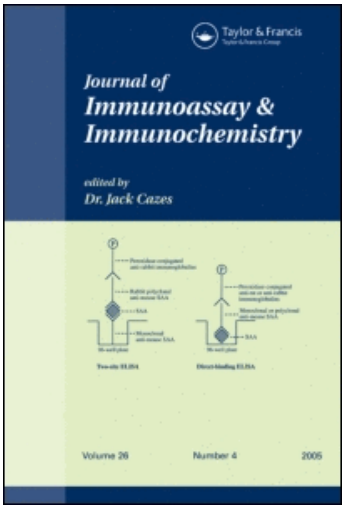

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A RADIOIMMUNOASSAY TO MEASURE CYCLOSPORIN A IN PLASMA AND SERUM SAMPLES

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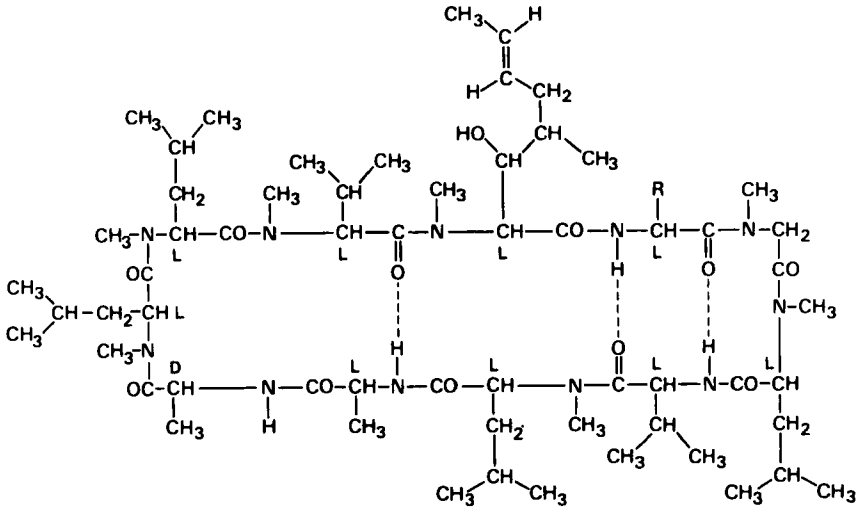
ABSTRACT

A radioimmunoassay for the immunosuppressant drug Cyclosporin A has been developed which makes possible the monitoring of the drug by direct measurements in clinical plasma and serum samples.

The antisera have been produced in rabbits using the hemisuccinate derivative of a structural analogue of Cyclosporin A as a hapten. The assay has both adequate specificity and sensitivity for Cyclosporin A to be suitable for the routine monitoring of therapy. Some degree of cross-reactivity has been shown to occur with four metabolites which were isolated from urine samples.

INTRODUCTION

Cyclosporin A (CyA) is a new fungal cyclic oligopeptide of 1203 daltons (1), which was found to be a powerful immunosuppressant in man (2). It is not myelotoxic, but in clinical studies some evidence of reversible nephrotoxicity has been reported (3). Such side-effects could possibly be circumvented if the dose regimen were individually adjusted for each patient by monitoring his plasma levels (4). Such a procedure could also be valuable to optimize the drug's immunosuppression in order



R = CH_2-CH_3 Cyclosporin A
= $CH(OH)CH_3$ Cyclosporin C

Fig. 1 Structural Formula of Cyclosporin A and Cyclosporin C

to achieve a maximal graft survival. For these purposes, a radioimmunoassay for CyA without any extraction steps was developed.

CyA and its structural analogues are not immunogenic. In order to produce antibodies they must first be coupled to carrier proteins to form immunogenic products. Since CyA itself does not possess a functional group which allows such a linkage, Cyclosporin C (CyC), which contains the amino acid threonine with an esterifiable hydroxy group and in addition is structurally similar to CyA (Fig. 1), was used as the hapten (5). In this paper the procedure for the RIA, its detection limit, specificity and reproducibility are described.

MATERIAL AND METHODS

Preparation of the Protein Conjugate

CyC-hemisuccinate was synthesized from CyC with succinic acid anhydride in a mixture of pyridine and 4-dimethyl-aminopyridine according to the procedure described by R. Traber (5).

1 mg CyC-hemisuccinate was dissolved in 1 ml 0.1M $NaHCO_3$ containing 10 mg guinea pig IgG. A total of 200 mg N-ethyl-N'-(3-dimethylaminopropyl)-carbodiimid dihydrochloride (Merck, Darmstadt) was added in three portions to the reaction mixture, which was kept at room temperature for 6 hours. The reaction mixture was then dialysed against 1 liter double distilled water, lyophilized and the crude conjugate stored at -20°C.

