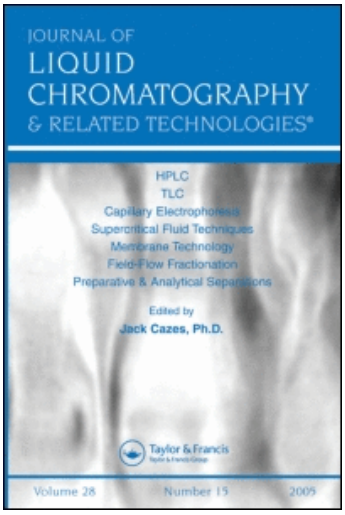

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DETERMINATION OF A MACROLIDE IMMUNOSUPPRESSANT INDOLYL-ASCOMYCIN DERIVATIVE IN PLASMA BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORESCENCE DETECTION

E. Woolf[a]; I. Fu[a]; B. Matuszewski[a]
[a] Department of Drug Metabolism, Merck Research Laboratories, West Point, PA, U.S.A.

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E. Woolf , I. Fu, B. Matuszewski

Merck Research Laboratories
Department of Drug Metabolism
West Point, PA 19486, USA

ABSTRACT

A method for the determination of a macrolide immunosuppressant, L-732,531, in plasma is described. Plasma samples are extracted using cyano solid phase extraction columns. The extract is analyzed by normal phase high performance liquid chromatography with a cyano column and a mobile phase of hexane/2-propanol/water (780:220:3). Detection is based on fluorescence at an excitation wavelength of 276 nm and an emission wavelength of 330 nm. The limit of quantification of the assay is 1 ng/mL. The assay was validated in the concentration range of 1 - 200 ng/mL when 1 mL aliquots of plasma are extracted.

INTRODUCTION

L-732,531 (Compound **I**, Figure 1) is a C_{32}-[1-(2-hydroxyethyl)-indol-5-yl]oxy synthetic derivative of a macrolide isolated from a mutant of the microorganism *S. hygroscopicus* var. *ascomyceticus.*[1-2]

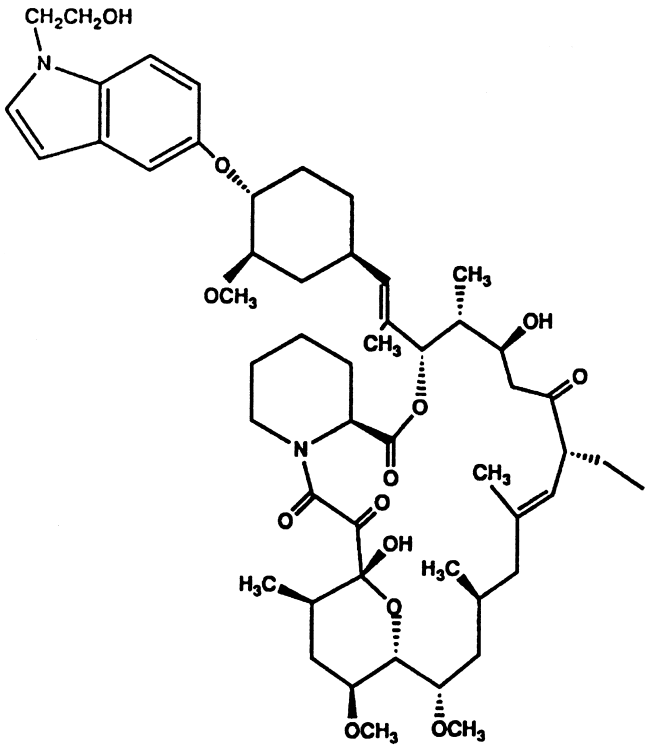

Figure 1. Chemical structure of compound **I.**

I is a potent immunosuppressant with activity equal to or better than tacrolimus,[3-4] a compound structurally similar to **I** in which the N-substituted indole group is replaced by a hydrogen atom. Compound **I** may be potentially useful in the field of organ transplantation as well as in the treatment of various autoimmune diseases. In order to support various pharmacokinetic studies with **I**, an HPLC assay with fluorescence detection to quantitate **I** in plasma has been developed and is presented in this publication.

