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Quantitative determination of a potent lipopolysaccharide antagonist, E5564, in rat and dog plasma by high-performance liquid chromatography with fluorescence detection

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Abstract

The assay method was established for the quantification of a potent lipopolysaccharide (LPS) antagonist, E5564, in rat and dog plasma using HPLC. E5564 and the I.S. (an analogue of E5564) were extracted and derivatized with 9-Anthryldiazomethane (ADAM reagent) to be given fluorescence. LC–MS analysis indicated that single molecule of E5564 was coupled with two molecules of ADAM reagent at one on each of the phosphorus groups. After solid-phase extraction, ADAM derivatives of E5564 and the I.S. were separated on an ODS column using methanol/ethanol containing sodium acetate as a mobile phase at 1.2 ml/min (gradient elution), and detected by a fluorescence detector (excitation: 254 nm, emission: 415 nm). The intra-day and inter-day precision were less than 14.4%, and accuracy were within ±13.0% in the concentration range of 30 to 20 000 ng/ml plasma in both species. E5564 was stable for at least 13 days in rat and dog plasma at −20°C, and the processed sample was stable for up to 14 days at 4°C. This validated method was successfully applied to the evaluation of the pharmacokinetics of E5564 in rats and dogs after single bolus intravenous doses.

Keywords: Lipopolysaccharide antagonist; E5564; Lipid A analogue

1. Introduction

Many of the cardiopulmonary derangements associated with Gram-negative sepsis result from the activation of monocytes and macrophages by endotoxin [also known as lipopolysaccharide (LPS)] with the resultant release of humoral mediators such as TNF-α. It is now well established that the lipid A portion of LPS is responsible for its endotoxic effects. It has been discovered that LPS or the lipid A portion from some nonenterobacterial species, such as *Rhodbacter capsulatus*, can block endotoxin-induced cytokine release from monocytes and macrophages. The structures of these nontoxic lipid A molecules have been tentatively determined and, in some cases, molecules with equivalent activity were synthesized. E5564, one of synthetic analogues of lipid A (see Fig. 1) demonstrated potent LPS antagonism in murine in vivo as well as in human blood in vitro systems [1]. Furthermore, E5564 is devoid of agonistic activity (in vitro and in vivo).

As mentioned above, E5564 is an analogue of lipid A, and can be detected by the LAL (Limulus amebocyte lysate) assay [2,3]. To evaluate the

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potency of E5564 on LPS antagonistic activity, it was necessary to establish the specific quantification method for E5564 in plasma samples containing LPS.

This paper describes the development of a HPLC method for quantification of E5564 in rat and dog plasma. The validated method was successfully applied to evaluate the plasma concentration profiles of E5564 in rats and dogs after intravenous administration.

2. Experimental

2.1. Chemicals and reagents

E5564 and the I.S. (an analogue of E5564) were synthesized at Eisai Merrimack Valley Laboratories (Andover, MA, USA) and those chemical structures are shown in Fig. 1. Purity of E5564 and impurity of I.S. determined by HPLC were 97.23 and 7.65%, respectively. Methanol (MeOH), ethanol (EtOH) and ethyl acetate (HPLC grade) were purchased from Wako Pure Chemicals (Osaka, Japan). 9-Anthryldiazomethane (ADAM reagent) was obtained from Funakoshi (Tokyo, Japan) and its chemical structure is shown in Fig. 1. Sodium acetate and sodium sulfite (Na_2SO_3, guaranteed grade) were obtained from Wako Pure Chemicals. One mol/l HCl (1 *M* HCl) and 0.01 mol/L NaOH (0.01 *M* NaOH) were obtained from Wako Pure Chemicals. Isolute™ Silica 500 mg/3 ml was obtained from International Solvent Technology (Hengoed, UK).

