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High-performance liquid chromatographic method for therapeutic drug monitoring of cyclosporine A and its two metabolites in renal transplant patients

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Abstract

A novel fast HPLC method was developed for the determination of cyclosporine A (CyA) and its two metabolites M17 (AM1) and M21 (AM4N) in blood. Whole blood was precipitated with zinc sulphate, extracted with diethyl ether, evaporated, dissolved in aqueous methanol and partitioned twice with *n*-hexane. Chromatography was carried out using a microbore RP-column under isocratic elution with acetonitrile–methanol–water (200:80:140, v/v/v) at 70°C and a detector set at 205 nm. Linearity for all three compounds was tested in the range of 1–1000 ng/ml. Recovery was 97–109%, and a coefficient of variation was 1.6–8.8% depending on the particular compound and its concentration. The method was used for a group of renal transplant patients having an inadequate response to CyA therapy in order to evaluate the possible role of CyA and its metabolites on the occurrence of hypertension and other toxicological events.

Keywords: Cyclosporine A

1. Introduction

Cyclosporine A (CyA) (Fig. 1), is a commonly used immunosuppressive drug with a narrow therapeutic window. Hence, its monitoring is of great importance in order to optimize CyA level within a range that minimizes both the risk of rejection and drug-induced toxicity. Among methods used for the determination of CyA blood levels [1–4], only HPLC methods can provide levels of the main metabolites. Various consensus panels concluded that routine measurement of metabolites is not necessary [5,6]. However, all agreed as well that there are some circumstances when the levels of metabolites would be helpful and justified further studies aimed to explore the role of metabolites from a clinical point of view [6]. These situations include, e.g., persistence of CsA levels caused by metabolic disorders [7] or clinical complications associated with high levels of CsA metabolites [8]. The primary

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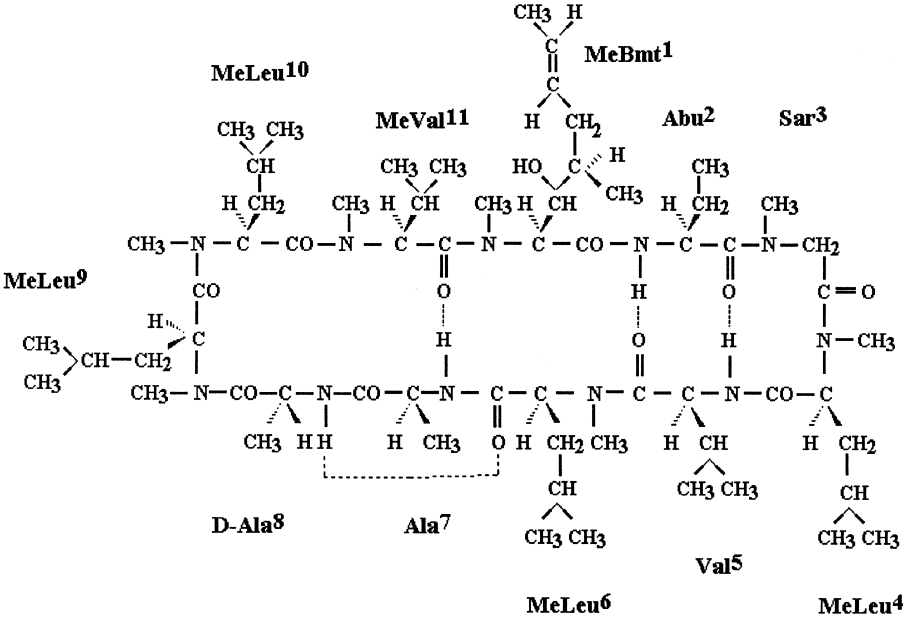

Fig. 1. Structure of cyclosporine A.

metabolites M17 (AM1), M1 (AM9), and M21 (AM4N), seem to be the most important ones from the clinical point of view with regard to their possible high concentration in some patients, to higher risk of nephrotoxicity and to metabolite/parent drug ratio [9,10]. A novel HPLC method has been developed in this work in order to evaluate whether these values can be individual for each renal transplant patient on a standard immunosuppressive therapy.