EXPERIMENTAL

Materials

Compound **I** was prepared by the Process Research & Development Department of Merck Research Laboratories (Rahway, NJ, USA). Acetonitrile,

hexane, and 2-propanol (Omnisolve HPLC grade) were obtained from EM Science (Gibbstown, NJ, USA). Methylene chloride (Optima grade) was from Fisher Scientific (Fair Lawn, NJ, USA). Drug-free heparinized human plasma was purchased from Sera-Tech Biologicals (New Brunswick, NJ, USA). 5-Methoxyindole was obtained from Aldrich (Milwaukee, WI, USA).

Solid-phase extraction (SPE) columns (20 mL wide-mouth format) packed with 100 mg of cyanopropylsilane bonded to silica gel were obtained on special order from J.T. Baker (Phillipsburg, NJ, USA).

Instrumentation

The HPLC system consisted of a Perkin-Elmer (Norwalk, CT, USA) model 410 pump, a WISP 715 automatic injector (Waters Assoc., Milford, MA, USA), a Jones Chromatography (Lakewood, CO, USA) column heater, and a Perkin-Elmer LC-240 fluorescence detector. A 500 psi back pressure regulator (Upchurch Scientific, Oak Harbor, WA, USA) was positioned between the pump and the injector to provide the back pressure needed for efficient pump operation. The detector output was connected to a PE-Nelson (Cupertino, CA, USA) Access-Chrom data system via a PE-Nelson 900 series interface.

Refractive indices were measured using an Abbe-60 refractometer (Bellingham & Stanley Ltd., England). The absorption and fluorescence spectra were taken with a diode-array spectrophotometer (HP 8452, Hewlett Packard, Wilmington, DE, U.S.A.) and a Hitachi (San Jose, CA, U.S.A.) F-4500 fluorescence spectrophotometer, respectively.

Chromatographic Conditions

The mobile phase consisted of a mixture of hexane (780 mL), 2-propanol (220 mL), and water (3 mL). The mobile phase was filtered through a nylon filter (0.20 μm) prior to use. While in use, the mobile phase was maintained under a blanket of helium. The flow rate was 1.2 mL/min through a Zorbax 5 μm normal phase CN column (150 mm x 4.6 mm I.D., MAC-MOD Analytical, Chadds Ford, PA, USA). Prior to equilibration with mobile phase, new HPLC columns were flushed at a flow rate of 0.5 mL/min with 2-propanol for 30 min. followed by water for 60 min. and concluding with 2-propanol for an additional 30 min. The column was maintained at 50°C. Injection volumes were 150 μL. Fluorescence detection at an excitation wavelength of 276 nm and an emission wavelength of 330 nm was used. The detector PMT voltage was set at 750 V while the detector response factor was set at 5. A detector attenuation setting of 256 was used.

Preparation of Standards

A 10 μg/mL solution of **I** was prepared by dissolving 1.0 mg of reference material in 100 mL of acetonitrile. A 1.0 μg/mL solution was prepared by diluting 5 mL of the 10.0 μg/mL solution to 50 mL with acetonitrile.

Working standards of 4, 2, and 1 μg/mL **I** were prepared by dilution of the 10 μg/mL stock solution with acetonitrile. Working standards of 0.4, 0.2, and 0.1 μg/mL **I** were prepared by dilution of the 1.0 μg/mL stock solution with acetonitrile. Working standards of 0.04 and 0.02 μg/mL **I** were prepared by dilution of the 0.4 and 0.2 μg/mL working standards. Working standard solutions were found to be stable for a minimum of 2 weeks when stored protected from light at room temperature.

Plasma standards were prepared by adding 50 μL of each working standard to 1 mL of drug-free plasma. The resulting standards ranged in concentration from 1 to 200 ng/mL.