2.2. Instruments and operating conditions

For the LC–MS analysis of the ADAM derivative of E5564, the HPLC system consisted of two-LC-10AD (dual-piston) solvent delivery systems, an SIL-$10A_{XL}$ autosampler equipped with a cooling system, a CTO-10A column oven and an SCL-10A system controller (Shimadzu, Kyoto, Japan). Mass spectrometric detection was carried out with a TSQ-7000 (Thermo Quest, CA, USA) using positive and negative ion electronspray ionizations as an LC-MS interface, and the capillary temperature was 275°C.

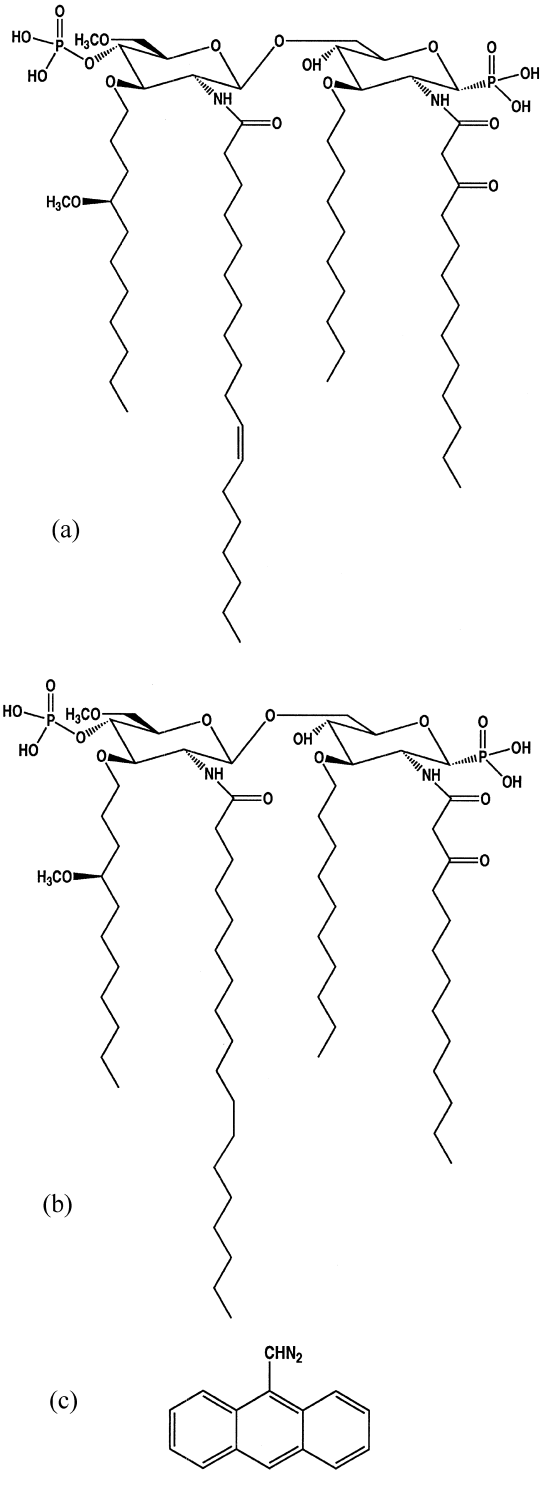

Fig. 1. Chemical structure of E5564 (a), the I.S. (b) and 9-anthrydiazomethane (ADAM) (c).

Five microliters of sample was injected on an L-column ODS (150×1.5 mm I.D.) supplied by Chemicals Inspection and Testing Institute (Tokyo, Japan) at 30°C. ADAM derivative of the E5564 was separated by isocratic elution at 0.2 ml/min with EtOH–MeOH (25:75, v/v) containing 0.1 *M* sodium acetate.

For quantification of E5564 in plasma, the HPLC system consisted of two-LC-10AD solvent delivery systems, an SIL-10A autosampler equipped with a cooling system, a CTO-10A$_{VP}$ column oven, an SCL-10A system controller (Shimadzu, Kyoto, Japan) and an FP-920 fluorescence detector (JASCO, Tokyo, Japan). The data were collected on a C-R7A integrator (Shimadzu, Kyoto, Japan).

