Journal of Chromatography, 419 (1987) 205–212
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3715

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY OF ERYTHROMYCIN AND ITS ESTERS USING ELECTROCHEMICAL DETECTION

D. CROTEAU, F. VALLÉE, M.G. BERGERON and M. LeBEL*

École de Pharmacie, Université Laval, Service d'Infectiologie, Centre Hospitalier de l'Université Laval, Quebec, Quebec G1V 4G2 (Canada)

(First received January 6th, 1987; revised manuscript received March 18th, 1987)

SUMMARY

A high-performance liquid chromatographic analysis of erythromycin and its esters in plasma, urine and saliva is presented. A diethyl ether extract of sample was chromatographed on a reversed-phase column and components of the column effluent were monitored by electrochemical detection at +0.9 V (vs. Ag/AgCl). The method sensitivity limit was 10 ng with inter-day coefficients of variation from 3.2 to 10.3%. In order to assess precisely the relative concentrations of erythromycin esters (ethylsuccinate or estolate) and their active by-product erythromycin base, it is necessary to adopt measures preventing their continuous hydrolysis in biological fluids and during sample preparation.

INTRODUCTION

Erythromycin, a macrolide antibiotic used since 1952, can be administered as acid-resistant but microbiologically inactive esters (estolate or ethylsuccinate). Pharmacokinetic studies differentiating between erythromycin base and its esters have been very few due to the lack of suitable assay which allows one to detect the intact pro-drug and its hydrolysed active product, erythromycin base.

Assay methods used in the past suffered major drawbacks such as poor specificity (microbiological) [1,2] or elaborate sample preparation (thin-layer chromatography, bioautography) [3,4]. High-performance liquid chromatography (HPLC) with fluorimetric determination of erythromycin [5], although very sensitive, required complex post-column derivatization and extraction. In view of the low molar absorption of erythromycin at conventionally used detection wavelengths (> 220 nm), HPLC with UV detection cannot be considered sensi-

0378-4347/87/$03.50

tive enough for pharmacokinetic studies. In spite of this, Stubbs et al. [6] described an HPLC analysis with UV detection at 200 nm suitable for erythromycin and its metabolites in human serum and urine. Nevertheless the sample preparation used in the assay (solid-phase extraction) would not prevent hydrolysis of erythromycin esters. Duthu [7] and more extensively Chen and Chiou [8] have described an HPLC assay with electrochemical detection of erythromycin using dual electrode cells. In this paper we describe a simple and sensitive HPLC method using a single electrode cell which may be useful in the quantification of erythromycin, its esters and various metabolites in plasma, urine and saliva.

EXPERIMENTAL

Materials

Erythromycin B, N-demethylerythromycin, erythrolosamine, 4″-acetylerythromycin and 6-O-methylerythromycin (A-56268) were kindly supplied by Abbott Labs. (North Chicago, IL, U.S.A.). Erythromycin A was purchased from Sigma (St. Louis, MO, U.S.A.). Erythromycin estolate was kindly provided by Eli Lilly (Indianapolis, IN, U.S.A.) and erythromycin ethylsuccinate was furnished by Novopharm (Montreal, Canada). Internal standard roxithromycin was kindly supplied by Roussel Canada (Montreal, Canada) and 9-deoxo-9*a*-aza-9*a*-methyl-9*a*-homoerythromycin (CP-62993) by Pfizer (Groton, CT, U.S.A.). Stock solutions (5 or 10 mg/ml) of erythromycin, its derivatives and roxithromycin were prepared in acetonitrile; these solutions were replaced monthly.

Sodium acetate, dipotassium hydrogen phosphate, acetonitrile, methanol and diethyl ether were all HPLC grade and purchased from Fisher Scientific (Fair Lawn, NJ, U.S.A.). Acetic acid was purchased from J.T. Baker (Phillipsburg, NJ, U.S.A.).

HPLC instrumentation

The chromatographic system consisted of a Waters Model 510 pump supplied with a high-sensitivity noise filter (Waters Lot No. 25200), a WISP 710B automatic injector and a Nova-Pak C_{18} column (Waters Assoc., Mississauga, Canada). The column effluent was monitored by a Waters M460 electrochemical detector in the oxidative mode with the amperometric cell potential set at +0.9 V (vs. Ag/AgCl reference electrode). The detector was interfaced with an HP-3390 data system (Hewlett-Packard Canada, Montreal, Canada). The mobile phase was pumped at a flow-rate of 1.1 ml/min (53 bar).

