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Short communication

A liquid chromatographic–tandem mass spectrometric method for the determination of YM466, a novel Factor Xa inhibitor, in rat plasma

Yuji Mano*, 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 sensitive and selective method for the determination of YM466, a novel Factor Xa inhibitor, in rat plasma was developed and validated using liquid chromatography–mass spectrometry with electrospray ionization. Plasma samples were pretreated by solid-phase extraction and chromatographed on a C18 column (75 mm × 4.6 mm i.d.) with a mobile phase consisting of 0.1% formic acid–methanol (70:30 (v/v)), and detected using selected reaction monitoring in the positive-ion mode. The lower limit of quantification was 0.4 ng/ml, and good precision and accuracy were achieved. The validated method allowed analysis of samples for the determination of pharmacokinetic profiles of YM466 in rats.

Keywords: Validation; LC–MS/MS; Factor Xa inhibitor; YM466; Rat; Plasma

1. Introduction

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 [1]. After oral dosages of YM466 to rats, YM466 was not subjected to metabolism [2], and exhibited anticoagulant activity [3,4]. In order to estimate the effective plasma concentration of YM466 in rats, a sensitive analytical method is necessary. Although, we have already established the bioanalytical method for YM466 in the plasma of rats and dogs using high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection [5], the lower limit of quantification (LOQ) using 1 ml of plasma was 10 ng/ml. This LOQ value may not be sufficient to determine pharmacologically effective plasma concentrations for YM466 in rats. This is because effective plasma levels of YM466 in squirrel monkeys were 10.5 ng/ml [6], and species differences in the pharmacological response should be considered. Furthermore, the clean-up procedure for the pre-treatment process (e.g. washing with two kinds of organic solvents) has been time-consuming. A more sensitive determination method along with a simpler pre-treatment process would be highly desirable for assaying YM466 in rats at lower doses. As reported previously, assay for Factor Xa inhibitors in rat plasma using mass spectrometry had an LOQ of 5 nM [7]. This paper reports the development and validation of a sensitive method specific for the determination of YM466 in rat plasma by liquid chromatography–tandem mass spectrometry (LC–MS/MS). In addition, we have applied this method to the determination of YM466 plasma concentration in rats after intravenous infusion.

2. Experimental

2.1. Materials

YM466 and internal standard (I.S.), YM-59892 were supplied from the Chemical Technology Laboratories (Ibaraki,

* 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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Japan) and Drug Discovery Research Laboratories (Ibaraki, Japan) of Yamanouchi Pharmaceutical Co. Ltd., respectively. For the preparation of calibration standards and quality control (QC) samples, heparinized blank plasma was obtained from drug-free male Sprague–Dawley (SD) rats. HPLC grade methanol and formic acid were obtained from Kanto Chemical (Tokyo, Japan) and Nacalai Tesque (Kyoto, Japan), respectively. Filtered and deionized water (Milli-Q system, Millipore, MA, USA) was used. All other chemicals used were of analytical grade or HPLC grade and were obtained from commercial sources.

2.2. Chromatographic conditions

The HPLC system consisted of a Waters 616 pump, a Waters 717Plus autosampler, and a Waters 600S controller, (Waters, Milford, MA, USA). The mobile phase was 0.1% formic acid–methanol (70:30 (v/v)) and the chromatographic separation was performed using a Symmetry C18 column (75 mm × 4.6 mm i.d., Waters) maintained at 50 °C at a flow rate of 0.3 ml/min.

2.3. Mass spectrometric conditions

A TSQ7000 triple quadrupole mass spectrometer with an API source (Thermo Finnigan, San Jose, CA, USA) was used. The API source was fitted with an electrospray ionization inlet for ionizing the analytes. Nitrogen gas was employed as sheath and auxiliary gas with pressure of 70 psi and 15 units, respectively. Electrospray voltage was set at 4.5 kV and the heated capillary temperature was maintained at 270 °C. In the positive ion mode, YM466 and its I.S. were monitored with selected reaction monitoring (SRM) by transmitting the protonated molecular ions at *m*/*z* 269.7 and 277.2, respectively. These ions were fragmented by collision activated dissociation with argon (0.267 Pa) at −15 eV, then, the product ions were monitored at *m*/*z* 208.6 for YM466 and I.S. The total LC–MS run time was 4 min.

