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DETERMINATION OF CYCLOSPORIN A IN WHOLE BLOOD BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY USING AUTOMATED COLUMN SWITCHING

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SUMMARY

A fully automated high-performance liquid chromatographic column-switching system is presented for the determination of cyclosporin A in whole blood. After blood proteins were precipitated with acetonitrile, the supernatant was automatically loaded on to a cyanopropyl column for initial separation, and then the fraction containing cyclosporin A was loaded on to a trimethylsilica column for final separation and quantitation. Cyclosporin A was detected by ultraviolet absorption at 205 nm. The minimum detectable concentration of cyclosporin A was 5 ng/ml in 100 μl of blood. The coefficient of variation of the method was 1.755, 1.748 and 0.655% in whole blood when spiked at the 170, 425 and 850 ng/ml levels, respectively. One assay was completed in 15 min.

INTRODUCTION

Cyclosporin A (CyA), an immunosuppressive agent, has been successfully used to prolong the survival of patients with various organ transplants. However, no established therapeutic or toxic range has been available, nor has it been clearly established whether whole blood, plasma and serum is appropriate for analysis [1,2].

Radioimmunoassay (RIA) of CyA in whole blood, plasma and serum appears to overestimate its actual concentration [3], presumably because the antibodies

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cross-react with one or more of its metabolites. Whole blood samples are reportedly preferred to plasma or serum, because CyA is partitioned unevenly between the blood cells and plasma. This relationship varies with time and temperature [4].

Recently published high-performance liquid chromatographic (HPLC) methods [5–7] have succeeded in improving the recovery and sensitivity and reducing chromatography time. However, they require a multi-step sample preparation and a disposable extraction column apparatus. The column-switching technique or coupled column chromatography [8–10] is known to increase the separation selectivity. In this paper, we describe an on-line column-switching method for measuring CyA levels in whole blood after deproteinization of the blood samples.

EXPERIMENTAL

Reagents

Acetonitrile was of HPLC grade (Katayama Chemical, Osaka, Japan). Distilled, deionized water was prepared by using a Milli Q water purification system (Millipore, Milford, MA, U.S.A.). Cyclosporin A was obtained from Sandoz (Basle, Switzerland).

A stock standard solution of CyA was prepared by dissolving 8.5 mg of CyA in 100 ml of acetonitrile. Further dilution of this stock solution was made with acetonitrile prior to preparing blood standards.

Apparatus

All analyses were performed using a liquid chromatograph equipped with two solvent-delivery pumps (LC-6A, Shimadzu, Kyoto, Japan), an automatic sampler (SIL-6A, Shimadzu), a variable-wavelength UV detector (at 205 nm) (SPD-6A, Shimadzu) and an integrator (C-R3A, Shimadzu). The two columns used were a sample preparative column (3 cm $\times$ 4.0 mm I.D.) (Shim-Pack CLC-CN, 5 μm, Shimadzu) and a reversed-phase analytical column (15 cm $\times$ 6.0 mm I.D.) (Shim-pack CLC-TMS, 5 μm, Shimadzu), which were kept at 60°C in a thermostated column oven (CTO-6A, Shimadzu). Low- and high-pressure six-port switching valves were placed in-line and operated by an HPLC system controller (SCL-6A, Shimadzu). Fig. 1 shows the arrangement of the apparatus.

Mobile phase

Four different water–acetonitrile eluents were used: (A) 65:35; (B) 50:50; (C) 20:80; and (D) 25:75 (v/v).

Protein precipitation from the whole blood sample

Whole blood was drawn into heparin-coated tubes and prepared for CyA analysis.

A 0.5-ml volume of blood (sample or blood standard) and 0.5 ml of acetonitrile were placed in a 5-ml glass tube and the stoppered tube was vortexed for 30 s, incubated for 5 min at room temperature and centrifuged at 3000 *g* for 5 min. The supernatant was transferred into a 2-ml automatic sampler vial and capped with

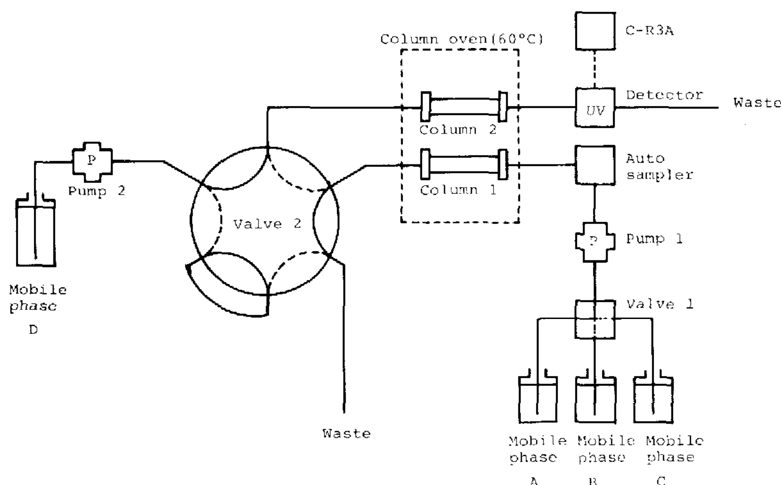

Fig. 1. Flow diagram of fully automated column switching for CyA in whole blood. Valves 1 and 2 are low- and high-pressure six-port valve switching units, respectively, and are operated by an HPLC system controller which is not shown in order to simplify the drawing. The solid and dotted lines in the high-pressure valve (valve 2) show the position 0 and 1 modes.

a septum. CyA blood standard was prepared by the same procedure, except that different amounts of CyA stock solution were added to the blood.

