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# LIQUID CHROMATOGRAPHIC DETERMINATION OF CYCLOSPORIN A IN SERUM WITH USE OF A SOLID-PHASE EXTRACTION

# COMPARISON BETWEEN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND RADIOIMMUNOASSAY LEVELS IN CLINICAL INVESTIGATIONS

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## SUMMARY

A simple reliable liquid chromatographic method for assay of cyclosporin A in serum or urine is described. Samples were cleaned up on a solid-phase extraction system (cyanopropyl column). The system involved a reversed-phase $C_{18}$ Ultrasphere column maintained at 72°C and an acetonitrile linear gradient (65 to 95%) in 0.14% triethylammonium phosphate. Liquid chromatographic analysis of radioimmunoassay standards shows that some samples contain a contaminant peak. Comparison of cyclosporin A levels obtained by radioimmunoassay and high-performance liquid chromatography in clinical investigations show that the former values are generally, but not always, higher than the latter, and that cyclosporin A is very differently metabolized depending on the patient, disease and treatment.

## INTRODUCTION

Cyclosporin A (CsA) has become intensively used in recent years as an immunosuppressive agent [1,2], and could prevent graft rejection in more than 80% of transplants. To avoid the toxic effects it is recommended that the cyclosporin

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concentration as measured by radioimmunoassay (RIA), should be maintained between 100 and 200 ng/ml in the serum and between 300 and 800 ng/ml in the whole blood [3,4] as the trough level 12 h after the last intake of the drug. Therefore, a fast and sensitive method is needed to monitor the CsA level in patient serum during drug administration.

Two methods are available for the assay of CsA concentrations in biological fluids: RIA and high-performance liquid chromatography (HPLC). RIA (Sandoz kit) allows numerous assays in series without any pretreatment of the samples, but the antibodies cross-react with CsA metabolites [5]. These metabolites have not all been identified and their toxicity is not well known. In fact, it seems that their activity is only 10% of that of the parent drug in vitro [6]. Furthermore they give a non-linear response to RIA [7]. HPLC requires prior purification of the biological sample. However, it is a more selective method as it differentiates the parent drug from its metabolites. It thus gives a better indication of the residual level of the active drug [8].

Several HPLC procedures have been reported, some using an isocratic mode [9–11]. Other, more complex, procedures require multi-step sample preparation [12] or column-switching [13,14]. These methods are applicable to serum and plasma or whole blood. Measurement of CsA in whole blood has been recommended [10] because only 30% of circulating cyclosporin is found in serum. However, the sole commercially available RIA kit was initially developed for plasma or serum, although it can now be used for whole blood [15].

We report here a selective and sensitive HPLC method, which involves solid-phase extraction and ion-pairing chromatography with an acetonitrile gradient in triethylammonium phosphate (TEAP). This method can be used with serum, plasma or urine, and is suitable for pharmacokinetic studies or for follow-up of transplant patients. We have been able to follow-up children with bone-marrow transplants and one adult with a heart transplant for several months. CsA was measured by HPLC and RIA, and results are compared and discussed.

## EXPERIMENTAL

### *Radioimmunoassay*

Radioimmunoassay was performed with cyclosporin RIA kit (Sandoz, Basle, Switzerland). Serum was separated from the blood cells at 37°C (see *Sample preparation*). The detection limit varies with the dilution of the sample from 10 to 40 ng/ml; the coefficient of variation was 2.94% for 125 ng and 15.68% for 62 ng CsA per assay.

### *High-performance liquid chromatography*

*Reagents.* All-glass vessels were siliconized with Rhodorsil emulsion 1 EP (Prolabo, Paris, France). All reagents were HPLC grade or reagent grade. Acetonitrile, methanol and orthophosphoric acid were purchased from Merck (Darmstadt, F.R.G.). Triethylamine was supplied by Fluka (Buchs, Switzerland).

Daily bidistilled water was obtained from a quartz distillator (MQS Bioblock, Strasbourg, France). Organic solvents were filtered before use through FH 0.5-

μm filters with a Millipore Pyrex system, and aqueous solutions were filtered through GS 0.22-μm filters. They were sonicated or helium-degassed before chromatography.

