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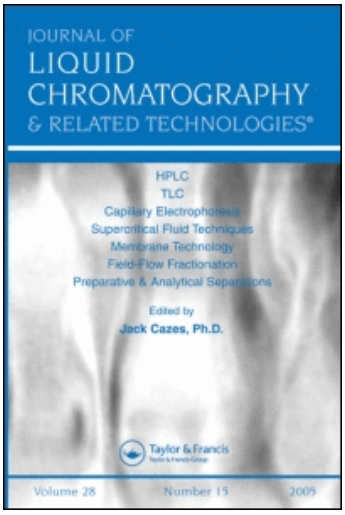

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Determination of Erythromycin in Human Plasma and Whole Blood by High-Performance Liquid Chromatography

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DETERMINATION OF ERYTHROMYCIN IN HUMAN PLASMA AND WHOLE BLOOD BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A simple, selective, sensitive, and rapid high-performance liquid chromatographic (HPLC) method is described for the quantitation of erythromycin (EM) in plasma and whole blood. The volume of plasma and whole blood and analytical time were only 0.2 ml and ~15 min. Analytical recovery of EM at concentrations of 0.2, 1.0 and 5.0 μg/ml after the complete extraction procedures with diethyl ether from alkalinized plasma and whole blood was > 75%. The coefficients of variation (CVs) for intra- and inter-

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day assay riabilities at 0.2, 1.0 and 5.0 μg/ml of EM were < 5.8% in plasma and < 6.3% in whole blood, respectively. The calibration curves for the respective analyte were linear (r = 0.994) over concentrations ranging from 0.1 to 5.0 μg/ml in plasma and whole blood. The lower detection limit for EM in plasma and whole blood was 0.1 μg/ml (CVs < 5.3%). This assay method is useful for the routine monitoring of EM in biological specimens.

INTRODUCTION

Erythromycin (EM) is a macrolide antibiotic reagent used widely in the treatment of infectious diseases caused by gram-positive bacteria.[1] It is known that EM is a specific effective drug for the treatment of diffuse panbronchiolitis (DPB), which is a type of chronic obstructive pulmonary disease with a grave prognosis.[2] Characteristics of the treatment for DPB are low-dose, 200 mg every 8 hours, and long therapeutic term (mean 19.8 months).[3] Despite this use, however, no clinical pharmacokinetic data on EM in patients with DPB have yet been investigated. It has been proposed that concentrations of EM between 0.5 and 3.0 μg/ml in plasma are effective for the treatment of infectious diseases.[4] Therefore, individual monitoring of the blood concentration and determination of appropriate EM dose in each patient are needed for the treatment of DPB.

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Various methods have been reported to quantitate EM levels in serum, plasma, urine, and/or tonsillor tissue.[5,6] Several high-performance liquid chromatographic (HPLC) methods have been described for the determination of EM in serum and urine.[7-12] EM and EM ethylsuccinate in serum were quantitated with fluorometric detection.[7] However, this last method has not been used widely, probably due to the complexity of the procedure and the instrumentation. Although the sensitivity limit at 0.25 μg/ml in serum was obtained with UV detection at 200 nm, method of Stubbs, et al. includes a solid-phase extraction and the use of 2-ml of the sample.[8] Because EM has a very weak UV absorbance, a wavelength of 200 nm was used to obtain sufficient sensitivity. At such a short wavelength, however the components in biological fluids often caused interference of the detectable peakes. Simple HPLC procedures using electrochemical detection have also been reported.[9-13]

In this article, we describe an improved HPLC method with electrochemical detection for the quantitation of EM in plasma and whole blood from a healthy subject and a patient with DPB. An alkaline mobile phase (final pH of 10.0) was used to stabilize EM.

