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Determination of minalrestat (an aldose reductase inhibitor) in rat, dog and human plasma by high-performance liquid chromatography

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Abstract

A high-performance liquid chromatographic (HPLC) method was developed for the quantification of minalrestat (a potent aldose reductase inhibitor) in rat, dog and human plasma. Minalrestat and internal standard (I.S.) were extracted from plasma by either solid-phase extraction (SPE) or liquid–liquid extraction (human plasma). Plasma extracts were chromatographed on a Hypersil ODS column with 3-μm packing with a mobile phase of acetonitrile–0.05 *M* potassium phosphate buffer, pH 3.0 (45:55, v/v) at 0.2 ml/min. The signal in the eluent was enhanced by UV-irradiation when passing through a photochemical reaction unit with a 10-m reaction coil, prior to detection by UV absorbance at 255 nm. The intra-day coefficients of variation was less than 9% in rat, dog and human plasma and the intra-day accuracy (%MRE) was within ±5% in all matrices tested. The inter-day coefficients of variation were less than 12% in rat and human plasma and the accuracy (%MRE) was within ±15%. Minalrestat was stable for at least 60 days in rat and human plasma and at least 30 days in dog plasma samples stored at −20°C. In human plasma samples, the analyte was stable for up to 5 cycles of freezing and thawing. This method has been applied successfully for the evaluation of the pharmacokinetics of minalrestat in rats, dogs and humans.

Keywords: Minalrestat; Aldose reductase inhibitor

1. Introduction

Minalrestat (2-[(4-bromo-2-fluorophenyl)methyl]-6 - fluorospiro[isoquinoline - 4(1H), 3′ - pyrrolidine] - 1,2′,3,5′(2H)-tetrone; ARI-509, WAY-121509; **I**) is a potent orally active aldose reductase inhibitor (ARI) that is being evaluated in clinical trials for the treatment of peripheral neuropathy, a diabetes-related complication. The inhibition of aldose reductase and polyol accumulation by **I** have been tested in various in vitro, in vivo and ex vivo models. In vitro IC_{50} of **I** for the inhibition of partially purified bovine lens aldose reductase activities was 1.4×10^{-8} *M* [1]. The ED_{50} of **I** for the inhibition of polyol accumulation in the sciatic nerves of STZ diabetic rats was 0.09 mg/kg/day [1]. When evaluated for inhibition of galactitol accumulation in galactose-induced galactosemic rats, the ED_{50} of **I** was 0.1 mg/kg/day. The compound **I** has been shown to inhibit aldose reductase in isolated red blood cells from both rats and dogs [1]. The partition coefficient (log ρ where ρ=octanol/pH 7.4 buffer) of **I** is 2.82. The lipophilicity of this compound may enhance distribution into the peripheral nerves.

To evaluate the pharmacokinetics of **I** in rats, dogs

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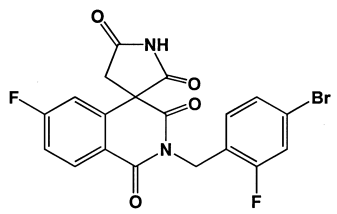
and humans, a high-performance liquid chromatographic (HPLC) method with on-line photochemical signal enhancement was developed for the quantification of **I** in rat, dog and human plasma. Photochemical reaction has been shown to enhance the ultraviolet absorption of heterocyclic compounds [2,3]. In this method, **I** and a structurally-related internal standard (WAY-123916. **II**) (Fig. 1) were extracted by solid-phase extraction from rat or dog plasma or by liquid–liquid extraction from human plasma. After HPLC separation, the effluent was passed through a photochemical reaction unit with a 10-m reaction coil which rendered close to two-fold enhancement of the signal of **I** prior to its detection at 255 nm. This paper describes the HPLC methods for the determination of **I** in plasma of all three species evaluated.

