Journal of Chromatography, 385 (1987) 357–361
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 991

Note

Simple and specific isocratic liquid chromatographic procedure for cyclosporine A determination in whole blood, compared with radioimmunoassay

JOSEF KLÍMA*, RICHARD PETRÁSEK and VLADIMÍR KOČANDRLE
Institute for Clinical and Experimental Medicine, Videňská 800, 140 00 Prague 4 - Krč (Czechoslovakia)

Although cyclosporine A (CA) is widely used in organ transplants, not enough is known about its absorption, pharmacokinetics and drug interactions. Serum and plasma have been employed in most previous pharmacokinetic studies and pharmacologic monitoring of CA. However, the plasma and serum concentrations are an inconstant measure of CA in the blood, because redistribution of the drug between erythrocytes and plasma or serum occurs *in vitro* during storage and processing of blood specimens. The concentration in plasma, obtained after incubation of blood at 20°C, was only 76% of the value found for blood incubated at 37°C[1,2].

The aim of our study was to monitor CA during a three-month period of treatment and to determine whether the whole-blood levels, estimated by high-performance liquid chromatography (HPLC) and radioimmunoassay (RIA), provide a relevant indicator of the toxic effects of CA and of interactions of erythromycin and ketoconazole with CA metabolism.

EXPERIMENTAL

Patients

Four heart-transplant patients were studied. They were treated with CA for between six weeks and three months. Perioperatively, the drug was administered by continuous infusion at a rate of 6 mg/kg per 24 h. After 48 h, CA was given orally, twice daily in a total dose ranging from 75 to 650 mg. The CA solution was taken before the morning meal (fasting state) and after the evening meal. The total daily dose was reduced over the course of three months, according to the CA blood levels and the helper/suppressor cell ratios in the peripheral blood. In addition to CA, all patients received prednisone (70 mg/day, decreased stepwise to 20 mg/day). Erythromycin and ketoconazole were used to prevent infections. The daily dose of erythromycin was 2 g/day and of ketoconazole, 200 mg/day. The administration of diuretic and antiarrythmic drugs was dependent on the clinical status of the patients.

Determination of cyclosporine

Blood samples for CA assay were drawn into heparinized tubes before the morning dose of CA and stored at 4°C. The CA levels in whole blood were measured once or twice a week, depending on the patients' clinical status, using RIA and

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HPLC. The blood samples for the two types of determination were collected at the same time. Conventional RIA for whole blood was carried out according to the manufacturer's instructions using the kit supplied by Sandoz (Basle, Switzerland). The simple, accurate and specific isocratic HPLC procedure developed by Kabra *et al.*[3] was adapted for use with Czechoslovak extraction and analytical columns. The CA was extracted from whole blood by the C_{18} solid-phase extraction procedure described[3], and an aliquot of the eluate was analysed on a glass column (15 cm × 3.3 mm) packed with Separon SGX CN (Laboratory Instruments Work, Prague, Czechoslovakia). A SP 8100 XR liquid chromatograph, equipped with a column oven, a Model SP 8440 XR variable-wavelength detector and a Data System SP 4200 (all from Spectra-Physics, San Jose, CA, U.S.A.), was employed. The column was mounted in an oven. The samples were injected into a Valco valve with a 100-μl sample loop, mounted on the chromatograph.

Acetonitrile and tetrahydrofuran, both of HPLC grade, were obtained from Fluka (Ulm, F.R.G.). Dimethyl sulphoxide (for gas chromatography) and hexane (for gas chromatography) were obtained from Merck (Darmstadt, F.R.G.). Absolute ethanol (190 proof) was obtained from Lachema (Brno, Czechoslovakia).

Vac Elut™ vacuum chamber and Bond-Elut™ C_{18}, 100 mg/1 ml, extraction columns were obtained from Analytichem International (Harbour City, CA, U.S.A.).

Water was redistilled in glass and filtered through 0.45-μm poly(tetrafluoroethylene) membrane filters (Sartorius, Göttingen, F.R.G.).

