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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF ERYTHROMYCIN PROPIONYL ESTER AND ERYTHROMYCIN BASE IN BIOLOGICAL FLUIDS

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SUMMARY

The simultaneous determination of erythromycin propionate and erythromycin base in serum and urine by high-performance liquid chromatography using oleandomycin as internal standard is described. The separation was achieved on a reversed-phase C_{18} column employing acetonitrile–0.05 *M* phosphate buffer (65:35), adjusted to pH 7.0, as the mobile phase with coulometric detection. Hydrolysis of the ester during blood sample collection was minimised by immediate high-speed centrifugation of collected blood samples, followed by separation and immediate freezing of the serum fraction. A solid-phase extraction procedure, combined with a simple phase-separation step was used prior to chromatographic analysis. The method has the necessary precision, sensitivity and accuracy to allow the simultaneous determination of both components in serum and urine following a single 500-mg oral dose of erythromycin estolate.

INTRODUCTION

Erythromycin is a macrolide antibiotic used mainly in the treatment of infections caused by gram-positive organisms. Erythromycin base is rapidly degraded in acidic media [1,2] necessitating the use of structurally modified erythromycin derivatives or acid-resistant dosage forms in order to decrease gastric inactivation of the drug.

Erythromycin estolate is the lauryl sulphate salt of the propionyl ester of erythromycin (Fig. 1) which is reported to be acid-stable due to its insolubility in acidic media [1]. In the less acidic environment of the small intestine, this salt form dissociates to the soluble propionyl ester, the majority of which is absorbed as the intact ester [3], and a lesser amount being absorbed as erythromycin base formed by hydrolysis of the ester in the intestinal contents. The inactive ester form therefore constitutes the major portion of the circulating drug which must be hydrolysed in vivo in order to produce the necessary antibacterial activity [4]. Studies

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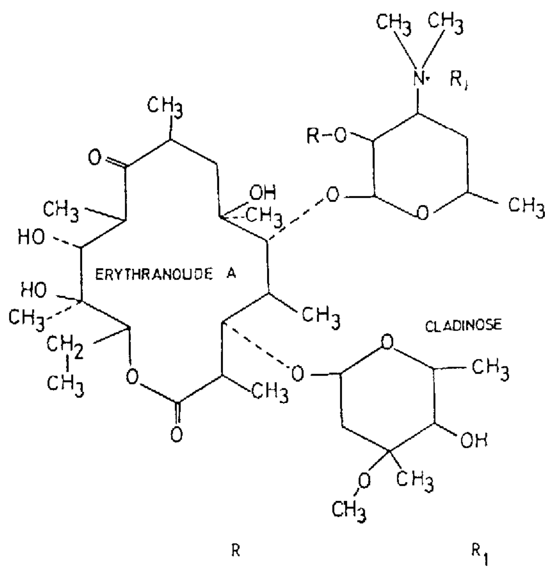

	R	R_1
Erythromycin base	H	
Propionyl erythromycin	CH_2CH_2 CO	
Erythromycin estolate	CH_2CH_2 CO	$C_{12}H_{25}OSO_3$

Fig. 1. Molecular structure depicting erythromycin estolate, its propionyl ester and the free base.

of the rate and extent of this hydrolysis have been hampered by the lack of a suitable and specific analytical method whereby post-sampling hydrolysis is minimized.

In 1969 Stephens et al. [3] used a two-step paper chromatographic–bioautographic technique to determine both erythromycin propionate and base in body fluids, whereas Easterbrook and Hersey [5] described a thin-layer chromatographic technique, also used in conjunction with bioautography. These procedures appear to effect the separation adequately but suffer from the general disadvantages associated with microbiological methods [6] as well as being tedious to perform with low sample throughputs. A liquid–liquid separation followed by fluorimetric detection of the derivatized species was reported by Tserng and Wagner [7] which has also been adapted to utilize a microbiological end-point [8–10] but lacks the selectivity of modern chromatographic methods. Recently two high-performance liquid chromatographic (HPLC) assays for erythromycin base in biological fluids employing dual-electrode coulometric detection operated in the oxidative screen mode have been reported [11,12] which show increased sensitivity for erythromycin base over spectrophotometric detection methods.

The objective of the present study was to develop a rapid, sensitive and precise HPLC method for the simultaneous determination of erythromycin propionate and erythromycin base in biological fluids. This will enable the elucidation of the actual in vivo situation with respect to the circulating levels of both erythromycin

base and erythromycin propionate following the oral administration of erythromycin estolate.

