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Determination of cyclosporine A in whole blood: Comparison of a chromatographic method with three different immunological methods

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Abstract

Cyclosporine A is potent immunosuppressive agent characterized by a narrow therapeutic range, inter- and intra-individual variability and a lack of correlation between drug dosage and blood levels. In view of these facts, blood levels of CyA should be routinely monitored to assess organ rejection and toxicity.

We evaluated CyA as well as its metabolites (AM9, AM19, AMl, and AM4N) in whole blood samples from 117 patients using commercially available immunological assays (AxSYM, EMIT, Dimension) and HPLC.

Cross-reactivity of the immunological assays was evaluated using different concentrations of the CyA metabolites (in vitro cross-reactivity) and by statistical analysis of patient data (in vivo cross-reactivity). Cross-reactivity was seen in all immunological assays, with differences in in vitro and in vivo cross-reactivity.

The statistical analysis showed a classical correlation between HPLC and AxSYM of $r^2 = 0.89$, HPLC versus EMIT of $r^2 = 0.93$, and HPLC versus Dimension of $r^2 = 0.93$.

The percentage metabolite cross-reactivity (%) by immunological assays for four metabolites at two concentrations each (250 and 1000 ng ml^{-1}) was lowest with the Dimension assay.

Of the immunological methods examined, the new Dimension for CyA determination can be relied on to produce results comparable to HPLC; other advantages are its simplicity, practicability and ease of handling.

Keywords: Cyclosporin A; Metabolites; Immunoassays; HPLC; Cross-reactivity

1. Introduction

Cyclosporine A (CyA) is one of the most effective immunosuppressive drugs used to prevent allograft rejection and to treat certain autoimmune diseases.

Therapeutic drug monitoring is essential to successful CyA therapy, especially because the clinical application of CyA is complicated by the drug's narrow therapeutic range, and by substantial intra- and inter-individual variations in drug absorption, distribution, metabolism and elimination [1,2]. On the one hand, nephro-, hepato- and neurotoxic side effects are well known and can lead to serious organ impairment [3–6]. On the other hand, the dosage must be carefully adjusted for each patient to avoid the risk of organ rejection through underdosage, leading in the worst case to loss of the transplanted organ.

As for other drugs, the techniques used to measure CyA have often been a compromise between operational constraints and selective, accurate measurement of the drug. High-performance liquid chromatography (HPLC), which is still considered the gold standard [7], has been replaced in most routine laboratories by immunoassays, because they are faster and easier than HPLC. In recent years, manufacturers have made every effort to further automate these immunoassays. Consensus documents on therapeutic drug monitoring of CyA recommend that laboratories select immunoassay methods that have the lowest CyA metabolite interference [8], whereby such interferences should not be greater than approximately 10% [9].

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Nevertheless, there still are some differences between results obtained by the HPLC and specific immunoassays, although most of the immunological methods are declared to be specific for the parent CyA. The explanation for this is the cross-reactivity between CyA and its metabolites [10–12], meaning that it is important to know the correlation of the immunological method with HPLC and to what extent the various CyA metabolites cross-react with the antibody used.

We compared the performance of a no-pretreatement monoclonal antibody-based immunoassay from Dade Behring for measuring CyA in whole blood, with the results from the monoclonal antibody-based immunoassay of AxSYM and EMIT and an HPLC method (CyA parent drug and metabolites). Beside this, the cross-reactivity of the four CyA metabolites in the different immunoassays was determined by spiking whole blood samples with different concentrations of metabolites and by statistical analysis of patient samples.

2. Materials and methods

2.1. Patient samples

The EDTA-anticoagulated whole blood samples were obtained from a group of patients undergoing combined immunosuppressive therapy after transplantation.

We analyzed 248 blood samples collected from 117 transplantation patients; kidney (KTX, 103), heart (HTX, 78), liver (LTX, 10), lung (PTX, 4), and bone marrow (BMTX, 53).

