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Note

Simple and rapid high-performance liquid chromatographic analysis of cyclosporine in human blood and serum

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Cyclosporine is a cyclic peptide (Fig. 1) of fungal origin which has proven to be a potent immunosuppressive agent in man [1]. Cyclosporine is effective in preventing rejection of transplanted organs including heart—lung, kidney, liver, pancreas and bone marrow [2].

Cyclosporine is a toxic drug with a narrow therapeutic index. Toxicity associated with cyclosporine includes non-specific immunosuppression, hepato

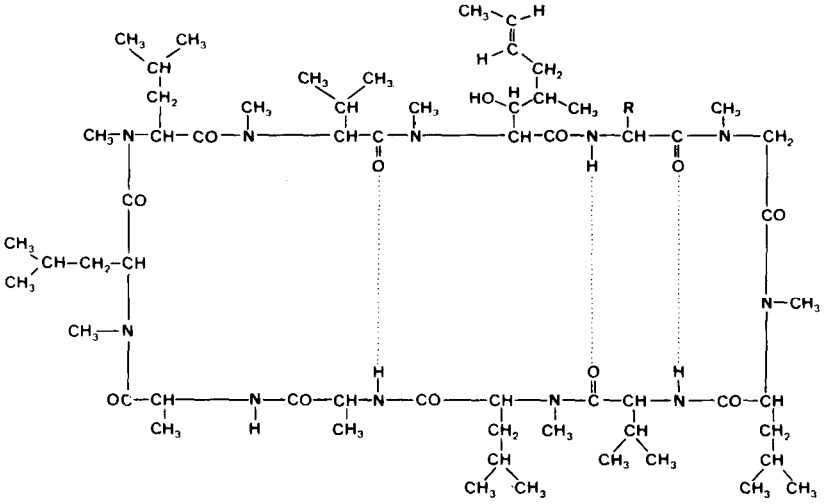

Fig. 1. Chemical structure of cyclosporine where R = $-CH_2CH_3$ and the internal standard, cyclosporin D where R = $-CH(CH_3)_2$.

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and nephro toxicity and lymphoma [3,4]. It has been suggested that optimal use of cyclosporine requires careful titration of dosage and that serum or blood concentration monitoring of cyclosporine is advisable [5]. While a definitive therapeutic range of blood or serum concentrations has not been established, it has been reported that levels between 100 and 250 ng/ml are associated with adequate immunosuppression without a significant incidence of toxicity [6].

While several methods have been suggested for measuring cyclosporine blood or serum levels, most of these are not sufficiently sensitive to measure levels below 100 ng/ml and several are highly complex requiring special attention and equipment [7—9].

We have developed a rapid high-performance liquid chromatographic (HPLC) procedure for measuring cyclosporine in either whole blood or serum. This procedure is relatively simple and rapid, and has a lower limit of sensitivity below 50 ng/ml.

EXPERIMENTAL

Chemicals and reagents

Cyclosporine and the internal standard, cyclosporin D (Fig. 1), are obtained from Sandoz (Basle, Switzerland). Glass-distilled diethyl ether, acetonitrile, *n*-hexane, and methanol are obtained from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). Tris(hydroxymethyl)aminomethane is purchased from Aldrich (Milwaukee, WI, U.S.A.).

Instrumentation and chromatographic conditions

A Waters Instrument (Milford, MA, U.S.A.) Model 6000A HPLC pump, Model 480 variable-wavelength UV detector, Model 710B sample injector and Model 730 data module are employed. The column used is a Brown-Lee Labs. (Santa Clara, CA, U.S.A.) RP-8 MLPC analytical cartridge (10 cm × 4.6 mm I.D.; particle size 10 μm). A 3-cm RP-8 guard cartridge is positioned at the head of the column. The column is maintained at 70°C with an Eldex (Menlo Park, CA, U.S.A.) column heater. The heater is left on at all times and a constant flow is maintained through the column. The flow-rate of the mobile phase is 0.6 ml/min which produces a precolumn pressure of about 34 bars (500 p.s.i.). The effluent from the column is monitored at a wavelength of 215 nm.

Mobile phase

The mobile phase consists of a simple mixture of acetonitrile–water (72:28). This mixture is filtered and degassed by vacuum and sonication. The mobile phase is continuously recycled and replaced about every two weeks.

