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Determination of erythromycin base and 2′-acetylerythromycin in human plasma using high-performance liquid chromatography with electrochemical detection

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SUMMARY

A high-performance liquid chromatographic method for the determination of plasma concentrations of erythromycin base and 2′-acetylerythromycin, an ester prodrug of erythromycin, is described. *tert.*-Butyl methyl ether extracts of 1-ml plasma samples (pH 10) were chromatographed on a C_{18} reversed-phase column A three-electrode coulometric detector (oxidation potentials +0.65 and +0.85 V) was used for quantitation. Oleandomycin was used as an internal standard. The method has good precision and accuracy, is linear in the range 0.25–7.5 mg/l and has proved to be suitable for pharmacokinetic studies in humans Correlation with a microbiological assay was satisfactory ($r \geq 0.95$), but the chromatographic method gave ca 30% higher values.

INTRODUCTION

Unpredictable and incomplete absorption has limited the clinical usefulness of the macrolide antibiotic erythromycin in the treatment of infections. One important reason for this variation in bioavailability is the acid lability of

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erythromycin base: it is inactivated by gastric acid and inactive anhydrous forms are produced. To overcome this, erythromycin is usually administered as enteric-coated preparations or as esters. Erythromycin esters serve as prodrugs, which are first absorbed from the gastrointestinal tract and then undergo gradual hydrolysis to free erythromycin base, the only antibacterially active form. Erythromycin acistrate (2′-acetylerythromycin stearate) is a new erythromycin derivative. 2′-Acetylerythromycin is absorbed into the circulation and is gradually hydrolysed to free erythromycin base in body fluids and in tissues.

Microbiological assays developed for erythromycin base are not suitable for the quantitative determination of erythromycin esters. Esters are microbiologically inactive, but are hydrolysed to erythromycin base during incubation on agar plates, thus leading to over-estimation of the antimicrobial activity.

Chemical methods are therefore required for the concomitant quantitative analysis of erythromycin base and its esters. At least eleven reports of high-performance liquid chromatographic (HPLC) methods for the determination of erythromycin base, some erythromycin esters and their degradation products have been published [1–11]. Most assays employ reversed-phase chromatography with silica-based C_{18} columns [2–11]. Ultraviolet [2,4,11], fluorimetric [3] and electrochemical [1,5–10] detection have been used for quantitation.

The aim of the present study was to develop and validate a sensitive, reproducible and rapid method for the quantitation of erythromycin base and the novel derivative, 2′-acetylerythromycin, in human plasma, for use in pharmacokinetic and bioavailability studies.

EXPERIMENTAL

Materials

Erythromycin base, 2′-acetylerythromycin, anhydroerythromycin base, anhydro-2′-acetylerythromycin, erythromycin hemiketal and 2′-acetylerythromycin hemiketal were obtained from the Research Centre of Farmos Group, Medipolar (Oulu, Finland). The internal standard, oleandomycin phosphate, was purchased from Sigma (St. Louis, MO, U.S.A.). Methanol and acetonitrile were of HPLC grade and were purchased from Rathburn (Walkerburn, U.K.). Anhydrous sodium acetate (analytical grade), glacial acetic acid 100% (analytical grade) and *tert.*-butyl methyl ether (spectroscopic grade) were obtained from E. Merck (Darmstadt, F.R.G.). Absolute ethanol was from Alko Oy (Rajamäki, Finland).

Standard solutions

Standard stock solutions were made by dissolving 50 mg of oleandomycin phosphate, erythromycin base and 2′-acetylerythromycin in 5 ml of ethanol.

Solutions were kept at −20°C and replaced every two weeks. The working solutions (100 mg/l in ethanol) were prepared weekly and stored at −20°C.

HPLC instrumentation

The chromatographic apparatus consisted of an HPLC pump (Model 2150, LKB, Bromma, Sweden), a pressure filter (Model LP-21, Scientific Systems, State College, PA, U.S.A.), a syringe loading sample injector with a 20-μl loop (Model 7125, Rheodyne, Berkeley, CA, U.S.A.), a Direct Connect® guard column (Alltech Assoc., Deerfield, IL, U.S.A.) packed with μBondapak C_{18} (Waters Assoc., Milford, MA, U.S.A.) and an Ultrasphere C_{18} reversed-phase column (25 cm×4.6 mm I.D., particle size 5 μm, Beckman Instruments, San Ramon, CA, U.S.A.).

