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Highly sensitive high-performance liquid chromatographic determination method for a new erythromycin derivative, EM523, and its major metabolites in human plasma and urine using post-column tris(2,2′-bipyridine) ruthenium(III) chemiluminescence detection

Hisako Monji*, Masashi Yamaguchi, Isamu Aoki, Hayao Ueno

Takeda Analytical Research Laboratories Ltd., Juso-Honmachi, Yodogawa-ku, Osaka 532, Japan

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Abstract

A method for the simultaneous determination of de(N-methyl)-N-ethyl-8,9-anhydroerythromycin A 6,9-hemiacetal (EM523, I) and its three metabolites in human plasma and urine has been developed using high-performance liquid chromatography (HPLC) with chemiluminescence (CL) detection. Plasma and urine samples spiked with erythromycin as an internal standard were extracted with a mixture of dichloromethane and diethyl ether under alkaline conditions. The organic layer was evaporated under a stream of nitrogen gas. The reconstituted sample was injected into an HPLC apparatus and separated on an ODS column using a gradient elution method. The eluate was reacted on-line with a mixture of tris(2,2′-bipyridine) ruthenium(II) and peroxodisulfate, and the generated CL intensity was detected. Optimization of the CL reaction conditions resulted in a sensitive and stable CL intensity for the determination of I and its metabolites. The recovery of each compound from human plasma and urine, and the sensitivity, linearity, accuracy and precision of the method were satisfactory. The lower limits of quantitation for each compound using 0.2 ml of plasma and 0.1 ml of urine were 1 and 10 ng/ml, respectively. This method has been used for the determination of I in samples from clinical trials.

Keywords: Erythromycin derivative; EM523; De(N-methyl)-N-ethyl-8,9-anhydroerythromycin A 6,9-hemiacetal

1. Introduction

A new erythromycin derivative, de(N-methyl)-N-ethyl-8,9-anhydroerythromycin A 6,9-hemiacetal (EM523, I; Fig. 1), with strong gastrointestinal motor-stimulating activity, like motilin but without the antibiotic activity [1–3], is now under development as an accelerator of digestive-tract motility. Yamashita et al. [4] have reported a high-performance liquid chromatographic (HPLC) method for the determination of I in human plasma and urine. A column-switching technique with UV detection is employed in this method to obtain the necessary sensitivity and selectivity, since I shows only weak UV absorption (ϵ=8000 at 210 nm) in a short wavelength region [4]. However, the method requires a large amount of human plasma (1 ml) to quantitate I to concentrations as low as 1 ng/ml. Furthermore,

*Corresponding author.

