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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY FOR CYCLOSPORIN MEASUREMENT: COMPARISON WITH RADIOIMMUNOASSAY

MARIO PLEBANI, MAURIZIO MASIERO, CARLO D. PALEARI, LAURA SCIACOVELLI, DIEGO FAGGIAN and ANGELO BURLINA*

Cattedra di Chimica e Microscopia Clinica, Laboratorio Centrale di Analisi, Università di Padova, Via Giustiniani 2, 35128 Padova (Italy)

SUMMARY

The large inter-patient variability in cyclosporin pharmacokinetics coupled with the agent's narrow therapeutic index with adverse effects resulting from supra-therapeutic levels, necessitates individualization of drug dosage and therapeutic monitoring of cyclosporin blood levels. The performance of a liquid chromatographic method for the measurement of cyclosporin was evaluated and the results obtained by this method and by a specific radioimmunoassay were correlated. The method described is sensitive, selective, reproducible and easier to perform than other chromatographic methods. It is suitable for the daily measurement of cyclosporin in batches of up to 40 samples and the results correlate well with another chromatographic method and with the specific radioimmunoassay.

INTRODUCTION

Cyclosporin A (CsA) is widely used as an effective and potent immunosuppressant in organ transplantation[1,2] and treatment with CsA has greatly improved the results of kidney, heart, bone marrow and liver transplantation. However, the narrow therapeutic index and the wide variability in its pharmacokinetics necessitate individualized dosage adjustment[3]. Well known adverse effects of drug levels above that range include nephrotoxicity, neurotoxicity, hypertension, gingival hyperplasia, anorexia, nausea and ileus[4]. On the other hand, levels below the appropriate range are associated with an enhanced risk of graft rejection or graft *versus* host disease in bone marrow transplants. For these reasons, careful attention to the CsA concentration in blood is essential for optimization of therapy.

Several techniques [radioimmunoassay (RIA) with polyclonal and monoclonal antibodies, high-performance liquid chromatography (HPLC) and fluorescence polarized immunoassay (FPIA)] for therapeutic monitoring of CsA have been developed[5]. RIA with polyclonal antiserum and FPIA measure CsA together with some cross-reactive metabolites that appear in blood after the drug is administered. In contrast, HPLC and the new RIAs with monoclonal antibodies specifically measure the parent drug, independent of its metabolites. Specific measurement of CsA seems to

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be recommended, as suggested by recent papers[6]. The aim of this work was to evaluate the performance of isocratic HPLC for the measurement of CsA and to compare the results with those obtained by a specific radioimmunoassay.

EXPERIMENTAL

Samples

Whole-blood samples were taken from 80 renal and 60 heart transplant patients who had received immunotherapy with CsA. CsA (5–20 mg/kg body weight) was given orally, once or twice a day, and all blood specimens were collected before the next dose. Samples were collected in tubes containing EDTA-K3 (Merck-Bracco, Milan, Italy) as an anticoagulant and stored at −20°C until analysed. All samples were analysed by three different methods for CsA measurement (specific RIA, reference HPLC and described HPLC).

Chemical and reagents

Cyclosporin A (CsA) and the internal standard, cyclosporin D (CsD), were obtained from Sandoz (Basle, Switzerland). Acetonitrile, hexane, methanol and water (HPLC grade) were purchased from Merck-Bracco.

Instrumentation and chromatographic conditions

A Bio-Rad HPLC instrument was employed (Bio-Rad Labs., Segrate, Italy), including a Model 1330 HPLC dual piston pump, Model 1306 variable-wavelength UV detector, Model AS-48 autosampler and Model 3392 A integrator. The column used was a reversed-phase C_8 mini-column (high-performance RP mini-column, 30 × 4.6 mm I.D., particle size 5 μm, from Bio-Rad Labs.), maintained at 70°C with a column heater (Bio-Rad Labs.). The flow-rate of the mobile phase (20 m*M* ammonium phosphate buffer in 56% aqueous acetonitrile, pH 6.2) was 1.2 ml/min. The effluent from the column was monitored at 210 nm.

Preparation of extraction columns

The extraction of CsA involved the use of 1.0-ml disposable cyano extraction columns (40-μm mean particle size) (Analytichem International, Harbor City, CA, U.S.A.). These columns were prepared by washing with 3 ml of 15% acetonitrile under vacuum. A Vac-Elut SPS 24 vacuum chamber, designed to accept 24 extraction column simultaneously, was purchased from Analytichem International.