The column effluent was monitored by a three-electrode coulometric detector (Model 5100A, ESA, Bedford, MA, U.S.A.). The guard cell potential was set to +0.95 V and the potentials of the screen electrode (detector 1) and the sample electrode (detector 2) were varied in order to find the optimal detection conditions. The chromatography was carried out at room temperature (range, 20–25°C). The 1000-mV output from detector 2 was connected to a linear strip chart recorder with two channels (BD41, Kipp & Zonen, Delft, The Netherlands). The chart speed was 5 mm/min.

Mobile phase preparation

The mobile phase was acetonitrile–methanol–0.1 *M* sodium acetate buffer (pH 5.0) (42:10:48, v/v). The buffer was prepared by titrating 0.1 *M* sodium acetate with 0.1 *M* acetic acid to the appropriate pH. The final pH of the mobile phase was adjusted to 6.30–6.35. The water used in mobile phase preparation was first distilled and then deionized in a Milli Q apparatus (Millipore, Bedford, MA, U.S.A.). The mobile phase was filtered through a 0.45-μm filter (type HVLP, Millipore) and degassed under reduced pressure. The mobile phase was pumped at a flow-rate of 1.20 ml/min (pressure 15 MPa) and was allowed to recirculate.

Sample preparation

Venous blood sampled were collected by venepuncture into prechilled polypropylene tubes with K_2 EDTA as anticoagulant, chilled in ice and centrifuged (10 min, 3000 *g*, 4°C) within 30 min to separate the plasma. Frozen human plasma samples kept at −70°C were thawed and centrifuged at 3000 *g* for 10 min at 4°C. An aliquot (1 ml) was pipetted into a 10-ml conical borosilicate tube. After addition of the internal standard, oleandomycin phosphate, (20 μl of a 100 mg/l solution in ethanol to produce a concentration of 2.0 mg/l in plasma), the samples were made alkaline (pH 10) with 60 μl of saturated potassium carbonate (pH 12) and mixed briefly. Then 5 ml of *tert.*-butyl methyl ether were added, and the tubes were stoppered and shaken in a reciprocating shaker for 15 min. After centrifugation (5 min, 800 *g*) 4 ml of the upper ether

layer were transferred into a 5-ml glass tube and evaporated to dryness under a stream of dry nitrogen at room temperature. Next, 200 μl of *tert.*-butyl methyl ether were added to rinse the inner wall of the tube to concentrate the sample; this was carefully evaporated, avoiding prolonged exposure to the nitrogen stream.

The dry residue was dissolved in 125 μl of the mobile phase, transferred into a 1-ml conical plastic tube and stored at −20°C until analysis (no longer than 48 h). The samples were mostly turbid and for that reason they were centrifuged just prior to analysis with a Beckman Microfuge Model 11 (Beckman Instruments) for 30 s at speed 11. The injection volume was 40 μl.

All glassware used in sample preparation was silanized with 5% (v/v, in toluene) SurfaSil® silanizing reagent (Pierce, Oud-Beijerland, The Netherlands).

Preparation of calibration curves

Calibration curves were made by spiking drug-free human plasma with erythromycin base or 2′-acetylerythromycin to yield concentrations of 0.25, 0.50, 2.0, 5.0 and 7.5 mg/l. The extraction procedure was carried out as previously described.

The lot of 2′-acetylerythromycin used as reference material contained ca. 7% erythromycin base as impurity. Because of this, calibration curves were prepared separately for erythromycin base and 2′-acetylerythromycin.

Calibration curves were obtained by plotting the peak-height ratio (erythromycin or 2′-acetylerythromycin/internal standard) on the ordinate and the respective drug concentration on the abscissa.

Stability

The stability of erythromycin and 2′-acetylerythromycin in plasma was studied with spiked plasma samples (5.0 mg/l) that were stored at −20°C or −70°C for four weeks. The samples were prepared as described above.