EXPERIMENTAL

Apparatus

The analysis was performed with a high-performance liquid chromatograph consisting of an M 6000 A pump and a WISP 710 B automated sample injector (Waters Assoc., Milford, MA, U.S.A.), a Coulochem 5100 A electrochemical detector fitted with a Model 5020 guard cell and a Model 5010 analytical cell (ESA International, Bedford, MA, U.S.A.) and a strip chart recorder (Model 561, Hitachi, Tokyo, Japan). The temperature of the column was maintained at 35°C with the aid of a Model LC-22 temperature controller (Bioanalytical Systems, West Lafayette, IN, U.S.A.).

Reagents

All reagents were of at least analytical grade. The acetonitrile was distilled-in-glass UV grade (Burdick & Jackson Labs., Muskegon, MI, U.S.A.). The separation was performed on a 25 cm×3.9 mm I.D. column packed with 10-μm C_{18} packing material (Techsil HPLC Technology, Cheshire, U.K.) preceded by a Guard-Pak (Waters Assoc.) guard column unit fitted with a cartridge containing 40–60 μm glass beads (Supelco, Bellefonte, PA, U.S.A.). Oleandomycin phosphate was obtained from Pfizer Labs. (Pietermaritzburg, South Africa) and propionyl erythromycin from Lilly Labs. (Indianapolis, IN, U.S.A.). Erythromycin base and erythromycin estolate were both U.S.P. standards.

Mobile phase

The mobile phase was prepared by mixing acetonitrile (650 ml) with 0.05 *M* phosphate buffer (350 ml). The phosphate buffer was prepared by adding 3.2 ml phosphoric acid to 1 l of water. Sodium hydroxide was then used to adjust the solution to pH 6.3. The solvent mixture (pH 7.0) was degassed and filtered through a 0.45-μm filter (Millipore, Type HVLP). The mobile phase, which was constantly degassed using an in-line vacuum degassing unit (Model ERC-3510, Erma Optical Works, Tokyo, Japan), was recycled until prolongation of retention times were observed.

Chromatographic conditions

The mobile phase was used at a flow-rate of 1.3 ml/min for the analysis of both serum and urine samples, with a resulting pressure of 103 bar. The electrochemical detector was operated in the oxidative screen mode with the upstream electrode set at +0.70 V, the downstream electrode at +0.90 V and the guard cell at +1.00 V.

Sample collection

Blood samples (5 ml) were collected from the forearm of human volunteers via an indwelling catheter. Approximately 3 ml were immediately transferred to a

PTFE centrifuge tube and centrifuged at 16000 *g* for 2 min in an Eppendorf Model 5414 high-speed micro-centrifuge (Eppendorf, Hamburg, F.R.G.). A 1-ml aliquot of serum was then accurately transferred to a glass tube which was immediately immersed into liquid air. The frozen sample was then transferred to an insulating holder and stored at $-15°C$ for a maximum of 6 h prior to analysis. Urine samples were collected during prescribed intervals, the volume recorded and a 0.5-ml aliquot immersed in liquid air prior to storage at $-15°C$.

Calibration standards

Stock solutions of erythromycin base (0.25 mg/ml) and erythromycin propionate (0.50 mg/ml) were prepared in acetonitrile. Three dilutions were prepared by adding 1.0, 2.0 or 4.0 ml of either stock solution to a 20.0-ml volumetric flask which was made up to volume with acetonitrile. On the morning of the study, aliquots of between 20 and 100 μl of these dilutions were added to 1.0 ml blank serum or 0.5 ml blank urine using a programmable automatic pipette (Microlab P, Hamilton, Bonaduz, Switzerland) producing calibration curves over the ranges 0.25–4.00 μg/ml (base), 0.50–8.00 μg/ml (ester) in serum and 1.00–20.00 μg/ml (base and ester) in urine. The standards were vortexed briefly, snap frozen in liquid air and stored at $-15°C$ in insulated holders. Aqueous oleandomycin phosphate solutions (20.00 μg/ml in serum and 40.00 μg/ml in urine) were prepared for use as the internal standard.

