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LC determination of YM466, a new factor Xa inhibitor, in rat and dog plasma

Yuji Mano *, Toshiko Aoki, Yumiko Kikuchi, Yoshiaki Soeishi, Takashi Usui, Hidetaka Kamimura

Drug Metabolism Laboratories, Yamanouchi Pharmaceutical Co., Ltd., 1-8 Azusawa 1-Chome, Itabashi-ku, Tokyo 174-8511, Japan

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Abstract

A specific method for the determination of YM466, a novel Factor Xa inhibitor, in rat and dog plasma was developed and validated. YM466 was extracted from plasma by solid-phase extraction and analyzed by UV-HPLC at an absorbance wavelength of 240 nm. The intra-day precision and accuracy ranged from 0.8 to 2.4% and 0.1 to 5.0% in rats, and from 1.6 to 2.4% and 0.0 to 4.1% in dogs, respectively. The lower limit of quantification was 10 ng/ml when 1 ml of plasma was used. No endogenous interference was observed in the plasma of rats and dogs.

Keywords: Analytical method; Factor Xa inhibitor; YM466; Plasma; Rats; Dogs

1. Introduction

Anticoagulants currently available for the treatment and prevention of thromboembolic diseases are coumarins (e.g. warfarin) and thrombin inhibitors (e.g. low molecular weight heparin and argatroban). However, it is generally recognized that the greatest problem related to the clinical use of these inhibitors is bleeding complications [1,2]. On the other hand, Factor Xa inhibitors have not shown a significant prolongation of bleeding time in animals, due to their lack of action on thrombin [3,4]. YM466, [*N*-[4-[(1-acetimidoyl - 4 - piperidyl)oxy]phenyl]-*N*-[(7-amidino-2-naphtyl)methyl]sulfamoyl]acetic acid mesilate, is a novel low-molecular weight anticoagulant with a potent anti-Factor Xa activity [5]. This drug is therefore expected to be useful in the treatment of several thrombotic disorders, such as deep vein thrombosis, disseminated intravascular coagulation and unstable angina. As YM466 has been assumed to have a strong pharmacological activ-ity from the in vitro pharmacological finding that the plasma concentration required to double the clotting time was 1.27 and 0.13 μM in rats and dogs [5], a sensitive analytical method is essential

* Corresponding author. Tel.: +81-3-5916-2163; fax: +81-3-3960-6220.
E-mail address: mano@yamanouchi.co.jp (Y. Mano).

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for the evaluation of its disposition in plasma and tissues. Although the bioanalytical method for DX-9065a, the preceding anti-factor Xa inhibitor, was established in human plasma using radioimmunoassay [6], it would be preferable that chemical assay methods by chromatography are adopted, from the viewpoint of specificity and simplicity. This paper reports the development and validation of a sensitive method specific for the determination of YM466 by high-performance liquid chromatography with UV-detection (UV-HPLC) and the application of this method to rat and dog plasma samples for the pharmacokinetic evaluation of the drug in a preclinical study.

2. Experimental

2.1. Materials

YM466 and internal standard (I.S.; YM-59892) were supplied by Yamanouchi Institute for Drug Discovery Research Laboratories (Ibaraki, Japan). Their chemical structures are shown in Fig. 1. For the preparation of calibration standards and quality control (QC) samples, heparinized blank plasma was obtained from drug-free experimental animals. Reagent grade diethyl ether, ethyl acetate, and HPLC grade acetonitrile were purchased from Kanto Chemical Co. Ltd. (Tokyo, Japan). Reagent grade 1-sodium octane sulfonate was from Nacalai Tesque (Kyoto, Japan).

