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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC COLUMN-SWITCHING METHOD FOR TWO CYCLOSPORINE METABOLITES IN BLOOD

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SUMMARY

Cyclosporine (CSA) is biotransformed to many metabolites which may contribute to its immunosuppressive and nephrotoxic activity. We report a rapid and sensitive, automated column-switching high-performance liquid chromatographic (HPLC) method for measuring CSA-M17 in whole blood; the method also separates CSA-M1. CSA metabolite standards were isolated by a preparative-scale HPLC method. Samples were prepared by protein precipitation with acetonitrile followed by dilution with water. CSA-M17 was initially separated on a C_8 column; final separation was on a C_{18} column. The inter-day relative standard deviation at 50 ng/ml was 8% ($n=3$). Limit of detection was 20 ng/ml.

INTRODUCTION*

Cyclosporine is a cyclic undecapeptide of fungal origin [1,2]. It is an immunosuppressive agent used to prevent graft rejection in solid organ transplant re-

*Abbreviations used: CSA = cyclosporine; CSC = cyclosporin C; CSA-M1 = (4-hydroxy-N-methyl-L-leucine[9]) cyclosporin; CSA-M17 = [(N-methyl)-2-amino-3,8-dihydroxy-4-methyl-6-octenoic acid[1]] cyclosporin; CSA-M18 = [(N-methyl)-2-amino-3,6-oxa-4-methyl-8-hydrooctanoic acid] cyclosporin; CSA-M21 = (N-desmethyl-L-leucine[4]) cyclosporin; HPLC = high-performance liquid chromatography; NMR = nuclear magnetic resonance; FAB-MS = fast atom bombardment mass spectrometry; R.S.D. = relative standard deviation.

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cipients [3] and graft-versus-host disease in marrow transplant recipients [4,5]. Although CSA is one of the most effective immunosuppressive agents, it has several adverse effects, particularly nephrotoxicity [6].

CSA is biotransformed in humans to at least thirteen metabolites [7–9]. All of the metabolites identified thus far show that the cyclic peptide structure is intact with minor oxidative changes of the amino acid side-chains. At least four CSA metabolites (CSA-M1, CSA-M17, CSA-M18, and CSA-M21, numbered according to ref. 8) are present in human blood [9–11]. Whole blood concentrations of one of the metabolites, CSA-M17, have been reported to be higher than those of parent drug [10,11]. Of the four metabolites tested for immunosuppressive activity, CSA-M17 and CSA-M1 have 20–100% of the in vitro immunosuppressive activity of CSA while CSA-M21 and CSA-M8 have considerably less activity [10,11].

Since CSA metabolites may contribute to the immunosuppressive and nephrotoxic effects of CSA, further studies are needed to determine the clinical benefit of monitoring blood CSA metabolite concentrations. Previously reported assays to measure CSA, CSA-M17, CSA-M1, CSA-M18, and CSA-M21 in whole blood samples use a single-column (either isocratic or gradient) HPLC method [10–13]. The major disadvantages of these methods are laborious sample treatments and lengthy assay times. Column-switching techniques, several of which have been developed for CSA but not for CSA metabolites, could overcome these limitations [14–19]. We report a column-switching HPLC method for measuring CSA-M17 in whole blood; the method also separates CSA-M1.

EXPERIMENTAL

Reagents

Small amounts of CSA-M17, CSA-M1, and CSA-M21 to be used for retention indices were graciously supplied by Dr. Gerald Maurer (Department of Pharmacokinetics and Biopharmaceutics, Sandoz, Basel, Switzerland). CSA and CSC were also supplied as HPLC standards by Sandoz. Acetonitrile, methanol, and acetic acid (J.T. Baker, Phillipsburg, NJ, U.S.A.) were HPLC grade. Water was deionized with a water purification system (Milli-Q system, Continental Water Systems, El Paso, TX, U.S.A.). Outdated whole blood and plasma were obtained from the local blood bank.

