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An explanation of the noncorrespondence between assessment methods of cyclosporin

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Abstract. Various methods of determining cyclosporin (CyA) levels in patients after kidney transplantation were compared. These included polyclonal antibody (pcAb-), specific and nonspecific monoclonal antibody (S- and NmcAb-) radioimmunoassays (RIA), and high performance liquid chromatography (HPLC). The results obtained by the various methods when compared showed some correlation but did not correspond. A probable explanation for part of this noncorrespondence is the presence of monoclonally crossreactive metabolites (CyA-M). Another reason was that the concentration of CyA in the standards supplied with the RIA kits was found to be higher than stated.

Key words: Cyclosporin determination in kidney transplantation - Cyclosporin determination radioimmunoassay, HPLC - Radioimmunoassay of cyclosporin - HPLC of cyclosporin.

Cyclosporin A (CyA) is widely used in transplantation as an immunosuppressive agent specific for T lymphocytes [1]. The efficacy of CyA immunosuppression is dependent upon the maintenance of a dosage within the therapeutic window sufficient to prevent rejection yet low enough not to be nephrotoxic. To accomplish this it is necessary to monitor CyA levels in patients' blood [5, 13]. CyA assay methods can be divided into two categories, namely, those that measure CyA alone and those that measure CyA and CyA metabolites (CyA-M). High performance liquid chromatography (HPLC) is one of the methods that measures only CyA, while the radioimmunoassay (RIA), which uses specific CyA monoclonal antibody (SmcAb), is also reported to be specific [10, 11]. The RIA method that uses polyclonal Ab (pcAb) or nonspecific monoclonal Ab (NmcAb) measures CyA as well as many of its metabolites [4, 10].

In this paper we compare and correlate various RIA methods with the HPLC method, and from our results we propose possible reasons for noncorrespondence between the different assays as well as between centres.

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Materials and methods

Samples

Centre A provided 50 frozen samples of ethylenediaminetetraacetate (EDTA)-treated whole blood, taken at random from postrenal transplant patients, together with single results of their pcAb-RIA assessment, ranging between 150 and 1350 ng/ml CyA.

Reagents and standards

Organic solvents were of HPLC grade or highest purity available. Methanol was obtained from Burdick and Jackson (Muskegon, Mich) and ethanol (Lichrosolv) from E Merck (Darmstadt, FRG). n-Hexane, heptane, isopropyl alcohol, and acetonitrile, as well as Sep-Pak cartridges and Millex filters were supplied by Millipore (Bedford, Mass). HPLC-grade water was prepared from reagent grade water (Elgastat UHQ, Bucks, UK) using the Norganic filtration system of Millipore. All solvents were filtered through appropriate Millipore filters. Cyclosporin A and D were obtained from Sandoz (Basle, Switzerland), dextran sulphate (500 kD) from Sigma (St. Louis, Mo), and magnesium chloride from E Merck. The chemicals were of the highest purity. For HPLC determinations stock solutions of CyA (1 mg/100 ml methanol) and CyD (0.5 mg/ml methanol) were prepared. For RIA the CyA standard provided with the kit was used. In the execution of the RIAs, the polyclonal "ciclosporin RIA-Kit" and

the "Sandimmun kit" from Sandoz were used. Both the specific and nonspecific monoclonal antibodies from the Sandimmun kit were employed.

Instrumentation and detection procedure

Chromatography. An LKB HPLC system (Bromma, Sweden), consisting of a solvent conditioner (model 2156), a pump (model 2150), and a controller (model 2152), was used. A variable wavelength monitor (Shimadzu SP-6AV, Kyoto, Japan) was used at 215 nm with a sensitivity setting of 0.01 AUFS to detect CyA. Peaks were measured at 0.5 s intervals and recorded by a Kipp and Zonen recorder (model BD 40 obtained from Delft, The Netherlands) at a chart speed of 5 mm/min.

Two column systems were used. One consisted of a 250 × 4.6 mm (5 µm) prepacked Lichrosorb-5-Si60 column obtained from Phenomenex (Rancho Palas Verdes, Calif). The isocratic mobile phase used at ambient temperature with this column was n-hexane: ethanol (85/15) and the flow rate 1 ml/min. The other system consisted of an LKB reverse phase C-18 column (Lichrosorb RP-C18; 5 µm particle size) used at 65°C together with a mobile phase of acetonitrile: water (75/25) at the same flow rate.