Cyclosporins A and D were a gift from Sandoz. Stock solutions were prepared by dissolving 20 mg of CsA or CsD in 100 ml of acetonitrile. Standard solutions (2 μg/ml) were obtained by diluting the stock solution in the mobile phase. Both stock and standard solutions were stable for at least six months at room temperature.

*Apparatus and chromatographic conditions.* HPLC was performed on a Waters chromatograph (Millipore, St Quentin en Yvelines, France) equipped with two 6000A pumps, a U6K injector, an M 720 system controller. Detection was provided by a Model 480 variable-wavelength UV detector set at 210 nm. The detector signal was recorded with an SP 4270 recorder (Spectra-Physics, Orsay, France).

Several reversed-phase $C_{18}$ analytical columns (25 cm×4.6 mm I.D.) packed with ODS were tested: Ultrasphere 5-μm (Beckman, Palo Alto, CA, U.S.A.); 218 TP 54-6, 300-Å, 5-μm Vydac (Separations Group, Hesperia, CA, U.S.A.); and Hibar 5-μm (Merck). The analytical column was protected by a guard column of 10-μm ODS (5 cm×4.6 mm I.D., Beckman). The mobile phase was a mixture of 65 m*M* orthophosphoric acid adjusted to pH 3.2 with triethylamine and acetonitrile (35:65, solution A; 5:95, solution B). A linear gradient was developed from 0 to 100% solution B for 15 min, followed by isocratic elution for 5 min, at flow-rate of 1 ml/min, sensitivity 0.05 a.u.f.s. The run time was 30 min with the Ultrasphere column and 20 min with 218 TP 54-6 Vydac or Hibar columns.

After use, the column was washed with bidistilled water (0.4 ml/min) and kept at 40°C to avoid large variations of temperature. After extensive use (ca. 50 injections), it is recommended that the column is washed with 200 ml of methanol to elute the more hydrophobic components retained.

*Sample preparation.* The blood was collected, kept at 37°C for 30 min, and centrifuged at 3000 *g* for 10 min at 37°C; serum was separated and frozen at −20°C until assay or purified immediately according to the method of Yee et al. [16]. We used a Baker 10 SPE extraction system (J.T. Baker, Phillipsburg, NJ, U.S.A.) and 3-ml cyano disposable extraction columns (40 μm). The columns were washed with two 3-ml volumes of acetonitrile and two 3-ml volumes of distilled water. Then 1 ml of sample (serum or urine) was applied, followed by 100 μl (200 ng) of CsD standard solution. The columns were washed with two 3-ml volumes of distilled water and 1 ml of methanol—water (40:60, v/v), and then dried under reduced pressure for 30 s. CsA and CsD were eluted with 2 ml of methanol, which was evaporated to dryness under a stream of nitrogen at 40°C. Before HPLC, the dry residue was dissolved in 200 μl of solution A in a microtube and centrifuged with an Eppendorf 5412 microfuge for 3 min. The supernatant was injected onto the chromatograph. The wash fractions were also evaporated to dryness and assayed for CsA to check that some CsA was not lost during the extraction procedure.

*Gas chromatography—mass spectrometry*

The assays were performed on an LKB 2091 apparatus (LKB, Bromma, Sweden) equipped with an SE 30, 250×0.34 mm I.D. column. Helium was used as the carrier gas. The separator and source temperatures were 253 and 264°C, respectively. Mass fragmentation was carried out in the electron-impact mode (ionization energy 22 eV). The chromatogram of amino acids was developed with a temperature programme from 80 to 280°C (3°C/min).

The peaks collected from HPLC were hydrolysed with 6 *M* hydrochloric acid for 24 h at 110°C in sealed tubes. The residue was evaporated to dryness under vacuum, and the amino acids were chromatographed on Dowex ($H^+$) 50W-X8 (100–200 mesh) and derivatized according to Desgres et al. [17].

*Subjects*

The pharmacokinetic studies were carried out on five children (6–15 years old) and one adult (33 years old) before bone-marrow grafting. All the subjects were treated with 200–284 mg of CsA intravenously. The samples were collected before and 30 min, 1, 2, 4, 6, 12 and 24 h after the injection.

The follow-up of CsA levels was performed in the five children after bone-marrow grafting. In accordance with the results of pharmacokinetic studies and their clinical status, the children received 20–100 mg CsA per day in continuous infusion at the beginning of the treatment then 30–100 mg orally every 12 h. The blood samples were collected on the morning when the children were receiving CsA continuously or 12 h after the last CsA intake when it was administered per os.