2. Experimental

2.1. Chemicals and reagents

Compounds **I** and **II** were synthesized at Wyeth-Ayerst Research, CN8000, Princeton, NJ, USA. Glacial acetic acid, heptane (Omnisolve) and ethyl acetate (Omnisolve) were obtained from EM Science (Gibbstown, NJ, USA). Monobasic potassium phosphate KH_2PO_4 (HPLC grade), phosphoric acid, 85% (Certified grade), methanol and deionized water (both HPLC grade) were obtained from Fisher Scientific (Pittsburgh, PA, USA). Trifluoroacetic acid (reagent grade) was obtained from Mallinckrodt (Chesterfield, MO, USA). Acetonitrile (UV grade) was obtained from Burdick and Jackson (Muskegon, MI, USA).

(A) Minalrestat, *I*



(B) Internal Standard (WAY-123916), *II*

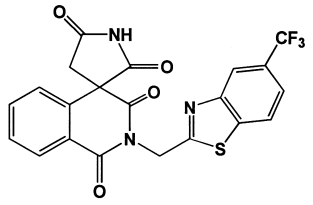


Fig. 1. Chemical structures of (A) minalrestat, **I** and (B) I.S.: WAY-123916, **II**.

2.2. Preparation of calibration standards and quality control samples

Stock solutions of **I** and **II** were prepared in acetonitrile at a nominal concentration of 100 μg/ml (223 μmol/l). Working I.S. solutions of concentrations (500 or 1000 ng/ml) were prepared in acetonitrile–water (30:70, v/v) by dilution of the stock solution of **II** with acetonitrile followed by addition of deionized water. For the determination of **I** in rat and dog plasma, intermediate solutions of **I** in acetonitrile–water (30:70, v/v) with nominal concentrations between 200 and 10 000 ng/ml were prepared by serial dilution of the stock solution. For the determination of **I** in human plasma, intermediate solutions of **I** with concentrations between 20 and 1000 ng/ml were prepared by dilution of **I** stock solution in acetonitrile and deionized water. All stock and intermediate **I** and **II** solutions were stored at 4°C.

The calibration curve for **I** in rat or dog plasma ranged between 20 and 1000 ng/ml. Plasma **I** calibration standards were prepared by adding 0.1 ml of working **I** solutions to 1 ml of control (drug-free) plasma. Quality control samples of 20, 200 and 1000 ng/ml were prepared by adding 0.1 ml of **I** intermediate solutions of concentrations 200, 2000 and 10 000 ng/ml to 1 ml of control rat or dog plasma. Test or study samples were added with 0.1 ml of acetonitrile–water (30:70, v/v) prior to extraction.

For the quantification of **I** in human plasma, the calibration curve ranged between 2 and 100 ng/ml and calibration standards were prepared by adding 0.2 ml of **I** intermediate solutions (20 to 1000 ng/ml) to 1.8 ml of control plasma. Quality control samples of 5, 20 and 100 ng/ml were prepared by adding 0.2 ml of **I** intermediate solutions of 50, 200

and 1000 ng/ml to 1.8 ml of control human plasma. All quality control samples were stored at −20°C prior to analysis. A separately weighed 10 mg of **I** reference standard was used in the preparation of the stock and intermediate solutions used in the preparation of quality control samples.

2.3. Solid-phase extraction of minalrestat from rat and dog plasma

Solid-phase extraction (SPE) cartridges (ODS, 1 ml, Chemical Separations) were conditioned by washing sequentially with 1 ml each of the following: (i) methanol; (ii) trifluoroacetic acid–acetonitrile (2:998, v/v); (iii) 0.1 *M* acetic acid; and (iv) deionized water. Calibration standard, quality control and test samples was loaded on a preconditioned SPE cartridge. The sample tube was rinsed with 0.5 ml of deionized water which was added to the SPE cartridge. The SPE cartridge was rinsed with the following solutions in sequence: (i) 1 ml of deionized water; (ii) 2×1 ml of acetic acid (0.1 *M*); and (iii) 1 ml of deionized water. After the final rinse with deionized water, **I** and **II** were eluted with 2×1 ml of 0.2% trifluoroacetic acid in acetonitrile. The eluent was evaporated to dryness under a stream of nitrogen in a water bath maintained at 50°C. The extract was reconstituted in 1 ml of HPLC mobile phase.