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MATERIALS AND METHODS

Reagents

EM was donated by Shionogi Co., Ltd. (Lot No. 88005, Osaka, Japan). Oleandomycin phosphate, kitasamycin, midecamycin, spectral grade dichloromethane and methanol were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). HPLC-grade acetonitrile with a 190 nm cutoff was also supplied by Wako. Water was purified twice in a glass still. All other chemicals were of reagent grade.

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Standard Drug Solutions

Standard EM was dried at 60°C for 3 hours under reduced pressure (< 5-mm Hg). The EM was placed in a desiccator to cool and 10 mg of the standard was accurately weighed in a 100-ml volumetric flask on an electronic balance (Libror AEL-200, Shimadzu). The EM was adjusted with distilled water to 100 ml. A stock solution (100 μg/ml) of the internal standard (IS, oleandomycin phosphate) was prepared in distilled water in the same manner. All stock solutions were stored at -20°C. Degradation over a period of 3 months was minimal. The stock solutions were diluted to appropriate concentrations when use.

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Apparatus and Chromatographic Conditions

The HPLC system consisted of a model LC-6A pump (Shimadzu Co., Ltd., Kyoto, Japan) equipped with a Rheodyne model 7125 syringe-loading sample injector (Rheodyne, Cotati, CA, U.S.A.), and a model C-R3A Chromatopac integrator (Shimadzu). Electrochemical measurements were performed using glassy carbon working electrodes set at +0.72 V versus an Ag/AgCl reference electrode (a model L-ECD-6A Shimadzu). The mobile phase, consisting of 50 mM KH_2PO_4 (pH 10.5) and acetonitrile (63:37, v/v), was pumped through the column at a speed of 1 ml/min. The mobile phase was passed through a 0.45 μm filter (Millipore, Bedford, MA, U.S.A.) prior to use, and degassed with an ERC-3322 degasser (Erma Co., Ltd., Saitama, Japan). The solvent was degassed by the reduced pressure. The analysis was performed on a reversed phase Asahipak octadecyl polymer (ODP) column (4.6 mm i.d. × 15 cm; particle size, 5 μm, Asahi Chemical Industry Co., Ltd., Kanagawa, Japan), equipped with a guard column packed with ODP-50G (4.6 mm i.d. × 10 mm; particle size, 5 μm, Asahi Chemical Industry Co., Ltd.). The ODP column packed with octadecyl-bonded polymer gel has been developed for reversed phase liquid chromatography by substituting the stearate ester

groups for the hydroxy groups of vinyl alcohol copolymer particles.[14-15] The ODP column can be used under an alkaline elution buffer.

Assay Procedures

Samples (0.2 ml) of plasma or whole blood were placed in 1.5-ml polypropylene tubes with safety lock (Eppendorf, Hamburg, Germany). Ten μl of IS (10 μg/ml), saturated sodium carbonate aqueous solution (20 μl) and diethyl ether (1 ml) were added to the plasma or whole blood and mixed vigorously for 30 seconds. Following centrifugation at 6000 g for 2 minutes, 0.75 ml of the diethyl ether was transferred to another polypropylene tube. The diethyl ether was evaporated to dryness under a nitrogen gas stream at room temperature for 10 minutes. The residue was reconstituted in 50 μl of the mobile phase, and an aliquot (20 μl) was injected into the HPLC column.

Quantification

To prepare a calibration curve, the standard samples of EM were arranged in drug-free plasma or whole blood by adding known amounts of EM to give final concentrations ranging from 0.1 to 5.0 μg/ml. The calibration curve for EM was obtained from the

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analyte/IS peak area ratio *versus* various concentrations of EM. All samples were prepared in triplicate. The concentrations of EM in plasma or whole blood samples were calculated from the calibration curve using linear regression least square analyses.

