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High-performance liquid chromatographic analysis of the anti-fungal agent SCH 56592 in dog serum

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Abstract

SCH 56592 is a novel triazole antifungal agent that is active both orally and intravenously. This compound is in phase II–III clinical trials for the treatment of systemic fungal infections. A high-performance liquid chromatographic (HPLC) method was developed for the analysis of SCH 56592 in serum of dogs, a species used for safety evaluation. The HPLC analysis involved protein precipitation with methanol followed by separation on a C_{18} column and quantitation by UV absorbance at 262 nm. The method was sensitive with a limit of quantification of 0.05 μg/ml in dog serum. The linearity was satisfactory as indicated by correlations of >0.996, in addition to visual examination of the calibration curves. The precision and accuracy were satisfactory as indicated by coefficients of variation (C.V.) ranging from 2.0 to 3.8%, and bias values ranging from −6.5 to 10%. Moreover, SCH 56592 was stable in dog serum after being subjected to three freeze–thaw cycles. The assay was shown to be sensitive, specific, accurate, precise, and reliable for use in pharmacokinetic or toxicokinetic studies. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: SCH 56592; Antifungal agent

1. Introduction

Fungal infections have substantially increased over the past two decades, and invasive forms are now an important cause of morbidity and mortality [1]. The rise in frequency of fungal infection has been due to an increasing number of immunosuppressed patients, for example individuals with hematological malignancies, organ and bone marrow transplants, and AIDS patients advancing to late-stage AIDS. Although amphotericin B is still used to treat these infections, a number of new systemic antifungal agents have been discovered during the past two decades. The azole antifungal agents are far less toxic than amphotericin B [1], and can usually be given by more than one route of administration.

SCH 56592, 4-[4-[4-[4-[[5-(2,4-difluorophenyl)-tetrahydro-5-(1H-1,2,4-triazol-1-ylmethyl)-3-furanyl]methoxy]phenyl-1-piperazinyl]phenyl]-2-(1-ethyl-2-hydroxypropyl]-2,4-dihydro-3H-1,2,4-triazol-3-one (Fig. 1), is a novel triazole antifungal agent that is active both orally and intravenously [2,3]. The in vitro and in vivo profiles of SCH 56592 in animal models showed significant advantages over existing agents in terms of potency and spectrum [3–5]. SCH 56592 was more active than itraconazole

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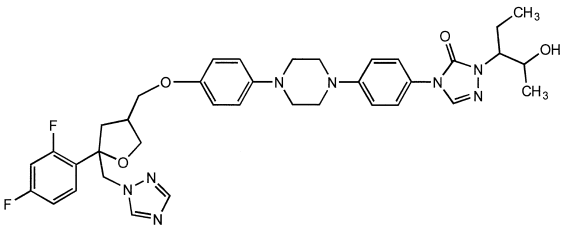
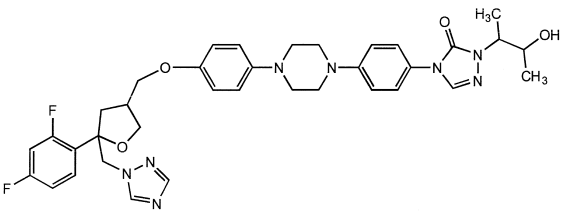


SCH 56592



SCH 56984 (Internal standard)

Fig. 1. Chemical structures of SCH 56592 and SCH 56984 (internal standard).

(ITZ) and fluconazole (FLZ) against all 283 strains tested and more active than amphotericin B (AMB) against 95% of these strains [3]. SCH 56592 was fungicidal against all strains of *C. krusei* that are resistant to FLZ [3]. SCH 56592 also was 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 infection animal models have shown that SCH 56592 appears to be more potent (lower minimal inhibitory concentrations and minimal fungicidal concentrations) than FLZ and ITZ against *Candida albicans, Aspergillus flavus* and *A. fumigatus* [7,8]. SCH 56592 was also very active against vaginal infections in hamsters when given orally or topically, and against *T. mentagrophytes* infections in guinea pigs when given orally or topically [9].