Production of Antisera in Rabbits and Estimation of the Titer

Groups of 2-3 New Zealand rabbits were immunized at approximately 3 week intervals for 3 to 4 months. 5 mg of the conjugate were emulsified in a solution composed of 0.5 ml saline, 1 ml Freund's complete adjuvant and 1 ml 2% Alugel (Serva). Approximately 1 ml of the emulsion was injected intramuscularly into the hind legs of each animal. The titer of the antisera, defined as the dilution of the antisera, which binds 50% of 0.5 ng ^{3}H-tracer, was measured from the second month of immunisation onwards. The crude antisera were stored at -20°C

^{3}H-Tracer

Tritiated Dihydro-CyA was used as radiolabel. Material with a specific activity in the range of 30-58 Ci/nmol was prepared by catalytic reduction of the olefinic double bond of

the β-hydroxy-ε,ζ, unsaturated amino acid of CyA (see Fig. 1) with tritium gas in dimethylformamide in the presence of 10% palladium on charcoal. The chemical and radiochemical purity of the ^{3}H-Dihydro-CyA (> 98% for all batches used in the RIA procedure) as well as its identity with the unlabeled reference compound were checked by HPLC, TLC and combined ^{3}H NMR/^{1}H NMR spectroscopy. About 60% of the tritium was attached to the ε,ζ, carbon atoms and 40% to the terminal methyl group.

Radioimmunoassay

Preparation of Solutions

Buffer:

For the binding experiments of 0.05 m TRIS buffer pH = 8.5 (TRIZMA -8.5, Sigma) containing 0.03% Tween® 20 (Merck) was used.

Antiserum Solution:

The original serum containing the antibody was diluted approx. 1:100 with TRIS-buffer (50 - 60% binding of ^{3}H-tracer).

^{3}H-Tracer Solution:

The stock solution of the radiolabel was first diluted in absolute ethanol to give a ^{3}H-tracer concentration of approx. 45 ng/ml (activity:≈2 μCi/ml). This solution was stored at -20°C for several months. Before use, this ethanolic tracer solution was diluted 1:10 with TRIS-buffer, which was supplemented

with 10% v/v control serum (for example, Validate®, General Diagnostics) to give a final concentration of approx. 43,000 dpm/0.1 ml.

Standard Solution and Clinical Samples:

0.2 ml of a solution containing 100 µg/ml CyA in absolute ethanol (Merck) was added to 4.8 ml of control serum under vigorous stirring. This solution was diluted stepwise with control serum to give standard stock solutions of 4000, 2000, 1000, 500, 250, 125 and 62.5 ng/ml CyA, which were stored in minimum aliquots of 50 µl at -20°C for months. Before use, these stock standards were diluted 1:50 with TRIS-buffer. To analyse clinical samples of very high or very low CyA concentrations, other standard solutions and sample dilutions should be prepared in an appropriate way.

Assay Procedure

The assay was performed with two different procedures (A and B) to show the possibility of adaptation to the needs of the investigator.

Procedure A:

The assay was carried out in 10 x 65 mm polystyrene tubes. Duplicate or triplicate aliquots of the standard solutions, or clinical samples, both containing equal amounts of serum or plasma (e.g. 2 µl/0.1 ml), were mixed with buffer, antiserum solution and ^{3}H-tracer solution. The tubes were incubated at 20°C for two hours, and then placed in a water

TABLE 1

Assay Flow Sheets in ml for procedure A and B

Tube	Control Serum Solution 1	Standard Solution 2	Sample Solution 3	Buffer A	Buffer B	Tracer Solution	Antiserum Solution
T	0.1	-	-	0.7	[1.2]	0.1	0.1
NSB	0.1	-	-	0.3	[0.8]	0.1	-
Bo	0.1	-	-	0.2	[0.7]	0.1	0.1
St	-	0.1	-	0.2	[0.7]	0.1	0.1
UNK	-	-	0.1	0.2	[0.7]	0.1	0.1

[ ] = volumes of procedure B
T = total radioactivity in solution
NSB = non-specific binding = radioactivity not absorbed by charcoal in the absence of the antibody
UNK = unknowns
St = standards
Bo = bound ^{3}H-tracer at zero dose of unlabeled CyA

bath of 4 - 7°C for 15 minutes. After this, charcoal suspension was added (see separation procedure).

