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Simultaneous high-performance liquid chromatographic determination of SCH 59884 (phosphate ester prodrug of SCH 56592), SCH 207962 and SCH 56592 in dog plasma

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Abstract

SCH 59884 is an IV prodrug of SCH 56592, the broad-spectrum azole antifungal agent that is active both orally and intravenously in animal models of infection. SCH 56592 is in phase III clinical trials for the treatment of serious systemic fungal infections. SCH 59884 is a carboxylate ester of SCH 56592 with γ-butyric acid phosphate. Following IV administration of SCH 59884, the compound is rapidly dephosphorylated to SCH 207962 which is then hydrolyzed to SCH 56592. A high-performance liquid chromatographic (HPLC) method was developed for the simultaneous determination of SCH 59884, SCH 207962 and SCH 56592 in plasma 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 260 nm. The lower limits of quantification were 0.1 μg/ml for SCH 59884 and 0.05 μg/ml for SCH 207962 and SCH 56592 in dog plasma. The linearity for the three compounds was satisfactory as indicated by correlation coefficients (r) of >0.98, back-calculated concentrations and visual examination of the calibration curves. The precision and accuracy were satisfactory as shown by coefficients of variation (CV) ranging from 2.4 to 10.6%, and bias values ranging from -8.4 to 13.3%. Moreover, SCH 59884 and SCH 207962 were stable in dog plasma after being subjected to three freeze–thaw cycles. SCH 56592 had been shown earlier to be stable under these conditions. The assay was shown to be specific, accurate, precise, and reliable for use in pharmacokinetic and toxicokinetic studies.

Keywords: Azole antifungal agent; HPLC analysis; IV Prodrug; Pharmacokinetics

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 the frequency of fungal infection has been attributed to an increasing number of

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immunosuppressed 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-[[(2*R*-*cis*)-5-(2,4-difluorophenyl)tetrahydro-5-(1H-1,2,4-triazol-1-ylmethyl)-3-furanyl]methoxy]phenyl-1-piperazinyl]-phenyl]-2-[(*S*)-1-ethyl-2(*S*)-hydroxypropyl]-2,4-di-

