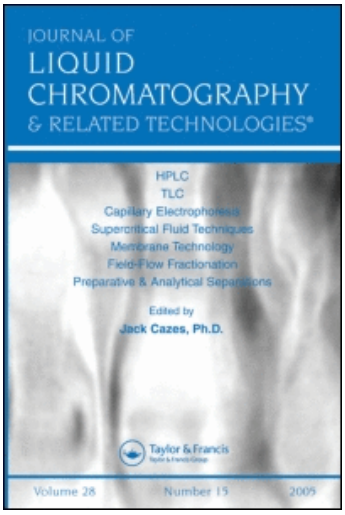

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Improved Liquid Chromatographic Analysis of Cyclosporine in Whole Blood with Solid Phase (Bond-Elut™) Extraction

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IMPROVED LIQUID CHROMATOGRAPHIC ANALYSIS OF CYCLOSPORINE IN WHOLE BLOOD WITH SOLID PHASE (BOND-ELUT™) EXTRACTION

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ABSTRACT

We describe a simple, precise, accurate, and specific isocratic liquid chromatographic procedure for determining cyclosporine in whole blood. The cyclosporine is extracted from 1 ml of hemolyzed blood with 200 µg of cyclosporin D added per liter as internal standard, by elution from a Bond-Elut™ C18 extraction column with 300 µl of ethanol. A 100 µl aliquot of the eluate, injected onto an octadecyl reversed phase cartridge column, is eluted with a mixture of acetonitrile and water at a flow rate of 1.0 ml/min and at 70 ° C. Detection is at 210 nm. The chromatography is complete in < 10 min. The minimum detection limit is 10µg/l. Analytical recovery of cyclosporine added to whole blood pool ranged from 95 to 113% for concentration upto 2000 µg/l. Between run CV's ranged from 6.2 to 6.3%. None of the numerous drugs and steroids tested interfered with the analysis.

INTRODUCTION

Cyclosporine is a selective immunosuppressive agent currently used to prevent graft rejection in human kidney, heart and liver transplantation (1-4). Although cyclosporine has markedly improved the outcome in allograft recipients, it is often associated with serious toxic effects, primarily renal and hepatic, which appears to be dose related in most patients(5-7). Therefore, the cyclosporine concentration is commonly measured to allow individualization of the dosage of cyclosporine in these patients so as to achieve therapeutically optimum concentrations in the blood and avoid dose associated toxicity.

The choice of specimen for assay is very important; serum or plasma give an inconsistent measure of cyclosporine in blood, because of in vitro redistribution of drug between erythrocytes and plasma or serum during storage or processing of blood samples. Various studies have confirmed a temperature dependent affinity of the drug for erythrocytes(8). In addition, the ratio of whole blood to plasma cyclosporine concentration is dependent on the hematocrit and lipoprotein content which frequently fluctuates widely in transplant recipients(9). Therefore, whole blood should be the sample of choice, and more widespread use of whole blood may expedite the development of a useful therapeutic range of cyclosporine.

Cyclosporine is assayed in whole blood or plasma by radioimmunoassay (RIA) or liquid chromatography (LC). The RIA method appears to overestimate the concentration of cyclosporine, presumably because the antibody cross reacts with some cyclosporine metabolites. Several investigators have reported that cyclosporine concentration values determined by RIA are 20 to 430% higher than those obtained with an LC

method(10,12). Thus clinical action based on values for cyclosporine determined by RIA may well be inappropriate.

Several LC procedures have been reported for the analysis of cyclosporine. Some procedures require labor-intensive multistep extractions(13, 14) to eliminate interferences from non-polar endogenous constituents; in some procedures the extraction efficiency is poor or suffers from lack of sensitivity for therapeutic monitoring (15). Complicated column switching(16) or time consuming gradient procedures(12) also have been reported. In most reversed phase methods there is a rapid deterioration of expensive analytical columns which are maintained at elevated temperature for optimum resolution and peak symmetry.

We describe here a LC method that is applicable to whole blood. It includes a simple sample preparation by solid phase extraction and chromatography on a cost-effective 4.6 X 33 mm cartridge column packed with 3 µm octadecyl reversed phase material.

MATERIALS AND METHODS

Instrumentation

We used a Series 10 liquid chromatograph equipped with a Model LC-100 column oven, a Model LC 95 variable wave length detector, and a Model LCI-100 recording integrator (all from Perkin-Elmer Corp., Norwalk, CT 06856). The 4.6 X 33 mm reversed phase column packed with 3 µm (Pecosphere 3X3 C 18, Perkin-Elmer) octadecyl phase, along with a Rheodyne 2 µm filter (Rheodyne, Cotati, CA 94928) was mounted in an oven. The sample was injected via a Model ISS-100 sampling system (perkin-Elmer).