2. Experimental

2.1. Chemicals

Methanol of HPLC gradient grade, acetonitrile for HPLC and hydrochloric acid were obtained from Merck, water, *n*-hexane and diethyl ether, all HPLC grade, were from Sigma–Aldrich and Fluka Chemie, Cyclosporine A, an official USP 23 standard, and cyclosporine D (98%, HPLC), were from Galena (Czech Republic). Metabolites M17, dihydro-M17, M21, and M1 were obtained from Galena, their sources were semisynthesis [11], producing fungus [12], and dog liver, respectively, and their purity was above 98% (HPLC) in all cases. Since metabolites M1 and dihydro-M17 were available in limited amounts, they were used only for HPLC calibration and the determination of cross-reactivity of immunoassays. Control samples (cyclosporin monoclonal whole blood) 150, 400 and 800 ng/ml were obtained from Abbott. Protein precipitation reagent was prepared from 10% solution of zinc sulphate–acetonitrile–methanol (50:20:30, v/v/v). Stock solutions of CyA, CyD, and metabolites were prepared in methanol at concentration 1 mg/ml and were kept at −20°C.

2.2. Preparation of calibration standards

Standard samples of metabolites M17 and M21 in concentrations 50, 100, 250, 500, and 1000 ng/ml and 4-times concentrated solutions of cyclosporine A (200–4000 ng/ml) were prepared from stock solutions by dilution with methanol. A working solution of the internal standard (cyclosporine D) 4000 ng/ml was used. All standard samples in appropriate con-

centrations were added into glass test tubes (200 μl for metabolites and 50 μl for both CyA and CyD) and the solutions were evaporated. Then, 200 μl of whole blood from non-medicated volunteer were added to each standard sample.

2.3. Sample extraction

For measuring CyA and its two metabolites, 200 μl of whole blood and CyD as internal standard were used. The protein precipitation reagent (0.6 ml) was added [13], the sample was vortexed and, after centrifugation, the supernatant was extracted with diethyl ether (3 ml). After centrifugation, the upper ether layer was transferred into a clean tube and evaporated to dryness under a stream of nitrogen. The residue was dissolved in methanol (200 μl) and 0.1 mol/l HCl (100 μl) and was partitioned twice with *n*-hexane (1 ml). Methanolic layer was used for analysis.

2.4. HPLC equipment and conditions

Chromatographic equipment (TSP) consisting of isocratic pump SP 1500, autosampler AS 1000 and UV 1000 detector set at 205 nm were used. The glass microbore column (1×150 mm) with reverse octadecyl phase (Tessek, Czech Republic) was maintained at 70°C in column heater. Isocratic elution was carried out with the acetonitrile–methanol–water mixture (200:80:140, v/v/v). The initial flow-rate of the mobile phase was increased from 110 μl/min (first 10 min) to 130 μl/min at the end of analysis (40 min).

2.5. Patients

Nineteen renal transplant patients were under combined immunosuppressive therapy with Sandimmun Neoral® at a starting dose of 10 mg/kg per day. Simulect™-Novartis Pharma (chimaeric mouse–human monoclonal antibody against the α chain of the IL 2 receptor) was given at a dose of 40 mg daily on days 0 and 4 day after transplantation. Blood was collected into tubes with EDTA K3 (Dispolab) before administration of the morning dose of CyA, and was kept frozen at −20°C until analysis. Samples were taken before surgery and on days 1, 2, 3, 4, 5, 6, 7, 10, 14, at weeks 4, 6, 8,12 and at months 4, 5, 6, 9 (continued every 3 months). When the CyA level was out of the therapeutic range, the dose scheme was corrected. In all 458 patient samples (14–34 from each patient), the concentration of CyA was correlated with specific RIA assay (CYCLO-Trac SP Whole Blood, DiaSorin, Stillwater, MN, USA).

2.6. Specific RIA assay for CyA

Blood radioimmunoassay for cyclosporine, Cat. No. 23 000 (CYCLO-Trac SP-Whole, DiaSorin) was used. The blood samples (200 μl) were extracted with methanol (800 μl) and, after centrifugation, 50 μl of supernatant were incubated with 100 μl of [^{125}I]CyA (CYCLO-Trac) and 1 ml of well-mixed Anti-CYCLO Trac SP for 1 h. The bound radioactivity was measured using a γ-scintillation counter Multigama 1261 (LKB, Wallac). The calibration range of the RIA (specific) method was 10–1380 ng/ml. Sensitivity was about 10 ng/ml. Within and between assay precisions were 3.2 and 2.8%, respectively, and recovery 97–112%. Cross-reactivity for CyA metabolites was calculated as 1.7, 0.8, 0.6, and 18.3% for M1, M17, M21, and dihydro-M17, respectively.