2.2. Study design

Whole blood samples were taken just before the morning administration of the respective cyclosporine dosage. The blood samples for routine CyA measurement were analyzed on the same day with the EMIT assay, the AxSYM assay and the Dimension assay. The samples were stored at 4 °C for HPLC analysis the next day, then frozen at −20 °C for a maximum of 4 months.

2.2.1. Instrumentation

High-performance liquid chromatography (HPLC) for the determination of cyclosporine A and its metabolites AMl, AM9, AM4N, AM19 was performed according to an HPLC method described in [13]. The fluorescence polarisation immunoassay (FPIA) was performed using an AXSYM analyzer and reagents for monoclonal AXSYM in whole blood from Abbott (Abbott Laboratories Diagnostics Division, North Chicago, IL, USA). The enzyme-multiplied immunoassay technique (EMIT) monoclonal CyA assay from Behring was run on Cobas miraS instruments, and the Dimension® Cyclosporine A assay was performed on Dade Behring Dimension X pand (Dade Behring, Inc., IL, USA).

The results were evaluated with classical correlation methods of Passing–Bablok (linear regression, sum of deviation squares) and the procedures recommended by Bland and Altman [14].

2.3. Quality control materials

Imprecision of all assay systems was determined using pooled whole blood samples. Three quality control samples – low, medium and high – were prepared in-house with pooled blood, using separate stock solution. The concentration levels were measured with HPLC for CyA in the range of 117, 297, and 391 ng ml^{-1}; for AM4N in the range of 28, 156, and 361 ng ml^{-1}; for AM1 203, 275, and 485 ng ml^{-1}; for AM9 52, 145, and 456 ng ml^{-1}; and for AM19 39, 139, and 231 ng ml^{-1}. The QC samples were aliquoted and transferred to polypropylene tubes (Corning Inc.) and stored at approximately −20 °C before use.

Accuracy was controlled daily with three different commercially available CyA controls, i.e. Cyclosporine Controls low, medium and high for Abbott AxSYM System (Abbott Laboratories); Lyphochek Whole Blood Control Levels 1, 2, and 3 (Bio-Rad Laboratories, Irvine, CA, USA) for the EMIT 2000 cyclosporine specific assay (two levels), and the Dade Behring Dimension® Cyclosporine A assay, also two levels.

2.4. Precision

Total imprecision was determined by assaying the quality control materials according to NCCLS protocol EP10A.

2.4.1. Cross-reactivity with CsA metabolites

To determine the cross-reactivity of CyA metabolites, 100 ng ml^{-1} CyA was added to a whole blood hemolysate. Additionally, CyA metabolites in a concentration of 250 and 1000 ng ml^{-1} were added to aliquots of this sample. Aliquots of these samples were analyzed five times with each immunological assay.

To determine cross-reactivity in patient samples, CyA parent drug and CyA metabolites were determined by HPLC, and CyA levels were measured by the various immunoassays.

3. Results and discussion

The concentration of cyclosporine (CyA) in whole blood has been used as a guide for dosage adjustment to reduce the frequency of side effects and still maintain the immunosuppressive potential.

The resulting slope and intercept from a method correlation between two methods (HPLC and immunological) with different specificity for CyA obviously depend to a great extent on the majority of metabolites present in the samples tested.

A well-known problem in the determination of CyA with immunoassays is the interference with CyA metabolites. International consensus reports recommend that assays for CyA should be specific for the parent compound and should have a “true” level of cross-reactivity not greater than approximately 10%, so as not to precipitate inappropriate clinical decisions [9,15,16]. Assay comparison has shown that all immunoassays have more or less cross-reactivity with CyA metabolites [4,17,18]. Most laboratories use immunoassays for the determination of CyA; they offer the easiest handling, and

Table 1
Intra-day precision of the immunological methods (AxSYM, EMIT, and Dimension), using quality control samples provided by the manufacturer ($n = 4$)

	Control level low			Control level high		
	70 ng AxSYM	100 ng EMIT	120 ng Dimension	600 ng AxSYM	400 ng AxSYM	310 ng Dimension
1	62	94	122	544	387	295
2	64	92	126	691	411	317
3	75	105	123	642	380	297
4	72	100	117	685	447	275
MW	68	98	122	640	406	296
S.D.	5.51	5.12	3.24	58.79	26.19	14.87
CV%	8.08	5.24	2.66	9.18	6.45	5.02

show a very acceptable imprecision at low as well as at high levels.

