ELSEVIER

Journal of Chromatography B, 690 (1997) 367–372

JOURNAL OF CHROMATOGRAPHY B

Short communication

Rapid, sensitive high-performance liquid chromatographic method for the determination of cyclosporin A and its metabolites M1, M17 and M21

Gholamali Khoschsorur[a,*], H.J. Semmelrock[a], S. Rödl[a], T. Auer[b], W. Petek[a], F. Iberer[b], K.H. Tscheliessnigg[b]

[a]*Department of Laboratory Medicine, Division of Surgery, University of Graz, Auenbruggerplatz 29, A-8036 Graz, Austria*
[b]*Department of Transplantation, Division of Surgery, University of Graz, Graz, Austria*

Received 7 December 1995; revised 26 July 1996; accepted 30 July 1996

Abstract

Cyclosporin A (CyA) and its metabolites seem to have nephro-, hepato- and neurotoxic side effects. Immunosuppressive therapy is a narrow path between the risk of rejection by underimmunosuppression and toxic organ damage by overdosage. Thus CyA dosage must be calculated to avoid the risks of organ rejection through underdosage and toxic organ damage through overdosage or accumulation of metabolites. In routine monitoring of CyA therapy, it can be important to measure not only the parent drug but also the metabolites. We describe a rapid and isocratic high-performance liquid chromatographic method for measurement of CyA and its metabolites M1, M17 and M21 in whole blood. CyA was detected by ultraviolet absorption at 212 nm with a CN analytical column maintained at 50°C and recycling of hexane–isopropanol as mobile phase for improved long-term column stability and efficiency. The minimum detectable concentration of CyA and the three metabolites was 10 ng/ml blood. Our modified HPLC method for the determination of CyA and its metabolites is a simple (isocratic), rapid (the retention times were 7.1 min for CYD, internal standard, 8.9 min for CyA, 11.0 min for M21, 12.9 min for M17 and 16.3 min for M1) and economical method suitable for measuring the concentration of the major metabolite, M17, and for routine monitoring of CyA-treated patients.

Keywords: Cyclosporins

1. Introduction

The effectiveness of cyclosporin in immunosuppression has been responsible for the enormous increase in the number of transplantations during the last 15 years. Cyclosporin A (CyA) is a highly lipophilic cyclic peptide, comprised of 11 amino acids, with selective and potent immunosuppressive activity as well as a number of untoward side effects. Therapeutic uses include transplantation of solid organs (kidney, liver, heart, lung, pancreas) and bone marrow, as well as the treatment of autoimmune and rheumatoid diseases and application in dermatology and pulmonology.

Cyclosporin is widely assumed to be metabolized exclusively in the liver but the principal enzyme (cytochrome P450IIIA) that produces the three major

*Corresponding author.

PII S0378-4347(96)00364-7

cyclosporin metabolites (M1, M17 and M21) in liver is also found in enterocytes [1,2].

The three main metabolites, M17, M1 and M21, are oxidized in positions 1, 9 and 4(N), respectively [3], and considered first-generation metabolites [4]. More than 30 different metabolites have been isolated [5,6], but not all of them have been identified. CyA and its metabolites have neuro- [7,8], hepato- and nephrotoxic [9,10] side-effects. CyA can cause gingival hyperplasia [11], hypertrichosis [12] and malignomas [13]. Recently, Sewin et al. [14] used HPLC to characterize the metabolite pattern associated with cyclosporin nephrotoxicity in liver-graft patients.

Cyclosporin is a hydrophobic substance and not directly soluble in blood. It binds to hydrophobic sites of cell surface proteins and plasma lipoproteins [15,16]. Cyclosporin in serum mainly bonds with lipoproteins and the bonding capacity depends on the (lipoprotein) (Lp) lipid component [17]. Patients with lipid metabolism disorders show distinctive changes in cyclosporin level and response to the drug [18].

