Determination of Erythromycin in Serum and Urine by High-Performance Liquid Chromatography with Ultraviolet Detection

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Abstract □ A high-performance liquid chromatographic analysis of erythromycin in human serum and urine with UV detection at 200 nm is presented. The method involves a solid-phase extraction procedure followed by a simple phase separation step and chromatography on a reversed-phase column. The method has sensitivity limits of 0.25 and 1.0 μg/mL in serum and urine, respectively, and is sufficiently sensitive to monitor concentrations of erythromycin in human serum and urine after the administration of a single 500-mg erythromycin stearate tablet.

Erythromycin, a macrolide antibiotic used mainly against gram-positive bacteria, has been in clinical use since 1952.[1] Previous pharmacokinetic data published on this antibiotic have been derived predominantly from microbiological assay techniques. These techniques, however, are relatively imprecise as well as nonspecific and tedious to perform.

In 1969, Stephens et al.[2] used a two-step paper chromatographic-bioautographic technique to determine erythromycin in blood, whereas Easterbrook and Hersey[3] described a TLC technique also used in conjunction with bioautography. These procedures, although enabling the determination of free base from the parent ester form of the drug, suffer from the same disadvantages associated with microbiological methods.

Tsuji[4] described a fluorometric determination of erythromycin, including erythromycin ethyl succinate and some major metabolites and degradation products, in serum by high-performance liquid chromatography (HPLC) and on-stream post-column derivatization and extraction. Although this method appears to be highly sensitive, it suffers from the disadvantage of requiring complex and expensive equipment. In addition, erythromycin is not well resolved from its metabolites and degradation products present in serum.

A recent paper by Chen and Chiou[5] described the HPLC analysis of erythromycin in biological fluids by electrochemical detection. A novel dual-electrode electrochemical detector, operated in the oxidative screen mode, was used. This procedure has the main advantage of high sensitivity, allowing the use of extremely small samples. To reduce background currents and enhance detector stability, however, it is necessary to maintain the HPLC system in continuous operation, even when it is not in use. Furthermore, their choice of erythromycin B as an internal standard may lead to anomalies during pharmacokinetic studies, since various amounts of this compound are usually present in batches of raw material.[6] A further recent report by Duthu,[7] who used the same detector system as Chen and Chiou, described the analysis of erythromycin in serum only, under different chromatographic conditions.

The objective of the present study was to develop a sensitive, reproducible, and rapid method to quantitatively determine erythromycin in serum and urine by HPLC with UV detection by employing a conventional variable-wavelength UV detector. The success of this method was largely due to

the efficiency of the solid-phase extraction procedure employed, which resulted in a clean sample extract. This enabled the utilization of a detection wavelength of 200 nm.

Experimental Section

Apparatus—The modular high-performance liquid chromatograph consisted of an M 6000A constant flow pump and a Wisp 710B automated sample injector (Waters Associates), a variable-wavelength UV detector (model 769; Kratos Analytical Instruments), and a strip chart recorder (model 561; Hitachi, Japan). The mobile phase was constantly deaerated with an in-line vacuum deaerating unit (model ERC-3510; Erma Optical Works, Japan). The separation was performed on a 15 cm × 3.9 mm i.d. column containing microparticulate bonded 5-μm octadecylsilane (C_{18}) material (Novapak; Waters Associates). The temperature of the column was maintained at 35°C with the aid of a model LC-22 temperature controller (Bioanalytical Systems).

Reagents and Materials—Reagents were of at least analytical grade. UV-grade acetonitrile was obtained from Burdick and Jackson Laboratories. Erythromycin base and oleandomycin phosphate were obtained from Abbott Laboratories and Pfizer Laboratories, respectively. Erythromycin B, the anhydro metabolite of erythromycin, erythralosamine, and erythromycin enol ether were obtained from Abbott Laboratories, and the de-*N*-methyl metabolite of erythromycin was obtained from Lilly Research Laboratories. The HPLC-grade water used in the mobile phase was purified through a Milli-Q system (Millipore Corp.).

Chromatographic Conditions—The mobile phase was prepared by mixing acetonitrile (300 mL) with 0.05 M phosphate buffer (700 mL). The phosphate buffer was prepared by adding 3.2 mL of phosphoric acid to 1 L of water. Sodium hydroxide was then used to adjust the solution to pH 6.30. The solvent mixture (pH 7.00) was deaerated and filtered through a 0.6-μm filter (type BD; Millipore Corp.).

