

1028

## ISOLATION OF 10 CYCLOSPORINE METABOLITES FROM HUMAN BILE

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### ABSTRACT:

Ten metabolites of cyclosporine were isolated from the ethyl ether extract of bile from four liver transplant patients receiving cyclosporine. Two of the metabolites were unique and previously unidentified. Liquid-liquid partitioning into diethyl ether with subsequent defatting with *n*-hexane was used for the initial extraction from bile. Separation of the individual metabolites (A-J) was performed using a Sephadex LH-20 column and a gradient high performance liquid chromatographic method. The molecular weights of the isolated metabolites were determined by fast atom bombardment/mass spectrometry. Gas chromatography with mass spectrometric amino acid analysis was also used to identify the amino acid composition and the hydroxylation position of metabolites A, B, C, D, and G. Proton nuclear magnetic resonance spectra were utilized to distinguish the chemical shifts of N-CH<sub>3</sub> singlets and NH doublets of

metabolites A, B, C, and D. Metabolites A, E, F, H, I, and J were reported previously in human urine and animal bile. Metabolites C and D are dihydroxylated compounds which cannot be clearly described as previously isolated compounds. Metabolites B and G are novel metabolites with a mass fragment which corresponded to a loss of 131 Da from the protonated molecular ion (MH<sup>+</sup>) in the fast atom bombardment/mass spectrometry, suggesting that the double bond in amino acid 1 has been modified. Metabolites B and G were primarily isolated from the bile of one of the liver transplant patients which contained abnormally high concentrations of these two metabolites. The method described is an efficient procedure for isolating milligram quantities of the major metabolites with greater than 90% purity.

Cyclosporine (CsA)<sup>1</sup> is a cyclic oligopeptide (fig. 1) which is isolated from the fungus *Tolypocladium inflatum* Gams (1). The drug is used clinically as an immunosuppressant in organ transplant patients (2) and for the treatment of autoimmune disorders (3). Cyclosporine is extensively metabolized in humans and in animals (4). Recent reports have demonstrated that several metabolites of CsA have *in vitro* immunosuppressive activity (5). Results obtained by radioimmunoassay, which measures both parent drug and metabolites, in transplant patients over time indicate an association between high blood concentrations and nephrotoxicity (6). The metabolites of CsA may therefore have both pharmacologic and toxicologic properties which warrant further investigation.

Bile is the major pathway of CsA metabolites in humans and animals. Eleven metabolites isolated from human urine and animal bile, urine, and feces have been fully characterized chemically (7). The terminology used for the identified metabolites uses the number assigned by Maurer and Lemaire (7), with metabolite number 17 (M17) being the primary ether-soluble cyclosporine metabolite. Metabolite 17 is hydroxylated in the number 1 amino acid position (fig. 1), whereas metabolite 1 (M1) is hydroxylated at the number 9 amino acid. The other CsA metabolites which have been reported most commonly are a demethylated metabolite 21 (M21) and a cyclic ether metabo-

lite (M18). Development of proper procedures for the isolation of CsA metabolites would be useful to obtain a sufficient amount of pure metabolites for standards for the quantitation of CsA metabolites and for pharmacologic testing.

Lensmeyer *et al.* (8) have recently reported a method for the isolation of CsA metabolites from bile and tissues. Although significant quantities of the metabolites were found in blood, bile, and urine, no resolution of the closely eluting peaks of M17, M1, and M18 was achieved using a cyano HPLC column. Christians and co-workers (9) also have reported a method of CsA metabolite isolation from bile using two sequential 250- $\mu$ m octyl columns. They reported seven structurally unidentified CsA metabolite peaks, and were able to purify five of the compounds for FAB/MS. The objective of this study was to develop a method of isolation of pure CsA metabolites from the bile of liver transplant patients and to identify the major metabolites in the bile of this patient population.

