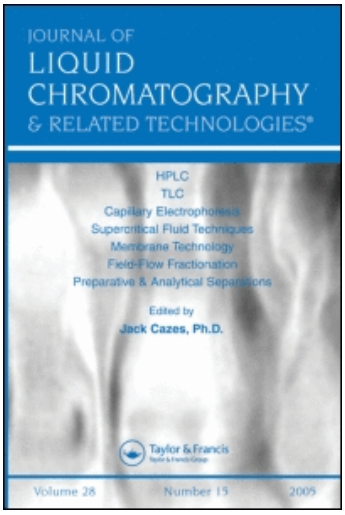

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

High Performance Liquid Chromatographic Isolation and Spectroscopic Characterization of Metabolites from the Bile of Rats Receiving Rapamycin (Sirolimus) Intravenously

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To cite this Article Wang, C. P. , Lim, H. -K. , Chan, K. W. , Scatina, J. and Sisenwine, S. F.(1997) 'High Performance Liquid Chromatographic Isolation and Spectroscopic Characterization of Metabolites from the Bile of Rats Receiving Rapamycin (Sirolimus) Intravenously', Journal of Liquid Chromatography & Related Technologies, 20: 11, 1689 — 1701
To link to this Article: DOI: 10.1080/10826079708006326
URL: http://dx.doi.org/10.1080/10826079708006326

J. LIQ. CHROM. & REL. TECHNOL., 20(11), 1689–1701 (1997)

HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC ISOLATION AND SPECTROSCOPIC CHARACTERIZATION OF METABOLITES FROM THE BILE OF RATS RECEIVING RAPAMYCIN (SIROLIMUS) INTRAVENOUSLY

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ABSTRACT

Ten major metabolites of rapamycin (M2, M3, M8, M9, M10, M11, M13, M14, M15, and M16) were isolated from pooled bile of intravenously dosed rats. Metabolites were extracted from the bile with ethyl acetate prior to isolation by HPLC using a Supelcosil SPLC-18, μm, 10 x 250 mm column. The mobile phase was a methanol/ammonium acetate linear gradient system. The isolated metabolites were characterized by negative ion FAB MS, ion-spray MS and ion-spray MS/MS analyses.

Metabolite M2 is oxygenated in the southern portion of rapamycin and the macrolide ring is opened. M3 is a structural isomer of rapamycin where the lactone ring is opened. M10 is oxygenated in the southern portion of rapamycin and the macrolide ring is intact. M13 is a monohydroxylation and demethylation metabolite and both biotransformations occurred at the southern portion. M8, M9, and M11 are monohydroxylation and demethylation metabolites. M14 and M15 are dihydroxylation metabolites. M16 is mainly a dihydroxylation metabolite.

INTRODUCTION

Rapamycin,[3S[3R*[S*(1R*,3S*,4S*)],6S*,7E,9S*,10S*,12S*,14R*,15E, 17E,19E,21R*,23R*,26S*,27S*,34aR*]]-9, 10, 12, 13, 14, 21, 22, 23, 24, 25, 26, 27, 32, 33, 34a-Hexadecahydro-9,27-dihydroxy-3-[2-(4-hydroxy-3-methoxy cyclohexyl)-1-methylethyl]-10,21-dimethoxy-6, 8, 12, 14, 20, 26-hexamethyl-23,27-epoxy-3H-pyrido[2,1-c][1,4]oxaazacyclohentriacontine-1, 5, 11, 28, 29 (4H,6H,31H)-pentone, (Figure 1), an antitumor and antifungal agent isolated from the fungus *Streptomyces* hygroscopicus,[1,2,3] has been found to have potent immunosuppressive activity while exhibiting little toxicity in primates.[4,5] Rapamycin is currently undergoing clinical trials as an immunosuppressive agent. The drug demonstrates a synergistic effect when co-administered with cyclosporine.[6,7]

We have recently reported the isolation and characterization of two major *in vitro* degradation products following incubation of rapamycin at 37°C in rat bile or ammonium acetate (pH 8.0).[8] Degradation product A was a macrolide ring-opened hydrolysis product of rapamycin where the ester bond linking C23 and C25 has been hydrolyzed. Degradation product B was a ring-opened isomer of rapamycin.

