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Development of a plasma high-performance liquid chromatographic assay for LY303366, a lipopeptide antifungal agent, and its application in a dog pharmacokinetic study

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Abstract

An HPLC assay for plasma analysis of LY303366 (I), a semi-synthetic lipopeptide antifungal related to echinocandin B (ECB), was developed to support the selection and subsequent preclinical development of I. The method involved extraction of I from plasma with the aid of solid-phase extraction (SPE) cartidges followed by reversed-phase HPLC with UV detection at 300 nm. The method is simple, selective and is applicable to dog, rat, mouse and rabbit plasma. Validation studies using dog plasma showed that the values obtained for parameters of linearity, precision and accuracy were within acceptable limits. Based on analysis of 0.3 ml of plasma, the lower limit of quantitation was 20 ng/ml. The method has been successfully applied to determine the pharmacokinetic parameters of I in the dog following intravenous (i.v.) and oral administration. Compared to first generation ECB antifungal agents, the results of the i.v. dog study indicated a 50% reduction in clearance of the drug from plasma (0.1 l/h/kg) and an 18-fold increase in the volume of distribution at steady state (1.8 l/kg). When administered orally, compound I had an absolute bioavailability of 9%; however, plasma levels remained above the MIC for *C. albicans* (0.005 μg/ml) through 48 h. Given the excellent potency of I and its broad spectrum of activity relative to first generation ECB antifungal agents, the assay results for I indicate the potential for its use as a broad spectrum i.v. and oral antifungal agent. © 1997 Elsevier Science B.V.

Keywords: LY303366; Lipoeptides; Echinocandin B

1. Introduction

LY303366, 1-[(4*R*,5*R*)-4,5-dihydroxy-N(2)-[[4″-(pentyloxy)[1,1′:4′,1″-terphenyl]-4-yl]carbonyl]-l-ornithine]echinocandin B (I, Fig. 1), is a cyclic lipopeptide of the echinocandin class being developed by Eli Lilly and Co. as an antifungal agent. Compound I is a second generation, semi-synthetic echinocandin fungicidal agent with in vitro and in vivo activity against *Candida*, *Aspergillus* [1] and *Pneumocytis* [2] organisms. The echinocandin lipopeptides most likely owe their primary mode of antifungal action to their inhibition of the biosynthesis of the fungal cell wall [1,3]. Compound I has been undergoing evaluation as a potential therapeutic agent for the treatment of fungal infections caused by these various organisms. To support the development

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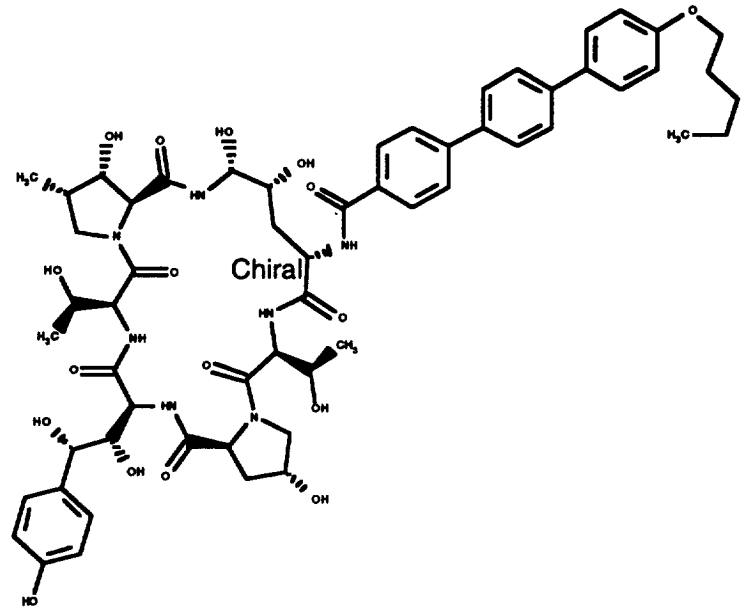
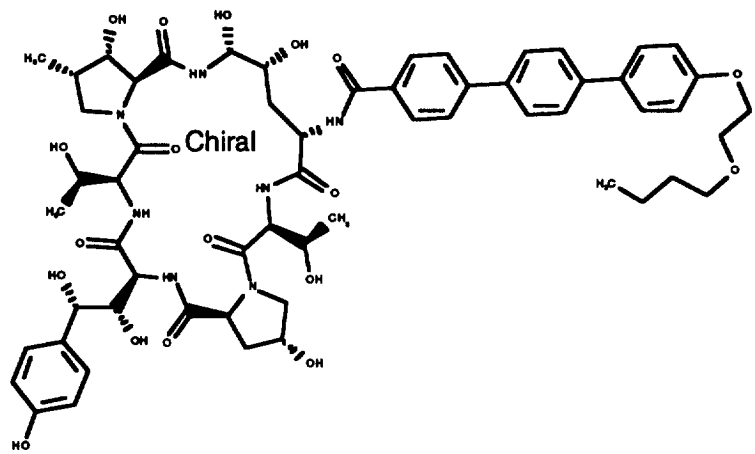


I



I.S.

