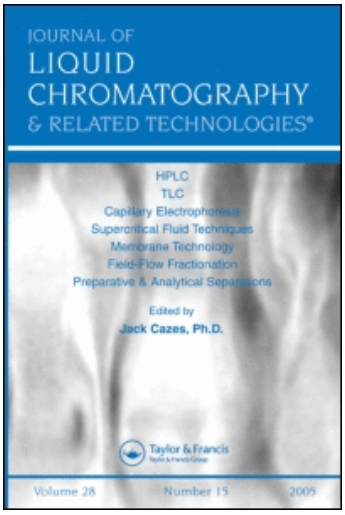

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DETERMINATION OF CYCLOSPORIN-A IN HUMAN WHOLE BLOOD BY REVERSED PHASE LIQUID CHROMATOGRAPHY WITH SINGLE-STEP EXTRACTION

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ABSTRACT

A simple, rapid and accurate reversed-phase liquid chromatographic method, utilizing a single-step extraction procedure, was developed for the determination of cyclosporin-A (CyA) in human whole blood. In this study, the drug was extracted from whole blood with diethyl ether and separated isocratically within 7 min using an octyl-bonded silica column and a mobile phase of acetonitrile, methanol, deionized water and isopropanol (57:18:25:1.5, v/v). The column temperature was maintained at 65 °C. Cyclosporin-A was quantified by absorbance at 208 nm, with cyclosporin-D as internal standard. A linear relationship between the ratio of peak area and the concentration of CyA from 40 ng/mL to 1200 ng/mL was obtained. The lower limit of detection of CyA was 20 ng/mL. Intra-day and inter-day coefficients of variation of assay for CyA at the 200 ng/ml level were 4.8% (n=7) and 7.8% (n=6), respectively.

The recoveries of CyA were 98.6% - 99.5% for whole blood. The method has been used to determine CyA in whole blood samples from ten volunteers and provided data on the pharmacokinetics of the drug.

INTRODUCTION

Cyclosporin-A (CyA) is a selective immunosuppressive agent. It has been found to be extremely effective in prolonging the survival of patients who received kidney, pancreas, lung, liver, or bone marrow transplants.[1,2]

Although CyA has remarkably improved the survival rate of organ transplant patients, it causes various adverse effects on the kidneys and liver. For most patients, these side effects have been found to be dose-dependent, and the therapeutic index of CyA is very narrow. Therefore, the determination of CyA concentrations in a patient's whole blood is necessary to optimize the dosage of CyA for optimum therapeutic action with minimum toxicity.

Consequently, a further point of interest was determination of CyA concentrations in whole blood. It would be necessary for study of the pharmacokinetics and bioavailability of CyA in humans and for improvement of formulation of the drug.

Since the affinity of cyclosporin with erythrocytes is highly temperature dependent, the CyA concentration in whole blood, serum, or plasma may vary due to changes in the temperature, storage, and processing conditions.[3,4] The CyA concentration must be determined in whole blood instead of in serum or plasma to obtain meaningful data.

Two procedures have evolved, to allow the estimation of CyA concentration: monoclonal radioimmunoassay (RIA)[5,6] and high performance liquid chromatography (HPLC).[7-12] Although RIA is more sensitive than the UV detection in HPLC methods, the reliability of the assay by RIA is very poor because of the cross-reaction of the metabolites of the parent drug.[13,14]

Therefore, HPLC has been extensively used to determine CyA in biological samples. Nevertheless, it is difficult to process the samples because of the complexity of constituents in whole blood.

Improved chromatography of CyA has been introduced, but some HPLC methods, based on a classical liquid-liquid extraction, or application of expensive solid-phase cartridges, still retain the major disadvantages, such as

laboriousness, multistep and time-consuming for sample preparation. These hardly meet the needs of simplicity and rapidity for clinical drug monitoring and pharmacokinic studies.

This report describes a simple, rapid and sensitive HPLC procedure for the determination of CyA in whole blood by using a rapid, single-step, diethyl ether extraction and a reversed-phase isocratic separation on a C_8 analytical column. It has been used to determine CyA in whole blood samples from ten volunteers who had taken CyA capsules and provided data on the pharmacokinetics of the drug.

EXPERIMENTAL

Apparatus

The HPLC system used was an HP 109-IM liquid chromatograph (Hewlett-Packard, Waldbronn, Germany), equipped with a Model K501 high pressure sample injector (20-μL loop; Shanghai Scientific Instruments Factory, Shanghai, China), thermostated column compartment and a diode array detector (Hewlett-Packard, Waldbronn, Germany) operated at 208 nm. Chromatographic separations were carried out on a Spherisorb octylsilane (C_8) column (200 x 4.6 mm I.D.; particle size 7 μm; Dalian, China) operated at 65 °C. Control of the instrument, data storage, evaluation, integration and reporting were performed with an HP Series 300 computer (Hewlett-Packard, Boeblingen, Germany).