In another report, three major metabolites have been isolated and characterized from the plasma of rats receiving rapamycin orally.[9] M2 is monohydroxylated in the southern portion of rapamycin and the macrolide ring is opened. M3 is the degradation product B and M5 is O-demethylated on the C41 moiety. This 41-O-demethyl rapamycin was one of the metabolites isolated by Christians et. al. from in vitro metabolism of rapamycin in microsomes from human liver and rat small intestinal microsomes.[10] The other was a hydroxylated metabolite but the position of hydroxylation was not determined.

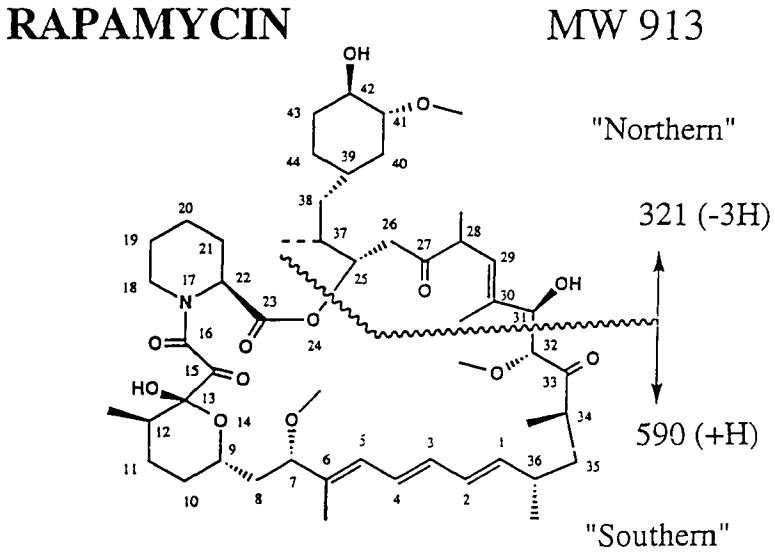

Figure 1. Chemical structure of rapamycin and its major fragmentation pathway.

However, the identification of *in vivo* biliary metabolites of rapamycin has not yet been reported. The present study was therefore conducted to isolate rapamycin metabolites from the bile of rats using a semi-preparative HPLC system and followed by structural characterization using mass spectrometry.

EXPERIMENTAL

Materials

Rapamycin was obtained from Wyeth-Ayerst Research, Rouses Point, New York. It was formulated in a vehicle consisting of 6% (v/v) ethanol, 5.6% (w/v) Polysorbate 80, 51.7% (w/v) PEG 300 and 36.7% (v/v) water at a concentration of 1.45 mg/mL for the doses used in the study. Ammonium acetate (HPLC grade) was obtained from J.T. Baker (Phillipsburg, NJ). All solvents used in the study were HPLC grade.

Animals and Treatment

Twenty-nine Sprague-Dawley rats (Hilltop Lab Animals, Inc., Scottdale, PA), weighing between 330 and 385 g were surgically prepared with implanted bile duct and jugular vein cannulas. Animals were not fasted overnight and received electrolyte supplemented water. Prior to dosing, control bile samples were collected. The animals were dosed slowly via the indwelling jugular vein cannula or tail vein when the jugular vein did not remain patent. The animals received rapamycin at a dose of 1.45 mg/kg. Bile was collected over dry ice at intervals of 0-4, 4-8, 8-12 and 12-24 hr after dosing. The samples were frozen at -20°C until analysis.

Instrumentation

The HPLC system consisted of a Waters 600E system controller and pump (Waters Associates, Milford, MA), a Waters 490E programmable multi-wavelength detector, a Waters U-6K manual injector and a Hewlett-Packard 3390A integrator. Separation of metabolites was achieved using Supelcosil SPLC-18, 5 μm, 10 x 250 mm column (Supelco, Bellefonte, PA). Negative electrospray ionization (ESI) single and multiple-stage mass spectra were acquired with a Sciex TAGA 6000E mass spectrometer upgraded to an API III and equipped with a home made ion sprayer.[11] FAB mass spectra were acquired using a Kratos MS 50 mass spectrometer equipped with a FAB ion source and operated in the negative ion mode.

