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SEMI-AUTOMATED HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF CYCLOSPORINE IN PLASMA AND BLOOD USING COLUMN SWITCHING

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SUMMARY

A sensitive, semi-automated high-performance liquid chromatographic method utilizing column switching is described for the determination of cyclosporine in plasma and blood. This method involves a short and improved manual protein precipitation of the sample followed by an automated clean-up of the supernatant After automatic loading of the clean supernatant onto an LC-8 column for initial separation, the segment containing cyclosporine is loaded (automatically) onto an LC-18 column for final separation and quantitation. Cyclosporine is detected by its ultraviolet absorption at 202 nm. The rate of analysis was four samples per h running 24 h per day (ca. 100 samples per day). The method is sensitive enough to measure with confidence cyclosporine concentrations of 8 μg/l in plasma and 20 μg/l in blood with a linear response up to 2500 μg/l using only 0.5 ml of sample. No internal standard is required. The method was applied continuously (24 h per day) to approximately 1000 samples without deterioration in method parameters.

INTRODUCTION

Cyclosporine (Sandimmune®), a novel immunosuppressive agent [1, 2], is a cyclic undecapeptide [3] currently used in the area of organ transplants. The clinical success of cyclosporine and the utility of measuring blood or plasma concentrations of the drug have been documented by several investigators [4—9]. The purpose of this report is to describe an improved semi-automated high-performance liquid chromatographic (HPLC) method for the determination of cyclosporine in plasma or blood.

Several methods have been reported and are summarized in Table I. The radioimmunoassay (RIA) method [10] was originally published for analysis of plasma but has been applied successfully to both blood and plasma analysis at

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TABLE I

COMPARISON OF PUBLISHED METHODS

Author	Ref.	Type of analysis	Sample type (vol. in ml)
Donatsch et al.	[10]	RIA	Blood/plasma (0.1)
Niederberger et al.	[11]	HPLC-Gradient	Plasma (1)
Lawrence and Allwood	[12]	HPLC-Isocratic	Serum (1)
Sawchuk and Cartier	[13]	HPLC-Isocratic	Blood/plasma (2)
Nussbaumer et al.	[14]	HPLC-Column switching	Blood/plasma (0.5)
Leyland-Jones et al	[15]	HPLC-Isocratic	Plasma (0.6)
Kahan et al.	[7]	HPLC-Isocratic	Blood/plasma (1)
Yee et al.	[16]	HPLC-Gradient (Isocratic?)	Serum (2)
Carruthers et al.	[17]	HPLC-Isocratic	Plasma (1)
Smith and Robinson (present publication)		HPLC-Column switching	Blood/plasma (0 5)

*Short = protein precipitation and injection; long = extraction and evaporation; very long = additional sample manipulation.
**By nature of the method the average sample preparation plus analysis time is short for a large number of samples.

detection limits better than reported [18]. This method is not totally specific in that cross-reactivity to selected metabolites has been shown [10, 17].

Most of the HPLC methods available have definite disadvantages. In general, sample preparations tend to be labor-intensive and/or chromatography times tend to be long, severely limiting sample through-put. In order to well define the pharmacokinetics of cyclosporine, a method should be able to define plasma or blood concentrations for at least three half-lives [19]. Based on the work of Beveridge et al. [20], detection limits of published HPLC methods seem to present problems and the methods of Niederberger et al. [11] and Nussbaumer et al. [14] have been up to now the most attractive.

In order that we might investigate the pharmacokinetics of cyclosporine in man, it was the objective of the following work to develop and validate an HPLC method with a detection limit of less than 20 μg/l and an improved analysis time over existing methods.

MATERIALS AND METHODS

Reagents

Chemicals used were cyclosporine (cyclosporin A) (Sandoz, E. Hanover, NJ, U.S.A.); methanol, acetonitrile and hexane (UV; HPLC grade, Fisher Scientific, Fair Lawn, NJ, U.S.A.) and glass-distilled water. Outdated human plasma (N.J. Blood Services, New Brunswick, NJ, U.S.A.) and whole blood (Sera-Tec Biologicals, North Brunswick, NJ, U.S.A.) were used in the preparation of standards.