Timing of column switching

The timing of automated column switching for the determination of CyA in the blood is shown in Fig. 2. At 0 min, valves 1 and 2 were positioned at 1 and 0, respectively, so that mobile phase A flowed through the preparation column (column 1) to waste through valve 2, and mobile phase D which connected to pump 2 flowed through the reversed-phase analytical column (column 2) to the UV detector. Through an autosampler, the sample was injected into column 1 on which partial separation of CyA and other substances in the biological fluids was achieved. Valve 1 was then turned to position 2 to pass mobile phase B 0.1 min after injection, but column 1 was still eluted with mobile phase A remaining in

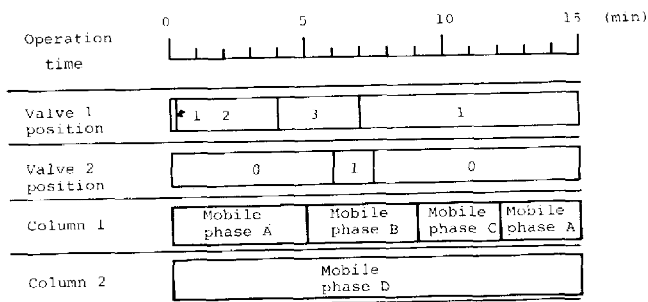

Fig. 2. Timing of automated column switching for determining CyA in whole blood. The time lag between switching of valve 1 and switching of mobile phase in the preparation column arises from the dead space between valve 1 and pump 1 (see Fig. 1).

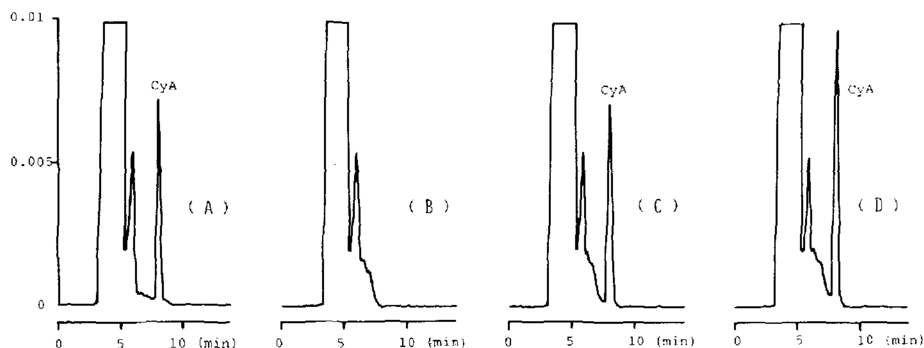

Fig. 3. Chromatograms of (A) CyA standard, (B) drug-free blank human blood, (C) human blood containing 425 ng/ml of CyA and (D) blood collected from a patient after an oral dose of a 10 mg/kg·day of CyA (the calculated concentration of CyA was 555 ng/ml).

the dead volume between valve 1 and pump 1. Valve 1 was then turned to position 3 at 4 min after injection to pass mobile phase C. At 6 min after injection when CyA began to elute from column 1, valve 2 was turned to position 1 so that the effluent from column 1 passed to column 2. CyA was concentrated at the head of column 2 by mobile phase B. At 7 min after injection, valve 1 was turned back to position 1. At 7.5 min after injection, valve 2 was turned back to position 0, and mobile phase D then flowed through column 2 where further separation of CyA and other substances in the biological fluid was achieved. Column 1 was then eluted with mobile phase C at 9 min to wash out the substances remaining in column 1. At 12 min after injection, mobile phase A flowed through column 1 to give equilibration for the next analysis. The injection interval for this procedure was 15 min.

Quantitation

The procedure was standardized by analysing 1–20 μl of CyA added to blank blood solution. The peak area of CyA was used to construct a calibration graph for blood samples. Linear regression was used to evaluate the statistical significance of the data.

RESULTS

Fig. 3 illustrates representative chromatograms of a CyA standard, drug-free blood, supplemented blood and patient blood. The retention time of CyA on a new analytical column was 8 min. Concentrations of CyA in the blood were determined from the calibration graph of the CyA peak.

Linearity

Linearity was tested by the measurement of blood standards. The peak area of CyA depended linearly on the concentration of CyA in the blood in the range 0–1700 ng/ml ($r=0.993$, $n=5$).

