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Drug Monitoring and Toxicology

Performance and Specificity of Monoclonal Immunoassays for Cyclosporine Monitoring: How Specific Is Specific?

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Background: **Immunoassays designed for the selective measurement of cyclosporin A (CsA) inadvertently show cross-reactivity for CsA metabolites. The extent and clinical significance of the resulting overestimation is controversial. A comprehensive assessment of old and new methods in clinical specimens is needed.**

Methods: **In a comprehensive evaluation, CsA was analyzed in 145 samples with the new CEDIA® assay and compared with the Emit® assay with the old and new pretreatments, the TDx® monoclonal and polyclonal assays, the AxSYM®, and HPLC. All samples were from patients with liver and/or kidney transplants.**

Results: **The CEDIA offered the easiest handling, followed by the AxSYM, which showed the longest calibration stability. The TDx monoclonal assay provided the lowest detection limit and the lowest CVs. The mean differences compared with HPLC were as follows: Emit, 9–12%; CEDIA, 18%; AxSYM, 29%; and TDx monoclonal, 57%. The CycloTrac® RIA paralleled the Emit results. In contrast to the mean differences, substantial (>200%) and variable overestimations of the CsA concentration were observed in individual patient samples. Metabolic ratios, estimates of the overall concentrations of several cross-reacting metabolites (nonspecific TDx polyclonal/specific reference method), correlated with the apparent biases of the various monoclonal assays. Metabolic ratios varied up to 10-fold, which translated into biases for individual samples between −7% and +174%. The higher the cross-reactivity of an assay was, the higher was the range of biases observed. The inter-individual differences markedly exceeded other factors of influence (organ transplanted, hepatic function).**

Conclusion: **Because assay bias cannot be predicted in individual samples, substantially erratic CsA dosing can result. The specificity of CsA assays for parent CsA remains a major concern.**

Regular monitoring of cyclosporin A (CsA)[1] in whole blood for dosage adjustment is considered mandatory. Despite the possible role of CsA metabolites in immunosuppression and toxicity *(1, 2)*, the consensus among clinicians and laboratorians is that specific methods for measuring the parent drug only should be used *(3–6)*. All assays utilizing monoclonal antibodies and designed for the selective measurement of CsA are usually labeled "specific". However, there is considerable debate about that specificity, its clinical relevance, and the comparability of results from different monoclonal immunoassays. A new pretreatment reagent for the Emit® (Emit-NPT) *(7)* and two new automated monoclonal immunoassays are presently being introduced: a fluorescence polarization immunoassay on the AxSYM® instrument from Abbott *(8)* and a cloned enzyme donor immunotechnique (CEDIA®) from Boehringer Mannheim.

Considerably different biases have been published and conflicting recommendations have been given concerning the replacement of HPLC by a certain assay, particularly for patients with hepatic dysfunction and those undergoing heart (HTx) or liver (LTx) transplantation *(9–22)*. Thus, it has remained difficult to achieve comparable results between transplantation centers.

In the present study, therefore, I evaluated the performance and particularly the specificity of all major monoclonal assays for CsA. Metabolite-to-parent ratios were estimated in all clinical specimens, using the TDx® poly-

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[1] Nonstandard abbreviations: CsA, cyclosporin A; Emit-NPT, Emit with new pretreatment; HTx, heart transplantation; TDx-m, TDx monoclonal; LTx, liver transplantation; Emit-MeOH, Emit with methanol extraction; KTx, kidney transplantation; MR, metabolic ratio; MR-H, metabolic ratio with HPLC as reference method; and MR-E, metabolic ratio with Emit as reference method.

clonal assay as a measure of the sum of metabolite and parent concentrations. The study includes RIA, TDx monoclonal (TDx-m), Emit with both pretreatments, AxSYM, and CEDIA from two clinical studies using HPLC as the reference method.

Materials and Methods

CLINICAL SPECIMENS

Whole blood trough samples were collected into tubes containing EDTA and analyzed on the same day with the Emit assay with methanol pretreatment [Emit-methanol (Emit-MeOH)] and TDx polyclonal assay. All other assays were performed on samples stored at 4 °C for a maximum of 3 days or frozen at −20 °C for a maximum of 4 months. All samples were assayed in duplicate with all assays except the TDx polyclonal and HPLC.

A total number of 145 randomly chosen samples from 78 patients after renal transplantation (KTx; 80 samples from 50 patients), LTx (63 samples from 26 patients), or combined transplantation (2 samples from 2 patients; LTx/KTx and KTx/HTx) were used in all assays. One hundred and eleven samples were obtained from outpatients, and 34 were obtained from inpatients.

The data of 6066 daily routine CsA measurements with Emit-MeOH and TDx polyclonal from 266 patients after LTx (1131 samples from 51 patients), KTx (4908 samples from 214 patients), and HTx (27 samples from 1 patient) were analyzed retrospectively. This provided data for the range of parent-to-metabolite ratios and thus the biases of monoclonal assays to be expected in large populations.

Data from a similar study (using RIA, Emit-MeOH, and TDx monoclonal and polyclonal assays) at a different transplantation center[2] (transplantation center 2) allowed me to compare the results with those obtained for other types of transplantation and different CsA target values, and provided comparative data for the RIA. The previous 1992 study contained 613 samples from 166 patients after LTx (330 samples from 46 patients), KTx (94 samples from 46 patients), HTx (87 samples from 42 patients), bone marrow (80 samples from 27 patients), lung (6 samples from 1 patient), pancreas (3 samples from 1 patient), and combined transplantation (13 samples from 3 patients).