A stock standard of cyclosporine was prepared by dissolving 12.5 mg of cyclosporine in 100 ml of methanol. Further dilutions of this stock solution

Sample preparation*	Analysis parameters			
	Detection limit (μg/l)	Selectivity	Standard-ization	Chromatography time (min)
**	1—20	Metabolite x-reactivity	External	**
Long	20	High	Internal	30
Long	100	(?)	External	5
Very long	25	High	Internal	10
Short	20	High	Internal	30
Long	100	High	Internal	20
Long	100	High	Internal	45
Long	30—50 (?)	High	Internal	15
Long	31	High	Internal	30
Short (semi-automated)	8	High	External	15

were made with methanol prior to preparing plasma and blood standards containing 20—2500 μg/l cyclosporine (less than 4% methanol).

Solutions

Mobile phases A and B. For mobile phase A, 5 l of acetonitrile—water (55:45) and for mobile phase B, 3 l of acetonitrile—water (75:25) are prepared daily. The solutions are filtered/degassed using a vacuum filter system (Millipore, Bedford, MA, U.S.A.) equipped with a 0.45-μm filter (Nylon 66, Rainin Instrument, Woburn, MA, U.S.A.).

Precipitation solution. Prepare 1 l of acetonitrile—water (97.5:2.5).

Precipitation procedure

Into 15-ml glass-stoppered test tubes pipet 0.5 ml of each blank, standard or subject plasma (or blood) sample. Add, by use of a Repipet® (Labindustries, Berkeley, CA, U.S.A.), 1.2 ml of the precipitation solution, stopper and mix for 30 sec using a Maxi-Mix® (Thermolyne, Dubuque, IA, U.S.A.). Centrifuge at 1000 *g* for 5 min.

Automated sample wash and chromatography

Decant the supernatant from each sample into a 2-ml glass autosampler vial (Cat. No. 3-3123, Supelco, Bellefonte, PA, U.S.A.) which is held in a 5-ml polystyrene sample cup (Technicon Instruments, Tarrytown, NY, U.S.A.), and cover with a 2.5 cm × 2.5 cm piece of aluminum foil. Place the sample cup containing the supernatant into the tray of the Sampler IV® (Technicon Instruments). Start the automated microprocessor-controlled (Altex 420, Altex, Berkeley, CA, U.S.A.) sample wash and chromatography process utilizing

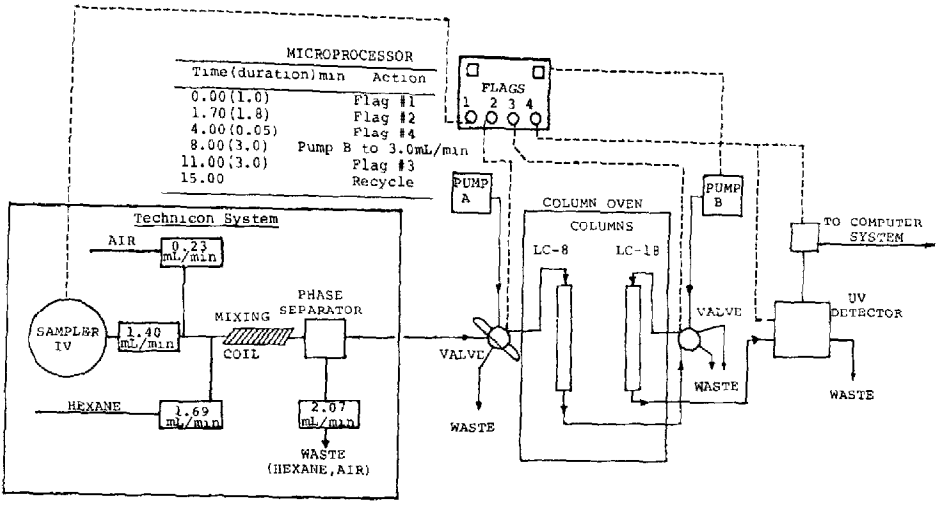

Fig. 1. Schematic of the automated sample preparation and chromatography system. (— — — —) Microprocessor controlled devices.

