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Use of high-performance liquid chromatographic and microbiological analyses for evaluating the presence or absence of active metabolites of the antifungal posaconazole in human plasma

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Abstract

Posaconazole (SCH 56592) is a novel broad spectrum triazole antifungal agent that is currently in phase III clinical trials for the treatment of systemic fungal infections. This study was initiated to determine if orally administered posaconazole to humans would result in the formation of active metabolite(s). Plasma samples from a multiple-rising dose study in healthy volunteers were analyzed by validated HPLC and microbiological methods. The HPLC analysis involved extraction with a mixture of organic solvent (methylene chloride–hexane) followed by separation on a C_{18} column and quantification by UV absorbance at 262 nm. The microbiological assay was performed utilizing an agar diffusion method using *Candida pseudotropicalis* ATCC 46764 as the test organism. Potency was determined by comparing the growth inhibition zones produced by the test sample to those produced by standard concentrations prepared in plasma. Individual and mean plasma concentration–time profiles were similar for both HPLC and microbiological assays. The area under the plasma concentration–time curves of the microbiological and HPLC results were similar with a mean (RSD) ratio of 105.5% (5.3%), indicating that there was no relevant biologically active metabolite of posaconazole in human plasma.

Keywords: Posaconazole; SCH 56592; Azoles

1. Introduction

Fungal infections have substantially increased over the past two decades, and invasive forms are now important causes of morbidity and mortality [1]. The rise in frequency of fungal infection has been attributed to an increasing number of immunosuppressed patients, for example individuals with hematological malignancies, organ and bone marrow transplants, and human immunodeficiency virus (HIV)-infected individuals advancing to late-stage acquired immunodeficiency syndrome (AIDS). Although amphotericin B remains widely used to treat these infections, a number of new systemic antifungal agents have been developed during the past two

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decades. The azole antifungal agents are better tolerated than amphotericin B [1], and can usually be given by more than one route of administration, benefitting a wide patient population.

Posaconazole (SCH56592), 4-[4-(4-[4-{[5-(2,4-difluorophenyl)tetrahydro - 5 - (1*H* - 1,2,4-triazol-1-yl-methyl) - 3 - furanyl]methoxy}phenyl - 1 - piperazinyl] phenyl) - 2 - (1-ethyl-2-hydroxypropyl)] - 2,4-dihydro-3*H* - 1,2,4 -triazol-3-one (Fig. 1), is a novel broad spectrum triazole antifungal agent that is active both orally and intravenously [2,3]. The in vitro and in vivo profiles of posaconazole in animal models showed significant advantages over existing agents in terms of potency and spectrum [3–5]. Posaconazole was more active than itraconazole (ITZ) and fluconazole (FLZ) against all 283 strains tested, and was more active than amphotericin B (AMB) against 95% of these strains [3]. Posaconazole was fungicidal against all strains of *Candida krusei* that are resistant to FLZ [3] and was also very active against other species including FLZ-resistant strains of *C. albicans*, *C. tropicalis*, some strains of *C. glabrata*, dermatophytes and many opportunistic fungi [5,6].

Results from several animal models of infection have shown posaconazole to be more potent than FLZ and ITZ against *Candida albicans, Aspergillus flavus* and *A. fumigatus* [7,8]. Posaconazole was also very active against vaginal infections in hamsters and against *T. mentagrophytes* infections in guinea pigs when given orally or topically [9].

Earlier, an HPLC method was developed and validated for the analysis of posaconazole in serum of dogs, a species used for safety evaluation [10]. The assay was shown to be sensitive, specific, accurate, precise, and reliable for use in pharmacokinetic or toxicokinetic studies. Posaconazole was stable in dog serum for at least for 348 days at −20 °C. Pharmacokinetics of posaconazole were evaluated in mice, rats, rabbits, dogs, and cynomolgus monkeys using the described method [11]. Posaconazole was orally bioavailable and showed dose-related increase in plasma concentrations in all species. The metabolism of [^{14}C]posaconazole was evaluated in dogs and rats [12]. Radioactivity was extensively distributed into tissues and most of radioactivity was found in feces. Metabolic profiles of rat and dog plasma indicated that there were no major metabolites in either species.

