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NEW AUTOMATED HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF CYCLOSPORIN A AND G IN HUMAN SERUM

P.E. WALLEMACQ* and M. LESNE

Department of Pharmacology, Pharmacokinetics Unit, University of Louvain, Avenue E. Mounier 73/55, B-1200 Brussels (Belgium)

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SUMMARY

An automated isocratic high-performance liquid chromatographic (HPLC) method is described for the determination of cyclosporin A and G in human serum. This method involves the use of an automated solid-liquid extraction procedure following rapid protein precipitation with acetonitrile. The use of a disposable C_8 extraction cartridge allows a good recovery of cyclosporine (87%) from serum and a detection limit of 20 ng/ml with good reproducibility using 0.5 ml of sample. This method can also be adapted to whole blood measurements. The choice of a 3-μm cyano analytical column and of the mobile phase hexane–isopropanol (85:15) permitted a low column temperature (50°C), a low flow-rate (0.6 ml/min) and a short run time (14 min). This method allows the accurate and fast routine monitoring of cyclosporine by HPLC, which is particularly important in hepatic transplantations.

INTRODUCTION

Cyclosporine (Cy A) (Sandoz OL 27-400 N, previously named cyclosporin A) is a cyclic, hydrophobic and neutral peptide of fungal origin [1, 2]. It has been shown to have a specific immunosuppressive activity in man, inhibiting the production of lymphokines such as interleukine 2. The time-dependent inhibitory effect of cyclosporine affects the early cellular events occurring in immunocompetent lymphocytes in response to the mitogenic stimulus by arresting them in the resting (G_0) or early inductive (G_1) phases of the cell cycle [3].

This unique property has contributed to the improvement of the treatment of transplanted patients [4, 5]. However, this drug produces several adverse effects, in particular nephrotoxicity [6]. Therefore, the optimal use of this drug requires careful monitoring of its plasma, serum or blood concentration [7]. This monitoring appears to be difficult because several parameters, such as its bioavailability, may show dramatic variations depending, e.g., on the food taken before

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TABLE I

PUBLISHED METHODS FOR THE DETERMINATION OF CYCLOSPORIN A

Reference	Sample	Extraction★	Chromatographic conditions			Detection limit (ng/ml)	Run time (min)
			Mode	Column, temperature	Mobile phase		
Sawchuk and Cartier [11], 1981	Plasma blood	Liquid–liquid, very long	Isocratic	C_{18}, 5 μm, 75°C	Acetonitrile–water	25	10
Nussbaumer et al. [12], 1982	Plasma blood	Protein precipitation, short	Column switching	C_{18}, 5 μm, 70°C	6-Solvent switching scheme	20	26
Leyland-Jones et al. [13], 1982	Plasma	Solid phase, long	Isocratic	Zorbax TMS, 5 μm, 55°C	Acetonitrile–water	100	20
Yee et al. [14], 1982	Serum	Solid phase, long	Gradient	Ultrasphere ODS, 5 μm, 70°C	Acetonitrile–water–trifluoroacetic acid	50	16
Carruthers et al. [15], 1983	Plasma	Liquid–liquid, long	Isocratic	C_8, 5 μm, 72°C	Acetonitrile–water–methanol	31	30
Smith and Robinson [17], 1984	Plasma, blood	Protein precipitation, short	Column switching	C_8, 5 μm, 75°C	Acetonitrile–water	8–20	15
Kates and Latini [18], 1984	Serum, blood	Solid phase, very long	Isocratic	C_8, 10 μm, 70°C	Acetonitrile–water	25–50	20
Takada et al. [19], 1985	Plasma, blood	Liquid–liquid, very long	Isocratic	CN, 40°C	Acetonitrile–water	100	>15
Aravind et al. [20], 1985	Blood	Charcoal adsorption, very long	Isocratic	C_{18}, 5 μm, 75°C	Acetonitrile–water–methanol	50	8
Hamilton et al. [21], 1985	Plasma blood	Protein precipitation, long	Column switching	C_{18}, 3 μm, 60°C	Acetonitrile–water	2–10	15
Lensmeyer and Fields [22], 1985	Plasma, blood	Solid phase, long	Isocratic	CN, 5 μm, 58°C	Acetonitrile–water	10–15	10
Shihabi et al. [23], 1985	Blood	Solid phase, long	Isocratic	CN, 5 μm, 56°C	Acetonitrile–water	50	>20
Gmur et al. [24], 1985	Serum	Protein precipitation, long	Column switching	C_{18}, 5 μm, 70°C	Acetonitrile–water	20	>20
Garraffo and Lapalus [25], 1985	Plasma, blood	Liquid–liquid, long	Isocratic	C_{18}, 4 μm, 75°C	Acetonitrile–water–trifluoroacetic acid	25	10
Kabra et al. [26], 1985	Plasma, blood	Solid phase, short	Isocratic	CN, 50°C	Acetonitrile–phosphate buffer	10	15
Kahn et al. [27], 1986	Blood	Liquid–liquid, long	Isocratic	C_8, 3 μm, 73°C	Acetonitrile–water–methanol–ammonium sulphate	10	8