a Proportioning Pump III® (Technicon Instruments) and the sample wash and chromatography set up shown in Fig. 1. Program the microprocessor (see Fig. 1) to automatically sample, wash (with hexane), separate (the supernatant from the hexane) and load the sample onto the 2.0-ml loop of the pneumatically actuated, remotely controlled injection valve (Model AH-CV6-UHPa-N60 with DVI; Valco Instruments, Houston, TX, U.S.A.). The sample contained in the loop of the injection valve is automatically injected onto an LC-8 column (Supelcosil LC-8, 150 × 46 mm, 5 μm particle size; Supelco) kept at 75°C in a thermostated column oven (Model LC250; Kratos, Westwood, NJ, U.S.A.). Cyclosporine is eluted with mobile phase A at a flow-rate of 3.0 ml/min using Pump A (Model 110A, Altex). The segment containing cyclosporine is automatically diverted, by means of a second injection valve (Valco Instruments), onto an LC-18 column (Supelcosil LC-18, 150 × 4.6 mm, 5 μm particle size; Supelco) also at 75°C and cyclosporine is eluted from this column with mobile phase B at a flow-rate of 1.0 ml/min using Pump B (Model 110A, Altex). Cyclosporine is detected by its UV absorption at 202 nm using a variable-wavelength detector (Model LC-75 with autocontrol; Perkin-Elmer, Norwalk, CT, U.S.A.). Peak height measurements, baseline integrations and calculations are performed by a computer system (HP-1000; Hewlett-Packard, Paramus, NJ, U.S.A.) equipped with a computer-automated laboratory system (CALS) software package (Computer Inquiry Systems, Waldwick, NJ, U.S.A.). Concentrations of cyclosporine in plasma and blood are determined by relating their peak height measurements to the standard curve concentration—response measurements run before, during and after analysis of unknown samples.

RESULTS

Chromatograms

No interfering peaks have been detected in the plasma or blood used for blank standards or from subjects who have been orally dosed with cyclo-

