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High-performance liquid chromatographic method for the simultaneous determination of cyclosporine A and its four major metabolites in whole blood

G. Khoschsorur*, W. Erwa, F. Fruehwirth, M. Stettin, A. Meinitzer, G. Hoebarth, S. Zelzer, G. Halwachs-Baumann

Institute of Medical and Chemical Laboratory Diagnostics, University Medical Center, Auenbruggerplatz 29, Graz A-8036, Austria

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Abstract

This study aimed to develop a simple and efficient optimized high-performance liquid chromatograph (HPLC) method for simultaneous determination of cyclosporine A (CyA) and its major, partly active metabolites AM1, AM9, AM4N, and AM19 in whole blood from transplant patients using cyclosporine D (CyD) as internal standard. The method used a CN analytical column maintained at 60 °C with hexan-isopropanol (93:7, v/v) as mobile phase; detection was at 212 nm. Linearity for all five compounds was tested in the range of 31–1500 ng ml^{-1} for CyA and of 31–1000 ng ml^{-1} for metabolites. The limit of detection was found to be 15 ng ml^{-1} for all compounds.

This modified, inexpensive method is also suitable for measuring cyclosporine A and metabolite concentrations in routine monitoring of patients undergoing treatment with CyA.

Keywords: Cyclosporine A; Metabolites (AM1, AM9, AM4N, AM19); HPLC

1. Introduction

Cyclosporine A, a potent and widely used immunosuppressant drug, is metabolised extensively in the liver, principally by the enzyme cytochrome P4503A [1,2]. The clinical application of (CyA) is complicated by substantial intra- and inter-individual variations in drug absorption, distribution, metabolism, and elimination [3]. The primary metabolites are the monohydroxylated AM9 (M1), AM1 (M17), and AM4N (M21) [4]. Further oxidation of AM1 and AM9 results in the dihydroxylated AM19 (M8) [5] (Fig. 1).

As immunosuppression with cyclosporine A in organ transplant patients involves considerable risks, regular and frequent drug monitoring is mandatory to prevent unexpectedly high trough concentrations of accumulated CyA metabolites, e.g. in liver recipients [6], and other side effects. High levels of the CyA metabolites, e.g. AM1 and AM9, may contribute considerably not only to immunosuppression but also to the toxic side effects of CyA [7].

Due to its narrow therapeutic spectrum, cyclosporine must be carefully monitored in post-transplant regimens, and optimal dosage adjustment depends on the analytical method used for monitoring. The assay should not only be reliable, fast, and simple but should also cover CyA metabolites. A comprehensive therapeutic strategy should include such monitoring as an effective tool for assessing individual dosage and as a key to developing an optimal individual dosage [8,9].

A literature survey [10–16] revealed that there are a number of methods available for individual determination of the metabolites, or for any two of these four metabolites in com-

* Corresponding author. Fax: +43 316 385 3419.
E-mail address: g.Khoschsorur@klinikum-graz.at (G. Khoschsorur).