Fig. 1. Chemical structures of I and I.S.

of the drug candidate, a method was developed to analyze I in plasma. This report describes the method and its validation using dog plasma. In addition, applicability of the method is shown in a pharmacokinetic study conducted in dogs following i.v. and oral administration of I.

2. Experimental

2.1. Materials

The test compound, I, and the internal standard (I.S.), 306168, were synthesized at Lilly Research

Laboratories (Indianapolis, IN, USA). The choice of the I.S. was based on matching of physico-chemical properties and chromatographic behavior of I with those of related compounds in our ECB library. The structures of I and I.S. are shown in Fig. 1.

HPLC grade acetonitrile, chloroform and concentrated ammonium hydroxide were obtained from Mallincrodt Specialty Chemicals (Paris, KY, USA). Monobasic ammonium phosphate was obtained from EM Science (Gibbstown, NJ, USA). Buffers and mobile phase were prepared using water purified through a Milli-Q system (Millipore, Bedford, MA, USA). Bond Elut solid-phase extraction (SPE) columns with phenylsilane (PH) packing (100 mg/1 ml; catalog No. 1210-2005) were obtained from Jones Chromatography (Lakewood, CO, USA). An International Sorbent Technology (IST) VacMaster-20 sample processing station was used in the extraction process (Jones Chromatography). A TurboVap, Model LV, evaporator (Zymark Corporation, Hopkinton, MA, USA) was used to evaporate SPE eluates to residue. Control beagle dog plasma (heparinized) was obtained from either in-house supply or from Marshall Farms (North Rose, NY, USA).

2.2. Buffer and extraction solutions

A 0.05 *M* ammonium phosphate pH 4.5 buffer (APB) was prepared by dissloving 11.5 g of monobasic ammonium phosphate in 1.9 l of Milli-Q water and adjusting to pH 4.5 using 30% ammonium hydroxide. The volume of the buffer was then brought to a volume of 2 l with Milli-Q water.

Extraction solutions and mobile phase were prepared by admixing acetonitrile with certain ratios of APB. One extraction solution was APB–acetonitrile (90:10) and was used to rinse the SPE columns after the sample was applied. An acetonitrile–APB (60:40) solution was used to elute the compounds from the SPE columns. For reconstituting the samples for analysis, an acteonitrile–APB (35:65) solution was used.

2.3. Preparation of standards

Stock solutions of I and I.S. were prepared in acetonitrile–APB (50:50) with each at a concentration of 1.0 mg/ml. The working solutions of I and I.S. were prepared by diluting stock solutions with APB as stated above. Plasma standards, in the range of 20 to 10 000 ng/ml, were prepared by aliquoting appropriate volumes of a stock or working solution of I and adjusting to 3 ml with blank (drug free) dog plasma. Reference control concentrations (30, 300 and 3000 ng/ml) were similarly prepared for monitoring the performance of the assay during each run. Plasma standards and controls were stored at −20°C until used or up to a maximum of 1 month. A working solution of the I.S. (10 μg/ml) was prepared on the day of analysis by diluting the stock solution with acetonitrile–APB (50:50).

3. Assay of compound I

3.1. HPLC instrumentation and operating conditions

Chromatographic analyses were performed using a Beckman System Gold (Fullerton, CA, USA) HPLC system consisting of a Model 126 solvent delivery system, Model 166 UV–Vis detector and a NEC 8300 controller. Samples were injected onto the HPLC with an Alcott Model 738 autosampler (Norcross, GA, USA). The HPLC peaks were identified by retention time with reference to standard compounds and quantitated using peak area ratio of I to I.S. The A/D data were collected and stored using Perkin–Elmer Nelson boxes (Model 941 interface, Cuperatino, CA, USA) and the Lilly Chromatography Computer System. The system utilizes an HP1000 A-series computer system (Hewlett Packard) with an RTE-A operating system and software written by the Laboratory Automation Group of the Analytical Development Department of the Lilly Research Laboratories. The chromatographic conditions were as follows: column: Zorbax SB-C8 (4.6×250 mm, 5 μm particle size) analytical column (Mac-Mod, Wilmington, DE, USA) with an in-line 2 μm filter; flow-rate: 1.0 ml/min; mobile phase: 45% acetonitrile and 55% APB; temperature: 30°C; detection: absorbance at 300 nm; run time: 14 min; retention times: I.S. and I, approximately 6 and 9 min, respectively.

