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Application of liquid chromatography–turbo ion spray tandem mass spectrometry for quantitative analysis of a potent motilin receptor agonist, EM574, and its metabolites in human plasma

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Abstract

A liquid chromatography–tandem mass spectrometry (LC–MS–MS) method for the simultaneous determination of a new potent motilin receptor agonist as erythromycin derivative, EM574 (erythromycin derivative), and its three metabolites, M-IV, M-V and M-VI, in human plasma was developed. The internal standards (I.S.s) used were deuterated EM574, M-IV and M-V. For the quantitation of M-VI, deuterated M-V was used. The analytes and I.S. were extracted from plasma samples with diethyl ether at neutral pH. A turbo ion spray interface was used as the ion source of LC–MS–MS, and the analysis was performed in the selected reaction monitoring mode. The lower quantitation limits for all the analytes were 0.05 ng/ml when 0.2 ml of plasma was used, and the standard curves were linear in the range 0.05 to 20 ng/ml. The method was precise; the intra- and inter-day precisions of the method were not more than 19.8% for all the analytes. The accuracy of the method was good with the deviations between added and calculated concentrations of each analyte being typically within ±11.2%.

Keywords: EM574; Motilin receptor agonist; Erythromycin derivative

1. Introduction

A new erythromycin derivative, de(*N*-methyl)-*N*-isopropyl-8,9-anhydroerythromycin A 6,9-hemiacetal (EM574, Fig. 1), has a potent motilin-like activity, but has no antibacterial activity, and accelerates gastric emptying in a dose-dependent manner in humans given this drug orally [1–4]. According to these characteristics, EM574 is currently under development as a prokinetic agent.

Two types of high-performance liquid chromatography (HPLC) methods for the quantitation of an EM574-related compound, EM523 [de(*N*-methyl)-*N*-ethyl-8,9-anhydroerythromycin A 6,9-hemiacetal; Fig. 1], using a column-switching technique [5] and post-column chemiluminescence detection with a combination of tris(2,2′-bipyridine)ruthenium (II), oxidizer, and light irradiation [6] were developed and reported previously. Since the chemical structures of EM523 and its metabolites are very similar to those of EM574 and its metabolites except for a single

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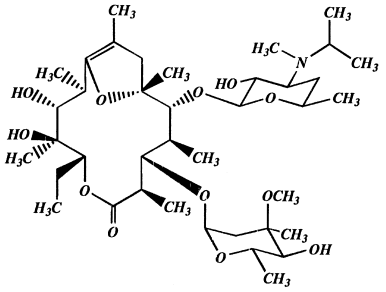
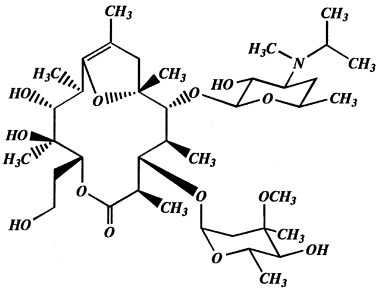
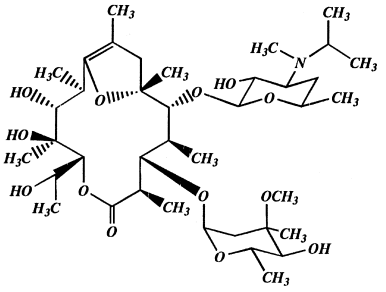


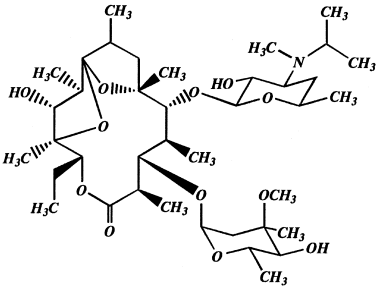
EM574



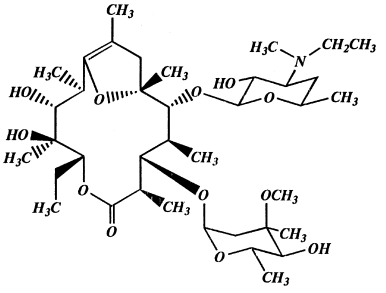
M-IV



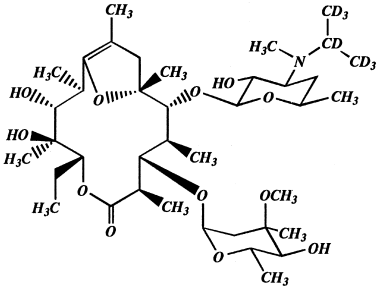
M-V



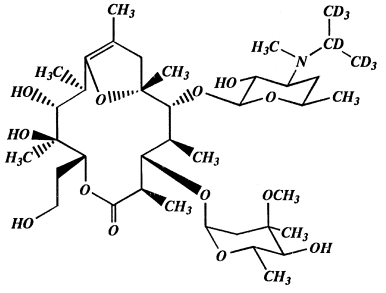
M-VI



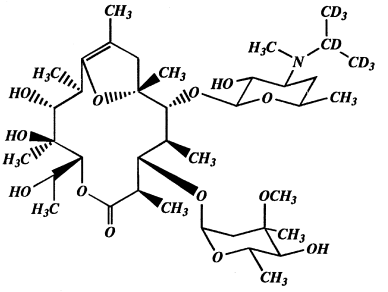
EM523



EM574-d_7



M-IV-d_7



M-V-d_7

Fig. 1. Chemical structures of EM574, M-IV, M-V, M-VI, EM523, EM574-d_7, M-IV-d_7 and M-V-d_7.