Extraction

Internal standard solution (100 μl) and 1.0 ml acetonitrile were added to the freshly thawed serum samples followed by vortex-mixing and centrifugation at 1600 *g* for 10 min in order to precipitate serum proteins. The supernatant was transferred to a culture tube containing 5.0 ml water and mixed well. To the 0.5-ml urine samples 100 μl internal standard solution and 5.0 ml water were added followed by thorough vortex-mixing prior to loading onto the extraction columns. The 1-ml C_{18} extraction columns (Bond Elut, Analytichem, Harbor City, CA, U.S.A.) were pre-washed prior to loading with 5.0 ml acetonitrile, followed by 5.0 ml water under atmospheric pressure. The diluted sample was then added to the extraction column with the aid of a 25-ml custom-made glass reservoir and a pasteur pipette. After the load solution had passed through the extraction column, it was washed with 20.0 ml water (serum) or 10.0 ml water (urine) followed by 3.0 ml acetonitrile–water (1:1). The compounds of interest were eluted into a 2-ml tapered collection tube with two successive 500-μl aliquots of acetonitrile–0.05 *M* phosphate buffer (3:2). The sample was then taken to dryness under vacuum in a rotary vacuum centrifuge (Savant Instruments, Hicksville, NY, U.S.A.) and the residue in the collection tube was reconstituted in 20 μl of water and vortex-mixed for 1 min. On addition of 100 μl acetonitrile, two layers formed as previously described [13]. This mixture was vortex-mixed for 1 min and then centrifuged for 1 min at 1600 *g* to ensure complete separation of the two layers. A portion of the top acetonitrile layer (60–80 μl) was then transferred to a WISP limited-volume insert (Waters Assoc.) using a micro-syringe. Aliquots (5–20 μl)

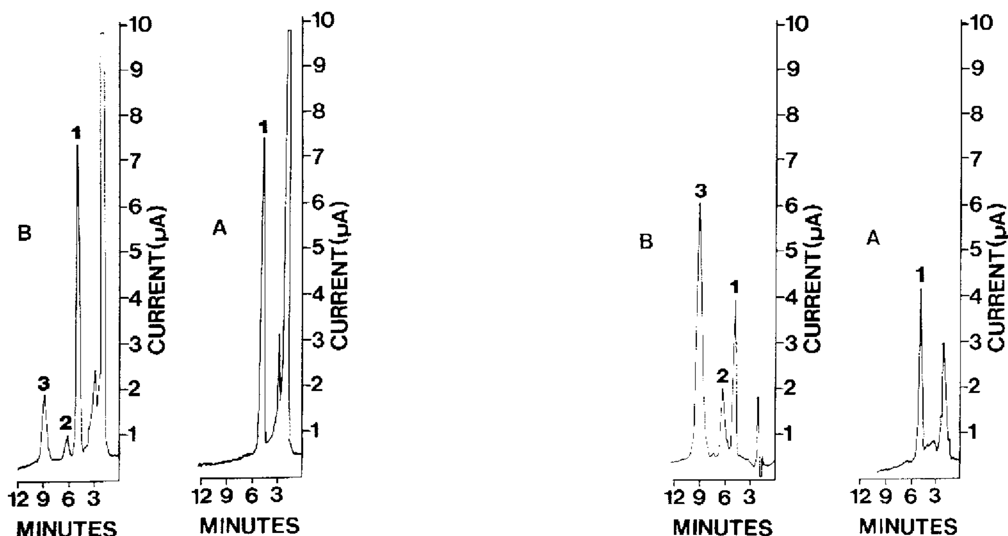

Fig. 2. (A) Chromatogram of blank serum extract (10 μl injected). (B) Chromatogram of an extract of serum (10 μl injected) 5 h following the oral administration of a tablet containing 500 mg erythromycin estolate. Peaks: 1 = internal standard; 2 = erythromycin base (0.2 μg/ml); 3 = propionyl erythromycin (1.3 μg/ml).

Fig. 3. (A) Chromatogram of blank urine extract (2 μl injected). (B) Chromatogram of an extract of urine (2 μl injected) 2 h following the administration of a tablet containing 500 mg erythromycin estolate. Peaks: 1 = internal standard; 2 = erythromycin base (3.0 μg/ml); 3 = propionyl erythromycin (15.0 μg/ml).

of this sample were injected onto the column. Relevant chromatograms are depicted in Figs. 2 and 3.

Clinical study

A clinical study involving a single volunteer was conducted to check the effectiveness of the analytical method to simultaneously measure concentrations of erythromycin base and the propionyl ester in serum and urine after the oral administration of a commercially available erythromycin estolate formulation (Ilosone 500; Lilly Labs.). The subject received one tablet of erythromycin estolate (500 mg base equivalent) after an overnight fast, accompanied by 250 ml water. Blood samples were drawn at 0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, 6, 8, 10, 12 and 24 h after ingestion of the medication. Urine samples were collected during the time intervals 0–2, 2–4, 4–6, 6–8, 8–10, 10–12 and 12–24 h. All samples were handled and stored as previously described. Serum samples were collected in duplicate for repeat analyses after 24 h at −15°C.