2.4. Liquid–liquid extraction of minalrestat from human plasma

A 2-ml volume of human plasma sample was transferred to a disposable glass extraction tube to which was added 0.1 ml of an anti-viral agent Panavirucide (Panbaxy, Vienna, VA, USA), 0.1 ml of working internal standard solution (500 ng/ml) and 0.1 ml of 1% trifuoroacetic acid solution. After thorough mixing, 10 ml of extraction solvent (heptane–ethyl acetate, 80:20, v/v) was added. The extraction mixture was Vortex mixed and then put on an Ebenbach shaker and shaken for 10 min at 70 strokes per min. The organic and aqueous phases were separated by centrifugation at approximately 1200 *g* for 10 min. A 9-ml aliquot of the upper (organic) layer was transferred into a 15-ml conical tube and evaporated to dryness under a stream of nitrogen in a water bath at 60°C. The dried extract was reconstituted in 0.25 ml of HPLC mobile phase.

2.5. Instrumentation and operating conditions

Reconstituted plasma extract (20 μl of rat or dog plasma extract or 50 μl of human plasma extract) was injected on a Hypersil ODS reversed-phase column (150×2.0 mm I.D., 3-μm packing; Chemical Separations) maintained at ambient temperature. Compounds were separated under isocratic condition with a mobile phase flow-rate of 0.2 ml/min. The mobile phase composition was acetonitrile–0.05 *M* potassium phosphate buffer (pH 3.0), 45:55, v/v. The HPLC sampling and solvent delivery system consisted of a Waters WISP 712 autosampler and an LDC/Milton Roy ConstaMetric III HPLC metering pump. After separation of the peaks of interest, the eluent was passed through an ICT photochemical reaction unit (Model Beam Boost, ICT, Frankfurt, Germany) with a 10-m reaction coil (I.D. 0.3 μm) surrounding an UV lamp. Analytes of interest were irradiated when passing through the reaction coil prior to entering the UV absorbance detector (LDC Analytical SpectroMonitor 3200 variable wavelength detector, Riviera Beach, FL, USA). Peaks were monitored at 255 nm with a sensitivity setting of 0.001 AUFS (rat or dog plasma samples) or 0.0005 AUFS (human plasma samples). Detected UV signals were recorded with a Hewlett-Packard 3392A Integrator. For the determination of **I** in human samples, the UV signals were also collected on the PE Nelson TurboChrom data acquisition system (ver. 3.0).

2.6. Pharmacokinetic studies of minalrestat in rats and dogs

This HPLC method was used in the assessment of the pharmacokinetics of a single 1 mg/kg intragastric dose of **I** in 55 Sprague-Dawley rats (body weights 200 to 250 g, Charles River, Kingston, NY, USA) and 6 beagle dogs (body weights 9 to 13 kg, Marshall Farm, NY, USA). Compound **I** was suspended in 60% polyethylene glycol, 10% ethanol (Mallinckrodt, Phillipsburg, NJ, USA) and 30% sterile water (Baxter Scientific Products, McGaw Park, IL, USA) at concentrations of 0.25 mg/ml (rat) or 2 mg/ml (dog). After the administration of **I** in

rats, blood samples (5 ml) were collected by cardiac puncture from five separate halothane-anaesthetized rats into heparinized tubes at 0.5, 1, 2, 4, 6, 8, 12, 15, 24 and 48 h post-dose. Blood samples were also collected from five control rats and designated as 0-h (predose) samples. After the administration of **I** in dogs, blood samples (5 ml) were collected from the femoral veins of study animals into heparinized tubes at 0.5, 1, 2, 4, 6, 8, 10, 15, 24, 48, 72, 96, 120, 144 and 168 h post-dose. Blood samples were also collected from each animal immediately prior to dosing (0 h). Plasma samples were obtained by centrifugation of the heparinized blood samples at approximately 1200 *g* and analyzed for the concentrations of **I**.

3. Results and discussion

3.1. Extraction efficiencies and limit of quantification of SPE vs liquid–liquid extraction of minalrestat and I.S.

The extraction efficiencies (expressed as peak height ratios of extracted samples vs unextracted standard solutions) of **I** and **II** from rat, dog and human plasma using either SPE or liquid–liquid extraction are summarized in Table 1. The efficiencies of **I** extraction from rat and dog plasma by SPE were 84% (rat) and 100% (dog), whereas the extraction efficiencies of the I.S. were 82% (rat) and 96% (dog). The extraction efficiencies of the liquid–liquid extraction procedure for human plasma were 47% (**I**) and 29% (**II**).

