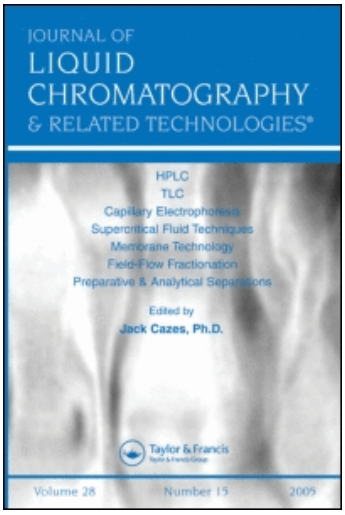

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A Gradient HPLC Method for Quantitation of Cyclosporine and Its Metabolites in Blood and Bile

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A GRADIENT HPLC METHOD FOR QUANTITATION OF CYCLOSPORINE AND ITS METABOLITES IN BLOOD AND BILE

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ABSTRACT

A quantitative gradient high performance liquid chromatographic method is reported for both cyclosporine and its metabolites. The identity of the metabolites was confirmed by radiolabelled products of rat liver perfusion studies and by chromatography of several pure standards. Recoveries of 95 and 97% were obtained for metabolite 17 and 1 using a diethyl ether extraction scheme in both blood and bile. Separation of cyclosporine and its metabolites in whole blood and plasma indicate that there is a significant temperature-dependent partitioning of these compounds between the erythrocyte and the plasma compartments of blood.

INTRODUCTION

Cyclosporine (CsA) is an effective immunosuppressive drug that has had a major impact on organ transplantation. Unfortunately, CsA has a number of untoward side effects, the most serious of which is a chronic nephrotoxicity. Therapeutic monitoring of CsA using HPLC is well established and efficacious due to variable absorption and metabolism of the drug as well as non-compliance and other difficulties in relating the dose to the therapeutic or toxic occurances. Radioimmunoassay (RIA) has also been extensively used for therapeutic monitoring. Unfortunately, the relationship between CsA concentrations in whole blood or plasma and either nephrotoxicity or therapeutic effect have been difficult to establish. As a result, the choice of a sample matrix and analytical technique has been controversial.

The role of CsA metabolites in immunosuppression and in toxicity has not been established. The RIA method detects the presence of metabolites in a non-linear and non-quantifiable fashion. As a result of this non-specific methodology, various authors have concluded that the metabolites of CsA are either the cause or not responsible for the CsA-associated nephrotoxicity. In addition, an erroneous conclusion regarding the enterohepatic circulation of CsA was reported based on RIA results. In bile, the concentration of metabolite appears to be on the order of 1 μg/ml; at least two orders of magitude greater than the concentration of CsA.

If monitoring of the CsA metabolites is to be useful in patient management, it must be measured in an accessible fluid such as blood or plasma. Several authors have reported that the CsA metabolites in blood are present primarily in the erythrocytes (1-3). Only Rosano and co-workers have actually reported quantitative results on whole blood CsA metabolite concentrations using an isochratic method employing a cyanopropyl column (3). In order to assess the relative importance of the metabolite concentrations, methods capable of accurate quantitation are required. We report here on a gradient method for quantitation of the major metabolites of CsA in whole blood and bile using a C18 column.

MATERIALS

The chromatograph used in this work was a Varian 5060 gradient HPLC (Walnut Creek, CA, USA) equipped with a column oven and a Perkin-Elmer LC-55 variable wavelength detector (Norwalk, CT, USA). Detection was carried out at 210 nm. The column was a 4.6x150 mm Varian MCH-5 reversed phase column. All separations were effected at 70°C due to the existence of various conformers of CsA (4). On-line radioactivity detection was accomplished with a Radiomatic Flow One Model CT (Tampa, FL, USA) scintillation counter. The scintillation fluid used was Aquasol-2 (NEN Research Products, Boston, Ma, USA) and was pumped at 4.5 ml/min and mixed with the column effluent before passage through a 0.2 ml cell.

All reagents were reagent grade and used as received unless otherwise stated. The diethyl ether, ethyl acetate, methanol, and acetonitrile were HPLC grade solvents (Burdick & Jackson, Muskegon, MI, USA). Water was freshly obtained from a Milli-Q deionization unit. Cyclosporine and cyclosporin D, the internal standard, were obtained from Sandoz (Hanover, NJ, USA). The tritium-labelled CsA, labelled on the aminobutyric acid residue ([^{3}H] OL 27-400),was donated by Sandoz (Basle, Switzerland). Samples of purified CsA metabolites 1, 17, 18, and 21 were also obtained from Sandoz.

