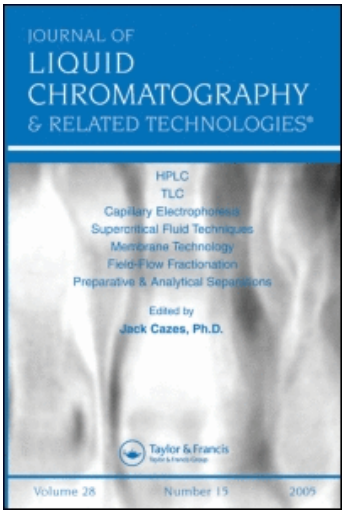


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# Assay of Erythromycin from Human Serum by High Performance Liquid Chromatography with Electrochemical Detection

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# ASSAY OF ERYTHROMYCIN FROM HUMAN SERUM BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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## ABSTRACT

A sensitive and selective method has been developed for the determination of serum concentrations of erythromycin A by high performance liquid chromatography with electrochemical detection. Erythromycin was extracted from alkalinized serum samples with methyl t-butyl ether. After evaporation of the ether, the samples were reconstituted in acetonitrile/ammonium acetate and washed with hexane. Aliquots were injected onto a Sepralyte diphenyl column. The mobile phase consists of acetonitrile/sodium perchlorate/ammonium acetate/methanol under isocratic conditions. Eluted peaks were detected by dual coulometric electrodes operated in the oxidative screen mode. The recovery of erythromycin from serum was 84%. Assay limit of quantification was 0.05 µg/ml serum, and dynamic linear range was 0.05-1.5 µg/ml. This method was used to quantitate both erythromycin and its gastric degradation products from human serum. Additionally, other macrolide antibiotics could be quantified by electrochemical detection. Analytical results for erythromycin compared favorably with those obtained with a standard microbiological assay.

## INTRODUCTION

The 14-membered ring macrolide antibiotic, erythromycin A, is routinely measured by microbiological assays (1-4). While having an appropriate limit of detection, bioassays lack specificity; active metabolites and prodrug esters of erythromycin, as well as other antibiotics, are likely to interfere. A number of high performance liquid chromatography methods employing spectrophotometric detection have been reported for the separation and quantification of macrolides (5-9). They were judged unsuitable for determination of serum concentrations of erythromycin expected from therapeutic doses of this antibiotic. Improved detection



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limits have been achieved by an HPLC-fluorescence assay (10-11) and used for the clinical serum determination of erythromycin, but the assay requires complex post-column derivatization.

Electrochemical detection has been successfully applied to trace analysis of electroactive compounds in clinical and environmental samples (12-14). This paper describes a practical and specific method for the quantification of erythromycin in serum based on HPLC with electrochemical detection. The method provides a sufficient lower limit of detection for clinical samples and is applicable to the quantification of other macrolides.

## MATERIALS

Stock solutions of erythromycin A (Pfizer Taito Lot No. 906-636005) and internal standard at 1 mg/ml, prepared in acetonitrile, were stable for several months at 4°C. Dilutions were made with acetonitrile/20 mM ammonium acetate (1/1). Hexane, acetonitrile, methanol, methyl t-butyl ether and water were all HPLC grade (Burdick and Jackson, Muskegon, MI, USA). All other chemicals were reagent grade.



## METHODS

### Instrumentation

A Spectra-Physics SP 8770 (San Jose, CA) liquid chromatograph was equipped with a 21 mm x 3.0 mm I.D. 40 µm glass bead guard column and a 25 mm x 4.6 mm I.D. column packed with 5 µm Sepralyte diphenyl (Analytichem International, Harbor City, CA). Samples were automatically injected using an HPLC autosampler (Micromeritics Model 725, Norcross, GA) equipped with a 100 µl sample loop. An ESA 5100A electrochemical detector (Environmental Sciences Associates, Bedford, MA) was interfaced with a Spectra-Physics 4100 computing integrator and peak height recorded.