3.2. SPE procedure

Plasma samples were extracted on an IST 20-position manifold using a Bond Elut phenyl (PH) SPE column. The SPE columns were conditioned with 1 ml of acetonitrile, 1 ml of water, followed by 2 ml of APB. A 0.3 ml aliquot of dog plasma sample was placed in a 12×75 mm glass test tube. To each plasma sample was added 50 μl of I.S. solution (10 μg/ml) and 0.5 ml of APB. The mixture was vortexed briefly and added to the SPE columns. The samples were slowly forced (vacuum <125 mmHg) onto the column packing. The test tubes were rinsed with 0.5 ml of APB and added to the columns. Subsequently, the columns were rinsed with 2 ml of APB followed by 2 ml of APB–acetonitrile (90:10) and thoroughly dried (1–2 min). A final rinse with 2 ml of chloroform was done at full vacuum (between 375–500 mmHg) until the columns were dry. Elution of I and I.S. was performed using 1 ml of acetonitrile–APB (60:40). The plasma eluate was evaporated to dryness using a TurboVap LV unit with a gentle stream of nitrogen and a 40°C water bath, followed by reconstitution of the residue in 0.4 ml of acetonitrile–APB (35:65). After mixing, 100 μl of the reconstituted extract was injected onto the HPLC column.

3.3. Validation studies

The degree of interference by endogenous constituents with the peaks of interest was assessed by visual inspection of chromatograms from processed control samples and each respective spiked sample. Lower limit of quantitation (LLQ, $S/N=3$) was established by evaluating both accuracy (percent deviation from the nominal <15%) and precision (R.S.D.<15%) of the observed concentration in at least six spiked samples. The intra-assay accuracy and precision of the method were evaluated by analyzing replicates at eight different concentrations. The inter-assay accuracy and precision were determined over at least three different days. Freezer stability of I was assessed by analyzing the dog plasma QC samples kept frozen at −20°C for 1 month. The extraction recovery of each analyte in dog plasma was determined by comparing processed standards versus neat standards within the same concentration range. Method linearity was based on evaluation of %R.S.D. and variability of slope for intra-day and inter-day analyses.

3.4. Dog pharmacokinetic study

Fasted female beagle dogs (age, 1–6 years; weight, 8–16 kg) were randomly assigned to two treatment groups (three per group). Group 1 was administered a single 5 mg/kg oral dose of I as an emulsion formulation. Group 2 dogs received a single 5 mg/kg i.v. dose of I as a solution formulation. The dogs dosed orally were immediately administered 50 ml of water following dosing. The study was conducted as a non-crossover design. Blood collection schedules were 0 (predose), 0.5, 1, 2, 4, 6, 8, 12, 16, 24, 48 and 72 h post-dose for dogs dosed orally. The i.v. time points were 0 (predose), 0.033, 0.083, 0.167, 0.25, 0.5, 0.75, 1, 2, 4, 8, 16, 24, 48 and 72 h post-dose. Blood samples, 3 ml of whole blood, were obtained by jugular venipuncture, immediately transferred to heparinized tubes and mixed thoroughly. The plasma samples were harvested and stored at −70°C until analyzed.

3.5. Data analysis

Plasma concentration data were analyzed using MIKAPC v1.11 (Lilly Clinic, Indianapolis, IN, USA) to provide the following pharmacokinetic parameters: maximum observed plasma concentration (C_{max}), time when C_{max} was observed (t_{max}), area under the curve from time zero to when the concentration was last quantifiable (AUC_{0-t}), area from time zero to infinity ($AUC_{0-\infty}$), systemic clearance (Cl), the volume of distribution at steady state (Vd_{ss}) and terminal elimination half-life ($t_{1/2}$). Absolute bioavailability was determined from the ratio of dose normalized AUC values obtained for oral versus i.v. administered drug.

