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ANALYSIS OF ERYTHROMYCIN IN BIOLOGICAL FLUIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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SUMMARY

A simple and sensitive high-performance liquid chromatographic assay was developed for the quantitative determination of major erythromycin components and their potential metabolites or degradation products in plasma and urine. An ether extract of alkalized plasma sample was chromatographed on a reversed-phase column and the components in the column effluent were monitored by an electrochemical detector. The recovery of the drug from extraction was virtually 100%. The detection limits for erythromycin A in plasma were 5—10 ng/ml and 30 ng/ml using 1 and 0.2 ml of sample, respectively. For urine samples, a simple one-step deproteinization with two volumes of acetonitrile was satisfactory for analysis. The method has been evaluated in plasma and urine from dogs receiving oral or intravenous erythromycin A. The standard curves for potential metabolites or degradation products were not constructed due to the lack of sufficient samples.

INTRODUCTION

Although erythromycin has been widely used for the treatment of various infectious diseases in the last 2—3 decades [1], detailed absorption and disposition kinetics of this antibiotic in the body are only limitedly known to date. This appears to be primarily attributed to the lack of a simple and sensitive method to quantitate the drug and its potential metabolites or degradation products, such as anhydroerythromycin, erythralosamine, and erythromycin enol ether [2].

The novel high-performance liquid chromatographic (HPLC) method of Tsuji [3] based on fluorimetric detection was highly sensitive and capable of separating major erythromycin components from potential metabolites or degradation products. This method apparently has not received wide use,

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probably due to the complexity of the procedure and instrumentation involved. For instance, the method required both a pre-column and an analytical column maintained at 70°C, a special system for post-column derivatization, and an on-line extraction before quantitation. About 1 to 3 ml of serum was needed and the sample preparation involved two 5-ml ether extractions followed by evaporation. In addition, its application to urine analysis has not been demonstrated.

The purpose of this paper is to describe a simple and sensitive HPLC method with electrochemical detection* which may be useful in the quantification of major erythromycin components and their potential metabolites or degradation products in both plasma and urine.

EXPERIMENTAL

Materials

U.S.P. erythromycin, des-N-methylerythromycin, erythralosamine and 4″-acetylerythromycin were kindly supplied by Abbott Labs. (North Chicago, IL, U.S.A.). Erythromycins A, B, C and anhydroerythromycin C were generously donated by Upjohn (Kalamazoo, MI, U.S.A.). Erythromycin ethylsuccinate and erythromycin estolate were purchased from Sigma (St. Louis, MO, U.S.A.). Anhydroerythromycin A and erythromycin A enol ether were synthesized from erythromycin A according to the method of Kurath et al. [4]. Sodium acetate, acetonitrile and methanol were of HPLC grade and were obtained from Fisher Scientific (Fair Lawn, NJ, U.S.A.). Anhydrous sodium carbonate and glacial acetic acid, also from Fisher Scientific, were of ACS reagent grade. Ethyl ether purchased from Eastman Kodak (Rochester, NY, U.S.A.) was used for extraction.

Stock solutions (1 and 5 mg/ml) of erythromycin A were prepared in acetonitrile. Erythromycin B was similarly dissolved in acetonitrile to make concentrations of 0.01—5 mg/ml. These stock solutions appeared to be stable for at least three months when kept in the freezer.

HPLC instrumentation

The chromatographic system was composed of a solvent delivery pump (ConstaMetric III, Laboratory Data Control, Riviera Beach, FL, U.S.A.), a syringe loading sample injector (Model 7125, Rheodyne, Cotati, CA, U.S.A.), and a μBondapak C_{18} reversed-phase column (30 cm × 3.9 mm I.D., particle size 10 μm, Waters Assoc., Milford, MA, U.S.A.). The chromatography was carried out at ambient temperature. The column effluent was monitored by using a dual-electrode electrochemical detector (Model 5100A Coulochem™, Environmental Sciences Assoc., Bedford, MA, U.S.A.) in the oxidative screen mode. The applied cell potential of the screen electrode was set at +0.7 V and the sample electrode at +0.9 V. The output from the detector was connected to a 10-mV potentiometric 25-cm recorder (Linear Instruments, Irvine, CA,

*It appears that the detectability of pure erythromycin by electrochemical detection was first noted by Environmental Sciences Assoc., Inc. and Dr. S.Y. Chu from Abbott Labs., North Chicago.