★Short, protein precipitation + injection; long, extraction + evaporation; very long, additional sample manipulation.

administration of the drug [8], the kind of graft undertaken and the age of the patient. The plasma therapeutic window at the trough level seems to be between 50 and 200 ng/ml by the radioimmunoassay (RIA) method. However, this range may also vary depending on the dose regimen adopted by the physician: the drug may be administered once a day, for instance after renal transplantation, or three times a day after cardiac transplantation, etc.

Cyclosporin G (Cy G) (Sandoz OG 37-325), recently developed by Sandoz (Basle, Switzerland) is a methyl derivative of cyclosporin A [9]. The greatest advantage of this derivative is the reduction of the nephrotoxicity observed on animal models. The immunosuppressive activity of Cy G seems to be about the same as that of Cy A. Sandoz are just beginning clinical trials of this new drug, which appears to be a potential successor to Cy A.

Up to now, two methods of cyclosporine determination have been described: RIA and high-performance liquid chromatography (HPLC). These assays can be performed on plasma or whole blood. Many laboratories tend to prefer whole blood determinations (mainly owing to the variation in the distribution of the drug in the serum, depending on the sample temperature and the hematocrit). However, the discussion is still open, and so far both methods of follow-up give the same results regarding the success of the treatment.

RIA [10] seems to be easier to perform in most laboratories, but this assay overestimates the cyclosporine concentrations in a non-linear manner because the antibodies cross-react with the metabolites. This situation may become particularly critical after hepatic transplantations where the amount of metabolites may increase significantly [7]. Two reasons can explain the continuing choice of the RIA method: (1) several investigators have correlated RIA cyclosporine concentrations with nephrotoxicity [3] and (2) the remaining immunosuppressive activity of the metabolites seems to be much lower but is not yet well known.

HPLC is selective and should be selected for pharmacokinetic studies and for monitoring of hepatic transplant patients in addition to RIA. Many difficulties have been reported, such as time-consuming extraction procedures, poor extraction efficiency, unsatisfactory sensitivity, rapid deterioration of the costly reversed-phase analytical columns accentuated by the high column temperature (75°C), complicated gradient procedures or column-switching techniques, all of which justify the number of publications that have appeared [11–27]. Table I illustrates the evolution of HPLC methods from 1981 to 1986. Many of the methods described so far may be difficult to apply to routine monitoring.

The purpose of this work was to improve the HPLC conditions in order to develop a routine, less expensive and semi-automated method for the determination of cyclosporin A and G. An automated HPLC sample preparation/injection system (Varian AASP) was used to reduce sample handling and tedious manual operations. The method described here can easily be applied to the dosage of cyclosporin G without changing any chromatographic conditions. It can be also used for whole blood measurements. This method combines normal-phase chromatography with the standard reversed-phase extraction after a rapid protein precipitation, without the problems of high temperatures and late-eluting peaks.