Mobile phase preparation

The mobile phase used in this study was 56 m*M* sodium acetate buffer–acetonitrile–methanol (56:50:4) in which the final pH was adjusted to 7.0 using concentrated acetic acid. In order to minimize the background noise the solvent mixture was pre-filtered with 0.22-μm Nylon 66 membrane filters (Fisher Scientific) and degassed using a magnetic stirrer in vacuo. The water used in the

mobile phase was purified through a Milli-Q system (Millipore, Mississauga, Canada).

Sample preparation

Frozen human plasma samples were thawed quickly (5 min) by placing the vials in warm water and aliquots (2 ml) were pipetted into 10-ml ground-glass stoppered conical extraction tubes. After the addition of internal standard (20 μl of roxithromycin solution, 750 μg/ml in acetonitrile), 5 ml of diethyl ether were added, the tubes were stoppered and then shaken vigorously for 3 min. Following centrifugation at 900 *g* for 5 min at 4°C, the upper layer was transferred into 13×100 mm disposable borosilicate tubes using a Pasteur capillary pipette and evaporated to dryness at 45°C under a stream of dry nitrogen (Reacti-Vap, Pierce, Rockford, IL, U.S.A.). The residue was reconstituted with 100 μl of acetonitrile and vortexed for 5 s to facilitate dissolution of the sample. A 40-μl aliquot of this sample was injected onto the column.

Standard curves were prepared by spiking erythromycin-free human plasma with 20 μl of concentrated acetonitrile solution of erythromycin base and estolate to yield 0, 0.5, 1, 2.5, 5, 7.5, 10 μg/ml or erythromycin base and ethylsuccinate to yield 0, 0.25, 0.5, 0.75, 1, 2, 3 μg/ml.

For urine samples (1.5 ml), the same preparation was used except that extraction with diethyl ether (4 ml) was preceded by the addition of 100 μl of saturated dipotassium hydrogen phosphate containing the internal standard roxithromycin at 750 μg/ml, increasing the pH from 6.5 to 8.5. Saliva samples (1.5 ml) were also extracted with diethyl ether (4 ml) and then centrifuged at 900 *g* for 15 min. Further steps are similar to plasma sample preparation.

RESULTS

Typical chromatograms of erythromycin, roxythromycin and erythromycin estolate or ethylsuccinate are shown in Fig. 1 (plasma) and Fig. 2 (urine). The retention times of various erythromycins and potential metabolites are listed in Table I. Although erythromycin base yielded an acceptable retention time, esters of erythromycin took much longer to eluate from the column (> 30 min). Attempts to shorten their retention time jeopardized erythromycin base analysis.

Linearity

Peak heights for drug and internal standard were recorded in integrator units and expressed as a ratio of drug to internal standard. In plasma the standard curves were linear from 0.25 to 10 μg/ml for erythromycin base and erythromycin estolate and from 0.25 to 3 μg/ml for erythromycin ethylsuccinate and yielded the following respective equations: $y=5.46x+0.27$ ($r=0.982$); $y=12.45x-0.07$ ($r=0.990$); $y=15.43x+0.002$ ($r=0.987$). In urine the standard curves were linear from 0.5 to 15 μg/ml and yielded the following equations for erythromycin base, estolate and ethylsuccinate, respectively: $y=2.63x+0.26$ ($r=0.989$); $y=23.59x+1.03$ ($r=0.988$); $y=11.84x+0.49$ ($r=0.982$).