This mobile phase was used at a flow rate of 1.0 mL/min for the analysis of both serum and urine samples, with a resulting pressure of 100 bar (1×10^4 kPa). The wavelength of detection was 200 nm, with a detector sensitivity of 0.01 AUFS and a time constant of 2 s.
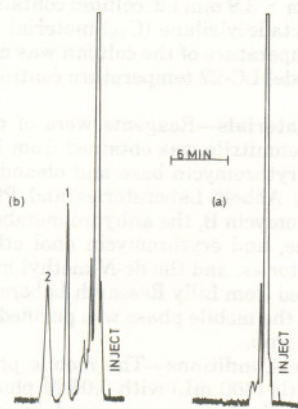
Extraction of Serum and Urine—Samples of serum (2 mL) or urine (1 mL) were mixed with 0.25 or 0.5 mL, respectively, of a 12-μg/mL aqueous oleandomycin phosphate internal standard solution. Addition of 1 mL of acetonitrile, followed by vortex mixing for 1 min and centrifugation for 5 min at 1600×g, resulted in deproteinization of the biological samples. The supernatant was transferred to a culture tube containing 8 mL of water for direct loading onto a prewashed, 1-mL, disposable octadecyl extraction column (J. T. Baker Chemical Co.) which had been prewashed under 10–15 mm/Hg vacuum (Baker 10 Extraction System coupled to a model A2-S Aspirator; Rikakikai Co., Japan) by successively passing 3 mL of acetonitrile and 3 mL of water through each. The diluted sample was then added to the extraction column with the aid of 15-mL sample reservoir (J. T. Baker Chemical Co.). The extraction column was washed with 5 mL of water and then with 5 mL of acetonitrile:water (1:1), with the vacuum maintained until dry. Erythromycin and the internal standard were eluted into 2-mL tapered collection tubes with two successive 500-μL aliquots of acetonitrile:0.05 M phosphate buffer (1:1). The sample was then dried under vacuum in a rotary vacuum centrifuge (Speed Vac Concentrator; Savant Instruments). The residue in the collection tube was reconstituted in 20 μL of water

and vortexed for 1 min. When 25 μL of acetonitrile was added, two layers formed due to the high concentration of salts in the aqueous layer. This mixture was vortexed for 1 min and then centrifuged for 1 min at $1600 \times g$. An aliquot (15–20 μL) of the acetonitrile phase was then transferred to a WISP limited volume insert (Waters Associates) with a microsyringe. A 5-μl aliquot of this sample was injected onto the column. Relevant chromatograms are depicted in Figs. 1 and 2.
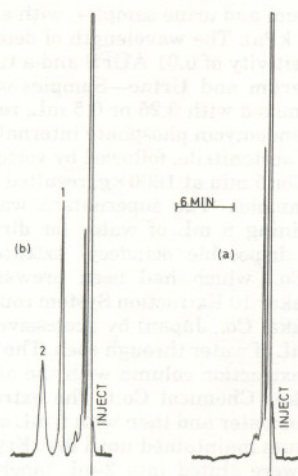
Clinical Study—A pilot trial in which one volunteer was used was conducted to check the effectiveness of the analytical method to measure concentrations of erythromycin base and any possible metabolites or degradation products in serum and urine after the oral administration of a commercially available erythromycin stearate formulation (Erythrocin 500; Abbott Laboratories). The subject received one film-coated tablet containing 500 mg of erythromycin stearate after an overnight fast, accompanied by 250 mL of water and immediately followed by a light breakfast. Blood samples were drawn at 0, 0.25, 0.5, 0.75, 1, 2, 3, 4, 6, 8, 10, and 12 h after ingestion of the medication. The serum was separated by centrifugation and frozen until analyzed. Urine samples were collected during the time intervals 0–2, 2–4, 4–6, 6–8, 8–12, and 12–24 h, and representative samples were frozen until analyzed.

Results

Linearity—Calibration curves, determined with five different concentrations of erythromycin base in serum and urine, were obtained by plotting the ratio of the peak height of erythromycin base to that of the internal standard versus their respective concentrations, and were linear over the concentration ranges studied. The calibration curve in serum (0.25–5.00 μg/mL) had a slope of 0.2938 and a y intercept of 0.0310, with a correlation coefficient of 0.9997, whereas the curve in urine (5.00–50.00 μg/mL) had a slope of 0.0686 and a y intercept of 0.0127, with a correlation coefficient of 0.9997.

Precision and Accuracy—Within-run precision was assessed by extracting six spiked serum and urine samples each at the upper and lower limits of the concentration ranges studied. The relative standard deviations were found to be 6.12% at 0.50 μg/mL and 2.10% at 2.00 μg/mL for serum and 4.2% at 5.00 μg/mL and 1.40% at 50.00 μg/mL for urine.

Spiked serum samples were found to be stable for at least 1 month and urine samples for 2 weeks when stored frozen at $-10°C$. Extracted urine samples were found to be stable at 4°C for 3 days when left on the extraction columns and for 1 week when eluted and stored dry at 4°C prior to reconstitution.
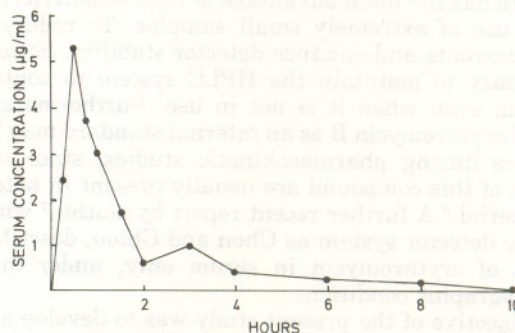
Extraction Efficiency—Spiked serum and urine samples were assayed in triplicate at four different concentrations. All samples were extracted as previously described, except that the internal standard was incorporated into the 25-μL acetonitrile aliquot used in the final reconstitution and phase separation step. The mean recovery values of 81% and 80% thus obtained for serum and urine, respectively, do not take into account the very small percentage of drug remaining in the aqueous layer. Partition studies of the distribution of erythromycin between the two phases obtained on reconstitution indicated that approximately 10% of the drug remained in the aqueous layer. The overall recovery values may thus be adjusted by 10%.

