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Short communication

Determination of erythromycin, clarithromycin, roxithromycin, and azithromycin in plasma by high-performance liquid chromatography with amperometric detection

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Abstract

In this study, a high-performance liquid chromatographic method was developed for the quantitative determination of erythromycin (EM), roxithromycin (RXM), and azithromycin (AZM) in rat plasma with amperometric detection under a standardized common condition using clarithromycin (CAM) as an internal standard. This method was also proved to be applicable for the determination of CAM by employing RXM as an internal standard. Each drug was extracted from 150 μl of plasma sample spiked with internal standard under an alkaline condition with *tert*.-butyl methyl ether. The detector cell potential for the oxidation of the drugs was set at +950 mV. The linearity of the calibration curves were preserved over the concentration ranges of 0.1–10 μg/ml for EM and RXM, and 0.03–3.0 μg/ml for CAM and AZM. Coefficients of variation and relative error were less than 9% and ±7%, respectively. The analytical method presented here was proved to be useful for the investigation of the pharmacokinetic characteristics of EM, CAM, RXM, and AZM in rats.

Keywords: Erythromycin; Clarithromycin; Roxithromycin; Azithromycin

1. Introduction

Macrolide antibiotics have been used for the treatment of bacterial infections caused by Gram-positive organisms. Although erythromycin (EM) have been widely used until 1980s, it is unstable to acid and its pharmacokinetics after oral administration are highly variable. Recently developed semi-synthetic derivatives of EM such as clarithromycin (CAM), roxithromycin (RXM), and azithromycin (AZM) are stable to acid and more extensively distributed to tissues.

For pharmacokinetic studies of macrolide antibiotics, analytical methods using radioactivity, a microbiological assay [1], or high-performance liquid chromatographic (HPLC) with UV [2], fluorescent [3], or electrochemical detection [4–15] have

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been employed. In recent years, HPLC methods with electrochemical detection have been widely used due to its high sensitivity and precision. In clinical settings, a more sensitive and simple method is required to determine the concentrations of several macrolide antibiotics in plasma. We have already reported a method for the determination of EM concentrations in rat plasma and liver by HPLC with electrochemical detection using a quite simple extraction procedure [14]. Kees et al. recently reported a highly sensitive method for the determination of EM, its ester, CAM, RXM, and AZM [15]. However, their method requires different mobile phases for these drugs, and changing of analytical conditions, such as mobile phases and columns, is laborious and requires additional stabilization times. In this study, therefore, we propose de novo HPLC method for the determination of EM, RXM, and AZM concentrations in rat plasma with amperometric detection, under a standardized, common analytical condition and extraction procedure. We also aimed to prove this method applicable for the determination of CAM by employing RXM as an internal standard.

2. Experimental

2.1. Materials and animals

EM base, CAM, RXM and AZM were kindly provided by Dai Nippon Pharmaceutical (Osaka, Japan), Taisho Pharmaceutical (Tokyo, Japan), Hoechst Marion Roussel (Tokyo, Japan) and Pfizer Pharmaceuticals (Tokyo, Japan), respectively. Acetonitrile (HPLC grade) and methanol (HPLC grade) were purchased from Wako Pure Chemical Industries (Osaka, Japan), and *tert.*-butyl methyl ether (HPLC grade) from Aldrich (Milwaukee, USA). All other compounds used were of reagent grade.

Male Sprague-Dawley rats weighing 250–370 g were purchased from Nippon Seibutsu Zairyou Center (Tokyo, Japan).

2.2. Standard solutions

EM, CAM, RXM, and AZM were dissolved in methanol at concentrations of 0.03, 0.3, 3.0, 30, and 300 μg/ml, and stored in dark at 4°C as stock solutions. These standard solutions were confirmed to be stable for one month.

2.3. Instrumentation and chromatographic conditions

The HPLC system consisted of a degasser DGU-4A (Shimadzu, Kyoto, Japan), a pump LC-10AD (Shimadzu, Kyoto, Japan), a column oven CTO-10AC (Shimadzu, Kyoto, Japan), an amperometric detector (L-ECD-6A) and a recorder C-R7A (Shimadzu, Kyoto, Japan). The separation was performed on a reversed-phase column YMC-Pack ODS-AP of 250×6.0 mm I.D., with the particle size of 5 μm (YMC, Kyoto, Japan). The assay was carried out at 30°C.