### Materials and Methods

Cyclosporine and trace amounts of purified metabolites M17, M18, and M21 were generously supplied by Dr. G. Maurer (Sandoz Inc., Basel, Switzerland). All organic solvents used in the procedure were liquid chromatographic grade (methanol and acetonitrile, J.T. Baker; hexane, Fisher Scientific; ethyl ether, Burdick Jackson, Muskegon, MI). The water used was Type 1 reagent grade water produced by using a Nanopure II, D3700 series cartridge deionization system (Barnstead Co., Boston, MA).

**Isolation of CsA Metabolites.** Bile was collected from the T-tubes of four adult patients who had undergone orthotopic liver transplantation. The bile was collected as untimed specimens, and the collection was discontinued after at least 300 ml of bile was obtained from each patient. For each patient's cumulative bile sample, one volume of fresh bile (pH = 7.5-8.2) was extracted in a separatory funnel with 1.5 volumes of

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<sup>1</sup> Abbreviations used are: CsA = cyclosporine, FAB/MS = fast atom bombardment/mass spectrometry.

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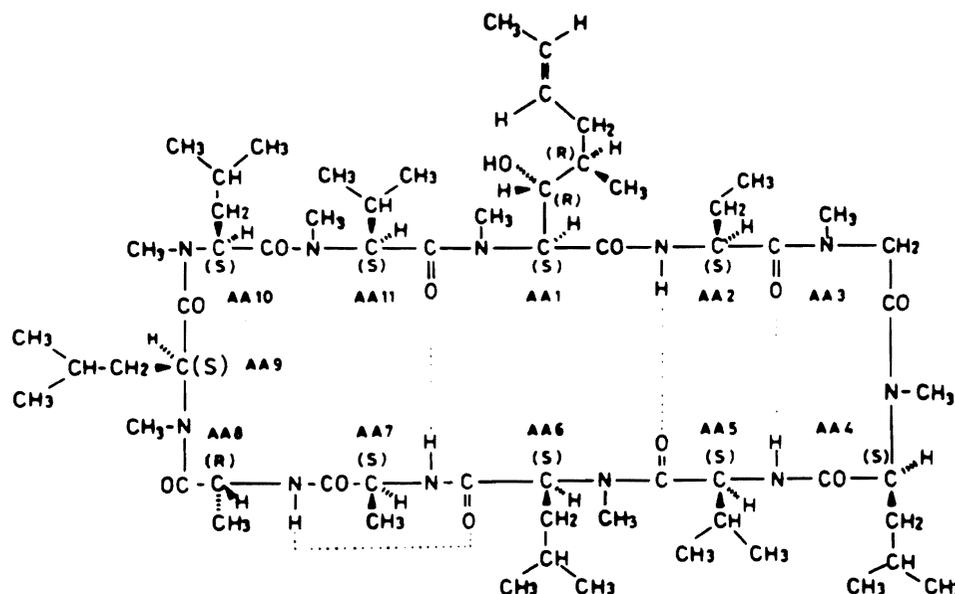


FIG. 1. Chemical structure of cyclosporine ( $C_{62}H_{114}N_{11}O_{12}$ ; molecular weight, 1202).

diethyl ether and shaken gently for 2 min. and the ether was decanted. This procedure was repeated twice more, and the ether fractions were combined and dried over anhydrous sodium sulfate. The ether extract was evaporated to dryness under reduced pressure at 30°C. The residue was redissolved in 200 ml of methanol:water (90:10), and defatted twice using 200 ml of *n*-hexane. The defatted methanol-water solution was then evaporated to dryness. The dried extract was dissolved in a minimal quantity of methanol, and was applied to a Sephadex LH-20 column (Sigma). Elution with methanol was performed to obtain the clear major metabolite fractions. The metabolites were separated by using a gradient HPLC system. The gradient HPLC system consisted of a Waters 600 Multi-Solvent Delivery System (Waters Associates, Milford, MA). The column was an LC-18, 25-cm, 5- $\mu$ m column (Supelco, Bellefonte, PA) heated to 70°C. The UV detector was set at 214 nm (Lambda-Max Model 481 LC Spectrophotometer, Waters Associates). The mobile phase consisted of a linear gradient of acetonitrile and water as shown in table 1. The eluates corresponding to the absorption peaks were collected into individual tubes. The eluate fraction was vortexed with an adequate volume of ethyl ether to fully partition acetonitrile from the aqueous to the organic layer. The acetonitrile/ethyl ether mixture was evaporated to dryness under nitrogen gas at the ambient temperature. The remaining aqueous solution was lyophilized to obtain the dried metabolites. The purity of the isolated metabolite fraction was tested by reinjection onto the HPLC column using the same HPLC conditions. The purity of that single peak was defined as the percentage of area for that peak (as integrated with a 3390A integrator, Hewlett-Packard, Avondale, PA) divided by the total area for peaks resulting from injection of that fraction.