Plasma Extraction Procedure

A plasma standard prepared as described above or a 1 mL aliquot of a plasma sample was pipetted into a 15 mL disposable polypropylene conical tube. Acetonitrile (1 mL) was added to the tube in four 250 μL portions; the tube was vortex mixed vigorously between additions of acetonitrile. The tube was then centrifuged at 2050 *g* for 10 min at 10°C. The resulting supernatant was decanted into a 100 x 16 mm disposable polypropylene culture tube. A 1.0 mL aliquot of water was added to the tube containing the supernatant. The contents of the tube were immediately poured into a cyano SPE column positioned on a 21 position vacuum manifold (J. T. Baker) equipped with stopcocks at each position.

The SPE column packing was previously conditioned by sequential washes of 1 mL of hexane, 1 mL of methanol, and 2 mL of water. The tube containing the supernatant was rinsed with 1 mL of water that was then added to the SPE column. The diluted plasma supernatant was aspirated through the column with the vacuum gauge set at about 10 inches of mercury.

Following sample application, the SPE sorbent bed was dried by aspirating air through the column for 1 minute. The dried SPE column was washed with 1 mL of hexane followed by 1 mL of 7.5% 2-propanol in hexane. The SPE column was air dried between the washes.

The analyte was eluted from the column into a 75 x 12 mm disposable borosilicate glass culture tube with 2 mL of 5% 2-propanol in methylene chloride. The elution solvent was evaporated at room temperature under a stream of nitrogen. The residue was reconstituted in 250 μL of HPLC mobile phase. The reconstituted sample was transferred to an autosampler vial containing a limited volume insert prior to injection into the HPLC system.

Fluorescence Quantum Efficiency

The absolute fluorescence quantum efficiency (ϕ_f) for **I** was determined relative to 5-methoxyindole (5-MI) as standard (ϕ_f = 0.63 in deaerated cyclohexane).[5] Because of the similarities of the emission spectra of **I** and the fluorescence standard (5-MI), the uncorrected fluorescence spectra of **I** and 5-MI were utilized for calculations of relative fluorescence intensities. The measurement of the relative ϕ_f was performed using a similar procedure as described earlier.[6] The fluorescence intensities in various solvents were corrected by multiplying the uncorrected intensities by η^2, where η is the refractive index of the solvent.

RESULTS

Assay Specificity

Figure 2 shows chromatograms of extracted drug-free plasma and a plasma standard sample containing 5 ng/mL **I**. A comparison of Figure 2A with Figure 2B illustrates that no endogenous peaks elute in the region of **I**.

Linearity

Weighted (weighting factor = 1/y where y=peak height) least-squares regression calibration curves, constructed by plotting the standard concentration of **I** versus peak height, yielded coefficients of regression typically greater than 0.999 over the concentration range 1-200 ng/mL **I** in plasma.

The use of the weighted least-squares regression generally resulted in less than a 10% deviation between the nominal standard concentration and the experimentally determined standard concentration calculated from the regression equation.

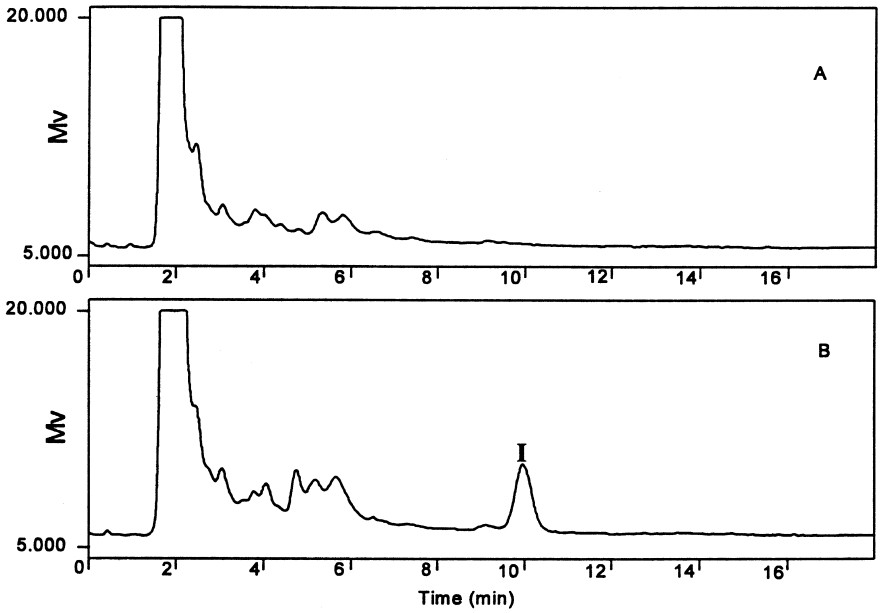

Figure 2. Representative chromatograms of (A) control human plasma and (B) plasma spiked with **I** at a concentration of 5 ng/mL.