The mobile phase consisted of acetonitrile and 0.05 *M* phosphate buffer (pH 7.2) (43:57, v/v) and pumped at a constant rate of 1.7 ml/min. The detector cell potential for oxidation was examined from +400 to +1050 mV to obtain current–voltage relationships, using a working electrode of glassy carbon and an Ag/AgCl reference electrode.

2.4. Sample extraction procedure

The internal standard (I.S.) was RXM (3 μg/ml in methanol) for the analysis of CAM, and CAM (3 μg/ml in methanol) for the analysis of other macrolides. The internal standard solution (200 μl of RXM or 150 μl of CAM) was pipetted into a 10-ml glass centrifuge tube and evaporated to dryness under a stream of nitrogen. An aliquot of 150 μl plasma, 10 μl of 1 *M* NaOH and 2 ml of *tert.*-butyl methyl ether were put into the tube. The mixture was shaken for 5 min by a mechanical shaker. After centrifugation at 1500 *g* for 10 min, the upper ether layer was transferred into another tube and evaporated to dryness as described above. The dried residue was dissolved with 50 μl of the mobile phase. The volume of injection into the HPLC system was 20 μl.

2.5. Calibration curves and assay validation

The drug-free plasma was added to the dried residue of the standard solution to obtain standard plasma used for calibration. The standard plasma of

0.1, 0.3, 1.0, 3.0 and 10.0 μg/ml were prepared for EM or RXM, and 0.03, 0.1, 0.3, 1.0 and 3.0 μg/ml for CAM or AZM.

Samples were submitted to the extraction procedure described above. Calibration curves were obtained by plotting the peak–height ratios (drug to the internal standard) on the ordinate and the respective drug concentrations on the abscissa.

Within-day assay precision and accuracy were assessed using five spiked plasma samples at concentrations of 0.3 and 10.0 μg/ml of EM or RXM and 0.1 and 3.0 μg/ml of CAM or AZM.

Between-day assay precision and accuracy were assessed using a spiked plasma samples during 5 days at concentration of 0.3 and 10.0 μg/ml of EM or RXM and 0.1 and 3.0 μg/ml of CAM or AZM.

The recoveries from plasma samples, at concentration of 10.0 μg/ml of EM or RXM and 3.0 μg/ml of CAM or AZM, were examined by comparing the peak areas with equivalent to pure compounds dissolved in methanol.

2.6. Pharmacokinetic study

Male Sprague-Dawley rats were anesthetized with diethylether, and polyethylene tubings were inserted and fixed into the femoral vein and artery. After recovery from anesthesia, EM (50 mg/kg), CAM, RXM, or AZM (20 mg/kg) was intravenously injected. Each drug was dissolved into a physiological salt solution (NaCl: 135 m*M*, $NaHCO_3$: 11.9 m*M*, KCl: 5.4 m*M*, $CaCl_2$: 1.8 m*M*, $MgCl_2$: 1.0 m*M*) by adding stoichiometrically equivalent phosphoric acid. Blood samples were collected from the femoral artery at 1, 3, 5, 15, 30, 60, 120, 180, and 360 min after the drug administration, and immediately centrifuged to obtain plasma. The plasma samples were then extracted and determined as described above.

3. Results and discussion

3.1. Chromatographic condition

The current–voltage relationships EM, CAM, RXM, and AZM were investigated. Peak height of each drug increased, as the applied potential increased from +400 to +1050 mV. The peak height became almost saturated at the potential of +950 mV. A detector cell potential of +850 to +1100 mV was reported to be required for the oxidation of macrolide antibiotics [4–15], being consistent with the result in this study. Although a higher peak was provided in the higher applied potential, the long time was required for stabilizing the HPLC system. Therefore, the potential was set at +950 mV for suitable determination of these macrolide antibiotics.