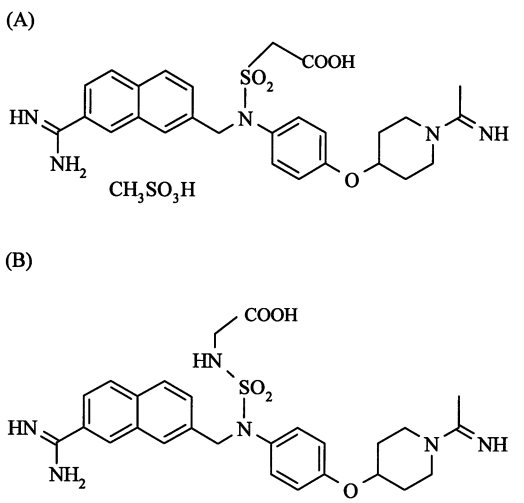

Fig. 1. Chemical structure of YM466 (A) and I.S. (B).

2.2. Apparatus and chromatographic conditions

The LC system consisted of a Shimadzu Model LC-10AT pump (Shimadzu, Tokyo, Japan) connected to a Shimadzu SIL-10A autosampler, a Shimadzu SPD-10AV spectrophotometric detector set at 240 nm and a Shimadzu C-R7A integrator. The eluent was a mixture of 50 mM phosphate buffer containing 5 mM 1-octane-sulfonate and acetonitrile (74:24, v/v) (the buffer was adjusted to pH 2.3). Separation was achieved on a TSK gel-ODS 80Ts (5 μm, 250 × 4.6 mm i.d.) reversed-phase column (TOSOH, Tokyo, Japan) at a column temperature of 40 °C. The HPLC system was operated at a flow rate of 1.0 ml/min.

2.3. Extraction procedure

To a 1.0-ml aliquot of plasma, 0.1 ml of internal standard solution (5000 ng/ml) and 1 ml of 50 mM phosphate buffer (pH 3.0) were added. The tube was mixed vigorously for 10 s, and then the residues were added onto a solid-phase extraction column, OASIS HLB (3 cc, 60 mg, Waters, MA, USA), conditioned with 2 ml of methanol and 2 ml of water. After washing the column with 2 ml of 10% ethanol, 2 ml of ethanol was employed to elute. The eluate was evaporated to dryness under reduced pressure, and the residue was reconstituted in 0.25 ml of 50 mM phosphate buffer (pH 2.3). In order to clean up the solution, 2 ml of diethyl ether was added. The tube was shaken for 10 min and then centrifuged for 10 min at 2200 rpm. The organic layer was removed, and following this, 2 ml of ethyl acetate saturated with water was added. The organic layer was again removed and a 0.05-ml aliquot was injected into the LC system.

2.4. Preparation of the calibration samples

A stock solution (0.1 mg/ml) of YM466 solution was prepared in 50 mM phosphoric acid for

calibration standards and QC samples. The stock solution was further diluted with 50 mM phosphoric acid to give samples of 10, 20, 50, 100, 200, 500, 1000 and 2000 ng/ml of YM466. These standard samples were treated as described above. The peak height ratios of YM466 to internal standard were plotted against nominal concentrations of YM466. The calibration curve was constructed by the least square method with 1/(peak height ratio) as a weighting factor.

2.5. Accuracy and precision

The intra-day accuracy and precision of the assay method were examined using rat and dog plasma containing 30, 750 and 1500 ng/ml of YM466. Six samples were determined for each concentration. The intra-day accuracy was expressed as the relative error, and precision as the relative standard deviation. The limit of quantification (LOQ) was defined as the lowest concentration with less than 20% of relative standard deviation and relative error between $\pm 20\%$ to obtain a predefined level of confidence [7].

2.5.1. Specificity

Rat and dog blank plasma samples from six drug-free male animals were extracted and assayed. The chromatograms were visually inspected for peaks from endogenous substances which might interfere with those of YM466 and I.S.

2.5.2. Recovery

The recovery of YM466 and I.S. from rat and dog plasma was determined at two concentrations (30 and 1500 ng/ml) for YM466 and one concentration (500 ng/ml) for I.S. The peak height ratios of YM466 added to blank plasma prior to the extraction procedure were compared with those in which YM466 was added after extraction (control).