Preparation of extraction columns

The extraction of cyclosporine involves the use of a Baker-10 SPE extraction system (J.T. Baker, Phillipsburg, NJ, U.S.A.). The columns used are the 3-ml cyano disposable extraction columns. These columns are prepared by washing with 6 ml of methanol and then 6 ml of water under vacuum. They are left approximately one half to three quarters full of water until the sample is added.

Extraction procedure

One ml of whole blood or serum is added to PTFE-lined, screw-capped tubes. To the blood or serum is added 450 ng of internal standard, 3 ml of 0.1 *M* Tris buffer (pH 9.8) and 10 ml of diethyl ether. They are rocked for 20 min on a labquake rocker (Lab. Industries, Berkeley, CA, U.S.A.) and centrifuged for 20 min to separate the ether and aqueous layers. The diethyl ether is pipetted into a clean tube and the blood or serum residue is discarded. To the diethyl ether is added 200 μl of 75% methanol in water. The diethyl ether is then evaporated at room temperature on an N-Evap (Organomation, Northburough, MA, U.S.A.) with a gentle stream of nitrogen. Only the diethyl ether is evaporated, leaving 150—200 μl of the methanol—water remaining. To this are added another 100 μl of methanol. The methanol—water is then transferred to a 3-ml Baker extraction column. The residue is eluted onto the column with water. The column is then washed with 3 ml of 25% acetonitrile in water and 6 ml of *n*-hexane. The columns are then dried by drawing through air for 4 min. Cyclosporine and cyclosporin D are then eluted off the column with three washings of 200 μl of methanol. The methanol is collected into disposable tubes and evaporated to dryness with a stream of nitrogen at 50°C. The resulting residue is dissolved in 200 μl of the mobile phase. An aliquot of 165 μl is then injected onto the column.

Preparation of calibration standards

Stock solutions of cyclosporine and cyclosporin D are prepared in methanol and stored in amber bottles at room temperature. A stock solution of cyclosporin D is prepared in a concentration of 30 ng/μl. The stock solution of cyclosporine is prepared in a concentration of 10 ng/μl. This solutions is used to prepare standards for the calibration curve. The calibration curve samples are prepared by adding amounts of cyclosporine (50—800 ng) to whole blood or serum from a normal volunteer. These are then treated as described in the extraction procedure.

RESULTS AND DISCUSSION

As shown in Fig. 2B the retention times of cyclosporine and the internal standard are 9.2 and 11.8 min, respectively.

An unidentified peak elutes with a retention time of about 19 min, but does not interfere with the quantification of the cyclosporine or internal standard. As shown in Fig. 2A, blood from normal subjects, not taking cyclosporine has no interfering peaks eluting at times which would interfere with the analysis. Fig. 2B is a chromatogram of an analyzed whole blood sample to which had been added 200 ng of cyclosporine and the internal standard. Fig. 2C shows a chromatogram of a blood sample from a patient who was taking cyclosporine. The concentration in this sample was 304 ng/ml.

The chromatography is sensitive to slight changes in conditions. As reported by others [10] a column temperature of 70—75°C is essential for optimizing sensitivity and resolution. We also found that the mobile phase must be at least 68% acetonitrile; 72% is optimal in our system for a new column. We observed that resolution is greatly decreased at mobile phase flow-rates above 1 ml/min.