The best separation was observed with phosphate buffer of pH 7.2 without interfering background peaks. Addition of ammonium acetate or tetrabutylammonium to mobile phase was reported to increase the background noise [15]. In our study, the same result was observed by the addition of 0.3% tetraethylammonium. Moreover, 10 mg/l of EDTA, which removes the effects of metal ions, also increased the background noise.

Fig. 1 shows typical chromatograms. The retention times of EM, CAM, RXM, and AZM were 6.8, 13.3, 16.3, and 9.6 min, respectively. No peaks interfering with the four drugs were found in the chromatograms from plasma samples and it was possible to measure the concentrations of EM, CAM, RXM, and AZM under the common HPLC condition. Moreover, each drug was well separated from each other, and simultaneous determination of three drugs with the rest one as an internal standard was feasible. Instead of using a common internal standard, we employed CAM as an internal standard for the determination of EM, RXM and AZM, and RXM for the determination of CAM. It was due to the difficulty of finding another suitable compound which can be separated well from these macrolides with sufficient precision.

3.2. Extraction procedure

tert.-Butyl methyl ether has been widely used to extract macrolide antibiotics from biological samples [7,8,14,15]. Among the four organic solvents investigated in this study, i.e., ethyl acetate, dichloromethane, chloroform and *tert*.-butyl methyl ether, the last one was found to provide the best extraction of EM, CAM, RXM, and AZM with the least interfering peaks from plasma samples. The recoveries with this extraction procedure were 107%, 106%, 112% and 94% for EM, CAM, RXM, and AZM. The