CyA inhibits a new immune response selectively at the molecular level as it suppresses interleukin-2 synthesis in the T-lymphocytes and thus the activation of T-helper lymphocytes and T-cytotoxic lymphocytes as well; T-suppressor lymphocyte function is retained [19]. It was shown in vitro that the primary metabolites 1 and 17 inhibit interleukin-2 formation in the same way as CyA [20]. A number of methods can be used to determine cyclosporin whole-blood levels. HPLC is currently the best method for quantifying CyA and some of its more important metabolites. Some of the reported methods, however, require time-consuming multi-step extraction procedures [21], some have unsatisfactory minimum detection limits [22–25], and some require expensive and elaborate equipment. All these methods have the disadvantage of lengthy and complicated preparation and retention times; above all, they do not provide information about metabolization rates in patients.

HPLC is the only technique that allows the specific quantification of cyclosporin and all its metabolites within a single run [4]. Our modified HPLC method for the determination of cyclosporin and metabolites M17, M1 and M21 is based on published methods [26,27].

Our HPLC method for the determination of CyA and its metabolites is a fast and economical method, which simultaneously provides information about the immunosuppressive effect and the metabolizing rate of CyA.

2. Experimental

2.1. Reagents

All chemicals were reagent or HPLC grade: *n*-hexane, 1-propanol, diethyl ether (Merck, Stuttgart, Germany). CyA and cyclosporin D (CYD, as internal standard) and metabolites M1, M17 and M21 were synthesized by Preclinical Research, Sandoz Pharmaceuticals (Basel, Switzerland). Cyclosporin was dissolved separately in ethanol to make standard solutions.

2.2. Patients

More than 5000 blood samples were taken from 334 patients; 65.8% had kidney transplants, 19% heart transplants, 2.6% liver transplants, 7.4% bone marrow transplants and 5.2%, autoimmune diseases.

CyA was administered twice a day during the examination period and EDTA whole blood samples were taken just before the morning administration of the respective cyclosporin dosage.

2.3. Instrumentation

For liquid chromatography, we used a high-performance liquid chromatograph (Merck-Hitachi) with an isocratic pump equipped with a wavelength detector, an integrator and an automatic sampling system. Separation was carried out on a 25 cm×4.6 mm I.D. normal-phase column (Sperisorb S5 CN, 250-4 phasesep, 5 μm) with the thermostatic chromatograph oven maintained at 50°C (all components by Merck-Hitachi). The flow-rate of the hexane–isopropanol mobile phase (90:10, v/v) was 1.45 ml/min. The column effluent was monitored at 212 nm. Chart speed was normally 1.25 cm/min.

2.4. Sample preparation and extraction

A 0.1-ml volumme of the CYD solution (as internal standard), 2 ml HCl (0.18 *M*) and approximately 7 ml diethyl ether were added to 1 ml of whole blood in a 15-ml screw-capped glass tube. After shaking and centrifugation (5 min each, 4200 g), the ether phase was transferred to another tube containing 2.5 ml NaOH (0.1 *M*). After shaking and short centrifugation, the ether phase was transferred to a conical glass tube and evaporated to dryness in a gentle stream of nitrogen. The residue was reconstituted with 0.2 ml mobile phase.

3. Results

Fig. 1 illustrates representative chromatograms from (A) drug-free blank human blood, (B) supplemented whole blood extracts and (C) a blood extract from a patient with a liver transplant. CyA was given intravenously at a dose of 3.5 mg/kg h. The analysis was carried out 24 h after CyA administration and revealed 228 ng/ml CyA, 192 ng/ml M21, 273 ng/ml M17 and 282 ng/ml M1.

