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Gel Chromatographic Analysis of Cyclosporin and Its Metabolites in Human Blood Compartments

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Abstract

Gel chromatography combined with specific and non-specific cyclosporin radioimmunoassays was adopted for quantitative analysis of cyclosporin and metabolites in free and protein-bound forms in blood compartments of kidney transplant patients. The analytical method was proved to be useful for the purpose, although plasma protein-bound forms of neither cyclosporin nor metabolites could be quantitated in the system. The present study also provided, by gel chromatographic analysis, additional examples to prove that concentrations of cyclosporin metabolites in blood compartments may not be deduced or inferred simply from those of cyclosporin.

Cyclosporin, a potent immunosuppressive agent, has been attracting much attention not only in pharmacological and therapeutic significance but also with biochemical and physicochemical interests in connection with the presence of its specific binding proteins belonging to the cyclophilin family. The marked uptake of cyclosporin into erythrocytes in blood under in-vitro or in-vivo conditions has been described (Lemaire & Tillement 1982; Atkinson et al 1983; Niederberger et al 1983; Rosano 1985; Annesley et al 1986; Legg & Rowland 1988; Hamberger et al 1988) and the presence of a cyclophilin-like cyclosporin binding protein was confirmed in human erythrocytes (Foxwell et al 1988). We have recently succeeded in isolation of two cyclosporin A binding proteins (both $\simeq$ 16 kDa) in human erythrocytes and identified one of the two as [Phe^{47}]-human cyclophilin A (Yamamoto et al 1995). Cyclosporin is also reported to bind to plasma proteins (Lemaire & Tillement 1982; Mraz et al 1983; Niederberger et al 1983; Gurecki et al 1985; Sgoutas et al 1986; Legg & Rowland 1987; Urien et al 1990; Hughes et al 1991). The cyclosporin binding plasma proteins were shown to be mainly lipoproteins and a hydrophobic interaction was suggested as the basis of their cyclosporin binding (Legg & Rowland 1987). The distribution of the drug in blood compartments has also been extensively discussed from the clinical point of view, since therapeutic drug monitoring is indispensable for immunosuppressive therapy with cyclosporin because of intra- and inter-individual variability of drug absorption and its narrow therapeutic window. In addition, cyclosporin undergoes biotransformation mainly in liver and small intestine and the metabolites also circulate, distributing in blood compartments (Maurer & Lemaire 1986; Rosano et al 1986; Lensmeyer et al 1989; Akagi et al 1991; Kivistö 1992; Christians & Sewing 1993). The binding of cyclosporin metabolites to blood components is different from that of cyclosporin and it is described to be a complex interplay of the type of metabolite, haematocrit, temperature and concentration (Lensmeyer et al 1989). Although the clinical and toxic effects of cyclosporin metabolites are not yet fully elucidated, some of them were shown to have immunosuppressive or toxic effects in-vitro and in-vivo (Christians & Sewing 1993), and it is said that the presence of cyclosporin metabolites in blood may not be disregarded from the clinical point of view.

The present study adopted the combined techniques of gel chromatography and radioimmunoassays using specific (monoclonal) and non-specific (polyclonal) antibodies to analyse quantitatively the distribution of cyclosporin and its metabolites in blood compartments in kidney transplant patients.

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Materials and Methods

Blood samples

Blood samples were taken from kidney transplant patients KN and HN. Patient KN of age 48 had received a kidney transplant 10 days before blood sampling and had been given orally 1·5 mg kg^{-1} cyclosporin drink solution (cyclosporin 5·0 g/50 mL) twice a day. Patient HN of age 20 who had received a kidney transplant 9 days before blood sampling had been given orally 2·5 mg kg^{-1} cyclosporin drink solution twice a day. On the day of blood sampling, the patients were given cyclosporin at 0800h after breakfast. Both patients had satisfactorily good graft function and no liver dysfunction.

Blood samples were collected into tubes containing ethylenediaminetetraacetic acid disodium salt (final concentration 0·1%). An aliquot of each sample was immediately centrifuged at 37°C for 5 min at 1600 *g* to separate plasma and erythrocytes, and the cells were lysed by freezing-thawing. The rest of the blood sample was also lysed.