2.4. Extraction procedure

To a 1.0 ml aliquot of plasma, 1.0 ml of 0.1% formic acid, 0.1 ml of I.S. (100 ng/ml), and working standard solution were added. The tube was mixed vigorously for 10 s, and then was centrifuged for 5 min at 1870 × *g*. The supernatant was applied to a solid-phase extraction column, Oasis HLB (3 ml, 60 mg, Waters), which had been conditioned with 2 ml of methanol followed by 2 ml of water. To the Oasis HLB, 2 ml of 10% methanol (v/v) was added for clean-up followed by elution by 1 ml of methanol. The eluate was then evaporated to dryness under a stream of nitrogen. The residue was reconstituted in 0.2 ml of 0.1% formic acid and 0.05 ml aliquots were injected for LC–MS/MS analysis.

2.5. Preparation of the standard and QC samples

A stock solution of YM466 was prepared by dissolving YM466 in 0.1% formic acid to yield a final concentration of 200 μg/ml. The solution was stored at 4 °C. The stock solution was further diluted with 0.1% formic acid to make working standard solutions of 4, 10, 20, 100, 200, 400, and 1000 ng/ml of YM466. To prepare QC samples, another stock solution of YM466 was prepared as described above. QC solutions were prepared at concentrations of 40, 120, 600, and 8000 ng/ml, and then diluted 100-fold using drug-free plasma. These standard and QC samples were treated as described above. The peak area ratios of YM466 to internal standard were plotted against nominal concentrations of YM466. The calibration curve was constructed by the least square method with $1/(\text{concentration})^2$ as the weighting factor.

2.6. Accuracy and precision

The intra-day accuracy and precision of the assay method were evaluated using rat plasma at four concentrations of 0.4, 1.2, 6, and 80 ng/ml. These QC sample concentrations were selected to cover the whole range of the standard calibration curve. Six samples were determined for each concentration. The intra-day accuracy was expressed as the relative error (RE) and precision as the coefficient of variation (CV). The LOQ was defined as the lowest concentration with less than 20% CV and RE within ±20%.

2.7. Specificity

Blank plasma samples from six individual male rats were extracted and assayed. The chromatograms were visually inspected for peaks from endogenous substances, which might interfere with those of YM466 and I.S.

2.8. Recovery

The extraction recoveries of YM466 and I.S. from rat plasma were determined at two concentrations (6 and 80 ng/ml) for YM466 and at one concentration (10 ng/ml) for I.S. The peak area ratios of the analyte added to blank plasma prior to extraction and after extraction (control) were compared, and results were expressed as a percentage of control.

2.9. Matrix effects

The potential matrix effects on the analysis of YM466 and I.S. were investigated using rat plasma. Rat plasma (1.0 ml) was extracted by the method described above, and the residue was reconstituted in 0.1 ml of YM466 and I.S. solutions (100 ng/ml). The peak areas of the two analytes and the peak area ratio of YM466 to I.S. were compared to those in unextracted samples (in the reconstitution solution, no matrix).

Table 1
Extraction recovery of YM466 and I.S. from rat plasma

Analytes	Nominal concentration (ng/ml)	Recovery (%)
YM466 (n = 3)	6	78.6
	80	71.1
I.S. (n = 3)	10	84.1

Data represent the mean (n = 3).

2.10. Drug administration

Male SD rats were given free access to standard pellet diets and water. YM466 was intravenously administered at infusion rates of 10, 30, and 100 μg/h/kg to rats (n = 6/dose). Blood was collected from the inferior vena cava using a heparinized syringe at 40 min after the start of infusion. Plasma was obtained by centrifugation at 1870 × g for 15 min and stored at −20 °C until the assay.