N-ethyl group on the desosamine moiety, it was considered that the above methods could be applied to the determination of EM574 and its metabolites, M-IV, M-V and M-VI. When the chemiluminescence detection method was applied to the quantitation of EM574 and its metabolites in human plasma, the lower quantitation limit was 0.1 ng/ml for all the analytes [6,7]. However, the method's procedure was complex and time-consuming, and the chromatographic analysis time for one sample was very long (about 50 min). In addition, the volume of plasma needed for the quantitation was relatively large (1 ml). As for the column-switching method, the chromatographic run time is relatively long (about 30 min) and the metabolites could not be determined by the method, as it is a very simple analytical procedure. Therefore, a more sensitive and rapid method for quantifying EM574 and its metabolites in human plasma is required to characterize the pharmacokinetic properties of EM574 in humans quickly and allow the clinical trials to proceed efficiently.

Recently, several liquid chromatography–mass spectrometry (LC–MS) and LC–MS–MS methods have been reported for the determination of erythromycin and its derivatives [8–12]. The sensitivity and speed of the mass spectrometric analysis were demonstrated clearly in these papers. Especially, the selected reaction monitoring (SRM) technique in LC–MS–MS allows a selective and specific quantitative analysis of the biological samples. Therefore, we tried to establish a quantitation method for EM574 using LC–MS–MS.

This paper describes the development of a highly sensitive LC–MS–MS method for the simultaneous determination of EM574, M-IV, M-V and M-VI in human plasma.

2. Experimental

2.1. Materials and reagents

Authentic samples of EM574, M-IV, M-V and M-VI (Fig. 1) were synthesized in the Pharmaceutical Production Division and Pharmaceutical Discovery Research Division, Takeda Chemical Industries (Osaka, Japan). Deuterium-labeled EM574, M-IV and M-V (EM574-d_7, M-IV-d_7 and M-V-d_7, respectively; Fig. 1) as internal standards (I.S.s) were prepared in the Pharmaceutical Discovery Research Division and Pharmaceutical Research Division, Takeda Chemical Industries. The isotope purity for all the deuterium-labeled compounds was 100%. HPLC-grade methanol and acetonitrile, and analytical grade diethyl ether, disodium hydrogenphosphate, sodium dihydrogenphosphate and ammonium acetate were purchased from Wako (Osaka, Japan). Drug-free human plasma samples were obtained from Nippon Bio-Supp. Center (Tokyo, Japan).

2.2. Instrumentation and conditions for LC–MS–MS analysis

The LC–MS–MS system consisted of a Waters Alliance 2690 separations module including in-line degasser, sample cooler, column heater and seal wash (Waters, Milford, MA, USA) and a Perkin-Elmer Sciex API365 triple quadrupole mass spectrometer with turbo ion spray interface (Toronto, Canada).

The HPLC column used was YMC J'sphere ODS-M80 (150×2.0 mm I.D., YMC, Kyoto, Japan). A mixture of 10 mmol/l ammonium acetate (neutral pH)–methanol–acetonitrile (11:20:70, v/v/v) was isocratically used as the mobile phase at a flow-rate of 0.2 ml/min. The column temperature was maintained at 40°C.

MS–MS detection was performed by the SRM from the protonated molecular ion ($[M+H]^+$) of analyte to its product ion under the positive ion mode. The product ion efficiency of the $[M+H]^+$ was optimized by varying the turbo ion spray, orifice and focusing ring voltages. The turbo ion spray, orifice and focusing ring voltages were set to 5000, 60 and 260 V, respectively. The flow-rates of curtain gas (N_2; 99.999% purity), nebulizer gas (zero grade air; 99.999% purity), and heater gas (zero grade air) were 1.25, 1.46 and 7 l/min, respectively, and the turbo probe temperature was 425°C. The collision-induced dissociation (CID) of the $[M+H]^+$ of analytes was performed at quadrupole 2 using N_2 gas at a pressure of 2.7×10^{-5} Torr (1 Torr=133.322 Pa) and a collision energy of −40 eV. These CID parameters were optimized by programmatically varying the collision gas pressure and collision energy to obtain the maximum intensity and repro-

ducibility of the product ion. The monitoring ions (precursor ion→product ion) for SRM analyses were set to m/z 744.5→586.4 for EM574 and M-VI, m/z 760.5→602.4 for M-IV and M-V, m/z 751.6→593.5 for EM574-d_7, and m/z 767.5→609.4 for M-IV-d_7 and M-V-d_7. The scan dwell time and multiplier voltage were set to 500 ms and 2.4 kV, respectively.

2.3. Preparation of standard solutions

Each stock solution of EM574, M-IV, M-V, M-VI, EM574-d_7, M-IV-d_7 and M-V-d_7 (each 100 μg/ml) was prepared by dissolving about 2 mg of each compound into 20 ml of acetonitrile. The stock solutions were refrigerated at 4°C and used within one month.

Equal volumes of each stock solution of EM574, M-IV, M-V and M-VI were mixed together and serially diluted with the mobile phase for LC–MS–MS analysis to provide working solutions with concentrations of 80, 40, 20, 4, 2, 0.8, 0.4 and 0.2 ng/ml for each compound. Besides, equal volumes of each stock solution of EM574-d_7, M-IV-d_7 and M-V-d_7 were mixed together and were serially diluted with acetonitrile to prepare the working I.S. solution (50 ng/ml for each compound). The working solution and working I.S. solution were prepared daily for analysis.