Reagents

All chemicals were of reagent grade. Acetonitrile, hexane, and methanol, all distilled in glass, were obtained from J.T. Baker Chemical Co., Phillipsburg, NJ 08865. Absolute ethanol (190 proof) was obtained from the University of California Storehouse, San Francisco, CA 94143. Mobile phase was prepared by mixing 630 ml of acetonitrile and 370 ml of deionized water.

Vac Elut™ Vacuum chamber and Bond Elut™ C18 extraction columns were purchased from Analytichem International Inc., Harbor City, CA 90710.

Drug Standards

The stock cyclosporine (Sandoz, Hanover, NJ 079360), standard used for calibration was prepared by dissolving 10 mg in 1 l of methanol/ water(1/1 by vol). The stock solution is stable for at least six months at 4 ° C. The stock internal standard, cyclosporin D (Sandoz), was prepared by dissolving 25 mg in 25 ml of methanol, and is stable at 4 °C for at least six months. The working internal standard, 200 µg of cyclosporin D per liter of acetonitrile/water (30/70 by vol), is prepared by diluting stock internal standard solution 5000 fold with the acetonitrile/water mixture. It is stable for three months at 4 ° C. The working cyclosporine whole blood calibration standards (200 and 400 µg/l) are prepared by diluting 2 and 4 ml of the stock calibration standard with 98 and 96 ml of a pool of drug free heparinized whole blood; these standards are stable for at least three months at -20 ° C.

Patient's specimens: Blood specimens are drawn into heparinized Vacutainer tubes. Cyclosporine is stable for at least 4 days in whole blood stored at 4°C.

Procedure

Pipet 2.0 ml of the working cyclosporin D internal standard into labeled 16 X 100 mm disposable glass tubes. Add 1.0 ml of appropriate calibration standard, control, or unknown into each tube, vortex-mix, and centrifuge all tubes for 5 min at 500 X g. For each sample, place a Bond-Elut C18 extraction column on the top of the Vac-Elut chamber, and attach a 5 ml reservoir to each column. Wash each column with 2 ml of 190 proof ethanol followed by 2 ml of water, and connect the vacuum to the chamber. Fill each reservoir with the hemolysed sample and allow the columns to drain thoroughly, then wash each column with 4 ml of wash solution(1/1 by volume, acetonitrile and water). Disconnect the vacuum, and place a rack of labeled 10 X 75 mm glass tubes in the Vac-Elut chamber. Pipet 300 µl of ethanol into each column and connect the vacuum. After collecting the eluates in the tubes, remove the rack from the vacuum chamber. Add 200 µl of water and 500 µl of hexane to each tube. Vortex-mix the contents of the tubes and centrifuge all tubes for 1 min at 500 X g. Aspirate off most of the upper hexane layer and pour the lower ethanol layer into a set of labeled auto-sampler vials, and inject 100 µl onto the liquid chromatograph. Elute the C18 analytical column with the mobile phase (acetonitrile/water, 63/37 by vol) at a flow rate of 1.0 ml/min, with the temperature at 70 ° C, and monitor the column effluent at 210 nm and 0.016 A full scale. Set the chart speed at 2 mm/min. Quantification of cyclosporine is based on the peak-height ratio of cyclosporine to cyclosporin D.

RESULTS

Optimum Conditions for Solid-Phase Extraction of Cyclosporine from Whole Blood

The optimum conditions for the extraction of cyclosporine were derived after we had investigated several solid-phase extraction methods(12,17).

The solid-phase extraction methods involving disposable cyano columns reported by Yee et al (12), and Kates and Latini (17) gave inconsistent and low recoveries (35-70%), and the late-eluting endogenous peaks prolonged the analysis. The solid phase extraction method of Lansmeyer (18) was not consistently reproducible in our experiments.