Metabolite isolation

Because the amounts of CSA-M17, CSA-M1, and CSA-M21 were not adequate for standardization, we isolated additional amounts from urine of patients receiving CSA. We used an "applejack" process [20] to concentrate CSA metabolites before small column extraction and semi-preparative HPLC. Patient urine (800-ml aliquots) was transferred into 1000-ml plastic plasma transfer sets (Fenwal Labs., Deerfield, IL, U.S.A.) and frozen at $-20\,^{\circ}C$ until used. A 60-μg amount of CSC was added to one bag before freezing to measure the efficiency of the overall isolation technique. To concentrate metabolites in the urine, 1600 ml (two bags) of urine were thawed at $6\,^{\circ}C$. The concentrate was collected (12 min

per fraction) with a Pharmacia fraction collector (Pharmacia, Piscataway, NJ, U.S.A.); the first 200 ml of concentrate were pooled. The pool was filtered sequentially through Whatman No. 4 filter paper (Whatman, Maidstone, U.K.), a GF/C glass filter, and a 0.45-μg pore-sized Nylon membrane (Supelco, Bellefonte, PA, U.S.A.). To measure efficiency of the disposable extraction columns, three of the 200-ml aliquots of concentrate (none of which came from the bag previously spiked) were spiked with 40 μg each of CSC prior to filtration and extraction.

CSA metabolites were further concentrated on 3-ml disposable extraction columns packed with cyanopropyl (CN) silane bonded silica gel (J.T. Baker). To extract 200 ml of filtered urine concentrate, twelve CN columns were each conditioned with 3 ml of acetonitrile, 3 ml of 0.1 *M* hydrochloric acid, and 3 ml of water, in that order. All solutions were eluted by vacuum. The urine was divided equally ($\sim$16 ml) among the columns and eluted, taking care to keep the columns wetted. The columns were each washed with 3 ml of water, 2$\times$3 ml of 0.1 *M* sodium hydroxide, 3 ml of 5% acetonitrile in 0.1 *M* sodium hydroxide, 3 ml of 0.1 *M* hydrochloric acid, and 3 ml of water. CSA and CSA metabolites were eluted with 3$\times$0.5 ml of methanol per column. The twelve methanol fractions were evaporated nearly to dryness with a gentle stream of dry nitrogen and consolidated into one vial before being taken to dryness.

We first developed the metabolite separation on an analytical-scale (0.46 cm I.D.) HPLC column before we transferred the gradient to a preparative-scale column. CSA metabolites were separated on a gradient HPLC system (IBM LC/9533, Danbury, CT, U.S.A.) equipped with a variable-wavelength UV detector (IBM 9523) set at 230 nm and a data integrator (IBM LC/9540). The sample was made up in 2 ml of 35% acetonitrile in water and filtered through a 0.45-μm Millex-HV filter (Millipore, Bedford, MA, U.S.A.). The sample was injected onto a Spherisorb 5-μm ODS-2 column (25 cm$\times$1.0 cm) heated to 70°C. The sample was eluted with a gradient starting at A–B–C 33:1:66 (A=acetonitrile, B=methanol, and C=10% acetonitrile in water). The ratio A–B–C was increased linearly to 50:1:49 at 45 min, increased to 51:1:48 at 55 min, increased linearly to 72:1:27 at 75 min, and held at that ratio until 80 min, the end of run. The flow-rate was 4.5 ml/min and the delay volume from the proportioning valve to the column head was about 7 ml. Fractions were collected every 0.5 min starting at 26 min. Fractions containing CSA-M17 were pooled, and the solvent was evaporated. The dried sample was re-chromatographed with the same HPLC system and chromatographic conditions described above except that fractions were collected every 0.25 min starting at 49 min. Fractions were combined from the portion of the peak with absorbance above 0.9 absorbance units. The rest of the fractions from the peak shoulders were saved for repurification. Solvent was again evaporated and residual solvent was removed under vacuum (100 mTorr for 16 h). The same procedure was used for CSA-M1.

Spectroscopic studies

^{1}H and ^{13}C NMR spectra were recorded on a Varian VXR 300 in deuteriobenzene at 300 and 75.4 MHz, respectively. The spectra were referenced to tetra-

methylsilane (0.0 ppm) for ^{1}H spectra and to deuteriobenzene (128.0 ppm) for ^{13}C spectra.