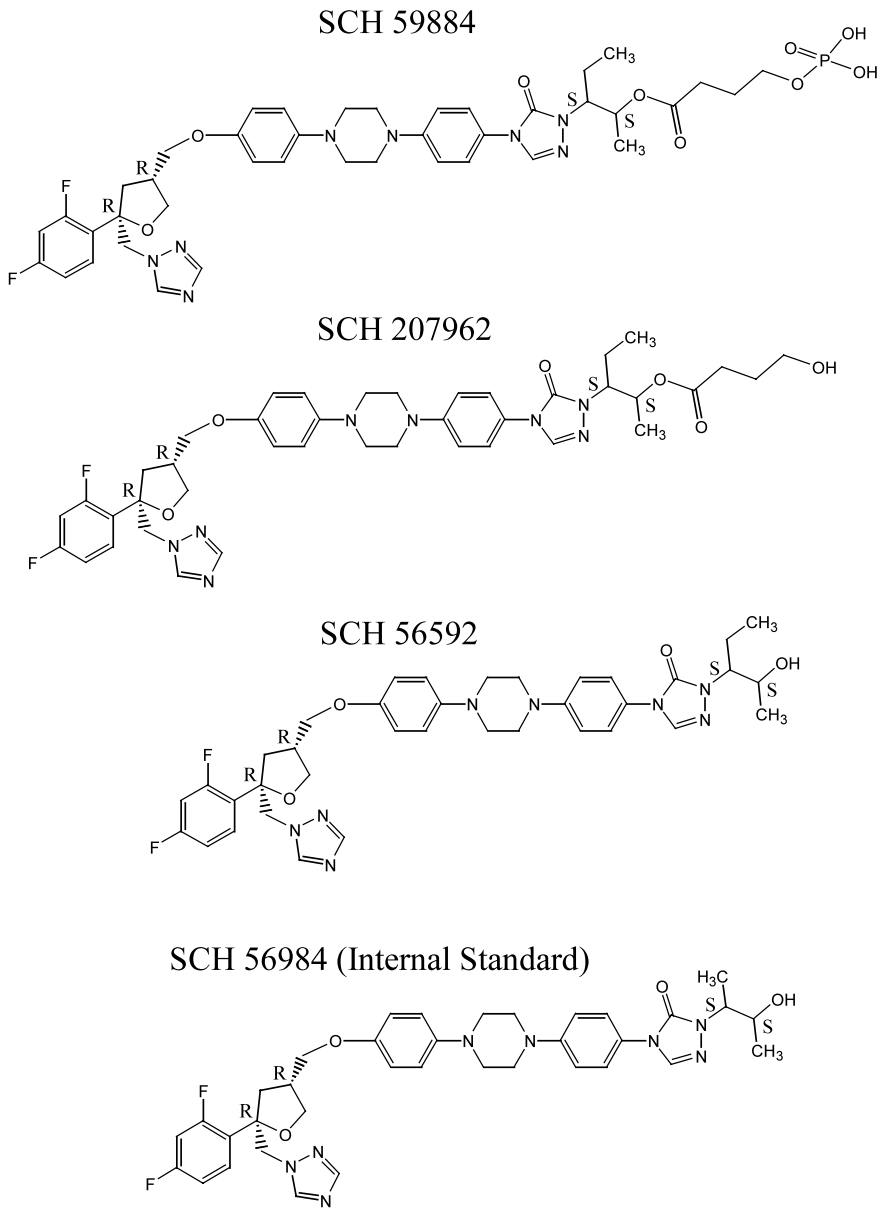

Fig. 1. Chemical structures of SCH 59884, SCH 207962, SCH 56592 and internal standard.

Table 1
Back-calculated concentrations and calibration curve parameters for the analysis of SCH 59884, SCH 56592 and SCH 207962 in dog plasma

Compounds		Nominal concentration (μg/ml)										Slope	Intercept	Correlation coefficient (r)
		0.05	0.1	0.2	0.5	1	2	5	10	20	50			
		Concentration found (μg/ml)[a]												
SCH 59884			0.11	0.20	0.43	1.03	2.04	4.95	10.57	20.86	48.62	0.29933	−0.005418	0.99429
			0.10	0.19	0.51	0.94	1.87	5.01	10.49	20.99	50.90	0.28666	−0.009550	0.99749
			0.10	0.21	0.50	0.96	1.89	4.91	10.64	20.48	50.31	0.28322	−0.009310	0.99676
			0.11	0.19	0.54	0.82	1.68	5.43	11.45	22.80	54.96	0.22591	−0.009080	0.98202
	Mean		0.11	0.20	0.50	0.94	1.87	5.08	10.80	21.28	51.20	0.27378	−0.008340	0.99264
	Precision (% CV)		6.2	4.3	2.4	9.1	7.7	4.7	4.3	4.9	5.3	–[b]	–	–
	Accuracy (% bias)		10.0	0.0	0.0	−6.1	−6.6	1.5	8.0	6.4	2.4	–	–	–
SCH 56592		0.050	0.10	0.21	0.55	0.86	2.22	4.23	10.96	20.88	53.86	0.41796	−0.009255	0.99021
		0.055	0.09	0.18	0.49	0.97	1.92	5.27	11.35	22.30	53.80	0.40462	−0.004960	0.99045
		0.053	0.10	0.17	0.49	1.03	1.97	5.16	11.18	20.91	51.54	0.41519	−0.003770	0.99394
		0.052	0.10	0.17	0.48	0.96	1.72	5.38	11.85	23.42	57.50	0.3257	−0.004470	0.98656
	Mean	0.053	0.10	0.18	0.50	0.96	1.96	5.01	11.34	21.88	54.18	0.39087	−0.005614	0.99029
	Precision (% CV)	4.0	6.2	8.5	5.8	7.5	10.6	10.5	3.4	5.6	4.6	–	–	–
	Accuracy (% bias)	5.0	0.0	−8.4	0.0	−4.4	−2.2	0.2	13.3	9.4	8.4	–	–	–
SCH 207962		0.051	0.10	0.20	0.48	1.04	2.06	4.91	10.15	19.50	51.29	0.37856	−0.004422	0.99910
		0.056	0.09	0.18	0.52	1.00	1.98	5.33	10.75	21.08	52.23	0.37528	−0.005100	0.99134
		0.054	0.09	0.18	0.49	1.01	2.00	5.19	10.94	21.25	50.92	0.37893	−0.004410	0.99973
		0.052	0.10	0.17	0.52	0.91	1.72	5.54	11.79	23.02	55.55	0.29143	−0.002220	0.98584
	Mean	0.053	0.10	0.18	0.50	0.99	1.94	5.24	10.91	21.21	52.50	0.35605	−0.004038	0.99400
	Precision (% CV)	4.2	6.2	7.8	4.0	5.5	7.7	5.1	6.2	6.8	4.0	–	–	–
	Accuracy (% bias)	6.5	0.0	−8.1	0.0	−1.2	−3.0	4.8	9.1	6.1	5.0	–	–	–

[a] $n = 2$ per day, for four separate days.
[b] Not appropriate to calculate for these parameters.