The stability during plasma processing was studied by taking blood samples from a human volunteer who had been treated with erythromycin acistrate tablets (400 mg three times a day) and incubating the whole blood at 0°C for 0, 0.5 and 1 h after which the blood was centrifuged and the plasma extraction carried out.

The stability in plasma was further studied with spiked plasma samples (5.0 mg/l) that were incubated at 0°C or at room temperature for 0, 0.5 and 1 h before being extracted as described above.

The stability of the compounds dissolved in the mobile phase was studied with reference materials. Samples were stored at four different temperatures (20, 4, 0 and −20°C) for 24 and 48 h.

Precision and accuracy

Intra-assay precision was assessed using six spiked plasma samples at three different concentrations: 0.5, 2.0 and 5.0 mg/l.

Inter-assay precision was studied using a control sample that was independently analysed eighteen times within a 25-day period. The control sample pool was prepared by pooling plasma from three volunteers who had ingested the erythromycin acistrate product. The control sample was divided into 1-ml aliquots and stored frozen at $-70°C$.

Accuracy was evaluated by calculating found erythromycin base and 2′-acetylerythromycin amounts in spiked samples from the calibration curve.

Recovery

Recovery was studied with plasma samples spiked with 1.0 and 5.0 mg/l erythromycin base or 2′-acetylerythromycin and extracted as previously described. The results were compared with equal amounts of pure compounds dissolved in mobile phase.

Pharmacokinetic study and comparison with microbiological assay

This method for determination of erythromycin base and 2′-acetylerythromycin in plasma was used in a comparative, multiple-dose bioavailability study of two erythromycin preparations: erythromycin base (250 mg) tablets and erythromycin acistrate (corresponding to 400 mg of erythromycin base) tablets in twenty healthy volunteers.

The agreement between this HPLC method and a microbiological method was studied with two sets of samples ($n=73$ and 39) from subjects taking the erythromycin base product. The microbiological assay employed the cylinder diffusion method with *Sarcina lutea* (ATCC 9341) as the test organism and Antibiotic Medium 11 as the culture medium. A mixture of bovine serum and phosphate buffer (pH 8.0) (1:4, v/v) was used to dilute the specimens, as well as to prepare the standard curve (from 0 to 0.8 mg/l).

RESULTS AND DISCUSSION

Chromatographic conditions

The optimal detector cell potential for the oxidation of oleandomycin, erythromycin base and 2′-acetylerythromycin was explored by using reference compounds dissolved in aliquots of mobile phase. The resulting hydrodynamic voltammograms are shown in Fig. 1. Based on these results, +0.65 V was chosen as the first (detector 1) and +0.85 V as the second electrode (detector 2) potential.

The retention times of oleandomycin, erythromycin base and 2′-acetylerythromycin were influenced by the acetonitrile concentration and the pH of the mobile phase. As shown in Fig. 2, the retention times decreased when the

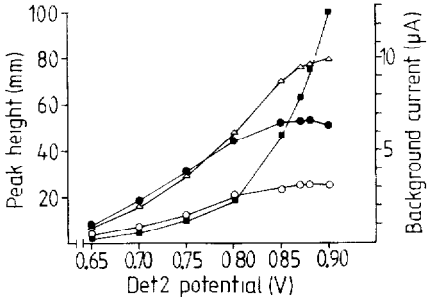

Fig. 1. Hydrodynamic voltammograms for oleandomycin (△), erythromycin base (●) and 2′-acetylerythromycin (○). The relationship between background current and applied potential at detector 2 is also shown (■) Mobile phase, acetonitrile–0.1 *M* sodium acetate buffer (pH 5.0)–methanol (42 48 10, v/v), final pH 6.35.

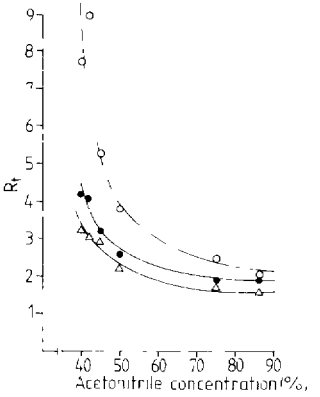

Fig. 2. Effect of acetonitrile concentration on the retention of oleandomycin (△), erythromycin base (●) and 2′-acetylerythromycin (○) at pH 6.35.

concentration of acetonitrile was increased. A mobile phase with 42% (v/v) acetonitrile was chosen as it gave sufficient separation within a reasonable elution time.