**Mass Spectrometric Analysis of CsA Metabolites.** The structures of the isolated metabolites were investigated by using the mass spectrometry protocols described by Hartman and Jardine (10). Briefly, FAB/MS data were collected on a VG 12-350 quadrupole mass spectrometer (VG

Masslab, Altrincham, United Kingdom). The FAB/MS matrix solvent used was a 5:1 mixture of dithiothreitol:dithioerythritol heated to liquid at 90°C and cooled to room temperature before use. The CsA metabolites (10 nmol) were dissolved in 1.0  $\mu$ l of acetonitrile and 0.5  $\mu$ l of water and applied to the FAB/MS matrix. Data were acquired in the multichannel analyzer mode, scanning from  $m/z$  1000–1500 at 10 sec/scan. The mass range was calibrated using CsA. Under these conditions, the CsA metabolites produced an intense protonated molecular ion ( $MH^+$ ), usually accompanied by a much less intense fragment ion resulting from loss of the side chain of amino acid 1 (–113 Da for CsA itself). Such FAB/MS analysis allows determination of the overall metabolic transformations (hydroxylations, *N*-demethylations, etc.) that have occurred for each metabolite.

To determine the specific transformation that has occurred with each amino acid type within the CsA molecule, an aliquot of each metabolite (50  $\mu$ g) was totally hydrolyzed to the component amino acids by heating at 115°C in 1 ml of 6 N HCl for 48 hr in evacuated glass tubes. The acid was then removed under vacuum down to 100  $\mu$ l and then 3 ml of acetonitrile was added and the solution was blown dry under nitrogen at room temperature. The amino acids were converted to isobutyl esters using 3 N HCl in isobutyl alcohol at 120°C for 30 min. After drying under nitrogen at room temperature, acylation was carried out using 50  $\mu$ l of heptafluorobutyric anhydride and heating to 150°C for 10 min. After drying under nitrogen at room temperature, the resulting heptafluorobutyrylated isobutyl esters of the metabolite amino acids were taken up in 25  $\mu$ l of carbon disulfide and then analyzed by GC/MS. GC/MS was carried out on a Kratos MS50-DS55 mass spectrometer/computer system (Ramsey, NJ), fitted with a Carlo Erba 4160 gas chromatograph (Milan, Italy). The GC column was a Hewlett-Packard 190891 cross-linked methyl silicone 25 m capillary column which was threaded through a heated GC/MS interface directly to the mass spectrometer electron ionization source that was operated at 70 eV.

**NMR Spectrometric Analysis of CsA Metabolites.** The  $^1H$  NMR spectra of metabolites A, B, C, and D in  $CDCl_3$  were determined at room temperature (22°C) by a Bruker/IBM AF-300 NMR Spectrometer (Bruker Instruments Inc., Silberstreifen, Federal Republic of Germany). The 90° pulse was 11.8  $\mu$ sec and the chemical shift reference was trimethylsilane as 0 ppm.