2.5.3. Stability

The effect of three freeze–thaw cycles on YM466 in plasma was assessed at the concentrations of 30 and 1500 ng/ml. One set ($n = 3$) was assayed on the day of preparation as described above with calibration standards and the results were referred as standard (100%). The remaining sets of samples were stored at -20 °C and were assayed after three freeze–thaw cycles and their concentrations were determined with the calibration curve. The results were expressed as a percentage of the standard.

Also the stability of the analyte in plasma stored at -20 °C in rat and dog plasma at 30 and 1500 ng/ml was evaluated. Replicate samples were processed as described above and one set was analyzed on the day of preparation (day 1). The remaining sets of samples were assayed over a period of 91 or 92 days and their concentrations were determined with the respective calibration curve. The results of day 1 were taken as standard (100%) and the subsequent results were expressed as a percentage of the standard.

The stability of the analyte during the analytical procedure was evaluated with six samples extracted from plasma containing 30 and 1500 ng/ml of YM466. Of each concentration six samples were chromatographied immediately after being prepared and the same samples, after having been stored for 72 h at 4 °C in the injector of chromatograph. The results of samples determined immediately after the preparation were referred as standard (100%) and those of samples stored for 72 h were expressed as a percentage of the standard.

The stability of YM466 in the stock solution stored at 4 °C was assessed with three samples. After storing it at 4 °C, the stock solution was diluted by 20 times with 50 mM phosphoric acid. Thereafter, it was assayed over a period of 103 days with the control, the newly prepared solution at the concentration of 5 μg/ml. The peak height ratios of the samples were compared with those of the control and expressed as a percentage of the control.

2.5.4. Drug administration

Male F344 rats and male beagle dogs were given free access to standard pellet diets and water. YM466 was administered orally at 10 mg/kg to rats ($n = 3$) and 1 mg/kg to dogs ($n = 4$) following an overnight fast. In rats, blood was collected from the inferior vena cava with a hep-

Table 1
Summary of linear regression data for the determination of YM466 in rat and dog plasma

Species	Slope	Intercept	Correlation coefficient
Rat, day 1	0.002507	−0.004220	>0.999
Rat, day 2	0.002427	−0.005198	>0.999
Rat, day 3	0.002421	−0.003613	>0.999
Mean ± S.D.	0.002452 ± 0.00004801	−0.004344 ± 0.0007997	
C.V. (%)	1.96	−18.4	
Dog, day 1	0.002732	−0.002508	>0.999

Table 2
Accuracy and precision for the measurement of YM466 in rat and dog plasma

	Nominal concentration (ng/ml)	Measured concentration (ng/ml)	C.V. (%)	R.E. (%)
Rat				
Intra-day ($n=6$)	10	10.5	2.8	4.5
	30	31.6	0.8	5.0
	750	752.9	2.4	0.2
	1500	1503.8	2.1	0.1
Inter-day[a] ($n=18$)	30	31.5	2.3	4.7
	750	752.9	1.4	0.2
	1500	1499.8	1.4	−0.2
Dog				
Intra-day ($n=6$)	10	9.9	1.5	−1.5
	30	31.3	2.4	4.1
	750	769.3	1.8	2.5
	1500	1501.5	1.6	0.0

[a] Six samples at each concentration were measured on three different occasions.

arinized syringe under diethyl ether anesthesia. In dogs, blood was collected from a forelimb vein using a heparinized syringe. Plasma was obtained by centrifugation at $1870 \times g$ for 15 min and stored at −20 °C until the assay.

3. Results

3.1. Linearity

The calibration curves were linear over the concentration range from 10 to 2000 ng/ml and the slope and intercept for the calibration curves are listed in Table 1. The slope and intercept of the calibration curves were similar in both species investigated.