U.S.A.). The mobile phase was pumped at a flow-rate of 1 ml/min (2000 p.s.i. or 140 bar), and the recorder chart speed was 10 cm/h [5].

Mobile phase preparation

The HPLC mobile phase used in the present study was acetonitrile—methanol—0.2 *M* sodium acetate (40 : 5 : 55), in which the pH of acetate buffer was pre-adjusted to 6.7 with 0.2 *M* acetic acid. Depending on the performance of a given column, however, the optimal composition of the mobile phase may have to be slightly modified. A proper pH of the acetate buffer was essential to the peak resolution. Furthermore, it was found that satisfactory chromatography may be achieved without the incorporation of methanol in the mobile phase for some columns from the same manufacturer. In order to minimize the background noise and to improve the sensitivity of the detection, it is important to prefilter all the mobile phase components with a 0.22-μm low-extractable membrane. The distilled water used in the mobile phase was purified through the Milli-Q system that contained an Organex-Q™ cartridge (Millipore, Bedford, MA, U.S.A.).

Sample preparation

Aliquots of plasma (0.2 ml) were pipetted into 13 × 100 mm disposable glass culture tubes. The screw cap was lined with a piece of aluminum foil to prevent possible leaching of chemicals and also adsorption of the drug onto the cap. After the addition of internal standard (10 μl of an erythromycin B solution of 0.01—0.1 mg/ml; other internal standards could also be used as will be discussed later), saturated sodium carbonate (20 μl) and ethyl ether (1 ml), each tube was vortexed for 20 sec. Following centrifugation at 800 *g* for 5 min, 0.75 ml of the diethyl ether layer was transferred to another disposable tube. The diethyl ether layer was then allowed to evaporate at room temperature in a vortex evaporator (Buchler Instruments, Fort Lee, NJ, U.S.A.) at reduced pressure for 10—15 min. Just prior to the HPLC analysis, the residue was reconstituted with 50 μl of mobile phase and vortexed for 10 sec to facilitate rapid dissolution of the sample. After brief centrifugation, a 20-μl aliquot was injected onto the column.

For urine samples, a 0.1-ml aliquot was transferred to the culture tube. After supplementing with the internal standard (10 μl of an erythromycin B solution of 0.2 mg/ml), the deproteinization was carried out by adding 0.2 ml of acetonitrile, a unique deproteinizing agent first extensively used in this laboratory [6—8 and others]. Following vortex mixing for 10 sec and centrifugation at 800 *g* for 2 min, about 20 μl of the supernatant was injected into the HPLC.

Standard curves were prepared by spiking blank human plasma with concentrated erythromycin A solution to yield 0.25, 0.5, 1.0, 2.5, and 5 μg/ml. Blank human urine was also supplemented with erythromycin A in concentrations of 2.5, 5, 10, 25, and 50 μg/ml. Due to the lack of sufficient samples, standard curves for erythromycin metabolites or degradation products were not constructed in the present study.

Reproducibility studies

Six replicate analyses of plasma samples at concentrations of 0.5 and 2.5 μg/ml for erythromycin A were performed as described above.

Drug interference studies

Potential interferences of drugs with the assay were studied by injecting aliquots of their stock solutions into the HPLC. The drugs tested included acetaminophen, adriamycin, butaperazine, chlorpheniramine, diazepam, disopyramide, estradiol, furosemide, gentamicin, griseofulvin, lidocaine, naphazoline, oleandomycin, primaquine, pronethalol, propranolol, quinidine·HCl, quinine, rifampicin, theophylline, thiopental, thyroxine, tolbutamide, trifluoroperazine, and trimeprazine.

RESULTS AND DISCUSSION

The electrochemical detector used in the present study is different from those commonly employed in the past in that it has series dual electrodes, which can be set in screen-mode of operation. In this mode, the first electrode was at a potential somewhat lower than the second electrode. The coulometric efficiency of the detector thus decreased background currents and eliminated undesirable components at the first electrode while quantitating erythromycin at the second electrode. In the assay development, the optimal cell potential was first explored using the pure compound dissolved in mobile phase. The resultant hydrodynamic voltammogram for the oxidation of erythromycin A is shown in Fig. 1. Based on this curve, +0.7 and +0.9 V were then chosen for the first and second electrode, respectively.