All patients had received dosages based on Emit-MeOH trough concentrations.

QUALITY CONTROL

Precision studies were performed using controls supplied by Bio-Rad Laboratories. External quality control was ensured through participation in the Cyclosporin International Proficiency Testing Scheme (Coordinator, Dr. D.W. Holt). Since April 1995, the maximum deviation from the method means has been 1.8 SD, with an average of 0.42 SD for Emit-MeOH (mean bias, −0.06) and 0.58 SD for the TDx polyclonal (mean bias, 0.4).

ASSAYS

The single-step whole blood CEDIA assay (Boehringer Mannheim) was performed on a Hitachi 912 instrument in its final marketed format. The monoclonal Emit-MeOH assay was supplied by Behring Diagnostics Inc. and was performed on two Cobas Mira Plus instruments. To evaluate the new pretreatment solution (Emit-NPT), we adhered to the original manual pipetting procedure. The Abbott Laboratories AxSYM monoclonal fluorescence polarization immunoassay and TDx-m use the same proprietary antibody. The CycloTrac® specific RIA from Incstar Corporation had been used in the previous 1992 study and was performed strictly as recommended by the manufacturer.

The HPLC kit (ClinRep®) from Recipe Merck served as a reference method specific for CsA, whereas the TDx polyclonal (parent and metabolites; Abbott) gives an overall estimation of the total concentration of CsA plus, to various degrees, several metabolites of CsA *(23)* (Table 1).

DATA ANALYSIS

Passing-Bablock analysis and Pearson correlation coefficients were used to compare the results obtained with different assays. Only the first result of duplicates was included. Wilcoxon–Mann–Whitney tests were performed when different populations were compared for a significant difference in means. Precision studies were done according to NCCLS document EP5-T2. The differences in CVs calculated from the duplicate patient results and reruns were tested by the Wilcoxon matched-pairs signed-ranks test.

The overall content of metabolites in each sample was estimated by calculating "metabolic ratios" (MRs) between the results of the TDx polyclonal and HPLC (MR-H = TDx polyclonal/HPLC) or Emit (MR-E). The individual deviation from the HPLC (or Emit) result was then calculated for each monoclonal immunoassay in each sample and compared with the MR calculated in the same sample.

Results

ANALYTICAL PERFORMANCE

Calibration stability. A two-point calibration had to be performed with each new reagent kit of the CEDIA. The average stabilities of the calibrations for the other assays were as follows: AxSYM >8 weeks *(8)*, TDx-m ~4 weeks, TDx polyclonal 3 weeks, Emit <1 week.

Linearity. There was a linear relationship between the expected and the measured concentrations for all assays when the highest calibrator was serially diluted: CEDIA; slope, 1.03 ± 0.02, intercept, −27.8 ± 6.9, $S_{y|x}$ = 8.0;Emit-NPT: slope, 1.00 ± 0.02, intercept, −14.4 ± 5.0, $S_{y|x}$ = 6.2; TDx-m: slope, 0.93 ± 0.01, intercept, −1.3 ± 2.1, $S_{y|x}$ = 4.0; AxSYM: slope, 1.03 ± 0.01, intercept, −4.3 ± 3.7, $S_{y|x}$ = 5.5.

[2] Analyses were done by myself and the same technicians as in the present study at the Institute of Clinical Chemistry, Klinikum Grosshadern, Ludwig-Maximilian University, Munich, Germany.

Precision. The total CVs of all control results during the study period are shown in Fig. 1A. The highest CVs were calculated for HPLC, the lowest over the entire range for the TDx-m. Both Emit versions and the CEDIA gave comparable CVs, although they were noticeably worse than the AxSYM at concentrations <300 μg/L. The results of the duplicate measurements of patient samples were grouped into concentration classes of 20 μg/L. The average within-batch CV was then calculated for each class (Fig. 1B). In addition, all samples were tested in a rerun with the Emit-NPT, the AxSYM, and the CEDIA, and total CVs were calculated (Fig. 1C). The lowest within-batch CVs were obtained with the TDx-m, followed by both Emit versions and, lastly, the AxSYM (>100 μg/L) and the CEDIA (<100 μg/L). There was no significant difference between both Emit versions and the CEDIA. The AxSYM performed significantly worse than both Emit versions (P = 0.022 and 0.008) *(8)* and the CEDIA (P = 0.024). Any other comparison between any two assays yielded highly significant differences between their within-batch CVs (P <0.0001). In contrast to the within-batch CVs, the total CVs of the AxSYM were significantly lower than both the Emit-NPT and the CEDIA when compared in patient samples (P <0.0001).