3. Results

Least square calibration curves for CyA, M17 and M21 were constructed by plotting the peak area ratio of substances versus internal standard to standard's concentrations in the range of 1–1000 ng/ml. The correlation coefficients were 0.999 for CyA and M17 and 0.998 for M21. A chromatogram of standard sample is shown in Fig. 2B. When blood from an untreated volunteer was analyzed, no interfering peaks occurred, as can be seen from chromatogram 2A. Chromatogram 2C shows retention time and position of metabolite M1. Accuracy and precision characterized by an average recovery and coefficients of variation, respectively, are summarized in Table 1. Four tested levels were included (n=9–12). The limit of quantitation, defined as the lowest concentration that yields coefficient of variations less

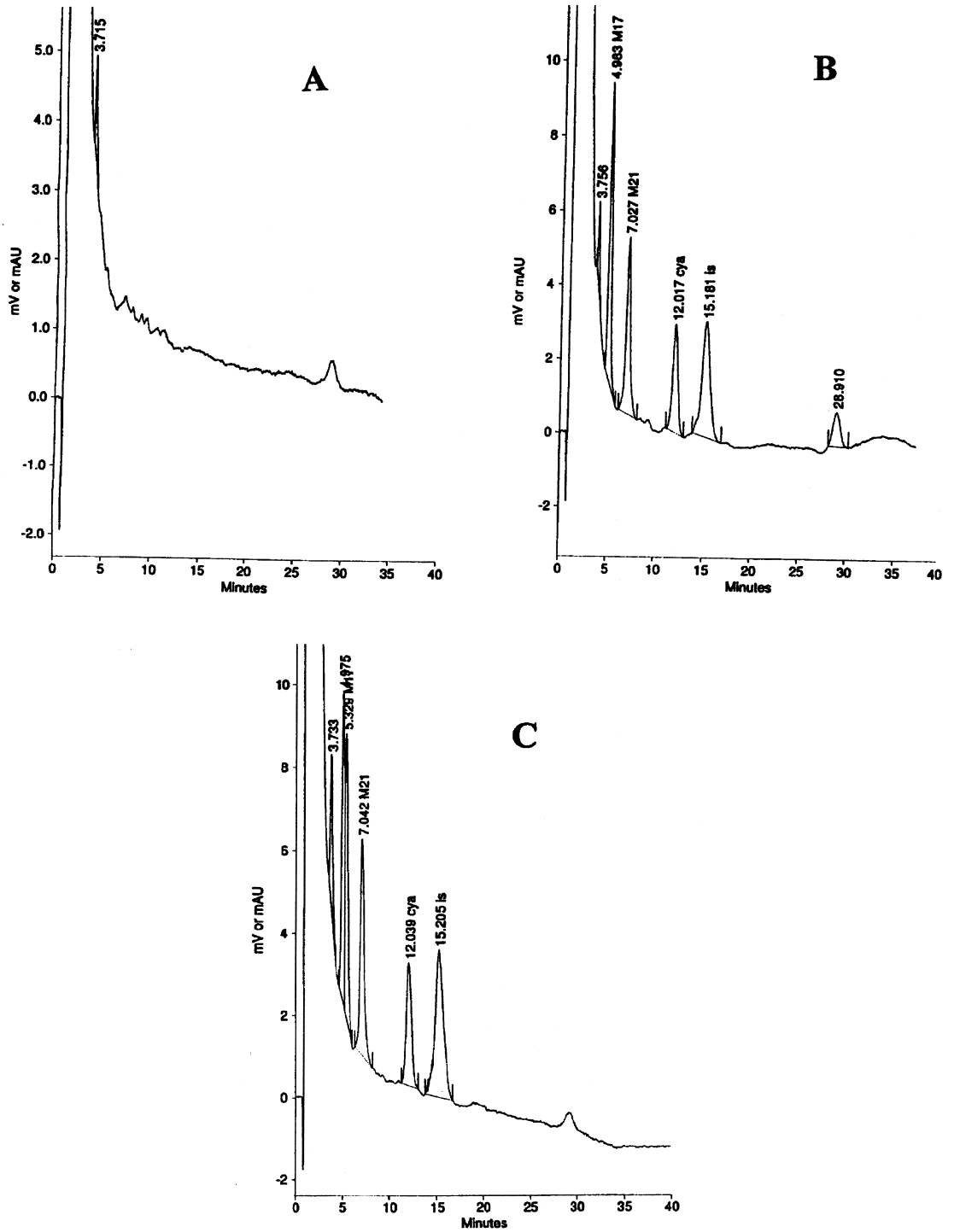

Fig. 2. Examples of chromatograms: (A) blood of untreated volunteer; (B) blood supplemented with 500 ng/ml of each of cyclosporins A and D (I.S.) and metabolites M21 and M17; (C) blood supplemented with 500 ng/ml of each of cyclosporins A and D (I.S.) and metabolites M21, M17 and M1.