To further check the reliability and intra-day precision of immunoassays, we used commercially available control samples of low and high level cyclosporine: level I (70 ng ml^{-1}) and level II (600 ng ml^{-1}) for AxSYM; level I (100 ng ml^{-1}) and level II (400 ng ml^{-1}) for EMIT; and level I (120 ng ml^{-1}) and level II (310 ng ml^{-1}) for Dimension. The intra-day precision (CV) for each available control ($n = 4$) was in the range 8.1–9.2% for AxSYM, for EMIT 5.2–6.5%, and for Dimension 2.7–5.0% (Table 1). This probably reflects the particular calibration curve on the day the within-run analyses were performed. Inter-day precision was evaluated by assaying quality controls with three different concentrations of cyclosporine on 14 different days (in series). The analytical performance of each method is summarized in Table 2. Inter-day coefficients of variation (CVs) for cyclosporine with three immunological methods were <12% and absolutely comparable with CV intra-day.

Kunzendorf et al. [19] reported that higher blood levels of AM1 and AM9 correlated with a decreased frequency of rejection episodes in human recipients of renal allografts. High CyA metabolite blood concentrations have been reported in association with CyA neurotoxicity and nephrotoxicity [2,20,21].

To determine the assays' specificity, cyclosporine metabolites were tested for cross-reactivity in the presence of CyA and metabolites in vitro as well as in vivo.

To evaluate the cross-reactivity of CyA metabolites in three assays, we added 250 and 1000 ng ml^{-1} of metabolites AM9, AM19, AM1, and AM4N to 100 ng ml^{-1} CyA drug-free whole blood. The cross-reactivities for the major CyA metabolite (AM1) were less than 4% and for the metabolite AM9 <15%. The results are summarized in Table 3 and compare well with other immunoassays (e.g. CEDIA) [22].

The results for routine patient samples (CyA parent drug and CyA metabolites) evaluated with HPLC and the various immunoassays were analyzed. Results are shown in Table 4 for 248 transplant recipients according to the transplanted organ: heart (HTX), bone marrow (BMTX), liver (LTX), lung (PTX), and kidney (KTX). In many cases, there were great differences between HPLC and AxSYM values; we found high concentrations of CyA metabolites that obviously caused false high CyA values through a cross-reaction with the CyA antibody. The distribution profiles of the differences, as shown in Table 4, also indicate that great differences can be attributed to the cross-reaction, because the differences seem to depend on the CyA concentration (and the corresponding metabolite concentration). Fig. 1 shows the comparability of CyA whole blood concentrations with AxSYM, EMIT, and Dimension, respectively, versus results from HPLC in samples of transplant recipients.

The individual data are presented in panels A/1, B/l and C/l of Fig. 1 for correlations between HPLC and AxSYM ($r = 0.89$, $y = 1.036x + 30.98$), HPLC and EMIT ($r = 0.93x$, $y = 1.087x + 10.64$), and HPLC and Dimension ($r = 0.93$, $y = 1.27x + 32.21$). This result compares well with literature data [18,23]. In panels A/2, B/2, and C/2 of Fig. 1, the absolute difference in CyA concentrations between the HPLC method is plotted against the mean CyA concentrations determined by HPLC and all immunoassays. The following differences can be observed with a probability of 95%: 22.9 ng ml^{-1} (HPLC versus EMIT) (Fig. 1, A/2), 36.0 ng ml^{-1} (HPLC versus AxSYM) (Fig. 1, B/2), 27.2 ng ml^{-1} (HPLC versus Dimension) (Fig. 1, C/2).