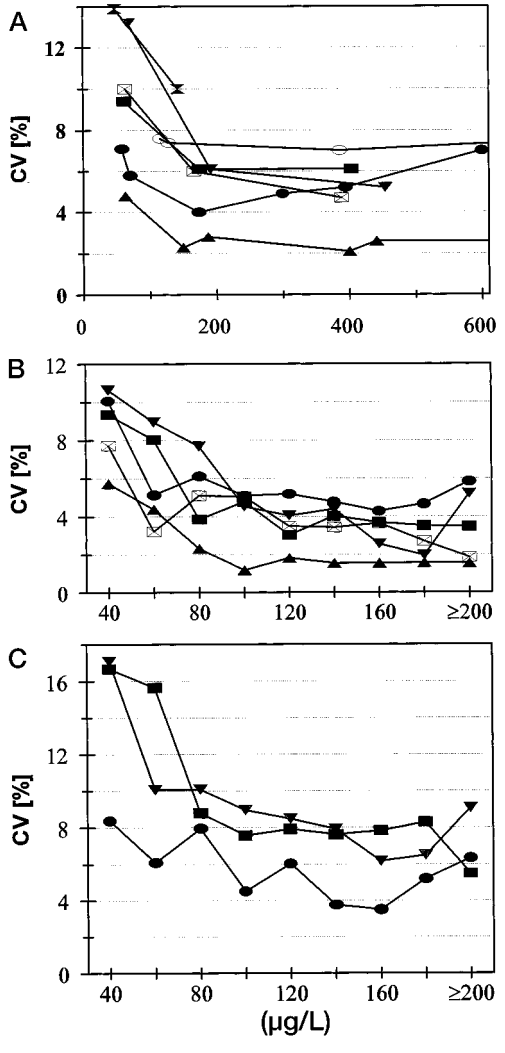

Fig. 1. Total CVs obtained from controls (*A*), and within-batch (*B*) and total CVs (*C*) calculated from patients duplicates and reruns.

Panels B and *C* grouped by CsA results (class means indicated). (⧗), HPLC; (○), RIA; (⊠), Emit-MeOH; (■), Emit-NPT; (▲), TDx-m; (●), AxSYM; (▼), CEDIA.

Sensitivity. The analytical limits of detection, defined as 3 SD above the mean for the zero calibrator (n = 10), were as follows: Emit, 20 μg/L; AxSYM, 12 μg/L; and TDx-m, 8 μg/L *(8)*. No zero calibrator was available for the CEDIA. Instead, I used the lowest calibrator (target value, 17.6 μg/L), which yielded a result of 31 μg/L. On the basis of a maximum tolerable CV of 10% *(4, 25)*, the functional sensitivity (limit of quantification) was estimated as 60–70 μg/L for the Emit and CEDIA and 40 μg/L for the AxSYM assay.

Recovery and cross-reactivity. Four unknown samples (processed base human serum with methylated human hemoglobin), provided by Abbott, were tested with the Emit-NPT, AxSYM (20 replicates), CEDIA, TDx-m, TDx polyclonal (10 replicates), and HPLC (4 replicates). CsA (target, 50 or 250 μg/L) had been added to each sample. The calculated recoveries were as follows: Emit-NPT, 125.8% and 107.5%; TDx-m, 117.8% and 118.6%; AxSYM, 114.0% and 114.3%; CEDIA, 143.4% and 122.9%; TDx polyclonal, 135.4% and 119.9%; and HPLC, 109.5% and 99.8%. The recovery of the HPLC was 102.0% and 101.5% when CsA (125 or 270 μg/L) was added to CsA-free whole blood. Two samples also contained 1000 μg/L metabolite AM1 and 500 μg/L AM9, respectively (Novartis). The calculated cross-reactivities for AM1 and AM9 in the presence of 250 μg/L CsA are shown in Table 1 (percentage of added metabolite appearing as additional apparent CsA concentration).

CONVENTIONAL DIRECT COMPARISON OF METHODS

The correlation coefficients and standard errors of the estimate achieved (Table 2) were better between the different monoclonal immunoassays (r = 0.93–0.98) than between HPLC and immunoassays (r = 0.89–0.93). This was attributable to the lower CVs and the positive bias of the various immunoassays when compared with HPLC (9–57%). The correlation coefficients with the polyclonal TDx were much lower for all the monoclonal assays and for HPLC (r = 0.63–0.78, data not shown).

Both Emit versions produced virtually identical results (y/x = 1.03). A good correlation had also been achieved-when the Emit-MeOH assay was compared with the RIA for the 1992 data set (mean y/x = 0.99).

There was an unexpected difference when the results of the present study were compared with those from the 1992 study. A strong positive bias of the TDx-m when compared with the Emit-MeOH was found in the 1997 population (mean y/x = 1.44) as opposed to a small bias in 1992 (mean y/x = 1.12). It is essential to appreciate that

Table 1. Metabolite cross-reactivity (%).

	Measured		According to package insert				
Method	**AM1**	**AM9**	**AM1**	**AM9**	**AM4N**	**AM19**	**AM1c9**
TDx polyclonal	148	17.1	96	19	62	9	<1
RIA[a]	ND[b]	ND	0.7	1.7	0.8	<0.1	ND
TDx-m[c]	9.3	23.5	6.7	19.4	NS	NS	NS
Emit[c]	2.2[d]	13.1	<0.3	7.3	<0.3	3.0	ND
AxSYM[c]	6.8	14.5	6.9	10.8	NS	NS	NS
CEDIA	5.4	29.3	5.1	11.9	15.5	0.2	0.5

[a] From Wallemacq et al. *(24)*.
[b] ND, not determined; NS, not significant (lower than CV).
[c] From Steimer *(8)*.
[d] Not significant.

there has been a shift in the relative performance of the Emit and TDx monoclonal over the last 5 years for unidentified reasons, possibly because of a shift in standardization of either of the two assays involved.

METABOLITE CROSS-REACTIVITY AND CONSECUTIVE BIAS OF MONOCLONAL ASSAYS

The comparison of MRs (as an estimate of metabolite-to-parent ratios) with the observed positive biases of the different monoclonal assays revealed a relatively strong correlation. Fig. 2 B suggests that the extent of the positive bias of the TDx-m compared with the HPLC [bias = (TDx-m − HPLC)/HPLC] is related to the amount of metabolites detected by the polyclonal assay. At an MR-H of 10 (TDx polyclonal/HPLC), the calculated function suggests a positive bias of 133%. On the other hand, samples with an MR-H of 2 display a positive bias of only 20%. Fig. 2C shows the smaller positive bias for the AxSYM (96% at an MR-H of 10). The Emit displays the lowest slope, followed by the CEDIA (Fig. 2, A and D).

