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Short communication

Determination of erythromycin concentrations in rat plasma and liver by high-performance liquid chromatography with amperometric detection

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Abstract

A simple method for the quantitative determination of erythromycin (EM) concentrations in rat plasma and liver by high-performance liquid chromatography with amperometric detection was developed. EM was extracted from 200 μl of plasma or liver homogenate sample under sodium hydroxide alkaline conditions with *tert.*-butyl methyl ether. Oleandomycin was used as an internal standard. The recovery rate of EM was up to 100%. The detector cell potential for the oxidation of EM was +1100 mV. The calibration curves were linear over the concentration ranges 0.1–20.0 μg/ml for plasma and 0.5–100.0 μg/g for liver. The method was applied to the determination of the plasma and liver concentrations of EM in rats after intravenous administration (50 mg/kg dose). The method presented here has proved to be of great use for the investigation of the pharmacokinetic characteristics of EM in small animals such as rats.

Keywords: Erythromycin

1. Introduction

Erythromycin (EM) is a macrolide antibiotic used for the treatment of bacterial infections caused by Gram-positive organisms. EM is the macrolide most frequently implicated in pharmacokinetic drug interactions. EM is known to inhibit the metabolism of various drugs, such as disopyramide [1,2], terfenadine [3] and cyclosporin [4], by an inactivation of microsomal oxidizing enzymes: P-450 (CYP) particularly in the liver [5]. This inhibition leads to elevated plasma concentrations of these drugs, which cause clinically significant adverse reactions. The extent of these interactions may be closely related to the kinetics of EM in liver, where the interaction occurs. Thus, to avoid these interactions, it is necessary to quantitatively investigate the pharmacokinetic properties of EM in plasma and particularly in the liver.

Methods using radioactivity or a microbiological assay have been widely used for pharmacokinetic studies of EM. However, these methods are of limited specificity and may lead to overestimation since interference by metabolites of EM could not be excluded. Several HPLC methods with UV [6–8], fluorescent [9,10], or electrochemical detection [11–

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17] were reported for the determination of the EM concentration in biological fluids. However, UV detection did not provide enough sensitivity for pharmacokinetic studies because of the low absorbence of EM. Though the fluorescent assay is sensitive, it requires a complicated apparatus for pre- or post-column derivatization and extraction. Even with the sensitive electrochemical detection, most of the methods require 1–2 ml of plasma sample [11–16], and they are not prefered for the investigation of pharmacokinetics in small animals. Moreover, a method for the determination of the EM concentration in the liver, which is prerequisite for the quantitative investigation of the drug interactions as mentioned above, has not been reported.

In this paper we propose a simple and sensitive method for the determination of EM concentrations in both plasma and liver in rats using small sample volumes (200 μl).

2. Experimental

2.1. Materials and animals

Erythromycin base (Fig. 1) was kindly provided by Dai Nippon Pharmaceutical (Osaka, Japan). Oleandomycin, acetonitrile (HPLC grade), methanol (HPLC grade), acetic acid, anhydrous sodium acetate and sodium hydroxide were purchased from Wako (Osaka, Japan). *tert.*-Butyl methyl ether (HPLC grade) was from Aldrich (Milwaukee, USA).

Sprague–Dawley rats weighing 250–350 g were purchased from Nippon Bio-Supp. Center (Tokyo, Japan).

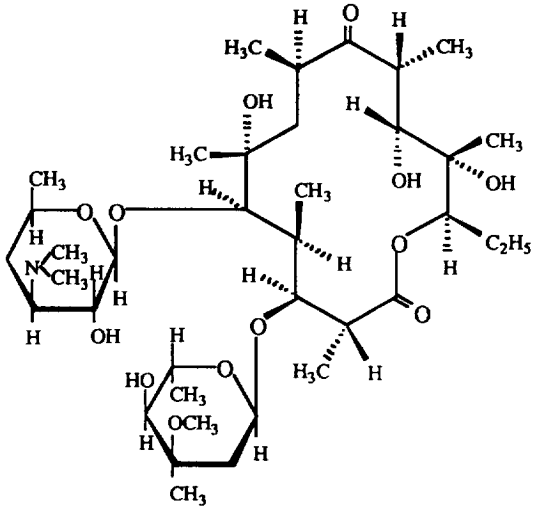

Fig. 1. Molecular structure of erythromycin base.

2.2. Standard solutions

Stock solutions (0.1, 1.0 and 50 μg/ml) of EM were prepared in methanol. Oleandomycin was similarly dissolved in methanol to a concentration of 3 μg/ml. These solutions were stored at 4°C.

2.3. HPLC conditions

The chromatographic apparatus consisted of an amperometric detector LC-4C, pump pm-60 (BAS, Tokyo, Japan), Chromatocorder 21 (System Instruments, Tokyo, Japan) and a C_{18} reversed-phase column: Cosmosil packed column 5-C_{18} (150 mm× 4.6 mm I.D., particle size 5 μm; Nacalai Tesque, Kyoto, Japan). The assay was carried out at room temperature. The mobile phase was acetonitrile–0.1 *M* sodium acetate buffer (pH 6.6) (50:50, v/v). The mobile phase was pumped at a flow-rate 0.6 ml/min. The amperometric cell potential for the oxidation of EM was +1100 mV (vs. Ag/AgCl reference electrode).