The high selectivity of the present HPLC method is illustrated by the chromatograms shown in Fig. 2. The peak shapes for most of the individual

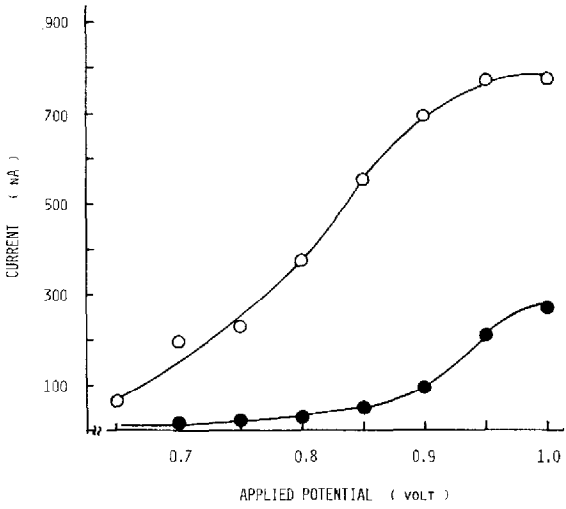

Fig. 1. Hydrodynamic voltammogram for the oxidation of erythromycin A. The lower curve denotes the corresponding background current. The on-column amount injected was 0.2 μg.

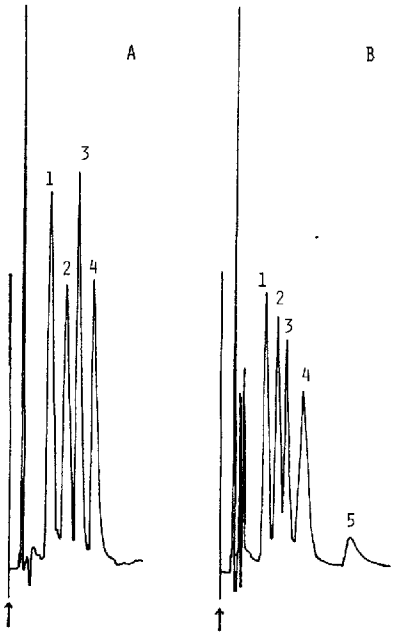

Fig. 2. HPLC chromatograms of various erythromycins, their potential metabolites and degradation products. (A) Peaks: 1 = erythromycin C; 2 = erythromycin A; 3 = anhydroerythromycin A or C; 4 = erythromycin B. (B) Peaks: 1 = des-N-methylerythromycin; 2 = erythromycin A; 3 = erythralosamine; 4 = 4″-acetylerythromycin A; 5 = erythromycin A enol ether. The arrows mark the point of injection.

components of the mixture appear to be symmetrical. In spite of the use of filter element installed before the detector cells, there is no significant peak broadening effect. The retention times for erythromycins A, B, and C were 9.6, 14.2, and 6.9 min, respectively. The retention times of various erythromycins, their potential metabolites and degradation products are listed in Table I. It was found that the powder form of U.S.P. erythromycin from

TABLE I

RETENTION TIMES OF VARIOUS ERYTHROMYCINS AND POTENTIAL METABOLITES OR DEGRADATION PRODUCTS

Compound	Retention time (min)
Erythromycin C	6.90
Des-N-methylerythromycin	7.74
Erythromycin A	9.60
Erythralosamine	11.10
Anhydroerythromycin A	12.36
Anhydroerythromycin C	12.60
4″-Acetylerythromycin A	14.04
Erythromycin B	14.22
Erythromycin ethylsuccinate	19.50
Erythromycin A enol ether	22.08
Erythromycin estolate	22.86

Abbott Labs. or Sigma was essentially free of erythromycin B. Therefore, erythromycin B was chosen as internal standard for this preliminary study. Alternatively, depending on the experimental condition and the availability of the compounds, other erythromycin derivatives such as erythromycin ethylsuccinate or estolate could be used as internal standard.