EXPERIMENTAL

Reagents

Acetonitrile, hexane and isopropanol, all of HPLC grade or Spectrograde, were purchased from Alltech (Arlington Heights, IL, U.S.A.). Millipore-treated water was used. Cyclosporin A (OL 27-400 N) and G (OG 37-325) were kindly supplied by Sandoz. The stock solutions of cyclosporin A and G contained 500 μg of pure powder per ml of HPLC-grade acetonitrile. Serum from a blood bank was used to prepare the standards.

HPLC equipment

The liquid chromatograph consists of an isocratic pump (Varian Model 2010) and a variable-wavelength UV detector (Varian Model 2050), which is connected to an integrator (Varian Model 4290). The analytical HPLC column is a Rosil cyano column (15 cm $\times$ 4.6 mm I.D.; particle size 3μm) from Alltech, protected by a 5-cm Alltech directly connected guard column dry-packed with CN pellicular material (30–40 μm) (Alltech). A 0.5 μm pore size directly connected pre-filter (Alltech) is placed before the guard column. A silica saturing column (15 cm $\times$ 4.6 mm I.D.) is connected between the pump and the injection module. The analytical column and pre-column are heated by a column heater (Varian).

Automated extraction–injection equipment

The new Varian AASP (advanced automated sample processor), an automated HPLC sample preparation– injection system and a Vac-Elut were used.

The AASP integrates HPLC sample preparation and injection into a single automated module. It automates the sorbent extraction method of chemical isolation and is rapidly replacing classical liquid–liquid extraction as the preferred technique for sample preparation. Further, it injects the isolated sample into the liquid chromatograph, eliminating the need for an additional injection valve or autosampler. The entire process is carried out on-line and automatically. There is a minimum of sample handling. At the heart of the AASP system, specially designed sorbent cassettes (Analytichem) selectively isolate and concentrate the compounds of interest from the original sample. Each cassette has ten cartridges containing up to twelve different bonded silica phases. The cartridges, in effect, become equivalent to the sample loop in the injection valve. The analyte is swept into the HPLC column.

In addition, a built-up pump delivers solvent (which can be different from the mobile phase) to the cassette, allowing many other applications (e.g., on-line derivatization and cartridge "rinsing"). Fig. 1 shows the hydraulics of the AASP, including the purge pump.

Among a choice of twelve different bonded phase AASP cassettes we selected the C_8 cassette, which gave the best results after preliminary assays. We also tried the C_{18}, C_8, C_2, 2-OH, CN and Si AASP cassettes. Studies of recovery and selectivity were made with 500 μl of serum containing tritiated Cy A and 20% of acetonitrile. After conditioning of the cassettes, the best results were obtained with

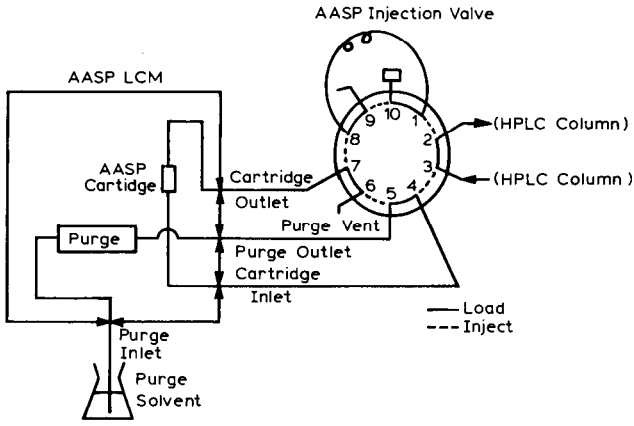

Fig. 1. Fluid system connections and hydraulics of AASP, including the purge pump.

the C_8 cassette, giving for the full extraction procedure (acetonitrile precipitation, retention on the cartridge, appropriate wash and elution with the mobile phase) a recovery of about 87% compared with 73, 70, 64, 64 and 20% for the C_{18}, C_2, 2-OH, CN and Si phases, respectively.