The limit of quantification (LOQ) of **I** in rat or dog plasma was 20 ng/ml and the limit of quantification in human plasma was 2 ng/ml. Even though lower extraction efficiencies were observed for **I** and **II** from human plasma samples, the use of a larger volume (2 ml for human plasma and 1 ml for rat or dog plasma), a smaller volume for the reconstitution of the extract (0.25 ml for human plasma extract vs 1 ml for rat or dog plasma extract) and a larger injection volume (50 μl for human plasma extract vs 20 μl for rat or dog plasma extract) compensated for the potential loss of assay sensitivity due to low extraction efficiency. These assay modifications also resulted in a 10-fold lower LOQ in human plasma vs the LOQ in rat and dog plasma. The liquid–liquid extraction method was used in the determination of **I** concentrations in human plasma samples from clinical studies.

3.2. Selectivity

In rat, dog and human plasma samples, no significant interference peak was found at the retention times of either **I** or **II**. Representative chromatograms of unextracted standards, extracted drug-free plasma and extracted **I** and **II** in human plasma are presented in Fig. 2 [(A): unextracted standard; (B): drug-free plasma extract; (C): 100 ng/ml spiked plasma extract]. In all the biological matrices tested, baseline separation of **I** and **II** was achieved.

3.3. Linearity

The regression lines relating the (**I** to **II**) peak height ratios to the concentration of **I** were calculated using weighted linear regression analysis with a weighting factor of $1/(\text{concentration})^2$. Typical calibration curves parameters of the standard curves for **I** in rat, dog and human plasma samples are summarized in Table 2. The linear quantification range of **I** from rat or dog plasma was 20 to 1000 ng/ml and the quantification range from human plasma was 2 to 100 ng/ml. Typical regression line coefficients of determination (R^2) were greater than 0.99.

Table 1
Extraction efficiencies of minalrestat and I.S. from rat, dog and human plasma

Plasma matrix	Minalrestat (**I**)	I.S. (**II**)	Extraction method
Rat	84%	82%	SPE
Dog	100%	96%	SPE
Human	47%	29%	Liquid–liquid