For the chromatographic separation, a mixture of 430 ml acetonitrile and 570

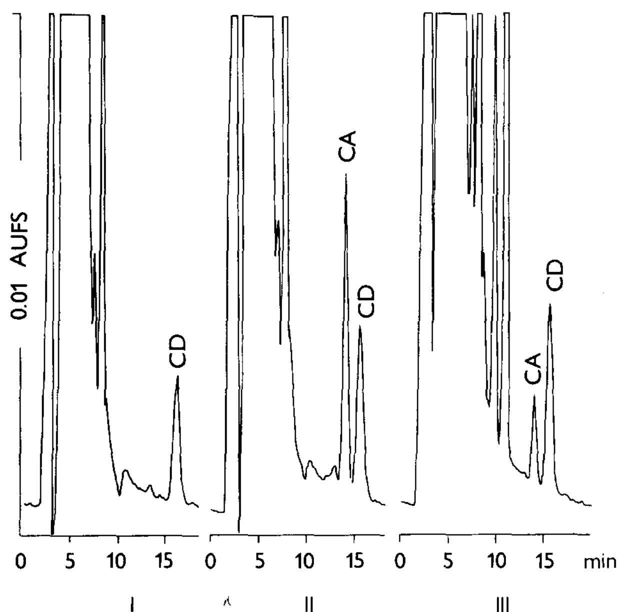

Fig. 1. Chromatograms of cyclosporine A-free whole blood containing 250 μg/l of cyclosporine D (CD) as internal standard (I), of 500 μg cyclosporine A (CA) per litre whole blood as calibration standard (II) and of a patient's blood containing 120 μg/l of cyclosporine A (III).

ml of 10 m*M* phosphate buffer (pH 7.0) was used. The mobile-phase flow-rate was 0.5 ml/min. The Czechoslovak CN-glass columns required only half as much acetonitrile as in the original method. Cyclosporine D (CD), 250 μg per 1 of acetonitrile–dimethyl sulphoxide (96:4, v/v) was used as internal standard. For calibration, standards of 250 and 500 μg/l of CA in whole blood were used, both for HPLC and RIA.

The wavelength for analysis was set at 210 nm and the sensitivity at 0.01 a.u.f.s.; the chart speed was 0.25 cm/min. The separation on the cyanoethyl column was achieved at a temperature of 50°C. This inexpensive column was replaced after every 100–150 analyses. The quantitative analysis of CA was based on the peak-area ratio of CA to CD.

RESULTS

The sensitivity of the HPLC method was better than 15 μg/l, and the recovery of CA, added to whole blood, ranged from 95 to 108%. The calibration curve was linear from 62.5 to 2000 μg/l; $y = 430.1\ x - 473.6$; correlation coefficient 0.9996. The intra-assay precision ranged from 6.6 to 6.9%. Chromatograms of cyclosporine-free whole blood, of a 500 μg/l CA calibration standard and of a patient's blood containing 120 μg/l CA are shown in Fig. 1. We also tested PRESEP C_{18} extraction cartridges from Laboratory Instruments Work. The recovery did not differ from that stated.

Blood levels of CA were measured by HPLC and RIA in 66 specimens during a 3-months following-up period. The means ± standard deviations (S.D.) of four patients after heart transplant, are shown in Table I, for the period without application of erythromycin and ketoconazole and for the period of therapy.

The ratios of the CA concentrations, estimated by RIA and by HPLC, were calculated. While the HPLC method selectively detects the parent CA compounds, the RIA kit also measures several cross-reacting metabolites, due to the low specificity of the polyclonal antibody reagent. This ratio gives an idea of the proportions of the parent CA and its metabolites[4].In all cases described above, the statistical analysis consisted of a paired *t*-test. In all clinical situations, there were statistically significant differences between the concentrations estimated by HPLC and RIA.