Scintillation counting. Samples containing ^{3}H-CyA (1 ml/5 ml Aquagel 1; ChemLab, Pinegowrie, South Africa) were counted for 10 min by a Packard New Tri-Carb Model 300C (Hewlett-Packard, Calif) liquid scintillation spectrometer.

Experimental procedures - liquid chromatography

Two extraction procedures were used. The one used in conjunction with the Si60-column system is based on the adsorption method described by Shibata et al. [16]. Briefly, 1 ml trough level hemolysed whole blood, obtained by freezing and thawing, was treated with 1 ml 0.1 m*M* dextran sulphate containing 50 m*M* $MgCl_2$ after being spiked with 100 µl CyD as internal standard. Before vortex mixing, 5 ml n-hexane was added to extract the cyclosporin. After 1 min of mixing, the phases were separated by centrifugation at 2000 *g* for 5 min at 20°C. The hexane extract was evaporated under airflow at 100°C. The residue was dissolved in 500 µl mobile phase and injected into the HPLC column via a 100 µl injection loop connected to a Rheodine sample injector. The peak height CyA/CyD ratio of the sample was computed and the CyA level deduced from a standard curve. The standard curve was compiled by adding separate known CyA concentrations plus the internal standard CyD to EDTA-treated normal whole blood, which was then subjected to the above procedures of extraction and chromatography. The CyA/CyD ratios obtained from the profile were plotted against their corresponding CyA values.

The other extraction procedure was a modification of the reverse phase method of Charles et al. [3], which was used in conjunction with the C-18 RP-column system [17]. Briefly, 1 ml of trough level whole blood was mixed with 2 ml methanol and spiked with CyD, mixed and centrifuged at 2000 *g* for 10 min at 20°C. The pellet was re-extracted with 1 ml 50% methanol in H_2O. The combined supernatants were transferred onto a Sep-Pak cartridge attached to a Millex SV-filter, which was prewashed with 10 ml methanol, followed by 10 ml water. The cartridge was flushed with 10 ml 70% methanol in H_2O before CyA-CyD were eluted with 2 ml 50% isopropanol in heptane. The sample was then subjected to RP-HPLC after the eluate was evaporated and the residue redissolved in 500 µl mobile phase.

Radioimmunoassay

Kit reagents were prepared as per instruction pamphlets for Sandimmun [14] and ciclosporin-RIA kits [15]. The procedure followed were also those described in the pamphlets. In short, for the NmcAb-RIA and SmcAb-RIA, 50 µl trough level lysed whole blood was added to 950 µl methanol, mixed and centrifuged at 1600 *g* for 5 min at 4°C. A 50 µl aliquot of the supernatant was used in the assay concerned. Samples were prepared in duplicate and each duplicate assayed in triplicate. Each standard was also assayed in duplicate. For the pcAb-RIA, 50 µl trough level lysed whole blood was added to 2.45 ml 50 m*M* Tris-HCl kit-buffer pH 8.5 containing 0.003% Tween 20, and 100 µl of this dilution was used in the assay.

Comparison of standards

The standard from the mcAb-RIA kit was subjected to HPLC to assess whether the variations observed between results obtained by the two methods could be attributed to differences inherent in the CyA standard. A series of standards was made from the CyA standard, included in the mcAb-RIA kit, in normal EDTA-treated whole blood spiked with CyD, which was then subjected to the Shibata method used for HPLC. The resultant CyA and CyD peak height ratios were read from the HPLC standard graph. These values were plotted against the stated concentrations of the mcAb kit standards.

To confirm the results, the reverse experiment was performed on the HPLC standards. A series of the pure CyA standards used for HPLC was prepared in normal EDTA-treated whole blood and subjected to the S- and NmcAb-RIA described above. The resultant concentrations were obtained from a RIA standard curve constructed using the RIA standards and method included in the kit and plotted against the real values.