2.4. Preparation of standard and quality control (QC) samples

The working solutions (50 μl) were evaporated to dryness under a stream of nitrogen gas at ambient temperature and residues were dissolved in 200 μl of drug-free human plasma to prepare the standard samples with concentrations of 20, 10, 5, 1, 0.5, 0.2, 0.1 and 0.05 ng/ml for each compound. Similarly, QC samples with three concentrations of 10 (high), 1 (medium), and 0.2 ng/ml in plasma (low) for each analyte were prepared from the stock solution and working solution described above. These samples were prepared at the beginning of the validation and were stored at −20°C.

2.5. Extraction procedure

A 0.2-ml aliquot of plasma sample was mixed with 20 μl of the working I.S. solution and 0.2 ml of 50 mmol/l phosphate buffer aqueous solution (pH 7.0). The analytes and I.S. were extracted with diethyl ether (3 ml) using a vortex-mixer. The diethyl ether extraction was performed twice. The combined organic layer was evaporated to dryness, and the residue was dissolved in 0.3 ml of ethanol and evaporated under a steam of nitrogen gas again. The residue obtained was reconstituted in 0.1 ml of the mobile phase and transferred to clean auto-sampler injection vial. A 20-μl portion of the solution was injected into LC–MS–MS system.

2.6. Calculation and validation

The standard curve was obtained by a $1/y$ weighted least-squares linear regression on the peak area ratios of each analyte to those of I.S. versus the concentrations of each analyte in the spiked samples:

$$y = a + b \times C_{\text{theor}}$$

where, y, C_{theor}, a and b are the peak area ratio, the analyte concentration, the y-intercept, and the slope, respectively. EM574-d_7, M-IV-d_7, M-V-d_7 and M-V-d_7 were used as the I.S.s for EM574, M-IV, M-V and M-VI, respectively. The concentration (C_{obs}) was calculated from the equation of the standard curve:

$$C_{\text{obs}} = (y - a)/b$$

The intra-assay precision and accuracy was determined by analyzing five sets of the spiked standard samples with concentrations of 20, 10, 5, 1, 0.5, 0.2, 0.1 and 0.05 ng/ml. The inter-assay validation was performed by analyzing QC samples in five replicates on three separate days.

2.7. Recovery

The extraction recovery of analyte from human plasma was calculated by comparing the peak area ratio (analytes versus their I.S.) of the extracted analyte to that of non-extracted analyte. The extracted analyte was prepared by the extraction procedure described above of the spiked plasma samples (10, 1 and 0.2 ng/ml; duplicate in each concentration). For non-extracted sample, the analyte was added in the drug-free plasma extract prepared by the same extraction procedure at the same final concentration of the extracted analyte. The extracted and

non-extracted samples were reconstituted with the mobile phase of HPLC and analyzed by LC–MS–MS. The percent recovery was calculated by dividing the peak area ratio of the extracted sample over that of non-extracted standard. The recovery of the I.S. from plasma (5 ng/ml) were determined by the same procedure as described above except for the reversal of the spiking order of the I.S. and its corresponding non-deuterium labeled compound: the final concentration of non-labeled compound in plasma was 10 ng/ml.

3. Results and discussion

3.1. Mass spectra

The full-ion mass spectra of EM574, M-IV, M-V and M-VI gave the $[M+H]^+$ appearing at m/z 744.5, 760.5, 760.5 and 744.5, respectively, indicating that both EM574 and M-VI and both M-IV and M-V have the same $[M+H]^+$. In the product ion mass spectra of the $[M+H]^+$ ions of EM574 and M-VI at the collision energy of -40 eV, two characteristic fragment ions were detected at m/z 586.4 and 186.2, which corresponded to a cladinose eliminated fragment and *N*-methyl-*N*-isopropyldesosamine moiety, respectively (Fig. 2A and B). The base ion peak in each product ion mass spectrum was observed at m/z 586.4. For M-IV and M-V, the $[M+H]^+$ ions appearing at m/z 760.5 were 16 units higher than those of EM574 and M-VI, because M-IV and M-V were the 15- and 14-hydroxyl derivatives of EM574, respectively. The product ion scan of $[M+H]^+$ of M-IV and M-V gave diagnostic fragment ions appearing at m/z 602.4 and 186.2 with a collision energy of -40 eV (Fig. 2C and D). These ions were produced by the same CID patterns as in the EM574 and M-VI, and the product ion at m/z 602.4 was detected as the base peak in the mass spectrum. On the basis of these observations, the monitoring ions on the SRM analysis were set to m/z 744.5→586.4 for EM574 and M-VI, and m/z 760.5→602.4 for M-IV and M-V in order to achieve the maximum analytical sensitivity which depends on the product ion intensity.

In the full-ion scan mass spectra of EM574-d_7, M-IV-d_7 and M-V-d_7, $[M+H]^+$ ions were detected at m/z 751.6, 767.5 and 767.5, respectively. Furthermore, the base product ions at m/z 593.5, 609.4 and 609.4, which were similar fragments to those in the respective non-deuterated compounds, were given by product ion scanning of $[M+H]^+$ of EM574-d_7, M-IV-d_7 and M-V-d_7, respectively, at the collision energy of -40 eV. Therefore, the monitoring ions for EM574-d_7, M-IV-d_7 and M-V-d_7 were set to m/z 751.6→593.5, 767.5→609.4, and 767.5→609.4, respectively.

