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A STABILITY-INDICATING LIQUID CHROMATOGRAPHIC METHOD FOR THE ANALYSIS OF ERYTHROMYCIN IN STORED BIOLOGICAL FLUIDS USING AMPEROMETRIC DETECTION

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ABSTRACT

A simple, sensitive and reliable high-performance liquid chromatographic procedure has been developed for the determination of erythromycin in human serum and urine using amperometric detection. A solid-phase extraction procedure was used followed by chromatography on a reverse-phase column. The mean recovery of erythromycin from serum and urine was 80%. Calibration plots for erythromycin base in serum and urine were linear over the ranges 0.25 - 5.0 µg/ml and 1.25 - 25.0 µg/ml respectively, with a sensitivity limit of 0.1 µg/ml.

This method allows both erythromycin and its principle degradation product, anhydroerythromycin, to be determined during a period of sample storage at 4°C and -15°C. The method is sufficiently sensitive and precise and is thus highly suited for use in both pharmacokinetic and stability studies.

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INTRODUCTION

Although erythromycin, a macrolide antibiotic, has been in clinical use since 1952 (1), detailed physico-chemical and pharmacokinetic studies have been hampered by the lack of suitable rapid, precise and selective analytical methods.

Whilst erythromycin base is known to degrade in acid media (2) there is a dearth of information regarding the compound's stability in stored serum and urine samples obtained from pharmacokinetic/bioavailability studies. Current official methods for the analysis of erythromycin involve the use of a microbiological assay (3) which is non-selective and cannot differentiate between active and inactive components of the drug. The authors have previously reported a high-performance liquid chromatographic (HPLC) method involving the use of a UV detector (4) but have also shown that the principle acid degradation product of erythromycin, anhydroerythromycin, is not readily detected by UV and requires electro-chemical detection (5). Recently, two HPLC methods of analysis for erythromycin in biological fluids have been published, both of which involve the use of a specific dual electrode coulometric detector (6, 7). The coulometric system, utilizes series dual electrodes operated in a screen mode whereby the upstream electrode irreversibly oxidizes most of the interfering compounds in the extracted samples prior to detection at the downstream electrode.

This system, however, suffers from the disadvantage that the flow-through nature of the porous electrodes render it prone to blockages with a subsequent increase in back-pressure and possible cell damage necessitating replacement of this relatively expensive component.

Although amperometric detectors are widely used in liquid chromatography, these systems generally require more extensive

sample preparation due to the lack of screening facilities. Using the solid-phase sample extraction procedure developed for HPLC-UV analysis (4), resulting extracts were sufficiently clean to enable the use of a simple single electrode amperometric detector for the detection of both erythromycin and anhydroerythromycin in serum and urine samples.

EXPERIMENTAL

Materials

Reagents were of at least analytical grade. UV grade acetonitrile was obtained from Burdick and Jackson Laboratories and HPLC grade water was purified through a Milli-Q system (Millipore Corp.) prior to use. Anhydroerythromycin was obtained from Abbott Laboratories and oleandomycin from Pfizer Laboratories whilst erythromycin base was a U.S.P. reference standard.

Apparatus

The liquid chromatographic system consisted of an M6000-A solvent delivery system and a WISP 710-B automated sample injector (Waters Associates). The mobile phase was constantly degassed with an in-line vacuum degassing unit (Model ERC-3510;Erma Optical Works). The separation was performed on a 15cm x 3.9 mm i.d. C_{18} column (Novapak; Waters Associates) preceded by a Guard-Pak (Waters Associates) guard column unit fitted with a cartridge containing 40-60μm glass beads (Anatech Instruments Inc.). A Metrohm 656 electrochemical detector fitted with a glassy carbon working electrode was used in conjunction with a model 641 VA current/voltage measuring device coupled to a Hitachi model 561 strip chart recorder. The temperature of the column was maintained at 35°C with the aid of a model LC-22 temperature controller (Bioanalytical Systems).

Chromatographic Conditions

The mobile phase was prepared by mixing acetonitrile (300 ml) with 0.05M phosphate buffer pH 6.30 (700 ml) followed by degassing and filtration through a 0.6 μm filter (Type BD;Millipore Corp.). The flow rate was set at 1.0 ml/min during anaysis of both serum and urine samples with a resulting pressure of 1500 p.s.i. The working electrode potential of the amperometric detector was set at 1.15 V versus a silver/silver chloride reference electrode. In-line UV detection was concurrently used for the purposes of comparison and operated under the conditions previously reported (4).