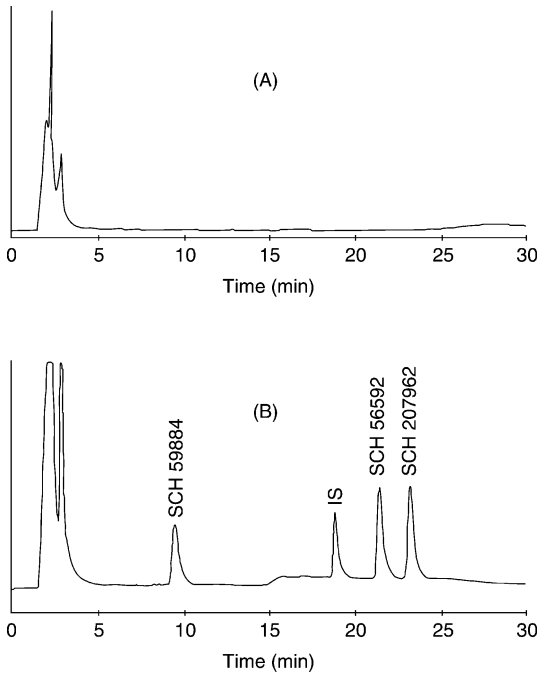

Fig. 2. Typical chromatograms of (A) blank dog plasma and (B) blank dog plasma spiked with 5 μg/ml of each of SCH 59884, SCH 207962, SCH 56592 and the internal standard (IS).

hydro-3H-1,2,4-triazol-3-one (Fig. 1), is a novel triazole antifungal agent that is active both orally and intravenously in animal models [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 [3–6]. SCH 56592 was more active than itraconazole (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 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 [5,7].

Since a large proportion of patients who develop serious systemic infections may need IV therapy, an intravenous formulation is necessary. SCH 56592 is highly bioavailable via oral administration in animals [8] but has poor aqueous solubility. Over 50 highly water-soluble prodrugs derived from amino acid esters, heterocyclic esters, phosphate-containing esters and polyether esters were synthesized and evaluated in detail [9]. SCH 59884 (Fig. 1) emerged as the lead compound from these studies and therefore was considered as an IV prodrug of SCH 56592. This compound is a carboxylate ester of SCH 56592 with γ-butyric acid phosphate [8,9]. SCH 59884 is inactive in vitro. However, SCH 59884 is dephosphorylated in vivo to the intermediate carboxylate ester SCH 207962, which is further hydrolyzed to SCH 56592. SCH 207962 and SCH 56592 are both active in vitro against 30 strains of *Aspergillus* and 109 strains of *Candida* [10].

Table 2
Intra-day precision and accuracy for the analysis of SCH 59884, SCH 56592 and SCH 207962 in dog plasma[a]

Compounds	Nominal concentration (μg/ml)	Concentration found (μg/ml)	Precision (% CV)	Accuracy (% bias)
SCH 59884	0.25	0.23	9.5	−8.0
	2.50	2.42	1.9	−3.2
	25.0	23.81	2.3	−4.8
	40.0	38.14	2.8	−4.7
SCH 56592	0.25	0.24	7.3	−4.0
	2.50	2.42	3.7	−3.2
	25.0	25.68	2.3	2.7
	40.0	39.84	1.7	−0.4
SCH 207962	0.25	0.24	7.1	−4.0
	2.50	2.38	2.7	−4.8
	25.0	23.49	3.1	−6.1
	40.0	36.10	5.1	−9.7

[a] All samples were analyzed on the same day, three replicate samples per day.