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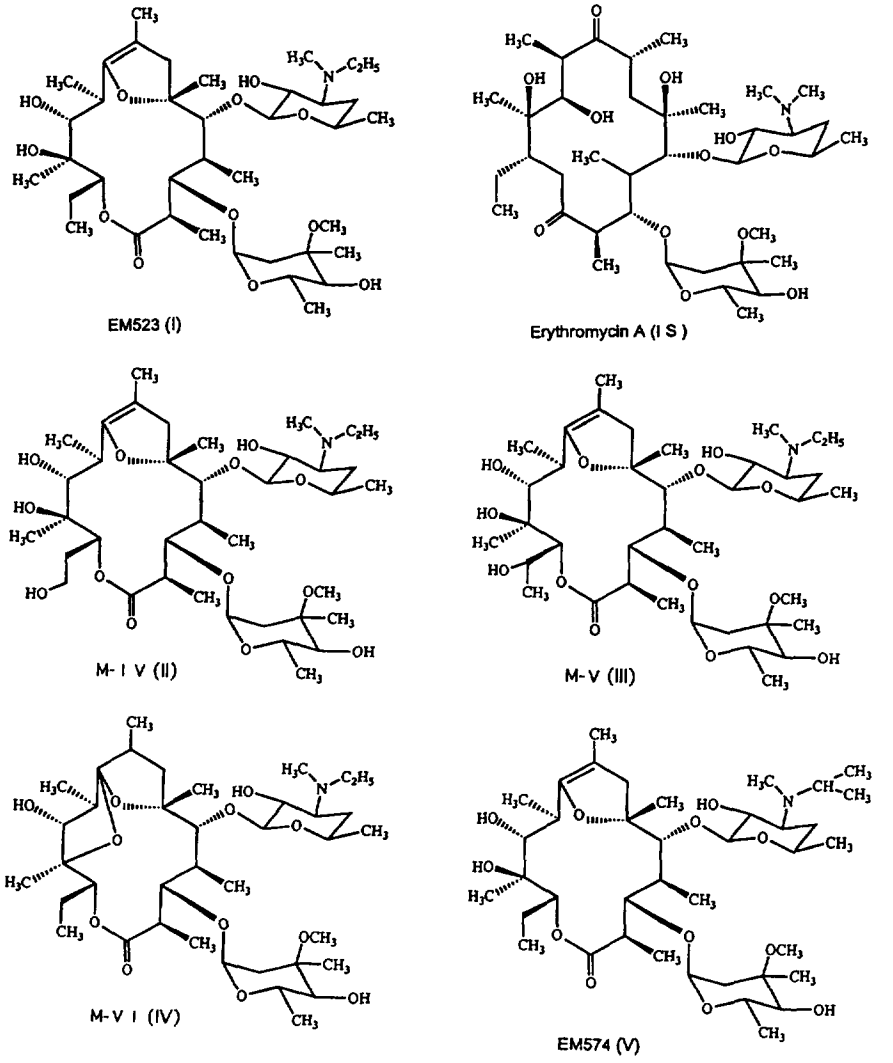

Fig. 1. Structures of erythromycin derivatives.

determination methods for active metabolites of I, which were found in animal plasma [5], have not yet been reported. In order to clarify the pharmacokinetics of I in humans and animals, the development of a simple and sensitive determination method for I and its metabolites in biological fluids was required.

Recently, a chemiluminescent (CL) method using tris(2,2′-bipyridine) ruthenium (II) [$Ru(bipy)_3^{2+}$] has become an attractive detection method for biochemical substances such as tertiary amines [6–8], oxalic acid [9] and derivatized amino acids [10], due to its high sensitivity and wide linear working range. The oxidation–reduction reaction scheme for CL from $Ru(bipy)_3^{2+}$ has been postulated by Rubinstein et al. [11].

$$Ru(bipy)_3^{2+} \rightarrow Ru(bipy)_3^{3+} + e^-$$

$$Ru(bipy)_3^{3+} + \text{reductant} \rightarrow \text{product} + [Ru(bipy)_3^{2+}]^*$$

$$[Ru(bipy)_3^{2+}]^* \rightarrow Ru(bipy)_3^{2+} + h\nu$$

The initial oxidation of $Ru(bipy)_3^{2+}$ to $Ru(bipy)_3^{3+}$ can usually be accomplished by electrochemical oxidation of the $Ru(bipy)_3^{2+}$ reagent. The oxidation is also afforded by UV or visible light irradiation in the presence of an oxidizing reagent such as potassium peroxodisulfate (PPS) [12,13]. The generated CL intensity is said to be proportional to the amount of reductants. This principle, however, has several difficulties: $Ru(bipy)_3^{3+}$ is a short-lived compound, so it has to be reacted with reductants just after the oxidation of $Ru(bipy)_3^{2+}$ and it has to be rapidly introduced into the detector. However, reliable equipment is not commercially available.

Considering that I and its metabolites M-IV (II, Fig. 1), M-V (III, Fig. 1) and M-VI (IV, Fig. 1), bearing tertiary amino moieties, might be reactive with $Ru(bipy)_3^{3+}$, we attempted to build a detection system from commercially obtained equipment and optimize the conditions of the CL reaction for HPLC analysis of these compounds.

This paper describes a sensitive HPLC method for the simultaneous determination of I and its metabolites in plasma and urine using CL detection with a combination of $Ru(bipy)_3^{2+}$, oxidizer and light irradiation. The application of the method for the samples from a clinical phase I study is also reported.

