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DETERMINATION OF CYCLOSPORIN A IN THE SERUM OF KIDNEY TRANSPLANT PATIENTS BY RAPID-FLOW FRACTIONATION AND NORMAL-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

High-performance liquid chromatography was used to determine cyclosporin A (CsA) concentrations in the serum of kidney transplant patients by rapid-flow fractionation (RFF) followed by silica gel normal-phase high-performance liquid chromatography (HPLC). The extraction of CsA from serum was achieved by RFF using a short diatomaceous earth column eluted with diethyl ether–*n*-hexane (50:50, v/v). The recovery was more than 80% at concentrations of 50–150 μg/l. The concentration of this compound was determined by HPLC using a conventional silica gel column with 3.3 *M* ammonia solution–ethanol–*n*-hexane (0.31:10.69:89, v/v) as eluent. Concentration calibration was made on the basis of the peak-height ratio of CsA to CsD as the internal standard. The coefficient of variation of this assay was less than 6.5% and the results were used for the therapeutic drug monitoring of CsA administered to kidney transplant patients. Measurements of the CsA concentrations in 160 serum specimens were also made by conventional radioimmunoassay (RIA) using commercial kits. The data obtained by RIA were on average 2.5 times those obtained by HPLC. Higher values in RIA were observed characteristically with patients with severe disfunction resulting from CsA hepatotoxicity. From the results, it appeared that HPLC rather than RIA provides more precise and reliable values for the concentration of this drug.

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INTRODUCTION

Cyclosporin A (CsA) is the most potential immunosuppressive agent [1] isolated from *Trichoderma polysporum* [2]. The drug consists of a cyclic undecapeptide with a molecular mass of 1202.6 and is slightly soluble in water but readily so in most organic solvents. It is clinically effective for the prevention of acute allograft rejection of transplanted organs and has an effect on graft survival rate that has been reported to be higher than that of prednisolone plus azathiopurine in conventional therapy [3–6]. However, because of its severe side-effects, therapeutic drug monitoring of CsA is recommended in most medical facilities. Concentrations of CsA exceeding the therapeutic range may lead not only to fatal lung infection owing to its excess immunosuppressive effect but also to renal and hepatic disfunctions owing to the toxic side-effects [7–11]. Concentrations below the therapeutic range may result in irreversible allograft rejection. The absorption and clearance of CsA are also highly variable, depending on the recipients [12–15]. Thus, the concentration of the drug in the blood should be maintained within the therapeutic range by administering it according to a schedule established by drug monitoring.

In this study, a highly sensitive assay method involving rapid-flow fractionation (RFF) for the clean-up of CsA followed by normal-phase instead of reversed-phase high-performance liquid chromatography (HPLC) for determining the concentration of this drug was developed. The application of this method to drug monitoring in four kidney transplant patients gave satisfactory results for the establishment of a dosing schedule.

EXPERIMENTAL

RFF apparatus

The apparatus has been described previously [16]. In brief, the system consisted of two glass columns for CsA extraction and washing the extract to eliminate acidic constituents. The support material in each column was diatomaceous earth granules of particle size larger than 50 μm and prepared from Celite powder No. 545 (Johns Manville, Denver, CO, U.S.A.) by precipitation in distilled water. The inner volumes of the extraction and washing columns were 3.5 and 0.8 ml, respectively. The two columns were connected to each other using a simple Teflon plug. The loading capacity of the extraction column was 0.5 ml of serum or plasma. The flow-rate to optimize the solvent polarity was 9 ml/min, using a KPW-10 (Kusano Scientific, Sumida, Tokyo, Japan) solvent-delivery pump for adjustment. For extraction and monitoring of the eluent, an SPD-2A (Shimadzu, Chiyoda, Tokyo, Japan) UV detector set at 215 nm and a Model 17105 syringe-loading sample injector (Rheodyne, Cotati, CA, U.S.A.) were used. An extraction chromatogram was obtained with an ES-22005 pen recorder (Chino, Chuo, Tokyo, Japan). In the routine operation of

RFF, solvent delivery was carried out through a solvent reservoir column with an inner volume of 14 ml, using nitrogen at a pressure of 1–2 kg/cm^2 to maintain the flow-rate at 0.7–0.9 ml/s.