Gel chromatographic analysis

Gel chromatography of blood samples was carried out at

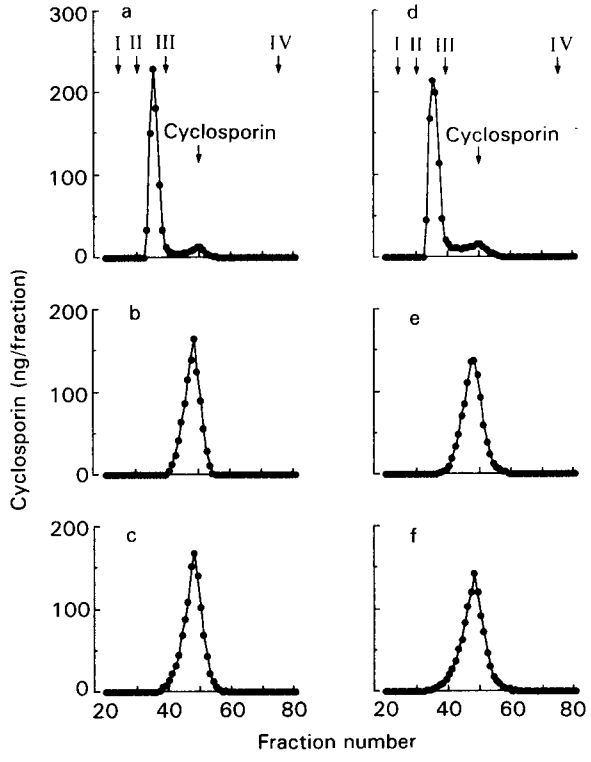

FIG. 1. Gel chromatography elution profiles of cyclosporin (1000 ng mL^{-1}) in whole blood (a and d), plasma (b and e) and serum (c and f). Cyclosporin concentrations in eluates were measured by cyclosporin non-specific (a, b and c) and specific (d, e and f) radioimmunoassays. The Roman numerals on arrows indicate the elution positions of bovine serum albumin (MW $\simeq$ 67000): I (Vo), chymotrypsinogen A (MW $\simeq$ 25000): II, ribonuclease A (MW $\simeq$ 13700): III and *p*-aminobenzoic acid (MW 137): IV (Vt) used for molecular weight calibration.

28 $\sim$ 30°C on a column of Sephadex G50 superfine (1·6 × 100 cm) with use of 50 mM Tris-HCl buffer (pH 7·4) containing 0·03% Tween 20 as eluent. Fractions of 2·5 mL each were collected and submitted to radioimmunoassays for cyclosporin.

Radioimmunoassays for cyclosporin

Specific cyclosporin radioimmunoassay was performed by the double antibody method using mouse monoclonal cyclosporin specific antibody (Lot. No. 9215030, Sandoz Corporation) and [^{125}I]-histaminylsuccinyl cyclosporin C (Lot. No. 9222014, INCSTAR Corporation) as tracer. The antibody had been proved to crossreact with cyclosporin metabolites, M1 and M21 (Maurer et al 1984) at rates of 3 and 5%, respectively. Non-specific cyclosporin radioimmunoassay was carried out with the use of sheep anti-cyclosporin serum (Lot. No. 5026, Sandoz Corporation) and [^{3}H]dihydrocyclosporin (Lot. No. 5026, Sandoz Corporation) as tracer. Cross-reactivities of the antiserum to cyclosporin metabolites were M1 44%, M8 19%, M13 21%, M17 50%, M18 17%, M21 4%, M25 < 1%, M26 < 4% and M203-218 7%. The intra- and interassay coefficients of variation were both within 7% for the specific assay and within 15% for the non-specific assay. For quality control of the assays, spiked cyclosporin (120 and 330 ng mL^{-1}) was measured in each assay and only when the errors of the measurement were within ±10%, were the assay data were accepted.

Table 1. Analytical recoveries of cyclosporin (1000 ng mL^{-1}) in human blood samples after gel chromatography.