3.2. Chromatography

Since monitoring ions were identical between EM574 and M-VI as described above, it was clear that chromatographic separation of these compounds should be needed for the LC–MS–MS quantification. Because of the same reasons, M-IV and M-V should be separated chromatographically. After several examinations of the SRM sensitivity of analytes using flow injection analysis, a mixture of acetonitrile–water (9:1, v/v) was found to be suitable for the HPLC mobile phase, indicating that the sensitivity tended to be higher by increasing the percentage of organic solvent in the mobile phase. As for the HPLC column, the reversed-phase column was selected for the analysis according to the hydrophobic properties of analytes. When the analytes were injected into the reversed-phase HPLC column with a mobile phase of acetonitrile–water in a preliminary test, all the compounds eluted as broad-tailing peaks from the column resulting in insufficient chromatographic separation between EM574 and M-VI, and between M-IV and M-V. Hence, ammonium acetate was added into water at the final concentration of 10 mmol/l to obtain sharp and symmetrical elution peaks of EM574 and its metabolites, and methanol was also added to the mobile phase to achieve an adequate separation of analytes. The optimization of mixture ratio of 10 mmol/l ammonium acetate solution, methanol and acetonitrile was performed by monitoring the peak intensities and chromatographic separation of EM574 and its metabolites under SRM conditions using LC–MS–MS with reversed-phase column. First, the optimal ratio of methanol and acetonitrile was determined when the ratio of 10 mmol/l ammonium acetate solution was fixed at 10% of the mobile phase. And then, the ratio of 10 mmol/l ammonium acetate was optimized. After the optimization of mixture ratio of 10 mmol/l ammonium acetate solution, methanol, acetonitrile, the

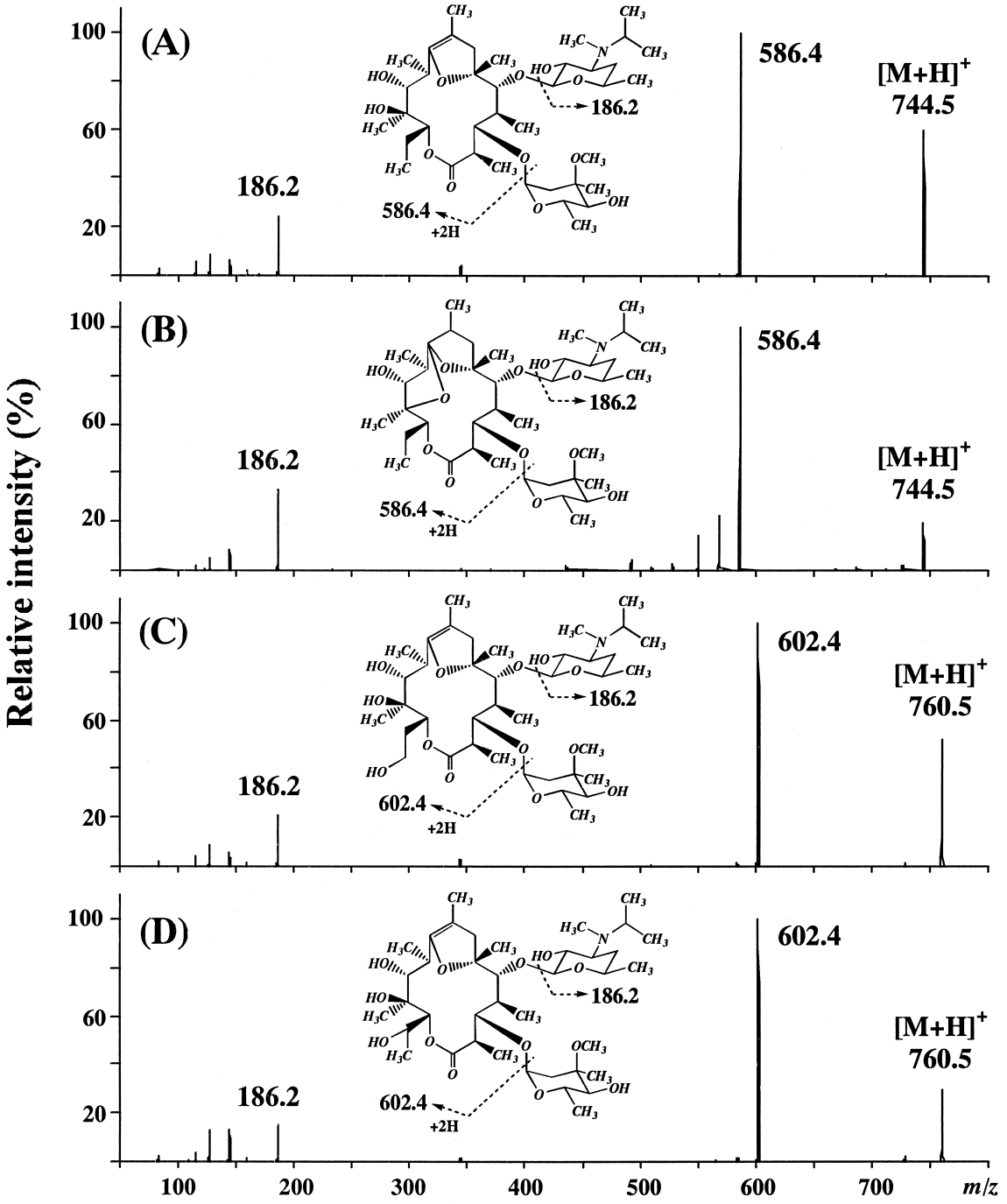


Fig. 2. Product ion mass spectra of $[M+H]^+$ of EM574 (A), M-VI (B), M-IV (C), and M-V (D) at a collision energy of −40 eV.

ratio 11:20:70 (v/v/v) was found to be appropriate for both sensitivity and chromatographic separation.