Table 3
Inter-day precision and accuracy for the analysis of SCH 59884, SCH 56592 and SCH 207962 in dog plasma[a]

Compounds	Nominal concentration (μg/ml)	Concentration found (μg/ml)	Precision (% CV)	Accuracy (% bias)
SCH 59884	0.25	0.24	7.8	−4.0
	2.50	2.40	1.4	−4.0
	25.0	25.48	4.4	1.9
	40.0	41.55	6.8	3.9
SCH 56592	0.25	0.23	4.2	−8.0
	2.50	2.43	7.0	−3.0
	25.0	24.88	13.2	−0.5
	40.0	42.83	7.2	7.1
SCH 207962	0.25	0.23	7.5	−8.0
	2.50	2.48	5.5	−1.0
	25.0	26.01	8.5	4.0
	40.0	41.32	11.4	3.3

[a] Samples were analyzed on four separate days, two replicate samples per day.

An analytical method for the simultaneous analysis of SCH 59884, SCH 207962 and SCH 56592 in various biological matrices was needed in order to evaluate the pharmacokinetics and toxicokinetics of this drug candidate in various species. This report describes an HPLC method developed for the simultaneous determination of SCH 59884, SCH 207962 and SCH 56592 in plasma of dogs, a species used for safety evaluation of this drug candidate.

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 59884, SCH 207962, 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

Stock solutions of SCH 56592, SCH 59884, and SCH 207962 were prepared in methanol at 200 μg/ml; the internal standard (SCH 56984) was prepared in methanol at 0.4 μg/ml. The stock solutions were stored at −20 °C. Ten calibration standards (at 0.05, 0.1, 0.2, 0.5, 1, 2, 5, 10, 20 and 50 μg/ml) were prepared in duplicate on each of four separate days for validation. Four sets of quality control samples (QC samples) at concentrations of 0.25, 2.5, 25 and 40 μg/ml were prepared in bulk from separate weighings, aliquotted and stored at −20 °C for use during the entire validation and sample analysis. A 0.2 ml aliquot of dog plasma standard, QC sample or unknown was placed into a 2.0 ml polypropylene microcentrifuge tube containing 0.1 ml of internal standard in methanol and 0.5 ml of methanol. The tubes

Table 4
Recovery of SCH 59884, SCH 56592, SCH 207962 and internal standard from dog plasma

Compounds	Concentration (μg/ml)	Mean % recovery[a]
SCH 59884	0.50	92
	2.50	88
	10.0	88
SCH 56592	0.50	82
	2.50	81
	10.0	92
SCH 207962	0.50	94
	2.50	92
	10.0	91
Internal standard	5.0	97

[a] $n = 3$.

Table 5
Three-cycle freeze–thaw stability of SCH 59884 and SCH 207962 in dog plasma

Compounds	Parameter	Nominal concentration (μg/ml)		
		0.50	2.50	40.0
		Observed concentration (μg/ml)[a]		
SCH 59884	Mean	0.49	2.46	40.58
	% CV	6.5	8.9	0.8
	% Change	−2.0	−1.6	1.5
SCH 207962	Mean	0.49	2.59	40.38
	% CV	7.1	5.6	1.0
	% Change	−2.0	3.6	1.0

[a] $n = 3$.

were vortexed for 5 min at high speed and centrifuged at $4500 \times g$ at room temperature for 5 min. The supernatant samples were transferred into microcentrifuge tubes and stored for a minimum of 12 h at −20 °C to complete the precipitation. The samples were vortexed for 1 min, and centrifuged for 5 min. A 0.2-ml sample of the supernatant was injected onto the HPLC column for sample analysis.

2.3. Chromatographic conditions

The HPLC system consisted of a Shimadzu LC-9A pump and a Waters 486 absorbance detector set at 260 nm. The separation was accomplished on an Ultrasphere ODS, 5 μm, 150 × 4.6 mm column which was preceded by an on-line filter using gradient elution with two solvent mixtures. Mixture A consisted of 0.1 M ammonium phosphate, acetonitrile, methylene chloride and triethylamine (650:350:5:0.5, v/v/v/v); and mixture B consisted of 0.1 M ammonium phosphate, acetonitrile, methylene chloride and triethylamine (500:500:5:0.5, v/v/v/v). The gradient profile was 100% A for 10 min, then a linear gradient to a 100% B by 12 min, remained at 100% B until 21 min from the initiation of the assay, followed by returning to 100% A by 23 min. The flow rate was 1 ml/min. The Waters LIMS Monitor (VT 340) Software System was used for data handling.