2. Experimental

2.1. Chemicals and reagents

Compounds I, II, III and IV were synthesized in the Discovery Research Laboratories of Takeda Chemical Industries (Tsukuba, Japan). Erythromycin was of biochemical reagent grade. Acetonitrile and dichloromethane were of HPLC grade. Diethyl ether was of pesticide residue analysis grade. Sodium hydroxide (1 *M*) solution was of volumetric analysis grade. Potassium peroxodisulfate (PPS) was of reagent grade. These reagents were purchased from Wako Pure Chemical Industries (Osaka, Japan). Tris(2,2′-bipyridine) ruthenium dichloride ($Ru(bipy)_3Cl_2 \cdot 5H_2O$, TBRC) was obtained from Sigma (St. Louis, MO, USA). All other reagents were of reagent grade or better and all were used without further purification. Water was distilled.

2.2. Instrumentation and conditions

The post-column HPLC analysis system is shown in Fig. 2. The system consisted of two LC-10AS pumps, an SIL-10A autosampler with cooling system,

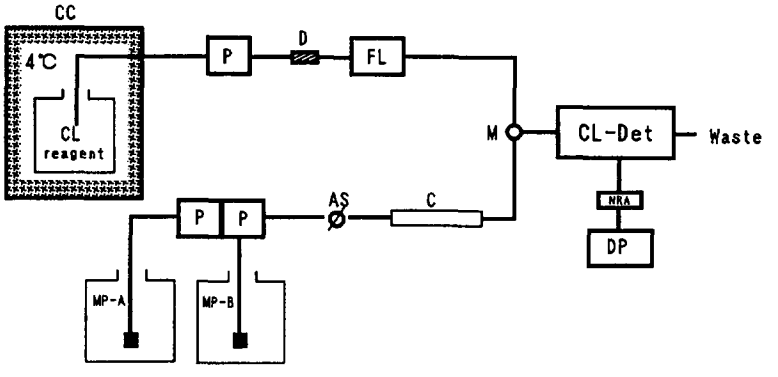

Fig. 2. Schematic diagram of the HPLC system. P = pump; AS = autosampler; C = column; CL-Det = chemiluminescence detector; NRA = noise-reducing apparatus; DP = data processor; CC = cooling chamber; D = dumper tube; FL = fluorescence spectrometer; M = mixing tee; MP-A, MP-B = mobile phases A and B, respectively.

an SCL-10A controller, a CTO-10AC column oven (all from Shimadzu, Kyoto, Japan), an L-6000 pump (Hitachi, Tokyo, Japan), an F-1000 fluorescence spectrometer (Hitachi), an 825-CL CL detector (Jasco, Tokyo, Japan), a UNI-3 noise-reducing apparatus (Union, Tokyo, Japan), an 805 data station (Nippon Waters, Tokyo, Japan) and a UC-65 cooling chamber (Tokyo Rikakikai, Tokyo, Japan). An Inertsil ODS-3 column (5 μm particle size, 150×4.6 mm I.D.; GL Science, Tokyo, Japan) was used at 50°C. The flow-rate of the HPLC mobile phase was 1 ml/min. Samples for HPLC were cooled at 7°C during analysis. The CL reaction reagent was cooled at 4°C and the flow-rate was 1 ml/min.

For the analysis of I, II, III and IV in plasma and urine samples, a gradient elution mode using two mobile phases was adopted: mobile phase A (MP-A), 20 m*M* monobasic potassium phosphate–acetonitrile (80:20, v/v) adjusted to pH 5.3 with 1 *M* NaOH; mobile phase B (MP-B), 20 m*M* monobasic potassium phosphate–acetonitrile (30:70, v/v) adjusted to pH 5.3 with H_3PO_4. Initially the mixing ratio of MP-A/MP-B was 88:12 (v/v), and the ratio was changed linearly to 58:42 (v/v) over 30 min. After the elution of analytes was completed, the column was washed with MP-B for 5 min and re-equilibrated under the initial conditions for 15 min before injecting the next sample.