Sample preparation

Standards were prepared in glass tubes with 500-μl serum samples obtained from a blood bank, containing 500, 250, 125, 62.5, 31.3 and 15.6 ng/ml Cy A and 250 ng/ml Cy G as internal standard (cyclosporine D could also be used as an internal standard, as illustrated in Fig. 2).

Blood samples were drawn into dry tubes from patients who had received Cy A orally or intravenously. The samples must remain for 2 h at room temperature before centrifugation in order to stabilize exchanges between blood cells and plasma. After separation of the serum, a sample of 500 μl of serum was used. Both standards and patients' samples were treated identically using glass tubes, by adding to the 500 μl of serum 600 μl of acetonitrile [21, 26] (containing the internal standard in order to obtain a final concentration of 250 ng/ml in serum) and 2 ml of hexane. All tubes were vortexed for 45 s, then centrifuged for 4 min at 1000 *g*. The upper hexane layer was discarded, and the acetonitrile layer containing the cyclosporine was diluted with 0.8 ml of water in the AASP cartridge reservoir in order for the final solution of serum extract to pass through the cartridge. Before the sample extraction, the AASP cassette should be conditioned by passing through it 1.6 ml of the mobile phase to remove any interfering chemicals from the cartridge.

After all the serum extract has passed through the cartridge, it was washed twice with 1.6 ml of a solution of 33% acetonitrile in water, followed by 1 ml of hexane. The cartridges were then dried by drawing air through them for 2 min.

Chromatographic conditions

Hexane–isopropanol (85:15, v/v) was selected as the mobile phase at a flow-rate of 0.6 ml/min. The column effluent was monitored at 212 nm. The UV detec-

tor sensitivity range was set at 0.02 a.u.f.s., the integrator chart speed at 0.25 cm/min and its attenuation range at 8. The analytical column should be heated progressively to 50°C at 1°C/min. It was found necessary to add a 0.5-μm pre-filter before the guard column in order to prevent rapid plugging of the guard column due to the multiple injections from the cartridges. The filter should be changed about once a month (depending on the use of the system) and the guard column every three to four months, ensuring a low back-pressure. To achieve better long-term column stability, a silica saturating column was used [22].

RESULTS

Chromatograms

No important interfering peaks were detected in the range of retention times studied in the serum used for standards or in the transplant patients' serum. The retention times of cyclosporin A and of the internal standard (Cy G) were ca. 11.7 and 9.8 min, respectively. Typical chromatograms obtained from 0.5 ml of blank serum and 0.5 ml of serum containing 125 ng/ml Cy A and 250 ng/ml Cy G are shown in Fig. 2. Fig. 3 shows chromatograms from patients' whole blood and serum extracts.

Linearity and reproducibility

Both peak-height and peak-area ratios (Cy A/Cy G) were studied. A good linear relationship was obtained with increasing cyclosporin A concentrations in the serum. Both methods of calculation could be used. The linearity tested between 31.3 and 250 ng/ml of Cy A is characterized by a correlation coefficient (r) of 0.999 for both methods. The linear regression performed on the line relating peak-height ratio to Cy A concentration corresponds to the equation $y=0.003x+0.018$ where the y-intercept is not significantly different from the origin, as shown in Fig. 4. The reproducibility was evaluated after extraction of several serum samples with pre-determined concentrations of Cy A (between assay). As shown in Table II, between 31.3 and 500 ng/ml Cy A the coefficient of variation ranged from 12.5 to 3.8% ($n=5$).

Precision and sensitivity

Table II shows good precision for the observed concentrations of Cy A; the relative error ranges from 0.03 to 4.0%. The sensitivity of the method was investigated by analysing serum samples spiked with 20 ng/ml Cy A. At this low concentration the signal-to-noise ratio was 2:1 and the coefficient of variation increased significantly.