CsA was also assayed for two months in one male adult (41 years old) after cardiac transplant. This subject received orally 55–75 mg prednisone and 200–400 mg CsA per day in two divided doses as initial dose. Blood samples were collected 12 h after the last drug intake. CsA was also assayed in 24-h urine collected from this patient.

## RESULTS

*Chromatographic data*

The first experiments were carried out isocratically with the Ultrasphere $C_{18}$ 5-μm column. The mobile phase was 65 m*M* TEAP in acetonitrile at a flow-rate of 1 ml/min. Various concentrations of acetonitrile were assayed (65, 75, 85%), but no isocratic procedure gave a good resolution of CsA and CsD; thus the next experiments were performed with the acetonitrile gradient described in Experimental. The efficiencies of three RP-$C_{18}$ columns were evaluated. Retention times were 17 and 18.6 min with the Beckman column, 9.03 and 10.57 min with the Vydac column and 10.6 and 12 min with the Merck column for standard solutions of CsA and CsD, respectively. CsA and CsD were resolved better on the Beckman and Vydac columns (data not shown); with the last two columns, no interfering peak was detected from CsA-free serum (Fig. 1A). The Beckman Ultrasphere column was chosen for use in all the experiments described below, because it can

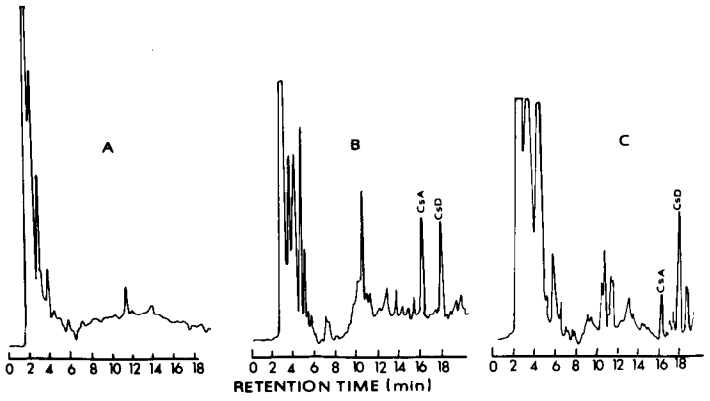


Fig. 1. Chromatograms obtained with an Ultrasphere column of extracted human serum as described in Experimental. (A) Blank human serum sample; (B) human serum sample spiked with 200 ng of CsA and CsD standard solutions; (C) human serum after administration of 700 mg of CsA to a patient (concentration found, 40 ng/ml).

be used at high temperature without loss of resolution after a daily use of four months, provided care is taken not to vary temperature as described in Experimental. Fig. 1B and C show chromatograms of a serum spiked with 200 ng of CsA and CsD and of a serum of a patient receiving 700 mg of CsA daily.

We prepared a working curve by adding 25–2500 ng of CsA and a constant amount of CsD to 1 ml of pooled drug-free serum. The samples were prepared in duplicate, extracted and chromatographed as described in Experimental. The concentration—response relationship is linear between 25 and 2500 ng, and the regression analysis data are: $r=0.997$, slope $=0.866$, $y$-intercept $=0.06$. This concentration range allows the detection of amounts currently encountered in serum during therapeutic monitoring.

The limit of detection, defined as the amount of injected standard CsA giving a signal-to-noise ratio greater than 3, is 20 ng.

The recovery was determined by comparing the peak area of CsA added to serum samples and extracted to the peak area of CsA standard solution directly injected. The recovery varies from $80 \pm 7.8\%$ (50 ng of CsA, $n=8$) to $85 \pm 6.6\%$ (200 ng of CsA, $n=8$). However, the recovery can vary with the sets of extraction columns. To overcome this problem, an internal standard (CsD) was added to the samples before extraction.

The within-day precision of the assay was established by replicate analysis of serum samples spiked with 50 and 200 ng of CsA. The coefficients of variation were 8.2% for 50 ng ($n=6$) and 6.3% for 200 ng ($n=8$). Day-to-day precision was measured on ten different days. The coefficients of variation were 10.4% for 50 ng and 8.6% for 200 ng.