Isolation of Rapamycin Metabolites in Rat Bile

The bile (0 to 4 hr), which contained the largest number and quantity of metabolites, was used for the isolation of metabolites. A total of 122 mL of bile was extracted with ethyl acetate. The ethyl acetate extracts were taken to dryness under nitrogen. The residues were dissolved in methanol/water (65/35) prior to injection onto the HPLC system. A Supelco semipreparative SPLC-18, 25 cm x 10 mm, 5 μm column was used for isolation of metabolites. The mobile phase gradient profile is described in Table 1. Detection was by UV absorbance at 276 nm. The HPLC eluates corresponding to the ten major metabolite peaks were collected manually. Methanol in the eluates was removed with a Savant AS-160 at room temperature under manual mode. The remaining aqueous residues were lyophilized to dryness. Metabolite fractions were then frozen at -80°C until analysis.

Table 1

Stepwise Gradient System Used for the Isolation of Rapamycin in Metabolites[a]

Time (min)	Flow Rate (mL/min)	Percent Methanol	Percent 0.05 M Ammonium Acetate
0.0	2.0	62	38
8.0	2.0	84	16
95	2.0	84	16
100	2.0	62	38

[a]Linear gradient was used between each time point; column was Supelco SPLC-18, 250 x 10.0 mm, 5 μm.

Structural Elucidation of Metabolites by Mass Spectrometry

The isolated metabolites were subjected to either FAB MS and/or ion spray MS and MS/MS analyses. Due to limited quantities, only M2, M3, M10 and M13 were analyzed by LC/MS/MS. In the FAB experiments, the primary beam was 1 mA of 7kV Xenon atoms. Resolution of the instrument was adjusted to 2000 (10% valley). Triethanolamine was employed as the matrix for the samples. Each sample was dissolved in methylene chloride, and an aliquot of the solution was mixed with the matrix on a copper probe tip before insertion into the mass spectrometer for analysis.

For infusion ESI/MS and MS/MS analyses, the sample was initially dissolved in either dichloromethane or absolute ethanol and an aliquot was removed for analysis. The organic solvent was then evaporated to dryness under nitrogen at room temperature. Prior to dissolving in 20% 4 mM ammonium acetate in methanol, the solution was infused at 4 μL/min into the mass spectrometer using a Harvard Syringe Pump. The mass spectrometer was calibrated in positive ion mode up to 2000 daltons with PPGs (polypropylene glycols) and switched to negative polarity prior to analysis. Final optimization of sprayer position and mass spectrometer conditions for maximum sensitivity was carried out by infusing with rapamycin standard (1 mg/mL). The sample was sprayed into the mass spectrometer at -3.2 kV and desolvation was carried out at declustering potential of 66 volts. Full scan ESI mass spectra were acquired by scanning Q1 at unit resolution from m/z 300 to 1000 at step size of 0.2 and dwell time of 0.57 seconds. Product ion mass spectra were obtained by setting Q1 to transmit deprotonated molecular anion $(M-H)^-$ at unit resolution

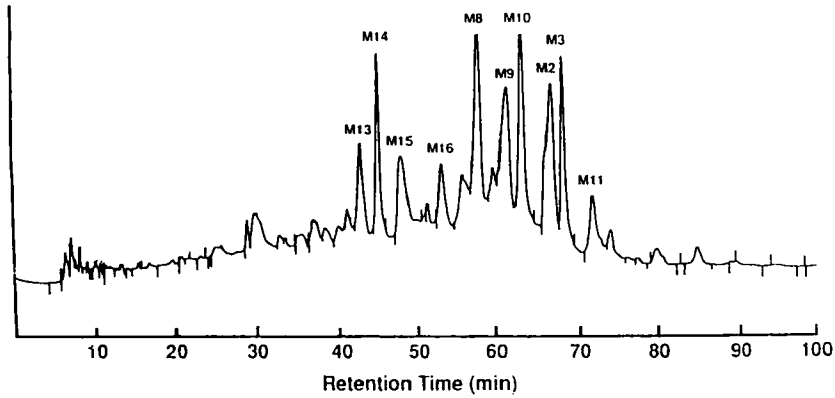

Figure 2. Semipreparative HPLC chromatogram showing separation of rapamycin metabolites labelled as M13, M14, M15, M16, M8, M9, M10, M2, M3 and M11 from analysis of pooled 0-4 hour post-dose bile from rats administered rapamycin intravenously at a dose of 1.45 mg/kg.

