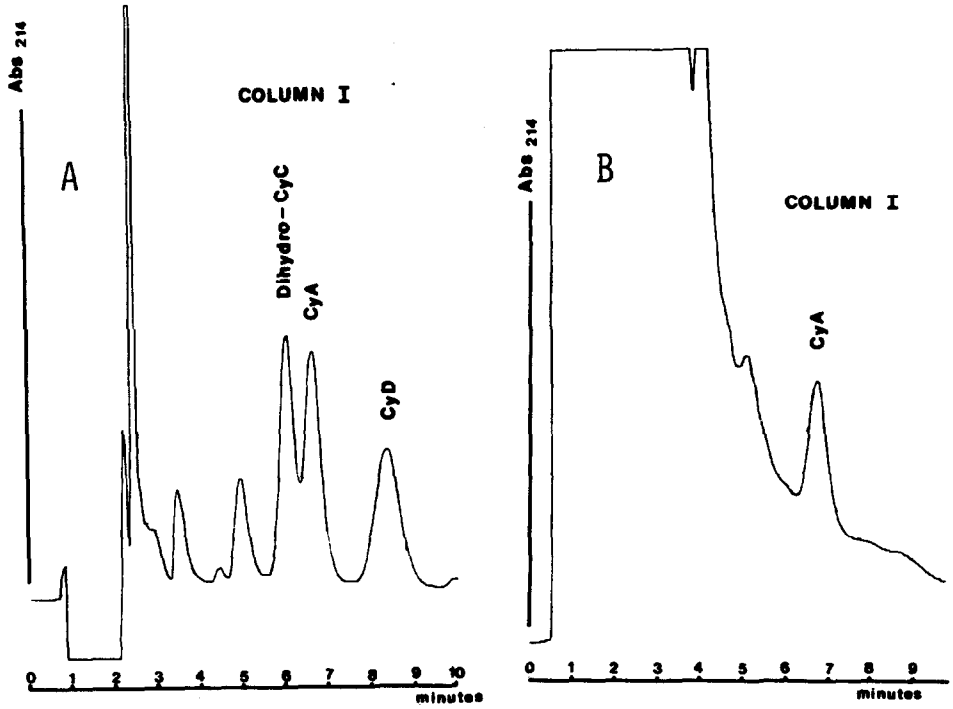

Fig. 2. (A) Separation of a mixture of three cyclosporines (ca. 1000 μg/l of each cyclosporine in methanolic solution) with column I. (B) Separation of a blood standard sample containing 1000 μg/l CyA with column I.

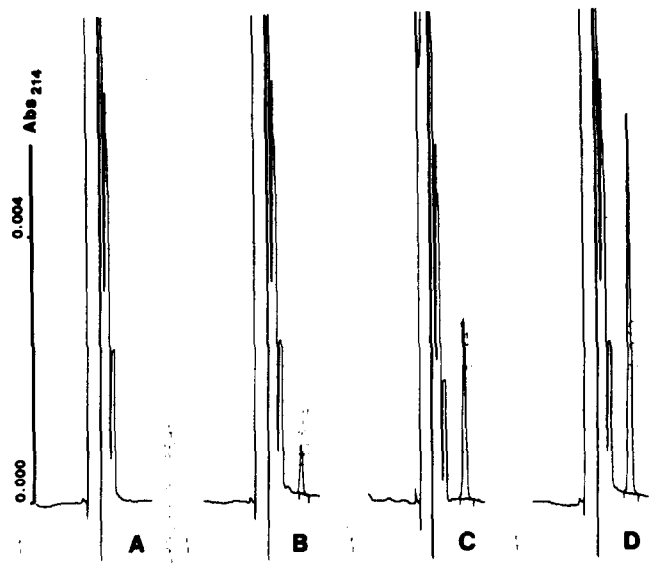

Fig. 3. Representative chromatograms obtained in the analysis of 0.5 ml of (A) blank blood, (B) a blood standard containing 50 μg/l CyA, (C) a blood sample from a kidney graft recipient containing 257 μg/l CyA and (D) a blood standard containing 500 μg/l CyA. Baseline, starting and end points of the peaks are written during data processing by the integrator.

were used to check the correct setting of the switching parameters (start of switching and duration). Reversal of acetonitrile concentrations in mobile phase (57.6%) and sample solute (42%), and the high separation capability of the RPSC 5 μm column I (Fig. 2B), result in a small CyA-containing fraction. Addition of trifluoroacetic acid to mobile phase B and washing with 95% acetonitrile while eluting CyA from column II allow continuous use of column I without further maintenance.

Typical chromatograms of the switched CyA fractions eluted from column II are shown in Figs. 3A—D. The large offset prior to CyA elution is caused by the injection of mobile phases A and B during automatic column switching. No interfering peaks were detected in the blood samples from healthy volunteers, in a number of patients not receiving CyA (pancreatitis, burns) and in graft recipients who showed CyA metabolites as assessed by RIA determination but had no unmetabolized native drug. No internal standard has been used. CyA concentrations in samples were calculated by comparing peak areas of samples with those of standards run daily.

Linearity

Cyclosporine blood standards were run daily, and these determinations gave a linear concentration—response relationship. Cyclosporine concentrations of 0, 10, 20, 100, 250, 500, 1000 and 1500 μg/l in blood were used for calibration, and from the data a mean slope of 0.2851 mV sec l μg^{-1} and a mean y-intercept value of 0.36 μV sec were calculated for the linear calibration curve by regression analysis. Linearity is achieved in the range 10—1500 μg/l as shown by a mean correlation coefficient of 0.998 (n = 22; days 1, 8 and 15).