2.3. Post-column reaction

In a light-protected glass bottle, reaction reagent (CL reagent) was prepared by dissolving 510 mg (0.7 mmol) of TBRC and 2.16 g (8 mmol) of PPS in 2 l of 20 m*M* phosphate buffer, pH 5, which was prepared by mixing 20 m*M* monobasic potassium phosphate and 20 m*M* dibasic potassium phosphate. The CL reagent was freshly prepared before analysis.

To reduce the pulsation caused by feeding of the CL reagent, PTFE dumper tubing (D; 0.2 mm I.D., tube length about 20 m) was positioned just in front of the fluorometer to pressure the tubing at about 30 kg/cm^2. Since the CL intensity was quenched by contact with metals, the use of metal tubing for the CL reagent line should be avoided as much as possible, and PTFE tubing that was completely protected from light with aluminum foil was used. Since the CL reaction is short lived (less than 1 s) [6], the tubing from the mixing tee (M) to the detector must be as short as possible.

Non-grated light from the xenon-lamp was used to irradiate the CL reagent in the flow-cell of the fluorometer to oxidize $Ru(bipy)_3^{2+}$ to $Ru(bipy)_3^{3+}$. If the irradiation for the activation into $Ru(bipy)_3^{3+}$ is too strong, it can cause great noise. The light strength was adjusted by changing the irradiation angle. A noise-reducing apparatus (UNI-3) was mounted on the detector. The eluted I, its metabolites and the internal standard (I.S.) from the column were immediately reacted with $Ru(bipy)_3^{3+}$ at the mixing tee, and the emission light was detected by the CL detector.

2.4. Preparation of stock and standard solutions

A stock solution containing a mixture of I, II, III and IV was prepared in acetonitrile (the concentration of each was 50 μg/ml). The stock solution can be stored at 4°C without loss of contents for at least five months. Standard solutions were prepared at 2, 10, 20, 100 and 200 ng/ml for the plasma assay and 10, 50, 100, 500 and 1000 ng/ml for the urine assay by serially diluting the stock solution with MP-A. Erythromycin (I.S.) was dissolved in acetonitrile (50 μg/ml) and further diluted with MP-A to prepare solutions of 400 ng/ml and 2.5 μg/ml for the plasma and urine assays, respectively.

2.5. Preparation of quality control (QC) samples

A stock solution was diluted with acetonitrile to prepare a 5 μg/ml solution. The solution (100 μl) was evaporated to dryness under a stream of nitrogen gas in a 10-ml glass tube. The residue was dissolved in 10 ml of drug-free human plasma (50 ng/ml, QC-1). QC-1 was further diluted with drug-free plasma to give final concentrations of 10 and 5 ng/ml, respectively.

Urine QC samples were prepared in a similar manner to those for plasma using a stock solution. The concentrations in urine QC samples were 500, 100 and 50 ng/ml. Aliquots of these QC samples were stored at −20°C until analyzed.

2.6. Calibration standards

Calibration standards were prepared by mixing 100 μl of each diluted standard solution with 200 μl of plasma or 100 μl of urine. The final concentrations of calibration standards were 1, 5, 10, 50 and 100 ng/ml for the plasma assay and 10, 50, 100, 500 and 1000 ng/ml for the urine assay.

2.7. Quantitation

Calibration curves were constructed by plotting peak-height ratios of each analyte to the I.S. obtained from calibration standards against corresponding concentrations. The weighted (1/concentration) linear regression lines were fitted over 100-fold concentration ranges. The concentration in samples was calculated from these curves.

2.8. Sample preparation

2.8.1. Plasma

To 200 μl of human plasma and QC samples were added 100 μl of MP-A, 20 μl of 0.1 *M* disodium hydrogenphosphate and 50 μl of the I.S. The mixture was extracted twice with 3 ml of a mixture of diethyl ether–dichloromethane (4:1, v/v). The organic layer was evaporated to dryness under a stream of N_2 gas at room temperature. The residue was dissolved in 200 μl of MP-A. A 150-μl volume was injected into the HPLC apparatus.

