

Determination of a cyclic guanine monophosphate phosphodiesterase inhibitor (SCH 51866) in rat serum using capillary zone electrophoresis¹

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Abstract

A capillary zone electrophoretic (CZE) assay was developed for the determination of *cis*-5,6a,7,8,9,9a-hexahydro-2-[4-(trifluoromethyl)phenylmethyl]-5-methyl-cyclopent[4,5]imidazo[2,1-b]purin-4(3H)-one, SCH 51866 (I), a cyclic guanine monophosphate phosphodiesterase inhibitor, in rat serum using acetonitrile deproteination as a clean-up step before injection. The calibration curve was linear over a serum concentration range of 0.5 to 10 µg/ml serum with a correlation coefficient (*r*) > 0.99. The limit of quantitation (LOQ) was established at 0.5 µg/ml. Fifty microliters of serum were used for analysis, which allowed serial bleeding (8 samples) from a single rat to characterize the pharmacokinetic profile of I after either oral or intravenous drug administration. In traditional pharmacokinetic and toxicokinetic studies in rodents, one animal provides only one serum sample since 1 to 2 ml of sample volume is required for chromatographic analysis, resulting in the use of a large number of animals per study. This assay yields a significant reduction in the use of animals, hence providing a large reduction in resources and time in drug discovery and development.

1. Introduction

Capillary electrophoresis (CE) has become an important separation technique due to its high resolution power, minimum sample volume requirement, various modes of separation and speed of analysis [1,2]. Among the different modes of the technique, micellar electrokinetic capillary chromatography (MEKC) is commonly used for the separation of a variety of drug

classes including antibiotics [3,4], non-steroidal anti-inflammatory agents [5], steroids [6], and analgesics [7]. Capillary zone electrophoresis (CZE) has also been used for the separation of different drug classes [8–10]. CE has recently gained popularity as a method for the quantitation of drugs and metabolites in biological samples [11–13]. We were interested in developing CE assays for pharmacokinetic studies in animals and man, due to their advantages over conventional chromatographic techniques (e.g. HPLC or GC).

cis - 5,6a,7,8,9,9a - Hexahydro - 2 - [4 - (trifluoromethyl)phenylmethyl] - 5 - methyl - cyclopent[4,5]imidazo[2,1 - b]purin - 4(3H) - one,

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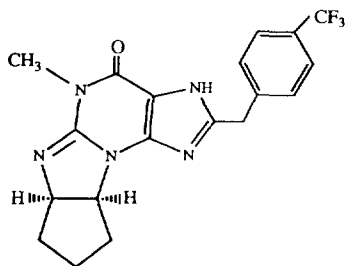


Fig. 1. Chemical structure of SCH 51866 (I).

SCH 51866 (I, Fig. 1), is a new compound under investigation for potential cardiovascular pharmaceutical use, due to its inhibitory activity against cyclic guanine monophosphate (cGMP) phosphodiesterase (PDE). A simple CZE assay was developed for the quantitation of I in rat serum and used in the determination of the pharmacokinetic profiles of I following either oral or intravenous administration to rats as part of the new drug discovery effort.

2. Experimental

2.1. Materials

Compound I was synthesized at the Schering-Plough Research Institute (Kenilworth, NJ, USA). Acetonitrile was of HPLC grade (Fisher Scientific Co., Fairlawn, NJ, USA). Water and 1 M sodium hydroxide solution of CE grade were purchased from Scientific Resources (Eatontown, NJ, USA). Sodium dihydrogen phosphate (NaH_2PO_4) and orthophosphoric acid (H_3PO_4) were purchased from Fluka Chemical Corp. (Ronkonkoma, NY, USA)

2.2. Capillary electrophoresis instrumentation and method

A Hewlett-Packard (HP) ^{3D}Capillary Electrophoresis System with diode-array detection was used. The electrophoretic separation was performed on a HP Extended Light Path (bubble) fused-silica capillary of 50 μm I.D. \times 64.5 cm (56 cm effective length). All buffers were filtered through a 0.45- μm Miller-HV filter (Millipore,

Bedford, MA, USA). Sample injection was performed under pressure (50 mbar) for 5 or 10 s and the voltage, temperature and current were continuously monitored during electrophoresis. The migration of I and other compounds was detected on-line near the negative electrode by monitoring UV absorbance at 210 and/or 280 nm. In the final optimized procedure, the applied voltage was 30 kV, injection time was 10 s, and the capillary temperature was controlled at 25°C. The standard running buffer was 10% acetonitrile in 100 mM phosphate buffer (PB), pH 2.5.