The effect of mobile phase pH on retention is shown in Fig. 3. The retention times of all compounds increased when the pH was increased. By a slight adjustment of the mobile phase pH, an unknown peak could be separated from the erythromycin base peak. The optimal pH value was 6.30–6.35, depending on the age of the column.

Typical chromatograms of oleandomycin, erythromycin base and 2′-acetylerythromycin are shown in Fig. 4. The lower limit of reliable detection of this method was 0.25 mg/l, based on peaks that could be easily manually measured (signal-to-noise ratio at least 3). The relative retention times of erythromycins and their potential plasma metabolites are listed in Table I.

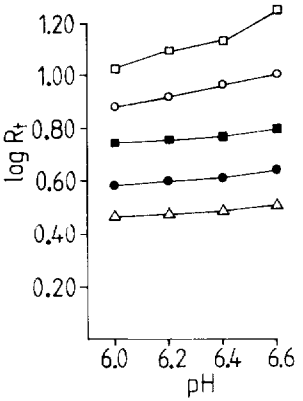

Fig. 3. Effect of pH on the retention of oleandomycin (△), erythromycin base (●), anhydroerythromycin (■), 2′-acetylerythromycin (○) and anhydro-2′-acetylerythromycin (□) in mobile phase containing 42% (v/v) acetonitrile.

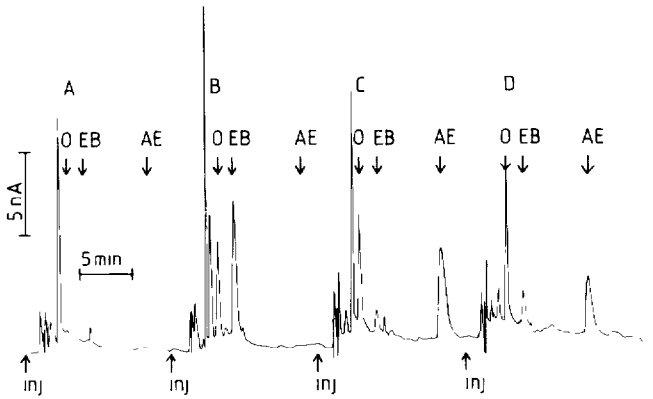

Fig. 4. Chromatograms of (A) drug-free plasma, (B) plasma spiked with oleandomycin (O, 2 mg/l) and erythromycin base (EB, 5 mg/l), (C) plasma spiked with O and 2′-acetylerythromycin (AE, 5 mg/l, containing 7% EB as impurity) and (D) plasma from a human volunteer after ingestion of 400 mg of erythromycin acistrate (with O as internal standard).

Linearity

Calibration curves (0.25–7.5 mg/l) in plasma were linear and yielded the following equations: $y=0.427x+0.001$, $r=0.9993$ for erythromycin base and $y=0.132x+0.023$, $r=0.9971$ for 2′-acetylerythromycin.

Stability

Erythromycin base in plasma was stable for at least four weeks at −70°C as well as at −20°C. 2′-Acetylerythromycin tended to be slightly more labile: 3% was hydrolysed to erythromycin base when stored at −70°C and 5% when stored at −20°C for four weeks ($n=2$ for all samples).

TABLE I

RELATIVE RETENTION TIMES OF ERYTHROMYCIN BASE, 2′-ACETYLERYTHROMYCIN AND THEIR DEGRADATION PRODUCTS AND OLEANDOMYCIN

The relative retention time is the retention time of the compound divided by void volume/flow-rate.