METHODS

Whole blood (1 ml) or plasma (1 ml) was acidified with 1 ml of 180 mM HCl and extracted with 10 ml of diethyl ether using vigorous shaking. It is important to carefully wash the extraction tubes with ether prior to use. The organic layer is transferred as quantitatively as possible to a second

washed tube and washed with 1 ml of 95 mM NaOH. The ether is quantitatively transferred to a third tube and dried under a room temperature stream of N_2. The residue was dissolved in 50 µl of methanol with vortexing, and diluted with 50 µl of water. Of the reconstitution volume, 80 µl was injected for analysis.

Bile was collected from three hour liver perfusion experiments, the detailed results of which will be reported elsewhere. The perfusion fluid contained both unlabelled and radioactive CsA. An aliquot of the bile was extracted with 10 volumes of diethyl ether, the ether layer removed and dried, and the residue taken up as above.

The gradient conditions employed were optimized for quantitative analysis. The flow rate was 1.0 ml/min throughout. Solvent A consisted of a 90/10 mixture of water and methanol. Solvent B consisted of a 90/10 mixture of acetonitrile and methanol. The methanol seemed to narrow the peaks slightly under both isochratic and gradient conditions. The gradient began with a 56/44 mixture of solvents A and B respectively. The solvent mixture was linearly ramped to 49 in 5 minutes, held for 5 minutes, then ramped to 76% in 23 minutes. After a ten minute hold, the system was purged with 100% strong solvent for 7 minutes before re-equilibration to the initial conditions. An injection can be made every 55 minutes.

The ability of the gradient method to quantify CsA, and by implication its metabolites, was assessed by comparison to an isochratic method used for analysis of clinical specimens over the last four years. It employs a 4.6x50 mm column packed with 3 µm Supelcosil LC-18 support material. The mobile phase is a 46/20/34 acetonitrile/methanol/water mixture. The separation is carried out at 70°C at a flow rate of 2 ml/min. An injection is made every 8 minutes using a Waters WISP autosampler. Detection is accomplished witha Perkin-Elmer LC-55 variable wavelength detector. Column life has averaged 1000 injections over the past 2 years (i.e., 20 columns).

The radioimmunoassay was performed as described in the kit from Sandoz.

RESULTS

The recovery of both CsA and its metabolites through the solvent extraction steps is an important aspect of the present procedure. Using both non-radioactive CsA and metabolites isolated from bile samples and radioactive metabolite isolated from the rat liver perfusion studies, the recovery of the CsA from blood using diethyl ether was greater than 96%.

A chromatogram of the elution pattern of CsA and its metabolites isolated from bile is shown in Figure 1. The retention times of CsA and the metabolites can be clearly validated using the liver perfusion products. Further validation was achieved by comparison of the retention times to samples of purified metabolite 1, 17, 18, and 21. Note that a cluster of three metabolites can be clearly resolved in the area of 26 minutes.