Table 1
Accuracy and precision

Compound	Theoretical concentration (μg/l)	Measured concentration (μg/l)±SD	Precision (coef. of variation) (%)	Accuracy (recovery) (%)
M17	50	54.8±4.4	8.8	109.0
	100	100.4±4.4	4.4	100.4
	250	242.8±16.1	4.1	97.0
	500	512.5±13.2	2.6	102.5
M21	50	55.0±2.6	4.8	110.0
	100	101.6±2.7	2.7	101.6
	250	252.7±10.0	2.7	101.1
	500	500.4±8.2	1.6	100.0
CyA	50	52.1±3.5	6.7	104.3
	100	102.8±4.7	4.5	102.8
	250	252.0±9.0	3.6	100.8
	500	507.2±12.1	2.4	101.4

than 20% and accuracy between 80 and 120%, was determined as 25 μg/l for all compounds.

For each analytical run, QC samples with declared concentration of CyA (low, medium or high) were used. Between run precision was calculated for all QC samples (coefficients of variation, were 6.7, 3.7 and 2% for concentration 150, 400 and 800 ng/ml, respectively). A typical chromatogram with CyA and both metabolites (high M17 and low M21) is shown in Fig. 3A. A chromatogram with CyA, M17, M21 and peak, which was identified as M1 according to its retention time, is shown in Fig. 3B. This metabolite was present in samples of only six patients. Metabolite/parent drug ratios for M17 and M21 were calculated in all patients. While the level of M17 is high and individual mean ratio M17/CyA significantly differs in many patients, as is shown in Fig. 4, the level of M21 is low and the M21/Cy A ratio is stable in all patients, and in the range 0.05–0.13.

Results obtained by HPLC were correlated with specific RIA assay (CYCLO-Trac, DiaSorin) in 458 patient samples. Although both methods correlate very well and the equation of linear regression model is near to ideal ($RIA_{CyA}=0.967\times HPLC_{CyA}-2.698$; $r=0.981$), there is a significant difference ($P=0.05$) between them (Fig. 5). Using the method of Hollis [14] revealed that results obtained by HPLC are about 8% lower.

4. Discussion

Although there are more than 30 various cyclosporine A metabolites described in the literature [15], most of them lack any immunosuppressive activity and do not exhibit toxicity in in vitro assays. Moreover, in order to distinguish two possible clinical events, i.e., persistence of CsA high levels without metabolism [7] or accumulation of metabolites [8], it is useless to analyze all of them, but some of them can serve as suitable markers. We have chosen primary metabolites M17 (AM1), with regard to its high concentration in blood [16], some immunosuppressive activity [17], and relatively slow clearance [16], and M21 (AM4N) which can contribute to the toxicity accompanying the CsA therapy [18]. Metabolite M1 (AM9) was detected only in several patients, probably due to its much faster clearance compared to M17 [16]. Hence M1 is less important as a marker with regard to its possible accumulation as well as to its lower immunosuppressive activity [17].

Following this approach, a novel HPLC method has been developed using a microbore column. Cyclosporine A and its metabolites can be analyzed in a relatively short time with high linearity. The accuracy of the method was in the range 97–110% for all compounds, and the precision of the assay was expressed by within-day and between-day varia-

