Journal of Chromatography, 385 (1987) 305–310
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 1020

LIQUID CHROMATOGRAPHIC DETERMINATION OF CYCLOSPORINE IN WHOLE BLOOD WITH THE ADVANCED AUTOMATED SAMPLE PROCESSING UNIT

POKAR M. KABRA* and JEFFERY H. WALL
Department of Laboratory Medicine, School of Medicine, University of California at San Francisco, San Francisco, CA 94143 (U.S.A.)

SUMMARY

We describe a rapid, precise, cost-effective, and accurate isocratic liquid chromatographic (LC) procedure for determining cyclosporine in whole blood. The cyclosporine is extracted from 0.5 ml of whole blood together with 200 μg of cyclosporin D, added per liter as internal standard, by using an Advanced Automated Sample Processing (AASP) unit. The on-line solid-phase extraction is performed on an octasilane sorbent cartridge which is interfaced with a Perkin-Elmer 83 × 4.6 mm I.D. cartridge column, packed with 3-μm octadecyl packing. The column is eluted with a mobile phase containing acetonitrile–water (13:7) at a flow-rate of 1.0 ml/min at a column temperature of 70°C. The column effluent is monitored at 210 nm. The absolute recovery of cyclosporine exceeded 87% and the linearity extended up to 2000 μg/l. Within-run and day-to-day coefficients of variation were less than 8%. The correlation between AASP–LC and manual Bond-Elut extraction–LC method was excellent ($r = 0.97$).

INTRODUCTION

Cyclosporine (cyclosporin A, Sandimmune™) is a selective immunosuppressive agent, currently used to prevent graft rejection in human kidney, heart, and liver transplantation. Although cyclosporine has markedly improved the outcome in allograft recipients, its use is often associated with low therapeutic index and serious toxic effects (primarily renal and hepatic), which appear to be dose-related in most patients. Therefore, the cyclosporine concentration is usually monitored to achieve therapeutically optimum concentrations in the blood and to avoid dose-associated toxicity.

The choice of specimens for assay is very important; serum or plasma give an inconsistent measure of cyclosporine, because of *in vitro* redistribution of drug between erythrocytes and plasma or serum during storage or processing of blood samples[1]. In addition, the ratio of whole blood to plasmas cyclosporine concentration is dependent on the hematocrit and lipoprotein content, which frequently fluctuates widely in transplant recipients[2]. Therefore, whole blood should be the sample of

0021-9673/87/$03.50

choice, and more widespread use of whole blood as a sample matrix 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. Varghese *et al.*[3] and other investigators have reported that cyclosporine concentration values determined by RIA are 20–430% higher than those obtained by LC methods[3–5], and similar discrepancies have also been observed in our laboratories[6]. Thus, clinical action based on values for cyclosporine determined by RIA may well be inappropriate.

Several LC procedures have been reported, but interferences from non-polar endogenous components or lack of sensitivity necessitated development of elaborate and complex liquid–liquid[4,7–9] or solid–liquid[5,10,11] extraction procedures before chromatographic analysis. The long chromatographic analysis times and the need for gradient elution of cyclosporine are two major disadvantages of many of these procedures[4,5]. The low and variable recovery of cyclosporine and interferences by a late-eluted peak are two major disadvantages of two reported solid-phase extraction procedures[5,10]. Recently, we developed a simple and fast solid–liquid extraction procedure for the analysis of cyclosporine[12]. However, heavy work loads in our laboratories prompted us to investigate a faster automated sample processor for analyzing a larger number of samples.

We describe here an LC technique that incorporates a solid–liquid extraction technique with an Advanced Automated Sample Processing (AASP) unit. The AASP device transfers extracted cyclosporine and internal standard retained on an octyl sorbent cartridge, directly to the LC system through an automated injection valve. The AASP sequentially routes the mobile phase through each cartridge, allowing the retained cyclosporine to be eluted as a tight band directly onto the head of the column. A major advantage of this technique, therefore, is that virtually 100% of the extracted cyclosporine present in the sample is introduced into the LC column. This allows us to detect <20 μg/l of cyclosporine in 0.5 ml of whole blood.