*Comparison of HPLC and RIA data*

*Comparison of HPLC and RIA standards.* The CsA levels of patients' serum measured by RIA and HPLC are different. The discrepancy might be related to the standard used in each procedure. To investigate this hypothesis, a standard

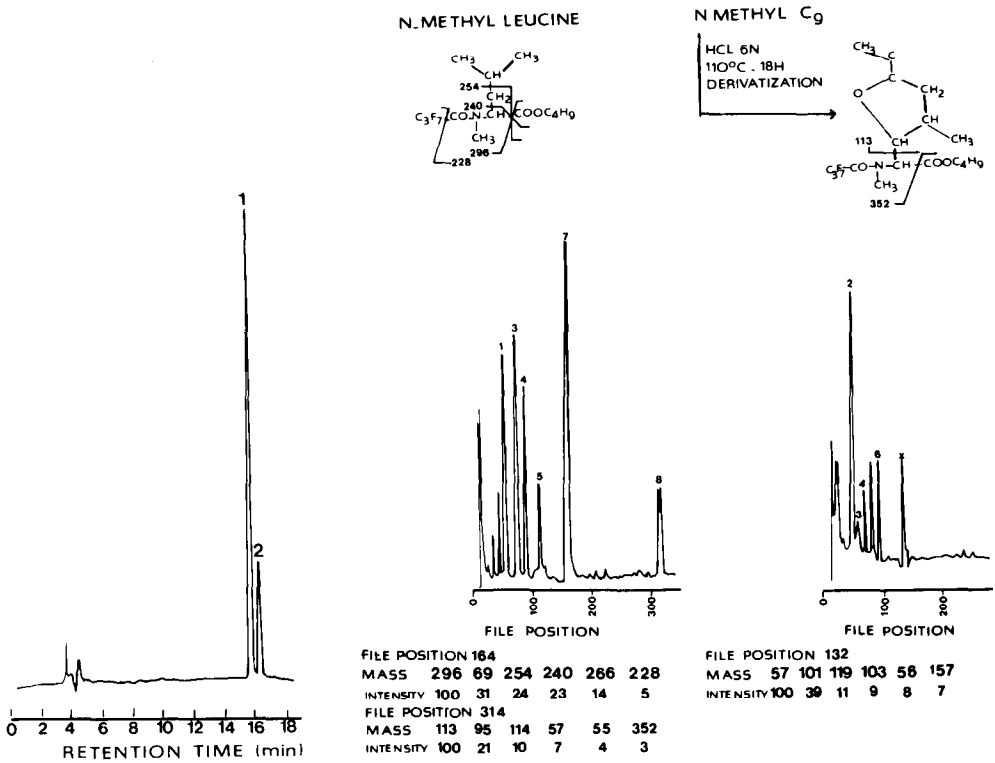


Fig. 2. Chromatogram of 4 $\mu$g standard CsA supplied in RIA kit (Sandoz). Peaks: 1 = contaminant; 2 = cyclosporin A (sensitivity 0.5 a.u.f.s.).

Fig. 3. Analysis by GC—MS of two peaks separated by HPLC from CsA RIA standard. Left: peak 2 = CsA; right: peak 1 = contaminant. Peaks: 1 = alanine; 2 = glycine; 3 = aminobutyric acid plus N-methylglycine; 4 = valine; 5 = N-methylvaline; 6 = leucine; 7 = N-methylleucine; 8 = N-methyl $C_9$; X = not identified.

RIA solution was injected under our usual chromatographic conditions. Two peaks were detected: the first was eluted before CsA, the second had the same retention time as CsA (Fig. 2). These two peaks were collected and analysed by GC—MS after acidic hydrolysis. The second peak gave the characteristic mass fragmentation of N-methylleucine and N-methyl $C_9$ amino acid of CsA, whereas in the first peak this characteristic mass fragmentation was not found. However, this compound is also of a peptide nature (Fig. 3). The first peak assayed in RIA did not respond to the immunological test, so it appeared that this peak (not found in all RIA standards tested) could not have played a large part in the discrepancies observed between HPLC and RIA values.

In order to study the possible loss of CsA during solid-phase extraction, we used HPLC to analyse the fractions discarded during extraction of several CsA-supplemented sera. We did not find any CsA in these fractions for sera spiked with 100 and 200 ng of CsA. For a 500-ng supplemented serum, only 10% was found in the water—methanol fraction.