## Results

The chromatogram of the separation of 10 CsA metabolites present in each of the four bile samples is depicted in fig. 2. A

TABLE 1

Linear HPLC gradient profile used in the LC-18, 25-cm column for cyclosporine metabolite separation

Time	Flow Rate	Acetonitrile:Water
min	ml/min	
0	0.9	43:57
105	0.9	58:42
108	0.9	73:27
113	0.9	73:27
115	0.9	43:57

total of 10 peaks were collected and analyzed from bile and were labeled A through J. The molecular weights and probable structure modifications of CsA are presented in table 2. Metabolites F, I, and J were obtained with greater than 95% purity in one HPLC separation. The purities of metabolites A, B, C, D, E, G, and H were higher than 90% in one HPLC separation. Metabolites A, B, C, D, E, and H were reappplied to the HPLC column using the same procedure, and were further purified to greater than 95%. Metabolite G was not purified further because of the small quantity obtained. Fig. 3 demonstrates the purity of metabolites F (M17), H (M1), I (M18), and J (M21).

**Metabolite Peak A.** The protonated molecular ion of this metabolite was observed in the FAB/MS at  $m/z$  1235 ( $MH^+$ ) corresponding to an increase of 32 Da in comparison to the protonated molecular ion ( $m/z$  1203  $MH^+$ ) of CsA. A fragment at  $m/z$  1106 resulted from the loss of 129 Da from the protonated molecular ion, instead of 113 Da as observed for CsA, indicating that an additional oxygen was contained in amino acid 1 (MeBmt) of compound A. In the  $^1H$  NMR spectrum (300 MHz,  $CDCl_3$ ), the presence of seven  $N-CH_3$  singlets and four NH doublets demonstrates that none of the methyl groups of the *N*-methylleucines was altered. GC/MS amino acid analysis indicated that one of the *N*-methylleucine residues was hydroxylated at the  $\gamma$ -carbon. This metabolite was tentatively identified as M8 according to the chromatographic scheme of Maurer *et al.* (4).

**Metabolite Peak B.** The FAB/MS of metabolite B indicated a molecular ion species at  $m/z$  1237 ( $MH^+$ ) corresponding to an increase of 34 Da over CsA. The fragment at  $m/z$  1106 resulting from the loss of 131 Da from the protonated molecular ion indicates that the additional oxygen and 2H were contained in amino acid 1 of compound B. The addition of 2H suggests that the double bond in amino acid 1 is saturated. The  $^1H$  NMR spectrum showed the signals of four NH doublets and seven  $N-CH_3$  singlets, indicating that *N*-demethylation has not occurred. GC/MS amino acid analysis revealed that one of the *N*-methylleucine groups had been hydroxylated on the  $\gamma$ -carbon. By comparison with the identified 11 cyclosporine metabolites, the loss of 131 Da from the  $MH^+$  suggests that metabolite B could be a new metabolite with a novel modification on the double bond of amino acid 1. This metabolite is a dihydroxyl + 2H derivative of CsA.

**Metabolite Peak C.** The protonated molecular ion at  $m/z$

1235 ( $MH^+$ ) was consistent with the addition of two atoms. One of the *N*-methylleucines was hydroxylated on the  $\gamma$ -carbon, as demonstrated in the GC/MS amino acid analysis. The  $^1H$  NMR spectrum showed seven  $N-CH_3$  singlets and indicated that no *N*-demethylation had occurred. The pattern of chemical shifts of these seven  $N-CH_3$  singlets in NMR was different from metabolite D. A fragment from the loss of a C7 fragment of amino acid 1 was not observed in the FAB/MS. This metabolite could be a dihydroxyl derivative of CsA.

**Metabolite Peak D.** The protonated molecular ion was at  $m/z$  1235 ( $MH^+$ ) which is an addition of 32 Da to CsA. The fragment ion at  $m/z$  1106 indicates that an additional oxygen was contained in the C7 fragment of amino acid 1 for metabolite D. GC/MS amino acid analysis indicated one of the *N*-methylleucine moieties was hydroxylated on its  $\gamma$ -carbon. The  $^1H$  NMR spectrum showed the presence of seven  $N-CH_3$  singlet and four NH doublet peaks, which indicated that no *N*-demethylation had occurred. This metabolite is a dihydroxyl derivative of CsA.

