*Journal of Chromatography*, 344 (1985) 422—427
*Biomedical Applications*
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2711

## Note

# Modified column-switching high-performance liquid chromatographic method for the measurement of cyclosporine in serum

D.J. GMUR, G.C. YEE* and M.S. KENNEDY

*Division of Clinical Research, Fred Hutchinson Cancer Research Center, 1124 Columbia Street, Seattle, WA 98104 (U.S.A.)*

(First received February 5th, 1985; revised manuscript received May 3rd, 1985)

Cyclosporine is a selective immunosuppressive agent used to prevent or treat graft rejection in solid organ recipients [1] and graft-versus-host disease in marrow transplant recipients [2]. Since the therapeutic and some of the toxic effects of cyclosporine are probably related to systemic concentrations, it is important to monitor cyclosporine concentrations in transplant patients receiving the drug [3].

Cyclosporine is a chemically neutral compound with a large molecular weight. A number of high-performance liquid chromatographic (HPLC) methods for measurement of cyclosporine concentration in biological fluids have been published [4]. However, most of these HPLC methods have serious limitations. Laborious clean-up extractions are usually required prior to HPLC measurement of cyclosporine in serum, plasma or whole blood. Furthermore, most of the methods are limited by low sensitivity (30–100 μg/l) or long analysis times (20—45 min) [4].

Smith and Robinson [5] recently published a semi-automated column-switching HPLC method with rapid sample preparation. The major advantages of this method are improved precision and sensitivity and shorter analysis times. While adapting this method to our existing HPLC system, we have modified the procedure and have noted that some equipment was unnecessary. Because these changes may reduce the high cost of adapting this method, we report these modifications here to assist others who want to adapt the column-switching method to their own instrument.

0378-4347/85/$03.30 

## EXPERIMENTAL

### *Reagents*

Acetonitrile was HPLC grade (J.T. Baker, Phillipsburg, NJ, U.S.A.). Water was deionized with a water purification system (Milli-Q reagent water system, Continental Water Systems, El Paso, TX, U.S.A.). Human serum used to make standards was obtained from commercial sources (Gibco Labs., Lawrence, MA, U.S.A.).

### *Modifications*

The method of Smith and Robinson [5] was modified to an existing microprocessor-controlled high-performance liquid chromatograph (Varian Model 5060 with CDS 401 microprocessor, Varian, Sunnyvale, CA, U.S.A.) (see Fig. 1).

Endogenous proteins in patient serum samples (0.66 ml) were precipitated with 0.8 ml of 15 m*M* phosphoric acid (J.T. Baker) in acetonitrile. The final pH of the acidified samples was between 5 and 6.

An autosampler (Model LC 9505, IBM, Danbury, CT, U.S.A.) equipped with a 1-ml sample loop injected 850 $\mu$l of the sample into the $C_8$ column (Spherisorb $C_8$, 150 × 4.6 mm, 5 $\mu$m particle size; ASI, Santa Clara, CA, U.S.A.). The in-line hexane extraction (Technicon autosampler IV and pump III, Technicon, Tarrytown, NY, U.S.A.) was initially used but was later found to be unnecessary.

The Varian HPLC system pumped mobile phase through the $C_{18}$ column (Spherisorb ODS-2, 250 × 4.6 mm, 5 $\mu$m particle size; ASI) and controlled the timed events. To elute late interfering peaks, the HPLC system briefly increased the flow-rate and percentage of acetonitrile.

An inexpensive HPLC pump (Milton Roy minipump, Laboratory Control Data, Riviera Beach, FL, U.S.A.) pumped mobile phase through the $C_8$ column at a flow-rate of 3 ml/min. A 10-ml sample loop (upstream from the injector) served as a pulse damper.

A switching valve (Rheodyne Model 7067 tandem enrichment injector, Rheodyne, Cotati, CA, U.S.A.) diverted solvent flow as needed.

Cyclosporine was eluted from the $C_8$ column with acetonitrile–water (55:45) at a flow-rate of 3 ml/min. Later, cyclosporine was eluted from the $C_{18}$ column with acetonitrile—water (80:20). The columns were heated to 70°C in a block heater.