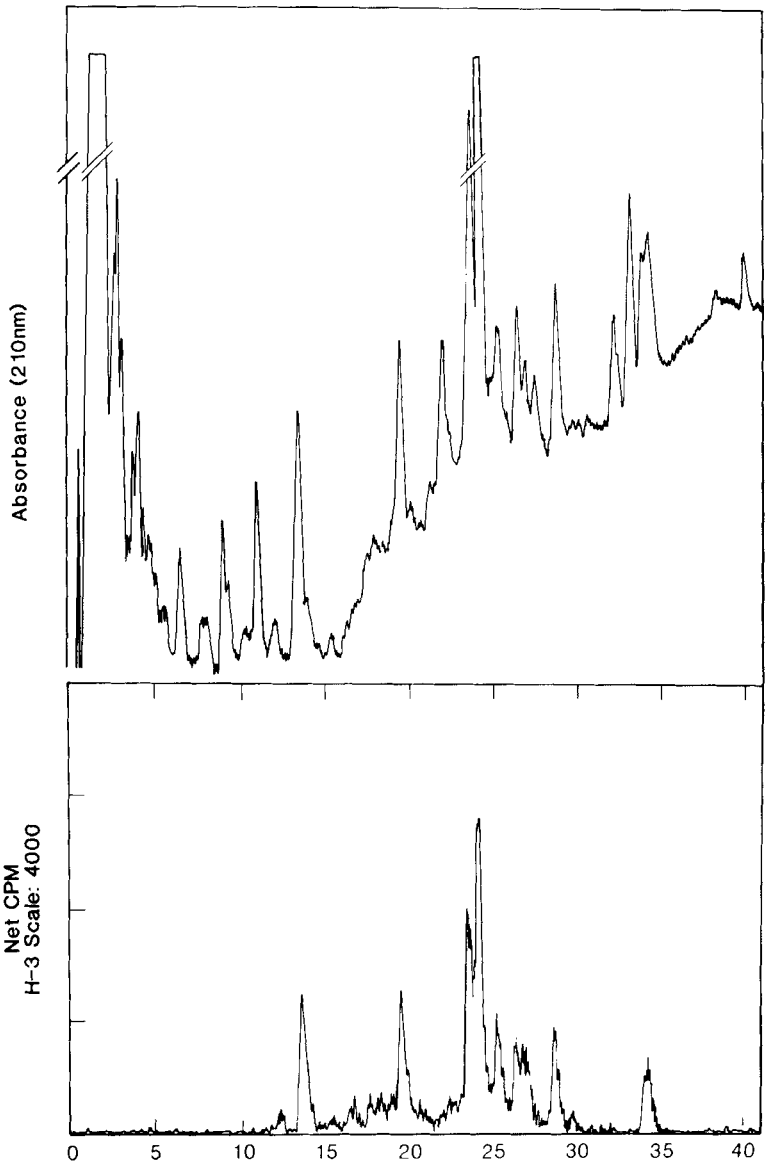

Figure 1. Chromatographic separation of cyclosporine and its metabolites from bile. Note the numerous peaks detected at 210 nm from the ether extraction. Note also that many of the major peaks seen in the 210 nm chromatogram are related to cyclosporine.

Whole blood obtained from ten renal dialysis patients prior to their dialysis treatment was subjected to the analysis to assess the interferences that might be encountered in the blood of transplant patients. Chromatograms of the best and worst cases observed are shown in Figure 2.

One minute fractions were collected during the course of the gradient elution run, evaporated to dryness, and taken up in a buffer solution containing protein and Tween surfactant. The fractions were then subjected to RIA analysis. The RIA analysis was also performed on the whole blood independent of the HPLC analysis. The results of this analysis are shown in Figure 3. Note the cross-reactivity of the antibody in the RIA procedure with the metabolites present at 10 (metabolite 10) and 21 (metabolites 17 and 1) minutes.

Whole blood was obtained from a renal transplant patient receiving CsA therapy and the plasma fractionated from the erythrocytes at both 37° C and at room temperature. The whole blood and both plasma fractions were subjected to the analysis reported above to determine the distribution of the metabolites between the two matrices at different temperatures. The chromatograms obtained are shown in Figure 4. It is apparent that the metabolites distribute between the plasma and erythrocyte compartments in a temperature dependent manner, with more metabolite present in the erythrocyte at a lower temperature. It appears that the distribution of metabolite 17 is more temperature dependent than CsA, but work is continuing on this topic in our laboratory.

DISCUSSION

Quantitation of the metabolites of CsA requires clear validation of the retention behavior of the compounds in question. As shown above, both radioisotopic metabolites generated in liver perfusion studies and pure metabolites have been used to confirm the retention times. The second aspect of quantitation, calibration, has proven to be a more difficult task. Because the "pure" metabolite is available in extremely small (e.g., < μg) amounts at present and because in our system it was clear that the metabolites were not truly pure, we were unable to accurately weigh standards to calibrate the assay. We have, therefore, made several assumptions in our attempt to measure a concentration of the individual metabolites relative to the parent CsA. First, the absorbance at 210 nm is due to the peptide bonds. We have assumed that the molar absorptivity of the metabolites and the CsA is the same since all of the known metabolites retain the cyclic undecapeptide structure. Second, since the solvent strength is the determinant of peak width in a gradient HPLC run, and since the signal to noise ratio precludes accurate determination of peak area, we have used a ratio of peak heights to estimate the concentration. This approach is strengthened by the fact that the peak width at half height for the 17 and 1 metabolites and the CsA are 0.14±0.05, 0.16±0.09, and 0.16±0.09 minutes respectively (n=5) . With similar peak widths, the height response should be proportional to concentration, unlike the situation in isochratic elution where peak width varies with elution time. When pure standards are available, the ratio should be readily converted to an absolute number. It should also be pointed out that at present even a relative quantitation will allow assessment of the importance of CsA metabolites in therapeutic monitoring.