Figs. 3 and 4 show typical SRM chromatograms of a human plasma sample prepared by the method described in Experimental. The chromatograms of drug-free human plasma were free from endogenous peaks at the retention times of each analyte and I.S. (Fig. 3). Chromatographic peaks of EM574, M-IV, M-V, M-VI and the I.S. spiked in plasma at the concentration of 0.05 ng/ml were resolved well by

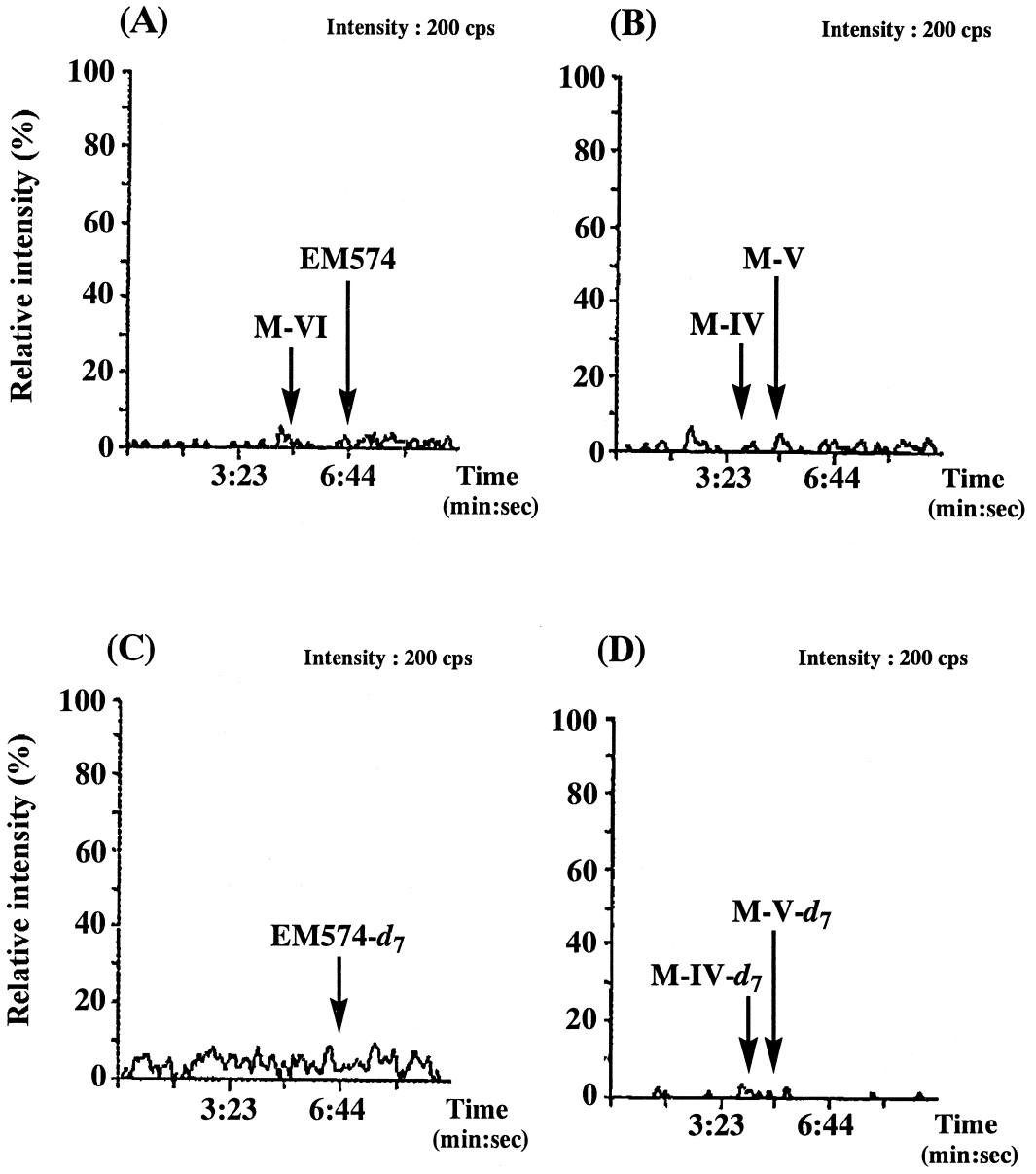


Fig. 3. Representative SRM chromatograms of extract from drug-free plasma. Detection mode for EM574 and M-VI (m/z 744.5→586.4) (A), for M-IV and M-V (m/z 760.5→602.4) (B), for EM574-d_7 (m/z 751.6→593.5) (C), and for M-IV-d_7 and M-V-d_7 (m/z 767.5→609.4) (D). Figures in parentheses indicate m/z values of precursor ion and product ion, respectively.