Since the pharmacological activity of a drug could result from the sum of biological activity of the parent compound and active metabolite(s), chemical analysis of plasma for parent compound(s) may not be sufficient to assess the efficacy of the compound and establish a pharmacokinetics–pharmacodynamic relationship. Formation of active metabolites, particularly in humans, would have a major impact on the design of safety, toxicokinetic and drug–drug interaction studies. Therefore, it is important to determine as early as possible in the program if active metabolites are formed. In the current report, plasma samples from a phase I clinical study were analyzed by high-performance liquid chromatographic (HPLC) and microbiological assays to determine if significant amounts of pharmacologically active metabolites of posaconazole were formed in humans.

2. Experimental

2.1. Reagents

Methanol, acetonitrile, methylene chloride, hex-

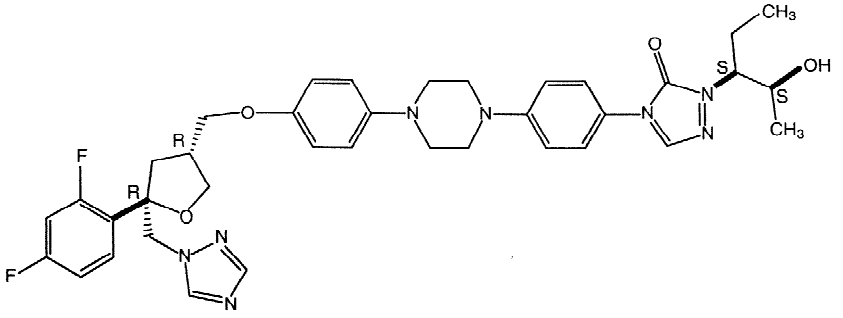

Fig. 1. Chemical structure of posaconazole.

ane, triethylamine, ammonium hydroxide and ammonium phosphate monobasic were purchased from Fisher Scientific (Fairlawn, NJ, USA). All reagents and solvents were analytical or HPLC grade. Posaconazole was provided by the Chemical Research Division, Schering-Plough Research Institute (Kenilworth, NJ, USA). The chemical purity of posaconazole was 98.6%. Cyclobenzaprine hydrochloride (internal standard) was purchased from Sigma (St. Louis, MO, USA). The stock solution of posaconazole was prepared in methanol at 1 mg/ml; the internal standard was prepared in water at 1 mg/ml. Stock solutions were stored at −20 °C.

2.2. Analysis of plasma samples by HPLC

Plasma samples were analyzed for posaconazole by HPLC using a previously developed and validated assay (unpublished data). The HPLC system consisted of a Waters model M-510 pump, a Zymark ZP 311-1 injector, and an Applied Bio-Systems 785A absorbance detector set at 262 nm. The separation was accomplished on a 5-μm Intersil ODS-2, 150× 4.6 mm column which was preceded by a R2, 10×2 mm guard column. The mobile phase consisted of 0.09 *M* ammonium phosphate buffer (pH 4.5)–acetonitrile–methylene chloride–triethylamine (1060:940:10:1, v/v), and was delivered at 1 ml/min. A 1-ml aliquot of human plasma was placed into a borosilicate 16×125 mm culture tube containing 200 μl of the internal standard solution. A 250-μl aliquot of 4.5 *M* ammonium hydroxide was added to the tube followed by 5 ml of hexane–methylene chloride (140:60, v/v). The tubes were shaken for 10 min and centrifuged for 5 min. The organic layer was evaporated to dryness at 40 °C under nitrogen and the residue was reconstituted in 300 μl of mobile phase. An aliquot of 50 μl was injected into the column. The retention times of the internal standard and posaconazole were approximately 4.1 and 10.9 min, respectively (Fig. 2). There were no endogenous peaks in control plasma that coeluted with posaconazole or the internal standard, indicating that the method was selective. The calibration curve was linear over a concentration range of 5–5000 ng/ml. The limit of quantitation was 5 ng/ml. Inter-day precision (RSD) and accuracy (bias) were in the ranges from 4.1 to 9.8% and from −3.2 to 3.4%, respectively; indicating that the method was satisfactory. The recovery (75–80%) was consistent over a concentration range of 5–5000 ng/ml. Posaconazole was stable in human plasma for at least 12 months at −20 °C. With each analytical run, duplicate quality control samples at seven different concentrations were analyzed, along with two standard curves prepared in plasma. The Millennium 2010 Chromatography Manager Software System was used for data acquisition and analysis.