TABLE I

REPRODUCIBILITY OF THE DETERMINATION OF CyA IN WHOLE BLOOD

Sample	CyA concentration (ng/ml)	Peak area	Standard deviation	Coefficient of variation (%)
1	170	284	4.983	1.755
2	425	558	9.752	1.748
3	850	1120	7.342	0.655

Sensitivity

The detection limit of the assay was determined as the lowest detectable concentration above the background with a variance of less than 10%. The sensitivity limit of the blood assay was 5 ng/ml in a 200-μl injection volume.

Recovery

The analytical recovery of CyA from blood ranged from 98.4 to 100.2% and was independent of CyA concentration in the range 320–850 ng/ml.

Reproducibility

The intra-assay reproducibility was determined by analysing ten replicate blood samples spiked with CyA at 170, 425 and 850 ng/ml. The results, expressed in terms of coefficients of variation, are given in Table I.

Clinical study

We measured the concentration of CyA in the blood from a patient with a kidney transplant. CyA was given intravenously at a dose of 4 mg/kg·h for 1 h. The blood concentration versus time during a 24 h interval for the patient is shown in Fig. 4.

DISCUSSION

Most reports have described blood or plasma levels of CyA determined by RIA. Although it has been suggested that this procedure is not highly specific for CyA, the dosage is adjusted by measured CyA levels with an RIA method. Its main drawback is the cross-reactivity of the antibody with some of the circulating metabolites of CyA.

Earlier HPLC methods for the determination of CyA in human plasma and serum required pre-treatment for extracting CyA into the diethyl ether phase [7,11] or into the solid phase prior to application to the reversed-phase HPLC system. These methods did not separate the background peak of blood samples from that of CyA. In addition, they required more than 60 min to complete an analysis. The low and variable recovery of CyA and interference by late-eluting peaks were two major disadvantages of the reported solid-phase extraction procedures [12,13].

Recently, the HPLC column-switching technique has proved useful for the

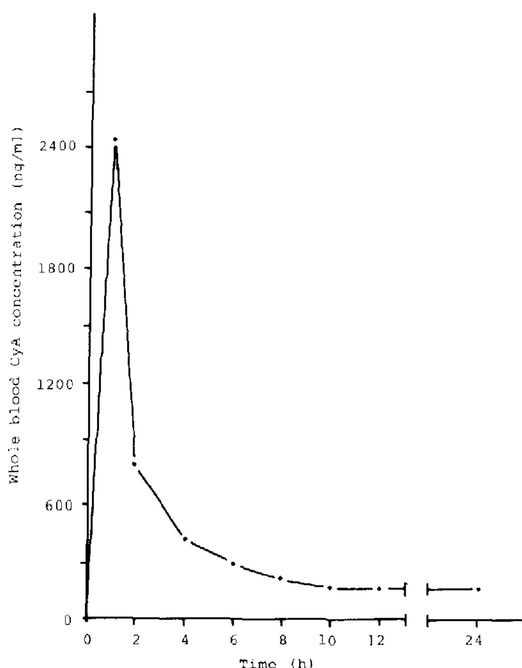

Fig. 4. Whole blood CyA concentrations determined by HPLC after intravenous infusion of the drug.

determination of water-soluble materials in biological fluids [14–16] and has been employed in measuring CyA in blood, plasma and serum [8–10]. However, it involves relatively expensive and sophisticated equipment (three pumps and two column-switching valves) and a large volume of sample (1000 μl). In this study, we used a relatively inexpensive system with two pumps and one column-switching valve and a small sample volume (200 μl). Complete separation of the background peaks of the blood samples from that of CyA was achieved by HPLC column switching using a cyanopropyl preparative column and a TMS reversed-phase analytical column. The 5 μm cyanopropyl preparative column used for the fractionation of CyA can be re-used. The use of this short column made it possible to concentrate the large volume of eluted fraction on the analytical column without disturbing the chromatogram. The concentration of the large volume of eluent on the short column permitted a rapid fractionation of CyA from the blood samples with very high efficiency, specificity and precision. The cyanopropyl column can be used for approximately 500 samples. The column can be easily packed with a small amount of packing material.

The analytical column (CLC-TMS, 5 μm) gives a good selectivity and sufficient resolution, and is usable under isocratic conditions with the water–acetonitrile (25:75, v/v) mobile phase. Another distinct advantage of the method is that the preparative and analytical columns are maintained at 60°C, compared with the 70–80°C required in another method [17]. The column life can be lengthened by heating the column slowly. The time required for a sample analysis, including sample precipitation and chromatographic analysis, is less

than 15 min. The sensitivity limit appears to be adequate for monitoring blood CyA concentrations in patients under regular treatment with this drug.

In general, cyclosporin D is not necessary as an internal standard in liquid chromatography. The simple sample preparation in this method allows one to employ external standardization, which simplifies the method and reduced the analysis time without losing linearity, precision or accuracy.

The method presented here provides fully automated sample purification in a single step. A large number of blood samples from patients can be loaded on to the autosampler after manual precipitation of protein. A sample cycle is completed in 15 min, and this system can be run continuously.

In conclusion, the method reported here employs (1) an automated sample wash, (2) column switching and (3) computer analysis, providing greater recovery, accuracy and sensitivity.

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