The electrochemical detector was equipped with an ESA Model 5020 guard cell placed in line before the injector in order to electrolyze components of the mobile phase (1.0 volt potential) which could contribute to the background current. The

ESA Model 5010 dual electrode cell was operated in the oxidative screen mode with electrochemical cell I voltage set at 0.7 V and cell II at 0.8 V. To prevent pressure build-up on the graphite electrodes, in-line filters were used. A 0.5 µm stainless steel filter (Rainin, Woburn, MA) was placed before the guard cell and a similar 2 µm filter before the injection loop. A 0.5 µm carbon filter (ESA No. 5100-A-50) was used before the dual electrodes. These filters were replaced periodically to maintain total system pressure below 2,500 psi. A mobile phase of acetonitrile/20 mM sodium perchlorate/20 mM ammonium acetate/methanol (50/32/8/10), pH apparent of 7.0, was filtered through a 0.2 µm Nylon 66 filter (Rainin). Mobile phase was pumped at 1.0 ml/minute, recycled into a 1 L reservoir and replaced weekly. When the response of the electrode decreased by 50%, the analytical cell was flushed with 6N $HNO_3$ for 30 minutes and washed overnight with acetonitrile/20 mM sodium perchlorate (1/1).

Cyclic voltammetry was conducted on a BAS Model CV-1B (West Lafayette, Indiana) equipped with a glassy carbon working electrode and Ag/AgCl reference electrode. Compounds were dissolved in mobile phase to give a final concentration of 0.5 mg/ml. Current was recorded over a voltage range of +1.3 to -1.0 volts.



## Microbiological Assay

An automated microbiological agar diffusion assay using *Micrococcus luteus* (ATCC #9341) was used as a bioassay for erythromycin A in serum (3). Neomycin assay agar and potassium phosphate buffer solution were used.

## Sample Preparation

Frozen human serum samples from males dosed with erythromycin were thawed at room temperature, and 0.25 ml aliquots were added to disposable 16 mm x 100 mm culture tubes. If samples were found to contain concentrations of erythromycin greater than 1.5 µg/ml by this assay, the samples were diluted with control serum, processed and analyzed again. After addition of saturated $K_2CO_3$ (50 µl), 1 ml of HPLC-grade water, fortified with internal standard, was added to the samples. (The internal standard was an analog of erythromycin.) The sample was immediately extracted with 5 ml of methyl t-butyl ether on a Vortex mixer for 30 seconds. The organic layer was transferred to another tube and evaporated to

dryness at 40°C using a Buchler Vortex evaporator. The residue was reconstituted in 1.0 ml acetonitrile/20 mM ammonium acetate (1/1) and the reconstituted sample washed with 1 ml hexane. The phases were separated by centrifugation and the aqueous layer removed and analyzed for erythromycin A by HPLC with electrochemical detection. Samples at this stage were stable for at least 48 hours. Five-point calibration curves were established using drug extracted from fortified serum samples.

## RESULTS AND DISCUSSION

### Chromatography

Of the several stationary phases tested (μBondapak $C_{18}$, μBondapak $C_8$, μBondapak CN, μBondapak phenyl, Sepralyte diphenyl, Absorbisphere $C_{18}$, Spherisorb $C_{18}$), the Sepralyte diphenyl column yielded minimal tailing of macrolide peaks. Buffers tested in the mobile phase with regard to their effects on background current and electrode response to erythromycin A were potassium phosphate, ammonium acetate, monochloro-acetic acid and sodium perchlorate. Only the sodium perchlorate did not lead to a loss in electrode sensitivity after several days of electrode use. However, a mobile phase supplemented with sodium perchlorate, with a pH below 6.5, caused the degradation of erythromycin. Consequently, a mixture of acetonitrile/20 mM sodium perchlorate/20 mM ammonium acetate/methanol (pH 7) (50/32/8/10) was used to prevent this degradation but still maintain electrode sensitivity to erythromycin for at least ten days. The low ionic strength of the buffer used in the mobile phase minimized background current from the detectors. As noted by previous investigators (8), retention time for erythromycin was greatly affected by the pH of the mobile phase; the lower the pH, the shorter the retention time. However, at higher pH the electrochemical oxidation of erythromycin was facilitated. Thus, the pH of the mobile phase was maintained between 7 and 7.5.