Sensitivity and Detection Limit—Under the conditions of this assay and based on a signal-to-noise ratio of 3, the detection limit for erythromycin base was 0.25 μg/mL in serum and 1 μg/mL in urine. The detection limit in serum may be increased by using a larger injection volume of 8–10 μL or by using a detector with a 0.005 AUFS setting. This was achieved in our laboratory with the aid of an amplifier-filter (model 1021A; Spectrum Scientific Corp.), which doubled the detector output.

Serum and Urine Profiles—The serum concentration-time profile and fractional urinary excretion plot from the clinical study are depicted in Figs. 3 and 4, respectively.

Discussion

The method described involves a rapid, accurate, and highly reproducible system for the determination of erythromycin in serum and urine after a single 500-mg oral dose. During the initial stages of selection of a suitable mobile

Figure 1—(a) *Chromatogram of blank serum extract.* (b) *Chromatogram of an extract of serum containing oleandomycin (peak 1) and erythromycin (peak 2).*

Figure 2—(a) *Chromatogram of blank urine extract.* (b) *Chromatogram of an extract of urine containing oleandomycin (peak 1) and erythromycin (peak 2).*

Figure 3—*Erythromycin serum concentration-time profile in a human volunteer after the administration of a single 500-mg erythromycin stearate tablet.*
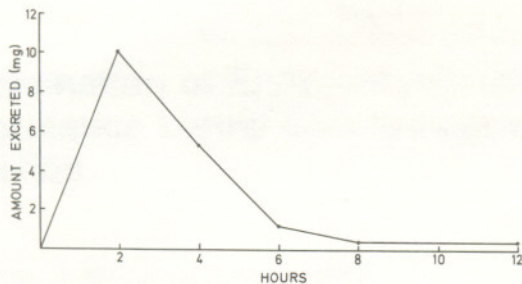
Figure 4—*Fractional urinary excretion plot of erythromycin after the administration of a single 500-mg dose of erythromycin stearate to a human volunteer.*

phase to separate erythromycin by reversed-phase HPLC, it was established that the drug did not elute from the C_{18} column when a buffer-free mobile phase was used. Hence, when a solid-phase extraction column was used, erythromycin could be selectively separated and retained while unwanted components were washed off with water and acetonitrile:water, and then the drug was eluted from the extraction column with an acetonitrile:buffer mixture. Utilization of this solid-phase extraction technique in the conventional manner as described in previous reports for various compounds[8,9] results in reasonably clean extracts and allows their determination and quantitation, provided that high sensitivity is not required. In the case of erythromycin, which has a relatively low UV molar absorptivity, further sample concentration was necessary. This was accomplished by evaporating the extraction column eluate, adding a 20-μL aliquot of water to dissolve the drug and residual buffer salts, and then selectively concentrating the erythromycin and internal standard into a 25-μL acetonitrile layer. This layer could be removed for injection as a result of successful phase separation during this final step. The resulting clean sample extract enabled the successful utilization of a conventional UV detector set at 200 nm. The many interferences normally seen when monitoring biological extracts at such low wavelengths were overcome, and selectivity was enhanced as a result of this phase-separation step. Injection of the aqueous layer of reconstituted extracts indicated extremely high levels of interference, in contrast to the acetonitrile layer, which allowed the unhindered quantitation of erythromy-cin. Retention times under the chromatographic conditions of this assay were as follows: erythromycin base, 6.2 min; oleandomycin phosphate, 4.2 min; de-*N*-methyl metabolite of erythromycin, 4.5 min; erythromycin B, 8.2 min; anhydroerythromycin, 11.0 min; erythromycin enol ether, 22.4 min; erythralosamine, 13.0 min. Despite similar retention times obtained for both oleandomycin and the de-*N*-methyl metabolite of erythromycin, serum and urine samples obtained from the clinical study and processed without the addition of the internal standard oleandomycin showed no evidence of the de-*N*-methyl metabolite of erythromycin.

In view of the low UV molar absorptivity of erythromycin at conventionally used detection wavelengths ($>$220 nm), HPLC with UV detection has not been considered feasible to date. Wavelengths below 220 nm, and especially at 200 nm, where the molar absorptivity is considerably greater, are associated with additional problems, such as interfering species co-extracted from biological samples. Our method overcomes these problems as a result of system optimization and the successful application of a highly specific, solid-phase extraction procedure, followed by a simple phase separation step to quantitatively determine erythromycin in serum and urine.

References and Notes

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