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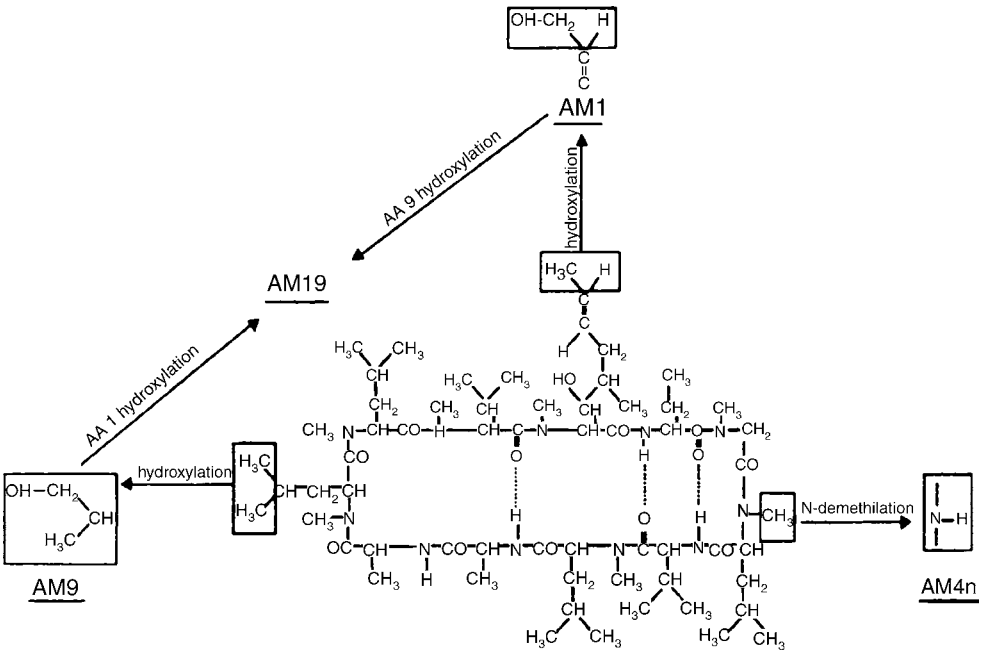

Fig. 1. Structure of cyclosporine A and metabolites (AM1, AM 9, AM19, and AM4N).

bination [1,17].

We describe an optimized high-performance liquid chromatograph (HPLC) method that is simple, fast, and efficient for simultaneous determination of CyA, AM1, AM9, AM4N, and AM19 in whole blood from transplant patients. This assay is based on a recently published method [18] and utilizes liquid-liquid extraction, isocratic HPLC separation and UV detection at 212 nm with a CN analytical column at 60 °C.

2. Experimental

2.1. Reagents and chemicals

Cyclosporine A, cyclosporine D (as internal standard) and metabolites AM9, AM1, AM4N, and AM19 were obtained from Novartis (Basel, Switzerland). Cyclosporines were dissolved separately in ethanol to make standard solutions. All other chemicals were HPLC grade: *n*-hexane, 1-propanol, diethyl ether, and ethanol were from E. Merck (Stuttgart, Germany).

2.2. Chromatographic conditions

For liquid chromatography, we used a high-performance liquid chromatograph with an isocratic pump (L-6000 A) equipped with a wavelength detector (L-4000), an automatic sampling system (AS-2000A), and a the thermostatic chromatograph oven (all HPLC components by Merck-Hitachi, Stuttgart, Germany). The detector signals were recorded with a computer interface with a Hewlett-Packard Chemstation. (A.05.01). The separation was on a 25 cm × 4.6 mm i.d. normal-phase column (Sperisorb S5 CN, 250-4 Phasesep, 5 μm), maintained at 60 °C. The flow rate of the hexane–isopropanol mobile phase (93:7, v/v) was 1.2 mL min^{-1}. The compounds were quantified by UV detection at 212 nm.

2.3. Preparation of standard solutions

Standard stock solutions of CyA and metabolites were prepared in ethanol at a concentration of 12.5 μg ml^{-1} and were kept at −20 °C. The internal standard cyclosporine D (CyD) was obtained by dissolving 2.0 μg ml^{-1} in ethanol.

2.4. Sample preparation and extraction

To 1 ml of whole blood in a screw-top test tube, 100 μl of a cyclosporine-D solution (internal standard) and 2 ml HCl (0.18N) are added and briefly agitated in a vortex machine. Then, approximately 7 ml diethyl ether is added and the samples agitated for 5 min and centrifuged for 5 min. The ether phase is put into another screw-top test tube containing 2.5 ml NaOH (0.1N), agitated for 3–5 min and then centrifuged. The ether phase, which is clear and colorless, is put into a tapered test tube and dried in a water bath at 37 °C in a nitrogen atmosphere. The residue is taken up in 120 μl mobile phase and an aliquote of 50 μl is autosampled for HPLC analysis.