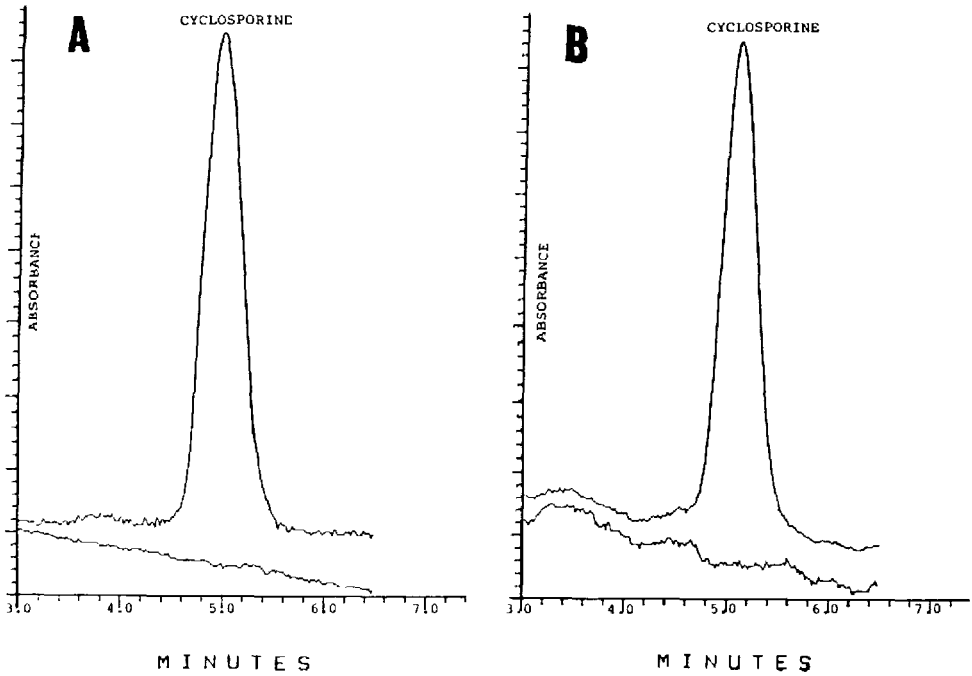

Fig. 2. Representative chromatograms obtained in the analysis of 0.5 ml of (A) blank plasma (lower trace) and plasma standard containing 200 μg/l cyclosporine and (B) subject plasma samples at zero h (lower trace) and 1.0 h (equivalent to 280 μg/l cyclosporine) after oral administration (1400 mg) of cyclosporine.

sporine. Fig. 2A shows a chromatogram of a plasma blank and 200 μg/l standard and Fig. 2B shows a zero-h and 1.0-h plasma sample from a normal volunteer who had received a single oral dose (1400 mg) of cyclosporine. The absence of a solvent front is due to a computer user-controlled rejection of the first 3 min of data following sample injection. This eliminates the large offset caused by the large volume of mobile phase A passing through the LC-18 column upon injection of the cyclosporine segment.

Linearity

Daily standardization curves (n = 16) for cyclosporine in both plasma and blood resulted in a linear concentration—response relationship. Cyclosporine concentrations of 0, 50, 200, 500, 1000 and 2500 μg/l in plasma and blood were used for standardization and regression analysis of the data resulted in mean slope and y-intercept values of 0.024 mV/μg/l and 0.020 mV for plasma and 0.016 mV/μg/l and 0.06 mV for blood, respectively. The corresponding mean correlation coefficients for the plasma and blood standard curves were 0.999 and 0.997, respectively.

Accuracy, precision and reproducibility

The accuracy of the method was evaluated by analyzing plasma and blood samples containing known amounts of cyclosporine. Using the t-value from a one-tailed Student's t-distribution table and the variance of absolute differences between the actual concentrations and the concentrations found (see Table II), the 95% confidence intervals for single determinations of cyclosporine in

LE II

IDATION OF METHOD APPLIED TO PLASMA AND BLOOD USING SPIKED SAMPLES

ed entration)	Mean ± S.D. (C.V., %)					Mean absolute difference from true value ± S D. (μg/l)	95% Confidence limit percent of true value
	Day 1 (n = 4)	Day 2 (n = 4)	Day 3 (n = 4)	Day 4 (n = 4)	Days 1—4 (n = 16)		
ıa							
	27 ± 3 (11.1)	31 ± 2 (6.5)	28 ± 2 (7 1)	32 ± 3 (9.4)	29 ± 3 (10.3)	2.4 ± 2.1	± 20.3
	117 ± 5 (4.3)	109 ± 1 (0.9)	108 ± 3 (2.8)	123 ± 7 (5.7)	114 ± 8 (7.0)	7.1 ± 4.0	± 12.1
	517 ± 20 (3.9)	473 ± 16 (3.4)	439 ± 7 (1.6)	455 ± 5 (1.0)	471 ± 32 (6.8)	29.0 ± 15.3	± 11.6
	1172 ± 22 (1.9)	1220 ± 6 (0.5)	1152 ± 29 (2.5)	1201 ± 33 (2.7)	1186 ± 35 (2.7)	36.8 ± 31.1	± 7.5
	2648 ± 41 (1.5)	2357 ± 67 (2.8)	2392 ± 23 (1.0)	2441 ± 30 (1.2)	2459 ± 123 (5 0)	94.8 ± 73.5	± 9.1
ı							
	29 ± 4 (13.8)	29 ± 1 (3.4)	26 ± 1 (3.8)	23 ± 2 (8.7)	26 ± 3 (11.5)	2.5 ± 2.4	± 26.8
	476 ± 22 (4.6)	585 ± 59 (10.1)	465 ± 33 (7.1)	519 ± 80 (15.4)	511 ± 68 (13.3)	52.4 ± 43.3	± 25.7
	1063 ± 81 (7 6)	1071 ± 66 (6.2)	972 ± 73 (7.5)	1093 ± 96 (8.8)	1048 ± 86 (8.2)	73.1 ± 64.5	± 18.7
	2200 ± 127 (5 8)	2388 ± 198 (8.3)	2575 ± 39 (1.5)	2497 ± 151 (6.0)	2409 ± 194 (8 1)	172.7 ± 75.0	± 12.7