Compound	Relative retention time
Oleandomycin	2.92
Erythromycin base	3.96
Anhydroerythromycin base	5.77
2′-Acetylerythromycin	9.08
Erythromycin hemiketal	11.11
Anhydro-2′-acetylerythromycin	12.71
2′-Acetylerythromycin hemiketal	35.83

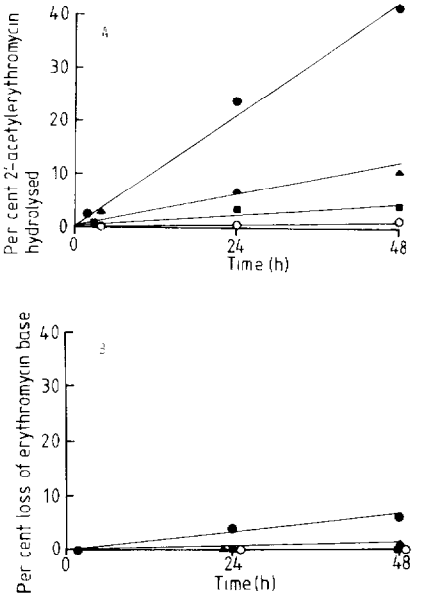

Fig. 5. (A) Stability of 2′-acetylerythromycin dissolved in mobile phase at room temperature (●), 4°C (▲), 0°C (■) and −20°C (○). (B) Stability of erythromycin base dissolved in mobile phase at room temperature (●), 4°C (▲), 0°C (■) and −20°C (○).

No loss of either erythromycin base or 2′-acetylerythromycin was seen when whole blood was incubated at 0°C for 0.5 or 1 h. Incubation of plasma at 0°C or at room temperature for 1 h did not influence the concentration of either drug ($n=4$ at both temperatures).

The stability of erythromycin base and 2′-acetylerythromycin dissolved in mobile phase is shown in Fig. 5A and B. 2′-Acetylerythromycin was rapidly

hydrolysed to erythromycin base (ca. 1%/h) when samples were kept at room temperature. Less than 2% of 2′-acetyl erythromycin was hydrolysed during 48 h when the samples were stored at −20°C. The loss of erythromycin base was 10% in 48 h at room temperature. No loss at all was seen when samples were kept at −20°C ($n=2$ for all samples).

Oleandomycin behaved unexpectedly when stored in the mobile phase at room temperature: its peak height seemed to increase. No change of the oleandomycin peak height was seen at +4°C or colder.

As a result of these experiments, blood samples were immediately placed on ice after collection and centrifuged at +4°C, and the plasma was promptly frozen to −70°C. Extracted samples dissolved in the mobile phase were stored at −20°C until analysis (no longer than 48 h). The rates of hydrolysis of 2′-esters of erythromycin are known to be medium- and temperature-dependent [7,12]. No extensive investigation of sample stability was attempted in the present study, but the satisfactory inter- and intra-assay precision (see below) obtained using this procedure for sample handling and storage indicated that hydrolysis of 2′-acetylerythromycin can be sufficiently controlled. Storage of plasma samples for periods exceeding four weeks was not investigated.

Precision and accuracy

Intra-assay precision is presented in Table II. The observed coefficients of variation (C.V.) at concentrations 0.5, 2.0 and 5.0 mg/l were 4.4, 1.9 and 4.4% for erythromycin base and 4.6, 7.4 and 6.6% for 2′-acetylerythromycin ($n=6$).

The accuracy of the determination at drug concentrations of 0.5, 2.0 and 5.0 mg/l is presented in Table III.

Inter-assay precision was evaluated by using a control sample containing 0.40 mg/l erythromycin base and 0.85 mg/l 2′-acetylerythromycin. The C.V.

TABLE II

ESTIMATION OF INTRA-ASSAY PRECISION USING SPIKED PLASMA SAMPLES

$n=6$ for each sample

Added (mg/ml)	Erythromycin/internal standard peak-height ratio							C.V. (%)
	Individual values						Mean	
Erythromycin base								
0.50	0.21	0.21	0.23	0.22	0.20	0.21	0.21	4.4
2.00	0.86	0.85	0.83	0.88	0.84	0.84	0.85	1.9
5.00	2.00	2.06	1.83	2.07	1.98	1.94	1.98	4.4
2′-Acetylerythromycin								
0.50	0.07	0.08	0.08	0.08	0.08	0.08	0.08	4.6
2.00	0.33	0.32	0.34	0.29	0.36	0.33	0.33	7.4
5.00	0.77	0.75	0.85	0.86	0.89	0.79	0.82	6.6

TABLE III

ESTIMATION OF ACCURACY USING SPIKED PLASMA SAMPLES

$n=6$ for each sample.