Procedure

We employed the method of Sivorinowsky *et al.*[7,8] for the HPLC measurement of CsA, with the following modifications. A 1.0-ml volume of of water and 2.0 ml of working internal standard solution (CsD in a 30% aqueous acetonitrile solution) are pipetted into 1.0 ml of whole-blood samples, controls and standards, followed by vortex mixing for 30 s and centrifugation for 10 min at 1000 *g* at 0°C. A 3.0-ml volume of blood supernatant is applied to the columns, which are drained under vacuum. Excess blood is washed off the column with 15% acetonitrile (three 1.0-ml volumes) and each column is rinsed with 4.0 ml of 50% acetonitrile to remove hydrophobic contaminants. The cyclosporins are eluted with 450 μl of ethanol into small

borosilicate test-tubes. The eluate is diluted with 200 μl of 10^{-3} *M* phosphoric acid and washed twice with 600 μl of hexane. After centrifugation (1 min at 500 *g*), the hexane is removed by aspiration and 100 μl of the eluate are injected into an isocratic HPLC system consisting of a reversed-phase C_8 minicolumn and the buffered acetonitrile mobile phase (pH 6.2) at 70°C. The quantitation is based on comparison of the CsA/CsD (internal standard) peak-height ratio in the unknown sample to the ratio in the whole-blood standard.

Interferences

We evaluated potential interferences in this analysis by chromatographing pure drug solutions and samples of whole blood from patients who had ingested various drugs.The drugs tested under these conditions were acetaminophen, aminotriptyline, caffeine, chloramphenicol, chlordiazepoxide, diazepam, ethosuximide, gentamicin, imipramine, pentobarbital, phenobarbital, phenytoin, primidone, salicylate, secobarbital, theophylline and cortisone analogues (prednisone, prednisolone and methylprednisolone). We also evaluated the interference of metabolite 17 of CsA (generously supplied by Dr. Maurer, Sandoz).

Recovery

CsA was added to a drug-free whole-blood pool in amounts equivalent to 100–1500 μg/l, and the analytical recovery was calculated.

Comparison methods

HPLC according to Carruthers *et al.*[9]. This method employs extraction with diethyl ether and chromatography on a 25 cm × 4.8 mm I.D. Ultrasphere-Octyl (5 μm) column (Beckman Analytical, Milan, Italy) maintained at 72°C.

RIA with monoclonal antibodies. We utilized a radioimmunoassay with a mice monoclonal antibody that did not react appreciably with the metabolites of CsA. The method utilizes an iodinated tracer and a double antibody for separating bound and free fractions (Cyclo-Trac sp, Incstar Corp., Stillwater, MN, U.S.A.).

Statistics

The comparison between methods was made by regression analysis with an *F*-test on variance; the difference in slope from unity was assessed by Student's *t*-test.

RESULTS

As shown in Fig. 1, the retention times of CsA and of the internal standard CsD are 6.0 and 8.5 min, respectively. No interfering peaks eluting at times that would interfere with the analysis were detected in samples from patients who had received CsA together with commonly used drugs. The precision of the method is shown in Table I. The results of the analytical recovery test carried out on samples spiked with CsA are illustrated in Table II. The lower limit of sensitivity (signal equal to twice the baseline noise) of our procedure is 25 μg/l.

In order to simplify the original method, we tried to eliminate the double extraction with hexane by introducing directly 1.0 ml of hexane (twice) into the extraction column. The results obtained were not significantly different from those

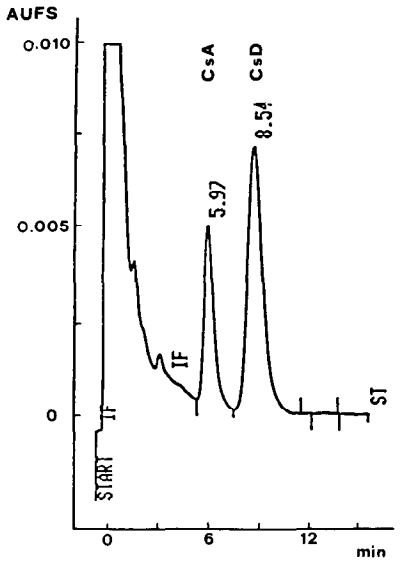

Fig. 1. Chromatogram of extracted whole-blood sample. CsA concentration, 460 μg/l.