	Cyclosporin recovered (ng)	
	Specific RIA	Non-specific RIA
Whole blood	999	846
Plasma	1037	970
Serum	1116	1124

RIA: radioimmunoassay. Analytical recoveries were calculated by integrating cyclosporin in peak fractions of the elution profiles of gel chromatography (Fig. 1).

Results

Fig. 1 shows the gel chromatographic elution profiles of cyclosporin (1000 ng mL^{-1}) in human whole blood, plasma and serum, respectively. The spiked cyclosporin in whole blood eluted mainly in a larger molecular size region (fraction No. 36), indicating the major protein-bound form of the drug in whole blood (Fig. 1a, d). From the eluting position, the molecular weight of the peak substance with cyclosporin was estimated to be approximately 17200 and the cyclosporin binding component was then confirmed as 16 kDa by sodium dodecyl sulphate-polyacrylamide gel electrophoresis. In plasma and serum, cyclosporin added was eluted exclusively in the position where cyclosporin in aqueous solution was eluted (Fig. 1b and e, Fig. 1c and f). The analytical recoveries of cyclosporin after chromatography, which were calculated from the peak areas, were satisfactory in both cases using monoclonal (specific) and polyclonal (non-specific) antibodies, as shown in Table 1. This supports analytical validity of the present procedure.

Whole blood, plasma and serum samples taken from a kidney transplant patient (KN) 3 h after administration of 1·5 mg kg^{-1} cyclosporin were respectively analysed by gel chromatography combined with specific and non-specific radioimmunoassays and the results are shown in Fig. 2. The values measured by non-specific radioimmunoassay were consistently greater than those by specific radioimmunoassay. The differences are attributable to cyclosporin metabolites that can be recognized by the present non-specific radioimmunoassay. The peak emerged in fraction No. 48 corresponds to cyclosporin, but in the whole blood and plasma samples there is an appreciable amount of metabolite(s) which co-eluted with cyclosporin (Fig. 2a $\sim$ b). On the other hand, the serum sample contained little metabolite that co-eluted with cyclosporin (Fig. 2c). The amounts of cyclosporin, bound and free, and metabolites, bound and free, in the blood samples which were calculated from the elution profiles are summarized in Table 2. In this calculation, the cross-reactivities of metabolites against the polyclonal antibody used were tentatively assumed to be as high as that of cyclosporin. Therefore, the values calculated for metabolites may be underestimated ones. As a whole, recoveries of cyclosporin and metabolites after gel chromatography ranged from 87·7% to 118·2%.

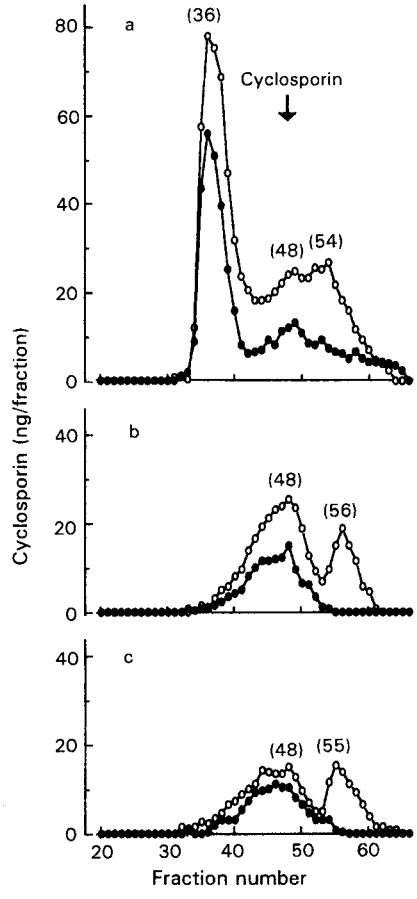

FIG. 2. Gel chromatography elution profiles of cyclosporin and its metabolites in whole blood (a), plasma (b) and serum (c) samples taken from kidney transplant patient KN, 3 h after cyclosporin ingestion. Concentrations of cyclosporin and its metabolites in eluates were measured by cyclosporin non-specific (○) and specific (●) radioimmunoassays.