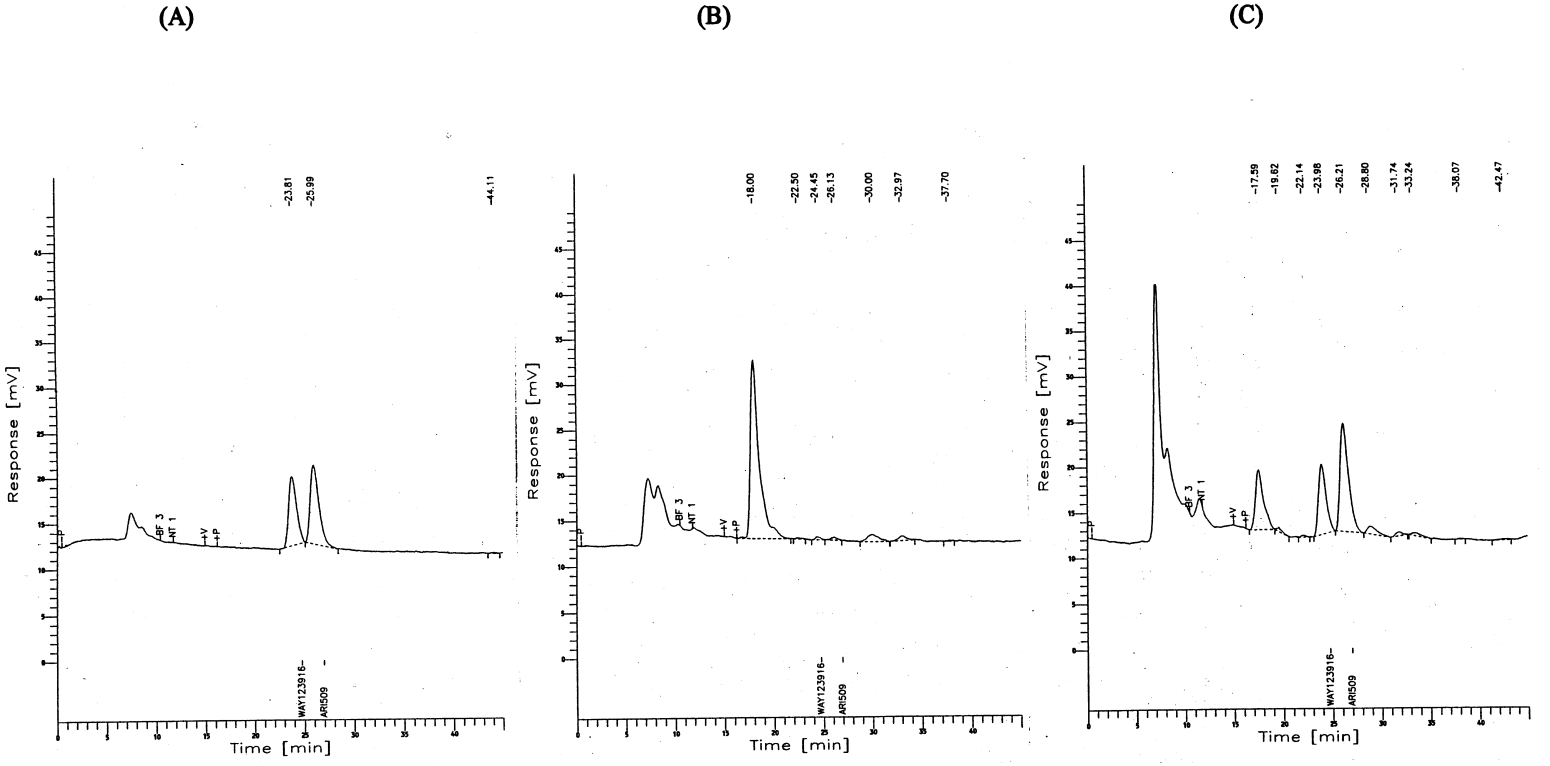


Fig. 2. Representative chromatograms of minalrestat (**I**) and I.S. (**II**) in human plasma: (A) unextracted standards of **I**, $t_R = 25.99$ min and **II**, $t_R = 23.81$ min.; (B) drug-free human plasma extract; (C) human plasma extract containing **I** (100 ng/ml), $t_R = 26.77$ min and **II**, $t_R = 24.06$ min.

Table 2
Intra-day precision and accuracy for the determination of minalrestat in rat, dog and human plasma calibration standards

Species	Concentration added[a] (ng/ml)	Concentration found (ng/ml)	Precision (%C.V.)	Accuracy (%MRE[b])
Rat	20	20.0	4.3	0.0
	50	50.3	2.9	0.6
	100	99.7	4.8	−0.3
	200	199.6	4.2	−0.2
	500	509.1	3.3	1.8
	1000	983.9	3.6	−1.6
$Y=0.004843+0.02445X$, $R^2=0.9977$, weighting factor $=1/C^2$, $n=30$				
Dog	20	20.2	5.6	1.0
	50	49.2	2.4	−1.6
	100	97.8	2.3	−2.2
	200	196.5	1.8	−1.8
	500	508.1	3.0	1.6
	1000	1028.4	4.4	2.8
$Y=0.04948+0.01195X$, $R^2=0.9976$, weighting factor $=1/C^2$, $n=30$				
Human	2	2.0	8.3	2.0
	5	4.8	3.1	−4.2
	10	9.8	4.7	−1.7
	20	19.9	3.0	−0.3
	50	50.5	2.7	1.0
	100	103.0	2.0	3.0
$Y=-0.008418+0.08675X$, $R^2=0.9962$, weighting factor $=1/C^2$, $n=30$				

[a] $n=5$ for each standard, results represent mean values.
[b] %MRE = (concentration found − concentration added)/concentration added × 100%.