The influence of erythromycin and ketoconazole was tested in three patients which were treated with these drugs. For the statistical analysis a non-pairing *t*-test was used. Fifty–four CA blood levels measured during a three-month follow up period were divided into two groups. The first group consisted of CA blood levels in patients without erythromycin and ketoconazole therapy and second group in those given therapeutic doses of both drugs. Both HPLC and RIA were used for the determination of CA and its metabolites. The RIA/HPLC ratios were calculated in order to analyse changes in CA metabolism. The results are shown in Table I.

The mean CA concentrations were significantly lower in the patient group without erythromycin and ketoconazole therapy, and the RIA/HPLC were higher. Erythromycin and ketoconazole therapy increased the mean CA concentration estimated by the HPLC and RIA methods, and the RIA/HPLC ratio decreased. This fact demonstrates that erythromycin and ketoconazole inhibit the biotransformation of CA.

TABLE I

CONCENTRATIONS (μg/l) OF CYCLOSPORINE A IN WHOLE BLOOD IN HEART-TRANSPLANT PATIENTS DURING A 3-MONTH FOLLOW-UP PERIOD

Statistical analyses: I, paired t-test; II, non-paired t-test.

	Period without therapy			*Period with therapy*			*3-Month period*		
	HPLC	*RIA*	*RIA/HPLC*	*HPLC*	*RIA*	*RIA/HPLC*	*HPLC*	*RIA*	*RIA/HPLC*
(I) Four patients, one without therapy and three with erythromycin and ketoconazole therapy									
No. of blood samples	54	54	54	12	12	12	66	66	66
Mean concentration	163.5	619.1*	4.9	812.9	1637.5*	3.4	281.6	804.2*	4.8
± S.D.	±108.6	±191.0	±2.6	±864.5	±940.0	±2.0	±447.0	±579.6	±2.7
Min. concentration	45	355	1.5	125	600	1.1	45	355	1.1
Max. concentration	455	1200	11.5	2685	3200	7.5	2685	3200	11.6
(II) Three patients with erythromycin and ketoconazole therapy									
No. of blood samples	42	42	42	12	12	12			
Mean concentration	139.1	566.1	5.5	812.9**	1673.5**	3.3**			
± S.D.	± 98.2	±155.3	±2.7	±864.5	±940.0	±2.0			
Min. concentration	50	355	1.6	125	600	1.1			
Max. concentration	455	1200	11.5	2685	3200	7.7			

* $p < 0.001$.
** $p < 0.00001$.

Normal blood concentrations of CA should lie between 50 and 455 μg/l as determined by HPLC and between 355 and 1200 μg/l as determined by RIA. All patients had normal concentrations of bilirubine during the 3-month follow-up period.

DISCUSSION

Our HPLC method estimated CA concentrations in whole blood and therefore the concentrations of CA were not altered by temperature-dependent redistribution between erythrocytes and plasma. The RIA method also used whole blood samples. The analytical conditions for HPLC and RIA were comparable. The results of this study confirm that the RIA method overestimates the concentration of CA. Variations in the CA blood concentrations between individual patients were observed. The difference between the CA concentrations estimated by HPLC and by RIA are connected with the existence of a cross-reacting antibody against CA and several metabolites. This ratio is altered by drug interaction. The RIA/HPLC ratios showed that erythromycin and ketoconazole interact with the transport and biotransformation system in the liver.

After oral administration, CA is absorbed via the lacteals of the small intestine and enters the lymphocyte-rich environment of the thoracic duct. From there it passes to the vascular tree, wherein greater than 90% is bound to the surface of lipoprotein particles. CA is extracted from the blood by the liver, metabolized at least in part by the hepatic cytochrome P 450 mixed oxidase system into 17 compounds by methylation, mono- and dihydroxylation and the secreted via bile into the small intestine. There it undergoes re-absorption and enterohepatic recycling[5].

Clinically, this implies that in most cases the CA dose can be reduced and the dosage intervals lengthened during application of drugs that interact with the biotransformation of CA in the liver. Since the biotransformation parameters differ widely per individual, changes in dosage should be guided by the whole blood levels. The comparison of CA whole blood levels estimated using HPLC and RIA makes it possible to analyse the individual biotransformation of CA to its metabolites.

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