Table 1

Recovery of I from Human Plasma and Within-day Variability of the Assay

Nominal Conc. (ng/mL)	Mean (n=5) Recovery %	Accuracy[a] (%)	Precision (C.V., %)
1.0	74.7	104.7	5.0
2.0	71.5	96.8	1.9
5.0	78.6	99.0	1.6
10.0	77.2	96.5	1.9
20.0	76.2	98.3	2.8
50.0	79.0	100.9	2.8
100.0	78.4	102.7	2.1
200.0	84.1	101.5	1.5
Mean ± S.D.	77.5 ± 3.7	100.1 ± 2.9	

[a] Calculated as [mean (n=5) observed concentration/nominal concentration] 100.

Table 2

Inter-day Variability of the Assay as Assessed by the Precision and Accuracy of the Calculated Standard Concentrations from the Regression Live on Each of Five Days

	Nominal Standard Concentration (ng/mL)								
Day	**1.0**	**2.0**	**5.0**	**10.0**	**20.0**	**50.0**	**100.0**	**200.0**	**Slope of Std. Curve**
1	1.1	2.0	4.9	9.7	19.9	48.2	100.0	202.4	882
2	1.0	2.1	4.9	10.1	19.0	50.5	101.5	199.1	797
3	1.1	1.9	4.8	9.9	19.5	50.4	99.7	202.1	854
4	1.0	1.9	5.1	9.9	19.5	50.4	99.7	200.5	895
5	1.0	2.0	5.0	9.8	21.1	52.1	99.1	198.2	837
Mean	1.0	2.0	4.9	9.9	20.2	50.0	99.7	200.5	853
Precision[a]	5.3	4.2	2.3	1.7	5.0	3.1	1.2	0.9	4.5
Accuracy[b]	100.0	100.0	98.0	99.0	101.0	100.0	99.7	100.0	

[a] Expressed as C.V. (%).
[b] Calculated as [mean (n=5) observed concentration/nominal concentration] x 100.

Extraction Recovery

The recovery of the extraction method was determined by comparing the responses of standards of **I** dissolved in mobile phase directly injected onto the HPLC column with those of extracted plasma standards. The results (Table 1) indicate that the mean recovery of the extraction procedure over the concentration range 1-200 ng/mL **I** in plasma is 77.5%.

Assay Precision and Accuracy

Replicate standards (n=5) were analyzed to assess the within-day variability of the assay. The mean accuracy of the assayed concentration as well as the coefficient of variation (C.V.) of the plasma replicate standards are shown in Table 1.

Standard curves over the assay range of 1 - 200 ng **I** per mL plasma were analyzed on each of 5 days to determine the between day variability of the assay. The daily, as well as, the mean results along with data on the slopes of the standard curves are presented in Table 2.

Table 3

Stability of the Analyte Toward Freeze-Thaw Cycles

Nominal Concentration (ng/mL)	Measured Concentration (ng/mL) ± S.D.[a]		
	1 Cycle	2 Cycles	3 Cycles
7.5	7.1 ± 0.1	7.4 ± 0.2	7.3 ± 0.1
125.0	119.4 ± 3.2	127.1 ± 1.1	125.3 ± 4.0

[a] n=5 for 1 cycle, n=3 for 2 and 3 cycles.