A whole blood sample of the kidney transplant patient (KN) was treated with methanol according to the pretreatment procedure of blood samples for cyclosporin radioimmunoassay using Cyclo-Trac SP RIA kit (INCSTAR Corporation). The whole blood sample and the methanol-treated sample were analysed by gel chromatography combined with non-specific radioimmunoassay (Fig. 3a ~ b). Analytical recovery of cyclosporin/metabolites in the whole blood sample after chromatography (Fig. 3a) was 99·2% and that of cyclosporin/metabolites in the methanol-treated sample was 84·5% (Fig. 3b). The elution profile of the methanol-treated sample (Fig. 3b) revealed the presence of a large amount of metabolites in the blood sample, supporting the contention that the first-eluting peak in Fig. 3a contained metabolites in protein-bound form together with cyclosporin.

Erythrocyte and plasma samples of a kidney transplant patient (HN) collected before and 1, 3, 5 and 7 h after ingestion of 2·5 mg kg^{-1} cyclosporin were submitted to gel chromatographic analysis (Fig. 4a–e). Cyclosporin/metabolites concentrations in the eluates were measured by non-specific radioimmunoassay, and the eluates of the samples from blood collected 3 and 5 h after drug ingestion, were

Table 2. Estimated cyclosporin and metabolites in free and bound forms in blood samples from a kidney transplant patient.

	Cyclosporin (ng)			[M]* (ng)		
	Bound	Free	Total	Bound	Free	Total
Whole blood	270·7	150·6	421·3	180·3	204·1	384·4
Plasma	0	175·9	175·9	0	151·5	151·5
Serum	0	115·0	115·0	0	133·5	133·5

*[M] cyclosporin metabolites that could be recognized by the present non-specific radioimmunoassay.

The blood samples were taken from patient KN 3 h after ingestion of 1·5 mg kg^{-1} cyclosporin. The volume of the whole blood sample used was 1 mL and the plasma and serum samples were obtained from 1 mL blood.

also measured by specific radioimmunoassay (Fig. 4-f ~ g). Recoveries of cyclosporin or cyclosporin A/metabolites after gel chromatography ranged from 95·3% to 119·2%. Again, no bound forms of cyclosporin and metabolites were detected in the plasma samples under the conditions used. Cyclosporin or cyclosporin/metabolites in the erythrocyte and plasma samples were calculated by integrating values of the peak fractions measured by specific or non-specific radioimmunoassay. The results are summarized in Table

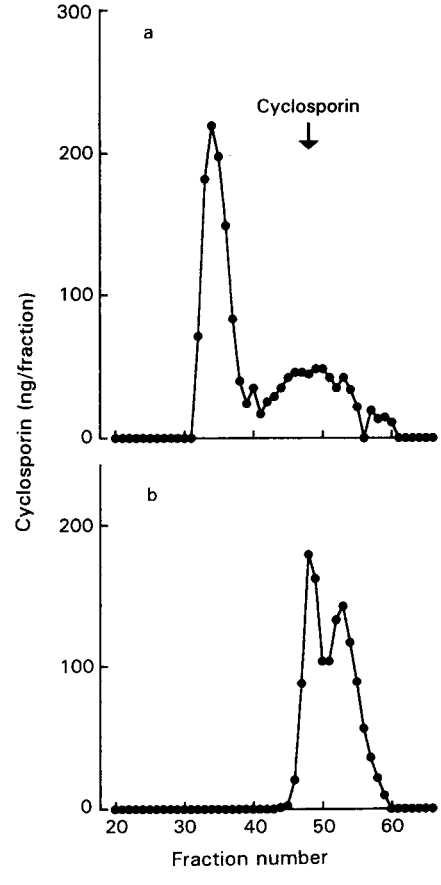

FIG. 3. Gel chromatography elution profiles of cyclosporin and its metabolites in whole blood (a) and methanol-treated blood (b) samples of kidney transplant patient KN. Cyclosporin and its metabolites in eluates were determined by cyclosporin non-specific radioimmunoassay.