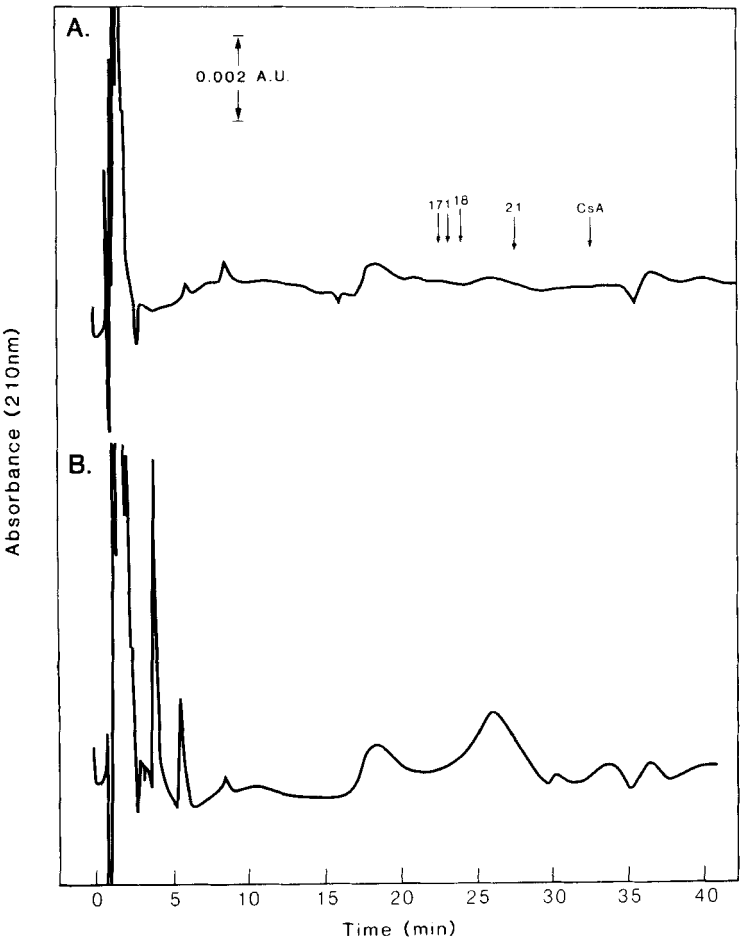

Figure 2. Chromatograms of the best (A) and worst (B) patterns observed in the blood of dialysis patients who were not receiving CsA. The elution times of the significant metabolites of CsA and CsA itself are indicated on the chromatogram.