The molecular mass of CSA-M17 was established by FAB-MS [21] (glycerol matrix, Xe ionization at 8 keV acceleration voltage) on a VG 70 SEQ mass spectrometer.

CSA metabolite assay

The assay for CSA-M17 in blood was developed from the assay for CSA in blood (described below). A stock solution of CSA-M17 was prepared by weighing 1.88 mg on a Cahn electrobalance and dissolving it in 25.0 ml of water–isopropyl alcohol (50:50). Whole blood standards were prepared by adding appropriate amounts of stock solution to outdated blood (to approximate patient values, the hematocrit was adjusted to 0.35 with outdated plasma of the same type). Standards were prepared at concentrations of 0, 50, 100, 250, 500, and 1000 ng/ml.

Sample preparation. Whole blood samples or standards (0.7 ml) were mixed with acetonitrile (0.9 ml) acidified with orthophosphoric acid (0.015 m*M*). The sample was vortexed, then centrifuged at ~10 000 *g* in a Beckman microfuge (Beckman Instruments, Palo Alto, CA, U.S.A.) for 1 min; the supernatant was decanted, then frozen for 30 min at −70°C in a chest freezer (SoLow Environmental Equipment, Cincinnati, OH, U.S.A.). The sample was thawed, then centrifuged again. An aliquot (1050 μl) of the supernatant was diluted with water (750 μl).

Chromatography. The sample was assayed with the same HPLC system described in our previous paper [17] except that a Valco ten-port electrically actuated switching valve (Valco Instrument, Houston, TX, U.S.A.) replaced the Rheodyne twelve-port tandem-switching valve. A portion of the sample (1.5 ml) was injected onto the Spherisorb 5-μm C_8 (15 cm × 0.46 cm) column (Phase Separations, Norwalk, CT, U.S.A.) and eluted with acetonitrile–methanol–water (50:1:49) at a flow-rate of 1.9 ml/min. From 7.5 to 10.5 min after injection, the effluent from the C_8 column was diverted onto the C_{18} (Spherisorb 5-μm ODS-2, 25 cm × 0.46 cm) column. The peaks of interest were eluted from the C_{18} column with acetonitrile–methanol–water (59:1:40) at a flow-rate of 1.0 ml/min. The mobile phases all contained 1-m*M* acetic acid, and both columns were heated to 75°C. At 21.8 min post injection, the automatic data reduction system began monitoring the C_{18} effluent to record the chromatogram; simultaneously, the next sample was injected. Data for the chromatogram were recorded for 4 min. Both peak height and peak area were used to calculate CSA-M17 concentration (by comparison with standards). Two standard curves were calculated, each day an assay was done, from six duplicate standards (0, 50, 100, 250, 500 and 1000 ng CSA-M17 per ml) interspersed among the unknowns.

As in the previously reported method for CSA, late-eluting peaks were cleaned from the C_8 column with a clean-up loop that injected 2 ml of C_{18} eluent onto the C_8 column after the switch. The C_{18} column was cleaned by simultaneously increasing the acetonitrile content of the mobile phase to 95% and increasing the flow-rate to 3.0 ml/min for 1.7 min after the chromatogram was recorded. For the

next 1.8 min the C_{18} mobile phase returned to beginning percentages, and, after the switch at 7.5 min, the flow-rate was returned to 1.0 ml/min.

Validation. Intra- and inter-day accuracy and precision of the assay were determined at three concentrations of CSA-M17 added to outdated blood (50, 100, and 500 ng/ml). Additional inter-day precision studies were done with patient blood pools collected from 24-h CSA time courses. The samples for the pools were divided into three groups according to time after infusion. Pools 1, 2, and 3 corresponded to samples collected fom the beginning (0–1 h after infusion), middle (1–10 h), and end (10–24 h) of the time course. These patient pools have been used routinely as quality controls for our CSA column-switching assay. The CSA values (mean ± S.D.) for pools 1–3 were 996 ± 91, 322 ± 30, and 145 ± 15 ng/ml ($n = 50$), respectively.

Forty-four whole blood trough (pre-dose) samples from six marrow transplant recipients receiving CSA therapy were used to further test the assay.