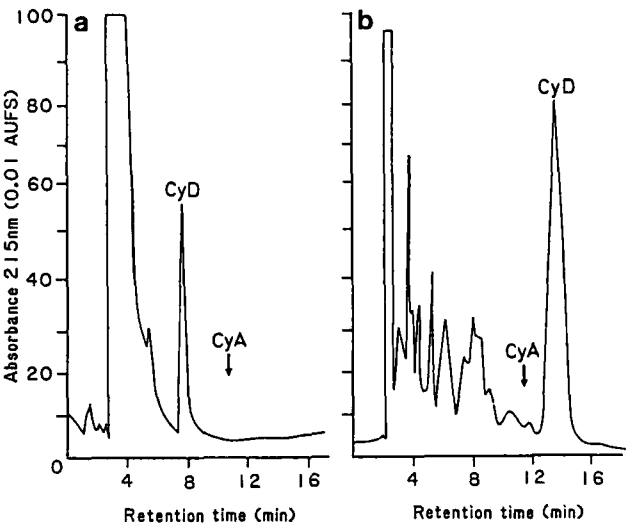

Fig. 1. High performance liquid chromatography (HPLC) profiles of human EDTA-treated whole blood spiked with cyclosporin D (CyD): **a** adsorption HPLC performed on Lichrosorb-Si60 column; **b** reverse phase HPLC performed on LKB-RP-C18 column

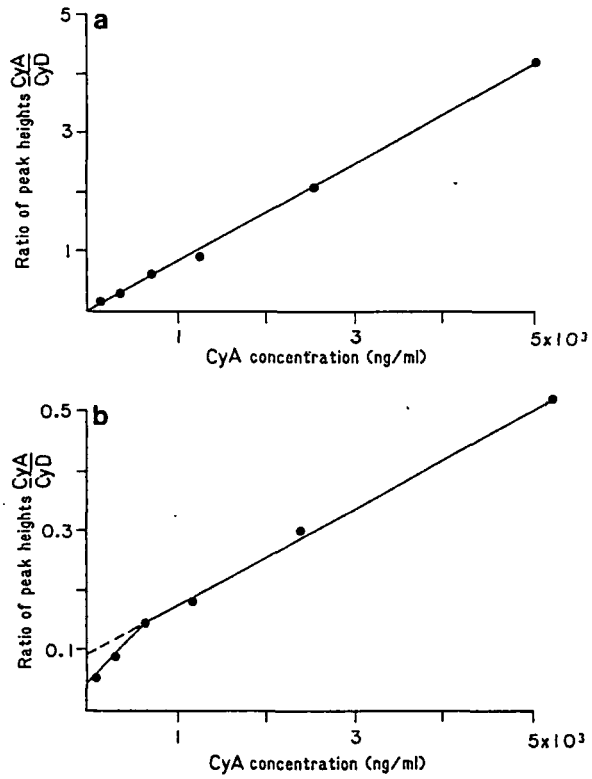

Fig. 2. HPLC standard curves obtained from whole blood spiked with CyA and CyD. **a** Adsorption HPLC performed on Lichrosorb-Si60 column; **b** reverse phase HPLC performed on LKB-RP-C18 column

Results and discussion

Although HPLC measures only CyA, it is subject to interference by other compounds having retention times that are the same as, or so close to, those of CyA and CyD as to make peak quantification inaccurate, especially at lower cyclosporin levels. When unspiked and CyA-spiked whole blood from the same normal individual were treated using Shibata's method, the baseline was free of interfering peaks as compared to the profile obtained using Charles' method (Fig. 1). When the peak ratios were plotted against the CyA values, the Shibata data produced a straight line having no intercept, while the data from Charles' method gave an intercept and deviated from linearity (Fig. 2) because the interference was greater at lower levels of CyA. This made the allocation of the baseline less accurate (Fig. 1b), which, in turn, affected the peak measurements (Fig. 2b). The Shibata procedure, being closer to ideal, was therefore chosen as the reference method.