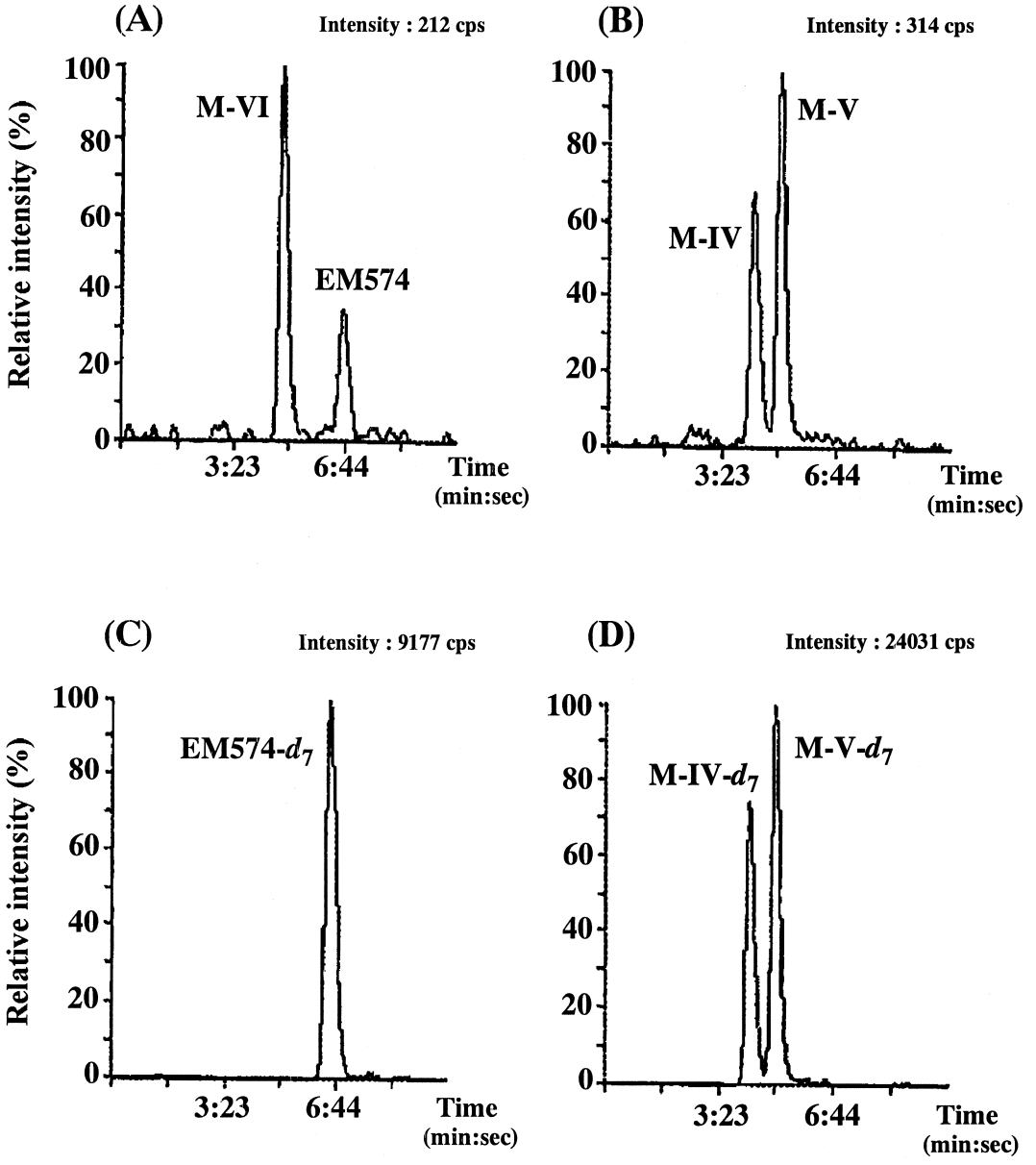


Fig. 4. Representative SRM chromatograms of extract from plasma spiked with EM574, M-IV, M-V, M-VI (0.05 ng/ml each), and I.S.s (5 ng/ml each). Detection mode for EM574 and M-VI (m/z 744.5→586.4) (A), for M-IV and M-V (m/z 760.5→602.4) (B), for EM574-d_7 (m/z 751.6→593.5) (C), and for M-IV-d_7 and M-V-d_7 (m/z 767.5→609.4) (D). Figures in parentheses indicate m/z values of precursor ion and product ion, respectively.

the SRM and chromatographic technique (Fig. 4). The I.S. did not interfere with any analyte. The retention times of EM574, M-IV, M-V and M-VI were 6.6, 4.3, 5.1 and 4.8 min, respectively. The analytical run time for one sample was 10 min.

3.3. Linearity, precision, accuracy and lower quantitation limit

The standard curves for EM574, M-IV, M-V and M-VI were obtained by the analysis of standard

Table 1
Linear regression parameters for the determination of EM574 and its metabolites in human plasma (intra-day assay, $n=5$)

Analyte	Concentration[a] (ng/ml)	Slope (CV, %)	Intercept	Correlation coefficient (r)
EM574	0.05–20	0.1621 (4.5)	−0.001748	0.99847
M-IV	0.05–20	0.1843 (4.1)	−0.000084	0.99930
M-V	0.05–20	0.1882 (3.0)	0.001522	0.99919
M-VI	0.05–20	0.1898 (6.0)	0.000983	0.99722

[a] 0.05, 0.1, 0.2, 0.5, 1, 5, 10 and 20 ng/ml.