MATERIALS AND METHODS

Instrumentation

We used a Series 2 liquid chromatograph, equipped with a Model LC-100 oven, and a Model LC-75 variable-wavelength detector (all from Perkin-Elmer, Norwalk, CT, U.S.A.) and a Hewlett-Packard 3390A integrator (Hewlett-Packard, Avondale, PA, U.S.A.). The 83 × 4.6 mm I.D. cartridge column, packed with 3-μm octadecyl packing material, was mounted in an oven. The samples were extracted and injected through a AASP unit (Varian, Walnut Creek, CA, U.S.A.), interfaced with the analytical column.

Reagents

All chemicals were of reagent grade. Acetonitrile and methanol, both distilled in glass, were obtained from J. T. Baker (Phillipsburg, NJ, U.S.A.). Mobile phase was prepared by mixing 650 ml of acetonitrile and 350 ml of deionized water. The wash solution (acetonitrile–water, 2:3) and purge solution (acetonitrile–water, 12:13)

were prepared by diluting 400 and 480 ml of acetonitrile, respectively, with deionized water to a final volume of 1 l. The Bond-Elut C_8 extraction cassettes were obtained from Analytichem International (Harbor City, CA, U.S.A.).

Drug standards

The stock cyclosporine (Sandoz, E. Hanover, NJ, U.S.A.) standard used for calibration, 10 mg in 1 l of methanol–water (1:1), is stable for at least three months at 4°C. The stock internal standard, cyclosporin D (Sandoz), 25 mg in 25 ml of methanol, is stable at 4°C for at least six months. The working internal standard, 200 μg of cyclosporin D per liter of acetonitrile–dimethyl sulfoxide (96:4) is prepared by diluting stock internal standard 5000 fold with the acetonitrile–dimethyl sulfoxide mixture. It is stable for three months at 4°C. The working cyclosporine calibration standards (200 and 400 μg/l) in whole blood are prepared by diluting 2 and 4 ml of 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 specimens: blood specimens are drawn into heparinized Vacutainer (Becton-Dickinson, Rutherford, NJ, U.S.A.) tubes. Cyclosporine is stable for at least seven days in whole blood stored at 4°C.

Procedure

Pipet 0.6 ml of the working internal standard into labeled 100 × 13 mm disposable glass tubes. Add 0.5 ml of appropriate calibration standard, control, or unknown to each tube, followed by 2 ml of hexane. Vortex-mix all tubes for 30 s, and centrifuge all tubes for 3 min at 3000 rpm (500 *g*). Aspirate the hexane layer and prepare a C_8 cassette by washing it with 1.5 ml of wash solution. Pour the supernatant from each tube into the cassette reservoirs and add 1.0 ml of water to each reservoir. Pass the diluted samples through the cassette, followed by 1.5 ml of wash solution. Load the cassettes onto the AASP unit and set up the following parameters of the analysis: Purge, acetonitrile–water (12:13) 80 strokes pre-injection and 10 strokes post-injection; valve reset, 0.8 min; cycle time, *ca.* 6 min, depending on current retention times of cyclosporin D and late eluted peak; chromatographic column, C_{18} cartridge, 83 × 4.6 mm I.D., packed with 3-μm sorbent; flow-rate, 1 ml/min; detector, 210 nm, 0.02 a.u.f.s.; oven, 70°C. The quantification of cyclosporine is based on the peak-height ratio of cyclosporine to cyclosporin D.

RESULTS

Optimum conditions for AASP extraction of cyclosporine from whole blood

The optimum conditions for the extraction of cyclosporine were derived by investigating several solid-phase extraction methods[6]. The solid-phase extraction methods involving cyano extraction columns gave inconsistent and low recoveries, and the late-eluted endogenous peaks prolonged the blood analysis. In our previously published procedure we extracted the whole blood supernatant by use of an octadecyl Bond-Elut column with good recovery and precision[6]. However, the use of a similar kind of sorbent cartridge resulted in poor resolution and broad peaks, because the mobile phase did not elute the cyclosporine as a tight band. Therefore, we selected an octyl cassette with a weaker retention sorbent than the octadecyl analytical col-

umn. The octyl sorbent cassettes transferred the extracted sample as a tight band to the head of the analytical column, as judged by the resolution and peak symmetry of cyclosporine and internal standard. The octyl sorbent cassettes were purged with 80 pre-injection strokes (25-μl stroke volume) to remove any interfering endogenous constituents.