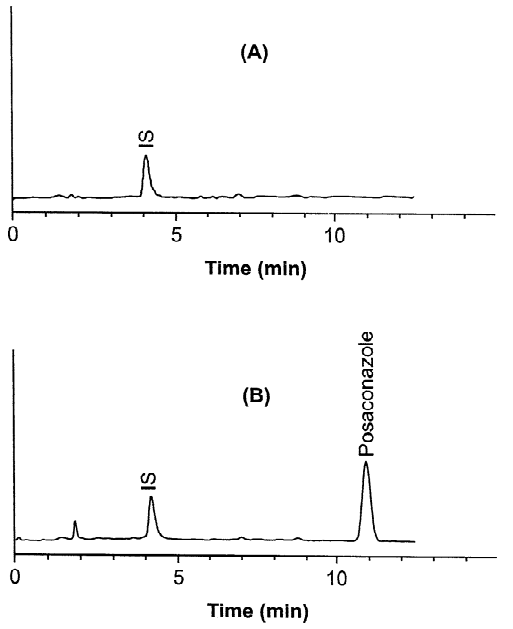

Fig. 2. Typical HPLC chromatograms of blank human plasma (A) and plasma from a subject at 317 h (B) following multiple dosing of posaconazole, both have been spiked with the internal standard (IS).

2.3. Analysis of plasma samples by microbiological assay

Posaconazole was analyzed by an agar diffusion assay utilizing *Candida pseudotropicalis* ATCC46764. Human plasma samples were plated undiluted in duplicate wells on three plates containing agar inoculated with *C. pseudotropicalis* ATCC 46764. These samples were incubated overnight (approximately 18–24 h) at 32–35 °C in a dry heat incubator. After incubation, the diameter of each zone of inhibition was measured and recorded. Each zone diameter was entered manually into a VAX 3100 computer programmed to perform the Code of

Federal Regulations (CFR 1990, 436.1059d) calculations. Results were reported as microbiological potencies in μg/ml based on a calibration curve prepared in plasma. The range of quantitation of microbiological activity was from 0.62 to 3.20 μg/ml. The limit of quantitation (LOQ) was 0.62 μg/ml and the limit of detection (LOD) was approximately 0.30 μg/ml.

2.4. Administration of posaconazole

Human plasma samples used were from a phase I multiple-rising dose safety and tolerance study. Eight healthy male and female volunteers received four tablets of posaconazole (100 mg/tablet) every 12 h for 14 consecutive days. Blood samples were collected at specified intervals following the morning and evening doses on day 14, in addition to earlier samples to determine plasma concentrations. Plasma samples were obtained by centrifugation of blood then stored at −20 °C pending analysis. These samples had been analyzed for posaconazole by HPLC prior to their use in this study.

2.5. Preparation of clinical samples and quality control samples

Two sets of samples from the same pools were prepared (one for HPLC and one for microbiological analysis). Because of volume limitation, samples with posaconazole concentrations >1.5 μg/ml were diluted with drug free pooled human plasma (2-fold dilution) prior to dividing them into the two sets. Quality control (QC) samples were prepared in human plasma at 0, 1, 2, 3, 4, 5, and 7 μg/ml. These samples were diluted 2-fold (with the exception of the 1 μg/ml) in order to match the dilutions made to the clinical study samples. One set of blinded samples (QC samples and clinical samples) were analyzed by HPLC and the other set of blinded samples were analyzed by the microbiological assay. Each day, two sets of QC samples at the seven concentrations including zero were analyzed along with the clinical samples.