Table 2
Precision and accuracy of the method (mean values of five determinations)

Analyte	Concentration	Intra-day		Inter-day	
	(ng/ml)	CV (%)	R.E. (%)	CV (%)	R.E. (%)
EM574	0.05	16.1	3.2	–[a]	–
	0.1	14.1	6.1	–	–
	0.2	10.9	3.6	5.4	−0.1
	0.5	5.7	−3.9	–	–
	1	13.8	−8.7	5.8	0.6
	5	4.7	−3.0	–	–
	10	2.6	3.6	3.8	−2.1
	20	7.6	−0.4	–	–
M-IV	0.05	19.8	0.4	–	–
	0.1	14.4	7.3	–	–
	0.2	11.9	4.3	6.0	2.8
	0.5	7.5	−6.2	–	–
	1	12.7	−4.8	3.0	1.7
	5	2.6	−0.9	–	–
	10	2.2	1.7	3.6	−1.3
	20	6.2	−0.3	–	–
M-V	0.05	18.8	−0.5	–	–
	0.1	14.7	6.7	–	–
	0.2	7.4	5.0	7.7	2.2
	0.5	7.0	−4.3	–	–
	1	14.1	−6.1	5.2	0.3
	5	2.7	−0.2	–	–
	10	2.1	1.8	4.5	−3.8
	20	5.3	−0.4	–	–
M-VI	0.05	16.1	11.2	–	–
	0.1	14.9	0.1	–	–
	0.2	8.5	0.8	6.1	2.1
	0.5	8.2	−4.1	–	–
	1	7.7	−2.1	4.8	−0.4
	5	6.3	−2.5	–	–
	10	4.4	−4.2	0.9	−2.4
	20	12.2	3.1	–	–

[a] –: Not examined.

solutions over the sample concentration range from 0.05 to 20 ng/ml (Table 1). The least-squares regression fit showed good linearity with correlation coefficients higher than 0.997 for each analyte. M-V-d_7 was best suited to the I.S. in preparation for standard curve of M-VI. This might depends on the close retention in the chromatography. The linearity of the standard curves for the four analytes added to human plasma was reproducible as indicated by coefficients of variation (CVs) in the slopes of the different standard curves.

Intra- and inter-day precision and accuracy data of the analytical procedure are presented in Table 2. The precision and accuracy were evaluated by CV and relative error (R.E.), respectively. The intra-day CV and R.E. were calculated from five observed concentrations at each spiked standard concentration, and those for the inter-day were determined by the mean observed QC concentrations ($n=5$) on three separate days. The intra-day CV and R.E. of analytes were not more than 19.8 and 11.2%, respectively. The inter-day variability indicated that CV and R.E. for analytes were 7.7 and 3.8%, respectively. Whereas, at the spiked concentrations of 0.2 and 1 ng/ml, the intra-day CV was slightly higher than the inter-day CV, it is considered that these results are not exceptional because the CV depends considerably on the instrumental conditions of LC–MS–MS (interface, mass analyzer, etc.) during sample analysis. From these results, it was clear that the method was reliable within that range and the lower quantitation limit was 0.05 ng/ml for all the analytes in plasma.

Table 3
Recoveries of EM574, its metabolites and I.S. from human plasma (mean values, $n=2$)

Concentration	Recovery (%)			
(ng/ml)	EM574	M-IV	M-V	M-VI
0.2	92.5	89.3	85.0	91.1
1	83.5	83.3	86.5	81.4
10	83.5	87.4	83.0	89.3
5 (I.S.)[a]	92.5	87.7	96.2	–

[a] EM574-d_7, M-IV-d_7 and M-V-d_7.

3.4. Recovery

The extraction recoveries of EM574, M-IV, M-V and M-VI from the human plasma in the concentration range 0.2–10 ng/ml were 83.5–92.5, 83.3–89.3, 83.0–86.5 and 81.4–91.1%, respectively (Table 3). Furthermore, the I.S. recoveries were 87.7–96.2%.

3.5. Application

The analytical method described was used for a clinical study of EM574. Concentrations of EM574, M-IV, M-V and M-VI in the plasma were measured for 24 h after oral administration of this drug at a dose of 20 mg. Figs. 5 and 6 show the concentrations of analytes in the plasma of a volunteer given this drug and the SRM chromatograms of the plasma at 3 h after dosing, respectively. In the SRM chromatograms of the plasma sample, EM574, M-IV, M-V and M-VI were detected clearly and well resolved, and M-IV concentration was highest among the four analytes. The concentration of each compound in the plasma attained a peak 3 h after dosing, and then declined to very low levels at 24 h postdose.

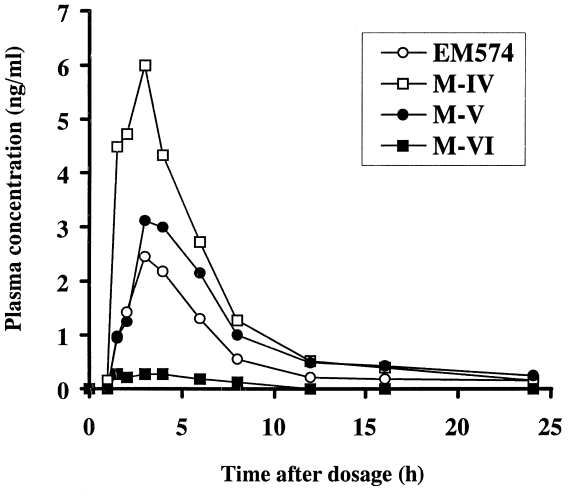


Fig. 5. Concentration of EM574, M-IV, M-V and M-VI in plasma of a volunteer given a 20 mg of EM574 orally.