The retention times were 7.1 min for CYD, 8.9 min for CyA, 11.0 min for M21, 12.9 min for M17 and 16.3 min for M1. No important interfering peaks

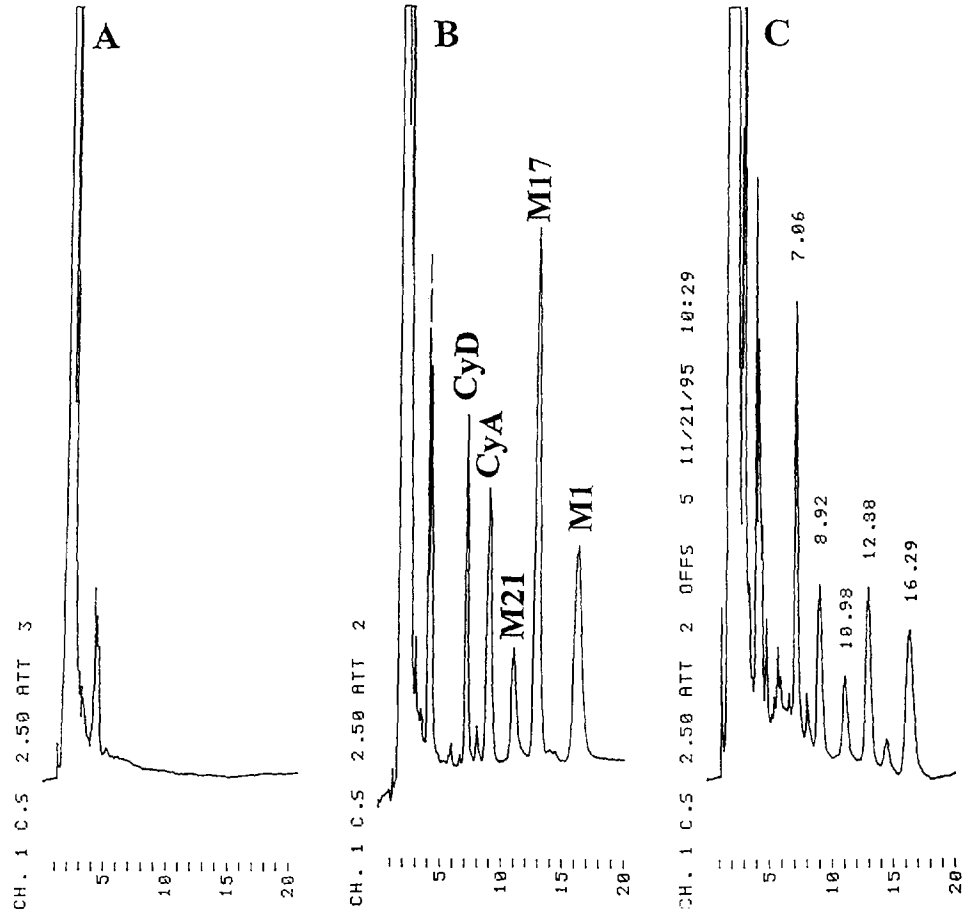

Fig. 1. Chromatograms of cyclosporin. Representative chromatograms of (A) drug-free blank human blood, (B) prepared whole blood standard containing CyD (internal standard, 1000 ng/ml), CyA (460 ng/ml), M21 (300 ng/ml), M17 (1000 ng/ml), M1 (500 ng/ml), (C) a patient's whole blood sample (CyA dose: 3.5 mg/kg.h, i.v.) of CyA (228 ng/ml) and metabolites M21 (192 ng/ml), M17 (273 ng/ml), M1 (282 ng/ml).

were detected in the range of the retention times of CyA, CYD, M1, M21 and M17.

3.1. Quantitation

The procedure was standardized by analysing 5–40 μl of CyA added to blank blood solution. The peak area of CyA was used to construct a calibration graph for blood samples. Linear regression was used to evaluate the statistical significance of the data. Linearity was tested by the measurement of blood standards. The peak area of CyA depended linearly on the concentration of CyA in the range from 10 ng/ml to 2000 ng/ml (r=0.995, n=10).