The Emit can, therefore, serve as an alternative reference method for the calculation of MRs (MR-E = TDx polyclonal/Emit). Both MRs correlated well [$r = 0.832$; MR-H = (1.16 ± 0.05)MR-E − (0.35 ± 0.20)]. After two outliers with HPLC results close to the detection limit (21 and 25 μg/L) were eliminated, the correlation coefficient was 0.885. The resulting slopes and intercepts when the positive bias of the other monoclonal immunoassays were correlated to MR-E are shown in Fig. 3. These ratios (MR-E) were available in a much larger set of samples and allowed the results of TDx-m and Emit from the present study to be compared with those from 1992. The calculated correlation coefficients and slopes and, thus, the dependence of TDx-m results on metabolites (MR-E) were

Table 2. Passing-Bablock regression analysis, ($S_{y|x}$), and Pearson correlation coefficient (r).

y[a]	x	y/x	Slope (95% CI)[b]	Intercept (95% CI)	$S_{y\|x}$	r
Present study 1997 (n = 145), KTx and LTx						
TDx polycl.	HPLC	4.41	6.90 (5.68–8.61)	−301 (−486 to −191)	284.8	0.628
Emit-MeOH	HPLC[c]	1.09	1.11 (1.02–1.22)	−7.0 (−16.3 to 2.8)	23.7	0.927
Emit-NPT	HPLC[c]	1.12	1.06 (0.98–1.15)	0.3 (−10.4 to 9.5)	21.9	0.926
TDx-m	HPLC[c]	1.57	1.50 (1.36–1.64)	2.0 (−13.1 to 14.9)	31.3	0.924
AxSYM	HPLC[c]	1.29	1.23 (1.12–1.35)	2.0 (−11.4 to 13.1)	27.7	0.907
CEDIA	HPLC	1.18	1.09 (0.98–1.21)	5.3 (−5.1 to 18.6)	26.9	0.891
TDx-m	Emit-NPT	1.41	1.37 (1.29–1.46)	2.5 (−6.4 to 12.1)	20.4	0.968
AxSYM	Emit-NPT	1.16	1.16 (1.10–1.24)	−2.3 (−11.0 to 3.9)	18.8	0.959
CEDIA	Emit-NPT	1.06	1.00 (0.93–1.09)	5.0 (−4.4 to 11.6)	19.9	0.939
TDx-m	AxSYM	1.24	1.17 (1.12–1.23)	5.6 (−1.6 to 12.2)	20.7	0.968
TDx-m	CEDIA	1.35	1.33 (1.23–1.44)	1.7 (−13.2 to 14.6)	29.6	0.931
AxSYM	CEDIA	1.11	1.14 (1.06–1.22)	−5.1 (−13.6 to 4.2)	21.7	0.944
Emit-NPT	Emit-MeOH[c]	1.03	0.95 (0.90–1.00)	7.0 (2.0–12.7)	13.7	0.973
TDx-m	Emit-MeOH	1.44	1.29 (1.23–1.35)	13.8 (7.1–20.7)	18.2	0.975
Previous study 1992 (n = 613), KTx, LTx, HTx, and bone marrow transplantation						
RIA	Emit-MeOH	0.99	1.07 (1.04–1.09)	−15.2 (−19.8 to −10.4)	35.9	0.977
TDx-m	Emit-MeOH	1.12	1.04 (1.01–1.06)	11.2 (6.5–16.7)	36.3	0.971

[a] y = slope x + intercept.
[b] CI, confidence interval; polycl., polyclonal.
[c] From Steimer *(8)*.