Typical chromatograms from blank human plasma, and plasma spiked with known concentrations of erythromycins A and B are shown in Fig. 3. No interferences with peaks of the drug and internal standard were found in the plasma samples. As shown in Table II, the standard curve for erythromycin A in plasma is linear over the concentration range studied (0.25—5 μg/ml), which is reflected by the constancy of response factors. Recovery of erythromycin from the extraction was virtually 100%. Excellent reproducibility was obtained for the present HPLC method; the coefficients of variation were 1.5% at 0.5 μg/ml, and 2.3% at 2.5 μg/ml. The intra-day precision was usually between 1.5 and 3.6%, while five replicate analyses in three days gave inter-day precision of 3.9%. As shown in Table II, assay of erythromycin without the use of internal standard may also be satisfactory since the coefficient of variation was only about 5%. To measure peak heights more accurately, it has been recommended that a micrometer [9] and a slower chart speed be used [5].

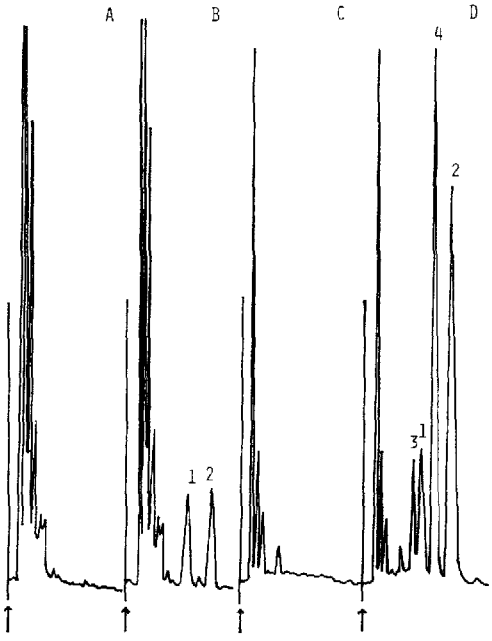

Fig. 3. Chromatograms of extracts from (A) human plasma blank; (B) human plasma spiked with 0.5 μg/ml of erythromycin A and 0.01 mg/ml of internal standard; (C) dog plasma blank; (D) dog plasma collected at 2 h after an oral dose of 500 mg erythromycin A. Concentrations of erythromycin A and internal standard were 1.1 μg/ml and 0.1 mg/ml, respectively. Peaks: 1 = erythromycin A; 2 = internal standard; 3 = unknown product; 4 = anhydroerythromycin A. The arrows mark the point of injection. The detector gain settings were 10 × 10 for A and B, and 50 × 1 for C and D.

TABLE II

RESPONSE FACTORS FOR ERYTHROMYCIN A IN HUMAN PLASMA

Spiked-plasma concentration (μg/ml)	Peak height* (cm)		Response factor I***	Response factor II§
	Erythromycin A	Internal standard**		
0.25	1.30	2.76	5.20	18.84
0.50	2.44	2.60	4.88	18.77
1.0	5.04	2.70	5.04	18.67
2.5	12.96	27.92	5.18	18.57
5.0	28.12	29.71	5.62	18.93
Mean ± S.D.			5.18 ± 0.28	18.76 ± 0.14
Coefficient of variation (%)			5.3	0.75

*Peak heights were measured based on 20-μl injection and normalized gain setting of 10 × 10.
**The internal standard spiked was 0.01 and 0.1 mg/ml for low (0.25—1.0 μg/ml) and high (2.5—5.0 μg/ml) concentrations of erythromycin A.
***Response factor I = peak height of erythromycin A divided by its concentration.
§ Response factor II = peak height ratio of erythromycin A and internal standard based on unit concentration of each compound.