Twenty microliters of the reconstituted plasma samples was injected on a YMC-Pack AP-313 (250×6.0 mm I.D.) column supplied by Yamamura Chemicals Co., Ltd. (Kyoto, Japan) at 30°C. ADAM derivatives of E5564 and the I.S. were separated under gradient condition at a flow-rate of 1.2 ml/min. The mobile phase (A) was methanol containing 5% (w/v) of sodium acetate, and (B) was methanol–ethanol (60:40, w/w) containing 1% (w/v) of sodium acetate. As an initial condition, the ratio of (A)–(B) was set at 70:30 (v/v). From 15 to 35 min after injection, a linear gradient was programmed from 70:30 to 50:50 (v/v). The ADAM derivatives were detected by fluorescence with excitation wavelength at 254 nm and emission wavelength at 415 nm.

2.3. Preparation of ADAM derivative of E5564 for LC–MS analysis

About 50 mg of Na_2SO_3 and 400 μl of ADAM reagent solution (0.8% w/v in MeOH) were added to 100 μl of E5564 solution (1.6 mg/ml in MeOH), and the mixture was incubated for 1 h at 40°C. Following the addition of 4 ml of ethyl acetate, the reaction mixture was applied to Isolute™ Silica (500 mg silica packed in the column). The column was washed with 2 ml of ethyl acetate–MeOH (95:5, v/v) then 4 ml of ethyl acetate–MeOH (75:25, v/v). ADAM derivative of E5564 was eluted with 2 ml of ethyl acetate–MeOH (60:40, v/v). The eluate was dried under a nitrogen stream at 40°C, then the residue was dissolved in 200 μl of EtOH–MeOH (50:50, v/v), and subjected to LC-MS analysis.

2.4. Preparation of calibration curve, standard samples and QC samples

E5564 and I.S. stock solutions were prepared in 0.01 *M* NaOH at a concentration of 1 and 0.5 mg/ml, respectively, and were stored at 4°C. Working I.S. solution (10 μg/ml) was prepared by dilution of the I.S. stock solution with 0.01 *M* NaOH. For the determination of E5564 in rat and dog plasma, standard solutions of E5564 at nominal concentrations between 150 and 100 000 ng/ml were prepared by serial dilution of the stock solution with 0.01 *M* NaOH just prior to use. I.S. solution and standard solutions of E5564 were prepared just prior to use.

The calibration curves for E5564 were prepared separately in rat and dog plasma within the range of 30–20 000 ng/ml. Calibration curve samples were prepared by adding 20 μl of standard solutions of E5564 to 100 μl of blank plasma. Standard samples of 30, 1000 and 20 000 ng/ml were prepared by adding 20 μl of standard solutions of E5564 to 100 μl of blank plasma, and were used for intra- and inter-day assay, and stability assay of processed samples. QC samples in duplicate at 100, 1000 and 10 000 ng/ml were prepared by adding 20 μl of standard solutions of E5564 to 100 μl of blank plasma, and were used to provide the basis of accepting or rejecting the analytical run of pharmacokinetic studies.

2.5. Treatment of rat and dog plasma sample

Ten microliters of working I.S. solution, 50 μl of 1 *M* HCl and 300 μl water for injection (Otsuka Pharmaceuticals, Tokyo, Japan) were added to 100 μl of plasma samples. The plasma samples were extracted with the mixture of 4.5 ml of ethyl acetate and 500 μl of EtOH–MeOH (50:50) by shaking them for 15 min, and were then centrifuged (1500 *g* 10 min). The organic layer was collected and washed with 500 μl of water by manually inverting the container five times. After centrifugation (1500 *g* 10 min), the aqueous layer was removed, and the organic layer was dried under a nitrogen stream at

40°C. The residue was dissolved in 100 μl of MeOH and approximately 20 mg of Na_2SO_3 and 400 μl of ADAM reagent in MeOH (0.4%, w/v) were added to the solution. Then the mixture was incubated at 40°C for 1 h. Following the addition of 4 ml of ethyl acetate, the reaction mixture was applied to an Isolute™ Silica column (500 mg silica packed in the column). The column was washed with 2 ml of ethyl acetate, and the derivatized E5564 and I.S. were eluted with 4 ml of MeOH–ethyl acetate (50:50, v/v). The eluate was dried under a nitrogen stream at 40°C. The residue was dissolved in 200 μl of EtOH–MeOH (50:50, v/v), and a 20 μl aliquot was subjected to HPLC analysis.