Analytical Variables

The recovery ratio of EM during the extraction procedures was obtained as follows: Ten μl of IS (10 μg/ml), saturated sodium carbonate aqueous solution (20 μl) and diethyl ether (1 ml) were added to the samples (0.2 ml) with known amounts of EM (0.2, 1.0 and 5.0 μg/ml) in plasma or whole blood, mixed vigorously for 30 seconds and analyzed by the HPLC after complete extraction, as described above. Meanwhile, 0.15 ml of the same concentrations of EM (0.2, 1.0 and 5.0 μg/ml) dissolved in water was added to 0.75 ml of diethyl ether containing 10 μl of IS (10 μg/ml), mixed vigorously and evaporated to dryness. The residue was analyzed in the same manner. The peak area ratios of these samples were compared with those of EM water solutions taken directly through the HPLC procedure. Reproducibility were evaluated by analyzing pooled plasma or whole blood samples

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containing 0.2, 1.0 or 5.0 μg/ml of EM after the complete extraction procedures every 6 days for 2 months, 5 times each day (every 2 hours), 5 injections of each sample.

Drug Interference Study

Patient with DPB normally take the following drugs in addition to EM; diltiazem hydrochloride, dipyridamole, orciprenaline hydrochloride, methacholine bromide, theophylline, ketotifen fumarate and clenbuterol hydrochloride. Therefore possible interference by those drugs with the quantitation of EM on the HPLC chromatogram was examined. An aliquot of the aqueous or methanol solutions of those drugs was injected directly into the HPLC.

Clinical Study

To test the clinical applicability of this method further, plasma and whole blood samples from a healthy male volunteer (36-year-old) and a female patient with DPB (54-year-old) receiving EM (Ilotycin®, an enteric-coated tablet containing 200 mg of EM, Shionogi Co., Ltd.) were analyzed by this HPLC method. The patient received one tablet 3 times a day (every 8 h) for 50 consecutive days. On the 51th day and after an

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overnight fast, the patient ingested one tablet and food was withheld for an additional 6 hours. A healthy volunteer also fasted overnight and food was withheld for 6 more hours. Blood samples from the healthy subject and the patient were obtained from the antecubital vein through a heparin lock (before and 0.5, 1, 2, 4, 6, 8, 10 and 12 hours after drug administration from the healthy subject and before and 0.5, 1, 2, 4, 6 and 8 hours after drug administration from the patient). The blood samples were centrifuged immediately at room temperature at 2000 g for 10 min. The fractionated plasma and whole blood samples were stored at -20°C until analysis.

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RESULTS AND DISCUSSION

Chromatography

As reported by previous investigators, the retention time of EM, varied greatly dependent on the pH of mobile phase under our HPLC conditions. It was shorter in the acidic mobile phase and longer in the alkaline mobile phase. The capacity factor (k') of EM and the effect of pH of the mobile phase is illustrated in Figure 1. Increasing the extent of

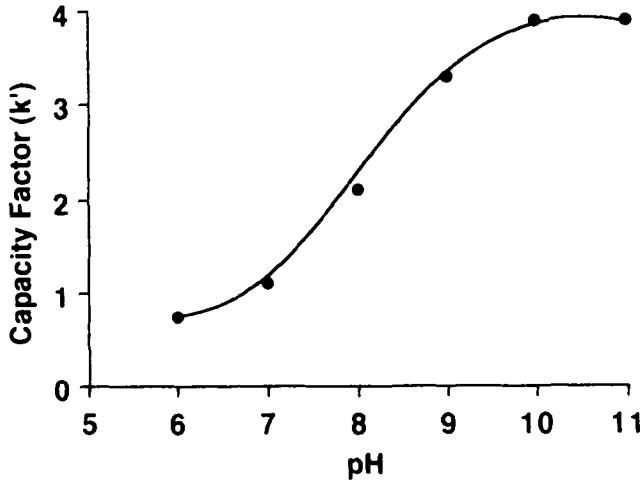

Figure 1. Effect of pH on retention for erythromycin on a ODP-50 column (5 μm, 15 cm × 4.6 mm inside diameter). A 50 mM potassium phosphate at various pH was used in the component of the mobile phase.