Table 2

Summary of MS Analysis of the Biliary Metabolites of Rapamycin in Rates

Metabolite Number	Mw[a]	Possible Metabolic Transformation[b]
M2	929	monohydroxylation
M3	913	ring opened isomer
M8	915	demethylation and monohydroxylation
M9	915	demethylation and monohydroxylation
M10	929	monohydroxylation
M11	915	demethylation and monohydroxylation
M13	915	demethylation and monohydroxylation
M14	945	dihydroxylation
M15	945	dihydroxylation
M16[c]	945	dihydroxylation
	931	hydrolysis product of rapamycin
	915	demethylationand monohydroxylation

[a]MW based on nominal monoisotopic mass.
[b]epoxidation across a double bond can be substituted for hydroxylation.
[c]this metabolite was contaminated with two other metabolites of Mws = 931 and 915.

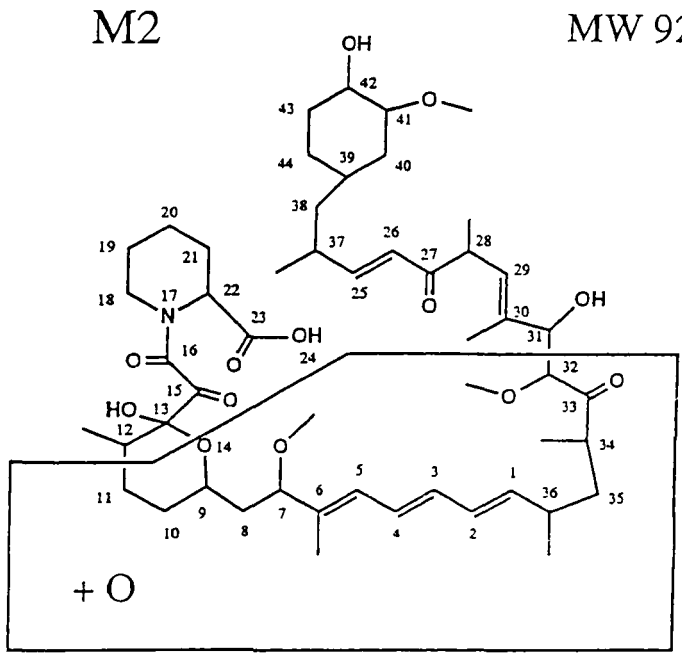

Figure 3. Proposed site of metabolic transformation of rapamycin to metabolite M2.

to Q2. The molecular anion was subjected to collision-activated dissociation (CAD) at collision energies of 50-53 eV and at collision pressure of 5 x 10^{12} atoms of argon/cm^3. The product ions were mass analyzed by scanning Q3 at unit resolution from m/z 40 to 1000 at step size of 1 and dwell time of 2.08 seconds. In each case, the mass spectrum was a sum of 10 scans.

RESULTS

Identification of Rapamycin Metabolites in Rat Bile

The major metabolites that were isolated by semipreparative HPLC system are labeled as M13, M14, M15, M16, M8, M9, M10, M2, M3 and M11 as shown in Figure 2. The notation of M2 and M3 is consistent with that used previously to identify metabolites in rat plasma.[9] The molecular weights (MW) and possible metabolic transformation of these metabolites were obtained from their mass spectra and summarized in Table 2.

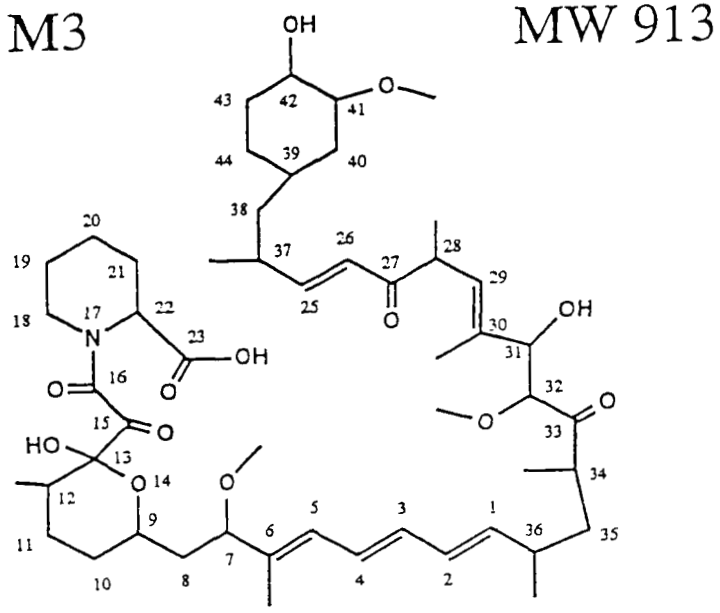

Figure 4. Proposed structure of M3.