2.4. Sample preparation

Arterial drug-free blood sample was collected through the abdominal aorta, heparinized and centrifuged (10 min, 1500 *g*) to separate the plasma. Liver was removed quickly after the collection of blood, and was then rinsed with an ice-cold physiological saline solution, blotted with a filter paper, weighed, and homogenized on ice with four volumes of ice-cold physiological saline solution in a blender homogenizer (Physcotron; Niti-on medical supply, Tokyo, Japan). Plasma samples and liver homogenates were stored at −20°C until assay.

2.5. Extraction procedure

Internal standard (100 μl of a 3 μg/ml solution in methanol) was pipetted into a 10-ml glass tube and

evaporated to dryness under a stream of dry nitrogen at room temperature. An aliquot (200 μl) of plasma or liver homogenate was put into the tube. After addition of 2 ml of *tert.*-butyl methyl ether, the mixture was made alkaline with 5 μl of 1 *M* NaOH, and then shaken for 5 min by a mechanical shaker. After centrifugation (10 min, 1500 *g*), the upper ether layer was transferred into a 10-ml glass tube and evaporated to dryness as described above. Next, the inner wall of the tubes was rinsed with 200 μl of methanol to concentrate the sample and evaporated off. The dry residue was dissolved in 30 μl of methanol. The injection volume was 10 μl.

2.6. Calibration and assay validation

A calibration curve was made by spiking drug-free rat plasma with concentrated EM solution to yield 0.1, 0.3, 1.0, 5.0, 10.0, 15.0 and 20.0 μg/ml. Drug-free rat liver homogenates were also supplemented with EM in concentrations of 0.1, 0.3, 1.0, 5.0, 10.0, 15.0 and 20.0 μg/ml in liver homogenates. Samples were submitted to the extraction procedure described above. Calibration curves were obtained by plotting the peak-height ratios (EM to 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 or liver homogenate samples at concentrations of 0.3 and 15.0 μg/ml of EM.

Between-day assay precision and accuracy were assessed using a spiked plasma or liver homogenate samples during 5 days at concentration of 0.3 and 15.0 μg/ml.

The recovery rate of EM was examined with plasma and liver homogenate samples. The results were compared with equal amounts of pure compounds dissolved in methanol.

2.7. Pharmacokinetic study

2.7.1. The time course of the EM concentration in plasma

Polyethylene tubing was inserted and fixed into the femoral vein and artery of a rat under ether anesthesia. After recovery from anesthesia, 50 mg/kg of EM was injected intravenously. Blood samples were collected at 1, 3, 5, 15, 30, 60, 120 and 180 min after the administration, and immediately centrifuged for 10 min at 1500 *g*. The plasma samples were extracted and determined as in the above procedure.

2.7.2. The time course of the EM concentration in liver

Polyethylene tubing was inserted and fixed into the femoral vein as mentioned above and then 50 mg/kg of EM was injected. The animals were killed by decapitation at 2, 5, 15, 30, 60 and 180 min after administration, and the liver was immediately removed and chilled on ice. The livers were homogenized and determined as described above.

3. Results and discussion

3.1. Chromatographic conditions

It has been reported that a detector cell potential of +850–+1100 mV is required for the oxidation of EM [14–17]. In this study, a detector cell potential of +1100 mV was found to be suitable enough for the determination of EM. Fig. 2(B) shows a typical chromatogram of extracts from liver homogenate containing 5.0 μg/ml of EM. The retention time was 8.5 min for the internal standard and 12.8 min for EM. No peaks interfering with EM were found in the chromatograms from liver and plasma samples.

3.2. Extraction procedure

Among the four organic extraction solvents, i.e., chloroform, isoamyl alcohol–hexane (98:2, v/v), ethyl acetate and *tert.*-butyl methyl ether, the last one was found to be the most suitable for the extraction of EM, because it gives the best recovery rate of EM without interfering peaks.

It has been reported that the plasma samples should be made alkaline up to pH 10 for the extraction of EM. However, addition of 5 μl of 0.25 *M* NaOH, which made the plasma samples pH 10, did not result in satisfactory recovery of EM from liver homogenate. With addition of 5 μl of 1 *M* NaOH, the recovery rate of EM reached up to 100% for both plasma and liver without any interfering peaks.

