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Note

Measurement of cyclosporin by high-performance liquid chromatography following charcoal adsorption from whole blood

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Cyclosporin is a powerful immunosuppressive agent which has significantly improved the survival of transplant organs with associated decreased morbidity of transplant recipients [1]. It differs from other immunosuppressive drugs in that it acts selectively on T-helper lymphocytes to inhibit their response to antigenic stimulation thereby impairing the development of both cell-mediated and humoral immune responses [2]. Because of its selective action, cyclosporin does not cause bone marrow suppression, a major life-threatening adverse effect of other immunosuppressive drugs.

Although cyclosporin has markedly improved the outcome in organ allograft recipients, it is associated with significant adverse effects, including nephrotoxicity, hepatotoxicity, neurological damage, and increased risk of lymphoma [1]. The most common and troublesome adverse effect is nephrotoxicity which appears to be dose-related in most patients [3, 4].

The great inter-individual variability in metabolism and clearance of cyclosporin leads to wide variation in blood concentrations and effect with the same dose. This is compounded by incomplete and widely variable bioavailability when cyclosporin is administered orally [1]. For these reasons it has become standard practice to use serum or blood concentrations to adjust doses during therapy to ensure concentrations consistent with adequate immunosuppression and below those associated with toxicity.

Measurement of cyclosporin in whole blood is preferable to measurement in serum or plasma for the following reasons. An average of only 30% of circulating cyclosporin is found in plasma [5]. Approximately 60% is found in

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the red blood cells and 10% in other cell components. Of the 30% in plasma, 21% is bound to lipoproteins, 7% is bound to plasma proteins, and 2% is unbound. Red blood cell uptake is very temperature-dependent; plasma concentrations may change by 65% when sample temperature is reduced below 37°C [6]. In addition, the ratio of whole blood to plasma cyclosporin concentration is dependent on the hematocrit which frequently fluctuates widely in transplant recipients [7]. It is not surprising, therefore, that plasma concentrations correlate poorly with whole blood concentrations as well as with clinical effect.

Currently available methods to measure cyclosporin employ either radioimmunoassay (RIA) or high-performance liquid chromatography (HPLC). The single commercially available RIA is applicable only to serum and plasma and exhibits considerable cross-reactivity with cyclosporin metabolites. HPLC methods may be used with serum, plasma, or whole blood. Satisfactory detection of cyclosporin using HPLC methods has been difficult due to the low extinction coefficient of cyclosporin at UV wavelengths which minimize background absorption. It also has been difficult to obtain a lipid-free extract of cyclosporin from whole blood prior to chromatography because it is a hydrophobic neutral cyclic peptide. Previously published HPLC methods [5, 8] have employed extraction with a lipophilic solvent such as diethyl ether or adsorption onto disposable solid-phase extraction columns followed by elution with an organic solvent prior to injection onto the analytical column.

A major disadvantage of the extraction procedures has been the co-extraction of lipid-soluble endogenous blood components which interfere with the chromatography of cyclosporin. Most assays have dealt with this problem by selecting chromatographic conditions which require a prolonged elution time of 20–40 min. Other investigators have attempted to reduce interfering substances by using an acidic hexane wash or partial column separation prior to switching to the analytical column for final separation [9, 10]. These methods tend to be cumbersome, time-consuming, yield poor recovery, and still require extended chromatography time to provide adequate resolution of cyclosporin from other eluting peaks. A recently published assay [11] has succeeded in improving recovery and reducing chromatography time. However, it requires a multi-step sample preparation and use of a disposable extraction column apparatus.

The purpose of this report is to describe a HPLC procedure for cyclosporin analysis in whole blood which uses charcoal for adsorption of the drug after the whole blood proteins are precipitated. Subsequent elution of cyclosporin from the charcoal provides an extract free of many interfering substances thereby decreasing chromatography time to less than 10 min without the need for a pre-column or wash-out of the analytical column between samples. The procedure is easily adaptable to the routine hospital laboratory, the lowest quantifiable concentration was 50 ng/ml, and it is accurate and reproducible.

MATERIALS AND METHODS

Chromatography

The analysis was performed on a Perkin-Elmer Series II HPLC instrument

equipped with an LC 75 UV/VIS variable-wavelength detector, interfaced with a Sigma-10 data system (Perkin-Elmer, Norwalk, CT, U.S.A.). All assays were performed using a 5-μm, 15 cm × 3.9 mm μBondapak C_{18} column (Waters Assoc., Milford, MA, U.S.A.) maintained at 75°C. The flow-rate was 2.0 ml/min and the eluent was monitored at 206 nm.

Reagents

Cyclosporin and cyclosporin-D (internal standard) were supplied by Sandoz Pharmaceuticals (East Hanover, NJ, U.S.A.). Charcoal (Norit SG) was obtained from Mathson, Coleman and Bell Manufacturing (Cincinnati, OH, U.S.A.). All other chemicals and organic solvents were HPLC or reagent grade. The mobile phase was acetonitrile–methanol–distilled water (45:30:25). This solution was prepared fresh and degassed under vacuum just prior to use.

Each stock standard of cyclosporin and cyclosporin-D was prepared in methanol to yield a concentration of 100 mg/l. Calibration standards (usually 250 ng/ml) were prepared in outdated hemolyzed blood bank blood from an intermediate standard in methanol, which was prepared from a 1:10 dilution of stock standard. Acetonitrile, hydrated with 2.5% water, was used to precipitate the blood samples. A charcoal suspension of 2 mg/ml in distilled water was prepared fresh weekly.