The statistical analysis according to Bland and Altman provides further information about differences that can be expected with a 95% probability when two methods are used for the determination of the CyA concentration in one blood sample. In spite of good correlation between the methods (calculated

Table 2
Comparison of results obtained by different methods for three pooled patient samples ($n = 14$)

	CyA concentration (ng ml^{-1})					
	Low		Medium		High	
	Mean ± S.D.	CV%	Mean ± S.D.	CV%	Mean ± S.D.	CV%
HPLC	117.2 ± 6.4	8.9	297.1 ± 7.4	6.3	391.4 ± 12.4	5.5
AxSYM	148.9 ± 12.1	8.2	342.2 ± 28.2	8.2	484.0 ± 28.8	5.3
EMIT	118.6 ± 14.4	12.2	312.7 ± 29.8	9.6	425.4 ± 36.2	8.5
Dimension	121.6 ± 6.9	5.7	309.9 ± 18.8	6.1	403.3 ± 25.8	5.3

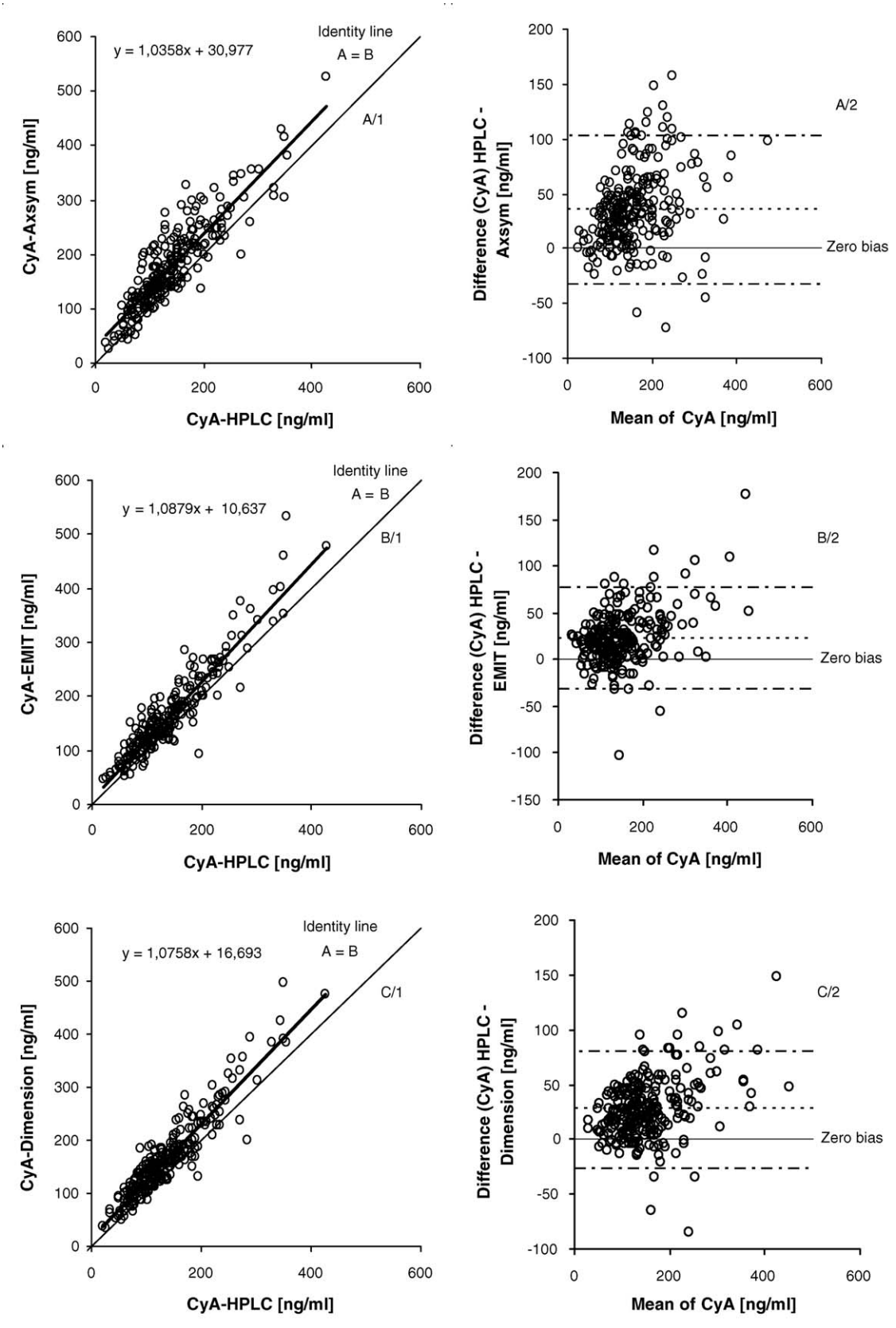

Fig. 1. Comparison of results for trough CyA concentrations in whole blood determined by HPLC and the different immunoassays. Panels A1 and A2 for EMIT; panels B1 and B2 for AxSym and panels C1 and C2 for Dimension. Panels on the right side show the absolute differences (Δ) between HPLC and one of the other methods (Bland–Altman), whereas panels on the left side show regression and correlation of cyclosporine A in whole blood determined by the different assays versus HPLC results ($n = 248$).

Table 3
Cross-reactivity with CyA metabolites in the presence of parent drug

Sample	CyA parent drug			
	100 ng ml^{-1} (analyte added)	AxSYM (ng ml^{-1}) (% cross-reactivity)	EMIT (ng ml^{-1}) (% cross-reactivity)	Dimension (ng ml^{-1}) (% cross-reactivity)
AM9	250	161.6 (13.3)	139.2 (7.7)	128.9 (1.6)
	1000	267.6 (13.9)	230.2 (11.4)	139.5 (1.5)
AM19	250	129.8 (0.5)	130.0 (4.1)	112.8 (−4.8)
	1000	149.1 (2.1)	149.4 (2.9)	130.6 (0.6)
AM1	250	127.3 (−0.4)	121.0 (0.5)	112.2 (−5.0)
	1000	164.6 (3.6)	127.2 (0.7)	114.3 (−1.0)
AM4N	250	130.3 (0.7)	132.0 (4.9)	139.6 (5.9)
	1000	157.24 (2.9)	182.0 (6.2)	206.5 (8.2)

Cyclosporine metabolites were tested for cross-reactivity in the assay by spiking each compound into whole blood sample in the presence of 100 ng ml^{-1} CyA ($n = 5$).