2.6. Pharmacokinetic studies of E5564 in rats and dogs

This assay method was used to assess the pharmacokinetics of E5564 in Sprague-Dawley rats (age: 7 weeks, body weights: 220–247 g, SLC, Shizuoka, Japan) and beagle dogs (age: 15 months, body weights: 11.61–14.89 kg, CSK Research Park Inc., Nagano, Japan) after single bolus intravenous administration at 0.3 mg/kg. E5564 (0.6 mg/ml) in 4.25 m*M* phosphate buffer (pH 7.4±0.3) containing 10% lactose (w/v, Mallinchrodt, Phillipsburg, NJ, USA) was prepared as a dosing solution. After intravenous administration of E5564 in rats via femoral vein at 0.3 mg/kg, approximately 0.25 ml of blood was drawn from jugular vein using a heparinized syringe at pre-dose and 5 and 30 min, 1, 4, 8, 12 and 24 h after administration. To avoid the influence of anesthetization, rats had been awake during experiment. After intravenous administration of E5564 in dogs via cephalic vein at 0.3 mg/kg, approximately 0.5 ml of blood was drawn from jugular vein using a heparinized syringe at pre-dose and 5, 15 and 30 min, 1, 4, 8, 24, 48, 96 and 168 h after administration. The blood sample was centrifuged at approximately 1500 *g* for 10 min at 4°C to obtain the plasma. Then 100 μl of plasma was transferred to a polypropylene tube and was stored at −20°C until analysis.

QC samples were incorporated into each analytical run. At least four of the six QC samples must be within 20% of their respective nominal values; two of the six QC samples (not both at the same concentration) may be outside the ±20% respective nominal value.

Plasma concentration–time data were analyzed by non-compartment pharmacokinetic methods (moment analysis) [4]. The elimination half-life at the terminal phase ($t_{1/2}$), the area under the concentration–time curve (AUC), the volume of distribution at steady state (V_{dss}) and the total body clearance (CL_{tot}) were estimated.

3. Results and discussion

3.1. Structural analysis of ADAM derivatives of E5564 by LC–MS

Although it is well known that the ADAM reagent reacts with compounds at their carboxyl moiety [5], the reagent was employed to target the phosphorus groups of E5564 in this study. Thus, the coupling of ADAM reagent to E5564 was evaluated by LC–MS. As shown in Fig. 2, the base peak of the ADAM derivative of E5564 was found the m/z 1716.5 in the positive ion mode that was estimated to be the single sodium adduct ion of E5564 coupled with two molecules of ADAM reagents. The ions of the derivative as free and double sodium adducts were also found. At ESI negative ion mode, m/z 1692.3 was found and corresponded to the mass number of E5564 coupled with two ADAM reagents without any sodium adduct. These data indicated that E5564 was coupled with two ADAM molecules.

3.2. Specificity

Typical chromatograms of blank rat plasma and plasma spiked with 1000 ng/ml of E5564 and the I.S. are presented in Fig. 3. In rat plasma, no significant interference peak derived from biological matrix was found at the retention time of either E5564 or the I.S.. E5564 and the I.S. were well separated on the chromatogram of plasma sample. For dog plasma, specificity was also confirmed. In the pre-dose plasma samples of the rat and dog, no significant interference peak was also found.