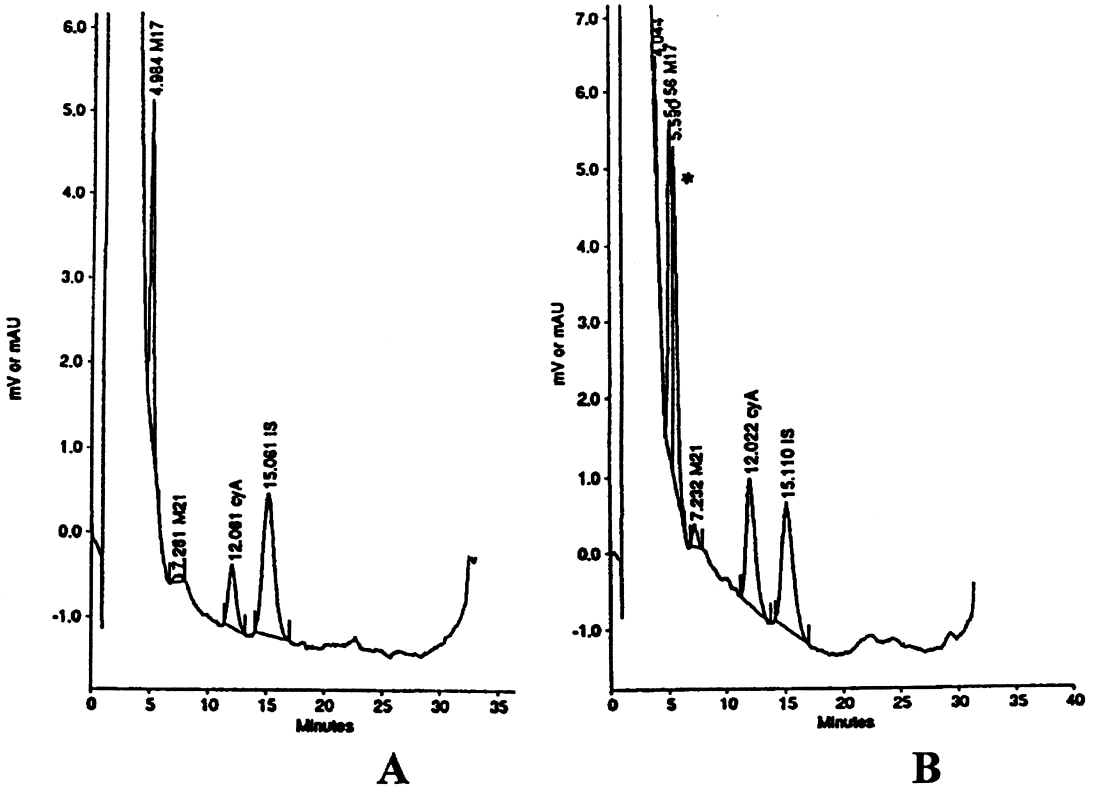

Fig. 3. Examples of chromatograms: (A) real sample CsA 218 ng/ml, M21 5 ng/ml, M17 492 ng/ml; (B) real sample with metabolite M1 (marked with asterisk), CsA 539 ng/ml, M21 12 ng/ml, M17 614 ng/ml.

tions between 1.6 and 8.9%. Several extraction procedures have been described for CyA including both liquid–liquid and solid-phase extractions [19–21]. In our method, a protein precipitation step preceding liquid–liquid extraction [13] and *n*-hexane cleaning was successfully used in order to remove any interfering substances [20]. Values obtained by HPLC were correlated with specific RIA method and were found to be about 8% lower. The over-estimate of the immunoanalytical method is well known and is in accordance with the published results [22].

The method was used to elucidate the relationship between CyA, and its metabolites M17 and M21, and the occurrence of toxicological events. The levels of M17 were usually higher than that of parent drug concentration, which is in accordance with the findings of other authors [19], and the ratio M17/CyA was highly variable. In contrast, the levels of M21 were low and the ratio M21/CyA was stable and low in all patients. Since M1 is typically encountered in some animal models [23], its occurrence can also reflect individual differences in activity, involvement, and/or amount of cytochromes in some patients [24,25]. Kahan et al. [26] demonstrated that the determination of pre-transplant pharmacokinetics and of individual metabolism makes it possible to prepare the post-transplant dosing scheme more precisely and positively affect the result of transplantation.

Assay of main metabolites of CyA in renal transplant patients on standard immunosuppressive therapy may provide further insight into the role of these metabolites, and contribute to the theory about lower tolerance of high metabolite blood concentrations in patients [27]. So this method may be used for TDM of patients with inadequate response to