TABLE I

REPRODUCIBILITY STUDIES

Parameter	$\bar{x}$ *(μg/l)*	*S.D. (μg/l)*	*Coefficient of variation (%)*
Within-run	150	3.5	2.3
precision	470	11.2	2.4
(n = 21)	805	21.7	2.7
Between-run	145	8.6	5.9
precision	468	23.8	5.1
(n = 11)	810	39.0	4.8

TABLE II

ANALYTICAL RECOVERY

Calculated value (μg/l)	*Observed value*[a] *(μg/l)*	*Recovery (%)*[b]
100.0	104.0 ± 4.0	104.0
200.0	205.0 ± 4.9	102.5
500.0	506.0 ± 13.4	101.0
1000.0	984.0 ± 26.4	98.4

[a] Mean of three determinations ± standard deviation.
[b] Mean value.

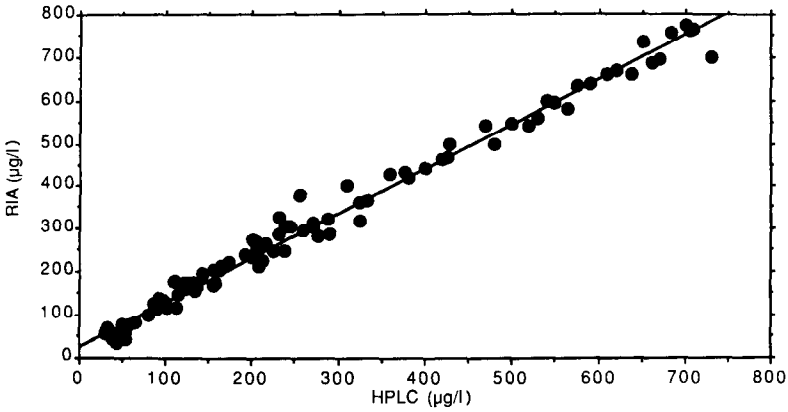

Fig. 2. Results of simultaneous analysis of whole-blood samples by HPLC and specific RIA [$n = 137$; $r = 0.974$ ($p < 0.0001$); $y = 1.037x + 27.17$ µg/l; $S_{y \cdot x} = 27$; the slope was not different from 1 ($p = 0.11$; n.s.)].

given by the original method ($p > 0.05$). No interference from metabolite 17 and the drugs tested was observed. A high correlation between the results obtained by our method (y) and that of Carruthers *et al.*[9] (x) was observed [$n = 135$, $r = 0.989$, $p < 0.0001$; $y = 0.920x + 10.2$ µg/l; $S_{y \cdot x} = 22$; the slope was not different from 1 ($p = 0.34$; n.s.)]. Moreover, a good correlation was found between our HPLC method and specific RIA with monoclonal antibodies (Fig. 2).

DISCUSSION

Numerous HPLC methods have been described for the measurement of CsA[10,11]. Despite their specificity, most HPLC determinations of CsA have definite disadvantages in comparison with RIA. In particular, long analysis times and difficulties in processing a large number of samples have been reported. Some procedures require time-consuming multi-step extractions; in some the extraction efficiency is poor; others have unsatisfactory detection limits. For these reasons, most laboratories prefere to monitor CsA by RIA.

The chromatographic method evaluated here is suitable for the daily measurement of CsA in batches up to 40 samples of whole blood. Whole blood was chosen because of the known problem of temperature-dependent separation[12,13] and because of recent recommendations of the National Academy of Clinical Biochemistry/American Association for Clinical Chemistry Task Force on Cyclosporin Monitoring[6]. The extraction is rapid and efficient, and chromatography is completed in 10 min per sample. With the use of a Vac-Elut chamber, designed to accept 24 extraction columns simultaneously, we are able to process 24 samples of whole blood in less than 30 min. The reproducibility is satisfactory, and 25 µg/l is an adequate sensitivity for therapeutic drug monitoring.

The principal advantage of our HPLC method is the rapid and simple extraction procedure compared with other published extraction procedures[10,11]. Moreover, the use of a mini-column is cost effective. The agreement observed between the results

obtained by our HPLC method and specific RIA demonstrates the accuracy of our method.

Although specific measurement of CsA is now recommended as a guide to therapy, some evidence exists that CsA metabolites are immunosuppressive[14] and even toxic[15]. Furthermore, differences exist in the metabolic handling of CsA in different groups of patients. The combination of specific and non-specific measurement of CsA provides a method for investigating the influence of metabolism on immunosuppressive therapy and its adverse effects[16]. Better information is derived from the concomitant identification and determination of CsA and its individual metabolites by HPLC. Recently, Lensmeyer *et al.*[17] described a chromatographic method for concomitant profiling of CsA and its metabolites in one assay. Preliminary results obtained with this selective measurement of CsA metabolites seems to prove the importance of such a determination for describing the interrelationship of CsA and its metabolites in therapy and toxicity[18]. We hope that new HPLC methods for concomitant measurement of CsA and its metabolites will soon be introduced into clinical practice.

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