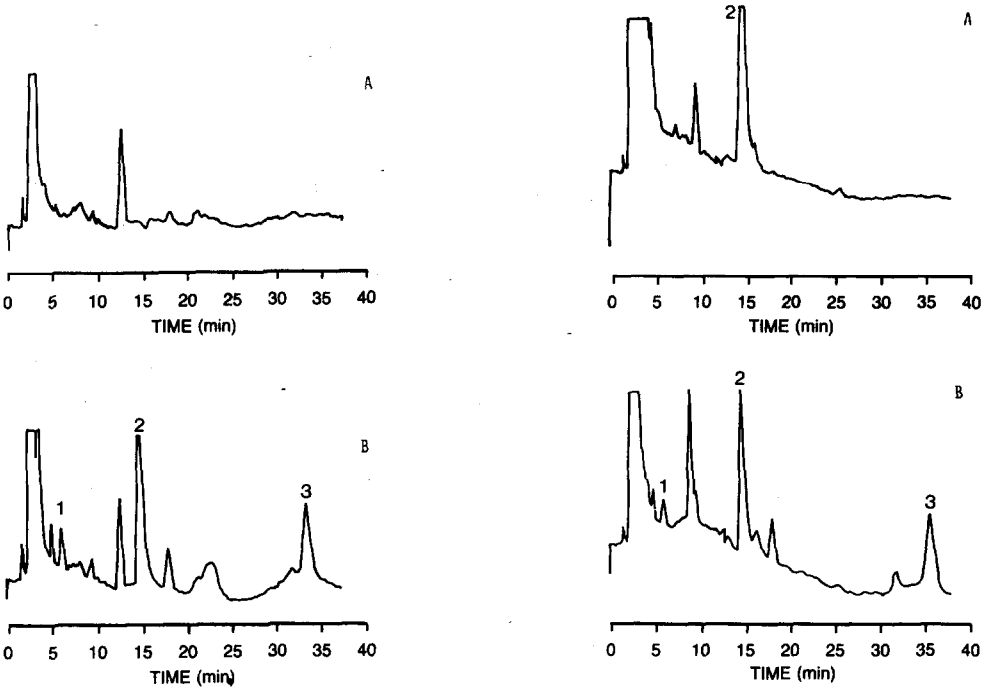

Fig. 1. HPLC results for the detection of erythromycin base and erythromycin estolate in plasma from (A) blank sample and (B) drug-supplemented sample. Peaks: 1 = erythromycin base (2.06 μg/ml); 2 = internal standard, roxithromycin (7.50 μg/ml); 3 = erythromycin estolate (5.91 μg/ml).

Fig. 2. HPLC results for the detection of erythromycin base and erythromycin ethylsuccinate in urine from (A) blank sample spiked with internal standard and (B) subject after an oral 600-mg dose of erythromycin ethylsuccinate (0–2 h period). Peaks: 1 = erythromycin base (1.20 μg/ml); 2 = internal standard, roxithromycin (15.0 μg/ml); 3 = erythromycin ethylsuccinate (6.88 μg/ml).

TABLE I

RETENTION TIMES OF VARIOUS ERYTHROMYCINS AND METABOLITES

Compound	Retention time (min)
CP-62993	3.8
Erythromycin base	6.0
Erythrolosamine	6.9
Erythromycin B	7.1
4″-Acetylerythromycin	7.4
6-O-Methylerythromycin	10.3
Roxithromycin	14.7
Erythromycin estolate	34.5
Erythromycin ethylsuccinate	35.5
N-Demethylerythromycin	Not detectable

Sensitivity and precision

The limit of detection of the assay has been evaluated to 10 ng, yielding a detector response approximately equal to three times the detector noise. Precision data were obtained by the repeated analysis of plasma samples and resulted in coefficients of variation of 3.2% for ethylsuccinate (six determinations at 4 μg/ml), 4.7% for estolate (seven determinations at 10 μg/ml) and 10.3% for erythromycin base (five determinations at 1 μg/ml).

Recovery

Plasma samples spiked to a final concentration of 10 μg/ml were taken through the entire sample preparation procedure described; these were compared with acetonitrile solutions taken directly through the HPLC procedure. Absolute recoveries of erythromycine base, estolate and ethylsuccinate were 55, 77 and 74%, respectively at pH 8.4 (thawed plasma samples).

Clinical study assay results

Figs. 3 and 4 represent plasma levels of erythromycin estolate, erythromycin ethylsuccinate and erythromycin base measured by HPLC following the administration of multiple doses of erythromycin esters in one volunteer.

DISCUSSION

Oxydation of erythromycin by this mode of detection occurs probably from the tertiary amine of the desoaminyl sugar moiety, N-demethylerythromycin being undetectable [7]. Electrochemical detection necessitates a mobile phase with a buffer in the low ionic range (10–100 m*M*) to provide adequate conductivity while minimizing the contribution to background current. Sodium acetate offered the best sensitivity in regards to the compounds of interests compared to sodium perchlorate and ammonium acetate which were tested.

As noted by previous investigators [5,7,8] the pH of the mobile phase was found to have great effect on retention times: thus increasing the mobile phase pH from 7.0 to 7.4 delayed erythromycin base by approximately 1 min and the esters up to 10 min (similar variations in the retention times could be observed with variations of the mobile phase composition). A pH of 7.0 was adopted in this study mainly to achieve good resolution of erythromycin base from interferences.