CSA assay

Several minor modifications in our column-switching method for CSA in serum [17] were made to allow us to measure CSA in whole blood. The sample preparation was as described above for CSA-M17. The mobile phase for the C_8 column was acetonitrile–methanol–water (56:1:43) at a flow-rate of 2.5 ml/min. Since methanol has been reported to sharpen CSA peaks [22], various mobile phase compositions were tried to determine which methanol concentration was most effective in this system. Mobile phases with 0, 1, 3, 5, 8, 10, 12, 25, and 50% methanol were tried with respective acetonitrile percentages of 58, 57, 56, 54, 52, 51, 50, 41, and 20. CSA retention times stayed within 0.5 min except at 0 and 50% methanol where the retention times were less. The mobile phase for the C_{18} column was acetonitrile–methanol–water (76:1:23) at a flow-rate of 0.9 ml/min. The column switch was from 8 to 12 min after injection and the C_{18} column was monitored for 3.5 min beginning 18.5 min after injection (6.5 min after the end of the switch). Two standard curves were calculated, each day an assay was done, from six duplicate standards (0, 50, 100, 250, 500, and 1000 ng CSA per ml) interspersed among the unknowns. Controls of CSA added to outdated blood and controls from patient pools (described above) were included in every assay.

RESULTS

Isolation of CSA metabolites by our preparative method was effective (Fig. 1), yielding an easily weighable amount of CSA-M17. About 5 l of patient urine and two months of time were required to isolate and purify that amount of CSA-M17. Recovery of CSC from a single bag of urine was 48% which shows that CSA metabolites were effectively concentrated by this technique. Recovery of CSC from freeze-concentrated urine was not very reproducible; it varied from 83% (from normal looking urine) to 28% (from some very green urine). The molecular mass of CSA-M17 was confirmed to be 1218 ($MH^+ = 1219$ and $M + Na^+ = 1241$) by mass spectrometry. The presence of a sodium ion indicates possible contamination with a sodium salt. The ^1H NMR showed a shift of the

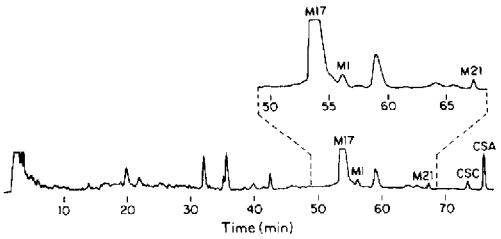

Fig. 1. Typical chromatogram from a preparative-scale separation of a CN column extract of freeze-concentrated urine. Inset is an enlargement of the area around CSA-M17.

vinyl methyl signal at 1.8 ppm for CSA (the ^{1}H NMR for CSA is from ref. 23) to a signal at 4.3 ppm for the vinyl hydroxymethyl group of CSA-M17. In addition the spectrum showed vinyl protons slightly shifted (5.84 and 6.06) and showed retention of all seven N-methyl groups. Hydroxylation of the vinyl methyl ($C\eta$) group caused a downfield shift ($\Delta\delta = -45.0$) of the corresponding ^{13}C NMR signal similar to that found by Maurer et al. [8]. NMR analysis also showed no measurable contamination from other CSA metabolites or any other protonated compounds. HPLC co-injection (Fig. 2) of the stock solution with CSC and CSA standards showed no extraneous peaks larger than 0.5% of CSA-M17 (within 1.0 min of the peak). The area response at 214 nm for CSA-M17 was 96% that of CSC and 91% of that of CSA on an equal molar basis. The area response of CSC was 95% that of CSA.