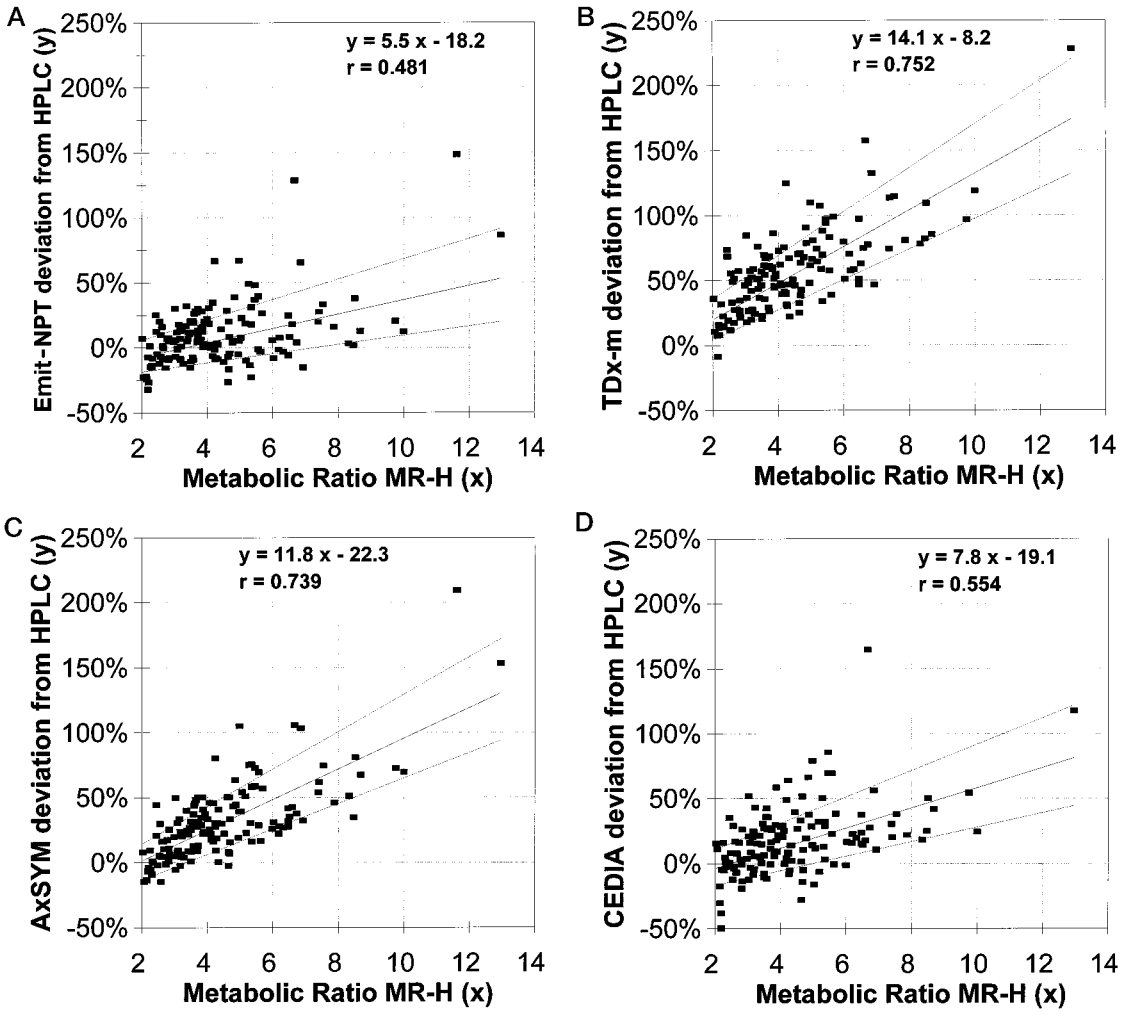

Fig. 2. MRs and consecutive positive bias (deviation) of monoclonal immunoassay results from HPLC (MR-H = TDx polyclonal/HPLC). 95% confidence intervals are indicated by *dashed lines*.

independent of the type of transplantation and very similar for both transplantation centers (range of slopes, 7.4–9.8 for the various transplantations in both centers). The differences of the intercepts between both transplantation centers, producing an additional positive bias of 25%, resemble those of the method correlation discussed above. They are consistent for all transplantation types and support the idea that a change of standardization rather than a change of metabolite cross-reactivity could be responsible for the unexplained change of relative performance between Emit-MeOH and TDx-m since 1992.

No significant correlation was found between MR-E and the RIA deviation from Emit-MeOH results (r = 0.175). This indicates no additional cross-reactivity for the RIA compared with the Emit.

TYPE OF TRANSPLANTATION AND HEPATIC DYSFUNCTION

Can MRs and, consequently, the bias of monoclonal assays in individual samples be predicted from the type of transplantation or other identifiable factors? The mean MR-E observed in 1992 at transplantation center 2 was 3.27 (n = 613) compared with 4.03 in the present study population (n = 145) and 3.83 in the routine samples (n = 6066). The observed mean MR-Es in the present study as well as in the routine population were very similar for both KTx and LTx (3.85 and 3.83) and for in- and outpatients (3.90 vs 3.76). The observed average MR-Es at transplantation center 2 were as follows: 4.25 for HTx (range observed in different samples, 1.36–13.8); 2.32 for bone marrow transplantation (0.97–5.73); 3.19 for LTx (0.95–13.5); and 3.54 for KTx (1.54–10.4). The differences between the transplantations, although significant (P <0.0001; exceptions were P = 0.02 for KTx vs HTx and P >0.05 for KTx vs LTx), were small compared with the variability of individual results between each subject or specimen irrespective of transplantation. The cumulative frequency distribution of the metabolite-to-parent ratio estimates (MR-E) of the three populations studied is shown in Fig. 3, together with the resulting positive bias

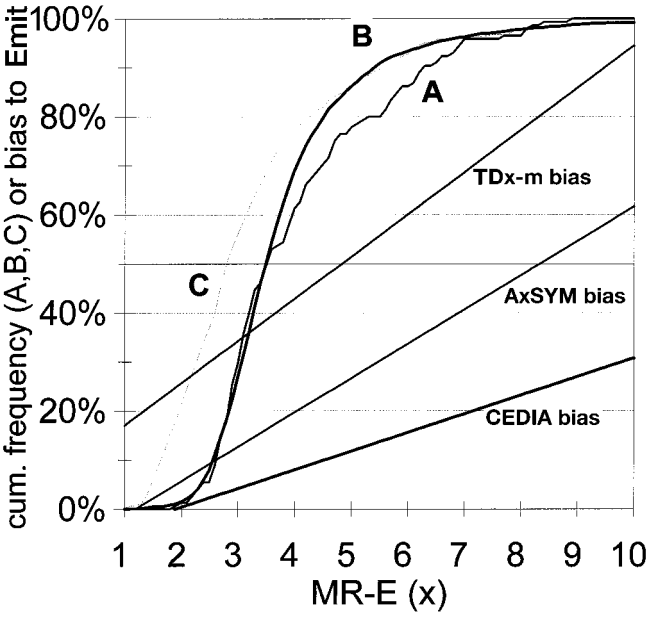

Fig. 3. Cumulative frequency of MRs (MR-E = TDx polyclonal/Emit).