3. Results and discussion

In the present study, a sensitive determination method for YM466 in rat plasma was established with LC–MS/MS. Although we have already established an HPLC–UV method [5], the present method using LC–MS/MS makes it possible to determine plasma concentrations 25 times lower. Furthermore, the pre-treatment procedures were much simplified and the analysis time was shortened, which resulted in greater efficiency.

In the HPLC–UV method, extensive washing after eluting analytes through the solid-phase column with two kinds of organic solvents was necessary for reducing interfering peaks which eluted at retention times close to the analytes, YM466 and I.S. In contrast, mass spectrometric detection enabled us to simplify the sample clean up due to its high specificity. Deleting the organic solvent washing process increased the extraction recovery of YM466 and I.S. from rat plasma slightly. The mean extraction recoveries of YM466 were 78.6% and 71.1% at 6 and 80 ng/ml, respectively. The recovery of I.S. was 84.1% (Table 1). In the previous method, the recoveries were 69.5% for YM466 and 80.5% for I.S.

The retention times of YM466 and I.S. were 3.1 min in the newly established method, while they were 17 min and 20 min, respectively, in the previous one. Along with these improvements, in the previous method, 1-octane-sulfonate, an ion-pair reagent, had to be added to the mobile phase to obtain sufficient retention of YM466 and I.S., which possesses hydrophilic properties. The slight alteration of organic solvent percentages in the mobile phase had a large impact on the retention times of analytes, which could lead to the specificity problems. The present method, with high specificity using LC–MS/MS, overcomes the uncertainty of the assay method mentioned above such as the poor ruggedness of the assay with respect to the retention times of the analytes. Representative chromatograms of rat blank plasma, plasma spiked with YM466 (1 ng/ml) and I.S., and rat plasma extracts after administration of 10 μg/h/kg of YM466 are shown in Fig. 1. No peaks derived from endogenous substances, which might interfere with the peaks of YM466 and I.S. were observed. In order to achieve the fast assay and similar retention times of YM466 and I.S., HPLC conditions with minimal retention of the analytes on the column were established.