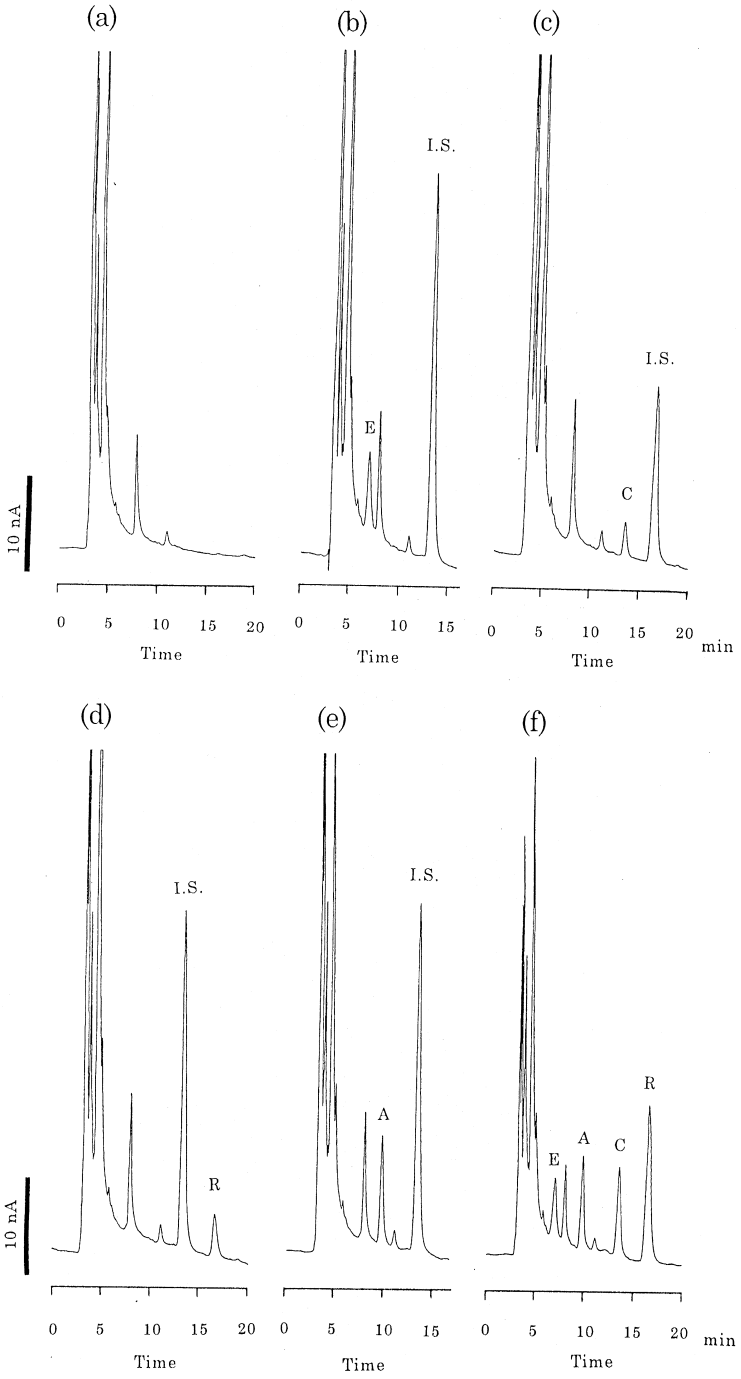

Fig. 1. A typical chromatograms of extracts from (a) rat blank plasma, (b) plasma spiked with internal standard (I.S.) and 1.0 μg/ml of EM, (c) plasma with I.S. and 0.3 μg/ml of CAM, (d) plasma with I.S. and 1.0 μg/ml of RXM, (e) plasma with I.S. and 0.3 μg/ml of AZM, and (f) plasma with 1.0 μg/ml of EM, 1.0 μg/ml of CAM, 4.0 μg/ml of RXM, and 0.3 μg/ml of AZM. E: erythromycin, C: clarithromycin, R: roxithromycin, A: azithromycin.

Table 1
Calibration lines used for the validation of within-day assay for determination of EM, CAM, RXM, and AZM in rat plasma[a]

Drug	Equations for calibration	r^2
EM	$y=0.2144x-0.0140$	0.99987
CAM	$y=0.3882x-0.0039$	0.99999
RXM	$y=0.1687x-0.0003$	0.99998
AZM	$y=0.6094x-0.0015$	0.99953

[a] y: concentration, x: peak–area ratio.

recovery rates in this study were better than or almost comparable to those previously reported [5,6,8,10,14,15].

3.3. Assay validation

The calibration curves for EM (0.1–10.0 μg/ml), CAM (0.03–3.0 μg/ml), RXM (0.1–10.0 μg/ml), and AZM (0.03–3.0 μg/ml) were quite linear ($r^2>$ 0.999; Table 1).

Within-day assay precision and accuracy are presented in Table 2. The observed coefficients of variation (C.V.) at all the concentrations examined were less than 9%. The accuracy was less than±7%. Between-day assay precision and accuracy are presented in Table 3. The C.V. values and accuracy at the same concentrations were within 8% and 4%, respectively. The limits of quantification were as-

Table 2
Within-day precision and accuracy for the determination of EM, CAM, RXM, and AZM in rat plasma

Drug	Concentration added (μg/ml)	Concentration found[a] (μg/ml)	Precision[b] (C.V.) (%)	Accuracy[c] (%)
EM	0.3	0.289±0.020	6.8	96.4
	10.0	10.6±0.20	2.1	106.0
CAM	0.1	0.103±0.007	6.3	103.2
	3.0	2.97±0.05	1.6	98.9
RXM	0.3	0.302±0.014	4.5	100.6
	10.0	9.77±0.12	1.3	97.7
AZM	0.1	0.0930±0.0075	8.1	93.0
	3.0	3.08±0.09	2.8	102.8

[a] Mean±SD (n=5).
[b] Coefficient of variation (C.V.)=SD/mean×100.
[c] Accuracy=found/added×100.

Table 3
Between-day precision and accuracy for the determination of EM, CAM, RXM, and AZM in rat plasma

Drug	Concentration added (μg/ml)	Concentration found[a] (μg/ml)	Precision[b] (C.V.) (%)	Accuracy[c] (%)
EM	0.3	0.301±0.011	3.6	100.3
	10.0	10.2±0.40	4.0	102.0
CAM	0.1	0.100±0.003	3.4	100.2
	3.0	3.04±0.04	1.4	101.3
RXM	0.3	0.309±0.019	6.2	102.9
	10.0	10.1±0.20	1.7	100.6
AZM	0.1	0.0966±0.0038	3.9	96.6
	3.0	3.11±0.24	7.6	103.6

[a] Mean±SD (n=5).
[b] Coefficient of variation (C.V.)=SD/mean×100.
[c] Accuracy=found/added×100.

sumed to be 0.03 μg/ml for CAM or AZM, and 0.1 μg/ml for EM or RXM, respectively.