A new uncoated capillary was first prepared by rinsing the capillary with 1 M NaOH at 60°C for 12 min, followed by rinsing at room temperature with 0.1 M NaOH for 3 min, water for 5 min, and 50 mM PB, pH 6.0 for 5 min. A standardized "pre-conditioning" procedure was applied before each injection: rinsing the capillary for 1 min with 0.1 M NaOH, 1 min with water, 1 min with 0.1 M H_3PO_4 , and 3 min with running buffer.

2.3. Sample preparation

The stock solution was prepared by dissolving an accurately weighed amount of I (10 mg) into 50% ethanol–water (v/v) to yield a concentration of 1 mg/ml. Calibration standards were prepared by diluting the stock solution with drug-free (control) rat serum to give concentrations of 10, 5, 2.5, 1, 0.5 and 0 $\mu\text{g}/\text{ml}$. Quality control (QC) samples were similarly prepared in control rat serum at three concentrations of I (0, 2 and 8 $\mu\text{g}/\text{ml}$). The serum samples were stored at –20°C pending analysis.

To 50 μl of the respective serum samples (calibration standards, QC samples, or unknown samples) was added 200 μl of acetonitrile in an Eppendorf tube. The tube was capped, vortex-mixed for 2 min and centrifuged at 8000 g for 5 min at room temperature using an Eppendorf Centrifuge 5415C (Brinkman Instruments, Westbury, NY, USA). The supernatant was transferred into a Microfilterfuge tube fitted with a 0.45- μm Nylon-66 filter (Rainin Instrument Co., Woburn, MA, USA). The pellet was re-ex-

tracted with 200 μl of acetonitrile as described above, the supernatant combined in the Microfilter tube, and centrifuged for 2 min. The filtered supernatant was dried in vacuo using a Speed-Vac AS 290 (Savant Instruments, Farmingdale, NY, USA) for 2 h and the residue reconstituted in 25 μl of 50% acetonitrile–20 mM PB, pH 5.0. The reconstituted solution was transferred into a 100- μl polypropylene vial, capped with a silicone snap cap, and placed in the sample tray for injection onto the CE system.

3. Results and discussion

3.1. Method development

The sample matrix plays an important role in CE. For analytes present in low concentrations, serum ions and proteins may interfere with the separation. Acetonitrile deproteination, which has been used successfully in both HPLC and CE [14,15], was chosen as a simple clean-up step for serum samples. Since I is relatively hydrophobic, 10% acetonitrile was added to the electrophoretic running buffer to enhance its solubility.

The effects of pH, ionic strength, organic modifier, temperature, running voltage and injection time (i.e. injection volume) on the separation were investigated.

First, the effect of the running buffer pH was studied in the range of pH 2.5 to 8.5 in phosphate buffer (PB) at a fixed concentration of 50 mM using a spiked sample of I (10 $\mu\text{g}/\text{ml}$) in 50% ethanol–20 mM PB, pH 5.0. It was found that the higher the pH, the shorter was the migration time and the lower was the theoretical plate number hence separation efficiency (e.g. $\sim 60\,000$ at pH 6.5). At pH 8.5, I co-migrated with the ethanol in the sample as a broad peak. Optimal separation was achieved at pH 2.5 based on peak height, theoretical plate number ($\sim 200\,000$) and migration time (~ 10 min). Consequently, pH 2.5 was chosen as the running buffer pH; this would minimize the effect of protein–capillary wall interaction [16,17]. Once the pH of the running buffer was established, rat serum samples spiked with I at various concentrations were used for further method de-

velopment since the objective was to quantitate I in rat serum.

The effect of the ionic strength of the buffer was then examined from 50 to 200 mM PB at pH 2.5 with 10% acetonitrile. Since the ionic strength of serum is relatively high (e.g. NaCl is ~ 150 mM), a high ionic strength buffer is desired to achieve good separation. However, this may lead to high current production and column overheating [18,19]. At an applied voltage of 30 kV, buffers having concentrations greater than 100 mM resulted in currents ranging from 120 to 140 μA which may lead to column overheating and eventually loss of column efficiency. Therefore, a buffer concentration of 100 mM, resulting in a current of approximately 70 μA , was chosen to achieve satisfactory separation and minimize column heating.