(*A*), transplantation center 1 (n = 145, present study); (*B*), transplantation center 1 (n = 6066, routinely performed between 1995 and 1997); (*C*), transplantation center 2 (n = 613, 1992 study). The relative deviations (biases) of TDx-m, AxSYM, and CEDIA from Emit results as a function of MR-E were as follows: TDx-m bias (%) = (8.6 ± 0.6)*x* + (8.5 ± 2.2), $r = 0.775$, $S_{y|x} = 10.2$; AxSYM bias (%) = (7.0 ± 0.5)*x* − (8.3 ± 1.8), $r = 0.776$, $S_{y|x} = 8.0$; CEDIA bias (%) = (3.8 ± 0.8)*x* − (7.2 ± 3.2), $r = 0.316$, $S_{y|x} = 13.7$. The diagram allows the derivation of the number of patients exceeding a certain metabolite-to-parent ratio (MR-E) and the positive bias arising from that MR-E. *cum.*, cumulative.

of the TDx-m, AxSYM, and CEDIA as compared with the Emit. There is a slight parallel shift towards lower MR-Es in transplantation center 2, where significantly higher CsA concentrations had been observed. In both transplantation centers, higher relative metabolite concentrations were observed at lower parent concentrations (Fig. 4). Very high MRs (e.g., MR-E >12) usually occur at CsA concentrations <100 μg/L. This is probably because of increased CVs for the specific reference methods (HPLC or Emit) at low CsA concentrations *(26)*.

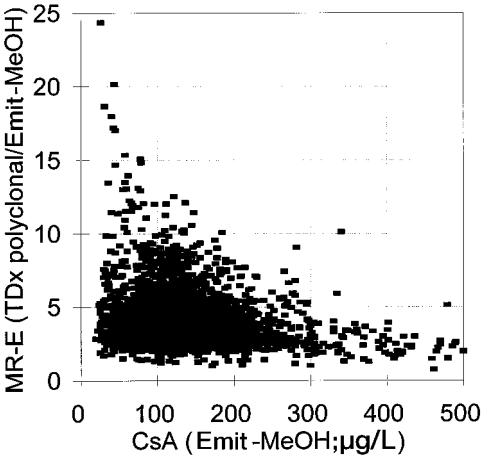

Fig. 4. Distribution of MRs (MR-E) in relation to CsA concentration (n = 6066).

MR-Es are stable in individual patients over long periods of time. At an average MR-E of 3.6 in 115 patients with at least five MR-Es measured (mean, 30 MR-Es), the mean SD within a patient was only 0.7, including the immediate post-transplantation phase. The whole population of patients, however, covered a wide range of individually kept MR-Es (2.1–7.2), irrespective of transplantation (Fig. 5), which pointed to the importance of genetic predisposition rather than environmental influences. Consequently, standard biochemical liver tests do not safely indicate MRs. Table 3 shows the three patients with the highest and the one patient with the lowest MR-E, the highest γ-glutamyl transferase (EC 2.3.2.2), the two highest alkaline phosphatase (EC 3.1.3.1), and the highest bilirubin values. When the correlation between 106 bilirubin and 107 γ-glutamyl transferase results available in the current study population and the MR-Es was calculated, it yielded very weak correlation coefficients of 0.315 and 0.390.

Discussion

The new CEDIA assay is a step toward further automation because no centrifugation step is required after the addition of the lysing reagent. The assay, therefore, offered the easiest handling, although a two-point calibration was necessary with every new reagent kit used (currently 100 tests). Within-batch and total precision was generally comparable to the Emit, although it was considerably worse at concentrations <100 μg/L. The average bias of the CEDIA relative to HPLC was 18%, the second lowest value after the Emit (9–12%).

No significant difference was observed when the patient results of both Emit versions were compared. Within-batch precision was second only to the TDx-m. The total CVs were significantly worse than those for the

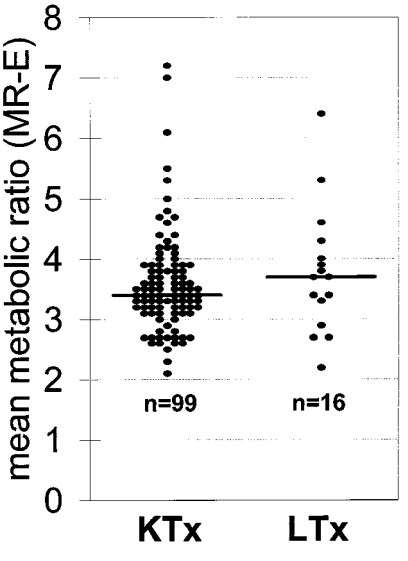

Fig. 5. Individual MR-Es for the KTx and LTx patient populations.

Each data point reflects the average MR-E (TDx polyclonal/Emit) for one patient and is the mean of 5–117 (mean, 30) consecutive determinations over a mean observation time of 469 days (12–962 days).

Table 3. MR-Es and biochemical liver tests in selected patients.

Patient	MR-E	GGT,[a] U/L 25 °C	ALP, U/L 25 °C	Bilirubin, μmol/L
1	8.9	84	255	15.4
2	8.3	419	500	37.6
3	8.1	61		13.7
4	1.9	136	114	10.3
5	5.2	3183	3121	25.7
6	5.7	2901	3158	32.5
7	3.9	485	1030	196.7

[a] GGT, γ-glutamyltransferase; ALP, alkaline phosphatase.

AxSYM and TDx-m methods, probably because of insufficient calibration curve stability. It has been suggested that CV improvement should be expected on automated systems because there would be fewer steps involving liquid handling *(7)*. This would primarily improve within-batch precision. No influence can be expected on the high total CVs caused by calibration instability.