3.2. Accuracy and precision

The intra- and inter-day accuracy and precision data in rat plasma are shown in Table 2 and intra-day accuracy and precision data in dog plasma are shown in Table 2. In rat plasma, the intra-day precision, expressed as coefficient of variance (C.V.) for each QC concentration, was 0.8, 2.4 and 2.1%, respectively at 30, 750 and 1500 ng/ml. The intra-data accuracy, expressed as relative error (R.E.), ranged from 0.1 to 5.0%. The C.V. values for inter-day accuracy were in the range from 1.4 to 2.3%, and the R.E. values for inter-day precision were from −0.2% to 4.7%. The C.V. and R.E. values at LOQ were 2.8 and 4.5%, respectively. In dog plasma, the intra-day C.V. and R.E. values, including LOQ samples, ranged from 1.5 to 2.4% and −1.5 to 4.1%,

respectively. These results satisfied the validation criteria since the accuracy and precision were within ±15 and 15%, except for the LOQ where those were within ±20 and 20%, respectively.

3.3. Specificity

The representative chromatograms of rat blank plasma, dog blank plasma, plasma spiked with YM466 (50 ng/ml) and I.S., and plasma obtained from a rat 1 h after oral administration of YM466 at a dose of 10 mg/kg are shown in Fig. 2. The retention times of YM466 and I.S. were 17 and 20 min, respectively. No endogenous peaks which might interfere with those of YM466 and I.S. were observed.

3.4. Recovery

The mean extraction recovery of YM466 was 70 and 72% from rat plasma ($n = 3$), and 74 and 76% from dog plasma ($n = 3$) at 30 and 1500 ng/ml, respectively. That for I.S. was 81 and 70%, from rat and dog plasma, respectively (Table 3).

3.5. Stability

The effect of three freeze–thaw cycles on the stability of YM466 in rat plasma was evaluated by assaying samples at concentrations of 30 and 1500 ng/ml. The mean values after three freeze–thaw cycles were 94 and 95% of the initial values (Table 4) and the stability of the analyte in rat and dog plasma stored at −20 °C for 91 days were above 94% for concentrations of 30 and 1500 ng/ml. YM466 in analytical samples showed no degradation at 4 °C for 72 h during the actual analysis. The mean value of YM466 in the stock solution at 4 °C for 103 days was 98% of the initial one.

4. Discussion

YM466 possesses a functional group causing amphoteric ion formation under physiological conditions, and exhibits a high hydrophilic property with a calculated $\log P$ value of < -4. Therefore, a liquid–liquid extraction was not applicable for the extraction of this compound. In addition to that, no fluorescence detection was available despite the fact that this compound possesses a naphthalen structure. Taking this into account, the selected method for the bioanalysis of YM466 was solid-phase extraction with ultraviolet detection. Although the combination of solid-phase extraction with ultraviolet detection often causes problems in the specificity of the assay method, this method minimized them by using a water-saturated organic solvent for the clean-up of the eluates. In fact, the eluate without any clean-up process occasionally showed interference peaks at the retention times of YM466 and I.S. At first, tri-*n*-butyl phosphate saturated with water was used as the organic solvent in the clean-up process, however, the residual volume was not constant after removing the organic layer. This led us to use two organic solvents, diethyl ether followed by ethyl acetate, as the solvents for the clean-up. The typical chromatograms of plasma samples shown in Fig. 2 show no interfering peaks of endogenous substances at the anticipated retention times of YM466 and its I.S. The metabolism study with the dosing of ^{14}C-YM466 suggested

Table 3
Extraction recovery of YM466 and I.S. from rat and dog plasma

Matrix	Compound	Nominal concentration (ng/ml)	Recovery (%)	C.V. (%)
Rat plasma	YM466	30	69.5 ± 8.5	12.2
($n = 3$)		1500	72.2 ± 10.6	14.7
	I.S.	500	80.5 ± 3.6	4.5
Dog plasma	YM466	30	73.8 ± 5.2	7.0
($n = 3$)		1500	76.4 ± 4.3	5.6
	I.S.	500	70.1 ± 19.6	27.9