DISCUSSION

The principle of the cartridge on-line injection allowed the total amount of cyclosporine retained on the bonded phase to be injected, but unfortunately owing to the inherent increase in the dead volume the peaks became wider. As initially the width of the cyclosporine peaks had been the reason for heating column up

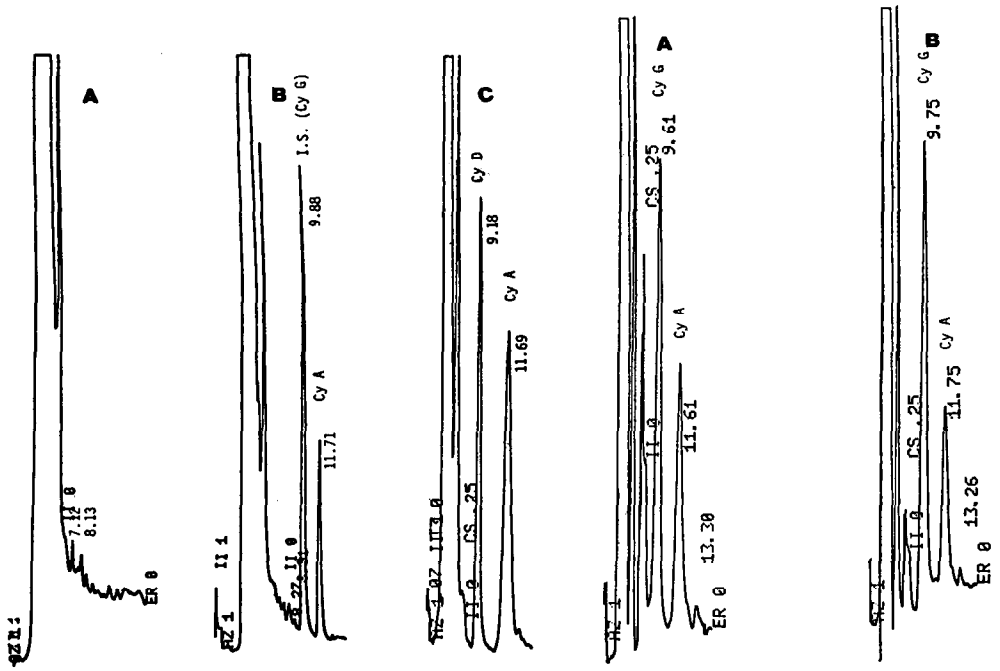

Fig. 2. Chromatograms of serum samples (500 μl). (A) Blank serum; (B) serum standard containing 125 ng/ml Cy A and 250 ng/ml Cy G (I.S., internal standard); (C) serum containing 250 ng/ml Cy D and 200 ng/ml Cy A.

Fig. 3. Chromatograms of whole blood and serum samples (500 μl) from patients treated with cyclosporine. (A) Whole blood sample containing 182 ng/ml Cy A; (B) serum sample containing 118 ng/ml Cy A.

to 75°C (Table I), this new difficulty had to be overcome. The choice of a short cyanopropyl column (3 μm) with such a mobile phase could correct this phenomenon, giving higher peaks with better shapes, and even avoiding heating the column to 75°C, which frequently caused rapid deterioration of the column.

The choice of serum instead of whole blood as a biological matrix for the development of this HPLC assay was based on its general acceptance by our clinical staff dealing with organ transplants. Greater interest in serum has also been stressed by Kahan [3]. However, preliminary assays on whole blood using the same procedure were performed with success (Fig. 3).

Most chromatographic methods described so far have used acetonitrile–water as the mobile phase. However, several interferences or late-eluting peaks have been reported [26] with acetonitrile in the mobile phase, probably owing to the presence of a plasticizer. The use of hexane–isopropanol as the mobile phase did not cause such problems. It may be necessary to increase the percentage of hexane in the mobile phase to 87% as the analytical column ages.

With the above chromatographic conditions, reversed-phase conditions no longer apply. Therefore, the order of the peaks is inverted and the internal standard is eluted first. For the determination of cyclosporin G, one can use Cy A as the internal standard without any change in the method.