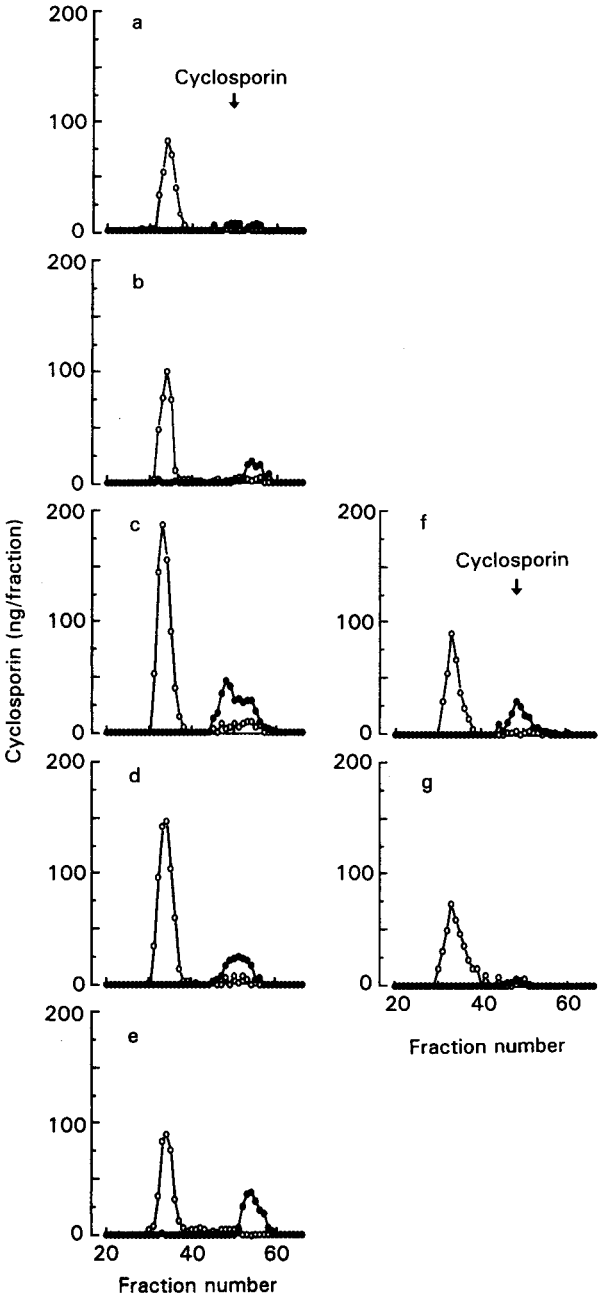

FIG. 4. Gel chromatography elution profiles of cyclosporin and its metabolites in erythrocytes (○) and plasma (●) of blood samples taken from kidney transplant patient HN before (a) and 1 (b), 3 (c and f), 5 (d and g), and 7 h (e) after ingestion of cyclosporin. Concentrations of cyclosporin and its metabolites in eluates were measured by cyclosporin non-specific (a ~ e) and specific (f and g) radioimmunoassays.

3. Based on the data, the amounts of cyclosporin A and metabolites in erythrocytes and plasma of the patient were estimated (Table 4).

Discussion

Gel chromatography combined with specific and non-specific cyclosporin radioimmunoassays was found to be useful for quantitative as well as qualitative analysis of both

Table 3. Cyclosporin and metabolites in blood samples from a kidney transplant patient, measured by specific and non-specific radioimmunoassays after gel chromatography.

After cyclosporin ingestion	Erythrocyte RIA		Plasma RIA	
	Non-specific (ng)	Specific (ng)	Non-specific (ng)	Specific (ng)
Before	288·6	n.d.	49·2	n.d.
1 h	379·3	n.d.	87·5	n.d.
3 h	743·1	341·6	329·9	149·8
5 h	636·8	230·3	170·4	19·7
7 h	401·2	n.d.	59·4	n.d.

n.d.: not determined. RIA: radioimmunoassay. The blood samples were taken from patient HN given 2·5 mg kg^{-1} cyclosporin. The values express radioimmunoassayable cyclosporin and metabolites in erythrocyte and plasma fractions from 1 mL of blood.

erythrocyte-bound and free forms of cyclosporin and its metabolites in human blood, although the polyclonal antibody presently used does not always cross-react with all possible cyclosporin metabolites to the same extent as it does with cyclosporin. It may be admitted that gel chromatography is not superior to HPLC technology in terms of analytical sensitivity and accuracy, but it involves much less of a problem regarding non-specific inconsistent adsorption of more hydrophobic compounds during chromatography as often seen in HPLC.