Table 4

Effect of Heat Treatment of Plasma Samples Containing I

Nominal Concentration (ng/mL)	Measured Conc. (Mean ± S.D., n=3) (ng/mL)	
	No. Treatment	56°C for 90 Minutes
7.5	7.0 ± 0.1	6.4 ± 0.1
125.0	118.6 ± 4.6	110.3 ± 2.2

Sample Stability

Samples containing **I** in plasma at concentrations of 7.5 and 125 ng/mL were prepared and frozen. The samples were analyzed after 1, 2, and 3 freeze-thaw cycles in order to determine the stability of the analyte towards repetitive freezing and thawing. The results are shown in Table 3.

Treatment at 56°C for a 90 minute period has been shown to deactivate hepatitis B and HIV viruses in plasma samples.[7-8] In order to determine whether **I** in plasma is stable under these deactivation conditions, plasma samples containing **I** at concentrations of 7.5 and 125 ng/mL were analyzed after heating at 56°C for 90 minutes. The results (Table 4) indicate that **I** undergoes less than 10% degradation during heat treatment.

DISCUSSION

Compound I is structurally similar to, and falls into the same therapeutic class as tacrolimus (FK-506).[1-4] FK-506 is currently being clinically evaluated for the treatment of rejection following organ transplantation.[9] The compound

has been found to be pharmacologically active at low nanogram concentrations, hence, it would be expected that an assay with low nanogram sensitivity would be necessary to support drug development studies of **I**.

While FK-506 shows no significant ultraviolet (UV) absorption above 220 nm, **I**, due to the presence of a N-substituted indole group, exhibits a maxima in its UV spectrum at 276 nm (ε = 7100 $M^{-1}cm^{-1}$ at pH 4.6 in 50/50 v/v% acetonitrile/20 mM phosphate buffer, 6300 $M^{-1}cm^{-1}$ in 50/50 v/v% acetonitrile/20 mM phosphate buffer, pH 9.4, 7300 $M^{-1}cm^{-1}$ in cyclohexane, and 7600 $M^{-1}cm^{-1}$ in mobile phase). Because of this relatively small UV absorption, the development of a sensitive assay for **I** in plasma at low ng/mL levels based on HPLC with UV detection was unlikely. Therefore, the fluorescent properties of **I** were evaluated.

It is well established that compounds possessing an indole group have a potential to be highly fluorescent.[10] However, the efficiency of fluorescence is very strongly dependent on a number of factors including the substitution of the indole ring, the nature of the solvent and its pH.[5,10,11] Since normal-phase mobile phase conditions were utilized in the assay (see below), the fluorescence properties of **I** were evaluated in organic solvents. Compound **I** was found to be highly fluorescent both in cyclohexane and mobile phase, with the same emission maximum (330 ± 5 nm) in both solvents. The fluorescence quantum efficiency was measured using 5-methoxyindole in cyclohexane as standard.[5] The ϕ_f values were found to be 0.11 and 0.18 in cyclohexane and mobile phase, respectively. These values were practically the same before and after deaeration of the solution with nitrogen, indicating that the lifetime of the excited singlet state of **I** is, like that of 5-MI, very short (<5 nsec). The decrease in fluorescence quantum yield of **I** in comparison with 5-MI could be due to intramolecular quenching of the indole fluorescence by the macrolide moiety and/or N-substitution of the indole nitrogen in **I**. The relatively high ϕ_f of **I** indicated the potential for the development of a highly sensitive HPLC assay with low nanogram sensitivity using fluorescence detection.

Initial attempts to chromatograph **I** at room temperature under reversed phase conditions (acetonitrile/water mobile phases) on C-18 (120 and 300 Å pore size), C-4 (300 Å pore size) and cyano (80 Å pore size) columns resulted in broad unsymmetrical peaks. The poor peak shape may be attributed to the fact that the molecule contains an α-ketoamide moiety that exists in solution as two interconverting rotamers (Figure 3). Similarly to FK-506, the rotamers are only marginally separated on the HPLC column.[12] Raising the temperature of the column to 70°C was found to significantly improve peak shape, however, the baseline stability of the detector was adversely effected and assay sensitivity was sacrificed.