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acetonitrile in mobile phase, the peak corresponding to EM moved forward on the HPLC chromatogram. Alkaline mobile phase (final pH of 10.0) was used stabilise of EM. First, cell potentials between +0.6 and +0.8 V were tried to detect EM. The maximum peak intensity of EM showed plateaux over 0.75 V (Figure 2). Although these potentials were suitable for the quantitation of EM, endogenenous plasma and whole blood constituents were also detected at these high oxidative potentials, which interfered with the quantitation of EM. Therefore, +0.72 V was used as

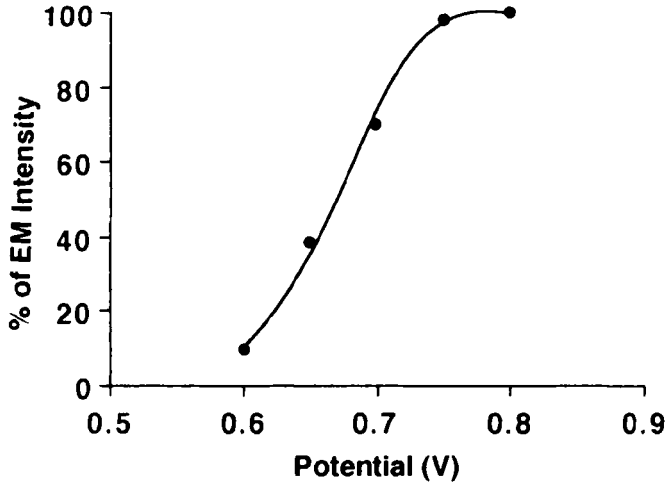

Figure 2. Effect of potential voltage on the peak intensity of erythromycin.

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the potential of the voltammograms. With our chromatographic system, both EM and IS exhibited symmetrical peaks (Figures 3 and 4) with base line resolutions and no disturbance from endogenous components in the plasma and whole blood from either a normal person or the patient with PDB. Oleandomycin phosphate, kitasamycin and midecamycin, all macrolide antibiotic reagents, were tested for an internal standard, with retention times of 5.7, 19.6 and 23.5 minutes, respectively. When oleandomycin phosphate was used as the internal standard, the analytical run time was within 15 minutes. The retention times of EM and IS were 12.1 and 5.7 min, and their capacity

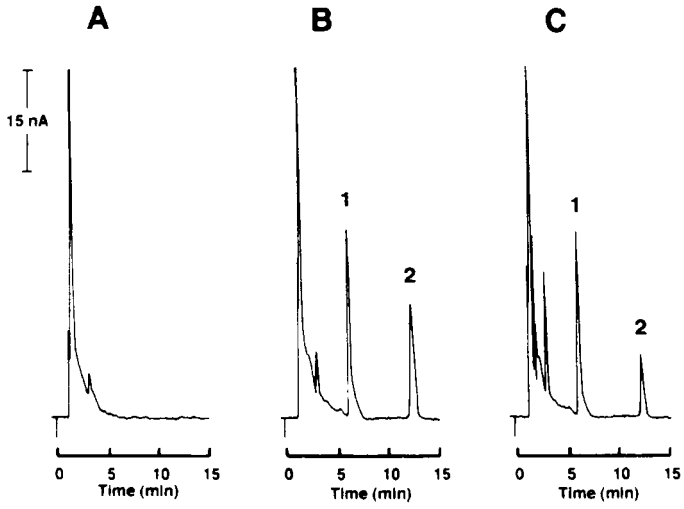

Figure 3. HPLC chromatograms of erythromycin in plasma. A: extract of blank plasma. B: extract of blank plasma supplemented with erythromycin (1.0 μg/ml) and IS (0.5 μg/ml). C: extract of plasma from a patient with DPB at 4 hours after dosing (200 mg), in which the concentration of erythromycin was estimated to be 0.7 μg/ml. Peaks: 1 = oleandomycin (IS) and 2 = erythromycin.