A 2-ml sample loop, connected into the switching valve upstream from the injector, was filled with 80% acetonitrile while the cyclosporine peak was diverted from the $C_8$ to the $C_{18}$ column. This volume of mobile phase emptied into the $C_8$ column upon return to initial conditions, which served to elute later interfering peaks.

Cyclosporine was measured with a fixed-wavelength detector (Model LC 9522; IBM) set at 214 nm.

## RESULTS AND DISCUSSION

Fig. 1 shows a typical chromatogram, as eluted from the $C_{18}$ column. The

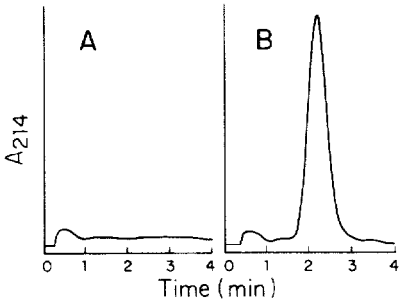


Fig. 1. Typical chromatograms of serum samples from a patient. (A) Baseline sample taken before cyclosporine was administered; (B) sample with a calculated cyclosporine concentration of 246 μg/l.

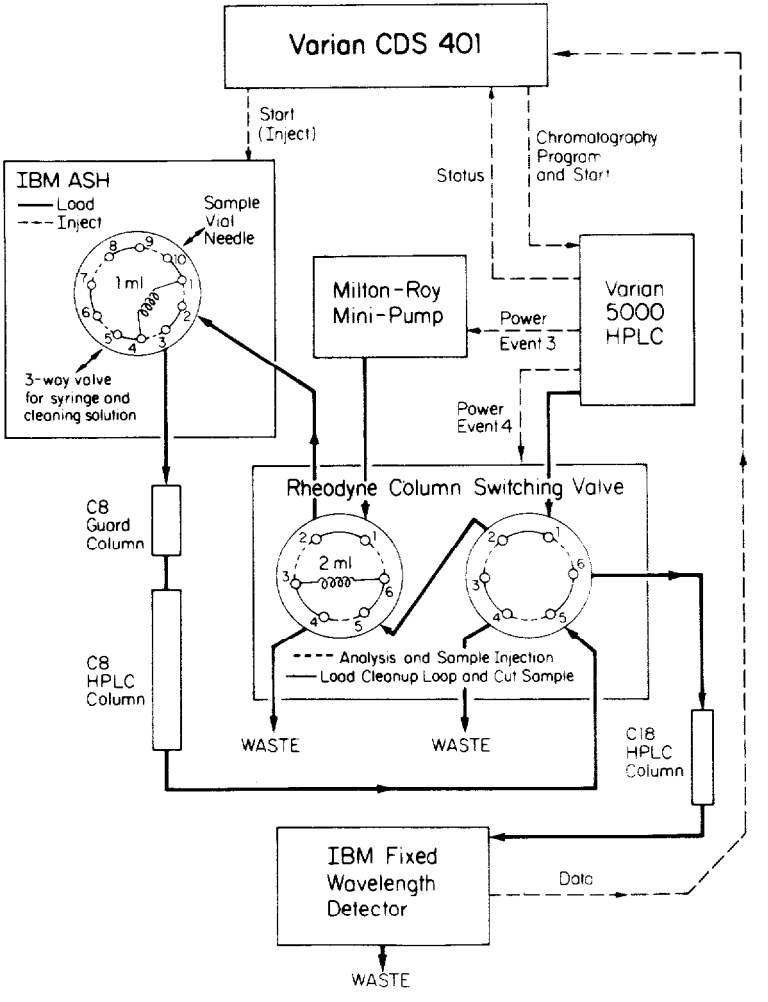


Fig. 2. Flow diagram for HPLC analysis of cyclosporine in serum.

modified HPLC method (Fig. 2) gave a configuration similar to that of Smith and Robinson [5]. The sequence of events used in our method was also similar (Table I). We prepared samples just as rapidly as the original method, but we changed the amount of acetonitrile and patient sample to make it similar to the $C_8$ mobile phase. We had no trouble with samples clogging the $C_8$ column, but periodic cleaning of the sample loop was necessary. The 2-ml volume of 80% acetonitrile, diverted from the Varian pump onto the $C_8$ column, eluted any interfering serum components but did not delay column re-equilibration. The analysis time was longer than that of the original procedure. The overall speed of analysis depended on the particular column (repacked columns sometimes varied from one to the next) and on the age of the particular column (retention times decreased with column age). The intra- and inter-day precision of our method were comparable to the original method [5] (Table II).