RESULTS

Linearity

Calibration curves with five different concentrations of erythromycin base and erythromycin propionate in serum and urine, obtained by plotting the ratio of

TABLE I

CALIBRATION DATA FOR SERUM AND URINE

Sample	Compound	Concentration range (μg/ml)	Slope	Intercept	Correlation coefficient
Serum	Base	0.25–4.00	0.3738	0.0020	0.9999
	Ester	0.50–8.00	0.1572	0.0074	0.9998
Urine	Base	1.00–20.00	0.1862	0.0212	0.9997
	Ester	1.00–20.00	0.0649	0.0047	0.9993

TABLE II

ACCURACY AND PRECISION OF THE METHOD FOR THE DETERMINATION OF ERYTHROMYCIN BASE AND PROPIONYL ESTER IN HUMAN SERUM AND URINE

Six samples were analysed at each concentration.

Sample	Base added (μg/ml)	Base found (μg/ml)	R.S.D. (%)	Ester added (μg/ml)	Ester found (μg/ml)	R.S.D. (%)
Serum	0.25	0.29	4.5	0.25	0.27	6.7
	1.00	1.04	1.2	2.00	2.01	3.2
	2.00	2.01	3.4	4.00	4.03	3.8
Urine	1.05	0.98	4.6	0.99	0.93	7.9
	10.48	10.69	3.5	9.88	10.27	7.5
	26.20	26.14	4.9	24.70	24.85	5.3

the peak height of these compounds to that of the internal standard were linear over the concentration ranges studies. The calibration data are summarized in Table I.

Precision and accuracy

Within-run precision and accuracy were assessed by extracting six spiked serum and urine samples over the range of concentrations studied. The results are summarized in Table II.

Extraction efficiency

Spiked serum and urine samples were analysed by extracting replicate ($n=5$) samples at five different concentrations without the addition of internal standard. After reconstitution, as much as possible of the acetonitrile (upper) layer was removed and transferred to a second collection tube and re-dried. Amounts of drug corresponding to a 100% recovery were spiked into similar tubes and taken to dryness. Both the extracted samples and standards were reconstituted in 20 μl acetonitrile containing oleandomycin as an external standard for comparison of the respective peak-height ratios. Mean recovery values of erythromycin base were 70 and 82.0% in serum and urine, respectively, while for the propionyl ester the recovery was distinctly lower in both serum and urine with mean values of 56 and 63%, respectively. Studies on the phase-separation step

suggest that approximately 20% of both the base and ester remain in the aqueous layer during the final phase-separation step with the remaining 10–20% being consistently lost during the solid-phase extraction procedure.

Sensitivity and detection limits

In HPLC with electrochemical detection, the term detection limit cannot be clearly defined due to the varying electrode sensitivities encountered during extended periods of analysis. During the least sensitive analysis periods, serum concentrations of 0.10 μg/ml erythromycin base and 0.25 μg/ml erythromycin ester were well within detection limits utilizing 10–20 μl injection volumes. However, during more sensitive periods similar results were obtained employing injection volumes of only 5–10 μl. Urine analysis was complicated by the large concentration ranges encountered (0.50–30.00 μg/ml) for both compounds, but the method proved both sufficiently sensitive and flexible to cope with these problems.

Clinical study and sample stability

The serum concentration profile and cumulative urinary excretion plots from the clinical study are depicted in Figs. 4 and 5, respectively. Serum samples re-

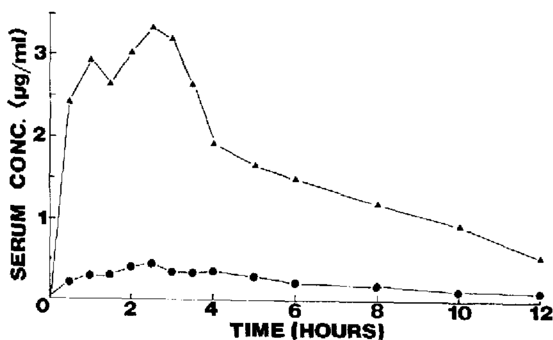

Fig. 4. Serum concentration–time profile of a human volunteer after administration of a single 500-mg erythromycin estolate tablet. (●) Erythromycin base; (▲) erythromycin propionate.