RFF operation

The following preliminary experiment was carried out with distilled water instead of serum. The column system was conditioned with 7 ml of ethanol and then with 7 ml of *n*-hexane. Following the injection of 0.5 ml of water through a plug, the extraction column was connected to the solvent delivery pump and the washing column to the UV detector. An appropriate mixture of diethyl ether and *n*-hexane pre-saturated with water was introduced as the mobile phase and the system was pre-conditioned at a flow-rate of 9 ml/min until a flat baseline was obtained at a sensitivity of 0.16 a.u.f.s. A 10-μl volume of the ethanol solution containing 20 μg of CsA was injected through the syringe-loading sample injector at a flow-rate of 9 ml/min. The solvent mixtures used were diethyl ether–*n*-hexane (40:60, 50:50, 60:40, 65:35, 70:30, 80:20, 90:10 and 100:0, v/v). The most appropriate solvent composition for the frontal extraction of CsA was determined from the extraction chromatograms obtained.

A preliminary experiment was then conducted using a serum specimen obtained from a healthy volunteer. A 0.5-ml aliquot of the serum spiked with 20 μg of CsA and CsD each was introduced into the pre-conditioned extraction column and 30 μl of sodium hydroxide solution (50 g/l) into the washing column. The drugs were extracted with diethyl ether–*n*-hexane (40:60, 50:50 and 60:40, v/v). The most appropriate solvent polarity and solvent volume were determined from the results of HPLC of the extracts. We were primarily concerned with how to obtain the extract with minimum amounts of contaminants with chromatographic retentions similar to those of CsA and CsD. A solvent composition of diethyl ether–*n*-hexane (50:50, v/v) and a volume of 14 ml were selected for the routine conduct of RFF. Under these conditions, the chromatographic determination and recovery of CsA and CsD were satisfactory.

Routine operation of RFF

The general procedure has been illustrated previously [16]. Into the washing column were introduced 30 μl of sodium hydroxide solution (50 g/l). After connecting the column to the extraction column, 0.5 ml of serum spiked with 200 ng of CsD as the internal standard was introduced into the extraction column through the plug. The reservoir column was attached to the extraction column and filled with 14 ml of diethyl ether–*n*-hexane (50:50, v/v), which was slightly over the optimized volume. This column was then connected to the nitrogen cylinder with its gauge set at 1–2 kg/cm^2 to flush the solvent out of the columns. The eluent was collected in a glass tube whose inner surface had previously been coated by methylsilylation to prevent chemical adhesion of the drugs. The entire procedure was carried out in less than 5 min. The

solvent was evaporated to dryness for later injection into the HPLC system. The columns were cleaned for subsequent use as described previously [16].

HPLC apparatus and conditions

A Shimadzu LC-5A continuous-flow solvent-delivery system, a Shimadzu SPD-2A variable-wavelength UV detector and a Chino EB-22005 pen recorder constituted our HPLC system. It was also provided with a Rheodyne Model 17105 syringe-loading sample injector. A conventional LiChrosorb Si 60 (5 μm) packed column (250 mm$\times$4 mm I.D.) was purchased from Cica-Merck (Chuo, Tokyo, Japan). The flow-rate was 1.0 ml/min and the wavelength was set at 215 nm with a sensitivity of 0.005 a.u.f.s. The solvent system was 3.3 *M* ammonia solution–ethanol–*n*-hexane (0.31:10.69:89, v/v).

RIA of CsA

Measurement of CsA by RIA was performed at the Special Reference Laboratory (Shinjuku, Tokyo, Japan) using commercially available polyclonal kits purchased from Sandoz (Basle, Switzerland).