Procedure B:

This assay procedure, which has been shown to be ideal for large numbers of samples, was carried out in 13 x 75 mm polystyrene tubes using different volumes of the same solutions as in procedure A. The tubes were incubated at 7°C over-night (18 - 20 h). In Table 1 the assay flow sheets for procedures A and B to estimate the binding of standards and clinical samples, non-specific binding (NSB), maximum binding (Bo) and "totals" (T) are summarized. Solution 1, 2 and 3 may contain 2, 5 or 10 µl serum/0.1 ml but the amount of serum in all 3 solutions must be identical.

Separation of Free CyA from Antibody-Bound CyA

The separation of free CyA from antibody-bound CyA for procedure A and B was performed identically, using a freshly prepared charcoal suspension which contained 1 g charcoal (Merck, No. 2186) in 100 ml TRIS-buffer without TWEEN 20 but supplemented with 0.5 % control serum. 0.5 ml of the charcoal suspension, which was kept under constant stirring in an ice-water bath, was added to the cold incubates and vortexed. The incubates were kept at 4°C - 7°C for 10 minutes and were then centrifuged at 2500 g for 5 minutes at the same temperature. 0.5 ml of the clear supernatant of the procedure A incubate, containing the bound ^{3}H-tracer, or 1.0 ml of the procedure B incubate, were pipetted into vials with 5 ml scintillation fluid and then measured in a β-counter. The radioactivity of the samples was measured either for 2 minutes or until 10 K was counted.

Calculation of Results

The relative binding was calculated according to the following formula:

$$\text{(per cent)} \quad {}^{B}/_{B_o} = \frac{\text{dpmB} - \text{NSB}}{\text{dpmBo} - \text{NSB}} \times 100$$

The concentrations of CyA in clinical samples were calculated from standard curves by use of the unweighted logit/log linearisation method (graphically or by computer).

RESULTS

Antiserum Titer

In order to set up standard curves the dilution of antiserum which results in a 50 - 60 % binding of 0.45 ng ^{3}H-tracer (approx. 43,000 dpm/incubate) was determined.

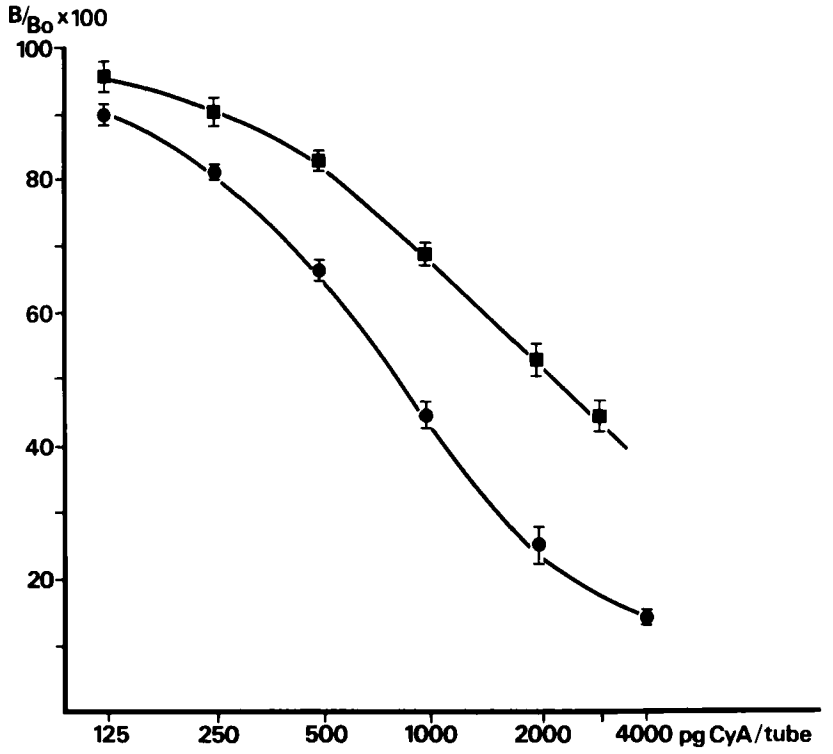


Fig. 2

Mean values ± SD from 5 standard curves carried out with antiserum 3 (0.8 µl/incubate) according to procedure A ■—■ and with antiserum 2 (1.2 µl/incubate) according to procedure B ●—●

This per cent of binding was achieved for all the antisera tested until now by using 0.5 to 1.5 µl of antiserum per incubate.