A comparison between centres using pcAb-RIA gave the best correlation (Table 2, column 5, row 4), along with the comparison between S- and NmcAb-RIA (Table 2, column 3, row 2). The correlation coefficients (r; Table 2) and the coefficients of variation (CV; Table 1) are comparable to those of Gibbons et al. [9]. To determine whether the r-values were statistically significant, the indices of determination (r^2) were calculated (Table 2). These r^2-values multiplied by 100 gave the percentage variation of y-values attributable to variation in x-values [2]. The CVs obtained showed a maximum of 20% variation within the x-values, so r^2-values of 80% and greater were significant, relating back to r-values of 0.89 and above. Using this criterion, only S- and NmcAb-RIA (Table 2, column 3, row 2) and the two centres using the pcAb-RIA (Table 2, column 5, row 4) truly correlated when a 10% tolerance within sample CVs (Table 1, column 1, row 1) was allowed. Table 2 shows that the RIA results did not correlate with HPLC. This was probably due in part to a reaction of the antibodies with crossreactive CyA-M, which we have previously shown to be inherent in the Sandimmun medication and which accumulate with time after transplantation [12].

When correspondence is good, the slope of the regression line should be unity and the intercept zero. In addition, the ratio should be unity and the difference of means zero. This held only for pcAb-RIA between centres (Fig. 3k). Intercepts are indi-

Table 1. Coefficients of variation (CVs) of different RIA methods and HPLC

	pcAb	SmcAb	NmcAb	HPLC
This report				
(between run)	9.8 (±1.9)[a]	18.5 (±4.4)	15.7 (±4.4)	5.0 (±0.5)
(within run)	7.1 (±1.5)	11.5 (±1.4)	13.8 (±2.4)	3.2 (±0.2)
Gibbons et al. [9]	19	17	-	5

[a] Mean (±SEM); $n=50$

Table 2. Percent indices of determination (r^2) and correlation coefficients (r) between different RIA and HPLC methods of measuring CyA

	HPLC	SmcAb	NmcAb	pcAb	Centre A[b]
HPLC	1	28 (0.66)[c]	61 (0.78)	64 (0.80)	59 (0.77)
SmcAb	52 (0.72)[a]	1	79 (0.89)	66 (0.81)	34 (0.58)
NmcAb	58 (0.76)[a]	41 (0.64)[a]	1	46 (0.68)	77 (0.88)
pcAb	45 (0.67)[a]	31 (0.56)[a]	46 (0.68)[a]	1	92 (0.96)

[a] Data published by Gibbons et al. [9]
[b] pcAb-RIA
[c] Correlation coefficients