Since many plasma components (i.e. lipids) were labeled by ADAM reagent, many interference peaks were found in the chromatogram. To separate these

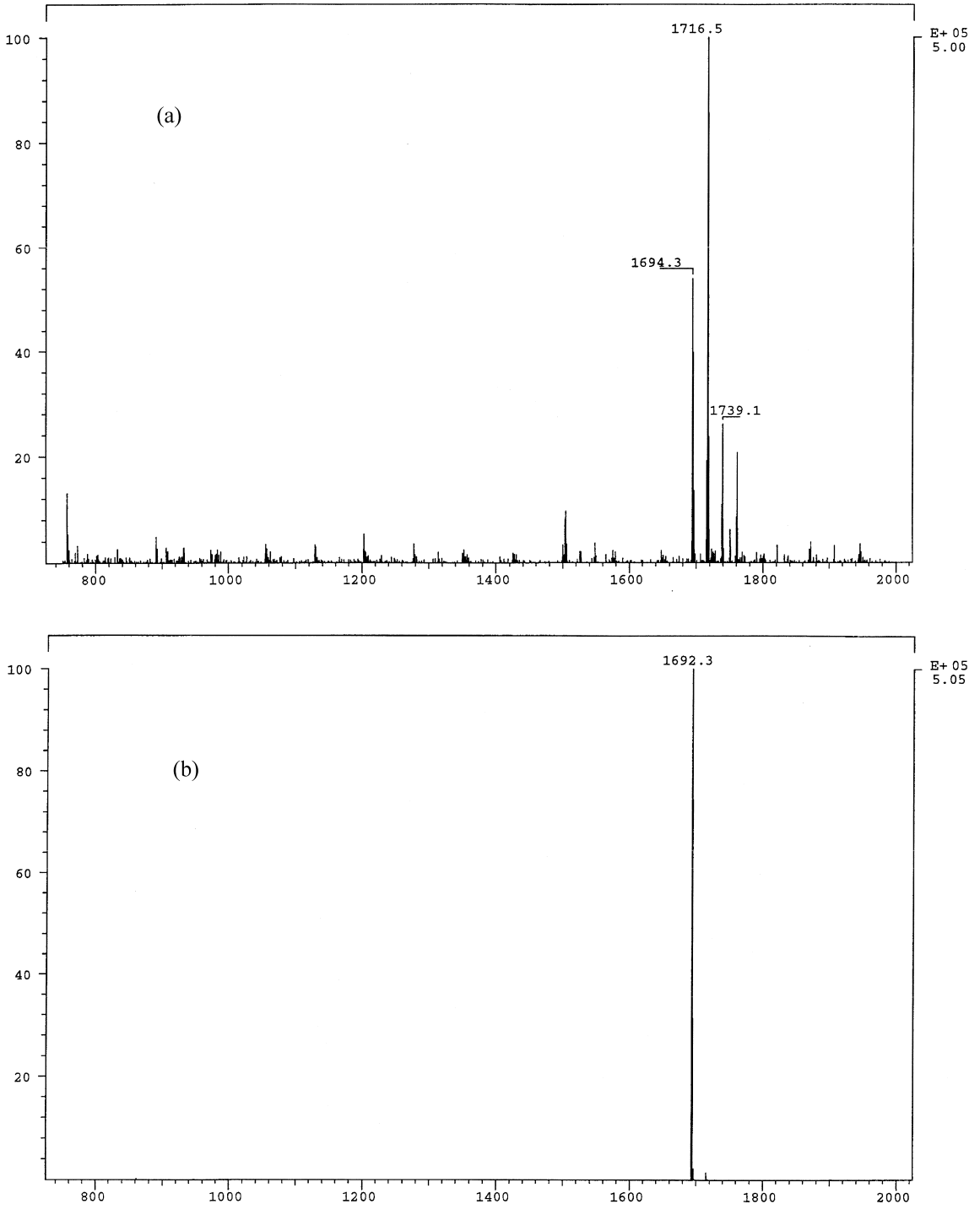

Fig. 2. Mass spectrum of the ADAM derivative of E5564 at positive (a) and negative (b) ion mode.