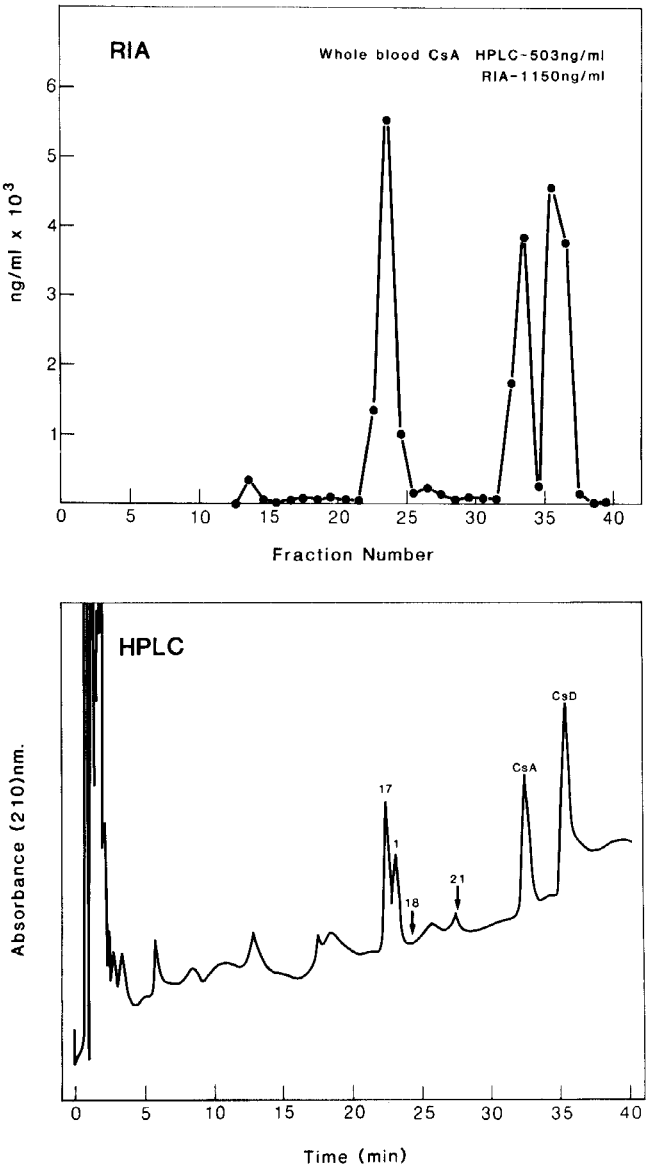

Figure 3. Chromatograms indiciating the cross-reactivity of the CsA metabolites in the Sandoz RIA procedure. Fractions were collected from the HPLC elution shown below, evaporated, and subjected to the RIA analysis. The numbers listed in the upper right corner of the figure are the concentrations measured in the original blood sample using the two techniques. Note that the data from the fractions agrees well with the assay on the original sample.