3. Results and discussion

Microbiological assays are typically used to screen for potency of new chemical entities in drug discovery. Occasionally, they have been utilized to determine the concentrations of active components in plasma or serum of dosed animals and humans, especially in early drug discovery/development and in therapeutic situations. These assays, however, tend to be variable, of low sensitivity and also are influenced by media composition and test organisms which make them less suitable for rigorous pharmacokinetic evaluation [13,14]. Capitalizing on the fact that microbiological assays measure all activity in the sample, while specific HPLC assays measure the parent compound only, analyzing the same samples by both methods would be a fast and accurate way to assess the presence or absence of active metabolite(s) in animals and humans. If the concentrations determined by microbiological assay are higher than those determined by HPLC, that would indicate the presence of active metabolite(s). If they are similar, that would suggest the absence of significant amounts of active metabolite(s). Once an active metabolite is recognized, it needs to be identified, synthesized and its activity needs to be tested. In many cases, the active metabolite becomes the candidate for development instead of the parent compound.

In order to evaluate the presence or absence of active metabolite(s) of posaconazole in humans, plasma concentrations from the high-dose group (400 mg, twice a day) of a multiple-rising dose study were determined by both HPLC and microbiological assays. To account for any consistent bias in the data due to the nature of the microbiological assay, we prepared pools of quality control samples at seven concentrations (including zero) within the expected range. These pools were divided into small samples, coded (code unknown to analysts) and stored frozen with the samples from the subjects dosed with posaconazole. With each analytical run, either HPLC or microbiological assay, a set of identical QC samples at the seven different concentrations was analyzed. The concentrations of QC samples as determined by both HPLC and microbiological assays are presented in Table 1. The concentrations of the QC samples determined by the microbiological assay were consistently higher (bias: 16–28%) than those obtained by the HPLC assay and somewhat varied from day to day. The HPLC method demonstrated the expected precision and accuracy with an

Table 1
Nominal and determined concentrations of quality control (QC) samples by HPLC and microbiological assay

Assays	Concentration found (μg/ml) (n=16)	Nominal concentration (μg/ml)					
		1	2	3	4	5	7
HPLC	Mean	1.06	2.26	3.24	4.48	5.46	7.85
	Precision (RSD, %)	4.72	5.55	6.44	4.76	4.63	4.21
	Accuracy (bias, %)	6.20	11.30	8.03	11.93	9.28	12.16
Microbiological	Mean	1.27	2.56	3.57	4.93	5.81	8.28
	Precision (RSD, %)	19.57	19.94	18.78	19.55	20.30	21.51
	Accuracy (bias, %)	26.70	27.80	18.87	23.40	16.10	18.23
Microbiological[a]	Mean	0.93	2.20	3.21	4.57	5.42	7.76
	Precision (RSD, %)	10.52	6.16	4.97	5.23	4.44	5.36
	Accuracy (bias, %)	−7.50	9.85	7.00	14.20	8.40	10.86

[a] Corrected data based on HPLC.

RSD range from 4.2 to 6.4% and a bias range from 6.2 to 12.2% (Table 1). However, the precision and accuracy of the microbiological assay were 18.8–21.5 and 16.1–27.8%, respectively (Table 1). Since the bias was obvious, it was determined that the bias must have been carried out to the samples from the subjects. Therefore, for each subject, we used the results of the QC samples that were analyzed with this subject in the same analytical run to correct for the bias in the microbiological data. This was done by linear regression between the QC data of the microbiological and HPLC assays and using the regression parameters to correct the microbiological data from each subject. After adjustment, the microbiological QC data demonstrated satisfactory precision and accuracy as shown by a RSD range from 4.4 to 10.5% and a bias range from −7.5 to 10.9%.