Added (mg/l)	Erythromycin base		2′-Acetylerythromycin	
	Found (mg/l)	Deviation (%)	Found (mg/l)	Deviation (%)
0.50	0.44	−12.0	0.46	−8.0
	0.44	−12.0	0.51	+2.0
	0.49	−2.0	0.51	+2.0
	0.46	−8.0	0.51	+2.0
	0 42	−16.0	0.52	+4.0
	0.43	−14.0	0.50	0.0
Mean	0.45		0.50	
C.V. (%)	5.6		4.2	
Accuracy (mean ± S.D.)		10.6 ± 5.0		3.0 ± 2.8
2.00	2.11	+5.5	2.04	+2.0
	2.09	+ 4.5	1.94	−3.0
	2.03	+1.5	2 06	+3.0
	2.14	+7.0	1.75	−12.5
	2.06	+3.0	2.19	+9.5
	2.05	+2.5	2.02	+1.0
Mean	2.09		2.00	
C V. (%)	2.1		7.3	
Accuracy (mean ± S D.)		4.0 ± 2.0		5.2 ± 4.7
5.00	5.02	+0 4	4.73	−5.4
	5.18	+3.6	4.60	−8.0
	4.59	−8.2	5.21	+4.2
	5 19	+3.8	5.22	+4 4
	4.88	−2.4	5 49	+9.8
	4.88	−2.4	4.84	−3.2
Mean	4.98		5.02	
C.V. (%)	4.4		6.8	
Accuracy (mean ± S.D)		3.5 ± 2.6		5.8 ± 2.5

was 9.5% for erythromycin base ($n=18$) and 10.1% for 2′-acetylerythromycin ($n=13$).

Recovery

The absolute extraction recoveries (corrected for volume loss) of erythromycin base, 2′-acetylerythromycin and oleandomycin were 82 ± 6, 81 ± 7 and 72 ± 5% (means ± S.D., $n=12$), respectively.

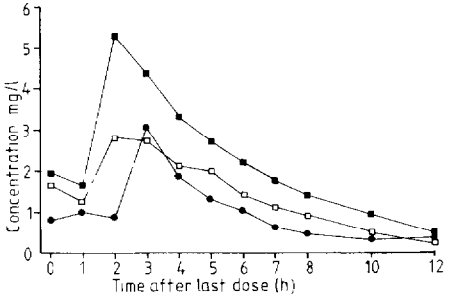

Fig. 6. Plasma erythromycin base (●) concentration after last dose of 250 mg of erythromycin base product and plasma erythromycin base (□) and 2′-acetylerythromycin (■) concentrations after last dose of erythromycin acistrate (corresponding to 400 mg of erythromycin base) product in one healthy volunteer.

Pharmacokinetic study and comparison with microbiological assay

Fig. 6 presents plasma levels of erythromycin base and 2′-acetylerythromycin after administration of multiple doses of erythromycin base and erythromycin acistrate in one volunteer and demonstrates the usefulness of this HPLC method in pharmacokinetic studies. Full results of this study will be presented separately.

Two batches of samples ($n=73$ and 39) from subjects taking the erythromycin base preparation were assessed in parallel with this HPLC method and with a microbiological assay. The results were linearly correlated ($r=0.95$ and 0.98), with regression lines of $y=0.711x+0.038$ and $y=0.797x-0.079$. Thus, the results produced by the different assays were comparable, but the microbiological assay systematically gave 20–29% lower concentrations. The reason for this difference may be related to different methods of calibration.

CONCLUSIONS

This HPLC method for determination of erythromycin base and 2′-acetylerythromycin in human plasma is precise, linear and simple, and has proved to be suitable for use in pharmacokinetic and bioavailability studies. It is also applicable for the determination of degradation products of erythromycin base and 2′-acetylerythromycin.

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