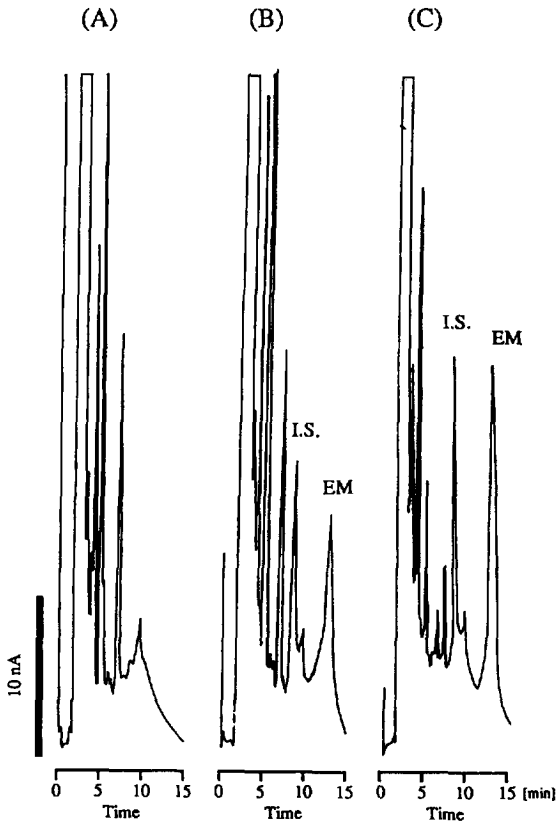

Fig. 2. Chromatograms of extracts from (A) blank rat liver, (B) liver containing 5.0 μg/ml of erythromycin (EM) and internal standard (I.S.) and (C) liver homogenate sample after intravenous administration of erythromycin 50 mg/kg.

3.3. Assay validation

Calibration curves for plasma (0.1~20.0 μg/ml) and liver (0.5~100.0 μg/g) were linear (r^2=1.000 and 0.999, respectively). The limit of detection, defined here as the concentration where the ratio of the peak height of analyte to background is 3:1, was 0.1 μg/ml for plasma and 0.5 μg/g for liver. These values are suitable to investigate the pharmacokinetics of EM in plasma and liver.

Within-assay precision and accuracy are presented in Table 1. The observed coefficient of variation (C.V.) at concentrations of 0.3 and 15.0 μg/ml in plasma and liver homogenate was less than 8%. The accuracy was not over ±10%. Between-assay precision and accuracy are presented in Table 2 and show the same results.

Table 1
Within-day precision and accuracy for erythromycin in plasma and liver samples

Concentration added (μg/ml)	Concentration found[a] (μg/ml)	Precision[b] (C.V.) (%)	Accuracy[c] (%)
Plasma			
0.3	0.279±0.003	1.14	−7.0
15.0	14.31±0.18	1.24	−4.6
Liver			
0.3	0.310±0.016	5.32	3.3
15.0	15.04±0.19	1.29	0.3

[a] Mean±S.D., n=5.
[b] Coefficient of variation=S.D./mean×100.
[c] Accuracy=(found−added)/added×100.

3.4. Pharmacokinetic study

The present method was applied to the pharmacokinetic study of EM in rats. Fig. 3 shows the time–concentration curves of EM in plasma and liver after the intravenous administration of EM (50 mg/kg). After the administration, EM is immediately distributed into the liver, and the concentration in the liver became almost parallel to that in the plasma with 30 min. The liver to plasma concentration ratio of EM was found to be 10–20 at the terminal phase.

Table 2
Between-day precision and accuracy for erythromycin in plasma and liver samples

Concentration added (μg/ml)	Concentration found[a] (μg/ml)	Precision[b] (C.V.) (%)	Accuracy[c] (%)
Plasma			
0.3	0.312±0.240	7.80	4.0
15.0	14.95±0.23	1.57	−0.3
Liver			
0.3	0.329±0.020	6.00	9.7
15.0	14.92±0.14	0.95	−0.5

[a] Mean±S.D., n=5.
[b] Coefficient of variation=S.D./mean×100.
[c] Accuracy=(found−added)/added×100.

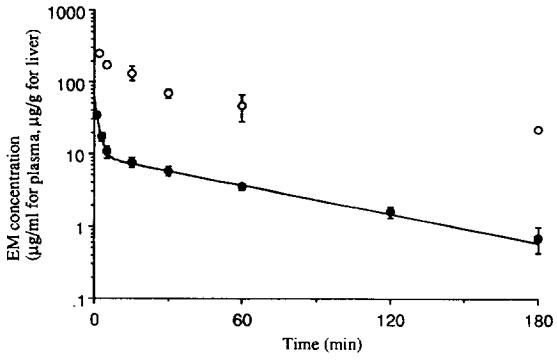

Fig. 3. Plasma (●) and liver (○) concentration vs. time profiles after intravenous administration of erythromycin (50 mg/kg). (mean±S.E.M.; plasma: $n=4$, liver: $n=3$).

This result was in accordance with the reported value determined by bioassay [18].

4. Conclusion

As the clinical range of EM is reported to be 0.5–2.5 μg/ml in plasma, the present method will be suitable for the pharmacokinetic study of EM in rats within the clinical range.

The HPLC method presented here is simple and has a good validation to allow the determination of EM concentrations in both plasma and liver with the same procedure using small sample volume (200 μl). Therefore, our method proved to be of great use for the investigation of the pharmacokinetic characteristics of EM in small animals such as rats.

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