3.2. Repeatability

Intra-assay precision was determined by analysing ten replicate blood samples spiked with CyA at 115, 230, 460 and 920 ng/ml, with M21 at 150, 300 and 600 ng/ml, M17 at 125, 500 and 1000 ng/ml and M1 at 100, 200 and 500 ng/ml. The standard deviation and the coefficient of variation (%) are shown in Table 1. Day-to-day analyses (up to 30 days) revealed a C.V. of 7.6% for CyA in a prepared whole-blood sample containing 230 ng/ml cyclosporin, 8.5% for M17 in a sample containing 500 ng/ml M17, and 6.1 and 7.7% for M1 and M21, respectively, in samples containing 100 ng/ml.

Table 1
Analysis of blood samples spiked with CyA, M21, M17 and M1 calculated from ten measurements of each sample

Spiked concentration (ng/ml)	Measured concentration (mean±S.D.) (ng/ml)	C.V. (%)	Relative error (%)
CyA			
115	116.4±5.7	4.9	1
230	228.3±7.4	3.2	−1
460	456.2±6.7	1.5	−1
920	927.1±11.1	1.2	1
M21			
150	151.6±6.7	4.4	1
300	303.4±12.2	4.1	1
600	594.0±13.0	2.2	−1
M17			
125	123.9±5.9	4.7	1
500	480.0±10.5	2.2	−3
1000	974.1±14.7	1.5	−3
M1			
100	97.6±4.3	4.4	−1
200	196.6±9.2	4.7	0
500	492.8±9.3	1.9	−1

3.3. Precision, sensitivity and linearity

Table 1 shows good precision for the observed concentration of CyA and its metabolites. Blood calibration curves shows good linearity between concentration and peak-height ratio over the range of 50 to 2000 ng/ml. The limit of quantitation and the detection limit were 20 ng/ml C.V. <10%) and 10 ng/ml, respectively (defined on the basis of the amount injected that caused an absorption three times the noise level of the baseline).

4. Discussion

In this study, we used HPLC to determine the whole blood levels of cyclosporin and some individual metabolites (M1, M17 and M21) in patients who had undergone immunosuppressive treatment.

According to Burckart et al. [28], individual dosage adjustment is absolutely necessary because of the small therapeutic spectrum, the differences in bioavailability and metabolic activity among patients and the different types of transplantation.

Awni et al. [29] reported that renal clearance of M17 in humans is four times higher than that of cyclosporin. CyA monitoring, including the CyA metabolites pattern, is efficient in detecting and preventing CyA metabolite toxicity. High CyA metabolite blood concentrations have a neurotoxic effect [30,31].

Early detection of metabolic differences of CyA in patients would permit adjustment of dosage in a more timely fashion, thereby minimizing episodes of rejection or toxicity [32].

Our HPLC method is suitable for precise assessment of the immunosuppressively active CyA concentration. Moreover, it is possible to assess the most important metabolite of CyA, M17, by prolonging the analysis by approximately 4 min.

The results show that the HPLC method described here allows a rapid, sensitive and reliable determi-

nation of CyA and its major metabolites. The total retention time for CyA, M21, M17 and M1 ranged around 17 min.

The rate of resorption of cyclosporin after oral administration shows great individual variations, as does the metabolisation rate in the liver. The whole blood level of CyA varies considerably from one patient to another, even when the same dosage is used, and depends on the type of transplantation performed. For optimal dosage adjustment, it is crucial to carry out analyses of cyclosporin and M17 at frequent intervals to achieve the desired immunosuppressive effect. The simultaneous determination of CyA and its metabolites has clinical relevance, because it allows the detection of metabolic disorders caused, for example, by drug-in-drug interactions or liver dysfunction.

The clinical findings show that, in measurements of CyA and its metabolites (M17), the level measured just before CyA administration allows a representative statement on the absorption, distribution and elimination patterns that may vary considerably from one patient to another.

Our HPLC method for fractionation of cyclosporin from blood samples is very efficient, specific and precise. The analytical column (Sperisorb S5 CN) provides good selectivity and sufficient resolution, and can be used under isocratic conditions with mobile phase (hexane–propanol). Another distinct advantage of the method is that the analytical column is maintained at 50°C. We assume that this method also allows determination of other CyA metabolites.