Standards and Reagents

Cyclosporin-A and cyclosporin-D were gifts from Fujian Institute of Microbiology. HPLC-grade acetonitrile and methanol (Linhai Chemicals Factory, Zhejiang, P. R. China) were used to prepare the mobile phase. All chemicals, except where otherwise stated, were of analytical grade, and water used in this assay was doubly distilled. Cyclosporin-A capsules were provided by Sandoz Pharmaceutical Ltd. (Basel, Switzerland).

Mobile Phase

The mobile phase consisted of acetonitrile, methanol, deionized water and isopropanol (57:18:25:1.5, v/v). This solution was passed through a 0.45-μm membrane filter (Millipore, Bedford, MA, U.S.A.) and was then degassed before use. The flow-rate of mobile phase was 1.40 mL/min with typical back pressure of 60 bar.

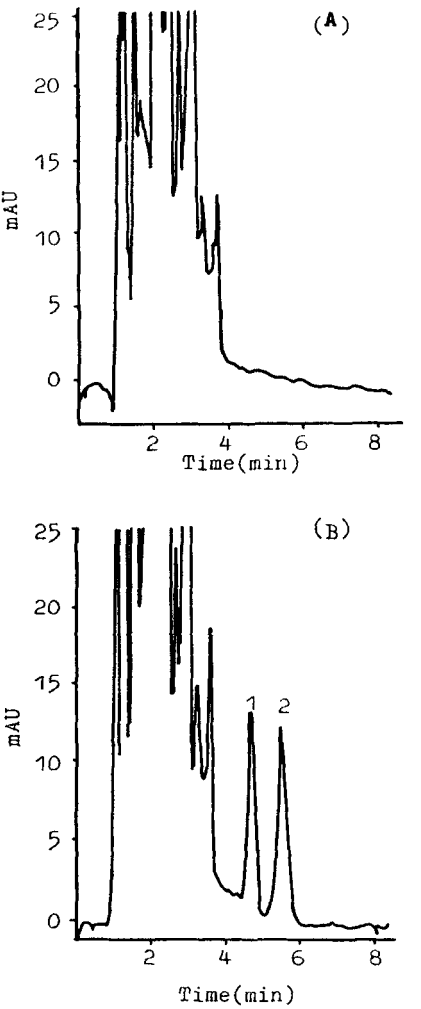

Figure 1. Chromatograms of cycosporin A in human whole blood. A. Blank whole blood; B. A whole blood sample collected 4h after oral administration of 200 mg of cyclosporin A. 1: Cyclosporin A; 2: Cyclosporin D (internal standard).

Preparation of Solutions

Cyclosporin-A and cyclosporin-D stock standard solutions (1.00 mg/mL) were prepared by dissolving 100 mg of cyclosporin-A and cyclosporin-D, respectively, in 100 mL of methanol and kept in a refrigerator.

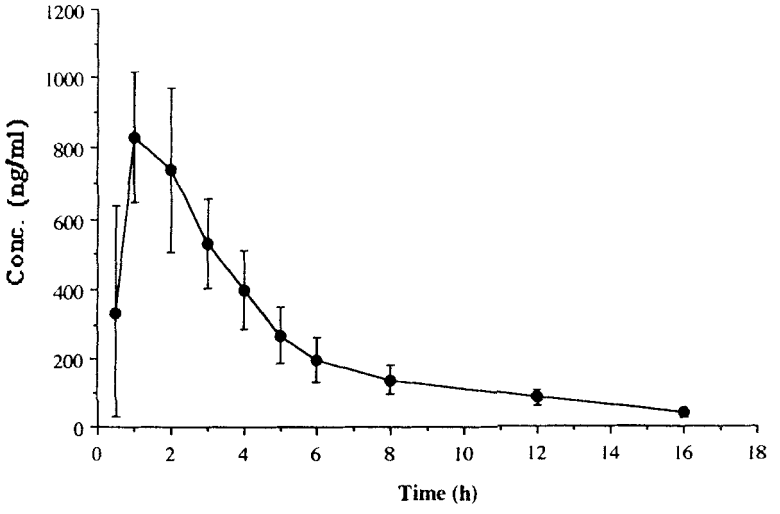

Figure 2. Mean whole blood concentration - time curve after oral administration of 200 mg of cyclosporin A to 10 healthy volunteers. (n=10, mean ± s).
•: Observed; —: Calculated; Concentration unit: ng/mL.