Organic modifiers such as acetonitrile, methanol or dimethylsulfoxide (DMSO), are used in CE to improve analyte solubility and to inhibit wall adsorption [20,21]. The effect of 10 to 20% acetonitrile in the running buffer was studied to optimize the separation. Comparable electrophoretic parameters were obtained within this concentration range with respect to migration time, peak area and peak height, except that 10% acetonitrile yielded a higher plate number. Therefore, the optimized buffer composition was 10% acetonitrile in 100 mM PB, pH 2.5.

The applied voltage was selected at 30 kV, which is also the highest achievable voltage with the HP $^{3\text{D}}$ CE System used, to yield rapid separation and high resolution. The temperature of 25°C (ambient) was chosen based on the potential instability of I at higher temperatures. The sample injection time was kept at 5 s at 50 mbar pressure for all the experiments described above, corresponding to a sample injection volume of ~ 4 nl. The effect of injection volume on the separation efficiency was studied by varying the injection time from 5 to 20 s. An increase in peak height was seen when the injection time was increased from 5 to 10 s, but no further improvement occurred when longer injection times were used. As the injection time (i.e. volume) increased, the peak width also increased, leading to a decrease in column efficiency (i.e. plate number). Therefore, an injec-

tion time of 10 s was chosen which corresponded to a sample injection volume of ~ 11 nl in order to provide optimum sensitivity and minimum loss of resolution.

The final optimized operation conditions for the separation of (I) in rat serum were as

follows: $T = 25^\circ\text{C}$; $V = 30$ kV; injection time 10 s; running buffer: 10% acetonitrile in 100 mM phosphate buffer, pH 2.5. A typical electropherogram of a rat serum sample spiked with I ($5 \mu\text{g}/\text{ml}$) and a diode-array UV spectrum of I are shown in Fig. 2. The migration time for I was