In our extraction procedure, initially we precipitated the blood proteins with acetonitrile before extracting the supernatant fluid by use of an octadecyl column (19). However, the absolute recovery was consistently higher (85% vs 75) by using the hemolyzed sample obtained by diluting the whole blood with 2 volumes of 30 % acetonitrile. Late eluting peaks which plagued the earlier solid phase extraction methods were eliminated from the chromatogram by using more hydrophobic octadecyl column. Additionally, by changing the elution solvent from methanol to ethanol, we were able to extract the cyclosporine quantitatively in <300 µl of ethanol. This eliminated the need to concentrate the eluate before the chromatographic analysis. The Bond-Elut octadecyl extraction columns were washed with acetonitrile/water (1/1 by vol) to remove the interfering endogenous constituents. The eluate was vortex-mixed with 500 µl of hexane to remove any neutral lipids. The Bond-Elut extraction columns could be regenerated for reuse by passing two column volumes of ethanol through them; 2-3 whole blood samples could be processed with each column without noticeable loss in the recovery of cyclosporine.

Analytical Chromatogrphy

We evaluated various chromatographic conditions (columns, the composition of the mobile phase, the pH of the mobile phase, the column temperature, and the detection wavelength) by injecting 10 µl of the extracted sample. We tried several commercially available reversed-phase

columns packed with 5 and 10 µm octyl and octadecyl packing material and found in accord with other investigators, that use of temperatures > 70 ° C was necessary for optimal resolution and peak symmetry, and for the required sensitivity. This has the serious disadvantage that useful column life is considerably shortened for the expensive analytical columns. By substituting for hydrophobic alkyl bonded reversed phase columns with a relatively polar cyano phase, the cyclosporine could be eluted at 50 ° C with a mobile phase containing 43 parts of acetonitrile(19). These milder conditions prolonged the useful life of column to about 500 analyses. However, the column to column reproducibility was poor and some lots of commercially available cyano columns were not suitable for this analysis. The acetonitrile concentration in the mobile phase was adjusted down (to as low as 37%) continously because the resolution between cyclosporine and internal standard deteriorated with time. The cyano guard cartridge was replaced after every 100 analyses. The high cost of replacing cyano analytical columns coupled with continously changing selectivity prompted us to reinvestigate commercially available octyl and octadecyl cartridge columns packed with 3 µm packing material. We investigated both 83 X 4.6 and 33 X 4.6 mm octyl and octadecyl cartridge columns (Perkin-Elmer, cost $ 75.00, and $ 39.00) and found that use of temperatures > 70 ° C was necessary for optimal resolution and peak symmetry. Our chromatographic time of < 10 min is about 20-30 minutes shorter than is the case of most of other LC methods. Because of the low cost of cartridge columns we eliminated the silica saturation column and guard column usually required with other LC methods. Even under the described conditions of 70 ° C temperature, the useful life of analytical cartridge was about 300-400 analyses.

Variations in mobile phase that we tested included various ratios of acetonitrile/water: 70/30, 65/35, 60/40, and 55/45 (by vol). The mobile

phases containing 70 to 60 parts of acetonitrile were used with octadecyl cartridge columns; the octyl columns were eluted with 50 to 60 parts of acetonitrile. The elution order of cyclosporine and internal standard remains unchanged, but retention times and resolution was increased by using the octadecyl columns. The endogenous peaks which eluted close to the cyclosporine peak with octyl column was moved with the solvent front by using the octadecyl column. The acetonitrile concentration was adjusted down (3 to 4 %) when 33 X 4.6 mm cartridge columns were used in place of 83 X 4.6 mm columns.

We elected to use elevated temperature (70 ° C) to optimize the resolution and peak symmetry for cyclosporine and internal standard. We also observed that a flow rate greater than 1.0 ml/min adversely affected the resolution and sensitivity. Although the wavelength of maximum absorption for cyclosporine was 195 nm in the mobile phase, we used 210 nm as the wavelength for analysis, because detection at 195 nm produced increased background absorbance just preceding the cyclosporine peak(Fig 1).

Analytical Variables

Detection limit: The limit of detection for the assay is <10 µg/l, when 1.0 ml of whole blood is extracted. The signal to noise ratio was >5 at 10 µg of cyclosporine per liter

Precision:

Repeated analysis of pooled whole-blood specimens containing cyclosporine at two different concentrations gave the results shown in