Sample preparation

Patient blood samples were collected in tubes containing EDTA as the anticoagulant. Hemolysis of samples was accomplished by the freeze/thaw procedure. If the samples were to be assayed on the day of collection, they were frozen at −70°C for at least 20 min and then thawed. Samples to be analyzed the next day were stored frozen at −70°C.

A 1-ml volume of working standard prepared in hemolyzed blood, control or hemolyzed patient sample was placed in a 12 × 75 mm disposable glass test-tube. The intermediate stock internal standard solution (50 μl) was then added to each tube and the contents were mixed. Hydrated acetonitrile (2 ml) was gradually added to each tube which was then vortexed vigorously for 30 sec. After centrifugation, the clean supernatant was transferred to a 10-ml conical centrifuge tube. A charcoal slurry was maintained in suspension by constant mixing on a magnetic stirrer. The slurry (5 ml) was pipetted into each tube to deliver approximately 10 mg of charcoal. All tubes were stoppered and agitated on an Eberbach bench-top shaker for 15 min at moderate speed followed by centrifugation at 1200 *g* for 10 min. The aqueous portion was removed leaving the charcoal sediment intact at the bottom of the tube. The tubes were kept in an inverted position over an absorbant pad for a few minutes to remove all remaining liquid. The charcoal was dispersed by vortexing for 10 sec followed by addition of 3.0 ml of ethyl acetate. The tubes were stoppered and shaken at moderate speed for 15 min, centrifuged at 1200 *g* for 10 min and the clear ethyl acetate was transferred to clean 12 × 75 mm disposable tubes. Each tube was dried under nitrogen at 40°C, the residue reconstituted with 100 μl of mobile phase, and 50 μl were injected onto the column.

RESULTS AND DISCUSSION

Inclusion of the charcoal adsorption/elution procedure produced a whole blood extract that contained fewer of the substances which require extended elution times with previously described methods [8, 12–14]. This allowed reduction of total chromatography time to 8 min (Fig. 1) with no requirement for a guard column or special treatment of the analytical column between injections. The cleaner extract also enabled the measurement of cyclosporin at 206 nm, a wavelength closer to its absorption maximum of 195 nm, with a corresponding increase in detection sensitivity. The potentially hazardous ether extraction step used by others [12–14] as well as the cumbersome column-switching techniques [15] are avoided .

The assay was linear over the range 50–2000 ng/ml. Within-run analysis was carried out with seven whole blood samples containing 200 ng/ml cyclosporin. The concentrations found were 204, 211, 208, 214, 185, 192, and 232 ng/ml (mean ± S.D., 206 ± 15.3 ng/ml; coefficient of variation, 7.4%). In order to evaluate day-to-day precision and determine the stability of stored whole blood cyclosporin samples, a whole blood standard containing 200 ng/ml was prepared. Aliquots of 1 ml were individually frozen and sequentially analyzed on the days shown in Table I. There was no apparent loss of measurable cyclosporin over a six-week time interval. EDTA was used as the anticoagulant to avoid the possibility of heparin-inactivating factor interfering with the assay [16]. Recovery based on comparison to a pure standard solution was 80%. This is greater than the 35–76% recoveries reported by others [8, 12, 14].

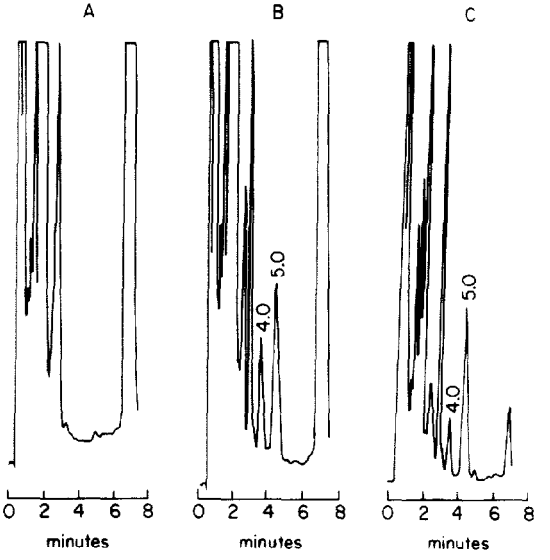

Fig. 1. Typical chromatograms obtained from outdated blood bank whole blood (A), prepared whole blood standard containing 250 ng/ml cyclosporin-D (B), a patient's trough blood sample of which the determined cyclosporin concentration was 117 ng/ml (C). The retention times for cyclosporin and the internal standard were 4 and 5 min, respectively. An unidentified endogenous peak which eluted at 7.5 min was well resolved from the peaks of interest.

TABLE I

DAY-TO-DAY ANALYSIS OF A PREPARED WHOLE BLOOD SAMPLE CONTAINING 200 ng/ml CYCLOSPORIN

Day	Determined concentration (ng/ml)	Day	Determined concentration (ng/ml)
1	193	21	181
2	182	22	193
3	198	23	170
4	216	24	189
10	214	34	222
14	188	35	175
15	195	36	223
17	189	37	195
18	225	38	213
Mean ± S.D.	198.0 ± 17 ng/ml		
Coefficient of variation	8.6%		

In order to assess the reliability of the assay, thirteen samples were aliquoted into two parts. One part was analyzed by the procedure described here and the other part was analyzed by an independent commercial laboratory using a different HPLC method. The correlation coefficient of paired assays between the two laboratories was 0.99. Prednisolone, gentamicin, tobramycin, salicylate, and acetaminophen, which are frequently administered to transplant patients, did not interfere with the assay. The method described here is currently being used in our laboratory for routine clinical monitoring of cyclosporin therapy. We have found it to be accurate, reproducible, efficient, and adequately sensitive for analysis of patient samples. It can easily be incorporated into those laboratories that have HPLC capability.

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