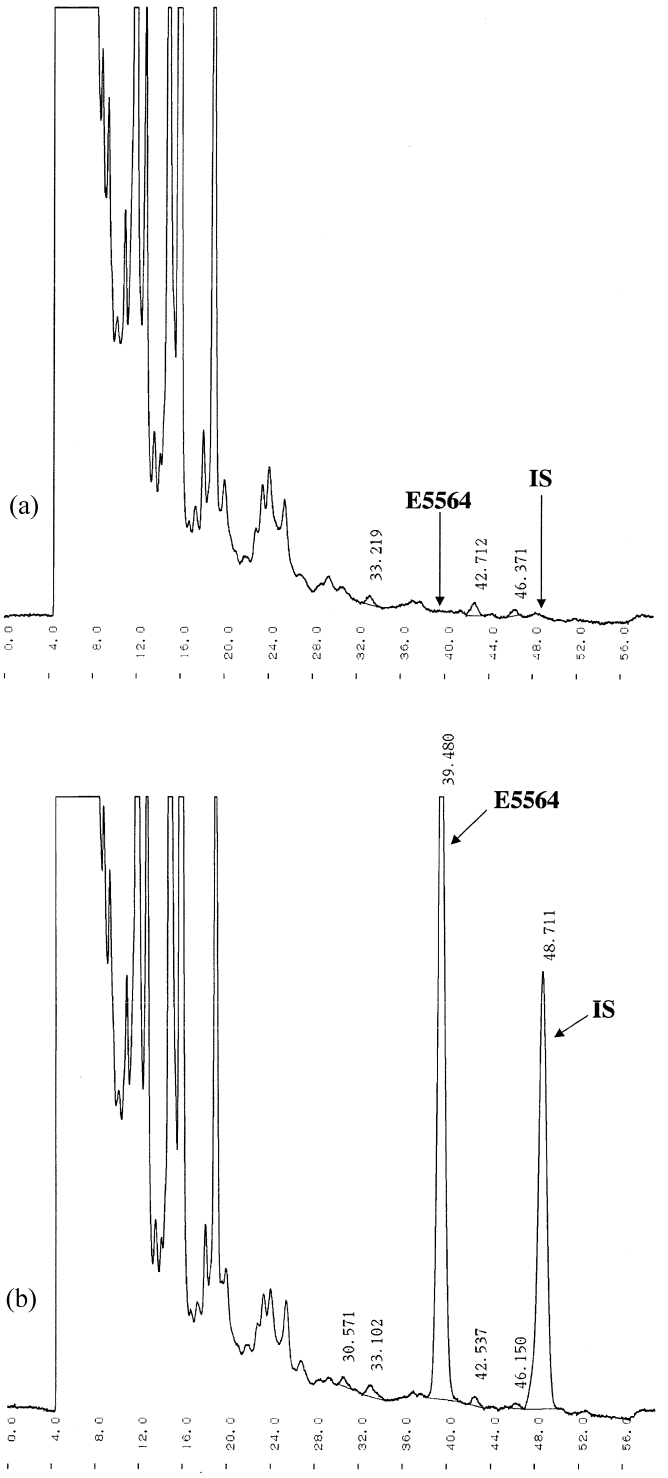

Fig. 3. Typical chromatograms of blank rat plasma (a), and blank rat plasma spiked with E5564 (1000 ng/ml) and the I.S. (1000 ng/ml) (b).

Table 1
Intra-day precision and accuracy for the determination of E5564 in rat and dog plasma

Species	Concentration added (ng/ml)	Concentration found[a] (ng/ml)	Precision (% C.V.)	Accuracy (% MRE[b])
Rat	30	31.0	4.8	3.3
	1000	1028.5	1.9	2.9
	20 000	17 693.7	2.7	−11.5
$Y=0.0013614+0.0016074X$, $r^2=0.9990$, weighting factor $=1/X^2$				
Dog	30	33.9	3.8	13.0
	1000	1034.5	1.7	3.5
	20 000	18 069.7	4.2	−9.7
$Y=0.0045302+0.0016032X$, $r^2=0.9990$, weighting factor $=1/X^2$				

[a] Results represent mean values of five replicates.
[b] % MRE = [(concentration found − concentration added)/concentration added] × 100%.

peaks from labeled E5564 and I.S., 56 min of chromatographic run time were required.