3.4. Precision and accuracy

Intra-day precision and accuracy for the quantification of **I** in rat, dog and human plasma samples were determined by analyses of five replicates of calibration standards in all three matrices. The results are summarized in Table 2. For rat and dog plasma, the concentrations of calibration standards were 20, 50, 100, 200, 500 and 1000 ng/ml. For human plasma, the concentrations of calibration standards were 2, 5, 10, 20, 50 and 100 ng/ml. The intra-day coefficients of variation of **I** calibration standards ($n=5$ in each case) were less than 5% in rat plasma, less than 6% in dog plasma and less than 9% in human plasma. The intra-day accuracies (expressed as %MRE: % mean relative error) for the determination of **I** were within ±5% in all three matrices evaluated.

The inter-day precision and accuracy were assessed in rat and human plasma on three separate days using quality control samples with concentrations spanning the range of the calibration curve, and the results are presented in Table 3. For rat plasma samples, the concentrations of the quality control samples were 20, 200 and 1000 ng/ml. The concentrations of quality control samples for human plasma were 5, 20 and 100 ng/ml. The inter-day C.V. ($n=15$) for the quantification of **I** in rat plasma was less than 6% at concentrations of 20, 200 and 1000 ng/ml and the accuracies (%MRE) were within ±15%. The inter-day precision (%C.V.) for the quantification of **I** in human plasma quality control samples was less than 12% and the accuracy (%MRE) was within ±15%. The intra- and inter-day precision and accuracy of this HPLC method are well within the recommended acceptance limits as outlined in the conference consensus report for analytical method validation [4].

3.5. Stability

The storage stability of **I** in biological matrices was evaluated after storage of dog plasma samples for up to 30 days and storage of rat and human

Table 3
Inter-day precision and accuracy for the determination of minalrestat in rat and human plasma

Species	Concentration added[a] (ng/ml)	Concentration found (ng/ml)	Precision (%C.V.)	Accuracy (%MRE[b])
Rat	20	22.5	5.9	12.5
	200	189.7	4.8	−5.1
	1000	955.9	2.6	−4.4
Human	5	5.6	11.3	12.2
	20	21.8	6.3	8.8
	100	108.7	5.2	8.7

[a] $n=15$ for each sample, results represent mean values.
[b] %MRE = (concentration found − concentration added)/concentration added × 100%.

plasma samples for up to 60 days at −20°C. The results, as summarized in Table 4, showed that **I** was stable in dog plasma for at least 30 days. Compound **I** was stable in rat and human plasma for at least 60 days. The stability of **I** in human plasma subjected to multiple cycles of freezing and thawing was evaluated at concentrations of 5 and 100 ng/ml. The results are summarized in Table 5. Compound **I** was found to be stable in human plasma samples through 5 cycles of freezing and thawing.

Table 4
Stability of minalrestat in rat, dog and human plasma stored at −20°C

Species	Concentration added (ng/ml)	Days in storage	Concentration found (ng/ml)	Remaining after storage[a] (%)
Rat	20	1	22.0 ($n=5$)	100
		44	23.3 ($n=2$)	106
		60	22.0 ($n=2$)	100
Dog	1000	1	1028.4 ($n=5$)	100
		30	1071.0 ($n=2$)	104
Human	5	1	5.8 ($n=5$)	100
		21	6.6 ($n=1$)	114
		60	6.6 ($n=3$)	114
	100	1	107.8 ($n=5$)	100
		21	126.4 ($n=2$)	117
		60	109.9 ($n=4$)	102

[a] % Remaining after storage = (mean concentration found after × days of storage − mean concentration on day 1)/mean concentration on day 1 × 100%.

Table 5
Stability of minalrestat in human plasma after multiple cycles of freezing and thawing

Concentration added (ng/ml)	Number of freeze–thaw cycles	Concentration found (ng/ml)	Remaining after freeze–thaw cycles[a] (%)
5	0	5.8 ($n=5$)	100
	3	6.1 ($n=2$)	105
	5	6.8 ($n=4$)	117
100	0	107.8 ($n=5$)	100
	3	102.4 ($n=2$)	95
	5	109.0 ($n=6$)	101

[a] % Remaining after freeze–thaw cycles = (mean concentration found after × freeze–thaw − mean concentration found after 0 cycle)/mean concentration found after 0 cycle × 100%.