Table 1

Within-Day and Between-Day Precision of the Method

Concentration Added (ng/mL)	RSD (%) Within-Day (n = 7)	Between-Day (n = 6)
200	4.8	7.8
500	2.0	5.0
800	2.9	3.4

Analytical Procedure

Two milliliters of whole blood was pipetted into a chemically clean screw capped glass tube and 16 µL of internal standard (CyD) solution (100 µg/mL) and 100 mg sodium fluoride were added. The mixture was homogenized on a vortex mixer for 15 sec, and 5 mL of diethyl ether was added and mixed for 2 min. Then, 4 mL of the organic layer was collected and evaporated to dryness with air at 50 °C.

The residue was reconstituted with 150 μL of the HPLC mobile phase and 400 μl of hexane. After vortex mixing, 20 μL of the sample solution were injected into the HPLC system.

RESULTS

Chromatographic Separation

Figure 1 shows typical chromatograms of whole blood samples. Under the chromatographic conditions described, CyA and CyD had retention times of approximately 4.6 min and 5.5 min, respectively. It can be seen, from Figure 1, that good separation and detectability of CyA in whole blood were obtained with minimal interference from whole blood components. Hence, it is relatively easy to estimate the peak area with accuracy.

Precision

The data for studies of within-day reproducibility, evaluated by assaying 7 whole blood samples containing different concentrations of CyA, and between-day reproducibility, evaluated by assaying the same concentration, 7 times over a 6-day period, are summarized in Table 1. The range of percentage of relative standard deviation (%RSD) was from 2.0% to 4.8% for within-day analyses and from 3.4% to 7.8% for between-day analyses, respectively.

Linearity and Detection Limit of the Method

A series of whole blood samples containing 40, 100, 200, 400, 600, 800, 1000 and 1200 ng/mL of CyA was prepared to study the relationship between the ratio of peak area of CyA to CyD and the concentrations of CyA under selected conditions. The results showed that the peak area ratio was linearly related to the CyA concentration for the range of 40 - 1200 ng/mL. The linear equation for the concentration versus the ratio of peak area was

$$Y=1.10 \times 10^{-3}X - 7.65 \times 10^{-3}$$

with a correlation coefficient of 0.9994. The detection limit was 20 ng/mL.

Table 2

Recovery of Cyclosporin A from Spiked Whole Blood (n = 9, mean ± s)

Concentration Added (ng/mL)	Concentration Measured (ng/mL)	Recovery (%)	RSD (%)
200	199.0 ± 16.8	99.5 ± 8.4	8.4
500	493.1 ± 24.8	98.6 ± 5.0	5.1
800	794.0 ± 36.6	99.3 ± 4.6	4.6

Extraction Efficiency

Extraction efficiencies of CyA and the internal standard were determined by comparing peak areas of the analyses from extracted standards from whole blood to those from a chromatographic standard solution prepared in mobile phase at the equivalent concentration and chromatographed directly. The data obtained for different concentrations of CyA are summarized in Table 2. The extraction efficiency of the internal standard (800 ng/mL) from whole blood was 97.7 ± 5.7% (mean ± S.D.; n=10).

Application

Ten healthy male Chinese volunteers, aged 22.1 ± 1.0 and weighing 63.3 ± 3.6 kg, entered the study. All volunteers gave their written consent and underwent physical examinations. There were no abnormal findings in liver and kidney functions, specifically. After 12 h of overnight fasting, the volunteers received an oral dose of single 200 mg CyA. Blood samples (2.0 mL) were taken before medication and after 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 8.0, 12.0 and 16.0 hours, and were then kept at 4 °C. Figure 2 illustrates the profile of whole blood concentration versus time for CyA in the 10 volunteers.

DISCUSSION

Investigations of analytical columns that were packed with different stationary phases were conducted to determine which one would give optimum analytical conditions for CyA. The results showed that all of the cyano-, octyl-, and octadecyl-bonded silica can be used as the stationary phase for analysis of CyA. Among them, cyanosilane required the lowest temperature (about 45 °C) as the analytical column was packed with it. Nevertheless, CyA could not be separated from CyD because the retentions were very much alike. When the

Table 3

Pharmacokinetic Parameters of Cyclosporin A after Administering a Single 200 mg Oral Dose to Ten Healthy Chinese Volunteers