### Detection

Macrolides exhibit a relatively high oxidation potential on glassy carbon electrodes ($\geq$1.20 volts). This made electrochemical detection of these compounds

more difficult because of oxidation of mobile phase and extraneous sample constituents. Amperometric response given by a glassy carbon electrode could detect $\geq$100 nanogram quantities of erythromycin A, but the detector rapidly lost sensitivity. Alternatively, coulometric response given by a porous graphite electrode could detect nanogram quantities of compound, but the detector generated a large background current, particularly critical in this application of high oxidation potential. Thus, it was necessary to use an electrochemical detection system with dual coulometric electrodes in the oxidative screen mode. In this manner, many components in the extracted serum samples were irreversibly oxidized at the upstream electrode (0.7 volt potential) and did not contribute to the detected current at the downstream electrode (0.8 volt potential). Operation of the electrochemical detector in the oxidative screen mode not only minimized sample background current but enhanced the selectivity as well. Slow loss of electrode response still occurred upon repeated injections (>1,000 injections) of serum extracts but could be regenerated by acid flushing of the electrodes.

Limit of detection for a standard solution of erythromycin was 0.5 ng injected. For analysis of human serum samples, a limit of quantification of 50 ng/ml was adequate (about 1 ng injected). Thus, the procedure reported here provided a similar limit of quantification as the HPLC-post column derivatization-fluorescence method (11) with potential for lower detection limits by the use of greater detector gain settings and extraction of larger sample volumes.



## Specificity

In spite of the use of the oxidative screen mode of the electrochemical detector, occasionally extraneous serum and reagent chromatographic peaks were detected. However, by judicious choice of mobile phase pH, the peaks did not interfere with the analytes of interest (Figure 1a). A drug-related peak was observed in the serum of subjects orally dosed with erythromycin suspension (Figure 1c) but was absent in the serum of subjects orally dosed with enteric coated erythromycin (Figure 1b). The retention time of this peak was identical with that of the internal spiroketal of erythromycin A (Table 1), the degradation product of erythromycin A that formed under the acid conditions of the stomach.

The assay method proved to be applicable to the quantification of several classes of macrolide antibiotics. The analyzed macrolide antibiotics which contained

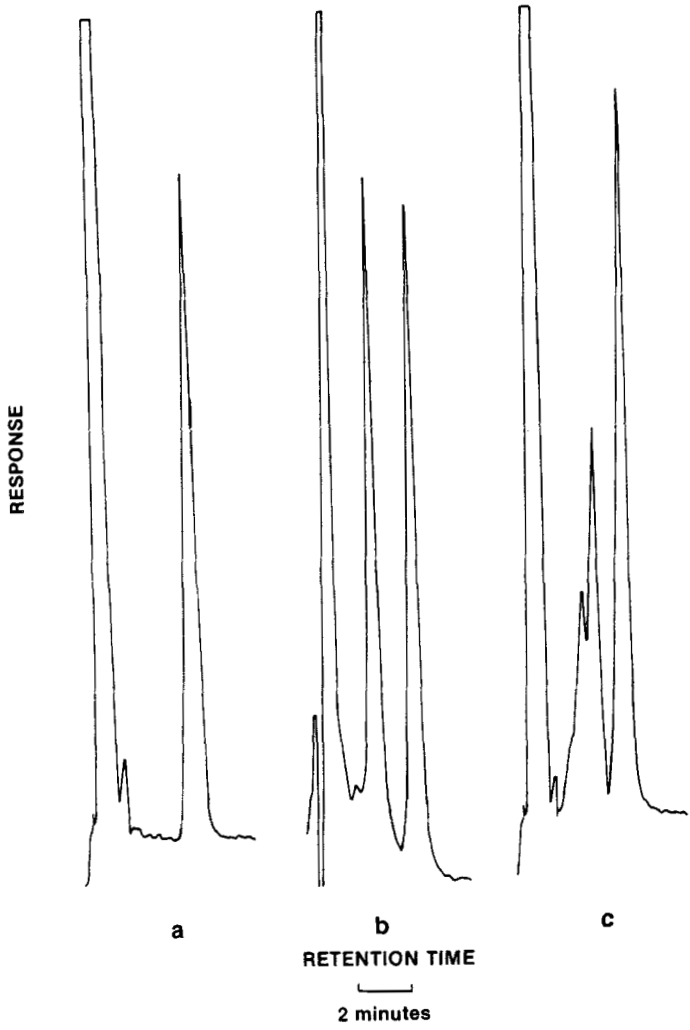


FIGURE 1. Chromatographic determination of erythromycin extracted from human serum. LC-EC conditions are noted in Methods.