2.8.2. Urine

To 100 μl of human urine and QC samples were added 100 μl of MP-A, 50 μl of 0.1 *M* disodium hydrogenphosphate and 50 μl of the I.S. The mixture was extracted in the same manner as for plasma. The residue was dissolved in 500 μl of MP-A. A 100-μl volume was injected into the HPLC apparatus.

3. Results and discussion

3.1. Pretreatment of samples

Compound I is a hydrophobic compound, and a mixture of *n*-hexane–acetone (6:1, v/v) was used as the extracting solvent in the previous method [4]. However, the recovery of metabolites was not satisfactory with this extracting solvent. Accordingly, several extracting solvents were investigated and satisfactory recovery for I and its metabolites from plasma and urine was obtained using a mixture of diethyl ether–dichloromethane (4:1, v/v) under alkaline conditions. Erythromycin has a structure similar to that of I and had properties that were similar to those of I regarding elution behavior when using an ODS column and in recovery from the samples. Thus, it was selected as the I.S. The I.S. was found to be stable (>98%) for 36 h in MP-A. Based on these results, the sample preparation method described in Section 2 was adopted.

3.2. CL conditions

Compound I and its metabolites show only weak UV absorption in the short wavelength region and have no appropriate functional groups to be derivatized to fluorescent compounds showing high sensitivity and selectivity. These properties previously prevented simultaneous and sensitive HPLC determination of each analyte in biological fluids. Recently, a sensitive and selective detection method using the CL reaction of tertiary amines with $Ru(bipy)_3^{3+}$ was reported for HPLC [6–8]. As I and its metabolites contain a tertiary amine in their chemical structures, CL detection was considered to be useful for our purpose.

$Ru(bipy)_3^{3+}$ is unstable and therefore it has to be prepared just before use by oxidizing $Ru(bipy)_3^{2+}$. The methods reported for oxidation have been electrochemical oxidation [7–10] and irradiation in the presence of an oxidizing reagent such as PPS [12,13]. The former method (used in most studies) requires a special oxidizing system [7–9] and the latter requires a laboratory-made cooling system to avoid the heat generated by the light source [12]. First, we examined the possibility of oxidation using PPS without light irradiation. However, the peak heights for I and its metabolites varied greatly with time, and high sensitivity proved unattainable. Therefore, routine analysis of many samples in a day would be difficult.

Recently, Morita et al. [13] reported a highly sensitive determination method for tetracyclines and tiamulin fumarate in pork meat using HPLC with

$Ru(bipy)_3^{2+}$–PPS CL reagent, in which $Ru(bipy)_3^{3+}$ was obtained by irradiation with light of the full wavelength range (non-grated light) from a xenon-lamp in the flow-cell of the fluorometer. In their investigation, it was found that the best results were obtained without irradiation, since irradiation with the xenon-lamp light increased the background noise.

We tested a similar system using non-grated light irradiation. A nearly constant peak height for I and a peak-height ratio (I.S. ratio) were obtained, indicating the possibility of using this technique for routine analysis. Moreover, details such as the flow-rate of the CL reagent, concentrations of PPS, TBRC and phosphate, pH of the CL reagent and light strength of the xenon-lamp were investigated.

Fig. 3 shows the effect of pH in the range of 3.5–7 on CL intensity. The highest CL intensity was observed at pH 5. The phosphate concentration in the CL reagent did not affect the CL intensity (data not shown). The CL reaction was not observed without PPS. The CL intensity increased with increasing PPS concentration up to 4 m*M*, but higher concentrations resulted in decreased CL intensity (data not shown). The CL intensity was dependent on the concentration of TBRC (up to 0.7 m*M*), but higher concentrations resulted in decreased CL intensity (data not shown). At concentrations higher than 0.7 m*M*, crystallization of TBRC was also observed in the refrigerated CL reagent. The highest *S/N* ratio was obtained with a CL reagent flow-rate of 1 ml/min (data not shown). A flow-rate of less than 1 ml/min resulted in slight enhancement of the CL signal, while background noise was also increased.