The whole blood supernate was vortex-mixed with 2 ml of hexane to remove any neutral lipids. Initially, we tried to extract the hemolyzed samples obtained by diluting the whole blood with two volumes of 30% acetonitrile. However, some of the hemolyzed samples did not flow freely through the sorbent cartridges. We also observed a late-eluted peak in the chromatogram from extracts obtained with octyl cassettes. This component was later identified as dioctyl phthalate by a diode-array detector. This contaminant peak was derived from the HPLC-grade acetonitrile used in the analysis. Various valve reset intervals from 0.2 to 0.6 min did not eliminate this interference from the chromatogram. A valve reset time of less than 0.6 min resulted in the loss of cyclosporine and decreased internal standard recoveries from octyl sorbent cassettes. The interfering peak was eluted between the internal standard and solvent front peak if the cycle time was set at 6.0 min. This adjustment of the cycle time allowed us to run the assay uninterrupted without any interferences. The cycle time was adjusted after determining the retention times of internal standard and late-eluted peak.

Analytical chromatography

We evaluated various chromatographic conditions (columns, composition of the mobile phase, and column temperature) by injecting the extracted sample. We tried commercially available octyl and octadecyl reversed-phase cartridge columns (Perkin-Elmer, cost US$75) and found, in accord with our previous experience[12], that a temperature >70°C was necessary for optimal resolution and peak symmetry. Because of the low cost of cartridge columns we eliminated the silica saturation column and guard column usually required in other LC methods for cyclosporine analysis. Even under the described conditions of 70°C temperature, the analytical column was useable for about 500 analyses.

Variations in mobile phase that we tested included acetonitrile–water, ratios of 70:30, 65:35, and 60:40. The mobile phases containing 65–70% of acetonitrile were used with an octadecyl column, while the octyl column was eluted with 55–60% of acetonitrile. The elution order of cyclosporine and internal standard remains unchjanged, but retention times and resolution were increased by using the octadecyl column. We also observed that a flow-rate greater than 1.0 ml/min adversely affected the resolution and sensitivity.

Analytical variables

Sensitivity. The limit of detection for the assay is <30 μg/l, when 0.5 ml of whole blood is extracted. The signal-to-noise ratio was >4 at 20 μg of cyclosporine per liter.

Precision. Repeated analysis of pooled whole-blood specimens containing cyclosporine at two different concentrations (288 and 118 μg/l) gave a within-day coefficient of variation (C.V.) from 6.3 to 7.8% (n = 19), while the day-to-day C.V. at a concentration of 440 μg/l was <4.5% (n = 8).

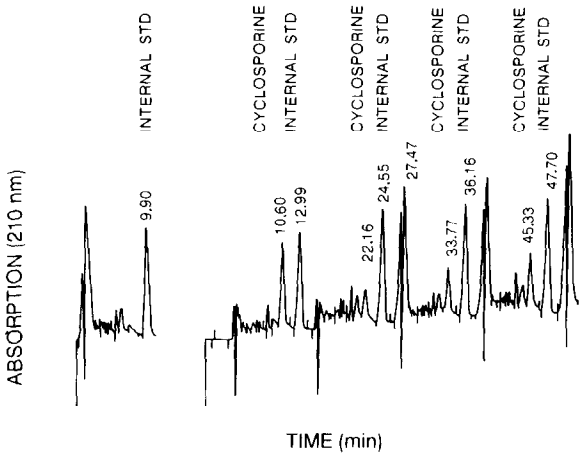

Fig. 1. Chromatograms of (from left to right) cyclosporine-free whole blood; whole blood, containing 400 μg of cyclosporine per liter; patient's blood, containing 40, 140 and 145 μg of cyclosporine per liter.

Background. We processed more than 20 different drug-free heparinized blood specimens to ascertain background peak interferences at the elution times corresponding to cyclosporine and internal standard. The background calculated from these samples was <20.0 μg/l, and it did not interfere with the analysis (Fig. 1).

Analytical recovery and linearity. Cyclosporine was added to a drug-free heparinized whole-blood pool in amounts equivalent to 62–2000 μg/l. A constant amount of cyclosporin D (internal standard) was added to each sample, which was then processed, as described above. Concentration and peak height ratios were linearly related over this range. Analytical recoveries for cyclosporine ranged from 98 to 104% (Table I). Absolute recovery of cyclosporine and internal standard averaged about 87%.