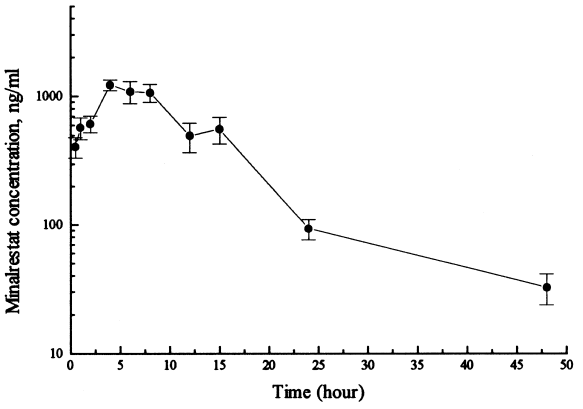


Fig. 3. Mean (±S.E.) plasma concentration vs time curve of minalrestat in Sprague-Dawley rats after a single intragastric dose of 1 mg/kg ($n=5$ at each time point).

*3.6. Pharmacokinetic evaluation of **I** in rats and dogs*

The plasma concentration vs time profiles of **I** after a single i.g. dose of 1 mg/kg in rats and dogs are depicted in Figs. 3 and 4, respectively. The C_{max} and t_{max} values were obtained by visual inspection of the plasma concentration vs time curves. The area under the plasma concentration vs. time curve ($AUC_{0-\infty}$) and the terminal half-life ($t_{\frac{1}{2}}$) were estimated by using the LAGRAN program [5]. The pharmacokinetic parameters in rats were estimated based on the mean concentration vs time curve with 5 animals at each time point. For dogs, the pharmacokinetic parameters of **I** were estimated in individual study animals ($n=6$).

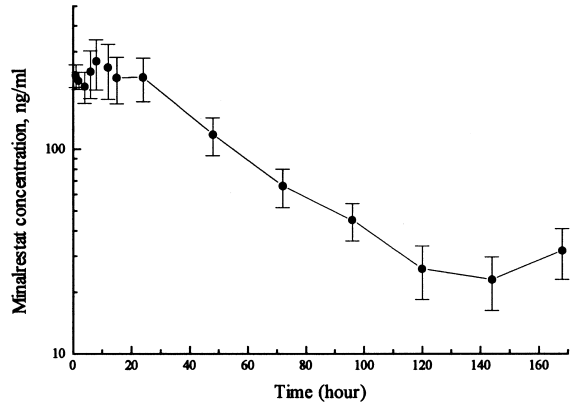


Fig. 4. Mean (±S.E.) plasma concentration vs time curve of minalrestat in beagle dogs after a single intragastric dose of 1 mg/kg ($n=6$).

The peak plasma concentration (C_{max}) of **I** in rats was 1.22 μg/ml, reached at 4 h post-dose (t_{max}). The estimated terminal half-life ($t_{\frac{1}{2}}$) of **I** in rats was 9 h and the estimated $AUC_{0-\infty}$ value was 14.0 μg●h/ml after a single 1 mg/kg dose of **I**. In dogs, the t_{max} of **I** ranged from 1 to 10 h, with a mean (±S.D.) value of 4.8 (±4.3) h. The plasma C_{max} of **I** in dogs was 0.36 (±0.11) μg/ml. The mean (±S.D.) of estimated terminal half-lives of **I** in dogs was 60.7 (±24.8) h and the estimated $AUC_{0-\infty}$ value was 18.3 (±7.9) μg·h/ml.

4. Conclusion

In the development of therapeutic agents through various stages of preclinical and clinical testing, analytical methods with various degrees of sensitivity and ruggedness requirements are developed. This HPLC method was initially developed to evaluate the preclinical pharmacokinetics of **I** in rats and dogs, both of which were used as preclinical models for the assessment of the pharmacology and toxicity of this compound, and shown to be of adequate sensitivity (LOQ of 20 ng/ml) for the quantification and pharmacokinetic assessment of **I** in both species. However, in support of clinical evaluation of **I**, an HPLC method of higher sensitivity (lower LOQ) and improved ruggedness was required. By slight modification of the extraction procedures, a 10-fold lower LOQ was achieved. Although liquid–liquid extraction was found to yield lower extraction efficiencies, the procedure was less tedious than the solid-phase extraction procedure and resulted in shortening of sample processing time. This HPLC method was applied successfully in clinical trials for the quantification of **I** in human plasma samples [6].

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