Performance and Detection Limit

Standard curves with antisera 2 and 3, and data from a typical standard curve performed with antiserum 2 are illustrated in Fig. 2 and Table 2. The lowest concentrat-

TABLE 2

Example of a Standard Curve Performed According to Procedure B with Antiserum 2

Tube	Sample	dpm	dpm - NSB	CV	%T
1 - 3	T	27026	-	0.9	100
4 - 4	Bo	17606	16973	1.5	62.8
7 - 9	NSB	633	-	3.6	2.3
	B Standard pgCyA/tube				$\frac{B}{Bo}$ x100
10 - 12	125	15694	15061	0.7	88.7
13 - 15	250	14283	13650	0.6	80.4
16 - 18	500	11648	11015	0.5	64.9
19 - 21	1000	7859	7226	1.2	42.6
22 - 24	2000	4764	4131	0.9	24.3
25 - 27	4000	2728	2095	3.1	12.3

T = total free radioactivitiy
Bo = maximum binding
NSB = non-specific binding
CV = coefficient of variation in %
B = binding of different CyA concentrations

ion of CyA which could be distinguished from "maximum binding values" (Bo) was calculated for antiserum 2 (5 standard curves on different days) to be at 91.3 % B/Bo (7). This represents a minimum detectable amount of 118 pg/assay tube and corresponds to 59 ng/ml (2 µl serum sample aliquot). If necessary, the detection limit of the assay could be decreased to 20 ng/ml if 5 or 10 µl serum aliquots were used in the standard solutions containing multiples of 125 pg CyA. Clinical samples with very high CyA-levels had to be prediluted with control serum.

TABLE 3

Intra- and Interassay Variations

Sample	Procedure	AS	n	Intraassay mean ± SD ng/ml CyA	Interassay mean ± SD ng/ml CyA
1	B	2	9	113.6 ± 10.2	123.7 ± 10.1
2	B	2	9	523.7 ± 19.6	586.0 ± 59.5
IS	A	3	9	500.0 ± 55	510.0 ± 65

IS = internal CyA-standard
AS = antiserum
n = number of measurements
1,2 = pooled clinical samples "low" and "high -level" CyA

Precision

The intra- and interassay variations were assessed by including pooled clinical samples with two different CyA plasma levels and serum samples, with a given CyA concentration (500 ng/ml) as quality controls in routine analytic work. The variations obtained from a series of tests are summarized in Table 3. The coefficients of variation were 3.7 - 11 % and 8.2 - 12.7 % from intra-assay and interassay analysis, respectively. These values were in the usual range of radioimmunoassays.

Specificity

The specificity of the antisera was determined by crossreactivity studies using Dihydro-CyA which was used in its tritiated form as the tracer and 4 CyA-metabolites which had been isolated from urine samples. Results obtained with antiserum 2 and procedure B are shown in Table 4. The crossreactivity was in the same range when antiserum 2 was used.

TABLE 4

Compound	% cross-reactivity	
	to displace 30% ^{3}H-tracer	to displace 50% ^{3}H-tracer
CyA	100	100
Dihydro-CyA	100	100
Metabolite 1	13	15
Metabolite 8	2	3
Metabolite 17	60	55
Metabolite 21	<1	<2

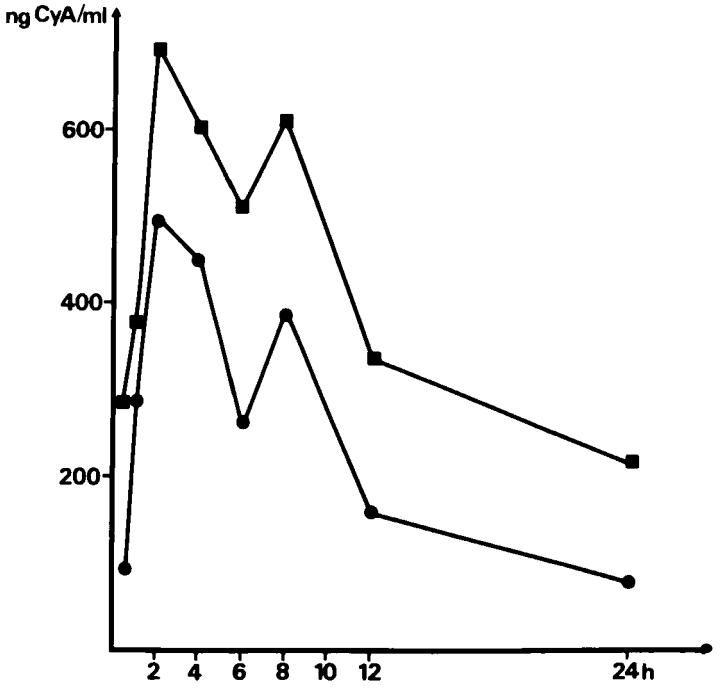


Fig. 3

Plasma concentrations of CyA in a patient (P.F.) after 35 daily doses of 12.5 mg/kg.