Loss in sensitivity (up to 60% in 24 h) after several injections of plasma extracts may be due to progressive poisoning of the working electrode. Therefore restoration of the glassy carbon working electrode's sensitivity was achieved by daily flushing of concentrated nitric and chromic acid followed by a 2–3 h period to allow background current to stabilize.

This HPLC assay can also be used for 6-O-methylerythromycin and CP-62993 with retention times of 10.3 and 3.8 min, respectively.

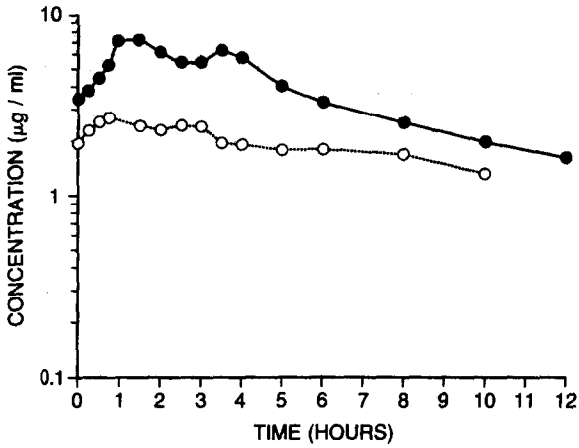

Fig. 3. Plasma levels of erythromycin estolate (●) and erythromycin base (○) following the administration of 500 mg erythromycin estolate every 8 h for ten doses.

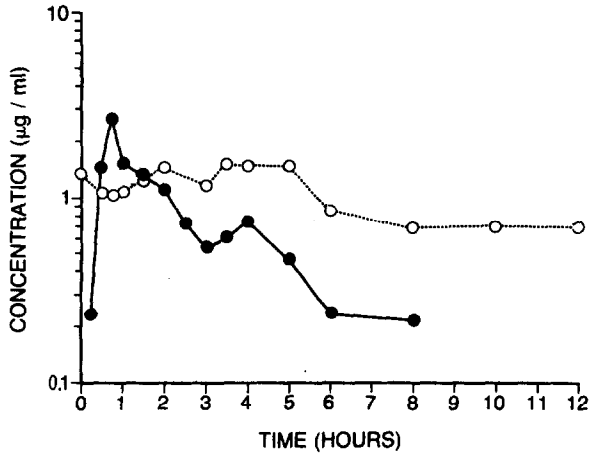

Fig. 4. Plasma levels of erythromycin ethylsuccinate (●) and erythromycin base (○) following the administration of 600 mg erythromycin ethylsuccinate every 8 h for ten doses.

Sample preparation

As both erythromycin esters and erythromycin base are present in blood after oral administration of estolate or ethylsuccinate, it was important to develop a sample preparation procedure that would prevent further hydrolysis of the esters. Thus simple aqueous dilution of concentrated urine samples could not be used, nor could eventual reconstitution with aqueous mobile phase be done as previously reported [6,7,8]. Therefore several solvents were tested for extraction efficiency including chloroform, methylene chloride, hexane and diethyl ether. Among these diethyl ether gave the greatest recovery and the least interferences. Use of 5% pentanol or isopropanol in diethyl ether (to reduce the adsorption onto glass) and protein precipitation with acetonitrile caused interferences with the compounds of interest, resulting in a lengthy evaporation time. Unlike erythromycin

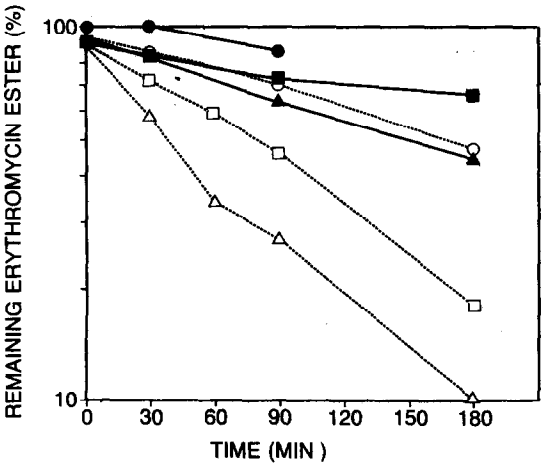

Fig. 5. Stability of erythromycin estolate (open symbols) and erythromycin ethylsuccinate (closed symbols) in plasma at 4°C (○,●), 25°C (□,■) and 37°C (△,▲).

base and related metabolites, estolate and ethylsuccinate were not extracted more efficiently from plasma at pH > 10, because they are more lipophilic. Also double extraction or salting out (using saturated solution of sodium chloride) did not substantially increase the recovery.