2.5. Calibrations curves

Standard stock solutions were prepared in ethanol and consisted of CyA and each of the metabolites (12.5 μg ml^{-1}). These solutions were added to drug-free human whole blood

to prepare seven non-zero concentrations in the range of 31.0–1500 ng ml^{-1} CyA (31, 62.5, 125, 250, 500, 1000, and 1500 ng ml^{-1}); range 31.0–1000 ng ml^{-1} for AM1, AM9, AM4N, and AM19, (31, 62.5, 125, 250, 500, and 1000 ng ml^{-1}), respectively, and six quality-control concentrations of CyA combined with its metabolites. The lines that best fit calibration standards were determined using linear least-squares regression analysis based on the peak height ratio of the analytes to internal standard (CyD) versus concentration of cyclosporine.

2.6. Patients' samples

Kidney, heart, liver, and bone marrow transplant patients received CyA with or without co-medications such as mycophenolate mofetil, sirolimus, and prednisolone for immune suppression. Samples of EDTA whole blood were taken just before the morning administration of the respective cyclosporine dosage.

2.7. Evaluation

CyA and its metabolites were evaluated automatically with the HP Chemstation. Linear regression was calculated with the quotients of the peak height for CyA, AM9, AM1, AM4N, and AM19 and the internal standard (CyD).

3. Results and discussion

The determination and clinical interpretation of CyA and its metabolic products are of clinical significance as they permit evaluation of metabolic disorders due to those substances. Complications can arise because: (i) The whole-blood level of CyA varies considerably from patient to patient, even when the same dosage is applied, and depends on the type of transplant. (ii) There can be drug-in-drug interactions or liver dysfunction. It is known that a number of pharmaceuticals can delay or accelerate the metabolism of CyA. (iii) The CyA metabolites accumulate differently and are present in significant amounts relative to the parent drug [19].

If the necessary immune suppressive effect and the prompt recognition of accumulation and elimination are to be achieved, a fast and easy HPLC method is clearly essential. To this end, we developed a method for determining CyA and its four major metabolites in whole blood from transplant patients. It is a simple, fast, and optimized HPLC method that is robust enough to be run on standard HPLC equipment.

The analytical column (Sperisorb S5 CN) provides good selectivity and adequate resolution, and can be used under isocratic conditions with mobile phase (hexane–propanol). The temperature is maintained at 60 °C, assuring optimum column performance, as well as better maintenance of the retention time.

Under the conditions used, the eluted peak of CyA, its metabolites and internal standard were distinctly separated. Unidentified peaks appeared which did not interfere with the peaks of interest. Fig. 2 shows representative chromatograms from (A) drug-free blank human blood, (B) supplemented whole blood (CyA: 125 ng ml^{-1}; AM4N: 125 ng ml^{-1}; AM1: 250 ng ml^{-1}; AM9: 125 ng ml^{-1}; and AM19: 125 ng ml^{-1}) and (C) a blood extract from a heart transplant patient. The analysis was carried out on the second day after transplantation and after CyA administration (200 mg b.i.d.) and revealed 170 ng ml^{-1} for CyA, 110 ng ml^{-1} for AM4N, 300 ng ml^{-1} for AM1, 126 ng ml^{-1} for AM9, and 95 ng ml^{-1}