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factors (k'), 8.43 and 3.86, respectively. The separation factor (α) between EM and IS was 2.18. Typical chromatograms of EM and IS taken after the extraction procedures from plasma and whole blood of a healthy subject and a patient with DPB are illustrated in Figures 3 and 4, respectively. The figures show chromatograms of extracts of blank plasma (3A) and whole blood (4A), and extracts of blank plasma (3B) and whole blood (4B) combined with EM (1.0 μg/ml) and

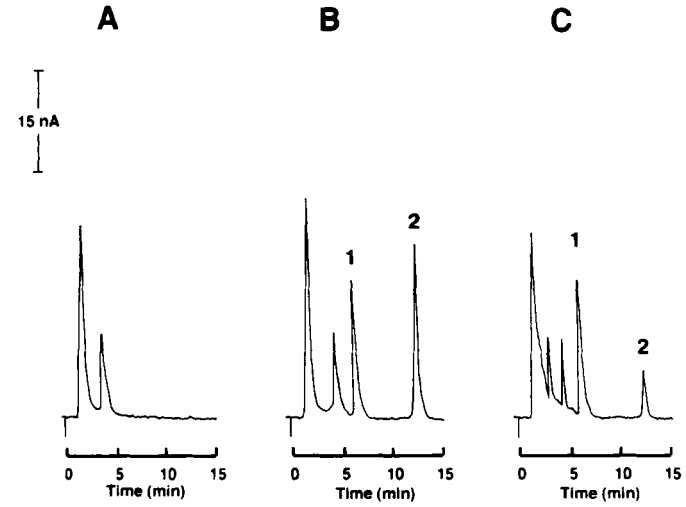

Figure 4. HPLC chromatograms of erythromycin in whole blood. A: extract of blank whole blood. B: extract of blank whole blood supplemented with erythromycin (2.0 μg/ml) and IS (0.5 μg/ml). C: extract of whole blood from a patient with DPB at 6 hours after dosing (200 mg), in which the concentration of erythromycin was estimated to be 0.6 μg/ml. Peaks: 1 = oleandomycin (IS) and 2 = erythromycin.

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IS (0.5 μg/ml). Figures 3C and 4C illustrate an example of a healthy subject and a patient with DPB after dosing. The calibration curves for EM, prepared as described above, were linear and passed through the origin: $y = 0.1625 x + 0.0971$, $r = 0.9941$ for plasma and $y = 0.3391 x + 0.0387$, $r = 0.9982$ for whole blood.

Drug Interference Study

The drugs (see Drug Interference Study *section in* MATERIALS AND METHODS) that the patient was taking in

addition to EM did not interfere with the quantitation of EM by this HPLC system.

Analytical Variables

Table 1 shows the amounts of EM and IS recovered from the plasma and whole blood after the extraction procedures at three different concentrations of EM. The mean recovery rate was over 80.1% from plasma and over 75.2% from whole blood. Corresponding rates for IS were over 76.5% and 46.3%. When a 200 μl sample of plasma or whole blood was used, the lower threshold of EM to be detected was as low as 0.1 μg/ml after complete extraction procedures (20 μl injection, signal/noise ratio of 3). However, EM in concentrations < 0.1 μg/ml could be detected when the plasma (or whole blood) sample and injection volume were increased. The coefficients of variation (CVs) for the detection limit variability from plasma and whole blood were 4.4 and 5.3%, respectively. The results of the analysis on reproducibility for intra- and inter-day assay variations are summarized in Table 2. These data suggest that the method is precise and accurate for routine clinical use.