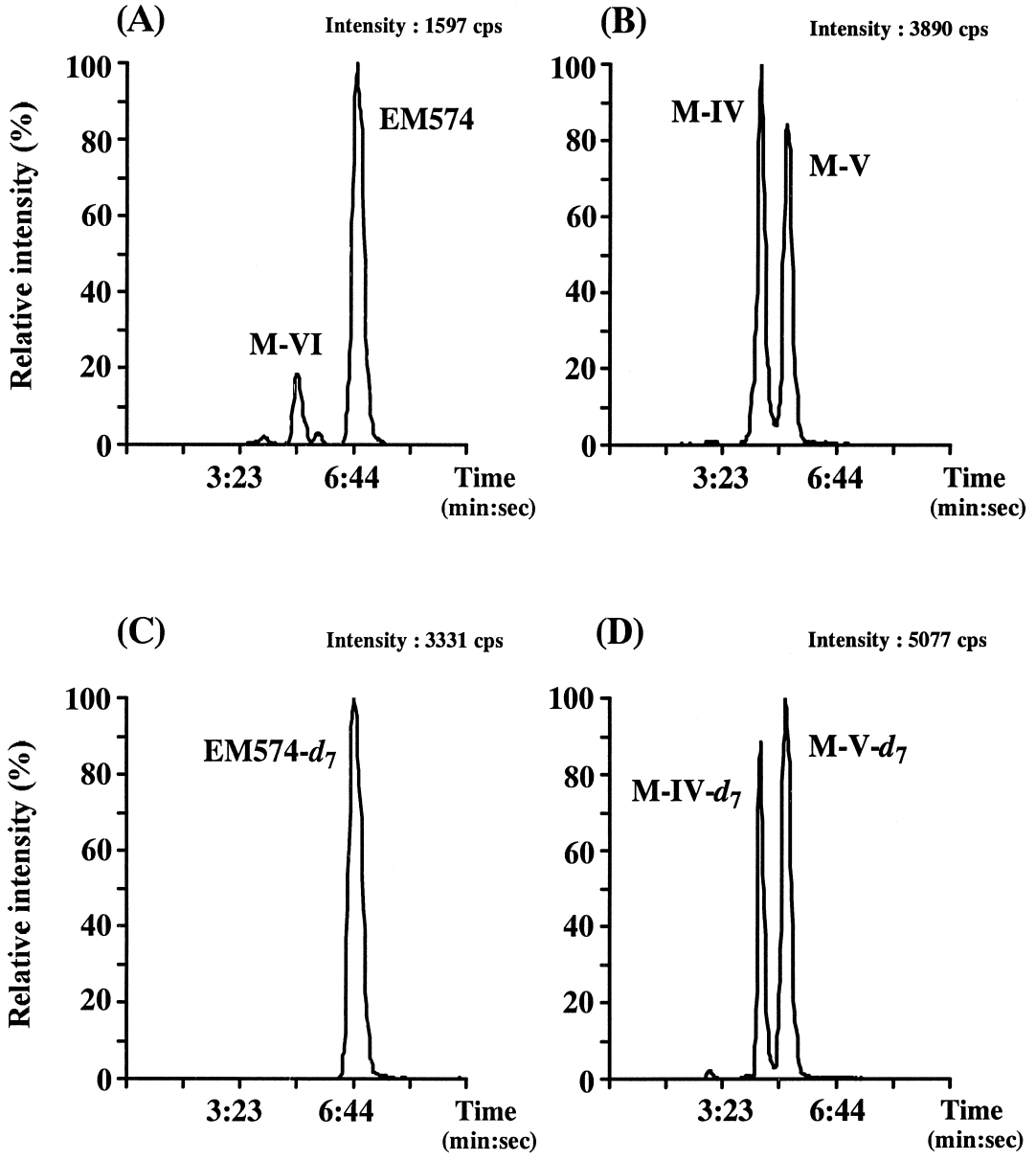


Fig. 6. SRM chromatograms of extract from the 3 h plasma of a volunteer given a 20 mg oral dose of EM574. Detection mode for EM574 and M-VI (m/z 744.5→586.4) (A), for M-IV and M-V (m/z 760.5→602.4) (B), for EM574-d_7 (m/z 751.6→593.5) (C), and for M-IV-d_7 and M-V-d_7 (m/z 767.5→609.4) (D). Figures in parentheses indicate m/z values of precursor ion and product ion, respectively.

4. Conclusion

This paper describes the development of a very sensitive and simple LC–MS–MS method with liquid–liquid extraction for determining EM574 and its metabolites in human plasma. The method established can quantify each analyte with a lower quantitation limit of 0.05 ng/ml using 0.2 ml of plasma, and proved to be fast and reproducible with an analysis time of less than 10 min for one plasma

sample. As the method has a very wide quantitation range, it should be able to elucidate the pharmacokinetic profiles of EM574 and its metabolites, in humans given this drug, at low and high doses.

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