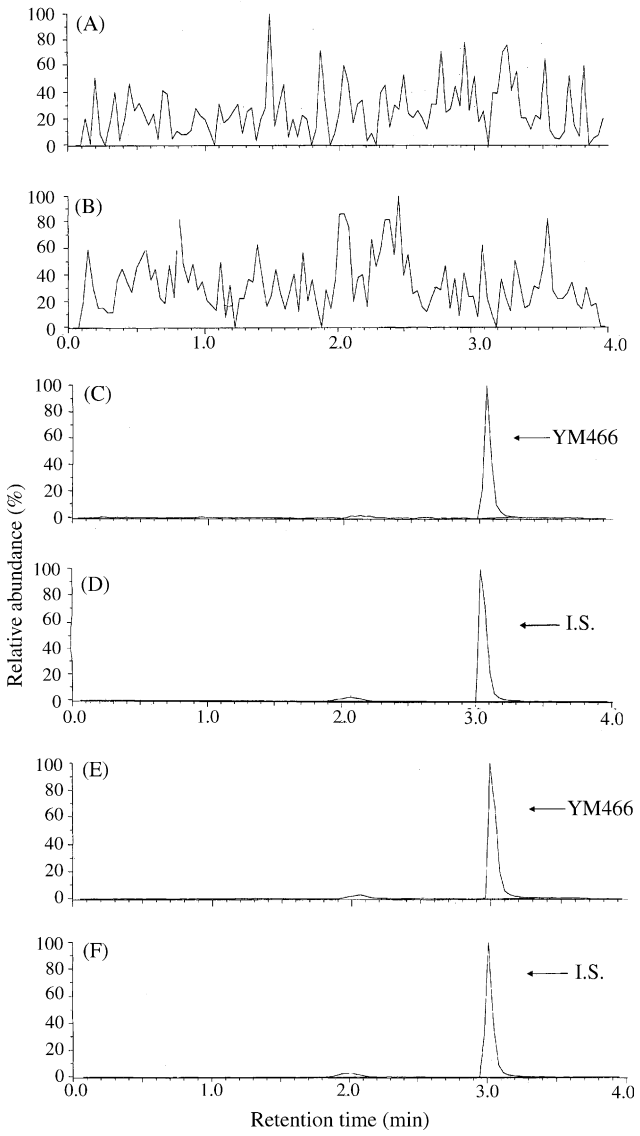

Fig. 1. Representative LC–MS/MS chromatograms of plasma extracts obtained from selected reaction monitoring at m/z 269.7 → 208.4 for YM466 (A, C, and E) and at m/z 277.2 → 208.4 for I.S. (B, D, and F). Upper figure (A and B): blank plasma. Middle figure (C and D): plasma containing 1 ng/ml of YM466 and 10 ng/ml of I.S. Lower figure (E and F): plasma extracts after 10 μg/h/kg of YM466.

Under such conditions the analytes may be susceptible to matrix effects, and co-eluting residual matrix components may have effects on the ionization of the target analytes. It has been reported that the solid-phase extraction pre-concentration step has magnified the matrix effect, and electrospray ionization was more vulnerable to matrix effects, as compared to atmospheric pressure chemical ioniza-

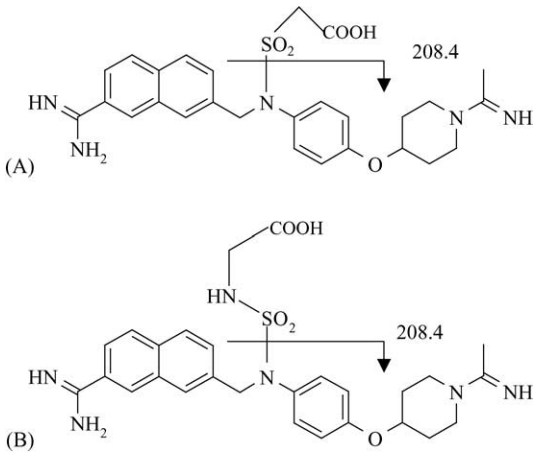

Fig. 2. Chemical structures of YM466 (A) and I.S. (B) Plus postulated structures of the product ions.

tion [8]. Thus, we investigated the potential for matrix effects on the analysis of YM466 and I.S. The absolute peak areas of YM466 and I.S. in the presence of the plasma extract were 74.8% and 70.4%, respectively, of those in the absence of plasma extract, suggesting slight matrix effects. However, the extent of suppression on the ionization of YM466 and I.S. was similar, and the peak area ratios of YM466 to I.S. were comparable, regardless of the presence of plasma extracts. These findings indicate the matrix effect had minimal impact on the analysis of YM466.

The high sensitivity achieved was attributable to the use of SRM detection, which can detect the selected fragment ion formed from its protonated molecular ion. The YM466 and I.S. protonated molecular ions were *m*/*z* 269.7 and 277.2, respectively, whilst the fragment ions of YM466 and I.S. from their protonated molecular ions were *m*/*z* 208.4 for both, indicating that the N–S bond in these chemicals was fragmented in the same manner (Fig. 2). In the development of this method, optimization was carried out regarding the collision energy and heated capillary temperature. The collision energy was set at −15 eV, as this study showed that deviation from this energy decreased the sensitivity of the assay. The temperature was set at 270 °C, as a lower temperature decreased the sensitivity. We tested different types of solid-phase extraction columns. The cartridges tested were Oasis HLB, Sep pak C18 (Waters) and Empore (3M Bioanalytical, St. Paul, MN, USA). Although the extraction recoveries were similar among all cartridges, Oasis HLB was selected due to its easy handling (e.g. there is no need to keep the cartridges wet throughout pre-treatment process). The chromatographic behavior of the analytes using a different mobile phase was also determined. The retention times of YM466 and I.S. was 4.9 and 7.0 min, respectively using a mobile phase consisting of 20 mM ammonium acetate–methanol (70:30 (v/v)) at a flow rate of 0.3 ml/min. However, the peak shape of the analytes was not satisfactory compared to that using 0.1% formic acid–methanol (70:30 (v/v)), which was selected as the mobile phase.