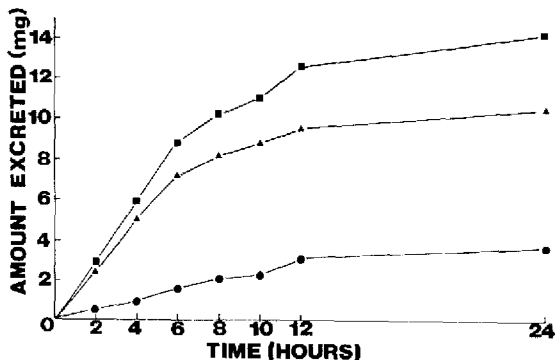

Fig. 5. Cumulative urinary plot of erythromycin following the administration of a single 500-mg erythromycin estolate tablet to a human volunteer. (●) Erythromycin base; (▲) erythromycin propionate; (■) total erythromycin (base plus ester).

analysed after 24 h storage at $-15\,^\circ C$ showed an approximate 5% increase in erythromycin base concentrations with an associated decrease in propionyl ester concentrations, which increased to approximately 40% after four days. Reconstituted samples were stable for up to 18 h in the automatic sample injector at ambient temperature (20–25 °C).

DISCUSSION

Erythromycin base exhibits interesting behaviour on C_{18} silica-based columns, being highly dependent on buffer ion concentration for its elution. This phenomenon which served as the basis for the extraction of erythromycin base from serum and urine during a previous investigation [13] has again been utilized in this assay procedure. The propionyl ester appears to be even more sensitive to the presence of buffer, necessitating the use of a 20-ml water wash preceding the acetonitrile–water wash in the extraction procedure. If less water is used, or if this water contains any buffer molecules (contamination from load solution, etc.), dramatic losses occur during the 3-ml acetonitrile wash. Attempts at utilizing a highly end-capped C_{18} analytical column (Novapak, Waters Assoc.) resulted in very long retention of erythromycin propionate at the optimum chromatographic conditions for erythromycin base analysis [13]. These results are similar to those reported by Cachet et al. [14] who found improved separation of erythromycin base and several related substances using aged C_{18} columns in which the polarity of the packing material had increased. Coulometric electrochemical detection as used by other workers for the analysis of erythromycin base in serum [11,12] provided sufficient sensitivity for the analysis of both compounds. This level of sensitivity could not be obtained using amperometric electrochemical detection [15] due to the low buffer content of the mobile phase required for their separation.

Various attempts at increasing the recovery of both drugs during the phase-separation step, including changes in buffer molarity, pH and acetonitrile proportions, proved unsuccessful. In spite of the relatively low recovery, the results were found to be highly reproducible (Tables I and II). The necessary levels of sensitivity were facilitated by the use of a solid-phase sample extraction procedure coupled with a simple phase-separation step. This resulted in sufficiently clean sample extracts which in combination with a coulometric detector operated in the oxidative screen mode provided the high degree of selectivity required to allow the quantitative simultaneous determination of both erythromycin propionate and erythromycin base. The final phase-separation step provided an unexpected advantage in that the hydrolysis of the ester in acetonitrile is almost negligible as against the rapid hydrolysis found in buffer solutions [16]. Sample degradation during the residence period in the automatic sample injector was thereby obviated. The modified collection of serum and urine samples coupled with the storage procedures described proved successful in limiting the hydrolysis of the ester prior to analysis. As was found by Tserng and Wagner [7] in contrast to Stephens et al. [3], the hydrolysis of the ester during storage of samples at $-15\,^\circ C$ was sufficient to affect the results, hence all serum samples were analysed within 6 h of sample withdrawal. The solid-phase extraction procedure is ideally

suited for this type of work in that many samples can be extracted simultaneously without constant operator attention. Using a system of custom-made support racks, serum samples from three volunteers a day were analysed in two batches of forty, at 6 and 12 h after drug administration. Application of this discriminatory assay technique may therefore be used to describe the in vivo situation more appropriately with respect to circulating levels of active and inactive drug.

In summary, the HPLC method presented here has the necessary precision, sensitivity and accuracy to allow the simultaneous determination of erythromycin base and erythromycin propionyl ester in serum and urine, and has proved extremely useful for the pharmacokinetic characterization of both compounds after the oral administration of erythromycin estolate to human volunteers.

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