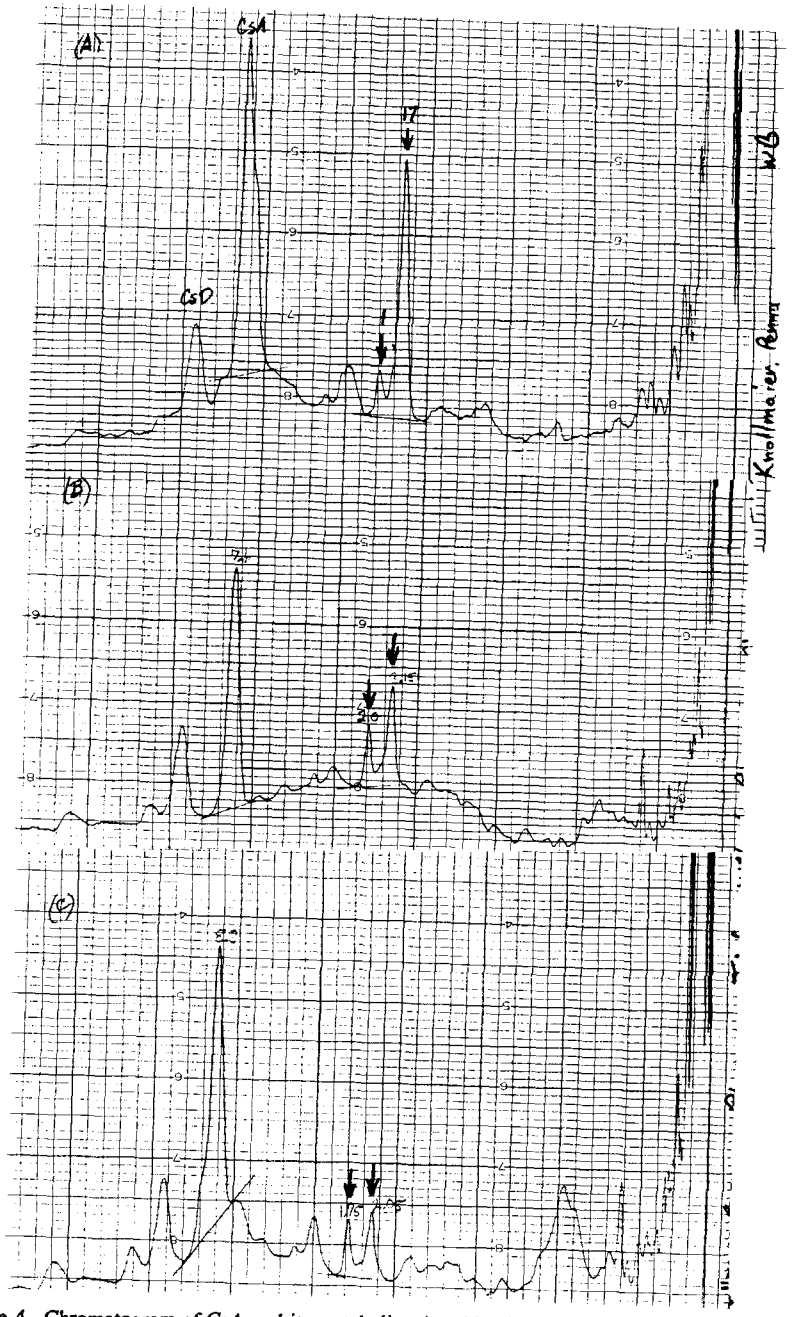

Figure 4. Chromatogram of CsA and its metabolites in a blood sample which was analyzed as whole blood (A), plasma separated at 37°C (B), and plasma separated at 22°C (C). Note the distribution of the metabolite and parent compound as a function of temperature.

The appearance of unidentified interfering compounds from the blood in the chromatogram is also a serious concern. Blood from renal dialysis patients was analyzed before their dialysis treatment to assess the problems posed by abnormal constituents in the blood of individuals with comprimised renal function. Blood from ten patients was analyzed. Even in the "worst" case, the metabolites can be clearly quantified in the presence of the broad baseline fluctuations seen (Figure 3).

The extraction of the metabolites also influences the quality and ease of analysis. As mentioned above, we obtain essentially complete extraction of the metabolits under the conditions used. We investigated several extraction procedures in the process of establishing this procedure. Rosano (3) recommends the used of alkalinized blood as the first extraction step rather than acidification of the specimens used here. In our hands both extraction schemes achieve equal recoveries. Use of alkalinized blood gives a "cleaner" ether layer in the initial extraction and may result in less frequent emulsion formation, although we have had no instances of emulsions in our studies. The final chromatogram appears essentially the same with both approaches, so we have continued to use the acid extraction in our work. Omission of the acid or base in the extraction does not affect recovery of the CsA or metabolites, but does result in the appearance of additional peaks in the chromatogram. Our evaluation of ethyl acetate as an extractant was less successful. The chromatogram contained many peaks when using blood or bile, as illustrated for the case of bile (Figure 1). It was impossible to quantitate any CsA related compounds using UV detection. It should be noted, however, that the use of diethyl ether restricts the measurement of metabolites to those which are quite non-polar.

Quantification of the CsA using the gradient method was comparable to the concentrations measured with an isochratic method. Seven samples taken at random from the analyses performed yielded a bias between the two methods of 22 ng/ml, which was not statistically significant at the 95% confidence interval. The coefficient of variation for three replicate injections of a spiked serum sample in the present system was 5.6%. A calibration curve generated with spiked whole blood samples was linear over the range from 0 to 1000 ng/ml. Retention times both within day and between days were consistant to 0.1 minute indicating the reproducibility of the solvent delivery system in generating the solvent compositions. Overall, the system appears to be quite robust in daily use.

We have been able to demonstrate clearly that the metabolites are associated with the erthrocytes in the blood matrix (Figure 4), in accord with the reports of others (1-3). The metabolites designated 17 and 1 partition between the red cells and the plasma in a temperature dependent fashion, as reported earlier for CsA (5). The non-specific nature of the RIA procedure is evident from Figure 3. The temperature-dependent partitioning of the metabolites explains the poor correlation between the RIA method and the HPLC methods observed for the whole blood matrix (6). Rosano and his co-workers have shown that the RIA does not give a linear relationship between the concentration of metabolite and the analytical response (3). Although this result was not surprising, it does show that the use of the difference between the RIA and HPLC results to

estimate the concentration of metabolite is an inadvisable and inaccurate approach. Thus, the development of procedures such as that reported here should help to resolve the controversy regarding the selection of plasma or whole blood as the matrix of choice for therapeutic monitoring (6). It is clear that if the metabolites are important in either toxicity or immunosuppression, the choice of serum or plasma separated at room temperature will seriously underestimate their contribution to these processes. Further work is required to clearly elucidate the mechanisms of both immunosuppression and nephrotoxicity.

The advantage of the present method over the isochratic methods reported previously (1, 3) are that the metabolites and parent CsA can both be quantified in a single 55 minute run and that the polar metabolites can be separated in the same chromatographic run, although at present they are impossible to quantitate accurately.

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