Table 2
Intra-day accuracy and precision for the measurement of YM466 in rat plasma

	Nominal concentration (ng/ml)	Measured concentration (ng/ml)	CV (%)	RE (%)
Intra-day (n = 6)	0.4	0.46 ± 0.03	7.6	13.9
	1.2	1.15 ± 0.17	14.8	−4.3
	6	5.51 ± 0.43	7.8	−8.1
	80	76.7 ± 7.06	9.2	−4.1

Data represent the mean ± S.D. (n = 6).

Table 3
Plasma concentration of YM466 at 40 min after the start of infusion to rats

Dose (μg/h/kg)	Plasma concentration (ng/ml)
10	10.0 ± 1.2
30	27.0 ± 1.6
100	72.9 ± 11.2

Data represent the mean ± S.D. (n = 6/dose).

The calibration curve was linear over the concentration range of 0.4–100 ng/ml, and the correlation coefficient was 0.989. The intra-day accuracy and precision data in rat plasma are shown in Table 2. The intra-day precision, expressed as CV for each QC concentration, was 14.8%, 7.8%, and 9.2% at 1.2, 6, and 80 ng/ml, respectively. The intra-data accuracy, expressed as RE, was −4.3% at 1.2 ng/ml, −8.1% at 6 ng/ml, and −4.1% at 80 ng/ml. These results were within the acceptance criteria (RE < ±15%, CV < 15%). The CV and RE values at the LOQ were 7.6% and 13.9%, respectively, which are also within the acceptance criteria (RE < ±20%, CV < 20%).

The present assay method was successfully applied to monitoring the plasma concentrations of unchanged drug after intravenous infusion of YM466 at doses of 10, 30, and 100 μg/h/kg to rats (Table 3). YM466 plasma concentrations were 10.0, 27.0, and 72.9 ng/ml, respectively, at 40 min after the start of infusion.

4. Conclusions

A sensitive method for the determination of YM466, a novel anticoagulant, in rat plasma by LC–MS/MS has been developed and validated. The method consists of sample preparation by solid-phase extraction, followed by detection in SRM mode using electrospray ionization. No interfering peaks were observed at the YM466 and I.S. elution times, and a sufficient specificity, good precision and accuracy was demonstrated in a calibration range of 0.4–100 ng/ml. This method is specific and applicable to the evaluation of the pharmacokinetic profiles of YM466 in rats.

References

[1] Y. Taniuchi, Y. Sakai, N. Hisamichi, M. Kayama, Y. Mano, K. Sato, F. Hirayama, H. Koshio, Y. Matsumoto, T. Kawasaki, Thromb. Haemost. 79 (1998) 543–547.
[2] Y. Mano, T. Sonoda, E. Nakamura, T. Usui, H. Kamimura, Biopharm. Drug. Dispos. 25 (2004) 253–260.
[3] T. Kawasaki, K. Sato, Y. Sakai, F. Hirayama, H. Koshio, Y. Taniuchi, Y. Matsumoto, Thromb. Haemost. 79 (1998) 410–416.
[4] K. Sato, T. Kawasaki, N. Hisamichi, Y. Taniuchi, F. Hirayama, H. Koshio, Y. Matsumoto, Br. J. Pharmacol. 123 (1998) 92–96.
[5] Y. Mano, T. Aoki, Y. Kikuchi, Y. Soeishi, T. Usui, H. Kamimura, J. Pharm. Biomed. Anal. 29 (2002) 631–638.
[6] Y. Iwatsuki, S. Kaku, Y. Moritani, Y. Taniuchi, F. Hirayama, H. Koshio, Y. Matsumoto, Y. Mano, T. Kawasaki, Drug Dev. Res. 58 (2003) 190–195.
[7] C. Chi, L. Liang, P. Padovani, S. Unger, J. Chromatogr. B 783 (2003) 163–172.
[8] R. Dams, M.A. Huestis, W.E. Lambert, C.M. Murphy, J. Am. Soc. Mass Spectrom. 14 (2003) 1290–1294.