Negative FAB mass spectrometric analysis was used to determine whether the macrolide ring is intact or open. For example, the observation of a deprotonated molecular anion $(M-H)^-$ is an indication of opening of the macrolide ring whereas a compound with an intact macrolide ring gave a molecular anion by capturing an electron (M^-).[12] Further insight into the possible sites of metabolic transformation was provided by tandem mass spectrometry (MS/MS) analysis. The product ion mass spectrum of rapamycin shows two complimentary diagnostic product ions at m/z 321 and 590 which correspond to the "northern" and "southern" portions of the molecule (Figure 1), respectively. The latter two product ions are useful for monitoring biotransformation of the rapamycin molecule by the mass shift technique.[13]

M2: The full scan mass spectrum of M2 shows the presence of a low abundance deprotonated molecular anion at m/z 928.8 which is 16 daltons more than rapamycin. This suggests a biotransformation by either aliphatic hydroxylation or epoxidation. The site of biotransformation can be located from the product ion mass spectrum which shows the complimentary fragment ions at m/z 321 and 606. The fragment ion at m/z 321 suggests that the "northern" portion of rapamycin is unchanged, and biotransformation at the

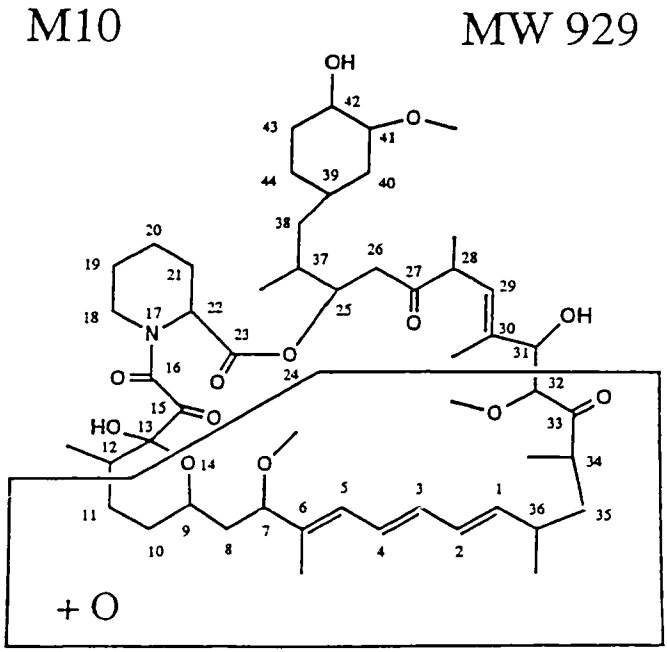

Figure 5. Proposed site of metabolic transformation of rapamycin to metabolite M10.

"southern" portion of rapamycin is inferred from the ion at m/z 606, which is 16 daltons higher than the corresponding fragment ion at m/z 590 from the parent compound. Furthermore, the base peak at m/z 240 indicates that the change is unlikely on carbons 12 to 23 since m/z 240 product ion corresponds to that portion of rapamycin molecule as shown in Figure 1.

The negative ion FAB mass spectrum obtained from this metabolite shows a $[M-H]^-$ at m/z 928.5, suggesting that it is in the macrolide ring opened form. The proposed site of metabolic transformation of M2 is depicted in Figure 3.

M3: This metabolite has the same deprotonated molecular anion (m/z 912.8) as rapamycin in the full scan ion spray mass spectrum. That this metabolite is isomeric with rapamycin is indicated by the similarity of its product ion mass spectrum to that of rapamycin. M3 has been identified and reported as a degradation product of rapamycin.[8] Figure 4 shows the structure of M3.