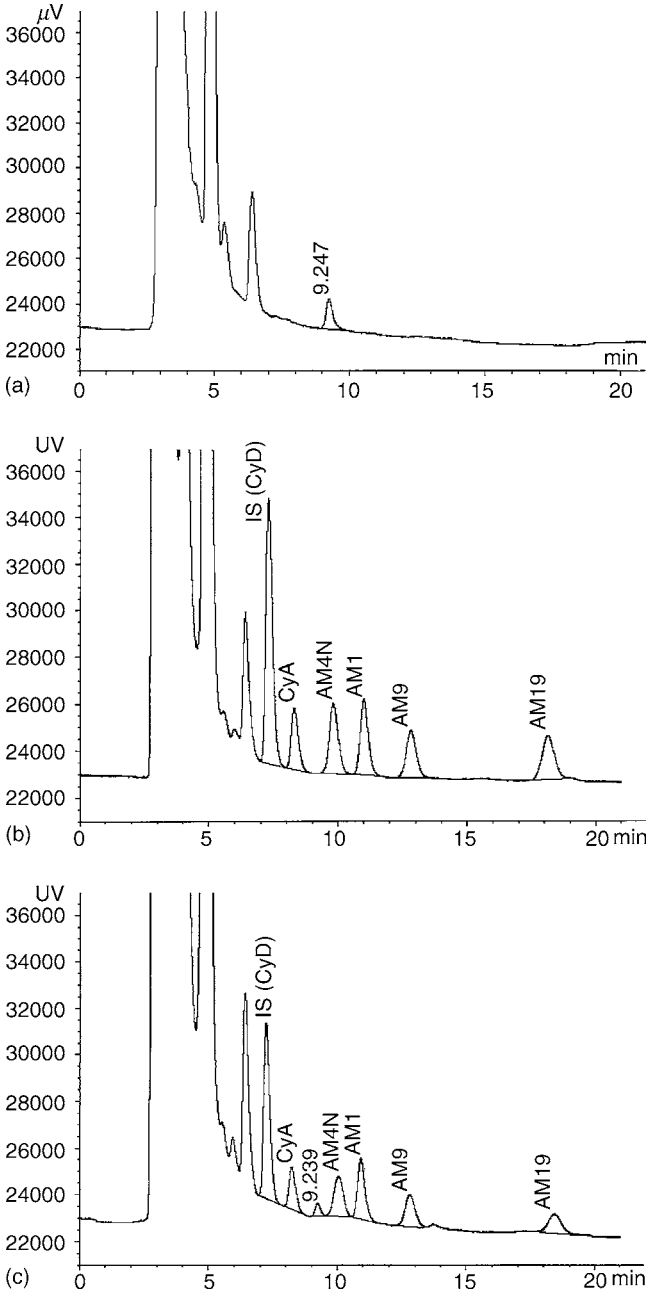

Fig. 2. HPLC chromatograms of CyA and its metabolites from: (A) drug-free blank human blood; (B) supplemented whole blood (CyA: 125; AM4N: 125; AM1: 250; AM9: 125; and AM19: 125 (ng ml^{-1}); and (C) a blood extract from a patient after heart transplant.

Table 1
Parameters of analytical performance for quantification of CyA, AM19, AM1, AM9, and AM4N in spiked and pooled whole-blood samples (n = 5)

Sample/analyte	Linearity (ng ml^{-1})	Sensitivity (ng ml^{-1})	Correlation coefficient (r)
CyA	31–1500	15	0.9963
AM19	31–1000	15	0.9986
AM1	31–1000	15	0.9984
AM9	31–1000	15	0.9973
AM4N	31–1000	15	0.9962

forAM19. The retention times were 7.2 min for CyD, 8.2 min for CyA, 10.1 min for AM4N, 10.9 min for AM1, 12.8 min for AM9, and 18.4 for AM19. No important interfering peaks were detected in the range of the retention times of CyA, CyD, AM1, AM9, AM4N, and AM19.

3.1. Linearity

Calibration curves in human whole blood were linear over a concentration range of 31–1500 (ng ml^{-1}) for CyA and range of 31–1000 (ng ml^{-1}) for metabolites. Each standard curve showed good linearity over the range of concentrations examined. The correlation coefficient was greater than 0.997 in all calibration curves (n = 5) and represented the ratio of the peak height of drug and the internal standard versus the concentration (Table 1). For linearity, regression analysis gave the following equations for CyA: $y = 1.680x + 1.369$, $r = 0.999$; for AM4N: $y = 2{,}085x + 4.500$, $r = 0.998$; for AM1: $y = 1.288x - 1.477$, r =0.998; for AM9: $y = 1.288x - 1.477$, $r = 0.997$; and for AM19: $y = 6.904x + 2.135$, r = 0.996; in which y is relative peak height and x is concentration in ng ml^{-1}. From these data, we defined the lower limit of quantification as the lowest calibration standard (31 ng ml^{-1}) and the upper limit of quantification as the highest standard for all components. The limit of detection, defined as the concentration that produces a signal-to-noise ratio of >3:1, was 15 ng ml^{-1} for all components.