plasma and blood were calculated at all concentrations. The results indicate that any single value would fall within ± 20.3% and 26.8% of its true value for plasma and blood, respectively.

The precision (within-day variability) and reproducibility (day-to-day variability) of the method are also demonstrated by the data in Table II. The coefficients of variation (C.V.) for the within-day variation at any concentration of cyclosporine in plasma ranged from 0.5% to 11.1% while the day-to-day variation for the same set of data ranged from 3.0% to 10.3%. For blood the within-day variation was 1.5% to 15.4% and the day-to-day variation for the same data ranged from 8.1% to 13.3%.

Sensitivity

The sensitivity of this method was evaluated by analyzing plasma and blood samples to which cyclosporine had been added in concentrations near the limit of sensitivity. The results from the analysis of these samples are shown in Table III. Although concentrations could be detected to 5 μg/l and 15 μg/l in plasma and blood, respectively, the precision and accuracy deteriorated at these concentrations. Method parameters remained consistent to 8 μg/l in plasma and 20 μg/l in blood. The difference in the limit of sensitivity is due to the lower recovery of cyclosporine from blood samples.

TABLE III

EVALUATION OF DETECTION LIMIT OF CYCLOSPORINE IN PLASMA OR BLOOD

Coefficients of variation (%) in parentheses.

Cyclosporine concentration (μg/l)	Mean response factor (μg/l/mV)	
	Plasma	Blood
5	61.61 (32.8)	—
8	36 16 (15.5)	—
15	39.88 (15.9)	118.28 (16.1)
20	34.31 (0.2)	77.99 (8.6)
50	35.69 (0.6)	63.39 (2.2)
200	38.58 (3.8)	62.96 (6.1)

Stability

The pooled plasma samples initially analyzed as part of the accuracy study were stored at 1°C for 12 weeks. A quadruplicate analysis of these samples after 4 and 12 weeks resulted in the mean values and C.V. values shown in Table IV. No apparent loss in cyclosporine was noted for the 12-week period.

Application of the method

Approximately 1000 plasma and blood samples from twelve subjects dosed with cyclosporine have been analyzed by this method. The method was applied on a 24 h per day basis. During this period, no deterioration in method parameters (peak shape, retention time) was noted. Typical blood concentrations after a single 1400-mg dose of cyclosporine are shown in Fig. 3. In most

TABLE IV

ANALYSIS OF PLASMA SAMPLES CONTAINING CYCLOSPORINE AFTER STORAGE AT 1°C FOR 12 WEEKS

Theoretical concentration (μg/l)	Mean ± C.V. (%)	
	4 weeks	12 weeks
30	34 ± 4.2	26 ± 6.5
117	104 ± 7.7	110 ± 0.5
480	494 ± 12.8	497 ± 2.6
1220	1204 ± 3.9	1323 ± 0.9
2460	2223 ± 6.8	2651 ± 1.7