In several complex metabolic disorders, such as chronic hepatic failure and drug interactions, it is difficult to find the optimal CyA dosage [33]. Rosano et al. [34] and Freed et al. [35] stated that metabolites M17 and M1 probably provide less than 20% of the total immunosuppressive effect in vitro, while the secondary metabolites M18 and M8 show little or no immunosuppressive activity. Kunzendorf et al. [36] reported that higher blood levels of M17 and M1 correlated with a decrease in the frequency of rejection episodes in human recipients of renal allografts. CyA monitoring, including the CyA metabolite patterns, is probably efficient in detecting and preventing CyA metabolite toxicity. According to an investigation carried out by Sandoz Laboratories, the metabolite M17 provides about 10% of the immunosuppressive effect of CyA. In contrast, Lucey et al. [37] reported that CyA metabolites appear to contribute to the immunosuppressive effect of CyA only to a negligible extent, but there is a strong association between blood CyA metabolite concentrations and CyA metabolism. Our results of CyA and M17 clearly show the extent of metabolism of CyA. A specific HPLC procedure, including metabolites M1, M21 and M17, may be a better tool for drug monitoring of CyA-treated patients to assess the interaction and relationship of CyA and metabolites in therapy and toxicity.

The method described here is currently being used in our laboratory for routine clinical monitoring of cyclosporin therapy. We have found it to be accurate, reproducible, efficient and adequately sensitive for analysis of patient samples.

1. Despite isocratic elution, retention time for the determination of the parent drug and the metabolites is 17 min, after which the next injection can be carried out.
2. The column temperature is maintained at 50°C, resulting in a longer life and a better reproducibility of the column.
3. Sample preparation is simple, inexpensive and rapid (12 min per sample; the preparation time decreases when several samples are prepared), which is very important in clinical routine. Because of the purity of the extracts, the column is exposed to minimal strain. Recycling of the mobile phase results in a further improvement of column life (approximately 1000 analyses per column and 2 l mobile phase). Furthermore, we carried out daily checks to determine the CyA and metabolite concentrations.
4. Several years of experience with this method have shown no interferences with other drugs such as antibiotics, except for Carbamazepin and Myoselin (in these two drugs, separation of CyA can be done by varying the hexane–isopropanol ratio of the mobile phase; however, the retention time is longer).
5. Because CyA metabolites, particularly M17, are gaining increasing importance, it is an advantage of our method that both the parent drug and the metabolites can be determined in one single run.

Acknowledgments

The authors wish to acknowledge Ing. Franz Frühwirth for his excellent technical assistance.