*Clinical data.* A pharmacokinetic test was carried out in six patients before

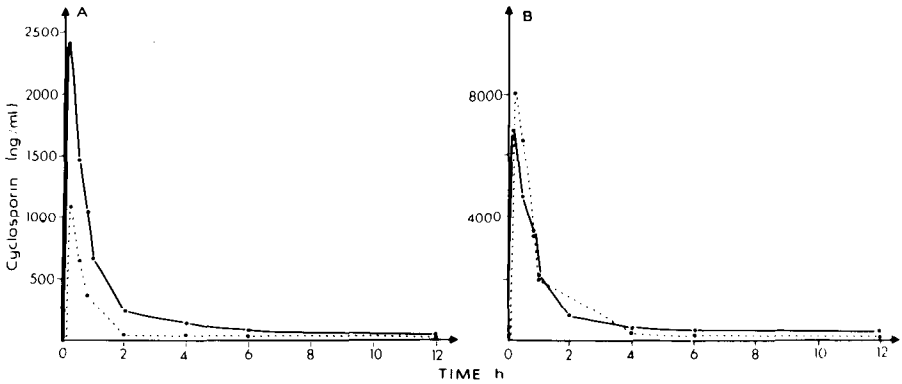


Fig. 4. Two examples of pharmacokinetic study after intravenous injection of CsA (200 mg). Solid lines are values measured by RIA and dotted lines are values measured by HPLC. (A) Plasma of a 12-year-old boy; (B) plasma of a 33-year-old woman.

bone-marrow transplant. All the patients had a peak concentration time of 15 min, and CsA serum levels returned to baseline values ca. 2–4 h after drug administration. The results obtained by RIA were about double those obtained by HPLC (Fig. 4A gives an example of the results) except in one patient (Fig. 4B).

For the five children, CsA serum levels have been followed up by HPLC and RIA for six months after bone-marrow transplant. Fig. 5 indicates the results of the regression analysis comparing RIA ($x$) and HPLC ($y$) values: $r=0.85$, slope $=0.44$, $y$-intercept $=0.59$ ($n=23$).

The serum cyclosporin concentration in the heart-transplant patient was also monitored by RIA and HPLC. Fig. 6 presents the results obtained during the follow-up. The three arrows indicate cardiac biopsies: biopsy No. 2 revealed an acute rejection episode with severe cellular infiltrates and myocardial cell necrosis; during this period, the serum CsA level measured by HPLC was lower than the limit of detection (less than 20 ng/ml), whereas the level measured by RIA was higher than the recommended therapeutic range (greater than 200 ng/ml). Results of regression analysis comparing RIA ($x$) and HPLC ($y$) values were: $r=0.89$, slope $=0.15$, $y$-intercept $=22.09$ ($n=36$).

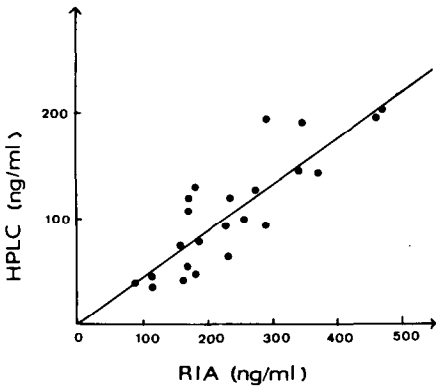


Fig. 5. Comparison of CsA serum levels measured by HPLC and RIA in five children after bone-marrow transplant: $r=0.85$; slope $=0.44$; $y$-intercept $=0.59$.