Kidney transplant recipients

Four patients aged from 19 to 53 years who had received renal transplants with living allograft and administration of CsA plus prednisolone were studied. They had been admitted to our hospital during 1985–1986. The initial dose of CsA was 12 mg/kg per day and, after two months, 4 mg/kg per day. Blood samples for therapeutic drug monitoring were collected at 8.00 a.m. before drug administration. The serum specimens obtained immediately after the collection of the blood at 37°C [17] were divided into two aliquots, one for HPLC and the other for RIA. They were stored at -20°C until used.

RESULTS

Optimization of extraction conditions

The results of column extraction by RFF are illustrated in Fig. 1. The elution profiles of CsA obtained using mixtures of diethyl ether and *n*-hexane were expressed in terms of the logarithmic relationship between the capacity ratio (k') and solvent composition. The frontal extraction of CsA was conducted with mixtures containing more than 70% diethyl ether. One k' unit corresponded to a solvent volume equal to the void volume of the column system, i.e., 4.5 ml. The optimum recovery of CsA should be possible using 6.3 ml of diethyl ether–*n*-hexane (70:30, v/v). Almost the same results were obtained with structurally similar CsD. However, with the above solvent composition, considerable amounts of contaminants present in the extract caused the chromatograms of CsA and CsD to be unsatisfactory. Consequently, the solvent composition diethyl ether–*n*-hexane (50:50, v/v) with a volume of 14 ml was

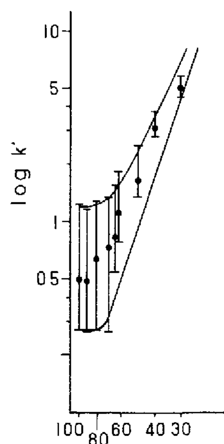

Fig. 1. Logarithmic relationship between peak retention and solvent composition in the diatomaceous earth column extraction of CsA. Solvent composition is denoted by % (v/v) of diethyl ether in diethyl ether-*n*-hexane. Retention of CsA is expressed in terms of peak position (●) and peak width (bar length) using k' values. One k' unit corresponds to 4.5 ml of solvent, the void volume of the column system Frontal extraction was achieved with a solvent containing more than 70% diethyl ether. However, 14 ml of diethyl ether-*n*-hexane (50:50, v/v) were used in the actual RFF procedure for satisfactory analytical results.

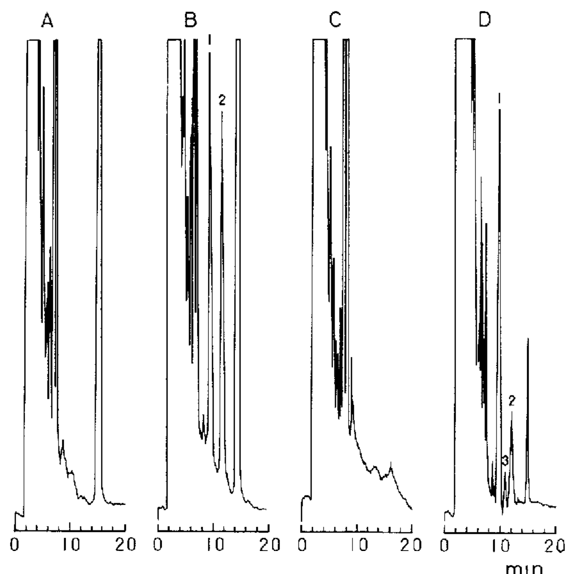

Fig. 2. Typical chromatograms of human sera for determining CsA. Peaks: 1=CsD; 2=CsA; 3=prednisolone coadministered with CsA to a patient. (A) Serum from a healthy female; the large peak at 15.0 min corresponds to 1-methyl-3-carboxamido-6-pyridone, a normal metabolite of nicotinamide; (B) serum spiked with 300 μg/l CsA and 200 μg/l CsD; (C) serum of a patient before CsA administration; (D) serum of a patient administered 12 mg/kg CsA per day. A blood sample was taken before CsA administration on the morning of the following day. The calculated amount of CsA was 79.8 μg/l, which corresponds to the trough concentration in this patient.

used, based on experimental results using serum specimens. The extract thus obtained afforded the satisfactory chromatogram shown in Fig. 2. The recoveries (mean ± S.D.) of CsA added in known amounts such as 50, 100 and 150 μg/l were 80.6 ± 1.0, 89.9 ± 4.8 and 102.1 ± 6.5%, respectively, ($n=4$). The coefficient of variation was less than 6.5%.