Patient	$T_{a1/2}$ (h)	$T_{\alpha 1/2}$ (h)	$T_{\beta 1/2}$ (h)	T_{max} (h)	C_{max} (ng/mL)	$AUC_{0\to\infty}$ (ng•h/mL)
1	0.80	0.55	4.44	1.62	571.4	2828.6
2	0.92	0.35	4.82	1.26	825.5	3102.3
3	0.96	0.12	4.73	0.90	833.3	4966.0
4	0.73	0.62	5.19	1.44	993.2	4682.0
5	0.96	0.64	5.45	1.62	1118.5	4804.1
6	0.88	0.17	4.89	0.72	1103.9	3945.8
7	1.87	0.07	2.91	1.08	806.2	4265.2
8	1.26	0.11	6.80	0.90	733.0	3232.5
9	0.69	0.55	6.16	1.44	726.4	3416.8
10	0.69	0.48	4.43	1.44	1088.0	4694.4
Mean	0.98	0.37	4.98	1.24	879.9	3993.8
± SD	0.35	0.23	1.05	0.33	186.8	796.3

analytical column was packed with octadecylsilane, the column temperature required was the highest. It was necessary to keep the temperature above 65 °C, or even higher than 72 °C, during the analysis.[10] It has been found that some problems occur with this column temperature condition, such as limited useful life of column and stability of the determination. One possible reason may be the formation of bubbles in the mobile phase. The best result for CyA was obtained with Spherisorb octylsilane (C_8) stationary phase. In this cause, the column temperature intervened cyanosilane and octadecylsilane stationary phase, and CyA and CyD can be fully baseline separated.

The effect of the composition of mobile phase on the chromatographic separation was investigated in this study. The results indicated that the optimum resolution for CyA, CyD and endogenous substances in whole blood was obtained when the mobile phase was composed of acetonitrile, methanol, water and isopropanol (57:18:25:1.5, v/v). The retention times of the CyA and CyD visibly increased with decreasing acetonitrile or increasing water content. Moreover, the responses of peak area tended to decrease, too. The retention time changed only slightly with varying methanol content.

Because of the cutoff wavelength of methanol, it is undesirable to increase methanol content. Additionally, the experimental results showed that addition

of isopropanol to the mobile phase increased the selectivity and stability of CyA since isopropanol changed the retention time of the endogenous peaks and had only a slight effect on the CyA peak.

The pharmacokinetics of CyA were studied in 10 healthy Chinese volunteers. After single oral administration of 200 mg CyA, the data obtained was fitted with a computer program: PKBP-N1.[15] Table 3 shows the pharmacokinetic parameters of ten volunteers to whom CyA was orally administered. The results suggest that the disposition of CyA was conformable to a two-compartment model. Peak concentration in whole blood occurred 1.24 h after ingestion and the mean peak concentration achieved was 879.9 ng/mL. This implied that CyA is absorbed rapidly in healthy individuals. Moreover, it was found that the standard deviations of the peak concentration and area under the curve (AUC) were large. This indicates that the individual variation is obvious after oral administration of CyA.

CONCLUSION

The method provides excellent recovery and good precision, and is simple and reliable in both chromatographic conditions and sample preparation. Furthermore, the analytical procedure is easy to handle and is very suitable for routine determination of a large number of samples because of the short time between sample injections. It has been successfully applied to the study of pharmacokinetics of CyA in whole blood samples obtained from 10 healthy volunteers during their participation in a clinical trial of CyA single oral dose.

REFERENCES

1. J. F. Borel, Transplant Proc., **21**, 810 (1989).

2. P. Wang, V. Meucci, E. Simpson, M. Morrison, S. Lunetta, M. Zajac, R. Boeckx, Transplant Proc., **22**, 1186 (1990).

3. M. Wenk, F. Follath, E. Abisch, Clin. Chem., **29**, 1865 (1983).

4. T. G. Rosano, Clin. Chem., **31**, 410 (1985).

5. P. P. Wang, E. Simpson, V. Meucci, M. Morrison, S. Lunetta, M. Zajac, R. Boeckx, Clin. Biochem., **24**, 55 (1991).

6. K. M. Parker, V. Rawlings, K. E. Thick, Clin. Chem., **37**, 2016 (1991).

7. T. P. Moyer, J. R. Charlson, L. E. Ebnet, Ther. Drug Monit., **8**, 466 (1986).

8. P. M. Kabra, J. H. Wall, P. Dimson, Clin. Chem., **33**, 2272 (1987).

9. B. G. Charles, R. L. G. Norris, P. J. Ravenscroft, Ther. Drug Monit., **10**, 97 (1988).

10. A. M. Rustum, J. Chromatogr. Sci., **28**, 594 (1990).

11. D. A. Svinarov, M. N. Dimova, J. Liquid Chromatogr., **14**, 1683 (1991).

12. P. M. Jones. K. Brune. Clin. Chem., **39**, 168 (1993).

13. T. M. Annesley, R. C. Coombs, P. J. Orsulak, Clin. Chem., **39**, 1050 (1993).

14. L. J. Langman, A. B. Leichtman, W. F. Weitzel, R. W. Yatscoff, Clin. Chem., **40**, 613 (1994).

15. Y. C. Yang, G. Chen, L. Yuan, Acta Pharmacol. Sin., **4**, 217 (1983).

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