Some changes were made in our previously published assay for CSA in serum samples to accommodate human blood samples. Addition of 1% methanol to the mobile phases sharpened peaks; other methanol concentrations were not as effective although there was a local optimum at 10% methanol. Addition of acetic acid slowed column-packing dissolution. Freezing and thawing the samples pre-

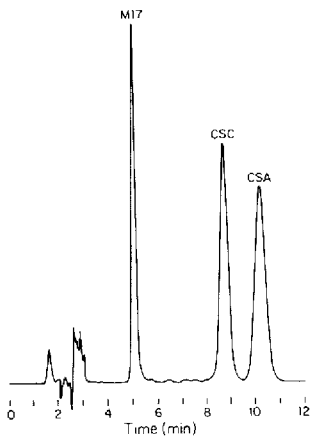

Fig. 2. Chromatogram of CSA-M17 (190 ng), CSC (250 ng), and CSA (250 ng). The sample was separated isocratically on a Spherisorb 5-μm ODS-2 (25 cm×0.46 cm) column. Mobile phase was acetonitrile–methanol–water (76:1:23) with 1 m*M* acetic acid. Compounds were monitored at 214 nm.

TABLE I

INTRA-DAY ASSAY PRECISION FOR CYCLOSPORINE METABOLITE NO. 17 ($n=10$)

CSA-M17 added (ng/ml)	CSA-M17 measured (mean ± S.D.) (ng/ml)	R.S.D. (%)
50	59 ± 5	8
100	98 ± 3	3
500	503 ± 10	2

TABLE II

INTER-DAY ASSAY PRECISION FOR CYCLOSPORINE METABOLITE NO. 17 ($n=3$)

CSA-M17 added (ng/ml)	CSA-M17 measured (mean ± S.D.) (ng/ml)	R.S.D. (%)
50	54 ± 4	8
100	96 ± 4	4
500	487 ± 18	4
Pool 1	245 ± 19	8
Pool 2	201 ± 8	4
Pool 3	116 ± 4	3

vented a later, secondary precipitation that occasionally plugged in-line filters and sample loops. Finally, the addition of water to the sample supernatant prevented peak broadening on the C_8 column, thus allowed a more reproducible elution time. When the column-switching assay was adapted to CSA-M17, the per sample analysis time was longer because of the need to resolve CSA-M17 and CSA-M1. No interference from late-eluting substances from previous injections was found in either assay.

Tables I and II show the accuracy and precision of the column-switching CSA-M17 assay. A typical standard curve had a y-intercept of 1.0 ng/ml with an S.D. of ± 10 ng/ml, indicating a sensitivity of about 20 ng/ml (~ 2 S.D. from 0). Fig. 3 shows typical chromatograms from an automated assay of patient unknowns. CSA-M1 was also eluted from the C_8 column and was well resolved from CSA-M17 ($R=1.3$) by the C_{18} column. The identity of CSA-M1 has been confirmed by retention time and by NMR spectroscopy. Both the ^1H and ^{13}C NMR spectra of our material agree with the data reported by Maurer et al. [8]. The ^1H NMR spectrum (D6-benzene as solvent) shows the loss of two methyl doublets at 0.91 and 0.84 ppm and the appearance of two singlets at 1.02 and 1.12 ppm (Maurer reports these signals at 1.22 and 1.25 ppm in $CDHCl_3$). Similarly the ^{13}C NMR spectrum shows the loss of a signal at 24.7 ppm and the appearance of a new signal at 69.0 ppm which corresponds to the hydroxylation of the Cγ of amino acid 9. More time and material will be required to isolate enough CSA-M1 to make a standard since it was present in lower concentrations than CSA-M17 in human urine (Fig. 1).

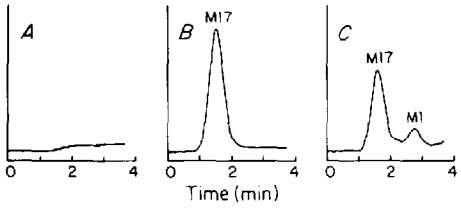

Fig. 3. Chromatograms from an automated column-switching assay for CSA-M17. (A) Blank blood sample; (B) standard of 500 ng/ml; (C) patient sample with a measured concentration of 310 ng/ml. The smaller peak is CSA-M1.