Determination of CsA

A three-point calibration graph for the determination of CsA concentration was constructed by plotting the ratio of the peak height of CsA to that of the internal standard CsD. Linear regression analysis was made using the equation $y=0.0028x+0.0143$ with a coefficient of regression $r=0.9999$, where x is the CsA concentration (μg/l) and y the CsA/CsD peak-height ratio. The intercept did not differ significantly from zero. The regression coefficient was the satisfactory for determining CsA at a concentration as low as 10 μg/l, which was the detection limit of our method.

Serum concentrations of CsA in kidney transplantation

A comparative study of HPLC and RIA was carried out using 160 serum specimens from the four patients. The results are shown in Fig. 3. The regression equation was $y=0.1993x+33.6087$ with a coefficient of regression $r=0.5465$ ($P<0.001$). The concentrations of CsA as determined by RIA were 2.5 times those by HPLC, on average. An increased serum concentration of CsA in patients with hepatic disfunction was clearly evident during the course of the drug therapy. This increase as observed by RIA considerably exceeded that detected by HPLC. The concentration ratio of that by RIA to that by HPLC reached a maximum of 7.5 in the range of moderate to severe disfunction.

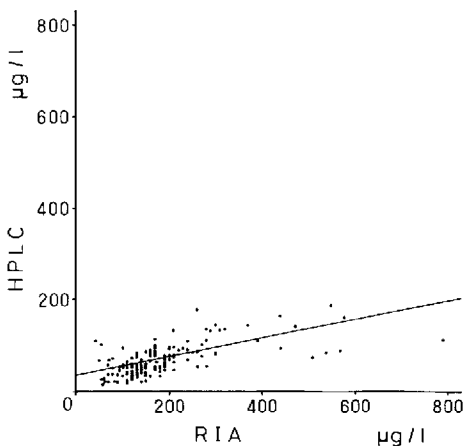

Fig. 3. Comparison of serum CsA concentrations measured by HPLC and RIA. The regression equation is $y=0.1993x+33.6087$; $r=0.5465$ ($n=160$, $P<0.001$).

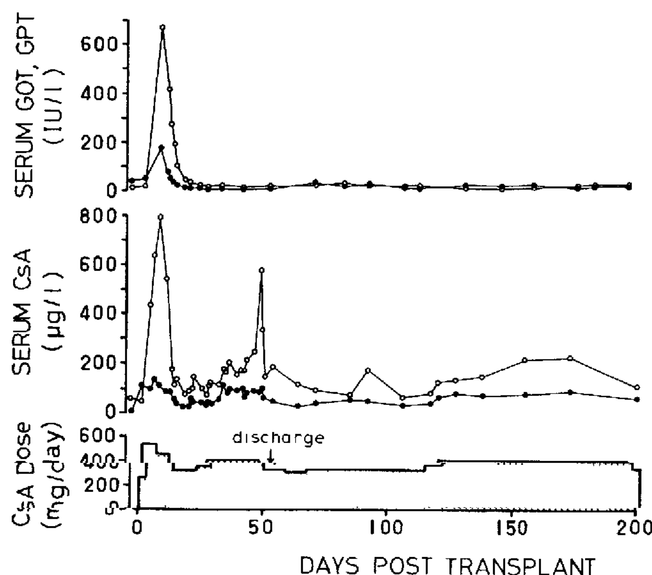

Fig. 4. Case study of CsA drug monitoring in a renal allograft recipient. The top graph shows kidney functions (○, GPT; ●, GOT), the middle graph CsA serum concentrations (●, HPLC; ○, RIA) and the bottom graph CsA dosage. It is clear that the higher levels of GOT and GPT correspond to the higher concentrations of CsA as measured by RIA. The CsA levels obtained by HPLC correlate well with dosage. Under good clinical conditions, the average value of the RIA/HPLC ratio was about 2.0.