Because of the coulometric detector design, the background current levels would limit the maximum gain that can be obtained. As a result, the detection limit for a drug was highly dependent on the background noise encountered. Practically speaking, based on a signal-to-noise ratio of 3, the lower limit of quantitation for erythromycin A using 0.2 ml plasma was approximately 30 ng/ml with a detector gain setting of 10 × 10. However, higher sensitivity could be achieved when 1 ml of plasma was used for extraction. The procedure was carried out as follows. After alkalization with 0.1 ml of saturated sodium carbonate, 2 ml of ethyl ether were added to the plasma sample. Following vortex mixing and centrifugation, the ether layer (about 1.5 ml) was evaporated to dryness. The residue was then reconstituted with 50 μl of mobile phase and centrifuged. An aliquot of 20 μl was injected into the HPLC. In this manner, the detection limit of erythromycin A could be 5—10 ng/ml with a gain setting of 10 × 10. The sensitivity of the present assay appears to be adequate for drug concentrations encountered clinically. There was no significant increase in the absolute peak height of erythromycin A when the diethyl ether—plasma volume ratio was increased to 4, suggesting a complete recovery of the drug with the volume ratio of 2.

Fig. 3 also shows a chromatogram from the plasma collected 2 h after oral administration of 500 mg erythromycin A to a 12-kg male mongrel dog. It appeared that peaks from two additional compounds were present in all of the samples analyzed. The peak eluting after erythromycin A had a retention time identical to that of anhydroerythromycin A. The other peak, eluting earlier, had a retention time different from those of potential metabolites or degradation compounds tested; its identity is presently under investigation. The plasma concentration—time profile of erythromycin A together with the peak heights of the two metabolites or breakdown products from the dog are depicted in

Fig. 4. The erythromycin levels were found to be 2.1 and 0.3 μg/ml at 1 and 6 h, respectively. It is of interest to note that peaks corresponding to anhydroerythromycin A and the unknown compound were both present after intravenous administration of erythromycin lactobionate to another dog. Detailed absorption and disposition kinetics of erythromycin in animals and humans are currently being pursued using the assay developed here.

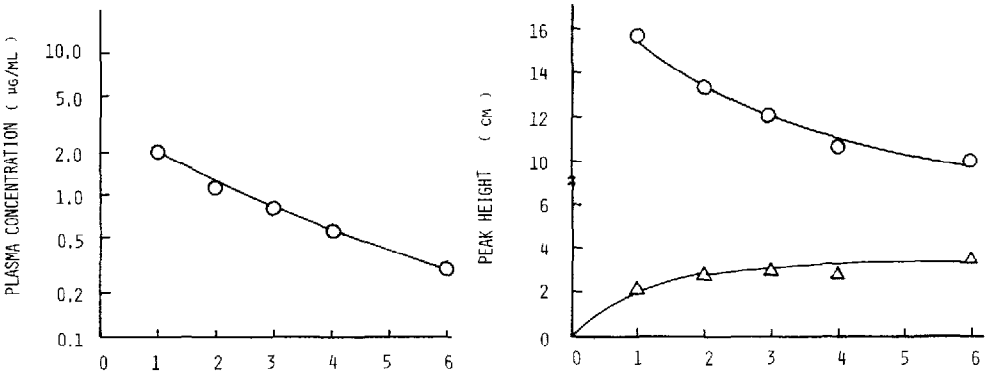

Fig. 4. Plasma concentration profile of erythromycin A (left) and peak heights of two metabolites or degradation products as a function of time (right; ○–○, anhydroerythromycin A and △-△, unknown compound) in a dog following an oral dose of 500 mg of erythromycin A.

The pH of the mobile phase was found to have a profound effect on both the retention volume and the resolution of the peaks. In general, the lower the pH, the shorter the retention time, and thus the higher the response. However, when the pH of acetate buffer in the mobile phase was lower than 6.0 a shoulder peak next to erythromycin A would appear; and in addition, the unknown product could not be resolved from the drug. To maximize the performance of the column, therefore, a pH of 6.7 of the buffer was adopted in this study.

One of the advantages of this assay is that a much smaller plasma volume (0.2 ml) can be employed for quantitation as compared with that used (1—3 ml) in the previous report [3]. The extraction procedure is also simpler and easier. Essentially complete recovery was obtained with 1 ml of diethyl ether for 0.2 ml of plasma samples. Several solvents were tested for extraction efficiency, including benzene, toluene, chloroform, ethyl acetate, hexane and diethyl ether. Among these, chloroform and diethyl ether offered the greatest recovery and the least interference. Diethyl ether was selected because of the easy handling of diethyl ether layer which was on the top of the aqueous solution. It has to be pointed out that alkalization of plasma samples by sodium carbonate was critical to the high extraction efficiency. The extraction procedure used in this study appeared somewhat similar to that employed earlier [3].