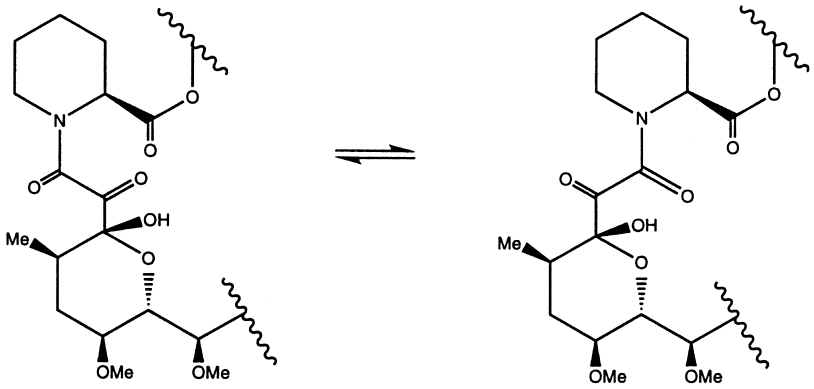

Figure 3. Structures of the rotamers of the α-ketoamide moiety of **I**.

Wallemacq[13] found that cyclosporin A, a member of the same therapeutic class as **I** that also exits in solution as two rotameric forms, could be chromatographed under normal phase conditions. Only a moderate (50°C) increase in column temperature was required under normal phase conditions for acceptable peak shapes to result. Compound **I** yielded symmetrical peaks when normal phase chromatography with a cyano column maintained at 50°C was used. Since baseline stability was adequate under these conditions, normal phase chromatography was chosen for the assay.

Initially, although peak shape was good under normal phase conditions, the retention time of **I** had a tendency to vary significantly within the course of a day. The retention time variation was believed to be due to the fact that the initially anhydrous mobile phase gradually absorbed moisture from the atmosphere over the course of the day, thus increasing its solvent strength. Preconditioning new HPLC columns by flushing them with water prior to use and the addition of a slight amount of water to the mobile phase to partially pre-saturate it eliminated the retention time variability.

Experiments with liquid-liquid extraction indicated that **I** could be extracted from plasma into diethyl ether. Chromatograms of ether extracts were free of interfering peaks, however, several problems were associated with the use of diethyl ether as an extraction solvent. In addition to being a safety hazard, the ether extracts were found to contain a significant amount of water. The water, which could not be removed by simple nitrogen evaporation, proved to be incompatible with the normal phase HPLC mobile phase. Hence, ether extracts had to be dried under vacuum before reconstitution in mobile phase. This extra drying step added approximately 1 hour to sample preparation time.

The use of solid phase extraction to isolate **I** from plasma was explored as an alternative to ether extraction. A Baker cyano SPE column containing 100 mg of sorbent was found to retain **I** from deproteinized plasma. Endcapped cyano SPE columns (Analytichem, Harbor City, CA, USA) were also found to give acceptable analyte recovery. However, non-end-capped cyano SPE columns and cyano SPE columns from other manufacturers did not give an adequate recovery of analyte. "Robotic format" SPE columns were used to eliminate the need for the additional solvent reservoirs that would be required to load a volume greater than 1 mL onto a conventional 100 mg SPE column. Protein precipitation was required to ensure good analyte recovery because **I** has been found to be highly bound to plasma proteins.

Once **I** was isolated on the SPE column, the packing could be washed sequentially with hexane and a mixture of 7.5% 2-propanol in hexane prior to elution with a mixture of 5% 2-propanol in methylene chloride. The organic solvent washes served both to remove water from the SPE column and to elute endogenous interfering species. The residue from the evaporation, under nitrogen, of the SPE column elution solvent proved to be dry enough to be compatible with mobile phase without requiring additional drying under vacuum.

CONCLUSION

The combination of solid phase extraction for analyte isolation and normal phase HPLC with fluorescence detection enables the determination of **I** in plasma with a limit of quantification of 1 ng/mL. The assay has been validated over the concentration range of 1 - 200 ng/mL. Plasma samples are stable towards freeze-thaw cycles. Treatment of samples at 56°C results only in minor analyte degradation and may be used for the decontamination of plasma samples from suspect populations.

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