Extraction of Serum and Urine

One millilitre of serum or urine samples were extracted as previously reported (4) after addition of 0.25 and 0.50 ml respectively of an aqueous oleandomycin phosphate internal standard solution. The concentration of the internal standard solution varied between 6.0 and 12.0 μg/ml depending on the calibration range required. A solid-phase extraction procedure using 1 ml C_{18} Bondelut extraction columns (Analytichem Int.) followed by a simple phase separation step during sample reconstitution was used. The upper acetonitrile layer was carefully transferred to a WISP limited volume insert (Waters Associates) with a microsyringe and between 3 and 5 μl injected onto the column.

Preparation of Stored Samples

Blank human serum (pH 7.2) was spiked with erythromycin base to yield final concentrations of 1.0 and 3.0 μg/ml. Samples of blank human urine (pH 5.6) were similarly prepared at erythromycin concentrations of 3.0 and 15.0 μg/ml. The samples were divided

into 10 ml aliquots and stored at 4°C and -15°C. Triplicate samples from each batch were analyzed after 1,2,4,8 and 12 weeks for both erythromycin base and anhydroerythromycin.

RESULTS AND DISCUSSION

Detection

Macrolide antibiotics have been reported (7) to have high oxidation potentials on glassy carbon electrodes (>1.20 volts). It was found that under the conditions of the present assay, a working electrode potential of 1.15 V provided the best compromise between detector response and background noise, enabling the detection of low concentrations of both erythromycin and anhydroerythromycin. Despite the normally large interferences associated with the use of high working electrode potentials, the solid-phase extraction procedure provided sufficiently clean extracts for the quantitation of erythromycin in human serum and urine over the required concentration range.

Chromatography

Figure 1 (A) depicts a blank serum extract while figure 1 (B) shows a typical chromatogram obtained for erythromycin base and the internal standard following the extraction of a serum sample containing 1.0 µg/ml erythromycin stored at 4°C for 8 weeks. Figure 2 shows chromatograms following extraction of a urine sample (15.0 µg/ml) stored for 8 weeks at -15°C. The advantage of electrochemical detection is clearly seen (Fig. 2A) since UV detection (Fig. 2B) is not able to detect the degradation product, anhydroerythromycin. Retention times for erythromycin base, anhydroerythromycin and internal standard were 6.3 min, 11.4 min, and 4.4 min respectively.