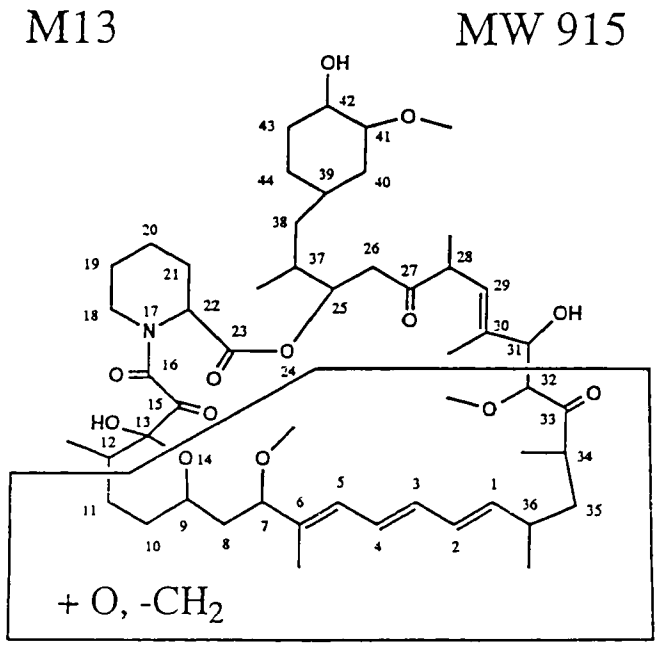

Figure 6. Proposed site of metabolic transformation of rapamycin to metabolite M13.

M10: The mass spectra of this metabolite are similar to those of M2 and, therefore, very likely to be produced from either aliphatic hydroxylation or epoxidation. Since the retention time of this metabolite is different from that of M2, this metabolite is likely to be isomeric with M2. Unlike M2 and M3, the M^- (m/z 929.5) is observed in the FAB mass spectrum, indicating that the macrolide ring is intact. Figure 5 shows the proposed structure of M10.

M13: The full scan ion spray mass spectrum contains a low abundance molecular anion at m/z 914.6 which suggests that this metabolite is likely to be a product of both hydroxylation/or epoxidation and demethylation. This speculation is corroborated by the complimentary product ions at m/z 321 and 592 which indicate that the "northern" portion of rapamycin is intact.

That both biotransformations must have occurred at the "southern" portion of rapamycin is deduced from the product ion at m/z 592. The abundant product ion at m/z 240 precludes any metabolic change occurring on carbons 12 to 23.

Demethylation is likely to occur at either of the two metabolic soft spots; for example, the methoxy group on carbon 7 or 32. There was not sufficient amount of sample for FAB MS experiment to determine if the macrolide ring is opened or intact. A proposed, closed ring structure of M13 is depicted in Figure 6.

DISCUSSION

A semipreparative HPLC procedure using a C-18 column has been successfully developed for the isolation of ten major biliary metabolites of rapamycin in rats (M13, M14, M15, M16, M8, M9, M10, M2, M3 and M11). Baseline separation of these metabolites was achieved with a reverse phase gradient system.

The major metabolic transformations of rapamycin in rats are aliphatic hydroxylation and O-demethylation. Ten major metabolites were isolated in the current study. Tandem mass spectral data were obtained on four samples and FAB MS data on three metabolites. These data have enabled us to propose possible structures for M2, M3, M10 and M13 as shown in Figures 3-6. Metabolites M2 and M3 are the major metabolites in rat plasma.[9] However, the other major plasma metabolite M5 (O-demethylated in the C41 moiety) and rapamycin were not found in rat bile at significant amounts in the current study. The pattern of metabolism of rapamycin is similar to that of FK506, another immunosuppressive agent, whose major metabolites are also produced via demethylation and oxygenation.[14]

ACKNOWLEDGMENT

We would like to acknowledge Professor Jack D. Henion at Cornell University for use of the Sciex TAGA 6000E for acquisition of the data. We wish to thank Mr. Joseph Cantone at Wyeth-Ayerst Research for acquiring the FAB mass spectra of the metabolites.

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† This work was presented at the Fourth International ISSX Meeting, Seattle, WA, August 1995.

Received July 30, 1996
Accepted August 27, 1996
Manuscript 4252