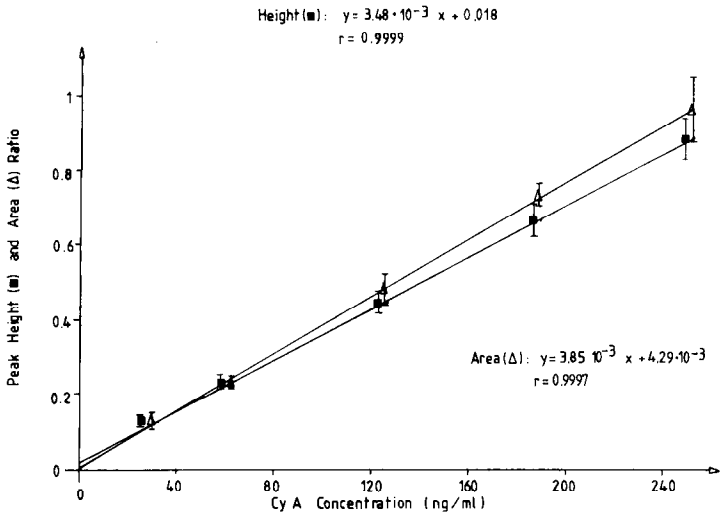

Fig. 4. Calibration graphs for cyclosporine (mean ± standard deviation; $n = 5$) based on peak-height (■) and peak-area (△) calculations.

It was demonstrated that the Cy A peak height decreased significantly when the flow-rate increased [28], so a flow-rate higher than 1.0 ml/min should be avoided if possible. The selection of a suitable UV absorption wavelength was also important; it had to be taken into account that the maximum UV absorption wavelength of Cy A in acetonitrile was 202 nm and in the hexane–isopropanol mobile phase 208 nm. However, the mobile phase may absorb in this range. One should avoid UV monitoring below 210 nm owing to the solvent absorption and to numerous interferences. On the other hand, above 215 nm there is a decrease in absorption of Cy A.

Conditioning the cassettes is important in order to remove several interfering peaks. The use of acetonitrile as a protein precipitation medium is more efficient than other solvents such as methanol [17], but it is necessary to use glass tubes and to avoid contact with most plastic surfaces because of the appearance of

TABLE II

BETWEEN-ASSAY COEFFICIENT OF VARIATION (C.V.), PRECISION AND SENSITIVITY OF THE ASSAY OF CYCLOSPORIN A BASED ON PEAK-AREA CALCULATIONS ($n = 5$)

Theoretical concentration (ng/ml)	Observed concentration (ng/ml)	Relative error (%)	Mean peak-erea ratio	σ (ng/ml)	Coefficient of variation (%)
500	499.4	0.12	1.92	0.15	7.8
250	248.8	0.48	0.96	0.08	8.2
188	191.3	1.75	0.74	0.03	3.8
125	125.1	0.03	0.48	0.04	8.6
62.5	60.0	4.0	0.23	0.02	7.2
31.3	31.6	0.95	0.13	0.02	12.5

interfering peaks with this solvent. Recycling of these cassettes several times is possible by washing them with isopropanol, apparently without any significant decrease in efficiency, but new calibrations may be necessary. This recycling, of course, reduces the cost of the analyses, which reaches a particularly competitive level.

This HPLC method is currently being used with RIA for the monitoring of Cy A after hepatic transplantation in the St. Luc University Hospital. Studies are carried out in order to assess the correlation between the two methods and parameters such as bioavailability of the Cy A within the population of patients. The automated HPLC method described here can be used for the assay of Cy A in most biological fluids (serum, whole blood, urine, bile, CRF, etc.). With the use of the AASP Vac-Elut for the extraction and wash, designed to accept cassettes of ten cartridges, we are able to process simultaneously ten serum samples in 20–25 min. The main advantage of this method for routine analysis is its reliable automation associated with good chromatographic performance. This automation allows us, for instance, to analyse during a day time 20–30 blood samples for clinical purposes, and during the evening or night other samples for research purposes. The reliability of the method has been demonstrated with the routine analysis of approximately 1000 samples.

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