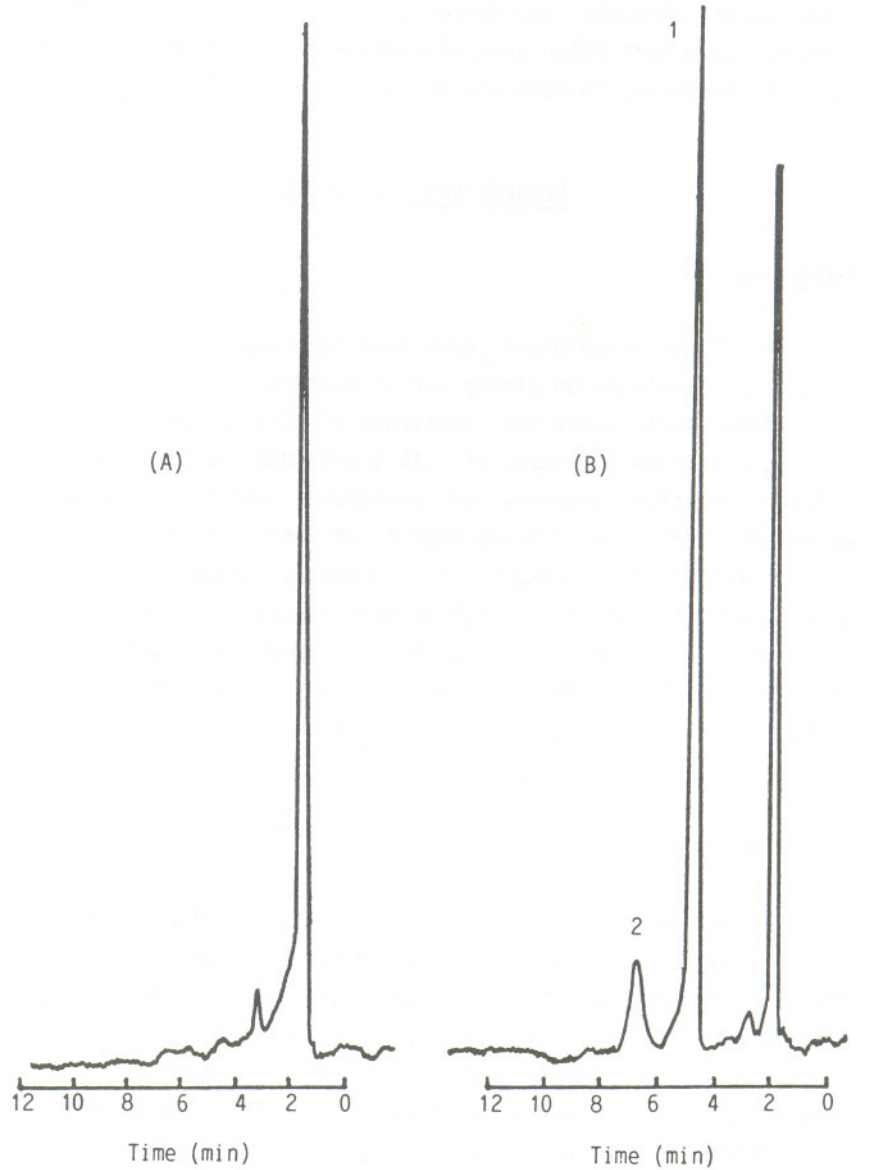

FIGURE 1 Blank serum extract (A) and (B) chromatogram obtained for erythromycin base (2) and internal standard (1) following the extraction of a serum sample containing 1 µg/ml erythromycin stored at 4°C for 8 weeks utilizing amperometric electrochemical detection.

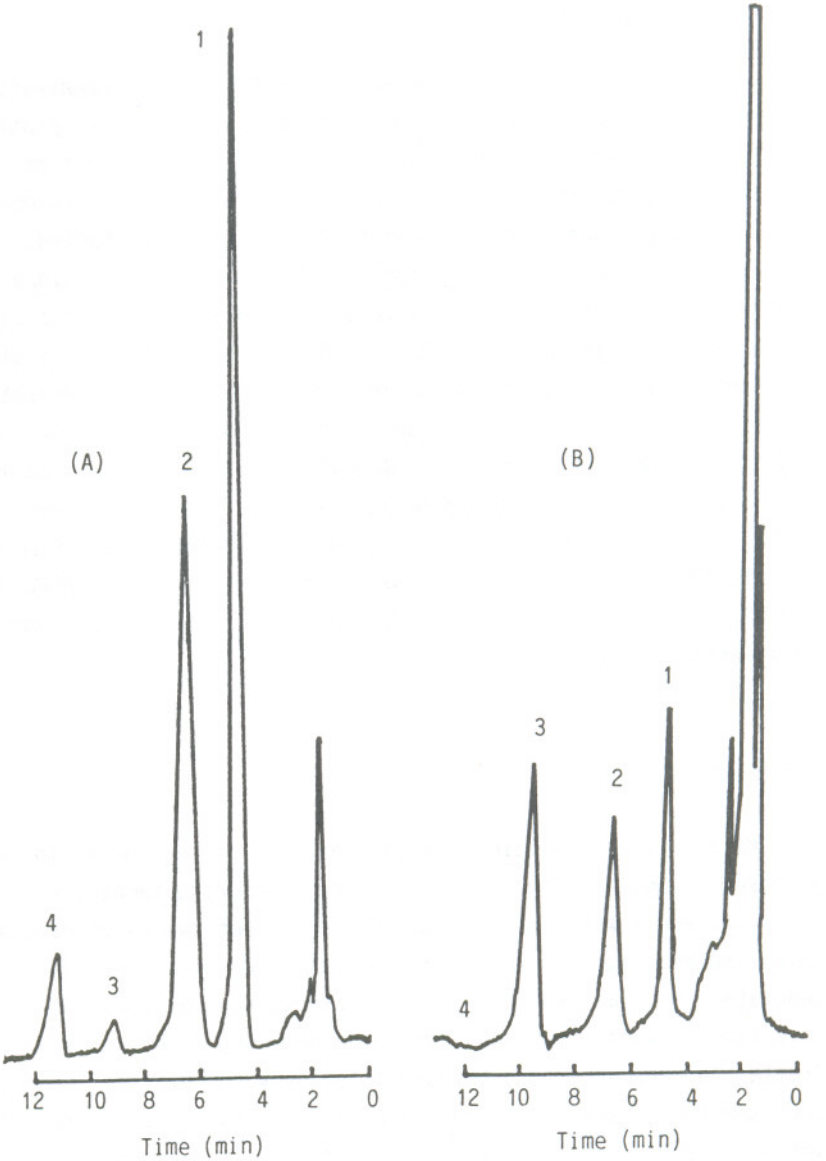

FIGURE 2 Urine sample showing degradation of erythromycin (2) to anhydroerythromycin (4) after storage for 8 weeks at -15°C monitored by (A) amperometric electrochemical detection and (B) UV detection at 200 nm. Peak (3) is a spurious peak found in all urine extracts and peak (1) is the internal standard.