4. Results and discussion

4.1. Chromatography

Initial development of the method indicated that blank plasma from several species including dog, rat,

rabbit and mouse had no significant endogenous peaks that would interfere with the analysis of I. Although the method has been used for sample analysis obtained from studies in rats, rabbits and mice, the validation studies reported here were performed with dog plasma. Typical chromatograms of blank dog plasma with I.S. added and dog plasma spiked with I and I.S. are shown in Fig. 2.

4.2. Assay validation

The recoveries of I and I.S. were determined at 30, 300 and 3000 ng/ml. As shown in Table 1, mean recoveries were >82% at all concentrations for both compounds. The recoveries were reproducible (R.S.D.<5%) and there was no evidence for concentration-dependence within the tested range. By using a non-weighted linear least squares regression analysis, standard curves were found to be linear in the range of 20 to 10 000 ng/ml, r^2>0.9994 for all curves. The results for the intra- and inter-day accuracy and precision for this range are shown in Table 2. R.S.D. was <12% at all concentrations and accuracy of the assay was quite acceptable as determined by the mean percentage difference of extrapolated values from the nominal (spiked) concentrations (Table 2). A concentration of 20 ng/ml was established as the LOQ based on acceptable estimates obtained for precision (R.S.D.<12%) and accuracy (% difference <5%) at this level. Based on reanalysis of spiked standards stored at −20°C, it was found that the drug was stable in dog plasma for at least 1 month. This amount of time was adequate for dog pharmacokinetic studies, since all plasma samples were analyzed within 1 week of sample collection.

Table 1
Extraction recoveries (n=3) of compound I and I.S. from dog plasma

Compound	Concentration (ng/ml)	Recovery (mean±S.D.) (%)
I	30	82±4
	300	86±2
	3000	88±2
I.S.	30	99±2
	300	99±2
	3000	97±2

4.3. Pharmacokinetic characteristics of compound I in the dog

Compound I plasma concentration data obtained following the two routes of administration are shown in Fig. 3. The results of the pharmacokinetic analysis of these data are summarized in Table 3.

Following a single i.v. dose of 5 mg/kg, the *Cl* of I (0.10±0.02 l/h/kg) was reduced by 50% when

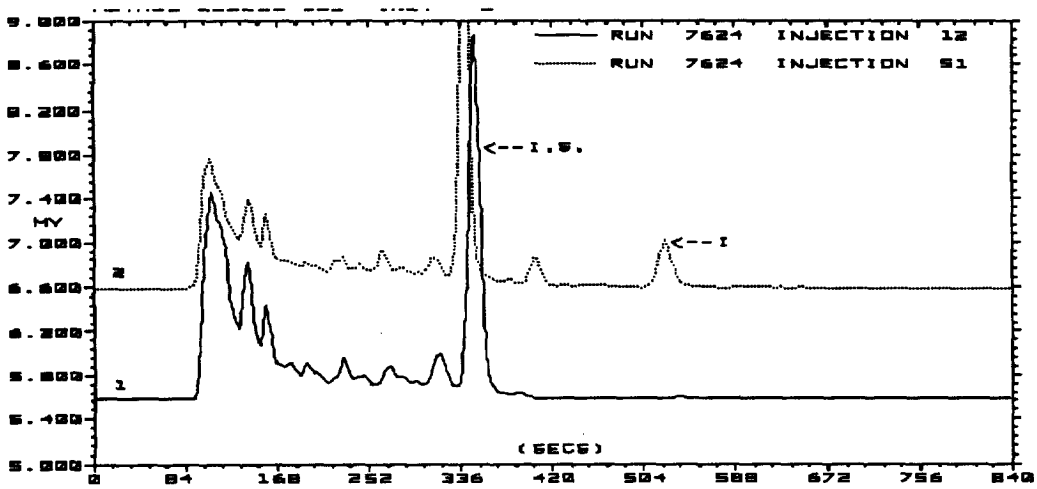


Fig. 2. Representative chromatograms (presented as overlays) of processed dog plasma samples: (1) blank plasma with I.S. (1670 ng/ml) added; (2) plasma spiked with I (200 ng/ml) and I.S.. Retention: I.S.=6 min; I=9 min.

Table 2
Intra-day and Inter-day accuracy and precision of the analytical method for compound I in dog plasma

Nominal concentration (ng/ml)	n[a]	Mean observed concentration (ng/ml)	Mean deviation (%)	Precision (R.S.D.) (%)	
				Intra-day	Inter-day
20	21	20.9±1.89	4.7	9.01	8.50
50	21	50.9±3.00	1.7	5.89	4.84
100	21	97.2±7.36	−2.8	7.57	8.84
200	21	197.5±10.3	−1.3	5.24	4.36
500	21	492.5±33.3	−1.5	6.76	11.2
1000	18	1009.9±25.8	1.0	2.55	3.38
5000	18	5024.5±10.6	0.5	0.21	6.58
10 000	18	10 037.5±93.6	0.4	0.93	0.70

[a] Six to seven replicates of each concentration were analyzed in three separate analytical runs on three separate days.