3.3. Linearity

The separate standard curves were constructed in the rat and dog plasma, and were obtained using the peak height ratios of E5564 to I.S. (Y) against the concentration of E5564 (X) by least square regression analysis (weighting factor: $1/X^2$). The concentration of E5564 in calibration curve samples were set at 30, 100, 300, 1000, 3000, 10 000 and 20 000 ng/ml in plasma. In this assay, the wide dynamic range (667 folds) required the weighting factor, and to make equal weighting for all cali-

Table 2
Inter-day precision and accuracy for the determination of E5564 in rat and dog plasma

Species	Concentration added (ng/ml)	Concentration found[a] (ng/ml)	Precision (% C.V.)	Accuracy (% MRE[b])
Rat	30	29.1	14.4	−3.0
	1000	988.2	4.2	−1.2
	20 000	18 997.4	4.1	−5.0
Dog	30	29.7	5.4	−1.0
	1000	1022.4	7.1	2.2
	20 000	19 016.6	11.0	−4.9

[a] Results represent mean values of four replicates.
[b] % MRE = [(concentration found − concentration added)/concentration added] × 100%.

Table 3
Stability of E5564 in rat and dog plasma stored at −20°C

Species	Concentration added (ng/ml)	Storage period (day)	Concentration found (ng/ml)	Remaining after storage[a] (%)
Rat	30	6	25.7	86
		13	28.9	96
	1000	6	937.8	94
		13	923.0	92
	20 000	6	18 523.1	93
		13	17 135.0	86
Dog	30	6	33.7	112
		13	34.1	114
	1000	6	966.6	97
		13	981.9	98
	20 000	6	18 393.7	92
		13	17 029.0	85

[a] Remaining after storage = [(mean concentration found after x days storage − concentration added)/concentration added] × 100%.

Table 4
Stability of E5564 in the processed samples

Species	Concentration added (ng/ml)	Storage period (day)	Concentration found (ng/ml)	Remaining after storage[a] (%)
Rat	30	3	28.0	93
		7	26.8	89
		14	28.2	94
	1000	3	1070.7	107
		7	1069.3	107
		14	1072.5	107
	20 000	3	19 992.5	100
		7	19 891.9	100
		14	20 393.5	102
Dog	30	3	28.4	95
		7	31.3	104
		14	30.1	100
	1000	3	1074.7	108
		7	1084.0	108
		14	1069.3	107
	20 000	3	19854.2	99
		7	19 986.3	100
		14	20 101.7	101

[a] Remaining after storage = [(mean concentration found after x days storage − concentration added)/concentration added] × 100%.

bration curves, the weighting factor was based on the values, X. The calibration curves for E5564 in rat and dog plasma were linear within the range of 30–20 000 ng/ml. The coefficients of determination (r^2) were greater than 0.99 (Table 1).

3.4. Precision and accuracy

The intra-day precision and accuracy for the quantification of E5564 in rat and dog plasma samples were determined by analyses of five replicates of the standard samples at 30, 1000 and 20 000 ng/ml. The results are summarized in Table 1. The coefficients of variation were less than 4.8 and 4.2% in rat and dog plasma, respectively. The accuracies (expressed as %MRE: % mean relative error) were within ±13.0% in both animals.

The inter-day precision and accuracy were assessed in rat and dog plasma on four separate days using quality control samples at 30, 1000 and 20 000 ng/ml, and the results are presented in Table 2. In rat plasma, the coefficients of variation were less than 14.4%. In dog plasma, the coefficients of variation of E5564 were less than 7.1%. The accuracies (%MRE) were within ±5.0% in both animals.

The intra- and inter-day precision and accuracy of this assay method meets the acceptance criteria as outlined in the conference consensus report for analytical method validation [6].

3.5. Stability

The stability of E5564 in rat and dog plasma frozen at −20°C was evaluated for up to 13 days at the concentrations of 30, 1000 and 20 000 ng/ml. The results, as summarized in Table 3, showed that E5564 was stable in rat and dog plasma for at least 13 days.