Mean plasma concentration–time profiles (144–408 h following initiation of dosing) of posaconazole

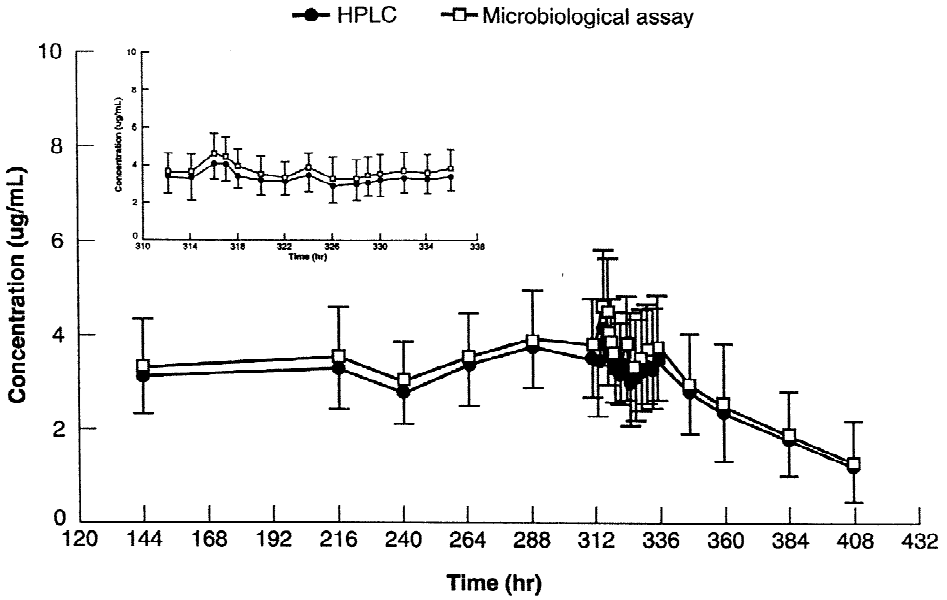

Fig. 3. Mean (n=8) plasma concentration–time profiles of posaconazole by HPLC and microbiological assay in healthy volunteers following a 400-mg dose twice daily. Each vertical bar represents one standard deviation from the mean. The insert is the expansion of time points from 312 to 336 h.

Table 2
Comparison of AUC (144–408 h) values of the individual subjects by HPLC and microbiological assays

AUC and ratio	Subject								Mean	RSD (%)
	A	B	C	D	E	F	G	H		
AUC (HPLC)	423	1028	839	637	813	843	467	953	751	29.3
AUC (microbiological)	428	1164	849	658	872	967	477	968	798	32.1
% AUC ratio microbiological/HPLC·100	101	113	101	103	107	115	102	102	105.5	5.3

by HPLC and adjusted microbiological assays are shown in Fig. 3. Individual plasma concentration–time profiles were similar for both HPLC and microbiological assays (data not shown). As shown in Fig. 3, from 144 to 408 h after the initiation of dosing, the mean values of the microbiological assay were similar and gave a pattern similar to that obtained with the HPLC method. The standard deviation bars from the two assays at each time point were overlapping. The area under the plasma concentration–time curve was calculated for each subject using both the HPLC and the microbiological assay data (Table 2). The results show similar areas under the curves (AUC) for both assays as shown by a mean AUC ratios of 105.5%, indicating that there was no relevant active metabolite of posaconazole in human plasma.

Earlier, studies with [^{14}C]posaconazole in dogs and rats demonstrated that there were no major metabolites in either species. These results are consistent with those of the present study.

In conclusion, the results of the present study indicate that following administration of posaconazole in humans, no significant amounts of active metabolite(s) have been detected in plasma. Therefore, concentrations of posaconazole as determined by the described validated assay might reflect the true pharmacological activity of this new triazole antifungal agent.

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