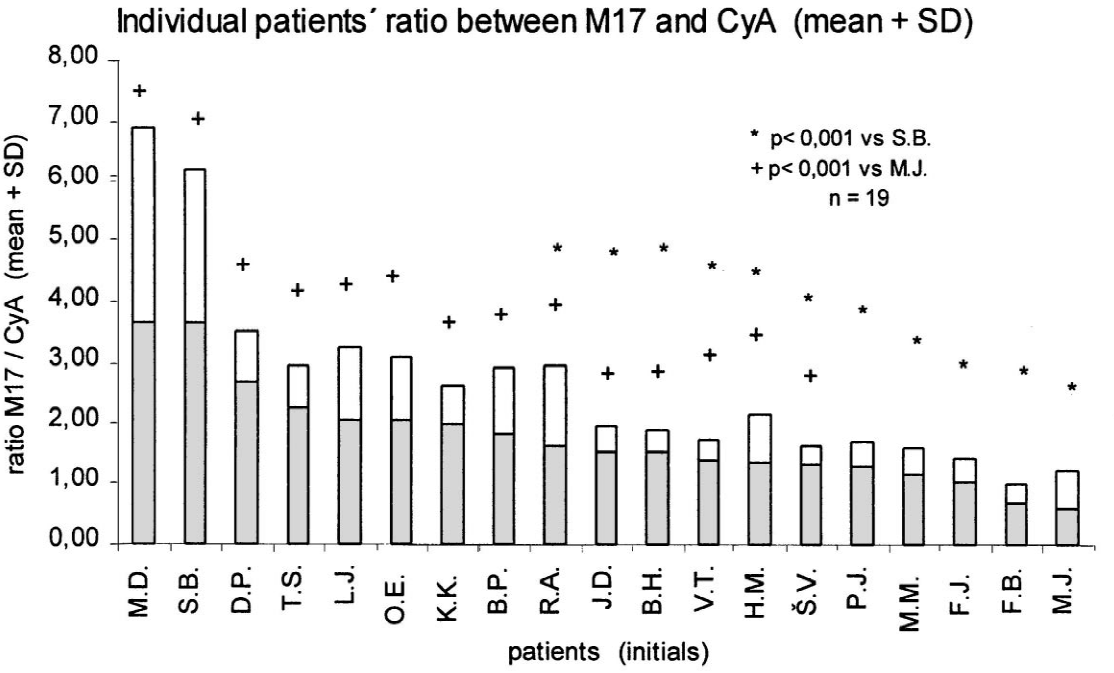

Fig. 4. M17/CyA ratio in individual patients: statistical significance is expressed against the highest (*) and the lowest (+) value.

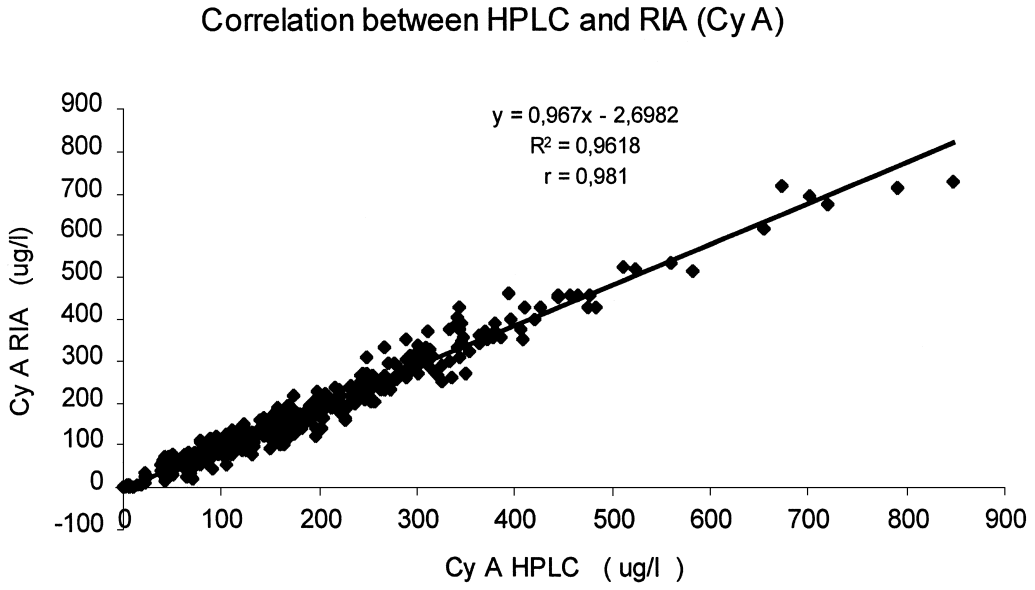

Fig. 5. Correlation between HPLC and specific RIA method $y=0.967x-2.698$ ($r=0.981$).

CyA therapy, and may help to elucidate the possible role of metabolites in the occurrence of hypertension and other toxicological events.

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