3.4. Pharmacokinetic study

The present analytical method was applied to a pharmacokinetic study of the four macrolide antibiotics in rats. Fig. 2 shows the time–concentration curves of EM, CAM, RXM, and AZM in plasma after i.v. administration of EM (50 mg/kg), CAM, RXM, and AZM (20 mg/kg). The pharmacokinetic parameters (i.e. elimination half-life and total clearance) of EM, CAM, and RXM are consistent with the values reported previously [16–18]. Although the elimination half-life of AZM was comparable to that reported previously, the total clearance was greater than the reported value [19], conceivably because the plasma concentrations of AZM were measured for only 6 h and the later phase post 6 h was not defined in our study. With respect to the plasma concentration of EM, we did not examine at 6 h after the injection since the concentration was assumed to be far lower than clinical range of 0.5–2.5 μg/ml. The present HPLC method was proved to be applicable to the pharmacokinetic studies of EM, CAM, RXM, and AZM.

In conclusion, a rapid and sensitive method with amperometric detection for the quantitative determi-

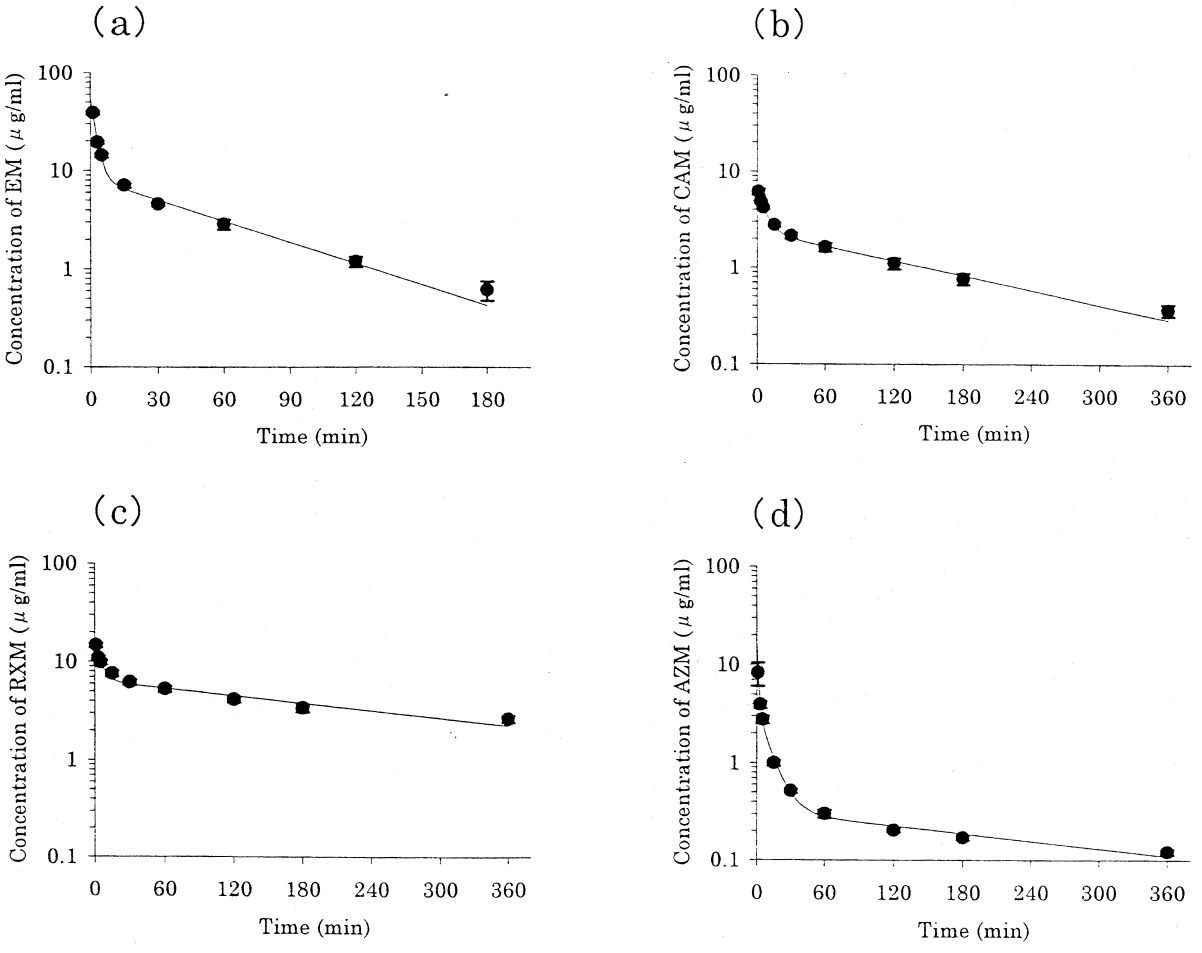

Fig. 2. Time courses of (a) EM, (b) CAM, (c) RXM, and (d) AZM concentrations in plasma after intravenous administration of the drugs to rats (EM: 50 mg/kg, CAM: 20 mg/kg, RXM: 20 mg/kg, AZM: 20 mg/kg). (mean±SEM, $n=3$). The solid lines represent the simulation curves calculated from pharmacokinetic analysis with a two-compartment open model.

nation of different macrolide antibiotics in plasma under a common analytical condition was developed. This method was demonstrated to be of quite useful for the investigation of the pharmacokinetic characteristics of macrolide antibiotics.