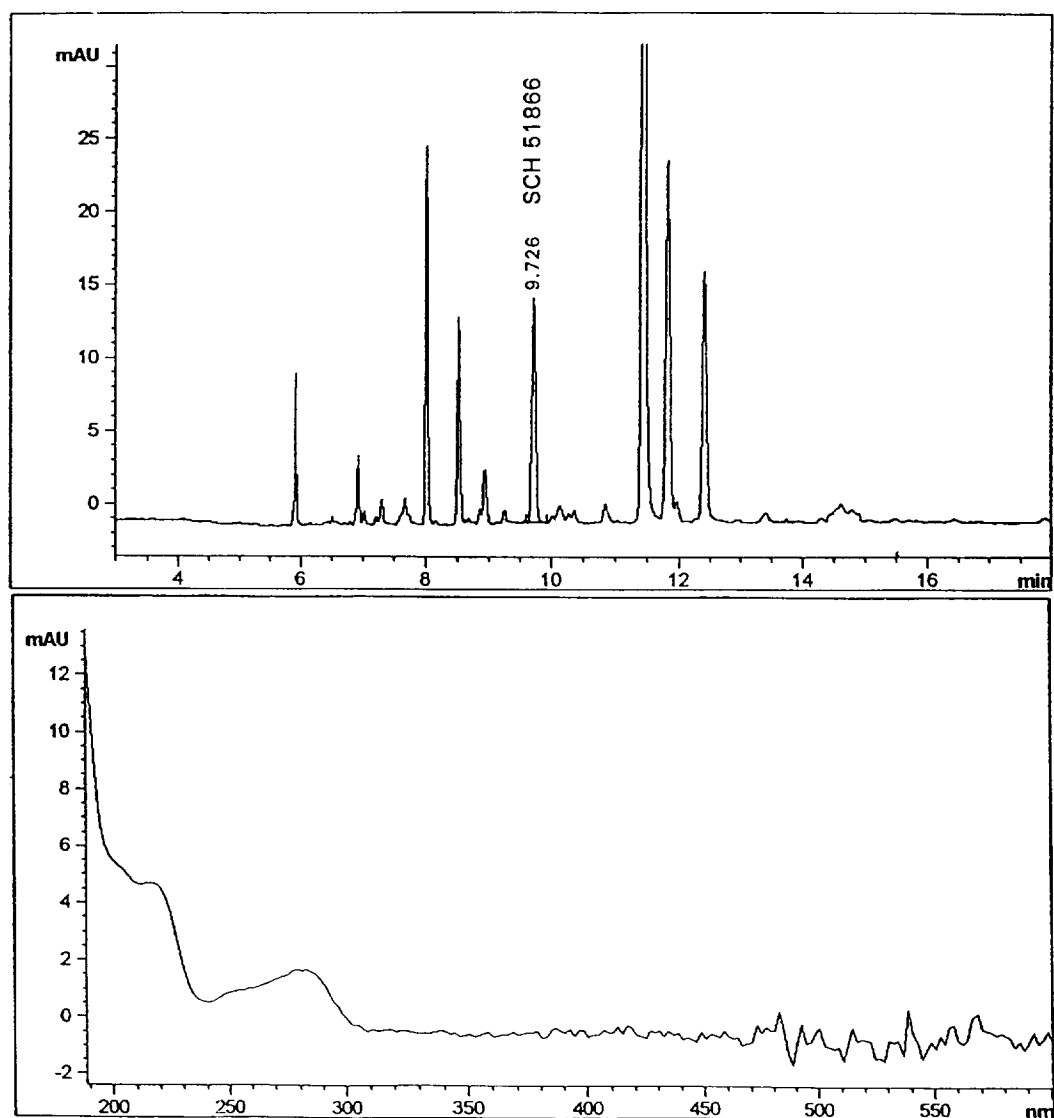


Fig. 2. Electropherogram of spiked rat serum sample containing $5 \mu\text{g}$ I/ml and the diode-array UV spectrum of I. Conditions: HP Extended Light Path (bubble) fused-silica capillary $50 \mu\text{m}$ I.D. $\times 64$ cm ($l = 56.5$ cm); 30 kV, 25°C , injection time 10 s, detection at 210 nm; running buffer 10% acetonitrile in 100 mM PB, pH 2.5.

reproducible as indicated by a coefficient of variation (%C.V.) of 1.9 ($n = 5$).

3.2. Calibration curve and limit of quantitation (LOQ)

The calibration curve for I in rat serum ($y = 11.223x - 1.41$) was established over a concentration range of 0.5 to 10 $\mu\text{g/ml}$ with good linearity ($r > 0.99$). The back-calculated standard concentrations also accurately matched the spiked (theoretical) values with a bias of $\pm 3.2\%$ or less (Table 1). The LOQ of the assay was established at 0.5 $\mu\text{g/ml}$. A typical electropherogram of spiked serum sample containing 0.5 μg of I/ml compared to that of a control rat serum sample is shown in Fig. 3, and indicates baseline separation of I at the LOQ from other serum components.

3.3. Determination of I in rat serum after either oral or intravenous administration

The utility of the assay in preclinical pharmacokinetic studies of I was demonstrated in a pilot study in which rats were dosed orally (10 mg/kg) or intravenously (3 mg/kg) and blood samples were collected from each animal at 1, 5, 15, 30, 60, 120, 180 and 240 min post-dose. A poly-

ethylene 50 (PE₅₀) catheter was inserted into the aorta of the rat, which enabled serial blood sampling of $\sim 150 \mu\text{l}$ blood per time point from a single rat to provide multiple samples since the sample volume required for analysis was only 50 μl . Serum samples were analyzed for I after acetonitrile deproteination and I was clearly separated from other serum components (Fig. 4). The concentration–time plots after oral or intravenous administration (Fig. 5) clearly demonstrate the suitability of the assay for pharmacokinetic studies in the rat. In conventional pharmacokinetic studies in rodents, one animal provides only one sample since 1 to 2 ml of sample volume is required for analysis, resulting in the use of a large number of animals. Thus this assay may have great significance in reducing animal numbers for pharmacokinetic studies, hence saving resources and time for pharmaceutical research and development.