Linearity and Recovery

Calibration plots determined with 5 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 the respective erythromycin concentrations and were linear over the ranges studied. The calibration line in serum (0.25 - 5.00 µg/ml) had a slope of 0.2274 and a y-intercept of 0.0044 with a correlation coefficient of 0.9999 whereas the urine line (1.25 - 25.00 µg/ml) had a slope of 0.0999 and a y-intercept of -0.0373 with a correlation coefficient of 0.9991. Three point standard curves for anhydroerythromycin in serum (0.30 - 6.00 µg/ml) and urine (3.00 - 12.00 µg/ml) showed good linearity with slopes of 0.2446 and 0.1279 and y-intercepts of -0.0193 and 0.0549 respectively. Correlation coefficients for serum and urine were 0.9995 and 0.9999 respectively. The mean recovery in all cases was found to be greater than 80% .

Precision

Within run precision for the analysis of erythromycin was assessed by extracting six spiked serum and urine samples each at the upper and lower limits of the concentration ranges studied and three spiked samples at the intermediate concentrations used to generate the calibration line. Intra-assay relative standard deviations ranged from 1-6% for both serum and urine (Table 1). Similar experiments were performed for anhydroerythromycin using the samples analysed to generate the regression lines in both serum and urine with relative standard deviations in the range of 1-5% (Table 2).

TABLE 1

Precision data for Erythromycin in serum and urine.

SERUM			URINE		
Erythromycin Conc. µg/ml	No. of Samples	R.S.D. %	Erythromycin Conc. µg/ml	No. of Samples	R.S.D. %
0.5	6	6.0	1.25	6	3.5
1.00	3	5.1	2.50	3	5.0
2.00	3	4.2	5.00	3	5.7
3.00	3	4.6	15.00	3	2.7
5.00	6	1.6	25.00	6	2.8

TABLE 2

Precision data for Anhydroerythromycin in serum and urine

SERUM			URINE		
Anhydro-erythromycin Conc. µg/ml	No. of samples	R.S.D. %	Anhydro-erythromycin Conc. µg/ml	No. of Samples	R.S.D. %
0.30	3	1.7	3.00	3	4.5
3.00	3	4.4	6.00	3	5.3
6.00	3	2.2	12.00	3	4.8

Sensitivity and Detection Limit

The sensitivity of the assay, defined as the minimum drug concentration corresponding to 3 times the signal-to-noise ratio, was found to be approximately 0.1 µg/ml for erythromycin. This limit is adequate for the analysis of samples following the oral administration of erythromycin in humans.

Long Term Storage of Samples

Erythromycin was found to be stable in human serum for 12 weeks when stored at both 4°C and -15°C. There was no presence of anhydroerythromycin in these samples. However, in urine, erythromycin was found to degrade after one week at 4°C with appreciable amounts of anhydroerythromycin being detected, while at -15°C the samples showed some evidence of anhydroerythromycin formation after 2 weeks with significant degradation occuring after 4 weeks.

CONCLUSIONS

The chromatographic system described provides a sensitive and precise method of analysis for erythromycin in serum and urine and is highly suited for use in pharmacokinetic/bioavailability studies. A simple single electrode amperometric electrochemical detector eliminates having to use more complex and maintenance intensive coulometric detectors making erythromycin analyses of biological samples readily accessible.

The results clearly indicate that the system described provides a suitable stability-indicating method for the analysis of erythromycin together with its principle degradation product, anhydroerythromycin, in stored samples. Whereas the drug was generally found to be stable in serum under the various conditions of storage, rapid decomposition was observed in the stored urine samples.

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REFERENCES

1. McGuire, J.M., Burch, J.L., Anderson, R.C., Baaz, H.E., Flynn, H.E., Powell, H.M. and Smith, J.W., "Ilotycin", A new Antibiotic, Antibiot. Chemother., 2, 281, 1952.

2. Flynn, E.H., Sigal, M.V., Wiley, P.F. and Gerzon, K., Erythromycin Properties and Degradation Studies, J. Am. Chem. Soc., 76, 3121, 1954.

3. U.S.P. XX1, Erythromycin Base, 391, 1985.

4. Stubbs, C., Haigh, J.M. and Kanfer, I., Determination of Erythromycin in Serum and Urine by High-Performance Liquid Chromatography with Ultraviolet Detection., J. Pharm. Sci., 74, 1126, 1985.

5. Stubbs, C., Haigh, J.M. and Kanfer, I., Aspects of Erythromycin Analysis and Pharmacokinetics., S. Afr. J. Sci., 81, 338, 1985.

6. Chen, M.L. and Chiou, W.L., Analysis of Erythromycin in Biological Fluids by High-Performance Liquid Chromatography with Electrochemical Detection, J. Chromatogr., 278, 91, 1983.

7. Duthu, G.S., Assay of Erythromycin From Human Serum by High Performance Liquid Chromatography with Electrochemical Detection., J. Liq. Chromatogr., 7, 1023, 1984.