The new AxSYM CsA assay performed very well, but showed a positive bias of 29% compared with HPLC and 16% compared with the Emit-NPT because of cross-reactivity. This is a definite improvement in specificity over the TDx-m (57%) and has been achieved by the modification of some of the assay conditions. However, interpreting results from different laboratories becomes even more difficult with yet another method not specific for the CsA parent on the market *(8)*. The performance of the assays is summarized in Table 4.

The specificity of monoclonal immunoassays for CsA has been addressed in the past mostly by direct comparison with HPLC or by measurement of purified metabolites. The former provides data about the mean bias of the population examined; the latter provides necessary basic information. Both methods are of limited value for explaining the different biases of monoclonal assays observed in single samples. Few data correlating the presence of metabolites in clinical samples and the irregular biases observed with monoclonal immunoassays are available. This is because HPLC procedures for measuring metabolite concentrations are labor-intensive and time-consuming. Ratios between nonspecific immunoassays and specific methods have been widely used to estimate overall metabolite concentrations in patient samples and to identify differences between certain populations. Large interindividual differences have also been observed, and the use of nonspecific assays has been discouraged because of these results *(27–31)*. Nonspecific immunoassays in combination with specific HPLC have seldom been used to assess the different biases observed in single clinical specimens when CsA is measured with monoclonal assays. These ratios are certainly inferior to HPLC for studies on CsA metabolism. Nevertheless, it is obvious from experimental studies *(17, 20, 24)* and theoretical consideration that the cross-reactivity of monoclonal antibodies is primarily detectable against the structurally related first-generation metabolites that are altered in only one position. Usually, AM1 and AM9 are also the most abundant metabolites in whole blood, with the AM1 concentration equaling or even exceeding that of CsA *(2, 23, 32, 33)*. The polyclonal TDx assay primarily detects first-generation metabolites *(2)* and is, therefore, useful for studying the metabolite cross-reactivity of monoclonal assays.

Table 4. Ranking of CsA assays for performance.

Method	Specificity for parent	Precision (total)	Sensitivity (functional)	Calibration stability	Ease of handling
HPLC	1	7	7		7
RIA	2	6	3		6
TDx-m	7	1	1	2	3
Emit-MeOH[a]	2	3	3	3	5
Emit-NPT[a]	2	3	3	3	3
AxSYM	6	2	2	1	2
CEDIA	5	3	3	5[b]	1

[a] Manual pipetting.
[b] Calibrated with each new kit (two-point calibration).

This study demonstrates that MRs (TDx polyclonal/HPLC or TDx polyclonal/Emit) correlate with the positive bias observed in all investigated monoclonal immunoassays as compared with the more specific method, including the latest developments with reduced cross-reactivity (TDx-m, AxSYM, CEDIA, and Emit). The individual bias observed in a sample depends predominantly on the individual content of metabolites. The data show that the cross-reactivity of all monoclonal assays, despite appearing negligible at first glance, can lead to substantial overestimation of the true CsA concentration in a number of patients. These patients could not be easily identified as being only patients with liver dysfunction or undergoing certain transplantations. The correlations and slopes describing the overestimation in single specimens were very similar when compared between two transplantation centers with different CsA target concentrations. A similar function has been reported for the TDx-m and TDx polyclonal/RIA (specific) ratios *(12)*.

The TDx polyclonal detects AM1 markedly better than AM9, thus explaining the higher correlation coefficients for the deviation of both TDx-m and AxSYM compared with those of Emit-MeOH/NPT and CEDIA. According to the results from purified metabolites, AM1 is the major problem for both monoclonal Abbott assays, whereas the other two assays cross-react more strongly with AM9 [manufacturers' inserts and Refs.*(17, 20)*].

Like many other authors *(12, 27, 30, 34, 35)*, I found highly significant differences in the mean MRs observed for different transplantations. This indicates a higher risk of metabolite accumulation, particularly in HTx. Nevertheless, the maximum difference observed for the various transplantation types (MR-E: bone marrow transplantation, 2.32; HTx, 4.25) is moderate compared with the large differences observed in individual samples from any transplantation *(27–31, 36–39)*. Consequently, the same applies to the different biases observed in monoclonal

assays. Not only single specimens but also the long-term mean MRs, which reflect an individual's genetic predisposition, showed more variability than was introduced by the type of transplantation (Fig. 5) *(29)*. The large interindividual differences agree with studies showing that the catalytic activities of cytochrome P450 3A, the enzyme family responsible for the formation of first-generation metabolites *(2)*, vary by at least 10-fold *(40, 41)*.

Similar to earlier reports *(37, 42–44)*, a very weak correlation between bilirubin and γ-glutamyl transferase and increased MRs was detectable in our population. Temporary increases in metabolite concentrations have been described, in particular, with severe hepatic dysfunction and immediately after transplantation *(10, 27, 35, 36, 45)*. Lacerda et al. *(46)*, however, found no correlation between hepatic metabolite concentrations and either serum bilirubin or the degree of cholestasis in liver biopsy specimens. Bleck et al. *(33)* and Christians et al. *(39)* showed that cholestasis was associated with a selective increase in concentrations of second-generation metabolites (AM19, AM1c9, AM1A, and AM11d). No differences in the concentrations of first-generation metabolites were observed in that study. Consequently, monoclonal assay results were not influenced (3H-RIA) as in another study assessing Emit and TDx-m *(26)*. The conclusion by Witzke et al. *(26)* that the contribution of metabolites to the TDx-m signal is predictable and constant is not justified. It ignores the presence of interindividual differences irrespective of hepatic function. To summarize the literature, there is convincing evidence for an association between hepatic dysfunction and increased predominantly second-generation metabolites. These are only partly detected by the TDx polyclonal and hardly pose a problem of cross-reactivity for monoclonal antibodies. This weak association is also superimposed by a large interindividual variability, which is probably determined genetically. Consequently, standard biochemical liver tests were unable to indicate the individual bias of monoclonal assays in our population. High MRs could be observed with either normal or abnormal hepatic function as reported by Tredger et al. *(43)*. Increased ratios may be anticipated, however, under co-therapy with interfering substances *(2, 47, 48)*.