3.2. Precision and recovery

Table 2 shows the precision and recovery of the assay in human blood. Intra- and inter-assay precision was determined with whole blood pools spiked with three different concentrations of the analytes. The intra- and inter-assay precision was expressed as the coefficient of variation (CV%).

Intra-assay precision for the CyA concentration range (125–1500 ng ml^{-1}) was 1.4–4.7%, and for inter-assay 1.6–4.9%. Intra-assay precision for all metabolites in the concentration range (31–500 ng ml^{-1}) was 2.0–9.4% for AM4N, 2.3–8.2% for AM1, 3.0–10.4% for AM9, and 2.2–11.2% for AM19. For the inter-assay for metabolites, the same concentrations were analyzed over a period of 6 days. The results are summarized in Table 2.

To determine recovery, known concentrations of CyA, AM1, AM9, AM4N, and AM19 were added to the pooled whole-blood samples and the concentrations determined in

Table 2
Parameters of analytical performance of the HPLC method for CyA and its metabolites (n = 6)

Sample/analyte	Analyte added (ng ml^{-1})	Intra-run (n = 6)			Inter-run (n = 6)		
		Analyte detected			Analytes detected		
		Mean ± S.D.	CV (%)	Recovery (%)	Mean ± S.D.	CV (%)	Recovery (%)
CyA	125	118.4 ± 5.6	4.7	94.7	120.8 ± 5.9	4.9	96.6
	500	465.2 ± 6.4	1.4	93.0	479.1 ± 7.7	1.6	95.8
	1500	1482.6 ± 37.6	2.5	98.8	1520.2 ± 38.9	2.6	101.3
AM4N	31	32.9 ± 3.1	9.4	106.1	29.4 ± 3.3	11.2	4.8
	250	260.0 ± 9.4	3.6	104.0	265.7 ± 9.1	3.4	106.2
	500	488.5 ± 9.8	2.0	97.7	494.9 ± 6.2	1.3	98.9
AM1	31	31.7 ± 2.6	8.2	102.2	33.7 ± 2.9	8.6	108.7
	250	245.5 ± 8.1	3.3	98.2	257.1 ± 9.4	3.7	102.8
	500	480.1 ± 10.8	2.3	96.0	490.8 ± 11.1	2.3	98.1
AM9	31	30.6 ± 3.2	10.4	98.7	33.6 ± 3.8	11.3	108.4
	250	255.0 ± 9.6	3.8	102.0	245.4 ± 8.1	3.3	98.1
	500	486.2 ± 14.8	3.0	97.2	504.7 ± 12.6	2.5	100.9
AM19	31	29.3 ± 3.3	11.2	94.5	30.5 ± 3.2	10.5	98.4
	250	259.9 ± 7.7	3.0	103.9	263.4 ± 7.0	3.8	105.3
	500	488.5 ± 10.9	2.2	97.7	480.5 ± 11.6	2.4	96.1

Table 3
Daily dose of CyA and trough levels of CyA and its metabolites AM1, AM9, AM4N, and AM19 in a 16-day pharmacokinetic profile based on whole-blood samples from a heart-transplant patient