The benefits of these modifications were several: lower cost of the autosampler, less expensive columns, increased column life with improved stability of performance, and less noise from the detector. An extra step-down transformer and low-voltage relay were required to integrate the Technicon autosampler with the external event on the Varian HPLC system. The autosampler required no further electronic modification; it was easily triggered

TABLE I

SEQUENCE OF EVENTS FOR THE COLUMN-SWITCHING METHOD

Abbreviations: ACN = acetonitrile, CDS 401 = microprocessor control station, CSP = cyclosporine.

| Time* (min) | Chromatographic condition or command | Purpose |
|---|---|---|
| 0 | Start | CDS 401 is monitoring CSP from a previous injection; sample is injected into a $C_8$ HPLC column |
| 0 | Flow-rate: 0.9 ml/min | Initial conditions for $C_{18}$ column |
| 0 | Percentage ACN: 80 | |
| 0 | Event 3 on | Minipump pumps 55% ACN at 3 ml/min. |
| 4 | — | CDS 401 stops monitoring CSP from a previous injection |
| 6 | Percentage ACN: 95 | Begin cleaning late eluting peaks from $C_{18}$ with a surge |
| 6.5 | Flow-rate: 3 ml/min | of 95% ACN |
| 7.6 | Percentage ACN: 80 | Re-equilibrate $C_{18}$ |
| 9.5 | Event 4 on | Divert solvent stream coming from the $C_8$ column (via |
| 9.5 | Flow-rate: 0.9 ml/min | minipump) onto the $C_{18}$ column; solvent stream from Varian pump diverted to waste through 2-ml loop |
| 12.5 | Event 4 off | The 2-ml volume of 80% ACN is sent into the $C_8$ column as conditions return to those at Start |
| 16 | End of program | Recycle the electronics to monitor the CSP from this sample and inject the next sample |

*These times may change slightly depending on the individual columns and the age of the columns.

TABLE II

INTRA- AND INTER-DAY VARIATION IN CYCLOSPORINE CONCENTRATIONS FROM SPIKED SERUM SAMPLES

| Nominal concentration (μg/l) | Intra-day ($n$ = 10) | | Inter-day ($n$ = 10) | |
|---|---|---|---|---|
| | Mean concentration (μg/l) | C.V. (%) | Mean concentration (μg/l) | C.V. (%) |
| 20 | 22 | 3.4 | 21 | 12.7 |
| 100 | 107 | 2.8 | 99 | 4.6 |
| 500 | 493 | 2.2 | 507 | 2.5 |

with contact closures on the Varian HPLC system. The cost of the hardware (Technicon autosampler and peristaltic pump, tubing, and glassware) used for the hexane extraction was several thousand dollars more than the cost of the autosampler.

As with all HPLC methods for cyclosporine, high column temperatures were required, which resulted in monthly replacement of columns. We found that both new and repacked Spherisorb columns were acceptable and were less than the cost of the columns used by Smith and Robinson [5]. The only disadvantage of the repacked Spherisorb columns was the inconvenience and cost of testing each set of columns. Although it was difficult to quantitate the longer column life due to acidification of the samples, major changes in the rate of dissolution of the $C_8$ packing were observed. Injection of 60—80 unacidified samples caused a 2—3 mm void in the $C_8$ column, resulting in erratic decreases in the slope of the standard curve (sometimes as much as 500%). As the $C_8$ column further deteriorated, the retention time of cyclosporine changed, which sometimes required adjustments in the cut times. The use of a silica saturator column alone upstream from the $C_8$ column did not prevent void formation. After we began to acidify the samples, void formation was eliminated, which resulted in increased stability of the standard curves and retention time. Acidification of samples alone did not change the retention time of cyclosporine nor did it affect the presence or absence of interfering peaks.