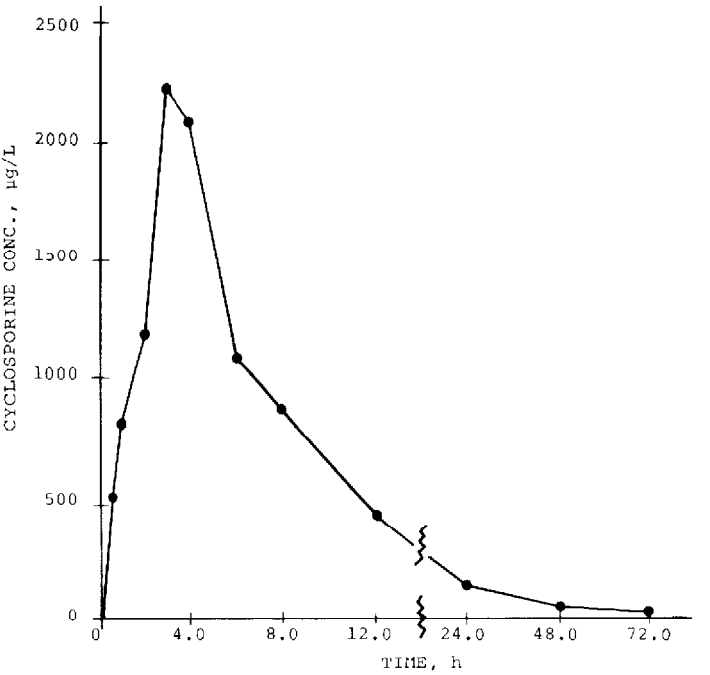

Fig. 3. Concentration of cyclosporine in blood as a function of time from a subject who received a single oral dose of 1400 mg.

cases, for doses down to 350 mg, plasma and blood concentrations above the detection limit of the method could be followed for at least three half-lives.

DISCUSSION

Although several HPLC methods are reported for the analysis of cyclosporine in plasma and blood, none has approached the level of automation that has been presented here. Following a simple manual protein precipitation of each plasma or blood sample, a fully automated analysis system completes sample preparation, chromatographic separation and data analysis at the rate of four samples per h. Moreover, the method could be applied continuously day and night at a rate of approximately 100 samples per day.

The use of acetonitrile as a protein precipitation medium is more efficient than methanol [21] and therefore, heating of the samples to obtain a clean supernatant as described by Nussbaumer et al. [14] was not necessary.

The use of a hexane wash for removing additional lipophilic material from the sample was introduced by Sawchuk and Cartier [13]. Incorporation of the hexane wash in the automated system provided a sample apparently free of most late eluting components. The remaining material was removed by increasing the mobile-phase flow-rate providing an adequate column clean-up without creating the longer analysis times associated with the step gradient [14] and gradient elution methods [11].

In general, use of an internal standard in liquid chromatography is not necessary. The simple sample preparation in this method allowed us to use external standardization simplifying the method and reducing the chromatography time without loss of linearity, precision or accuracy.

Based on the analysis of samples spiked with drug around the detection limit of the method, concentrations of 8 μg/l and 20 μg/l for plasma and blood, respectively, could be measured with confidence. Concentrations below this limit could be detected albeit with a loss in precision and accuracy.

Maintenance of the semi-automated system is limited to routine column care — which is performed daily and consists of replacement of the frit and the packing material at the top of each column — and weekly replacement of pump tubes on the Technicon Proportioning Pump. Total maintenance time is approximately 30 min daily for column care and 5 min weekly for pump tube replacement. The cost of the instrumentation used for this system was approximately $35,000 which does not include the HP-1000 computer system, but does include a gradient HPLC system (with a microprocessor), Technicon Proportioning Pump and Sampler IV which are found in many laboratories.

In conclusion, the method reported here employs (a) an automated sample wash; (b) column switching and (c) computer analysis providing greater sensitivity and greater sample through-put than previously reported. The reliability of the method, the sensitivity of the method and utility of the method have been adequately demonstrated with the routine analysis of approximately 1000 samples.

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