References

[1] S.C. Bell, J.W. Hamman, W.E. Grundy, Appl. Microbiol. 17 (1969) 88–92.
[2] C. Stubbs, J.M. Haigh, I. Kanfer, J. Pharm. Sci. 74 (1985) 1126–1128.
[3] K. Tsuji, J.F. Goetz, J. Chromatogr. 147 (1978) 359–367.
[4] M.L. Chen, W.L. Chiou, J. Chromatogr. 278 (1983) 91–100.
[5] C. Stubbs, I. Kanfer, J. Chromatogr. 427 (1988) 93–101.
[6] N. Grgurinovich, A. Matthews, J. Chromatogr. 433 (1988) 298–304.
[7] S. Laakso, M. Scheinin, M. Anttila, J. Chromatogr. 526 (1990) 475–486.
[8] R.M. Shepard, G.S. Duthu, R.A. Ferraina, M.A. Mullins, J. Chromatogr. 565 (1991) 321–337.
[9] S.-Y. Chu, L.T. Sennello, R.C. Sonders, J. Chromatogr. 571 (1991) 199–208.
[10] T.D. Rotsch, M. Spanton, P. Cugier, A.C. Plasz, Pharm. Res. 8 (1991) 989–991.
[11] M. Janecek, M.A. Quilliam, M.R. Bailey, J. Chromatogr. 619 (1993) 63–69.
[12] H. Toreson, B.-M. Eriksson, J. Chromatogr. B 673 (1995) 81–89.
[13] M. Hedenmo, B.-M. Eriksson, J. Chromatogr. A 692 (1995) 161–166.
[14] E. Hanada, H. Ohtani, H. Kotaki, Y. Sawada, T. Iga, J. Chromatogr. B 692 (1997) 478–482.
[15] F. Kees, S. Spangler, M. Wellnhofer, J. Chromatogr. A 812 (1998) 287–293.
[16] T. Suwa, Y. Kohno, H. Yoshida, K. Fukushima, Chemotherapy. 36 (1988) 205–212.
[17] S. Morimoto, T. Nagate, K. Sugita, T. Ono, K. Numata, J. Miyachi, Y. Misawa, K. Yamada, S. Omura, J. Antibiot. 40 (1990) 295–305.
[18] Y. Esumi, Y. Zin, S. Ban, S. Ninomiya, K. Hayashi, T. Yokoshima, T. Yamamoto, M. Hirayama, K. Saitoh, K. Okui, Chemotherapy 36 (1988) 148–163.
[19] R.M. Shepard, F.C. Falkner, J. Antimicrob. Chemother. 25 (1990) 49–60.