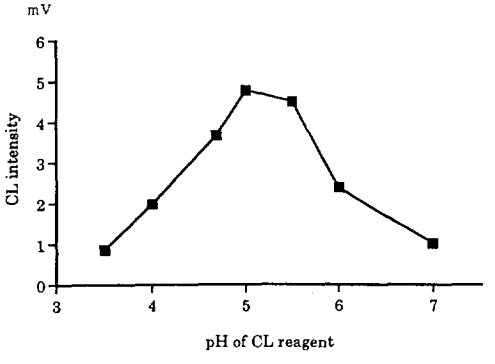

Fig. 3. Effect of the pH of the CL reagent on CL intensity. The isocratic elution mode was employed with acetonitrile–20 m*M* potassium phosphate (36:64, v/v; pH 5.3) as the mobile phase.

3.3. Chromatographic conditions

On the chromatograms obtained from isocratic HPLC with an ODS column, interference was observed at the retention times of I and its metabolites. To separate the analytes from the endogenous materials in plasma and urine that caused the interferences, a gradient elution method was employed. Fig. 4 shows the chromatograms of drug-free plasma and urine and of spiked samples. Each analyte was

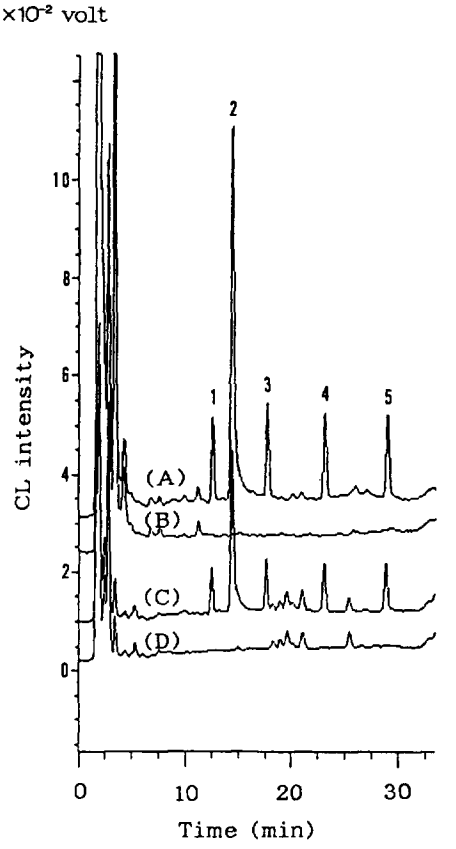

Fig. 4. Chromatograms of (A) human urine spiked with I; its metabolites (100 ng/ml of each compound) and the I.S. (1.25 μg/ml); (B) drug-free human urine; (C) human plasma spiked with I, its metabolites (10 ng/ml of each compound) and the I.S. (100 ng/ml) and (D) drug-free human plasma. Peaks: 1 = II; 2 = I.S.; 3 = III; 4 = IV and 5 = I.

Table 1
Linearity of calibration curves for I and its metabolites added to human plasma and urine

Compound	Range	Equation parameters[a]		
		Slope	y-Intercept	Correlation coefficient
Plasma				
I	1–100 ng/ml	0.0340(1.2)	−0.0018	0.99990(0.0)
II	1–100 ng/ml	0.0320(1.0)	−0.0023	0.99990(0.0)
III	1–100 ng/ml	0.0383(1.0)	−0.0067	0.99985(0.0)
IV	1–100 ng/ml	0.0346(1.0)	−0.0024	0.99995(0.0)
Urine				
I	10–1000 ng/ml	0.00231(1.9)	0.00038	0.99992(0.0)
II	10–1000 ng/ml	0.00229(0.8)	−0.00142	0.99996(0.0)
III	10–1000 ng/ml	0.00266(1.5)	−0.00211	0.99996(0.0)
IV	10–1000 ng/ml	0.00242(1.4)	0.00119	0.99995(0.0)

[a] Peak-height ratio (Y) = slope × concentration (X) (ng/ml) + Y-intercept.
Values in parentheses are coefficients of variation (%) ($n = 5$). Each parameter was calculated with weighted (1/concentration) linear regression.

separated from the endogenous compounds and could be determined with high sensitivity.