4. Conclusions

The use of CZE was examined for the determination of I in rat serum. Parameters considered in the development of this assay included: pH and concentration of the running buffer, applied voltage, organic modifier, temperature,

Table 1
SCH 51866 calibration curve summary

Calibration curve No.	SCH 51866 ($\mu\text{g/ml}$)					Parameters	
	0.5 ^a	1.0	2.5	5.0	10.0	Slope	r
1	0.520	0.924	2.49	5.06	9.74	9.53	0.999
2	0.502	1.05	2.45	5.07	8.91	10.8	0.998
3	0.500	0.900	2.46	5.05	10.1	11.2	1.00
4	0.488	1.02	2.28	5.05	10.2	13.2	0.999
Mean	0.502	0.972	2.42	5.06	9.74	11.2	1.00
C.V. (%)	2.63	7.38	4.01	0.157	6.05	13.5	0.066
Bias (%)	0.480	-2.76	-3.18	1.16	-2.61		

^a Spiked (theoretical) concentration.

Bias (%) = $(\text{mean} - \text{theoretical}) / (\text{theoretical}) \cdot 100$. C.V. and Bias were based on non-truncated mean values.

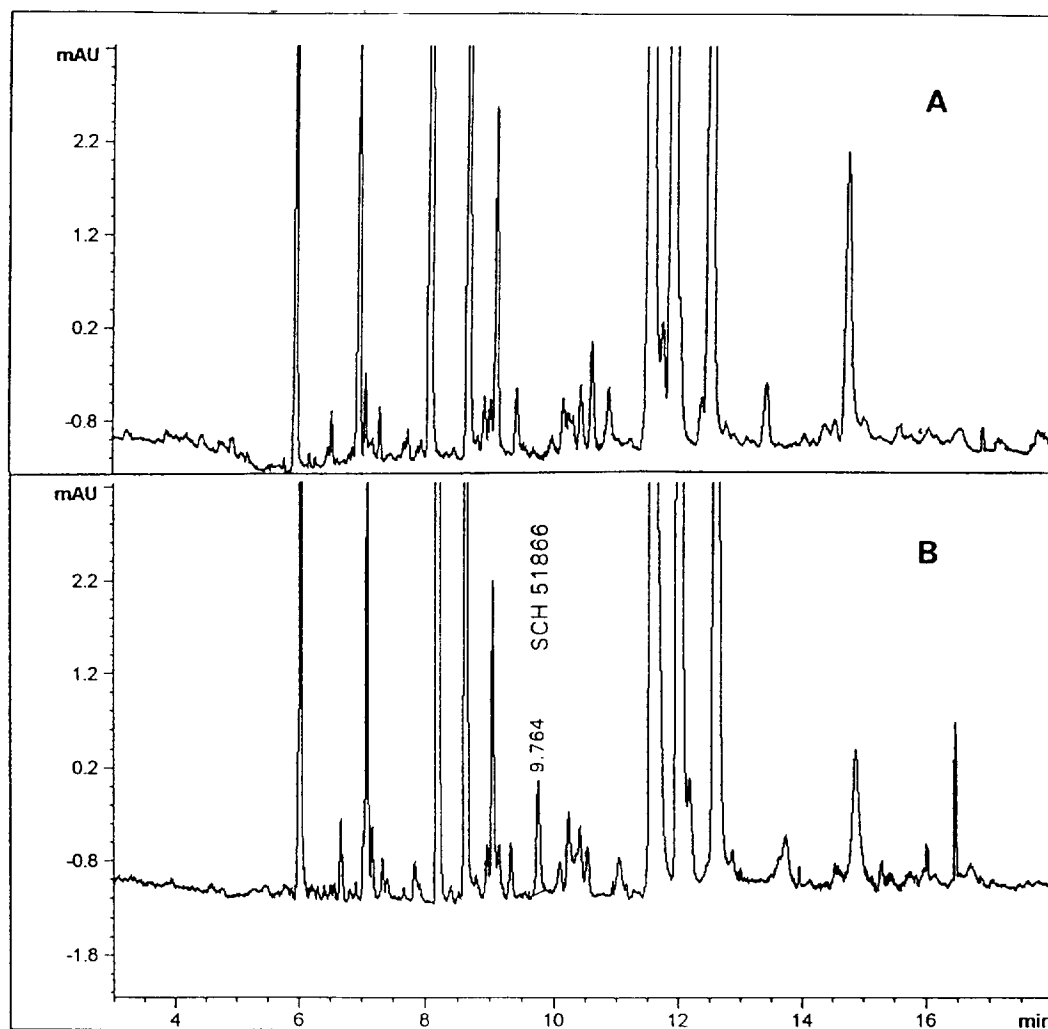


Fig. 3. Electropherograms of (A) a blank rat serum sample and (B) a spiked rat serum sample containing $0.5 \mu\text{g I/ml}$. Conditions are the same as in Fig. 2.