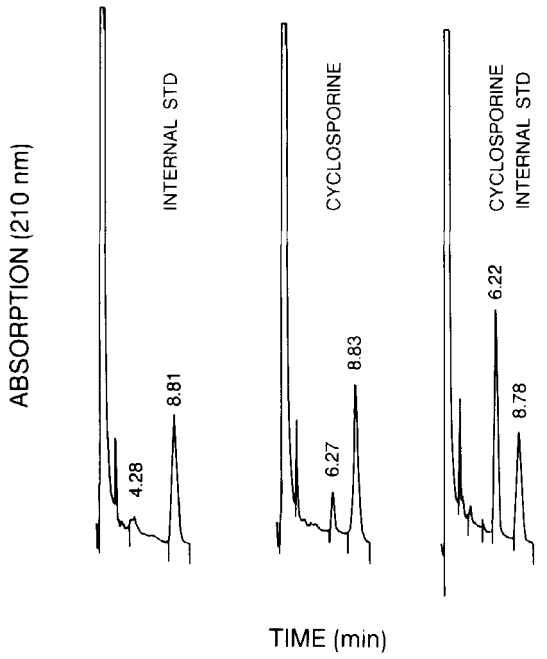

FIGURE 1. Chromatogram of (left) cyclosporine-free whole blood; (middle) a patient's blood containing 84 µg of cyclosporine per liter; (right) a patient's blood containing 670 µg of cyclosporine per liter.

TABLE 1

Precision of Assays for Cyclosporine in Whole Blood

Within-day (n=10)		Day-to-day (n=19)	
Mean (and SD), µg/l	CV, %	Mean(and SD), µg/l	CV, %
99.0 (4.5)	4.6	127 (8.0)	6.3
431.0 (12.3)	2.9	296 (18.3)	6.2

Table 1. Within-day CV's ranged from 2.8 to 4.6%, day-to-day CV's from 6.2 to 6.3%.

Analytical Recovery and Linearity:

Cyclosporine was added to a drug-free heparinized whole-blood pool in amounts equivalent to 100-2000 µg/l. A constant amount of internal standard was added to each sample, which was then processed as described above. Concentrations and peak area ratios were linearly related over this range. Analytical recoveries for cyclosporine from low therapeutic to toxic concentrations ranged from 96-115% (Table 2). Absolute recovery of cyclosporine and internal standard ranged from 80 to 85%.

Comparison of LC with RIA Measurements

We analyzed several serum samples by the RIA procedure and compared them with LC measurement. As previously reported, the result of our LC analysis were considerably lower than those by RIA(19). This is consistent with similar LC-RIA comparisons reported by others (11,12).

Interference

We evaluated potential interference in this analysis by chromatographing pure drug solutions and (or) samples of whole blood or plasma containing various drugs. Drugs tested but not detected under these conditions were: acetaminophen, amitriptyline, caffeine, carbamazepine, chloramphenicol, chlordiazepoxide, diazepam, ethosuximide, gentamicin, imipramine,

TABLE 2

Analytical Recovery of Cyclosporine from Whole Blood

Added	Recovered	
	Concn, µg/l	Recovery, %
100	95.5	95.5
500	522.0	104.4
1000	1016.0	101.6
1500	1616.0	108.0
2000	2259.0	116.0

pentobarbital, phenytoin, primidone, procainamide, quinidine, salicylate, secobarbital, and theophylline. Steroids (prednisone, prednisolone, methyl prednisone, and methyl prednisolone) did not interfere with the analysis.

DISCUSSION

The principal advantage of our LC method is faster and simple extraction procedure as compared with those published extraction procedures involving liquid/liquid (11,13-14) or solid-phase extraction procedures(12,17-18). The long chromatographic times and the need for gradient elution of cyclosporine are two major disadvantages of many of these LC procedures (12). The low and inconsistent recovery of cyclosporine and interferences by late eluting peaks are two major disadvantages of two reported solid phase extraction procedures. Batch-to

batch irreproducibility of cyano extraction columns was a major problem with other solid phase extraction procedure in our hands (18).

In contrast, our (solid phase) extraction procedure for cyclosporine from whole blood is relatively rapid and simple. The octadecyl extraction columns facilitate rapid extraction of cyclosporine from whole blood with very high efficiency, specificity, and precision. We did not observe any interference from late eluting peaks which plagued most of other LC procedures. With use of the Vac-Elut™ chamber designed to accept 10 extraction columns simultaneously, we are able to process 10 samples of whole blood in <30.0 min.

Another distinct advantage of our LC method is that the analytical column costs only $ 39.00, compared with the average cost of 200-300 dollars for other reversed phase columns. Eventhough the cartridge column is maintained at 70 ° C, the useful life of our column is still about 300-400 analyses. Because of the low cost of analytical column, we did not use any silica saturation and guard column generally required with the other LC procedures. Our chromatographic time of <10 min is considerably shorter than is the case for most of the other LC methods. The reliability, the sensitivity, utility, and cost-effectiveness of the method have been adequately demonstrated with the routine analysis of approximately 5000 whole blood samples.

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