References

[1] T. Kronbach, V. Fischer and U.A. Meyer, Clin. Pharmacol. Ther., 43 (1988) 630.
[2] P.B. Watkins, S.A. Wrighton, E.G. Schuetz, D.T. Molowa and P.S. Guzelian, J. Clin. Invest., 80 (1987) 1029.
[3] R.M. Wenger, Transplant Proc., 22 (1990) 91–104.
[4] U. Christians and K.F. Sewing, Pharmacol. Ther., 57 (1993) 291.
[5] G. Maurer and M. Lemaire, Transplant Proc., 18 (Suppl. 5) (1986) 25.
[6] P.E. Wallemacq, G. Lhoest and P. Dumont, Biomed. Environ. Mass Spectrom., 18 (1989) 48.
[7] U.J. Brockmüller, F. Jochimsen, F. Keller, G. Walz, I. Roots and G. Offermann, Lancet, i (1988) 1223.
[8] A. Davenport, E.J. Will, A.M. Davison and J.W. Ironside, Lancet, ii (1988) 333.
[9] K. Kohlhaw, K. Wonigeit, U. Frei, K. Oldhafer, K. Neumann and R. Pichlmayr, Transplant. Proc., 20 (1988) 572.
[10] K. Leunissen, G. Baumann, R. Bosman and J.P. Van Hoof, Lancet, ii (1986) 1398.
[11] G.P. Wysocki, W. Gebhardt, H. Kopsa and J. Spona, Oral Surg., 55 (1983) 274.
[12] J.B. Schmidt, W. Gebhardt, H. Kopsa and J. Spona, Exp. Clin. Endocrin., 88 (1986) 207.
[13] I. Penn and M.E. Brunson, Transplant. Proc., 20 (1988) 885.
[14] K.D. Sewin, U. Christians and K. Kohlhaw, Transplant Proc., 22 (1990) 1129.
[15] W. Mraz, A. Graf, W.D. Lillner, W. Land, D. Preis, W. Sieber and H. Zottlein, Transplant Proc., 15 (1983) 2426.
[16] D.W. Holt, J.T. Marsden, A. Johnston, M. Bewick and D.H. Taube, Br. Med. J., 293 (1986) 1057.
[17] S. Rödl and G. Khoschsorur, Transplant Proc., 22 (1990) 287.
[18] H.L. Verrill, R.E. Girgis, R.E. Easterling, B.S. Malhi and W.F. Mueller, Clin. Chem., 33 (1987) 423.
[19] I. Wich, Transplant. Proc., 18 (1986) 15.
[20] B.M. Freed, T.G. Rosano and N. Lempert, Transplantation, 43 (1986) 123.
[21] J.R. Sawchuk and L.L. Cartier, Clin. Chem., 27 (1981) 118.
[22] B. Kahan, C. Von Buren, S.N. Lin, Y. Ono, G. Agostino, S.J. Legrue, M. Boileau, W.D. Payne and R.H. Kerman, Transplantation, 34 (1982) 36.
[23] L.G. Lensmeyer and L.F. Barry, Clin. Chem., 31 (1985) 196.
[24] H. Hosotsubo, J. Takezawa, N. Taenaka, K. Hosotsubo and I. Yoshiya, J. Chromatogr., 383 (1986) 349.
[25] G. Hamilton, E. Roth, E. Wallisch and F. Tichy, J. Chromatogr., 341 (1985) 411.
[26] S.G. Garruthers, D.J. Freeman, J.C. Koegler, W.Howson, P.A. Keown, A. Laupacis and C.R. Stiller, Clin. Chem., 29 (1983) 180.
[27] P.E. Wallemacq and M. Lesne, J. Chromatogr., 413 (1987) 131.
[28] G.J. Burckart, J. Brady, B.P. Griffith, T.R. Hakala, R.L. Hardesty, S. Iwatsuki, R.J. Ptachcinski, J.T. Rosenthal, T.E. Starzl, D.H. Vanthiel and R. Venkataramanan, Transplant. Proc., 18 (Suppl. 5) (1986) 457.
[29] W.M. Awni, B.L. Kasiske, K. Heim-Duthoy and K.V. Rao, Transplant. Proc., 20 (Suppl. 2) (1988) 115.
[30] A. Davenport, E.J. Will, A.M. Davison and J.W. Ironside, Lancet, ii (1988) 333.
[31] K. Leunissen, G. Baumann, R. Bosman and J.P. Van Hooff, Lancet, ii (1986) 1398.
[32] G.L. Lensmeyer, D.A. Weibe and I.H. Carison, Clin. Chem., 33 (1987) 1851.
[33] U. Nattermann, W. Steiner, W. Land, Transplant. Med., 4 (1990) 8.
[34] T.G. Rossano, B.M. Freed, J. Gerilli and N. Lempert, Transplantation, 42 (1986) 262.
[35] B.M. Freed, C. Stevens, C. Brooks, S. Cramer, N. Lempert and T.G. Rosano, Transplant. Proc., 23 (1991) 980.
[36] U. Kunzendorf, J. Brockmöller, F. Jochimsen, I. Roots and G. Offermann, Lancet, i (1989) 734.
[37] M.R. Lucey, J.C. Kolars, R.M. Merion, D.A. Campbell, M. Aldrich and P.B. Watkins, Lancet, 335 (1990) 11.