**Metabolite Peak E.** The protonated molecular ion was at  $m/z$  1205 ( $MH^+$ ), which is an addition of 2 Da over CsA. This is considered to be the addition of one oxygen (+16 Da) and the loss of one methyl group (-14 Da). The fragment occurred at  $m/z$  1092, or a loss from  $MH^+$  of 113 Da, which indicated that no hydroxylation occurred on the side chain of amino acid 1. This metabolite was tentatively identified as M13 according to the chromatographic scheme of Maurer *et al.* (4).

**Metabolite Peak F.** The protonated molecular ion was at  $m/z$  1219 ( $MH^+$ ), which is an addition of 16 Da relative to CsA. The fragment at  $m/z$  1090 indicated that the additional oxygen was contained in the C7 fragment of amino acid 1. This metabolite exhibited the identical HPLC chromatographic retention time as authentic M17 obtained from Maurer. This metabolite was thus identified as M17.

**Metabolite Peak G.** The protonated molecular ion peak was at  $m/z$  1221 ( $MH^+$ ), which is an addition of 18 Da relative to CsA. The fragment at  $m/z$  1090 indicated that the additional oxygen and 2H were contained in the C7 fragment of amino acid 1. The loss of 131 Da was similar to the C7 fragment loss from metabolite B. Thus, metabolite G could be a previously unidentified CsA metabolite with a novel modification in amino acid 1 similar to that observed in metabolite B. This metabolite is a monohydroxyl + 2H derivative of CsA.

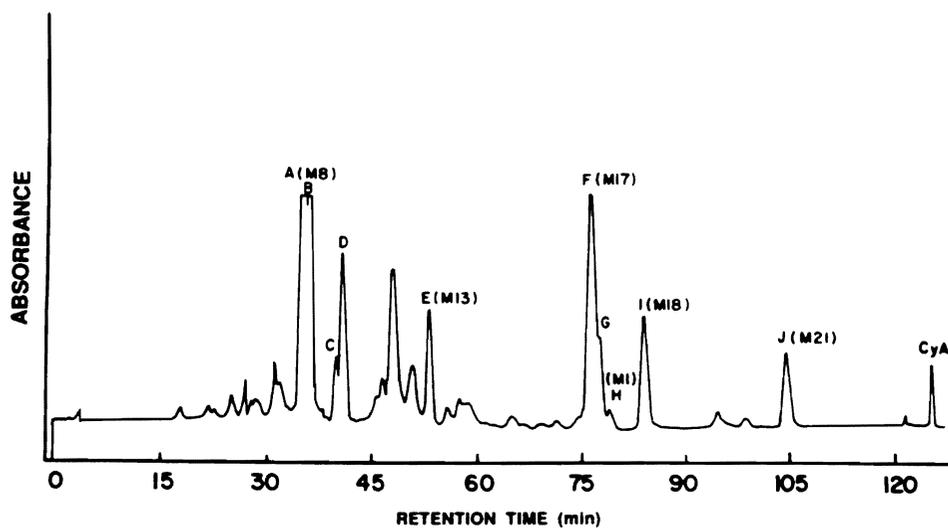


FIG. 2. HPLC chromatogram of the separation of 10 metabolites of CsA from the ether extract of the bile of a liver transplant patient.

Metabolite  
M17  
M18  
M21  
CsA  
MeBmt  
M1 of CsA  
Metabolite  
M19 indicates  
this metabolite  
the oxygen was  
The HPLC  
authentic M17  
considered to  
Metabolite  
metabolite was  
oxygen relative  
that the addition  
of amino acid  
the same as  
was, therefore  
Metabolite  
molecular ion  
fragment ion  
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TABLE 2

Properties of isolated metabolites of CsA

Metabolite	Observed Molecular Weight	HO-MeBmt <sup>a</sup>	HO-N-MeLeu	CsA Derivative
A (M8)	1234	Y	Y	Dihydroxyl
B	1236	Y	Y	Dihydroxyl + 2H
C	1234		Y	Dihydroxyl
D	1234	Y	Y	Dihydroxyl
E (M13)	1204			Monohydroxyl-demethyl
F (M17)	1218	Y	N	Monohydroxyl
G	1220	Y	N	Monohydroxyl + 2H
H (M1)	1218	N	Y	Monohydroxyl
I (M18)	1218	Y	N	Monohydroxyl
J (M21)	1188	N	N	N-Demethyl
CsA	1202	N	N	

<sup>a</sup> MeBmt, (4R)-4-[(E)-2-butenyl]-4-N-dimethyl-L-threonine (amino acid I of CsA); N-MeLeu, N-methylleucine.