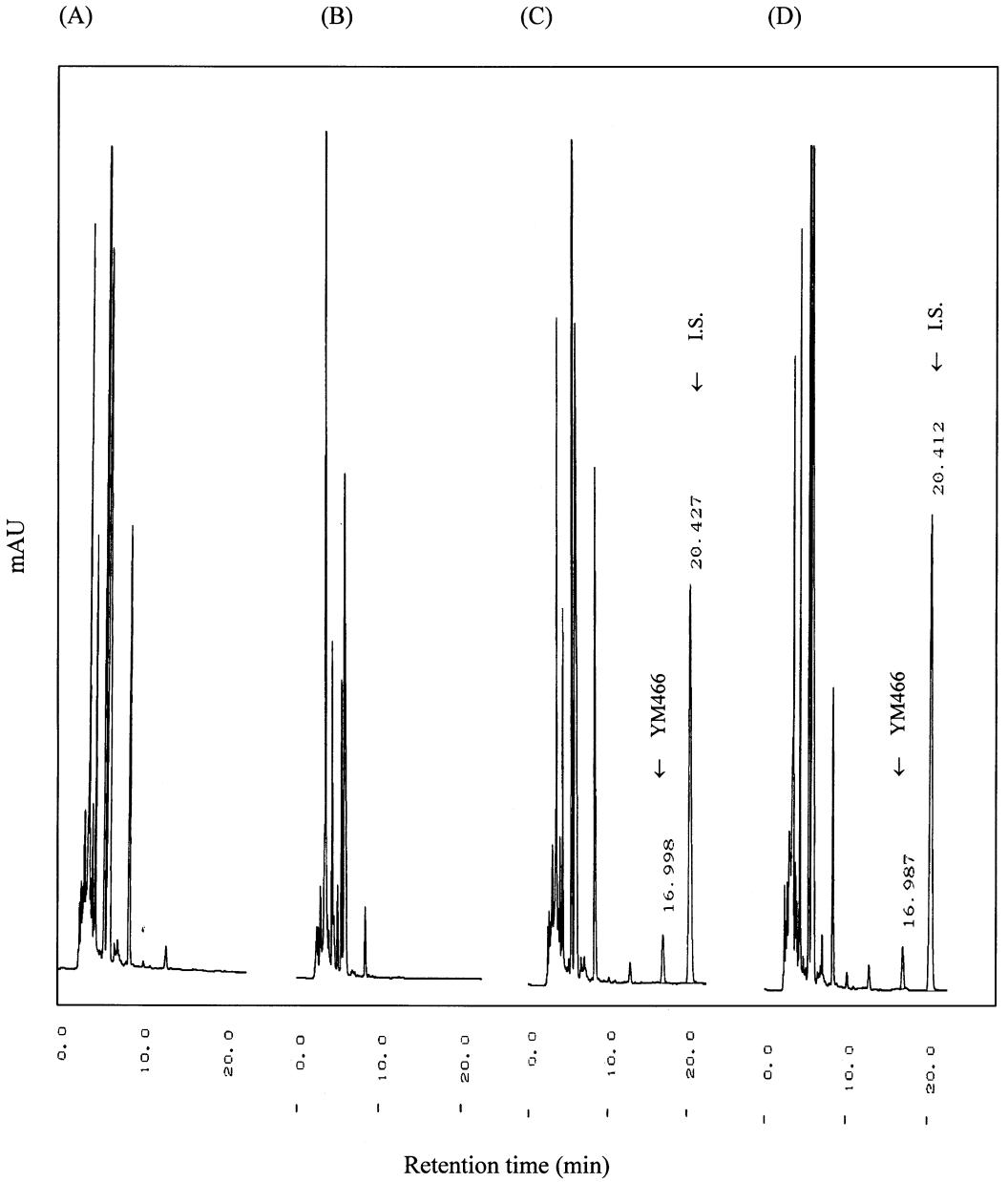

Fig. 2. Representative chromatograms from rat blank plasma (A), dog blank plasma (B), rat plasma spiked with YM466 (50 ng/ml) and I.S. (500 ng/ml) and rat plasma 1 h after oral administration of 10 mg/kg of YM466 spiked with I.S. (D).

that YM466 was not subjected to metabolism in rats, therefore, no interference with the peaks of metabolites was anticipated.