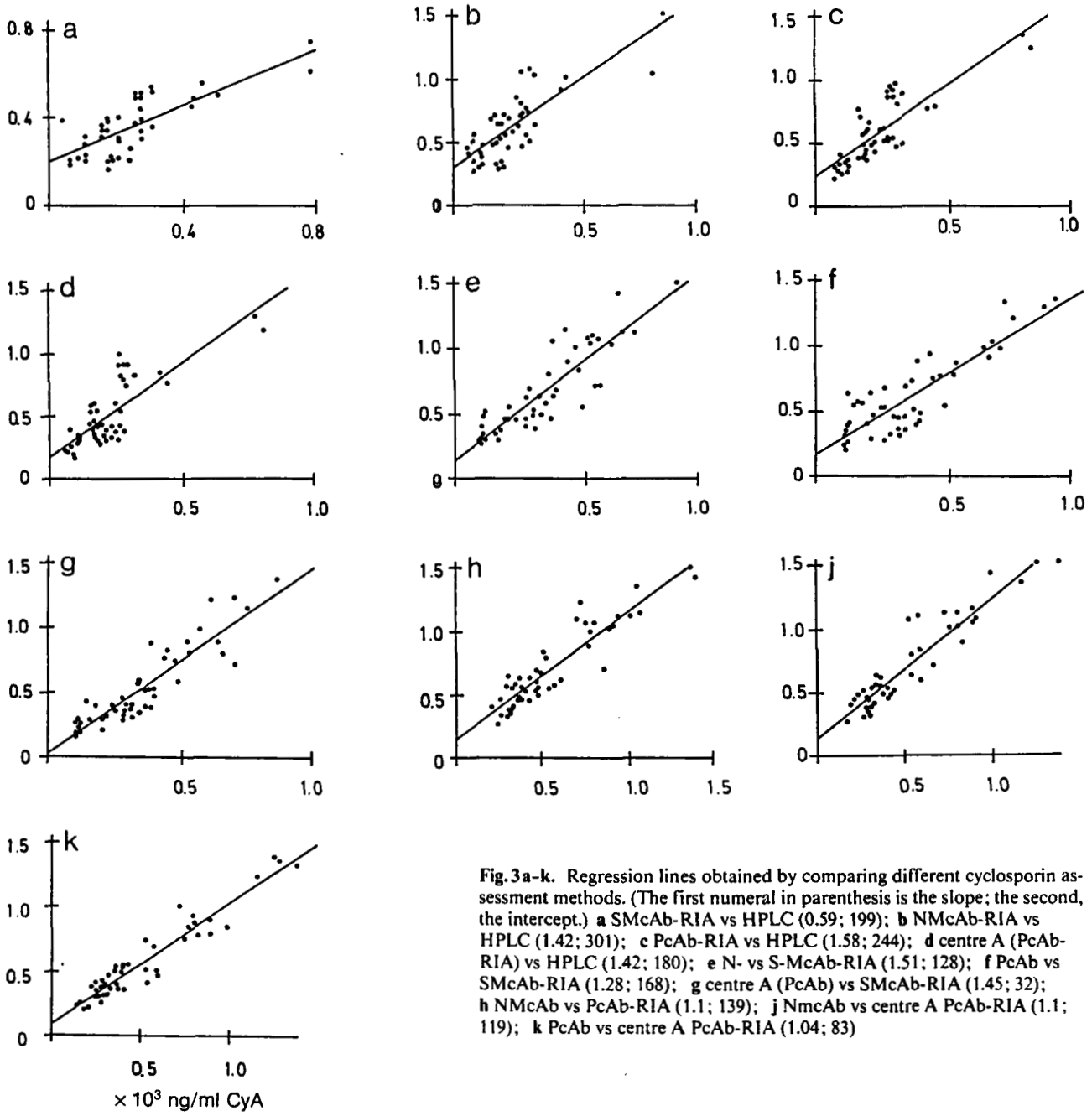

Fig. 3a–k. Regression lines obtained by comparing different cyclosporin assessment methods. (The first numeral in parenthesis is the slope; the second, the intercept.) **a** SMcAb-RIA vs HPLC (0.59; 199); **b** NMcAb-RIA vs HPLC (1.42; 301); **c** PcAb-RIA vs HPLC (1.58; 244); **d** centre A (PcAb-RIA) vs HPLC (1.42; 180); **e** N- vs S-McAb-RIA (1.51; 128); **f** PcAb vs SMcAb-RIA (1.28; 168); **g** centre A (PcAb) vs SMcAb-RIA (1.45; 32); **h** NMcAb vs PcAb-RIA (1.1; 139); **j** NmcAb vs centre A PcAb-RIA (1.1; 119); **k** PcAb vs centre A PcAb-RIA (1.04; 83)

cative of overestimation. The mean intercept values in Fig. 3 are actual, showing the amount by which one method would give a higher value for CyA than the other. The intercept obtained between centres using pcAb-RIA was close to zero, as expected. However, when their difference of means was analysed statistically, the variation was significant ($P<0.05$; Table 3, column 5, row 4). This agrees with results obtained by Frey et al. [6]. The difference might be attributed to some degradation of the CyA within the sample caused by repetitive freezing and thawing and a longer storage time. This protocol of handling could also account for the relatively high coefficients of variation as compared with those of Frey et al. [7].

Since the CyA values varied from patient to patient with each method used, the results had to be compared on a ratio basis, either directly (Table 4) or by using a common divisor (Table 3). The mean direct ratio of two methods showed possible correspondence but gave no indication as to whether the difference between them was statistically significant.

Table 3. Percent difference of means of RIA methods of measuring CyA with HPLC values as common divisor

	HPLC	SmcAb	NmcAb	pcAb	Centre A[a]
HPLC	-	61.38	199.57	182.32	134.64
P-value		0.05	0.01	0.01	0.01
SmcAb		-	138.19	120.94	73.26
P-value			0.01	0.01	0.05
NmcAb			-	17.25	64.93
P-value				NS	NS
pcAb				-	47.68
P-value					0.05

[a] pcAb-RIA

Table 4. Mean direct ratios of CyA concentrations from all patients assayed by different RIA methods and HPLC

	HPLC	SmcAb	NmcAb	pcAb
HPLC	1	1.58	1.98	2.83
(SEM)		0.17	0.18	0.16
SmcAb		1	1.30	2.08
(SEM)			0.08	0.18
NmcAb			1	0.71
(SEM)				0.04

When HPLC values were taken as a common divisor, the biometrical problem was overcome and non-correspondence could be assessed.