As mentioned above the use of an internal standard proved to be necessary since electrode sensitivity diminished slowly during sample analysis. Among various antibiotics tested (josamycin, spiramycin, oleandomycin, chloramphenicol, ticarcillin, etc.) roxithromycin, a new semi-synthetic macrolide, was chosen with retention time of 14.7 min and a recovery from extraction of 68%.

In vitro stability

In vitro stability of erythromycin estolate and ethylsuccinate was studied in plasma at 4, 25 and 37°C (see fig. 5). Plasma pools (pH 7.5) brought to 4, 25 and 37°C were spiked with erythromycin estolate or erythromycin ethylsuccinate to a final concentration of 10 μg/ml and assayed for erythromycin esters at different time. Due to the alkalinisation of plasma during the incubation period, sample pH was uniformly adjusted to 8.5 with 100 μl of saturated dipotassium hydrogen phosphate prior to extraction.

From Table II it can be shown that erythromycin ethylsuccinate hydrolysis in plasma was two to five times faster than erythromycin estolate. Thus at 37°C, in vitro half-life hydrolysis of erythromycin ethylsuccinate was 1 h, while that of erythromycin estolate was 3 h.

Use of EDTA or sodium citrate as anticoagulant agent in order to obtain plasma from fresh blood is common in clinical practice. Both are also known as inhibitors of aryl esterases, enzymes that could participate in the transformation of erythromycin esters into erythromycin base [9–11]. Erythromycin base formation is not inhibited by the presence of EDTA or sodium citrate in plasma (Fig. 5). Thus enzymatic activity (aryl esterase type) involved in the hydrolytic process of

TABLE II

HALF-LIFE HYDROLYSIS IN PLASMA OF ERYTHROMYCIN ESTOLATE AND ERYTHROMYCIN ETHYLSUCCINATE AT 4, 25 AND 37°C

Temperature (°C)	$t_{\frac{1}{2}}$ hydrolysis in plasma (min)	
	Estolate	Ethylsuccinate
4	382.9	181.6
25	384.3	77.1
37	180.9	55.2

erythromycin esters in plasma is probably minimal versus physicochemial hydrolysis.

This HPLC method is the first developed using electrochemical detection that measures simultaneously erythromycin base and erythromycin esters and that can be automated. It has been used for pharmacokinetic studies in our laboratory [12]. Finally, in order to assess precisely the relative concentrations of erythromycin esters (ethylsuccinate or estolate) and their active by-product erythromycin base, it is necessary to adopt measures preventing their continuous hydrolysis in biological fluids and during sample preparation.

REFERENCES

1 S.C. Bell, J.W. Hamman and W.E. Grundy, Appl. Microbiol., 17 (1969) 88.
2 J.V. Bennett, J.L. Brodie, E.J. Benner and W.M.M. Kirby, Appl. Microbiol., 14 (1966) 170.
3 G. Richard, C. Radecka, D.W. Hughes and W.L. Wilson, J. Chromatogr., 67 (1972) 69.
4 K.Y. Tserng and J.G. Wagner, Anal. Chem., 48 (1976) 348.
5 K. Tsuji, J. Chromatogr., 158 (1978) 337.
6 C. Stubbs, J.M. Haigh and I. Kanfer, J. Pharm. Sci., 74 (1985) 1126.
7 G.S. Duthu, J. Liq. Chromatogr., 7 (1984) 1023.
8 M.L. Chen and W.L. Chiou, J. Chromatogr., 278 (1983) 91.
9 F.M. Williams, Clin. Pharmacokin., 10 (1985) 392.
10 E.G. Erdos, C.R. Debay and M.P. Westerman, Biochem. Pharmacol., 5 (1960) 173.
11 A. Marton and W. Kalow, Can. J. Biochem. Physiol., 37 (1959) 1367.
12 D. Croteau, F. Vallée, M. LeBel and M.G. Bergeron, Clin. Pharmacol. Ther., 41 (1987) 239.