The response of the fixed-wavelength detector in mV was similar to the response from the variable-wavelength detector (set at 202 nm). However, because we monitored cyclosporine at 214 nm, the major advantage was less noise and a higher signal-to-noise ratio.

The major disadvantage of our method is that it may not be adaptable to other biological fluids, such as whole blood. We have not analyzed many whole blood samples, but we have observed that the supernatant from blood was darker and contained more interfering compounds than in serum. Plasma samples were analyzed with no special problems.

The column-switching method of Smith and Robinson [5] can probably be adapted to other existing HPLC systems. Initially, we had four major problems: (1) the existing autosampler could not inject more than 250 μl; (2) the existing variable-wavelength detector did not have adequate sensitivity at 214 nm; (3) the single-pump HPLC system could not simultaneously pump solvents through two columns; and (4) we did not have a column-switching valve. Unfortunately,

all of these problems required purchase of additional equipment. Once all the equipment was purchased, we began to solve the problem of how the equipment would be controlled. System control was shared by the Varian 5000 and Vista CDS 401, but it would have been better to have had one piece of equipment control all of the other pieces of equipment. The final problem that we faced was to develop a method for the different pieces of equipment to communicate with one another. Ideally, if a problem developed, both the HPLC system and the detector should be shut off. This would prolong the lifespan of the detector lamp and HPLC columns and save solvent. We were never able to completely solve this problem. For example, when a plug developed in the $C_8$ line or the autosampler jammed, the rest of the system continued to run. When a plug developed in the $C_{18}$ line, the Varian pump shut off but the columns stayed heated, the minipump continued to run, and the detector stayed on.

As with any complex method, routine quality control and maintenance are necessary. The solvents must be clean; contamination from the plastic protective ring around the graduated cylinder used to mix the acetonitrile—water mobile phase was a problem. We periodically replaced frit filters in all columns (daily), cleaned the sample loop (every six months), repacked the guard column (weekly), and replaced piston and valve seals in the pumps (every six months). To maintain precision and accuracy, we sometimes include several standard curves. Several internal and external control samples are included in every run.

After analyzing about 1600 samples with our modified column-switching HPLC method over a twelve-month period, we would recommend this type of method to any laboratory that regularly analyzes a large number of serum cyclosporine concentrations. However, we also would emphasize that adapting this method to existing instruments is not an easy task. We hope that our experience will be helpful to other laboratories.

ACKNOWLEDGEMENTS

This investigation was supported by Grant Nos. CA 15704, CA 30924, CA 18029, CA 18221, and CA 33252 from the National Cancer Institute, National Institutes of Health, DHHS and by Sandoz. The authors wish to thank Mr. Randy Schaffer and Dr. Tom Smith for helpful discussions.

REFERENCES

1 D.J. Cohen, R. Loertscher, M.F. Rubin, N.L. Tilney, C.B. Carpenter and T.B. Strom, Ann. Intern. Med., 101 (1984) 667—682.
2 R. Storb, H.J. Deeg, E.D. Thomas, C.D. Buckner, R.A. Clift, N. Flournoy, M.S. Kennedy, K. Doney, F.R. Appelbaum, J.E. Sannders, P. Stewart, H. Shulman, K.M. Sullivan and R.P. Witherspoon, Transplant. Proc., 15 (1983) 2620—2623.
3 G.C. Yee and M.S. Kennedy, in W.E. Evans, J.J. Schentag and W.J. Jusko (Editors), Applied Pharmacokinetics: Principles of Therapeutic Drug Monitoring, Applied Therapeutics, San Francisco, CA, 2nd ed., in press.
4 W.T. Robinson, H.F. Schran and E.P. Barry, Transplant. Proc., 15 (1983) 2403—2408.
5 H.T. Smith and W.T. Robinson, J. Chromatogr., 305 (1984) 353—362.