Accuracy. We analyzed about 50 samples by the Bond-Elut extraction procedure[12], and compared them with AASP procedure. In general, the results by both methods were comparable. Results of the regression analysis comparing AASP (y) and Bond-Elut (x) assays were: $n = 50$, $r = 0.970$, slope $= 0.904$, and y-intercept $= 19.4$ μg/l.

TABLE I

ANALYTICAL RECOVERY OF CYCLOSPORINE FROM WHOLE BLOOD

Cyclosporine concentration (μg/l)		*Recovery (%)*
Added	*Recovered*	
125	127	102
250	260	104
500	505	101
1000	1032	103
1500	1481	99
2000	1971	98

Interference. We evaluated potential interferences in this assay by chromatographing pure drug solutions and (or) samples of whole blood containing various drugs. Drugs tested but not detected under these conditions were: acetaminophen, amitryptyline, caffeine, carbamazepine, chloramphenicol, chlordiazepoxide, diazepam, ethosuximide, gentamicin, imipramine, pentobarbital, phenytoin, primidone, procainamide, salicylate, secobarbital, and theophylline. Steroids (prednisone, prednisolone, methyl prednisone, and methyl prednisolone) did not interfere with the analysis.

DISCUSSION

The principal advantage of AASP is that it is a faster, cost-effective, and simpler extraction procedure than those involving liquid–liquid[4,7–9] or solid-phase extraction[5,10,11]. The low and inconsistent recovery of cyclosporine and interferences by late eluted peaks are major disadvantages of some of the reported solid-phase extraction procedures. In contrast, the AASP procedure for cyclosporine is relatively rapid and to a large extent automated. The octyl sorbent cassettes allow rapid extraction of *ca.* 100 whole-blood samples with very high efficiency, sensitivity, and precision.

Another distinct advantage of our LC method is that the analytical column costs only US$ 75, compared with the average cost of US$ 200–300 for other reversed-phase columns. Even though the cartridge column is maintained at 70°C, our column is still useable for about 500 analyses. Because the octyl sorbent casettes were interfaced with analytical column, we did not use any saturation of guard column to protect the analytical column.

Currently our AASP method suffers from two major deficiencies: the presence of a late-eluted peak derived from the HPLC-grade solvents and the occasional presence of extraneous peaks, originating from reagents or standards stored in plastic containers.

At present we are adjusting the cycle time of the AASP in such a way that the late-cluted peak is eluted between the internal standard and the solvent fronmt peak. This allows us to run the AASP assay uninterupted without any interferences (Fig. 1). Additionally by storing all reagents and standards in glass containers, we can eliminate the other extraneous peaks. Experiments are underway to eliminate these problems by modifying the chromatographic procedure.

REFERENCES

1 M. Wenk, F. Follath and E. Abisch, *Clin. Chem.*, 29 (1983) 1865.
2 T. G. Rosano, *Clin. Chem.*, 31 (1985) 410.
3 Z. Varghese, M. K. Chan and L. V. Steele, *Lancet*, i (1984) 1407.
4 S. G. Carruthers, D. J. Freeman and J. C. Koegler, *Clin. Chem.*, 29 (1983) 180.
5 G. C. Yee, D. J. Gmur and M. S. Kennedy, *Clin. Chem.*, 28 (1982) 2269.
6 P. M. Kabra, J. H. Wall and N. Blanckaert, *Clin. Chem.*, 31 (1985) 1717.
7 W. Niuederberger, P. Schaub and T. Beveridge, *Clin. Chim. Acta*, 182 (1980) 454.
8 R. J. Sawchuk and L. L. Cartier, *Clin. Chem.*, 27 (1981) 1368.
9 H. T. Smith and W. T. Robinson, *J. Chromatogr.*, 305 (1984) 353.
10 R. E. Kates and R. Latini, *J. Chromatogr.*, 309 (1984) 441.
11 G. L. Lensmeyer and B. L. Fields, *Clin. Chem.*, 31 (1985) 196.
12 P. M. Kabra and J. H. Wall, *J. Liq. Chromatogr.*, in press.