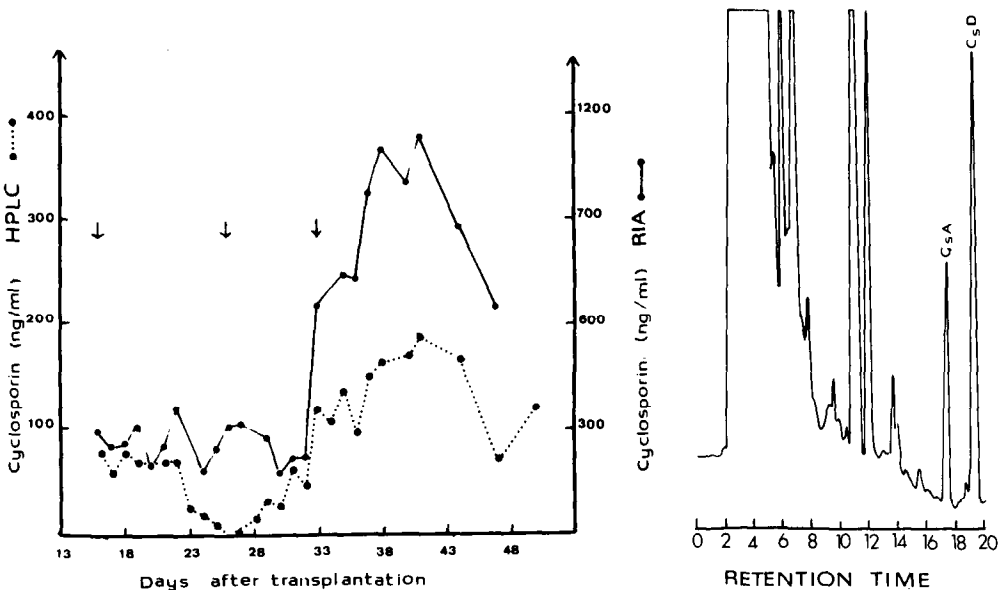


Fig. 6. CsA serum levels in an adult patient after heart transplantation. The three arrows indicate heart biopsies.

Fig. 7. Chromatogram of extracted human urine. The patient has been treated with 500 mg of CsA per 24 h (concentration found, 258 ng/ml). The urine samples were spiked with 500 ng of CsD standard solution.

CSA was assayed in this patient by HPLC of some 24-h urine samples. The values found represent ca. 0.12% of the CsA administered. Fig. 7 shows a chromatogram obtained from urine.

## DISCUSSION

Close control of CsA levels is needed to prevent toxic effects and to maintain a sufficient baseline value. We developed an HPLC technique with a low detection limit (20 ng/ml) and good efficiency, using a slightly modified TEAP buffer previously used for chromatography of polyamines [18]. The good selectivity of the system allowed the detection of a contaminant peak eluting near CsA, in a standard from the RIA kit. This peak could be collected and analysed by GC—MS. It was clearly shown that this product was different from the native CsA molecule, although its hydrophobicity was quite similar. The aggregation or intermolecular bonding [19] of CsA standard, kept in ethanol, could not explain this first peak because this product did not contain the characteristic $C_9$ amino acid of CsA. Furthermore, it did not give any cross-reaction with CsA antibodies. So it appears that this contaminant could not have played a part in the discrepancy observed between HPLC and RIA results.

The efficiency of the extraction procedure is similar to that obtained with a Bond-Elut $C_{18}$ column recently described [10]. We avoided protein precipitation

before extraction in order to minimize the loss of CsA, which is bound particularly to lipoproteins [20].

The good sensitivity of our method allows us to monitor trough plasma levels in clinical samples. The pharmacokinetic curves have been generally drawn after oral administration of CsA [21,22]; a pharmacokinetic study after intravenous administration shows that the concentration peak is reached in 15 min and that CsA levels return to baseline values in ca. 2–4 h. Like other investigators [10,22], we found RIA values to be higher than HPLC values. However, in one patient these results were reversed. This patient had received several courses of chemotherapy before the CsA pharmacokinetic study, and a liver dysfunction can be assumed. It seems that this patient has exhibited an increased nephrotoxicity to the drug.

The correlation coefficients obtained when the values measured by RIA and HPLC are compared are good ($r=0.85$ in marrow transplants, $r=0.89$ in heart transplant). The results obtained with serum give a better correlation than those obtained with whole blood [22]. If HPLC measures parent drug and RIA parent drug plus metabolites, one observes that CsA metabolism is quite variable. In patients receiving a marrow transplant (leukaemic patient), the previous treatment by chemotherapy seems to decrease the rate of CsA metabolism (slope of regression curve, 0.44), whereas in a patient receiving a heart transplant the CsA is more extensively metabolized (slope of regression curve, 0.15). In this patient, CsA administration was monitored from the serum CsA level measured by RIA. Unfortunately, RIA results (greater than 300 ng/ml) did not correlate with clinical status in this case, and acute rejection was observed on the 26th day after transplant (confirmed by biopsy). CsA levels measured by HPLC correlate better with clinical status, since on the day of acute rejection, CsA was less than 20 ng/ml. The rejection episode was treated with three daily doses of 300 mg of CsA in order to stabilize the serum CsA level between 100 and 200 ng/ml (HPLC) and with pulsed methylprednisolone therapy (1 g daily intravenously for three days). This patient has not received rabbit antithymocyte globulin. Blood levels of urea and creatinine were less than 14.9 mmol/l and 124 μmol/l, respectively. Liver tests (alanine aminotransferase, aspartate aminotransferase, bilirubin) were usually normal. Urinary elimination of CsA was slight and represented only 0.12% of the dose administered. This was consistent with values found elsewhere [23].