SCH 56592 is a hydroxylated derivative of SCH 51048, the previous lead compound in this series [10]. In an effort to further improve the oral absorption properties of SCH 51048, synthesis of its novel analogs having polar side chains was undertaken. While introduction of relatively basic or acidic side chains resulted in loss of activity, incorporation of hydroxyl groups led to greatly improved in vivo activity [11]. SCH 56592 was the lead compound that emerged from these efforts.

HPLC methods with ultraviolet detection have been widely used to determine the plasma and serum concentrations of azole antifungal agents such as FLZ [12], ITZ [13], and miconazole [14]. We report here on the development and validation of sensitive, precise and accurate HPLC method for the analysis of SCH 56592 in dog serum. The method was used to evaluate the pharmacokinetics of this drug candidate in dogs

2. Experimental

2.1. Reagents

Methanol, acetonitrile, methylene chloride, diethyl amine and ammonium phosphate monobasic were purchased from Fisher Scientific (Fair Lawn, NJ, USA). SCH 56592 and SCH 56984 (internal standard) were provided by the Chemical Research Division, Schering-Plough Research Institute (Kenilworth, NJ, USA).

2.2. Calibration standard and sample preparation

A stock solution of SCH 56592 was prepared in methanol at 200 μg/ml; the internal standard was prepared in methanol at 0.4 μg/ml. The stock solutions were stored at −20°C. Eight calibration standards (at 0.05, 0.1, 0.5, 1, 2, 4, 8, and 20 μg/ml) were prepared in triplicate on each of the three validation days. Three sets of quality control samples (QC samples) at concentrations of 0.1 (low), 1.5 (medium) and 15 μg/ml (high) were prepared in bulk from separate weighings, aliquoted and stored at −20°C for use during the entire validation and sample analysis. A 0.6-ml volume of the internal standard solution in methanol was added to 0.2 ml of dog serum, vortexed for 30 s at high speed and centrifuged at 4500 *g* at room temperature for 5 min. A 0.05-ml sample of the supernatant was injected onto the HPLC column for SCH 56592 analysis.

2.3. Chromatographic conditions

The HPLC system consisted of a Shimadzu LC-9A pump and a Waters 486 absorbance detector set

at 262 nm. The separation was accomplished on a 5-μm Ultrasphere ODS, 150×4.6 mm column which was preceded by an on-line filter. The Millennium 2020 Chromatography Manager Software System was used for data handling. The mobile phase consisted of 0.09 *M* ammonium phosphate monobasic acetonitrile–triethylamine (530:470:0.5, v/v/v), and was delivered at 1 ml/min.

2.4. Administration of SCH 56592

Six male beagle dogs were dosed orally with a 200-mg experimental tablet of SCH 56592 (approximately 15 mg/kg) for pharmacokinetic evaluation. Blood samples were collected at intervals after dosing, centrifuged and serum stored at −20°C pending analysis.

2.5. Pharmacokinetic analysis

Serum concentrations equal to or above the lower limit of quantitation (LOQ, 0.05 μg/ml) were used for pharmacokinetic analysis using model-independent methods [15]. The maximum serum concentration (C_{max}) and time of maximum serum concentration (T_{max}) were the observed values. The area under the serum concentration–time curve from time zero to the time of the final measurable sample [AUC(tf)] was calculated using the linear trapezoidal method.