The stability of E5564 in the processed samples of rat and dog plasma at 4°C was evaluated for up to 14 days at 30, 1000 and 20 000 ng/ml. The results, as summarized in Table 4, showed that E5564 was

Table 5
Pharmacokinetic parameters of E5564 in rats and dogs after intravenous administration of E5564 at 0.3 mg/kg[a]

Parameters		Rat (n = 4)	Dog (n = 3)
$t_{1/2}$	(h)	5.2±0.2	50.4±2.2
V_{dss}	(ml/kg)	81.1±2.7	77.1±5.7
AUC	(μmg·h/ml)	20.4±0.76	271.0±28
CL_{tot}	(ml/h/kg)	14.8±0.5	1.13±0.12

[a] Each value shows the mean and S.E.M.

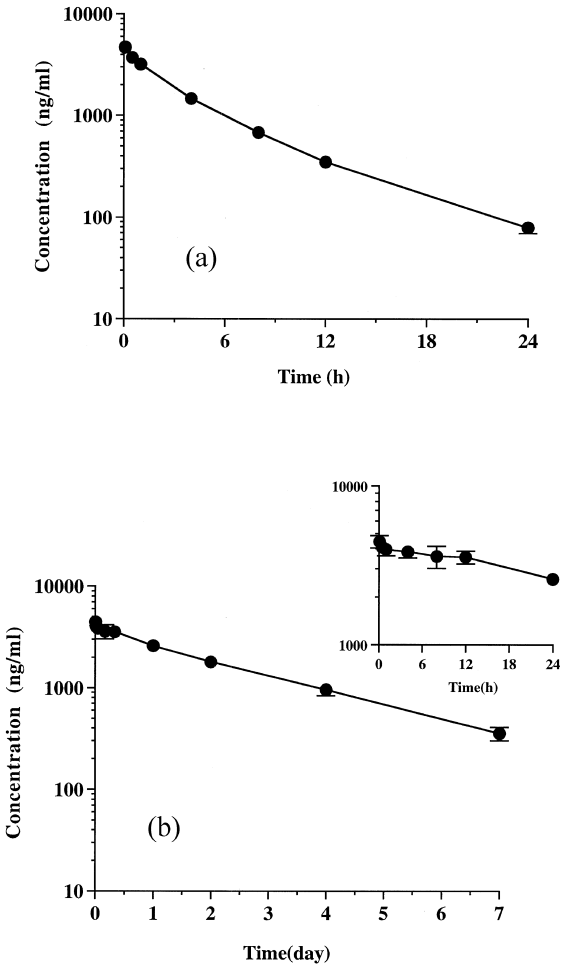

Fig. 4. Mean plasma concentration time profile of E5564 in rats (a) and dogs (b) after an intravenous administration of E5564 at 0.3 mg/kg ($n=4$ for rats and $n=3$ for dogs). Each point represents the mean and S.E.M.

stable in the processed sample of rat and dog plasma for at least 14 days.

3.6. Pharmacokinetic profile of E5564 in rats and dogs

The plasma concentration time profiles of E5564 after single bolus intravenous administration of E5564 at 0.3 mg/kg in rats and dogs are shown in Fig. 4. Pharmacokinetic parameters obtained by moment analysis were shown in Table 5. Accuracy of all QC samples were within ±4.3 and ±6.5% for rats and dogs, respectively.

The plasma concentration of E5564 decreased biphasically both in rats and dogs. The distribution volume of E5564 was similar in both species, whereas the elimination of E5564 in plasma in dogs was much slower than that in rats.

4. Conclusion

In this paper, we developed a sensitive assay for the determination of E5564 and applied it to evaluate the pharmacokinetic profiles of E5564 in rats and dogs. This method was validated within the range of 30 ng/ml to 20 μg/ml of E5564 in rat and dog plasma. Pharmacokinetic profiles of E5564 in rats and dogs were successfully determined.

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