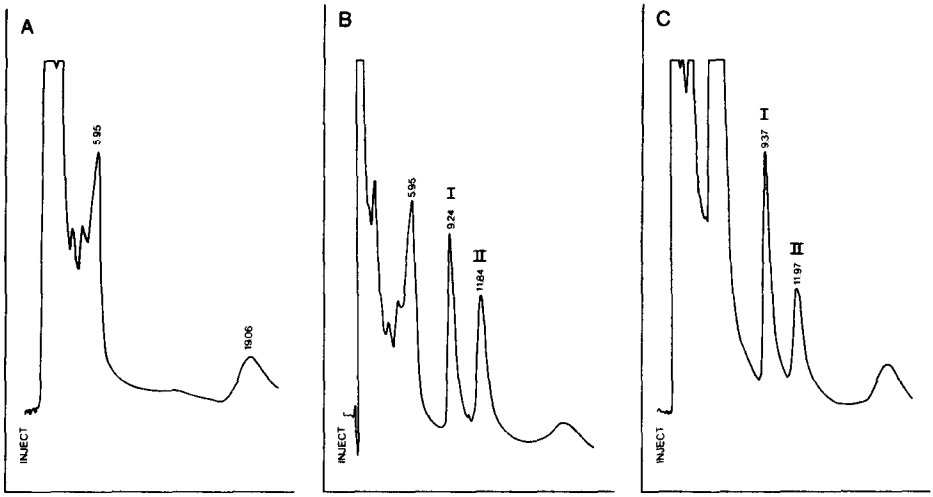

Fig. 2. Chromatograms of extracted whole blood samples. (A) Blood from normal healthy volunteer, not taking cyclosporine; (B) blood to which has been added 200 ng/ml of cyclosporine and the internal standard; (C) blood from a patient who was taking cyclosporine (the concentration in this patient sample is 304 ng/ml). Peaks: I = cyclosporine; II = cyclosporin D, internal standard.

For our conditions the optimal flow-rate is 0.6 ml/min. The flow-rate and column temperature are maintained constant at all times. Under these conditions, columns are stable and functional for at least six months.

Standard curves are prepared by plotting peak height ratios (cyclosporine/internal standard) against the amount added initially. These curves are linear over the range of interest, 50—800 ng. During the developmental phases of this procedure, curves were prepared daily. The variability in slope from seven daily standard curves was 21% (coefficient of variation, C.V.). The intercepts were not significantly different from zero.

The lower limit of sensitivity of the reported procedure is less than 50 ng/ml of blood. While our lowest calibration standard was 50 ng, our analytical range of interest was greater than 100 ng/ml. The peak height of a 50-ng standard was more than ten times the noise level, and samples with levels as low as 25 ng could easily be analyzed without modification. Analysis of samples with low concentrations (<50 ng/ml) of cyclosporine are best analyzed by using less internal standard and increasing the detector sensitivity. Fairly low sensitivity (0.05 a.u.f.s.) is sufficient for the range in which we have primary interest. To analyze samples with high concentrations (>800 ng/ml) we generally take smaller aliquots of blood (200 or 500 ml) and extract as described above.

The reproducibility of the procedure was evaluated by extracting and analyzing replicate blood samples containing 50, 200, 400 and 800 ng of cyclosporine. These data are summarized in Table I. The coefficients of variation, expressed as percent, for the 50, 200, 400 and 800 ng replicates are 3.3%, 7.6%, 7.2%, and 4.7%, respectively.

The extraction efficiency was determined by comparing peak heights of extracted standards with directly injected standards. This was carried out for both whole blood and serum. The recovery of cyclosporine was 34.7% and

TABLE I

REPRODUCIBILITY AT GIVEN PLASMA CONCENTRATIONS

Concentrations (ng/ml)	*n*	C.V. (%)
50	4	3.3
200	6	7.6
400	5	7.2
800	6	4.7

34.5% from whole blood and serum, respectively. Despite this low recovery, sensitivity was not a problem.

Several aspects of this analytical procedure deserve further amplification. The disposable CN columns which are used for the extraction of cyclosporine can be reused if washed with methanol (ca. 10 ml) between uses. We reuse these columns three times before discarding them, since we have noted that some columns tend to loose efficiency after three uses. Another consideration regarding the use of the CN columns is the eluting of the sample onto the column. The columns are at least half full of water when the sample, in ca. 300 μl methanol, is added. The columns are then filled with water to dilute the methanol and to facilitate mixing. If the methanol layer is drawn through the column without mixing or dilution, the cyclosporine will not be retained on the column.

A rather important step in the extraction is the addition of 200 μl of 75% methanol in water to the diethyl ether before drying. If the diethyl ether is dried completely, there is difficulty in redissolving the residue containing the cyclosporine and the internal standard. While the diethyl ether is evaporated the sample is not taken to dryness; the methanol—water mixture containing the drug and internal standard remain. This simple step helps overcome difficulties of low recovery which are encountered in the extraction.

In order to increase our capability, we are employing an automatic injector so that samples can be injected overnight. We have found that the use of plastic sample holders leads to several interfering peaks on the chromatogram. We therefore are using all glass sample holders to avoid this problem.