3. Results and discussion

Initially, the UV spectrum of SCH 56592 was determined which showed a maximum absorbance (λ_{max}) at 262 nm. Therefore, the analysis were carried out at 262 nm. The HPLC analysis by UV absorbance showed that the limit of quantitation (LOQ) was 0.05 μg/ml of serum, which provided adequate sensitivity to conduct pharmacokinetic studies in animals. Typical chromatograms of drug-free dog serum and serum spiked with SCH 56592 and the internal standard (SCH 56984) are illustrated in Fig. 2. The retention times of the internal standard and SCH 56592 were approximately 6.7 and 8.7 min, respectively. There were no endogenous peaks in serum of six undosed dogs that coeluted with SCH

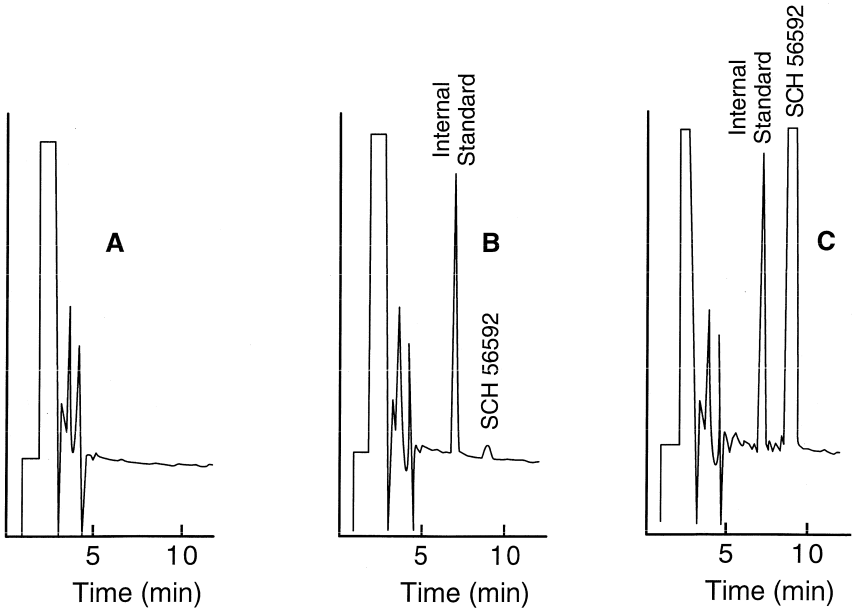


Fig. 2. Typical chromatograms of blank dog serum (A) and blank dog serum spiked with both SCH 56592 at the LOQ (B) or at 20 μg/ml (C) and the internal standard.

56592 or the internal standard, indicating that the method was selective. The linearity was evaluated over a concentration range of 0.05–20 μg/ml. Linear regression parameters of the peak height ratios versus concentrations along with back-calculated concentrations of nine calibration curves are presented in Table 1. The results showed highly reproducible calibration curves with correlation coefficients of >0.99, indicating that the response was linear over the concentration range studied. Intra-day precision and accuracy were evaluated at SCH 56592 concentrations of 0.1, 1.5 and 15 μg/ml. Five samples were analyzed at each concentration on the same day. The results showed satisfactory intra-day precision and accuracy as indicated by coefficients of variation (C.V.) of ≤4.0% and bias of ≤6.3% (Table 2). Inter-day precision and accuracy were evaluated at the same concentrations as above and the samples were analyzed on three separate days. The results demonstrated satisfactory inter-day precision and accuracy as shown by C.V. and bias values of ≤5.0% and ≤5.3%, respectively (Table 3). The LOQ, defined as the lowest concentration in the calibration curve that could be determined with acceptable precision and accuracy, was 0.05 μg/ml. At this concentration, the precision and accuracy from back-calculated concentrations were satisfactory (C.V.= 2.1%, bias=6.0%; Table 1). The recovery was consistent over a concentration range of 0.1–15 μg/ml (>73%; Table 4). The recovery of the internal

Table 2
Intra-day precision and accuracy for the analysis of SCH 56592 in dog serum

Nominal concentration (μg/ml)	Concentration found (mean) (μg/ml)[a]	Precision (% C.V.)	Accuracy (% bias)
0.1	0.10	4.0	0.0
1.5	1.48	2.0	−1.3
15.0	14.1	1.7	−6.3

[a] $n=5$.

Table 3
Inter-day and accuracy for the analysis of SCH 56592 in dog serum

Nominal concentration (μg/ml)	Concentration found (mean) (μg/ml)[a]	Precision (% C.V.)	Accuracy (% bias)
0.1	0.10	5.0	0.0
1.5	1.47	2.5	−2.0
15.0	14.2	3.7	−5.3

[a] $n=5$.