Therapeutic drug monitoring of CsA

A typical clinical course of CsA concentrations at trough levels as determined by HPLC and RIA is illustrated in Fig. 4. The changes in glutamic–oxaloacetic transaminase (GOT) and glutamic–pyruvic transaminase (GPT) values are also presented. The concentrations obtained by HPLC changed in accordance with the changes in the amount of CsA administered daily during therapy, but those obtained by RIA showed a good correlation with hepatic indices such as GOT and GPT. Similar results were observed in the other patients.

DISCUSSION

Therapeutic drug monitoring of CsA in renal transplantation has been recommended for establishing its appropriate immunosuppressive effects on acute allograft rejection [18]. Considering the severe side-effects of this drug on liver and kidney functions, its serum concentration should be kept within the effective therapeutic range, which has been shown to vary considerably by an individual genetic matching scale between host and graft.

Several methods, including RIA [19,20] and reversed-phase HPLC [21–36] have been reported for determining CsA concentrations in plasma, serum or

whole blood. The antibody used in RIA, however, cross-reacts with the metabolites of CsA, whose immunosuppressive activity and toxic effects have not been completely clarified. These cross-reactions result in overestimated concentrations of the drug in human biofluids [18]. It has been reported that RIA gives abnormally high levels of CsA in patients with hepatic disfunction [9,18], owing to such cross-reactions. A newly developed monoclonal antibody (Sandoz) requires much time for completion of the antigen–antibody reaction and still affords higher levels of CsA [37]. In chromatography, only reversed-phase techniques have been developed and the use of normal-phase techniques has not been reported so far.

As reported here, we have developed a normal-phase HPLC method to be conducted in conjunction with RFF for sample pre-treatment. We have already applied such a combination of techniques to determining plasma glucocorticoids [16,38] and testosterone [39,40]. With CsA the most important point is that this method makes possible the elimination of serum constituents that prevent a satisfactory chromatogram from being obtained. Fig. 1 shows the optimized solvent composition and volume for treating a serum sample prior to the chromatographic determination of CsA. This sophisticated procedure in the clean-up stage eliminates the tedious and time-consuming trial-and-error experiments required in ordinary chromatographic assays. The typical chromatograms in Fig. 2 demonstrate that the method provides satisfactory results.

The results of the method for determining CsA in blood samples from patients demonstrate the marked differences in values obtained by RIA and HPLC. The higher concentrations obtained by RIA are probably due to the cross-reactivity of the antibody with CsA metabolites. The differences become steadily greater in patients with liver disfunction, as is evident from Figs. 3 and 4. Changes in CsA showed a complete correlation with those in GOT and GPT. With hepatotoxicity, it was difficult to establish a suitable schedule for CsA administration through therapeutic drug monitoring by conventional RIA. In such a case, a reduced dose may increase the risk of graft rejection. The CsA concentration determined by HPLC does not change very much and its fluctuation is sufficiently consistent with the daily dose administered.

The results show that the therapeutic CsA trough concentrations before administration in the morning range from 50 to 100 μg/l following a schedule of 7–10 mg/kg per day. There was no significant elevation in serum CsA in hepatic disfunction patients, as can be seen from Fig. 4. Concentrations could be predicted from the doses administered. The elevated concentrations in liver disfunction as obtained by RIA are probably the result of cross-reactions with CsA metabolites secreted into the systemic circulation on account of biliary atresia.

It is worth noting that a new fluorescence polarization immunoassay developed by Dainabot (Tokyo, Japan), in spite of its technical improvement, gave higher concentrations than those obtained by normal-phase HPLC [41].

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