2.4. Administration of SCH 59884

Four male beagle dogs were administered SCH 59884 at an intravenous dose of 26.85 mg/kg (15 mg/kg SCH 56592 equivalent) for pharmacokinetic evaluation. Blood samples were collected at intervals after dosing, centrifuged and plasma stored at −20 °C pending analysis.

2.5. Pharmacokinetic analysis

Plasma concentrations equal to or above the lower limit of quantitation were used for pharmacokinetic analysis using model-independent methods [11]. The maximum plasma concentration (C_{max}) and time of maximum plasma concentration (T_{max}) were the observed values. The area under the plasma concentration–time curve from time zero to the time of the final measurable sample ($AUC_{0-48\ h}$) was calculated using the linear trapezoidal method.

3. Results and discussion

Typical chromatograms of drug-free dog plasma and plasma spiked with SCH 59884, SCH 207962, SCH 56592 and the internal standard (SCH 56984) are illustrated in Fig. 2. The retention times of SCH 59884, internal standard, SCH 56592 and SCH 207962 were approximately 9.3, 18.8, 21.5 and 23.3 min, respectively. There were no endogenous peaks in plasma of undosed dogs that coeluted with SCH 59884, SCH 207984, SCH 56592 or the internal standard, indicating that the method was selective. The linearity was evaluated over a concentration range of 0.1–50 μg/ml for SCH 59884, and 0.05–50 μg/ml for both SCH 207962 and SCH 56592. Linear regression parameters of the peak height ratios versus concentrations along with back-calculated concentrations are presented in Table 1. The results showed highly reproducible calibration curves with correlation coefficients of > 0.98, indicating that the response was linear over the concentration ranges studied. Intra-day precision and accuracy were evaluated at concentrations of 0.25, 2.5, 25 and 40 μg/ml. Three samples were analyzed at each con-

centration on the same day. The results showed satisfactory intra-day precision and accuracy as indicated by coefficients of variation (CV) of $\leq$ 9.5% and bias values of $\leq$ 9.7% (Table 2). Inter-day precision and accuracy were evaluated at the same concentrations as above and the samples were analyzed on four separate days. The results demonstrated satisfactory inter-day precision and accuracy as shown by CV and bias values of $\leq$ 13.2 and $\leq$ 8.0%, respectively (Table 3). The LOQ, defined as the lowest concentration in the calibration curve that could be determined with