- ● CyA plasma concentrations determined by HPLC
- ■ CyA plasma concentration determined by RIA (antiserum 2, procedure B)

For each metabolite the percent cross-reactivity was measured under assay conditions at various metabolite concentrations. The cross-reactivity was defined as $x/y \cdot 100$, where x represents the amount of CyA and y the amount of the different metabolites which are required to displace 30 and 50% of the ^{3}H-tracer.

Comparison of RIA with HPLC Measurements

The plasma concentrations of CyA in a patient were measured by a HPLC technique recently published by Niederberger et al (6), and compared with those obtained with the RIA procedure described. The pharmacokinetic profiles determined with these two techniques are illustrated in Figure 3.

DISCUSSION

We have shown that by using CyC-hemisuccinate as the hapten, antibodies can be produced in rabbits which cross-react with CyA. This, together with similar binding and displacement characteristics of the radioligand ^{3}H-Dihydro-CyA and CyA, made the development of a radioimmunoassay procedure possible. The method is rather simple with a small intra- and interassay variation of about 10%. The conjugate which was chosen to produce antibodies was expected to yield a rather broad specificity. This was confirmed by the fact that the antibodies crossreact with various metabolites of CyA. Of the four metabolites tested, three showed rather low cross-reactivity compared with CyA. However, a slightly more polar metabolite than the parent drug - metabolite 17 - showed a cross-reactivity of 50 - 60%. This would explain why plasma concentrations determined by the radioimmunoassay are occasionally higher than those determined by HPLC, where only the parent drug is

measured. Further information on the regions in the Cyclosporin molecule critical for the interaction with the antibody can only be given when the structures of these metabolites have been elucidated. Even though, the rations between the CyA values obtained by these two different techniques obviously change as a function of time after drug administration, the pharmacokinetic profiles determined are nevertheless quite similar. The detection limit of the RIA, which is in the range of 20 - 60 ng/ml at present, is certainly adequate to determine pharmacokinetic profiles or to monitor plasma CyA-levels in patients. Preliminary data from different series of human plasma samples indicate that the peak concentrations of CyA are reached 2 - 6 hours after drug administration.

Although two different assay procedures (A and B) have been described, we suggest that each investigator perform the assay strictly according to one of the procedures, and to keep all conditions constant. It should be stressed that the characteristics of the standard curves as demonstrated in Fig. 2 are determined by the antiserum used and not by the procedure chosen.

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REFERENCES

1) Rüegger, A., Kuhn, M., Lichti, H., Loosli, H.R., Huguenin, R., Quiquerez, C., von Wartburg, A. Cyclosporin A, ein immunosuppressiv wirksamer Peptidmetabolit aus Trichoderma polysporum (Link ex Pers.) Rifai. Helv. Chim. Acta 1976; 59: 1075.

2) Calne, R.Y., White, D.J.G., Thiru, S., Evans, D.B., McMaster, P., Dunn, D.C., Craddock, G.N., Pentlow, B.D., Rolles, K. Cyclosporin A in patients receiving renal allografts from cadaver donors. The Lancet 1978; II: 1323.

3) Calne, R.Y., Rolles, K., Thiru, S., McMaster, P., Craddock, G.N., Aziz, S., White, D.J.G., Evans, D.B., Dunn, D.C., Henderson, R.G., Lewis, P. The Lancet 1979; II: 1033.

4) Calne, R.Y. Immunosuppression in clinical organ grafting. Trends in Pharmacol. Sci. 1978; 1: 21.

5) Traber, R., Kuhn, M., Rüegger, A., Lichti, H., Loosli, H.R., von Wartburg, A. Die Struktur von Cyclosporin C., Helv. Chim. Acta 1977; 60: 1247.

6) Niederberger, W., Schaub, P., Beveridge, T. High-performance liquid chromatography determination of Cyclosporin A in human plasma and urine. J. Chromatography 1980; 182: 454.

7) Rodbard, D., Statistical estimation of minimal detectable concentration ("sensitivity" for radioligand assays). Analyt. Biochem. (1978), 90, 1.