For the ruggedness of the assay, the influence of the mobile phase (percentage of acetonitrile) on the analytical procedure was investigated. Alter

Table 4
Stability of YM466 in plasma and analytical samples

Matrix	Treatment	Nominal concentration (ng/ml)	Percent of initial value (%)	C.V. (%)
Stock solution ($n=3$)	Stored at 4 °C for 103 days	100 000	98.0 ± 1.2	1.2
Analytical sample ($n=6$)	Stored at 4 °C for 72 h	30	99.3 ± 4.1	4.1
		1500	100.1 ± 1.3	1.3
Rat plasma ($n=3$)	Three freeze–thaw cycles	30	94.0 ± 1.2	1.2
		1500	94.8 ± 0.5	0.5
Rat plasma ($n=3$)	Stored at −20 °C for 91 days	30	96.0 ± 0.2	0.3
		1500	100.0 ± 1.2	1.2
Dog plasma ($n=3$)	Stored at −20 °C for 92 days	30	98.5 ± 2.0	2.0
		1500	94.0 ± 3.3	3.5

ations in the percentage of acetonitrile in the mobile phase from 24 to 33% significantly changed the retention times of YM466 (from 17 to 9.3 min) and I.S. (from 20 to 12 min). In the mobile phase, 1-octane-sulfonate was used as an ion-pair reagent for the appropriate retention of YM466 and I.S. since the percentage of acetonitrile was found to be too low for the considerable retention without 1-octane-sulfonate, where the low percentage of the organic solvent in the mobile phase frequently made the assay less rugged.

The stability of YM466 in plasma showed no degradation at −20 °C over at least 91 days and three freeze–thaw cycles stability showed that plasma samples did not show any loss of YM466. YM466 in the analytical samples was stable for at least 72 h at 4 °C during the actual analysis and the stock solution for YM466 was stable for at least 103 days at 4 °C. These stability studies demonstrate that YM466 is stable in plasma and during the actual analysis.

The utility of the assay was demonstrated by monitoring the plasma concentrations of unchanged drug after oral administration of YM466 at doses of 1 and 10 mg/kg to dogs and rats (Fig. 3). In rats, plasma concentration reached a maximum level of 221 ng/ml at 0.25 h after administration, then declined with a half-life of 1.9 h. In dogs, the maximum plasma concentration of 182 ng/ml was achieved at 0.5 h after administration.

5. Conclusion

A simple and sensitive method for the determination of YM466, a novel anticoagulant, in rat and dog plasma by HPLC was developed and validated. The method consists of sample preparation by solid-phase extraction, followed by the clean-up with organic solvent, chromatographic separation and UV detection. No interfering peaks were observed at the elution times of YM466 and I.S., and a sufficient specificity and good precision and accuracy over the concentration range of 10–2000 ng/ml was demonstrated. This method is accurate, reproducible, specific

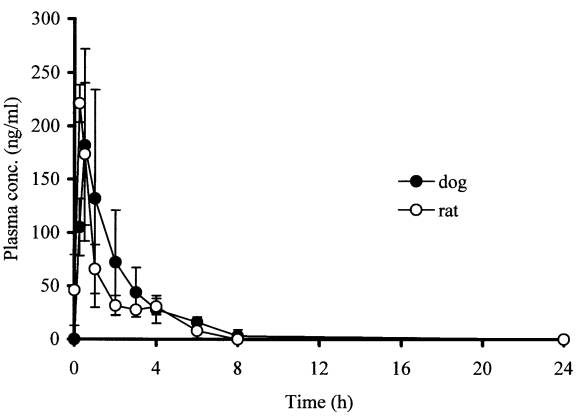

Fig. 3. Plasma concentration–time profiles of unchanged drug after oral administration of YM466 to rats and dogs at doses of 10 and 1 mg/kg, respectively. Values represent mean ± S.D. of three rats and four dogs.

and applicable to the evaluation of the pharmacokinetic profiles of YM466 in rats and dogs. The assay enabled us to evaluate more precisely the pharmacokinetic profiles of YM466 in these animals at low doses and proved to be useful in the analysis of more than 200 samples.

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