The results of interference studies showed that none of the drugs tested interfered with the analysis of erythromycin.

Since the drug concentrations in urine samples are usually much higher than found in plasma, a simple one-step deproteinization method was sufficient for electrochemical detection. Fig. 5 shows chromatograms from blank human urine, urine spiked with known concentrations of erythromycins A and B, together with that from dog's urine collected after receiving erythromycin A. A similar peak pattern was observed in the dog's urine and plasma. The response factor data for erythromycin A in urine are shown in Table III. Linearity was found within the concentration range 2.5—50 μg/ml. The coefficient of variation of response factors was found to be 5.0% in the absence of internal standard, indicating the adequacy of performing the assay without an internal standard.

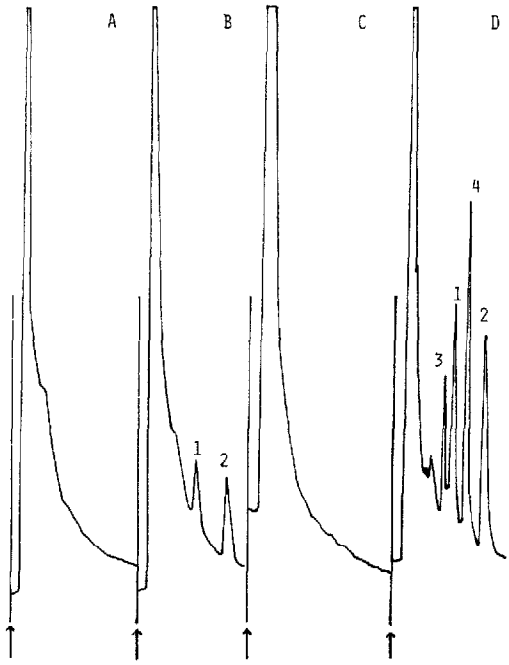

Fig. 5. Chromatograms from (A) human urine blank; (B) human urine spiked with 10 μg/ml of erythromycin A and 0.2 mg/ml of internal standard; (C) dog urine blank; (D) dog urine collected up to 6 h after an oral dose of 500 mg erythromycin A. Concentrations of erythromycin A and internal standard were 0.33 and 5 mg/ml, respectively. Peaks: 1, erythromycin A; 2, internal standard; 3, unknown product; 4, anhydroerythromycin A. The arrows mark the point of injection. The detector gain settings were 50 × 1 for A and B, and 5 × 1 for C and D.

With a daily or frequent use of electrochemical detector, it is often advised that the HPLC system be maintained in operation condition to reduce the background currents and to enhance detector stability. Long-term exposure of bonded silica columns to a high-pH mobile phase, however, may significantly shorten the column life. In order to preserve optimum column performance, the column was removed from the system when not in use (e.g. overnight),

TABLE III

RESPONSE FACTORS FOR ERYTHROMYCIN A IN HUMAN URINE

Spiked-urine concentration (μg/ml)	Peak height* (cm)		Response factor I	Response factor II
	Erythromycin A	Internal standard**		
2.5	0.45	2.37	0.180	15.13
5.0	0.91	2.41	0.182	15.04
10.0	1.91	2.46	0.191	15.53
25.0	4.92	2.48	0.197	15.87
50.0	10.10	2.58	0.202	15.66
Mean ± S.D.			0.190 ± 0.009	15.45 ± 0.35
Coefficient of variation (%)			5.0	2.28

*Peak heights were measured based on 20-μl injection and the gain setting was 50 × 1.
**The concentration of internal standard spiked was 0.2 mg/ml.

while the chromatograph was maintained with the mobile phase recirculating at a rate of 0.2 ml/min, and also with the electrode activated. The column was then flushed with 20 void volumes (about 30 ml) of water and methanol prior to storage. Just before the start of the assay, the column was first flushed with 30 ml of water and then equilibrated with mobile phase at a flow-rate of 1 ml/min for 0.5 h. The system could be quickly restored to operating conditions by reconnection of the pre-equilibrated column to the chromatograph.

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