The method described here is relatively easy. Its sensitivity and reliability have been demonstrated by routine follow-up of patients treated with CsA. It may lead to an efficient monitoring of patients receiving CsA, by means of combined RIA and HPLC, and may allow detection of possible disorders during the course of treatment. Combining the two techniques will be a way to detect and identify some metabolites occurring during particular episodes of a post-transplant period.

REFERENCES

1 B.D. Kahan, C.T. Van Buren, S.N. Lin, Y. Ono, G. Agostino, S.J. LeGrue, M. Boileau, W.D. Payne and R.H. Kerman, Transplantation, 34 (1982) 36–45.
2 J.F. Borel and K.J. Lafferty, Transplant. Proc., 15 (1983) 2219–2229.

3 G. Thiel, F. Harder, R. Lörtscher, M. Brünisholz, M. Laudmann, J. Brunner, F. Follath, M. Wenk and M. Mihatsch, Klin. Wochenschr., 61 (1983) 991–1000.
4 A.J. Wood and M. Lemaire, Transplant. Proc., 17 (Suppl. 1) (1985) 27–32.
5 P. Donatsch, E. Abisch, M. Homberger, R. Traber, M. Trapp and R. Voges, J. Immunoassay, 2 (1981) 19–32.
6 D.J. Freeman, A. Laupacis, P.A. Keown, C.R. Stiller and S.G. Carruthers, Br. J. Clin. Pharmacol., 18 (1984) 887–893.
7 T.G. Rosano, B.M. Freed and N. Lempert, Clin. Chem., 31 (1985) 936.
8 S.G. Carruthers, D.J. Freeman, J.C. Koegler, W. Howson, P.A. Keown, A. Laupacis and C.R. Stiller, Clin. Chem., 29 (1983) 180–183.
9 R. Garrafo and P. Lapalus, J. Chromatogr., 337 (1985) 416–422.
10 P.M. Kabra, J.H. Wall and N. Blanckaert, Clin. Chem., 31 (1985) 1717–1720.
11 R.J. Sawchuk and L.L. Cartier, Clin. Chem., 27 (1981) 1368–1371.
12 R.E. Kates and R. Latini, J. Chromatogr., 309 (1984) 441–447.
13 H.T. Smith and W.T. Robinson, J. Chromatogr., 305 (1984) 353–362.
14 D.J. Gmur, G.C. Yee and M.S. Kennedy, J. Chromatogr., 344 (1985) 422–427.
15 G. Randall and P. Jacobs, Exp. Hematol., 13 (1985) 874–878.
16 G.C. Yee, D.J. Gmur and M.S. Kennedy, Clin. Chem., 28 (1982) 2269–2271.
17 J. Desgres, D. Boisson and P. Padieu, J. Chromatogr., 162 (1979) 133–152.
18 B. Brossat, J. Straczek, F. Belleville, P. Nabet and R. Metz, J. Chromatogr., 277 (1983) 87–99.
19 J. Kirschbaum, S. Perlman and R.B. Poet, J. Chromatogr. Sci., 20 (1982) 336–340.
20 M. Maire and J.P. Tillement, J. Pharm. Pharmacol., 34 (1982) 715–718.
21 T. Beveridge, A. Gratwohl, F. Michot, W. Niederberger, E. Nüesch, K. Nussbaumer, P. Schaub and B. Speck, Curr. Ther. Res., 30 (1981) 5–18.
22 G.L. Lensmeyer and B.L. Fields, Clin. Chem., 31 (1985) 196–201.
23 G. Maurer, Transplant. Proc., 17 (Suppl. 1) (1985) 19–25.