Table 1
Back-calculated concentrations and calibration curve parameters for the analysis of SCH 56592 in dog serum

	Nominal concentration								Slope	Intercept	Correlation coefficient (r)
	0.05	0.1	0.5	1	2	4	8	20			
	Concentration found (μg/ml)										
Day 1	0.046	0.11	0.55	1.04	2.10	3.89	7.61	18.30	0.745371	0.011543	0.9967
	0.046	0.11	0.55	1.07	2.02	4.03	7.50	18.30	0.745297	0.014585	0.9969
	0.046	0.11	0.55	1.04	2.05	4.11	7.54	18.00	0.743520	0.015915	0.9964
Day 2	0.048	0.11	0.52	0.95	1.98	4.27	7.97	19.30	0.710598	0.010672	0.9989
	0.048	0.10	0.54	0.96	2.00	4.02	7.81	19.50	0.708858	0.009744	0.9991
	0.048	0.11	0.52	1.01	1.98	4.15	7.83	18.90	0.718699	0.008615	0.999
Day 3	0.048	0.11	0.54	1.02	1.97	4.13	7.78	18.70	0.776689	0.009909	0.9985
	0.048	0.11	0.54	1.00	2.00	4.15	7.80	18.50	0.757875	0.012770	0.9984
	0.047	0.11	0.56	1.00	1.99	4.16	7.72	18.40	0.745573	0.012760	0.9977
Mean	0.047	0.11	0.54	1.01	2.01	4.10	7.73	18.70	0.739164	0.011835	0.998
Precision (%C.V.)	2.1	2.7	2.6	3.8	2.1	2.6	2.0	2.6	–[a]	–	–
Accuracy (% bias)	−6.0	10.0	8.0	1.0	0.5	2.5	−3.4	−6.5	–	–	–

[a] Not appropriate to calculate these parameters.

Table 4
Recovery of SCH 56592 and internal standard from dog serum

Concentration (μg/ml)	Mean % recovery[a]
0.1	76.6
1.5	74.5
15.0	73.2
Internal standard, 0.4 μg/ml	73.3

[a] $n=3$.

standard at the concentration used (0.4 μg/ml) was 73.3%.

In the method development phase, we have attempted to use acetonitrile to precipitate serum protein. The chromatogram showed that the SCH 56592 peak was asymmetric (tailing). Also, the assay was developed using 0.2 ml serum, three volumes of methanol were added to give a total volume of the extracted supernatant of approximately 0.7 ml, of which only 0.05 ml was injected. Therefore, the sample volume may be reduced to 0.05 ml serum which would result in approximately 0.15 ml volume of the supernatant, sufficient for two injections in HPLC. Consequently, if the sample volume is a limitation, the analysis can be performed with only 0.05 ml without any loss of sensitivity. However, we used 0.2 ml volume for convenience.

The stability of SCH 56592 in serum was evaluated through three freeze–thaw cycles at concentrations of 0.1, 1.5 and 15 μg/ml. The samples were thawed in a water bath at room temperature and frozen within 5 min of thawing in each cycle. After three cycles, the changes from nominal concentrations were −10.0, −1.3 and −6.0% for the 0.1, 1.5 and 15 μg/ml, respectively, demonstrating that SCH 56592 was stable in serum through three freeze–thaw cycles (Table 5). Long-term stability was determined following 348 days of storage at −20°C at the concentrations above. The changes from the nominal concentrations ranged from 6 to 12% indicating that SCH 56592 was stable in serum for at least 348 days (Table 6). In-process stability was performed for up to 48 h after sample processing. Three sets of processed serum samples at the above SCH 56592 concentrations were analyzed after storage at room temperature for 48 h. The changes from nominal concentrations were 0.0, 0.0 and −2.7% for the 0.1, 1.5 and 15 μg/ml concentrations, respectively, demonstrating that SCH 56592 was stable under the conditions evaluated.