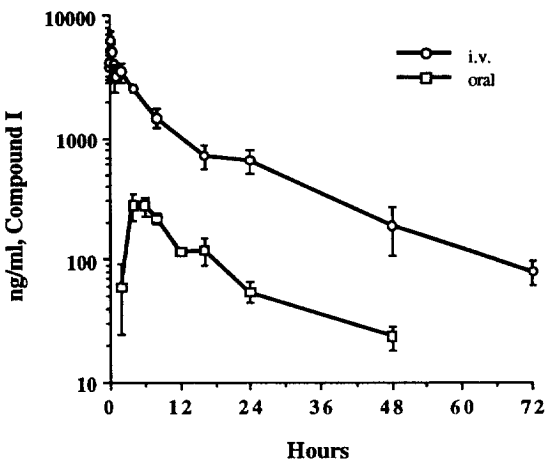


Fig. 3. Average plasma concentration (±S.D.) versus time plots for Compound I after i.v. or oral administration of 5 mg/kg of body weight to female dogs (n=3).

compared to cilofungin (0.2±0.01 l/h/kg) [4,5]. The Vd_{ss} was 1.76±0.11 l/kg compared to 0.1±0.01 l/kg for cilofungin [4]. These results suggest a wide distribution and provide evidence for extensive tissue uptake of I. The average $t_{1/2}$ for the terminal phase was 15.6 h, which was several times longer than the 1.3 h observed for cilofungin.

When compound I was administered orally as a single 5 mg/kg dose, C_{max} averaged 307±61 ng/ml and t_{max}, 4.7±1.2 h. The average terminal $t_{1/2}$ was 12.4 h, which was not significantly different from the estimated i.v. half-life (Student's t-test, $p<0.05$).

Based on a ratio of AUCs obtained following oral versus i.v. administration, the absolute bioavailability of I in the dog was estimated to be about 9%. Nonetheless, the excellent potency [1] and half-life of I resulted in plasma levels in excess of *C. albicans* MIC (0.005 μg/ml) [1] for at least 48 h following

Table 3
Summary of the pharmacokinetic parameters of compound I determined in dogs following intravenous and oral administration of 5 mg/kg[a]

Treatment route	C_{max} (ng/ml)	t_{max} (h)	Terminal $t_{1/2}$ (h)[b]	Cl (l/h/kg)	Vd_{ss} (l/kg)	AUC $_{0-\infty}$ (ng·h/ml)	F (%)[c]
i.v.	6161 ±1219	0.167 ±0	15.6	0.10 ±0.02	1.76 ±0.11	49 409 ±10 286	–
Oral	307 ±61	4.7 ±1.2	12.4	–	–	4477 ±768	9

[a] Values given are mean±S.D. for n=3.
[b] Reported as harmonic mean.
[c] $\%F=(AUC_{po}/AUC_{i.v.})\times 100\%$.

oral dosing. Coupled with its superior potency and expanded spectrum of activity relative to cilofungin, the above results provided by this method clearly indicate the potential for an enhanced clinical efficacy of compound I and its use as a broad spectrum i.v. and oral antifungal agent [5].

5. Conclusions

The method described in this report for the assay of I in plasma was developed to obtain pharmacokinetic characteristics of this compound to support its identification as the lead clinical development candidate during SAR studies and, further, to support its subsequent preclinical development. The method is simple, specific and adaptable to plasma from several species. Validation studies, performed with dog plasma as the matrix, demonstrated that a concentration of 20 ng/ml can be quantitated with satisfactory precision and accuracy. Compound I was found to be stable in dog plasma when stored at −20°C up to 1 month. The suitability of the method was demonstrated by analyzing plasma samples from a pharmacokinetic study in dogs. The results of this study indicated a reduced clearance and greater distribution compared to cilofungin, a first generation ECB antifungal [5]. When dosed intravenously, disposition of I was characterized as biphasic with a terminal half-life of 15.6 h. Availability of the analytical method helped in identifying the critical issues related to I development. The drug is currently being tested in clinical trials using this method with slight modifications to monitor compound I plasma levels.

Acknowledgments

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