and sample injection time (volume). The final optimized system yielded baseline separation between I and endogenous components in serum and resulted in a total run time of 20 min. The method required only a simple acetonitrile de-proteinization step for sample preparation/clean-up and required only $50 \mu\text{l}$ of serum sample for analysis. The linear calibration curve range and the LOQ of the assay were suitable for the

determination of I in rats post oral (10 mg/kg) or intravenous (3 mg/kg) doses. Therefore, the assay was deemed suitable for pharmacokinetic studies of I in the rat. Furthermore, serial bleeding/sampling ($150 \mu\text{l}$) of the rats demonstrated significant advantages in reducing the use of the large number of rodents usually needed for pharmacokinetic studies, resulting in savings of animal resources and time.

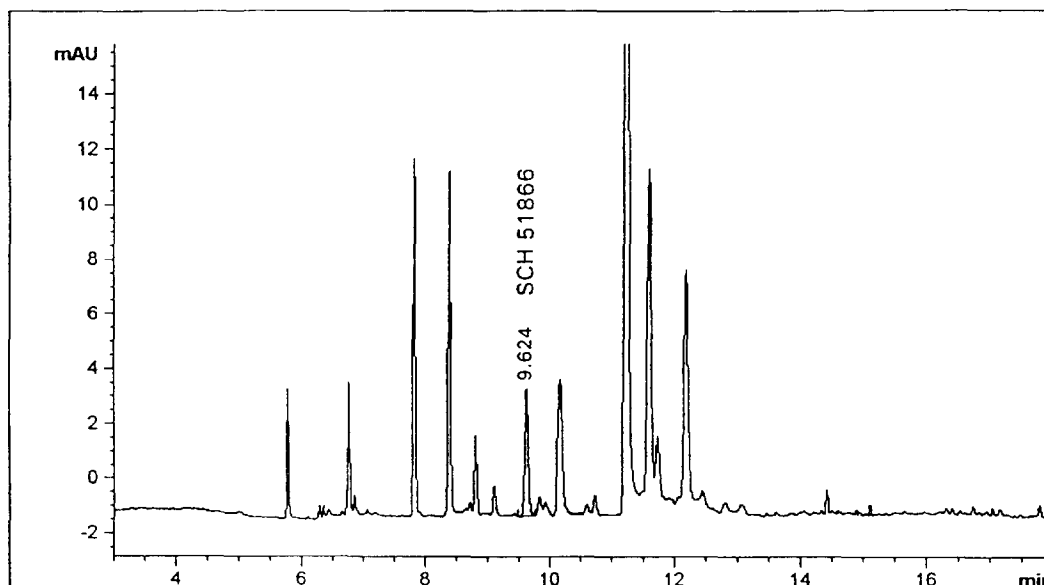


Fig. 4. Electropherogram of a 2-h serum sample from a rat dosed orally with I (10 mg/kg). Conditions are the same as in Fig. 2.

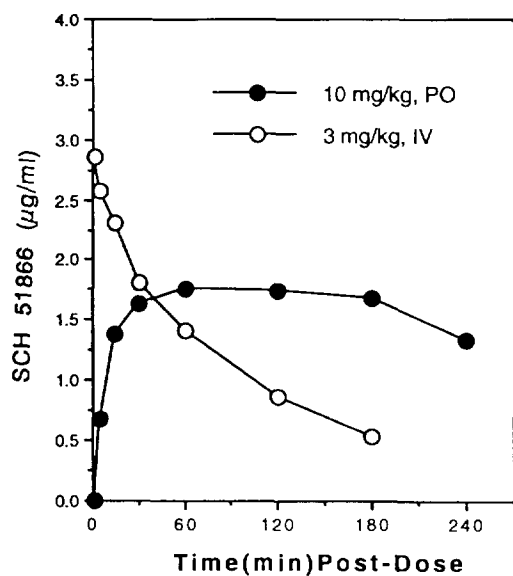


Fig. 5. Concentration–time plot of I in serum. Blood samples were collected using serial bleeding procedure from a single rat either dosed orally (10 mg/kg) or intravenously (3 mg/kg).

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