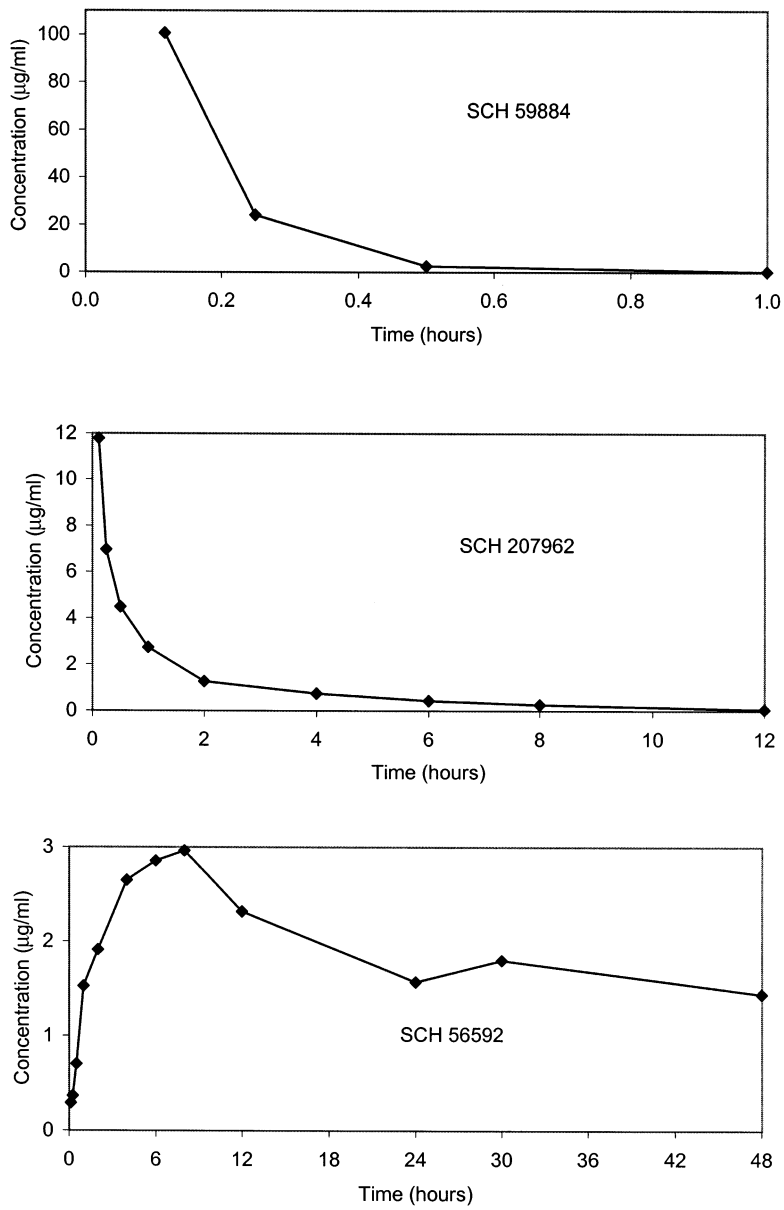

Fig. 3. Mean ($n = 4$) plasma concentration–time profiles of SCH 59884, SCH 207962 and SCH 56592 in dogs following intravenous administration of SCH 59884 at a dose of 15 mg/kg SCH 56592 equivalent.

acceptable precision and accuracy, was 0.1 μg/ml for SCH 59884 and 0.05 μg/ml for both SCH 207962 and SCH 56592. At this concentration, the precision and accuracy from the back-calculated concentrations were satisfactory (CV ≤ 6.2%, bias ≤ 6.5%; Table 1). The recovery values at a concentration range of 0.5–10 μg/ml for SCH 59884, SCH 56592 and SCH 207962 were ≥ 88, ≥ 81 and ≥ 91%, respectively (Table 4). The recovery of the internal standard at the concentration used (5 μg/ml) was 97%.

The stability of SCH 59884 and SCH 207962 in plasma was evaluated through three freeze–thaw cycles at concentrations of 0.5, 2.5 and 40 μg/ml. The samples were thawed in a water bath at room temperature and frozen within 5 min of thawing in each cycle. The results for the analysis of these samples are shown in Table 5. There were no significant changes in the concentrations demonstrating the stability of SCH 59884 and SCH 207962 through three freeze–thaw cycles. Similar results were reported earlier with SCH 56592 [12]. In-process stability was performed for up to 12 h after sample processing. Plasma samples at a concentration range of 0.25–40 μg/ml for SCH 59884 and SCH 207962 were prepared and stored at −20 °C. The samples were thawed, processed and injected immediately (0 h) and at 12 h after sample processing, during which time they were kept in the autosampler at 5 °C. After 12 h, the changes from 0-h values were < 3.4% for SCH 59884 and < 6.7% for SCH 207962, demonstrating that SCH 59884 and SCH 207962 were stable under the conditions evaluated.

The analytical method was used to characterize the pharmacokinetic profile of SCH 59884, SCH 207962 and SCH 56592 in the dog following intravenous administration of SCH 59884 at a 15 mg/kg dose (Fig. 3). The linear range of the calibration curve for the SCH 59884 concentration was extended for SCH 59884 to 0.1–200 μg/ml in order to accommodate the high initial concentrations observed in dog plasma following IV administration. The relationship between detector response and the concentration of SCH 59884 was linear and reproducible from 0.1 to 200 μg/ml of dog plasma. The plasma concentration–time profile showed a rapid disappearance of SCH 59884 ($t_{1/2} < 30$ min) and a rapid appearance of SCH 207962, while SCH 56592 profiles were similar to those observed following oral administration of SCH 56592. The mean C_{max} of SCH 56592 was 3.0 μg/ml which was attained at a mean T_{max} of 8 h. The area under the plasma concentration–time curve ($AUC_{0-48\ h}$) of SCH 56592 was 91.2 μg h/ml.

4. Conclusions

An HPLC assay for the simultaneous determination of SCH 59884, SCH 207962 and SCH 56592 in dog plasma was developed and was shown to be accurate and reliable over a concentration range of 0.1–200 μg/ml for SCH 59884 and 0.05–50 μg/ml for both SCH 207962 and SCH 56592. The method was used to determine plasma concentrations of SCH 59884, SCH 207962 and SCH 56592 in the dog following the intravenous administration of SCH 59884.

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