The majority of the literature concerning blood or plasma levels of cyclosporine has been based on a radioimmunoassay (RIA) procedure. While it has been suggested that this procedure is not highly specific for cyclosporine, the suggested plasma level guidelines for dosage adjustment are based on this method. We, therefore, analyzed several samples, both whole blood and serum, which had been analyzed by the RIA procedure. These samples were drawn from patients who had been taking cyclosporine for periods ranging from two weeks to several years. The results of this comparison are shown in Fig. 3. In general, the results of our HPLC analysis were considerably lower than those of the RIA analysis. This is consistent with similar HPLC—RIA comparisons reported by Carruthers et al. [9] and by Donatsch et al. [11]. In some patient samples, we noted a peak which eluted with a retention time similar to that of the internal standard. Since this interfering peak precluded our use of the cyclosporine D as an internal standard for those few samples in which it was

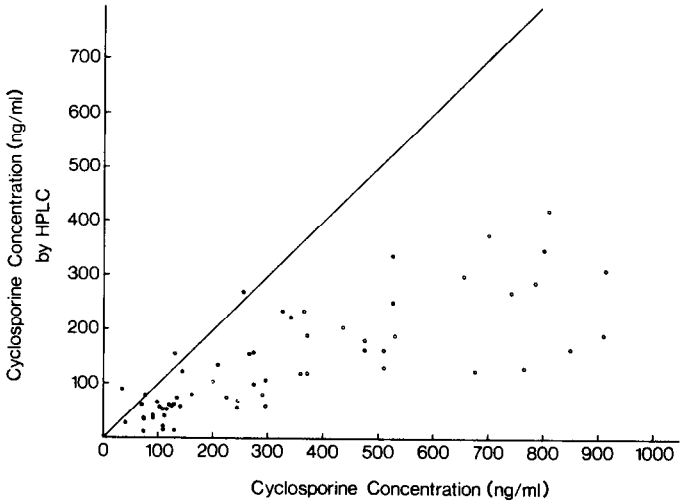

Fig. 3. Results of simultaneous analysis of whole blood (○) and plasma (●) samples by RIA and HPLC.

present, they were quantitated by means of a standard curve based on peak height of cyclosporine alone. These curves were linear over the range studied. It is interesting to speculate that this interfering peak may be due to a metabolite of cyclosporine. Since this peak eluted more slowly than the parent drug, it is probably less polar than cyclosporine. Its identity is currently being pursued.

While other methods have been developed for measuring cyclosporine in plasma or serum samples, they generally tend to be time-consuming and are not directly adaptable to analyzing whole blood. We developed the method presented here to facilitate the rapid and simple analysis of large numbers of whole blood samples from patients receiving cyclosporine following organ transplantation. The method reported here is sensitive, selective and equally adaptable for whole blood, plasma or serum samples. The advantage of this method over other procedures is that it is simple to perform and both the extraction and chromatography are rapid.

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REFERENCES

1 D.J.G. White, Drugs, 24 (1982) 322.
2 R.Y. Calne, Brit. J. Surg., 67 (1980) 765.
3 G.B.G. Klintmalm, S. Iwatsuki and T.E. Starzi, Lancet, i (1981) 470.

4 A.G. Bird and S.M. McLachlan, Lancet, ii (1980) 418.
5 D.J.G. White, D. McNaughton and R.Y. Calne, Transplant. Proc., 15 (1983) 454.
6 P.E. Oyer, E.B. Stinson, S.W. Jamieson, S.A. Hunt, M. Billingham, W. Scott, E.P. Bieber, B.A. Reitz and N.E. Shumway, Transplant. Proc., 15 (1983) 1247.
7 K. Nussbaumer, W. Niederberger and H.P. Keller, J. High Resolut. Chromatogr. Chromatogr. Commun., 5 (1982) 424.
8 G.C. Yee, D.J. Gmur and M.S. Kennedy, Clin. Chem., 28 (1982) 2269.
9 S.G. Carruthers, D.J. Freeman, J.C. Koegler, W. Howson, P.A. Keown, A. Laupacis and C.R. Stiller, Clin. Chem., 29 (1983) 180.
10 R.J. Sawchuk and L.L. Cartier, Clin. Chem., 27 (1981) 1368.
11 P. Donatsch, E. Abisch, M. Homberger, R. Traber, M. Trapp and R. Voges, J. Immunoassay, 2 (1981) 19.