a) Extract of human serum prior to erythromycin dose (sample fortified with internal standard).

b) Extract of human serum after oral dose enteric coated erythromycin (erythromycin and internal standard).

c) Extract of human serum after oral dose of erythromycin suspension (erythromycin, spiroketal and internal standard).

Table 1
Relative Retention Time and Relative Peak Area of
Various Macrolide Antibiotics and Erythromycin Derivatives

| Macrolide Antibiotics | Relative Retention Time | Relative Peak Area | Erythromycin A Derivatives | Relative Retention Time | Relative Peak Area |
|---|---|---|---|---|---|
| Erythromycin A | 1.00 | 1.00 | Dihydro | 0.96 | 1.02 |
| Erythromycin B | 1.19 | 0.93 | Anhydro | 1.26 | 1.06 |
| Erythromycin D | 1.02 | 1.04 | Enol Ether | 2.70 | 1.16 |
| Tylosin | 0.51 | 0.69 | Spiroketal | 1.25 | 1.66 |
| Josamycin | 0.66 | 0.94 | Descladinose | 0.59 | 1.70 |
| Oleandomycin | 0.88 | 0.79 | N-demethyl | 0.88 | ND* |
| | | | N-didemethyl | 0.80 | ND* |
| | | | 2-Propionate | 1.01 | 0.75 |
| | | | 2-Ethylsuccinate | 1.00 | 0.92 |

*N.D. = not detected by electrochemical detector. Retention time was determined by U.V. detection at 214 nm of a 10 µg injection.



a basic sugar were detectable by electrochemical oxidation (Table 1). Additionally, other erythromycins, erythromycin esters and erythromycin degradation products could be separated and quantified by this procedure. Only the two N-desmethyl erythromycins were undetectable (Table 1). Cyclic voltammetry on glassy carbon electrode confirmed the absence of an oxidation potential below 1.3 volts for these latter two compounds, which suggested the importance of the tertiary amine of the desosaminyl sugar in determining the oxidation potential of the molecule.

## Linearity

Peak heights for drug and internal standard (I.S.) were recorded in electronic integrator units and expressed as a ratio of drug to I.S. Three point standard curves were analyzed with authentic samples. A five point standard curve validated assay linearity between 0.05-1.5 µg/ml, with correlation coefficients of 0.9920-0.999.

## Recovery

Preparation of serum sample for injection onto the HPLC-electrochemical detection system required only a simple extraction procedure. The recovery of

Table 2
Erythromycin Standard Curve from Fortified Human Serum

| Erythromycin A Concentration (ng/ml) | Intraassay Precision* (n = 4) | | Interassay Precision** (n = 5) | |
|---|---|---|---|---|
| | ng/ml | RSD | ng/ml | RSD |
| 50 | 60 | 8.7 | 60 | 30 |
| 150 | 160 | 5.1 | 160 | 22 |
| 500 | 460 | 3.3 | 470 | 5.4 |
| 1,000 | 1,000 | 4.7 | 1,000 | 5.1 |
| 1,500 | 1,510 | 2.0 | 1,500 | 3.0 |

* Samples were prepared, processed and analyzed on the same day.
** Samples were processed and analyzed on consecutive days.



erythromycin (determined by comparing the response of known drug amounts with the extracted fortified samples) averaged 84% over the dynamic range of the assay (n = 20).

## Precision

Instrumental precision was 3.5% (n = 5), determined by repeated injections of 50 ng erythromycin. Intraassay relative standard deviation ranged from 3% to 9%. The maximum interassay relative standard deviations varied from 3% to 30% (Table 2). In order to correlate the HPLC assay data with those of the microbiological assay, serum samples of subjects dosed with erythromycin were assayed by both procedures. Comparable values were obtained by these assays, giving a correlation coefficient of >0.99 and a slope of 0.87.

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