3.4. Linearity, recovery, precision, accuracy and the limit of quantitation

The calibration curves were obtained by analyzing calibration standards in the concentration ranges of 1–100 ng/ml for plasma and 10–1000 ng/ml for urine. Good linearity was observed between the peak height ratio (Y) and the corresponding plasma and urine concentrations (X) (Table 1). The absolute recovery from plasma was 99.6–105.2%, 99.7–105.0%, 98.4–102.3%, 99.5–104.3% and 93.8–106.2% for I, II, III, IV and I.S., respectively. The absolute recovery from urine was 96.3–107.1%, 97.8–101.6%, 98.2–100.4%, 98.3–106.1% and 93.1–101.5% for I, II, III, IV and I.S., respectively. Table 2 shows the accuracy and precision of the quantified values for calibration standards back-

Table 2
Intra-day precision and accuracy for the determination of I and its metabolites added to human plasma and urine

Concentration added (ng/ml)	Mean concentration found (ng/ml)			
	I	II	III	IV
Plasma				
100.0	100.1 (1.2)	100.0 (0.8)	100.5 (1.0)	100.0 (1.1)
50.0	50.2 (1.6)	50.3 (1.5)	49.9 (1.5)	50.0 (1.7)
10.0	9.7 (2.5)	9.7 (4.6)	9.5 (4.4)	10.0 (2.0)
5.0	5.0 (1.7)	5.0 (2.1)	5.0 (1.7)	5.0 (2.0)
1.0	1.0 (4.0)	1.0 (5.0)	1.1 (8.1)	1.0 (4.8)
Urine				
1000	993 (2.2)	996 (1.1)	999 (1.9)	996 (1.7)
500	507 (1.6)	504 (0.6)	503 (1.0)	504 (1.1)
100	100 (1.8)	100 (1.8)	99 (1.5)	100 (1.8)
50	50 (0.6)	50 (0.6)	50 (1.2)	50 (1.0)
10	10 (1.5)	10 (5.0)	10 (4.5)	10 (4.3)

Values in parentheses are coefficients of variation (%) ($n = 5$).

Table 3
Inter-day precision and accuracy for the determination of I and its metabolites in quality control samples of human plasma and urine

Concentration added (ng/ml)	Mean concentration found (ng/ml)			
	I	II	III	IV
Plasma				
50.0	47.2 (5.6)	46.5 (8.0)	44.8 (8.3)	43.4 (5.7)
10.0	9.8 (2.1)	9.5 (3.2)	9.5 (1.6)	9.0 (1.1)
5.0	4.9 (1.2)	4.8 (2.4)	4.7 (3.3)	4.5 (1.3)
Urine				
500	502 (4.4)	514 (5.0)	509 (3.3)	524 (7.5)
100	100 (5.5)	99 (2.7)	102 (6.8)	98 (9.7)
50	47 (4.4)	47 (2.1)	50 (6.2)	50 (3.0)

Values in parentheses are coeffecients of variation (%) ($n=3$).

calculated from the calibration curves for plasma and urine. Table 3 shows the inter-day precision after analysis of QC samples for three days. These results indicated that the method would be acceptable for the quantitation of plasma and urine concentrations in clinical studies. The lower limit of quantitation of the present method was 1 ng/ml for plasma and 10 ng/ml for urine samples. The use of a large sample amount (e.g. 1 ml) may offer higher sensitivity, because no interference was observed at the retention time of any analyte (Fig. 4).