**Metabolite Peak H.** The protonated molecular ion at  $m/z$  1219 indicated that an additional oxygen molecule was attached to this metabolite. The fragment ion at  $m/z$  1106 indicated that the oxygen was not attached to the C7 fragment of amino acid I. The HPLC retention time of this metabolite was same as authentic M1 obtained from Maurer. Thus, metabolite H is considered to be M1.

**Metabolite Peak I.** The protonated molecular ion peak of this metabolite was at  $m/z$  1219 ( $MH^+$ ) and again had one additional oxygen relative to CsA. The fragment ion at  $m/z$  1090 indicated that the additional oxygen atom was attached to the C7 fragment of amino acid I. The HPLC retention time of metabolite I was the same as authentic M18 from Maurer, and this metabolite was, therefore, identified as M18.

**Metabolite Peak J.** The metabolite J produced a protonated molecular ion at  $m/z$  1189 ( $MH^+$ ) or 14 Da less than CsA. The fragment ion at  $m/z$  1076 indicated that no hydroxylation had occurred on amino acid I. The HPLC retention of this metabolite was the same as authentic M21 obtained from Maurer. This metabolite was assigned as M21.

### Discussion

Ten metabolites of CsA have been isolated from the ether extract of bile of liver transplant patients receiving CsA therapy. The method described is an efficient procedure for obtaining milligram quantities of CsA metabolites for structural characterization and to perform *in vitro* activity tests. By making 36 injections from approximately 300 ml of bile onto the HPLC column (see fig. 2), 1.4 mg of M17 with a purity of greater than 95% was obtained. The metabolite content of bile varies among patients. The bile used in this isolation was chosen because of its content of new metabolites B and G.

Although none of the compounds isolated by Christians and co-workers (9) appear to be similar to the B and G metabolites reported herein, the two peaks which they found difficult to purify are likely to represent our recently reported aldehydic metabolites (11). Metabolites F, H, I, J were identified as M17, M1, M18, M21, respectively, as assigned by Maurer and co-workers (4). Metabolites A and E are also most likely M8 and M13 as identified by Maurer. The presence of two new metabolites (B and G), which have not been reported previously, suggests