Clinical Application

To test the clinical applicability of this HPLC method further, plasma and whole blood samples from a

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TABLE 1
Analytical Recoveries of Erythromycin from plasma and Whole blood

	Plasma			Whole Blood		
Compound	**Conc, μg/ml**	**Mean ± SD, %**	**CV, %**	**Conc, μg/ml**	**Mean ± SD, %**	**CV, %**
Erythromycin	5.0	88.2 ± 2.1	5.0	5.0	85.4 ± 3.2	6.1
	1.0	80.9 ± 3.3	5.2	1.0	78.7 ± 3.9	7.2
	0.2	80.1 ± 5.1	6.4	0.2	75.2 ± 3.5	7.2
Oleandomycin[a]	10.0	76.5 ± 4.8	6.3	10.0	46.3 ± 5.4	7.5

[a] Internal standard, n = 5

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TABLE 2
Analytical Precision and Accuracy for Determining of Erythromycin in Plasma and Whole Blood

Plasma		Whole Blood	
	CV, %		CV, %
Conc, μg/ml		Conc, μg/ml	
Intra-assay variation (n = 5 each)			
0.2	5.8	0.2	6.2
1.0	3.4	1.0	3.2
5.0	4.0	5.0	1.0
Interassay variation (n = 5 each)			
0.2	4.1	0.2	5.6
1.0	2.3	1.0	6.3
5.0	4.2	5.0	5.1

healthy volunteer and a patient with DPB after oral administration of 200 mg of EM were examined. Figures 5 A and B illustrate the analytical results for EM in the plasma and whole blood. The pharmacokinetic profiles of EM in the plasma and whole blood of each individual was some what different (Figure 5). The elimination half-lives of EM in plasma and whole blood in a healthy subject and patient were 3.0 and 2.2 hours and 3.2 and 2.5 hours, respectively. The plasma half-life in a healthy subject was generally compatible with the values already reported.[16] The maximum plasma and whole blood concentrations of EM were 0.6 and 1.0 μg/ml in a healthy subject and 0.9 and 1.6 μg/ml in the patient. The concentration of EM in whole blood was always higher than in plasma in both the healthy subject and the patient with DPB. Recent studies have shown that EM is taken into the intracellular space by both human polymorphonuclear leukocytes (PML) and alveolar macrophages and accumulate at 15 to 24 times the extracellular levels.[17-19] Masaki, et al. have reported the penetration of EM into human PML in vitro,[20] in which the intracellular concentration of EM was 6.6 times higher than the extracellular concentration. The differences of EM concentrations in plasma and whole

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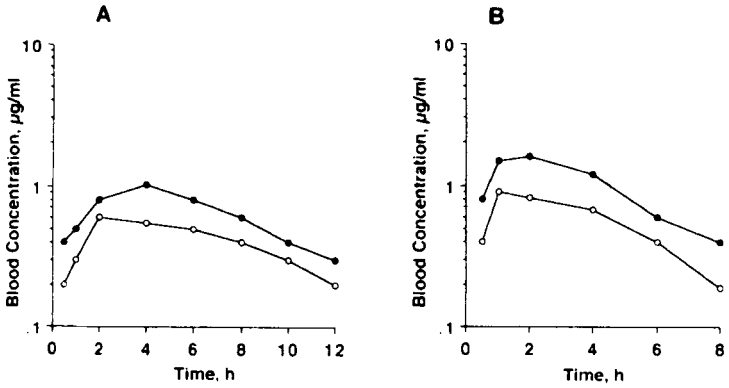

Figure 5. Plasma (O) and whole blood (●) concentration-time curves for erythromycin in a healthy volunteer (A) and a patient with DPB (B) following an oral administration of 200 mg of erythromycin.

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blood have not been fully elucidated. Probably, showing EM appearing in the whole blood at higher levels than in the plasma may be due to the intracellular uptake and accumulation by PML and/or macrophages. Aldhous, et al. have reported that infected patients have higher plasma EM concentrations than normal individuals.[21] In this case, the higher concentration of EM in the patient may be due to repeated administrations of EM.

The HPLC procedure presented in this article will allow a rapid, specific and sensitive quantitation of EM and is suitable for pharmacokinetic studies and the routine monitoring of EM in both plasma and whole blood.

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