with the classical method), clinically relevant discrepancies may occur.

Hamwi et al. reported that CyA concentrations in LTX patients determined by EMIT showed a higher deviation from HPLC results than all the other patient groups [24].

We found high deviations not only in LTX but even more so in BMTX patients. This difference (immunological assays versus HPLC) is not only due to AM1, but other metabolites may have contributed as well.

Steimer [17] found that AM1 is the major problem for the AxSYM, whereas Emit cross-reacts more strongly with AM9. Nevertheless, he also found that inter-individual differences have a stronger impact on immunological CsA determination than the type of transplantation. He concluded that the large inter-individual differences might be due to variations of catalytic activities of cytochrome P450 3 A, the enzyme family responsible for the formation of first-generation metabolites [17].

Our study confirms these reports. The in vitro cross-reactivity studies (spiked whole blood samples) as well as the observations in patient samples have shown cross-reactivity with the four main CyA metabolites. These are more pronounced in the in vitro studies than in the patient samples. The reason for this is that in patient samples, despite AM1, which has a concentration in blood as high as the CyA parent drug, the amount of CyA metabolites is not as high (3–8% of the total amount of CyA and its metabolites in blood, i.e. <10–47 ng ml^{-1}) as the concentrations used in the cross-reactivity studies. For the clinical interpretation of CyA results, it has to be kept in mind that the AxSYM immunoassay shows the most pronounced interference with AM1 ($p < 0.001$), whereas the Dimension system has a much weaker interference with this metabolite ($p = 0.012$). In contrast to AxSYM, the Dimension system also showed weak interference with AM19 ($p = 0.028$). In patient samples, only EMIT showed no significant interferences with any of the CyA metabolites. These results are also reflected by the data on correlation of the various immunoassays with CyA determination by HPLC.