Precision and reproducibility

The precision of the method was evaluated by analysing blood standard samples ranging from 10 to 1500 μg/l CyA. A set of standards was used to establish a calibration curve and then standards containing 10—1500 μg/l CyA were analysed at days 1, 8 and 15. Within-day variability is very low, the coefficient of variation ranging from 0.5% to 1.75% (n = 12; triplicate measurements of CyA standards with 20, 250, 500 and 1000 μg/l CyA), and results shown in Table I demonstrate the high reproducibility (low day-to-day

TABLE I

ANALYSIS OF BLOOD SAMPLES SPIKED WITH CYCLOSPORINE A

Calculated from duplicate measurements of each CyA blood standard at days 1, 8 and 15.

Spiked concentration (μg/l)	Measured concentration (mean ± S.D., μg/l)	Relative error (%)
10	9.38 ± 0.14	6.2
20	18.9 ± 0.36	5.7
100	95.9 ± 1.93	4.8
250	251 ± 0.18	0.1
500	496 ± 6.35	0.8
1000	1019 ± 24.7	1.9
1500	1490 ± 6.41	0.67

variation) of the method (seven CyA blood standards measured at days 1, 8 and 15 in duplicate).

Sensitivity

The sensitivity of the method was investigated by analysing blood samples spiked with 10 or 20 μg/l cyclosporine. These concentrations could be measured with high precision and accuracy (Tables I and II). The relative error is less than 7% for blood samples to which cyclosporine has been added in a concentration of 10 μg/l, and the mean response factor is the same. The mean absolute response was 3140 ± 64 μV sec for 10 μg/l CyA and peak areas as low as 600 μV sec, which corresponds to 2 μg/l CyA, could be clearly dissolved and integrated. Pure and helium-degassed mobile phases were required to achieve a low detection limit.

TABLE II

SENSITIVITY OF DETECTION OF CYCLOSPORINE A IN BLOOD

CyA concentration (μg/l)	Response factor (× 10) ($\mu g\ sec^{-1}\ l^{-1}\ mV^{-1}$)	Coefficient of variation (%)
10	31.82	2.5
20	34.60	3.4
100	35.70	2.1
250	34.83	0.3
500	34.95	1.9
1000	35.06	0.4

Application of the method

The method has been used for analysis of more than 1000 blood samples from heart, liver, pancreas, bone marrow and kidney graft recipients. CyA trough levels were monitored daily and compared with concentrations measured by the RIA method. The ratio between HPLC and RIA concentrations may vary from 1.1 up to 10.1 for CyA in kidney and liver graft

TABLE III

CONCENTRATION OF CYCLOSPORINE A IN BLOOD FROM A PATIENT

The patient, who had a liver graft, received a single (160 mg CyA = 2.3 mg/kg) or multiple oral doses (4 × 50 mg CyA).

Time (h)	CyA concentration (μg/l)	
	Single dose	Multiple doses
1	570	280
4	1695	630
6	1140	555
8	928	1060
10	642	635
12	347	380

recipients [12]. Table III shows the results of CyA determinations after a single oral dose (time = 0 h) or reduced multiple oral doses (given at 0.5 and 10 h), which resulted in lower CyA peak values.

The HPLC method is extremely useful for rapid determination of CyA in critical clinical situations (signs of acute nephro- or hepatoxicity). A sample analysis can be performed within 1 h and is specific for the unchanged drug, which seems to be the main toxic component [13].

DISCUSSION

Several HPLC methods for the determination of CyA in blood have been published (for a review see ref. 10), but they need relatively expensive and complicated equipment and daily maintenance of the separation columns. The new method presented here provides fully automated sample analysis after only manual sample purification performed in a single step. A large number of blood samples from graft recipicients can be loaded onto the autosampler after manual precipitation of proteins. A sample cycle is finished in 15 min, and this CyA determination system could be run continuously overnight. In most cases, perioperative monitoring of CyA in our laboratory could be finished the same day for all patients, and dosages could be adjusted the same evening for those receiving the drug twice daily (ca. hundred kidney and ten liver transplantations per year).

Acetonitrile treatment of the blood is efficient in precipitating most of the contaminating material and in extracting CyA. For the new method described here, a hexane wash to remove lipophilic material [16], which is an essential step in any other HPLC method reported so far, is not necessary. Sample purification is reduced to one simple precipitation step. Therefore no other HPLC grade chemicals, as used in manual extraction methods (diethyl ether, methanol, hexane) [14, 15] or automated sample wash (hexane) [10], are necessary. High-speed micropore columns (5 μm and 3 μm, respectively) with small dimensions allow for low flow-rates and minimal consumption of reagents, and lead to high reproducibility and increased sensitivity. Minimal sample handling and acetonitrile extraction resulted in high and reproducible recovery of CyA from blood and made possible external standardization without loss of accuracy. Concentrations as low as 10 μg/l CyA in blood could be measured with confidence. This makes the method suitable for routine monitoring of graft recipients who receive maintenance doses of CyA and show high metabolization rates.

By changing the separation conditions of column I, the method may be adapted for determination of CyA metabolites.

In conclusion, the new method reported here employs (a) a simple and short one-step sample purification, (b) sample separation on micropore columns (5 μm and 3 μm, respectively) which could be easily monitored for both columns used, (c) column switching, (d) fast analysis and sensitive detection in 15 min providing rapid sample throughput, (e) minimal technical equipment and minimal routine care for the HPLC system. The instrumentation consists of a standard gradient HPLC system with one additional pump for regenerating column I, and a column-switching device. The method is suitable for meeting

the growing demand for rapid and sensitive monitoring of unchanged CyA in blood samples from graft recipients with a minimum of sample handling and of technical equipment.

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