The difference in means between HPLC and SmcAb-RIA was significant at a *P*-value of 0.05 (Table 3, column 2, row 1). To explain this unpredicted finding, the standard used in the mcAb RIA was subjected to HPLC, and vice versa. The graph obtained by plotting concentrations read off the HPLC standard curve against actual concentrations of mcAb-RIA standard (Fig. 4) gave a slope of 0.82 and intercept of 188, comparable to the values obtained with HPLC vs SmcAb-RIA of patients' samples (Fig. 3 a and Table 3, column 2, row 1). Since the HPLC standard curve, prepared using pure CyA, gave a straight line through the origin and thus would not underestimate CyA concentrations, we conclude that the concentration of CyA in the standard of the Sandimmun mcAb-RIA kit is higher than stated. If the mcAb-RIA method was correct, the graph obtained by plotting the values of pure CyA assessed by mcAb-RIA against their actual concentrations should go through the origin (Fig. 5). However, it gave a negative intercept of 135, again indicating the possibility of a higher than stated concentration in the kit standard. This understatement would produce a shifted mcAb-RIA standard curve, leading to lower readings in patients' samples than those obtained using HPLC. However, the mcAb-RIA gave higher results than HPLC, due to the presence of crossreactive metabolites [4, 8]. A previous paper [12] showed that the Sandimmun medication has inherent monoclonally crossreactive metabolites and that CyA-M accumulate with time after transplantation. The net result of this crossreactivity and the understatement of mcAb-RIA standards would be a lower estimation by SmcAb- than by pcAb-RIA of the concentration of CyA in patients' blood but still an overestimation in comparison with HPLC.

In light of these results, we conclude that the introduction of an international CyA calibration standard would contribute greatly towards better reproducibility between centres and correspondence between methods.

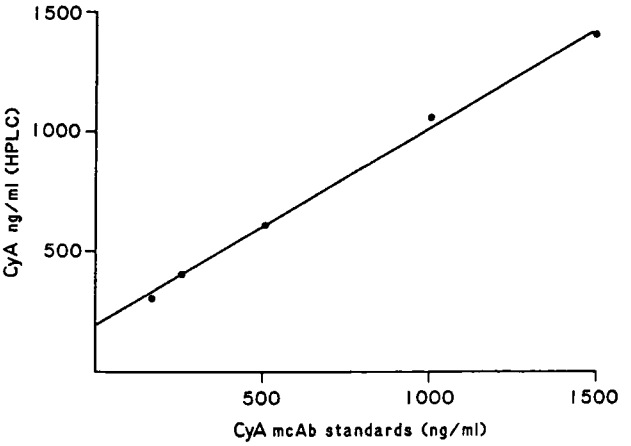

Fig. 4. Regression line obtained by plotting HPLC values against stated concentrations of CyA standards included in mcAb-RIA kit. Slope = 0.82; intercept = 188; $r = 0.96$

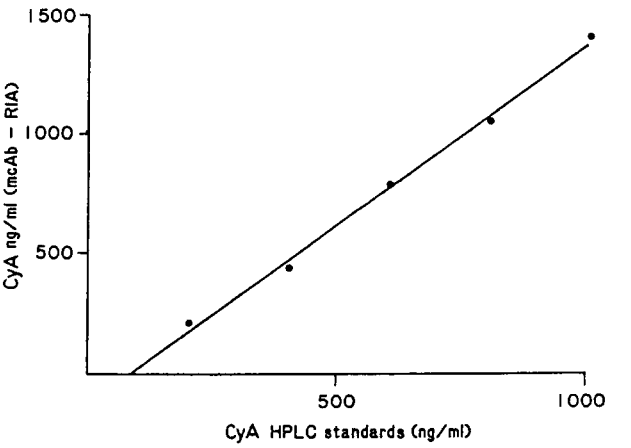

Fig. 5. Regression line from values obtained by mcAb-RIA using the kit's own CyA standard to construct the standard curve, plotted against actual concentrations of HPLC-pure CyA. Slope = 1.51; intercept = −135; $r = 1.0$

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