As shown by Tredger et al. [23] and Kimura et al. [25], the EMIT immunoassay showed the best correlation with HPLC parent drug determination ($r = 0.93$).

The correlation of the new non-pretreatment Dimension CyA immunoassay was identical to that of EMIT, although the mean values were slightly higher than with EMIT. The AxSym immunoassay showed the highest CyA values in patient samples

Table 4
Results of CyA determination with the various immunoassays and HPLC (CyA parent drug and four metabolites)

TX patients	Patients (samples)	HPLC, MW (±S.D.)					AxSYM	EMIT	Dimension
		CyA	AM1	AM9	AM4N	AM19			
BMTX	13 (53)	140.5 (53.0)	177.8 (150.4)	29.0 (26.7)	10.5 (3.6)	20.0 (19.7)	164.2 (53.9)	158.7 (50.4)	156.9 (48.4)
% cross-reactivity							16.8	729	11.7
HTX	44 (78)	129.6 (57.3)	268.9 (158.8)	61.4 (47.4)	14.6 (13.3)	53.6 (72.6)	167.2 (71.5)	149.9 (73.7)	161.8 (75.6)
% cross-reactivity							29.0	15.7	24.8
LTX	3 (10)	112.4 (60.1)	164.5 (58.0)	27.6 (22.2)	10.6 (1.8)	18.70 (16.95)	124 4 (59.9)	119.7 (61.1)	124.7 (67.0)
% cross-reactivity							10.6	6.5	70.9
KTX	56 (103)	145.7 (74.1)	217.7 (141.5)	45.5 (36.1)	12.9 (9.4)	33.70 (38.78)	188.4 (85.1)	173.3 (85.3)	175.3 (83.2)
% cross-reactivity							29.3	18.9	20.3
PTX	1 (4)	199.5 (75.3)	232.7 (109.0)	74.5 (53.2)	10.0 (0.0)	21.00 (19.05)	252.4 (81.4)	248.0 (84.3)	249.5 (79.6)
% cross-reactivity							26.5	24.3	25.0
All patients	117 (248)	139.0 (65.5)	223.4 (150.5)	46.7 (40.4)	12.8 (9.9)	36.23 (50.54)	175.0 (76.2)	161.9 (76.4)	166.3 (75.5)
% cross-reactivity							25.9	16.4	19.6

compared to HPLC, which might be explained by the strong interference with CyA metabolite AM1.

Statistical analysis of patient data has not only shown that CsA metabolites interfere with CsA determination by immunoassay. Since *p*-values of the linear mixed-effect model (including between-patient variance) are higher than those of the naive model (linear regression model), the between-patient variance has an important influence on CsA determination by immunoassays. This between-patient variance might include preanalytical variables (time of blood collection), interferences of co-medications (in vivo interference on CsA metabolism in the liver (http://medicine.iupiu.edu/flockhart/), in vitro interference of drugs upon CsA analysis; as listed in the package insert), nutritional differences [26], time after transplantation [23] and other as yet unknown variables. There are differences in the CsA metabolite concentrations depending on the transplanted organ. Mainly HTX seems to carry a higher risk of metabolite accumulation, as also mentioned by others [17]. Our group of HTX patients showed the highest concentrations of AM1, which are 51% of the total amount of CsA parent drug and CsA metabolites.

We conclude that of the immunological methods examined, the new Dimension for CyA determination can be relied on to produce results comparable to HPLC, and has the further advantages of simplicity, practicability and easy handling. It should be emphasized, however, that the results of EMIT or Dimension are not in accordance with those of HPLC in the cases of bone marrow and liver transplantation.

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