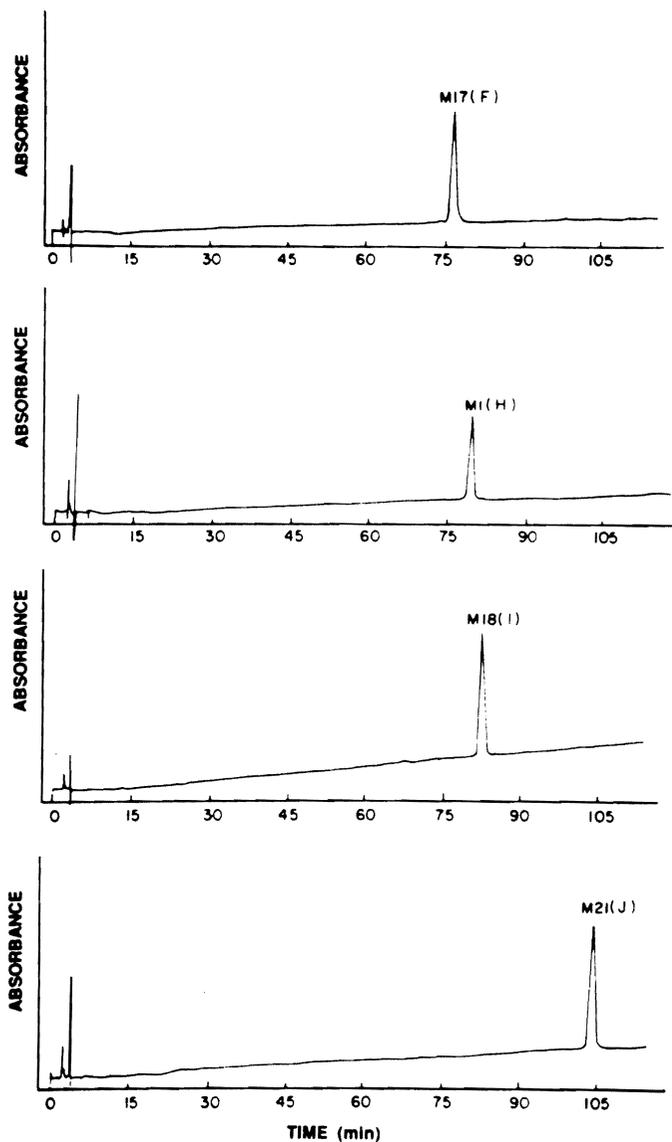


FIG. 3. HPLC chromatograms of the isolated cyclosporine metabolites F (M17), H (M1), I (M18), and J (M21) on an LC-18 25-cm column.

that CsA metabolism in liver transplant patients could be different from previous animal models or normal human subjects. These 10 CsA metabolites can be detected in varying concentrations in each of the four bile collections from liver transplant patients. Twenty-five other peaks present in the chromatogram of fig. 2 could be additional CsA metabolites but were not investigated. The full assignments of the positions of structural modifications of metabolites A, B, C, D, E, and G cannot be achieved, as it was not possible to obtain a sufficient quantity of metabolite to perform a <sup>13</sup>C NMR spectrum at this time.

The relative concentrations of new metabolites B and G were particularly high in the bile of one of the liver transplant patients with impaired liver function. The pharmacologic/toxicologic activities of these new metabolites will be investigated when sufficient quantities of these compounds can be obtained. We have observed unusually large peaks with identical retention times of Metabolites B and G in the blood of liver (12), heart, and bone marrow transplant patients using a similar chromatography system. Liver dysfunction occurs frequently in these pa-

tient populations, and may contribute to the formation of the new metabolites.

Pure metabolites F (M17), H (M1), and J (M21) obtained by using this method have been tested for *in vitro* immunosuppressive activity on T-lymphocyte cultures. These results have shown metabolite F to have substantial immunosuppressive activity against biopsy-grown lymphocytes and to have synergistic inhibitory effect with CsA on lymphocyte proliferation (13, 14). Ethyl acetate extraction of the bile after ethyl ether extraction produces small amounts of metabolites A through E and several more unidentified polar peaks in the chromatogram of the same HPLC system. The major polar metabolite of CsA is the carboxylated metabolite (15). However, none of the polar CsA metabolites appears to have *in vitro* immunosuppressive activity (16).

In addition to immunosuppressive activity of the metabolites, recent reports suggest that CsA metabolites are associated with CsA nephrotoxicity (6). Further studies of the pharmacologic/toxicologic importance of the CsA metabolites in transplant patients is critical for a complete understanding of the action of cyclosporine and differences in response among transplant populations.

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METABOLISM  
1988

QUANTITATIVE  
CYSTEINE  
OXIDATION

#### ABSTRACT:

In this study, the relative roles of cysteine conjugate formation and cysteine oxidase activity in the metabolism of 2-hydroxy-

Studies from our laboratory indicate that the metabolic pathway for the original observation of 2,3,4,5-tetrahydro-2H-pyridine (10) and its electrophilic protein adducts (9) are a number of studies and mutations interpreted as a metabolic pathway (13). However, it is clear that other non-toxic cysteine production and transformation of metabolites of cyclosporine (16-18) are cysteine conjugates.

Abbreviations:  
2,3,4,5-penta-  
hydro-L-cysteine  
2,2,2-tetrafluoro-

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