The distribution of MRs shows that 5% of all patients had an MR-E >6.4 and thus a deviation from the Emit >64% with the TDx-m and 37% with the AxSYM assay. Samples from patients with low MR-Es showed little or no bias. The resulting bias is higher when HPLC is used as the specific reference method (TDx-m >92%, AxSYM >61%, CEDIA >36%, Emit >21%). From this study, it is obvious that the average bias obtained from conventional method correlation studies is only valid for patients with average MRs. The CsA concentration in all other patients will be over- or underestimated. Reference ranges have been established, mostly using specific HPLC methods *(49)*. The suggestion to modify reference ranges according to the results of method correlation studies *(10)* does not seem to be justified. The practice of adopting new methodology and reporting reference ranges 0–67% higher than the one for HPLC *(6)* disregards the different bias shown for every patient. Measurements by one technique cannot be adjusted to allow use of a therapeutic range determined for another method *(4, 27, 31, 37)*. According to the results of this study, this is also true for monoclonal CsA assays.

The individualization of drug dosages is usually done by adjusting a standard dose e.g., according to the body weight. If additional individualization is required, therapeutic drug monitoring can usually help to achieve safe, sufficient drug concentrations in each patient. This is only possible, however, if the measured value is close to the true value or shows the same bias for all patients. The differences in bias for different patients in this study exceeded all other factors of influence described in the literature and sometimes even exceeded the published therapeutic ranges (Emit vs HPLC, −7% to 53%; TDx-m vs HPLC, 20–174%; AxSYM vs HPLC, 2–130%; CEDIA vs HPLC, −3% to 81%). The problem with cross-reactivity is not the overall bias observed, but the extended range of biases. The following illustrates this point: a result of 150 μg/L obtained by TDx-m in an individual sample can mean a true value (HPLC) of 55 or 125 μg/L when the two extremes observed in our 145 sample population are used. This is comparable to giving the same dose of a drug that is usually individualized by body weight to both a 55-kg and a 125-kg patient. The analytical error must be added on top of that. Under such circumstances, one must question whether drug-monitoring of CsA is justified at all. It at least implies that inappropriate dosage adjustments could be made from the use of such methods *(50)*.

Except for the immediate post-transplant period in HTx and LTx patients, it has been advocated that TDx-m results satisfactorily parallel those of HPLC *(10, 20)*. Few data were available to confirm or deny this assertion *(22)*. Adding to the confusion are the many different biases, slopes, and intercepts that have been published in method comparison studies, even those comparing the same methods, in particular for the TDx-m assay *(9 to 22, 51)*. Cross-reactivity to CsA metabolites has been identified as the principal cause of these biases, and doubt has been raised early about the equivalence of HPLC and monoclonal immunoassays *(38)*. Separate biases for different transplantations have been calculated *(10–12)*. Apart from misuse of the terms bias and slope *(22, 52)*, this is probably because of the presence or absence of samples with high MRs and the concentrations at which these are observed. In the present study, the addition of just five theoretical results with an MR-E of 10 to the study population (assumed concentration either 50 or 200 μg/L) caused a change in slope and intercept of 0.1 and 10 (TDx-m vs Emit-NPT, n = 145). Both the number of samples with high metabolite-to-parent ratios and the CsA parent concentrations at which these are observed are highly

influenced by chance in small study populations. In a random sample of 100 specimens, the number of specimens with an MR-E >6.4 varies between 2 and 11 (95% confidence interval; expected mean, 5). When the weight of these specimens on the slopes and intercepts of regression curves is considered, many of the varying results reported for comparisons of the same assays in different studies can be explained.

The extent of the contribution of CsA metabolites to immunosuppression or toxicity has been discussed at length *(1, 2, 22, 53)*. Presently, monitoring of metabolites is not recommended as a standard procedure *(6)*. Specific assays should be used if assessment of the immunosuppressive activity or toxic potential of the metabolites is deemed necessary *(20)*. It could not be shown that specific assays could predict acute rejection better than polyclonal assays *(54–56)*. However, the consensus is that analytic methods used should be specific for CsA because of the well-defined activity and toxicity of CsA and the improved interlaboratory comparison, which results from measuring standardized, chemically defined structures. This point of view is strongly supported by the results of this study.

The results of this study also imply a possible influence of assay technology on pharmacokinetic studies *(22)* and in randomized concentration-control clinical trials *(57)* and are, therefore, at variance with Aspeslet et al. *(58)*, who stated that specific fluorescence polarization immunoassay and RIA methods provided valid results irrespective of their cross-reactivity.

To conclude, there is still a need for an easy, fast, and truly specific assay with high precision and sensitivity. The consensus guidelines for assessing the specificity of CsA assays *(4, 6)* (slope to HPLC ≤10% from the line of identity; intercept ≤15 μg/L, $S_{y|x}$ ≤15 μg/L) should be revised because they do not ensure sufficient specificity. It is necessary to demonstrate that there is no correlation between any observed bias and the individual content of metabolites in patients. Similar problems may be encountered in the monitoring of other drugs [e.g., Tacrolimus *(59, 60)*].

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