Day	CYA-dose (mg day^{-1})	CyA (ng ml^{-1})	AM1 (ng ml^{-1})	AM9 (ng ml^{-1})	AM4n (ng ml^{-1})	AM19 (ng ml^{-1})
1	100/150	28	75	14	11	<10
2	200/200	46	180	21	11	41
3	200/300	115	410	132	13	128
4	300/250	180	490	160	<10	157
5	250/150	300	555	121	<10	183
6	100/100	305	460	156	14	198
7	100/100	220	540	79	17	133
8	100/100	230	750	135	12	215
9	100/100	140	520	62	<10	143
10	300/300	105	425	64	14	32
11	300/200	275	417	163	15	112
12	200/225	200	465	120	16	94
13	225/225	245	805	155	18	201
14	225/225	300	903	225	13	305
15	225/175	375	1000	290	13	337
16	225/175	315	664	169	21	190

five replicates. The mean recovery was 95.5% for CyA; for AM4N, 102.6%; for AM1, 98.8%; for AM9, 99.3%; and for AM19, 98.7% (Table 2).

As an additional check on the reliability of our method, we used commercially available control samples of CyA (level low 100 ng ml^{-1}, and level high 400 ng ml^{-1}, from Serobac, UTAK, Vienna, Austria) as external quality control. The results of analyses of these commercial controls compared with values provided by the manufacturer for CyA (n = 8). The total coefficient of variation (CV) for CyA was 3.2–4.3%.

This method was used to profile CyA and its metabolites for 16 days after heart transplant in a patient who also received MMF, to follow the daily course of CyA and the accumulation of the various metabolites (Table 3).

The stability of cyclosporine metabolites in whole-blood samples was evaluated by comparing triplicate assays in freshly spiked samples, prepared daily at four different concentrations, and in samples spiked with the same concentrations and stored under different conditions. No significant loss was observed after storage at room temperature for 2 days or at 4 °C for 10 days.

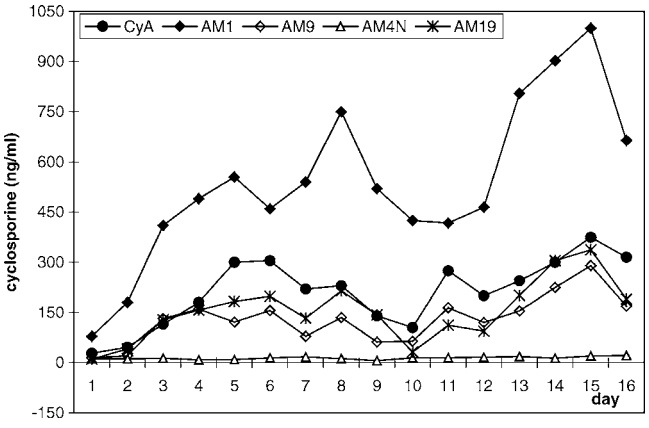

Fig. 3. Concentration profile of CyA and four metabolites over a period of 16 days after a heart transplant patient.

The applicability of the method was successfully proved by determination of the concentrations of CyA and its metabolites. Fig. 3 illustrates the trough levels of CyA as well as the metabolites AM1, AM9, AM4N, and AM19 in a 16-day pharmacokinetic profile based on whole-blood samples from this heart-transplant patient. This figure shows that there is a relative accumulation of metabolites during treatment with cyclosporine; this applies particularly to the AM1 metabolite, whose concentration can exceed that of the original substance (Fig. 3).

This method solves the problem of ongoing CyA adjustment with C2 values. The CyA concentration should be measured 2 h after administration of the medication to better predict exposition and clinical events than the trough level (C0). Our method can readily detect the values of up to 1200 ng ml^{-1} that are to be expected [20], while almost all of the immunological assays have an upper limit of maximally 800 ng ml^{-1}.

4. Conclusion

We present a simple, fast and sensitive HPLC method for the determination of CyA and its four metabolites in one cycle. This is the fastest method at present to quantify CyA and its major metabolites. In clinical routine, it provides additional information on the extent of the immune suppressive effect. Further, accumulation of CyA metabolites in excess of the toxic threshold can be recognized in a timely manner, as can errors in uptake and administration of the drug.

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