TABLE III

CYCLOSPORINE AND CYCLOSPORINE METABOLITE NO. 17 CONCENTRATIONS IN MARROW TRANSPLANT RECIPIENTS

Compound	Concentration (ng/ml)		
	Range	Median	Mean ± S.D.
CSA	25–662	207	265 ± 165
CSA-M17	<20–506	174	219 ± 125

CSA-M17 concentrations were calculated by peak height because the peak shape of CSA-M17 was rather wide (30 s width at half-height) and because of the presence of an interfering peak between CSA-M17 and CSA-M1 in patient samples (Figs. 1 and 3). Concentrations of CSA and CSA-M17 in the 44 whole blood trough samples are shown in Table III. CSA-M17 concentrations exceeded CSA concentrations in 10 of the 44 samples. The median CSA-M17-to-CSA concentration ratio was 0.82 (range 0.37–2.16).

DISCUSSION

We have developed a column-switching HPLC method for CSA metabolites because of our need for a rapid and sensitive assay to study the clinical importance of blood or plasma CSA metabolite concentrations. Our assay has both advantages and disadvantages compared to the assays reported by Rosano and co-workers [10,11] and Bowers and Singh [12]. Rosano and co-workers reported the sensitivity of their assay to be 20 ng/ml, but data concerning the accuracy and precision of their assay for metabolites were not provided. Bowers and Singh did not report a sensitivity for their assay, but they reported an R.S.D. of 5.6% at an unspecified CSA concentration. The advantage of these two methods is the ability to measure both CSA and many CSA metabolites with the same injection. An additional advantage of the method of Rosano and co-workers is the use of a lower column temperature (65 versus 75°C). The advantages of our assay are the relatively simple sample preparation and the short assay time, which allows rapid analysis of samples. One disadvantage of our assay is the inability to measure CSA and CSA metabolites simultaneously. However, even though we assay

the same blood sample twice, once to measure CSA and once to measure CSA-M17, the total analysis time is still considerably shorter (41 min) than the assay by Rosano and co-workers (>60 min) and Bowers and Singh (55 min).

Because a column-switching method does not lend itself to generating metabolite profiles, the choice of which metabolites to measure must be considered carefully. We chose to measure CSA-M17 and CSA-M1 in blood for three reasons. Firstly, of the metabolites tested thus far, CSA-M17 and CSA-M1 are present in the highest concentrations in human blood [9–11]. CSA-M17 concentrations have been reported to be two-fold higher and CSA-M1 concentrations slightly lower than CSA concentrations in human blood [11]. Secondly, of the metabolites tested to date, CSA-M17 and CSA-M1 have the highest in vitro immunosuppressive activity; in one assay — inhibition of interleukin-2 production in a mixed leukocyte culture — CSA-M17, CSA-M1, and CSA have about the same activity [11]. Thirdly, CSA-M17 and CSA-M1 can be measured simultaneously on the same injection due to similar retention times on both HPLC columns; we only lack a CSA-M1 standard. We do not want to make any assumptions about CSA-M1 UV absorption characteristics due to the unequal UV absorbance response of CSA-M17, CSC, and CSA.

The performance of our column-switching method for CSA metabolites is similar to that reported for column-switching methods for CSA. Our inter-day R.S.D. values of 8 and 4% at 50 and 100 ng/ml, respectively, compare favorably to those reported for CSA [14–19]. The sensitivity of our CSA-M17 assay (20 ng/ml) is comparable to those reported for column-switching assays for CSA [14–19]. The sample preparation in our method is slightly more difficult than some of the CSA assays that use a protein precipitation step and dilution with water [14,16,18,19]. Our sample assay time is less than those reported by Fiore [18] (24 min) and Nussbaumer et al. [14] (26 min), but is more than those reported by Hamilton et al. [16], Smith and Robinson [15], or Hosotsubo et al. [19] (15 min).

In summary, several studies suggest that CSA metabolites contribute to the immunosuppressive and nephrotoxic activity of CSA. Although CSA is biotransformed to many metabolites, two of the metabolites (CSA-M17 and CSA-M1) have significant in vitro immunosuppressive activity and are present in relatively high concentrations in human blood. The clinical importance of CSA metabolites in blood is not clear, and studies to determine their clinical importance are limited by the lack of a rapid HPLC assay for CSA metabolites. We report here a rapid, sensitive column-switching HPLC assay to measure these two CSA metabolites in human blood. As further information on other CSA metabolites is reported, this assay can probably be modified to measure other CSA metabolites.

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