The binding affinities of cyclosporin and metabolites to erythrocytic proteins were found to be high enough for the compounds to behave in the form of complexes with the binding proteins during gel chromatography. In addition, it was confirmed that the antibodies, monoclonal and polyclonal, both can recognize cyclosporin or cyclosporin/metabolites in erythrocytic protein-bound forms. Several elegant studies on the binding of cyclosporin to plasma proteins have also been described, but no protein-bound forms of cyclosporin or/and metabolites were detected in gel chromatograms of the plasma samples examined under the conditions currently used.

Although the cyclosporin binding to plasma proteins is known to be temperature-dependent (Lemaire & Tillement 1982; Niederberger et al 1983; Legg & Rowland 1987), substantial binding (≃ 70%) was still observed even at 4°C (Niederberger et al 1983). In this context, the present observation may imply that the binding affinity of cyclosporin or metabolites to plasma proteins, if any, was too low for the compounds, as compared with the binding to erythrocytes, to behave in protein-bound forms during gel chromatography under the neutral conditions used. This may suggest more possible significant implications of erythrocytic protein-bound forms of cyclosporin and metabolites in the clinical and toxic effects of those compounds, although little is known about the effect of cyclosporin- or metabolite-containing erythrocytes on biological processes in in-vivo systems.

Evidences have been accumulated for complexity of distribution patterns of cyclosporin and metabolites in human blood compartments. In a kidney transplant patient's blood examined in this study, cyclosporin in plasma was 48·1% of total cyclosporin in blood, while in

Table 4. Amounts of cyclosporin and metabolites in erythrocyte and plasma samples from a kidney transplant patient.

After cyclosporin ingestion		Cyclosporin (ng)	[M]* (ng)
3 h	Erythrocyte	341·6	401·5
	Plasma	149·8	180·1
5 h	Erythrocyte	230·3	406·5
	Plasma	17·7	150·7

*[M] = cyclosporin metabolites that could be recognized by the present non-specific radioimmunoassay. The values were calculated from the data summarized in Table 3 and correspond to the amounts of cyclosporin or [M] in erythrocyte and plasma fractions from 1 mL blood.

serum, cyclosporin was only 27·3% at a time of the maximum concentration of cyclosporin/metabolites. The contents of metabolites in plasma and serum of the patient showed almost the same percentages, 39·4% and 34·7%, of the total in blood. As has already been accepted, cyclosporin and metabolite levels either in plasma or in serum could not represent their blood concentrations. It should also be emphasized that concentrations of cyclosporin and metabolites in plasma and serum are not necessarily in parallel ratios. In a kidney transplant patient (KN) who showed a rapid adsorption (Phillips et al 1988) of cyclosporin within 3 h, the content of metabolites in blood was retained at higher level even in elimination phase when cyclosporin clearly decreased, particularly in plasma.

Many efforts have been devoted to establish reliable measures for therapeutic monitoring of cyclosporin. For development of cyclosporin radioimmunoassay, high specificity of anti-cyclosporin antibody seemed to be an absolute necessity and cyclosporin metabolites in blood have been almost abandoned during therapeutic monitoring. Although the complete profile of cyclosporin metabolites in blood has not been elucidated, it is more than conceivable, as Christians and Sewing (1993) and others have claimed, that cyclosporin metabolites may contribute to cyclosporin pharmacology and toxicity, and high metabolite blood concentrations in patients should not be tolerated. The present study provided, by gel chromatographic analysis, additional examples to prove that concentrations of cyclosporin metabolites in blood compartments may not be deduced or inferred simply from those of cyclosporin.

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