3.5. Application to a clinical phase I study

This method has been used for the determination of I and its metabolites in human plasma and urine after intravenous administration of I. The clinical specimens were obtained by Shimizu Pharmaceuticals. Fig. 5 shows the mean plasma concentration curve for I after intravenous administration of 4 mg of I to six volunteers. Compound II was detected in plasma at a concentration around the lower limit of quantitation in two out of six volunteers. The amounts of the other metabolites were less than the lower limits of quantitation. Compound I and all the metabolites were detected in urine. The mean urinary excretion within 24 h was 9.41%, 0.81%, 1.11% and 0.44% for I, II, III and IV, respectively (Fig. 6). Over 300 clinical samples were successfully analyzed using this system. The validity of this method was confirmed by comparison with the previously reported HPLC method using UV detection [4]. The concentrations of I in 36 clinical plasma samples determined by the two methods after administration of I were almost identical (Fig. 7).

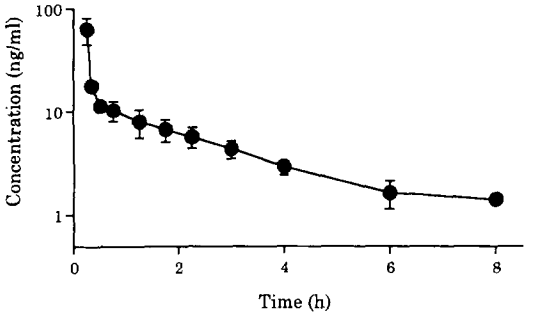

Fig. 5. Mean plasma levels of I after intravenous administration of 4 mg of I to volunteers ($n=6$). Each point and bar represent the mean ($n=6$) and the standard deviation, respectively.

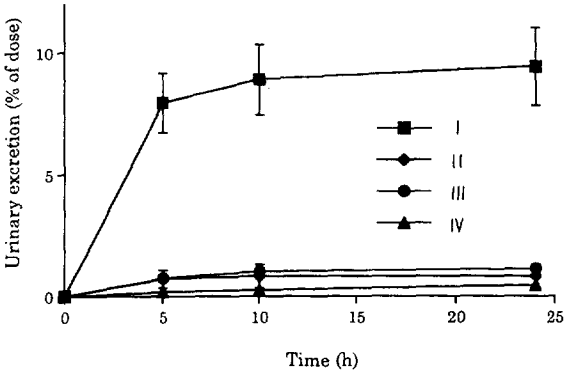

Fig. 6. Mean cumulative urinary excretion of I and its metabolites after intravenous administration of 4 mg of I to volunteers ($n=6$). Each point and bar represent the mean ($n=6$) and the standard deviation, respectively.

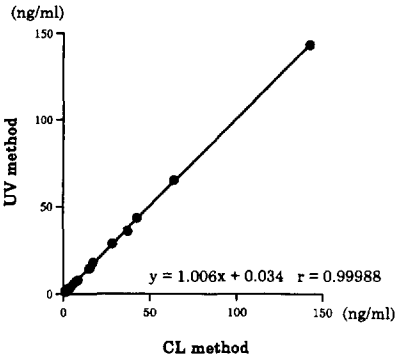

Fig. 7. Correlation of plasma concentrations of I determined by the previous method (UV) and the present method (CL) ($n = 36$).

This HPLC system using CL detection was also used for the determination of de(N-methyl)-N-isopropyl-8,9-anhydroerythromycin A 6,9-hemiacetal (EM574; V in Fig. 1), the chemical structure and the pharmacological activities of which are similar to those of I. Using 1 ml of plasma and by modifying the gradient elution program in the present method, 0.1 ng/ml of V and its metabolites could be determined. The results will be reported elsewhere.

4. Conclusion

By optimizing the conditions for the oxidation of $Ru(bipy)_3^{2+}$ to $Ru(bipy)_3^{3+}$ in the presence of PPS, stable CL intensity and high sensitivity were obtained for an HPLC method for the simultaneous determination of I and its metabolites in human plasma and urine. This method has been successfully used for the determination of I in clinical samples, and over 300 samples have been analyzed within two months without any trouble, indicating the ruggedness of the method. This system might be widely applicable for the determination of other tertiary amines.

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