Table 5
Three-cycle freeze–thaw stability of SCH 56592 in dog serum

Freeze–thaw cycle	Parameter	Observed concentration (μg/ml)[a]		
		0.1[b]	1.5[b]	15[b]
First	Mean	0.11	1.56	14.8
	% C.V.	6	4	1
	% Change	10	4	−1.3
Second	Mean	0.11	1.57	15.3
	% C.V.	11	4	4
	% Change	10	4.7	2
Third	Mean	0.09	1.48	14.1
	% C.V.	16	5	3
	% Change	−10	−1.3	−6

[a] $n=3$.
[b] Nominal concentration.

The analytical method was used to characterize the pharmacokinetic profile of SCH 56592 in the dog following oral administration of a 200-mg experimental tablet of SCH 56592. SCH 56592 was quantifiable in serum between 2 and 72 h after oral administration (Fig. 3). The mean C_{max} of SCH 56592 was 1.5 μg/ml which was attained at a mean T_{max} of 24 h. The area under the serum concentration–time curve ($AUC_{0-72\ h}$) was 60.4 μg.h/ml (Table 7).

In conclusion, an HPLC assay for the determination of SCH 56592 in dog serum was developed and was shown to be accurate and reliable over a concentration range of 0.05–20 μg/ml with an average C.V. of 2.6% and an average bias of 4.7%. The method was used to determine serum concen-

Table 6
Stability of SCH 56592 in dog serum following storage at −20°C for 348 days

Nominal concentration (μg/ml)	Observed concentration (μg/ml)[a]		
	Mean	% C.V.	% Change
0.1	0.11	11.0	10.0
1.5	1.41	3.0	−6.0
15.0	13.2	0.5	−12.0

[a] $n=6$ for each concentration.

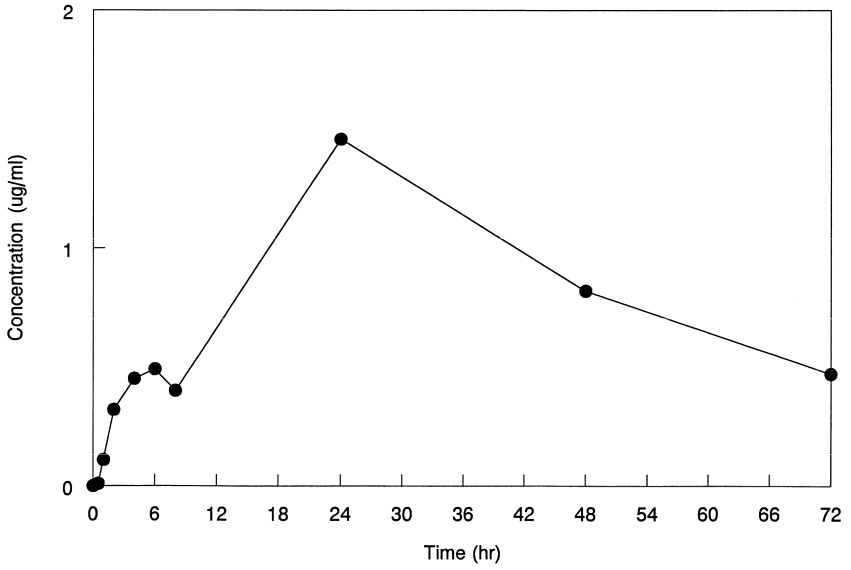


Fig. 3. Mean (n=6) serum concentration–time profile of SCH 56592 in dogs after 200-mg oral dose.

Table 7
Mean pharmacokinetic parameter of SCH 56592 in beagle dogs following oral administration of a 200-mg tablet

Parameter (unit)	Mean (n=6)	% C.V.
C_{max} (μg/ml)	1.5	48
T_{max} (h)	24	0
AUC (0–72 h) (μg/h/ml)	60.4	49

trations of SCH 56592 in the dog following oral administration of a 200-mg tablet.

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