Analysis of Erythromycin A and Its Metabolites in Biological Samples by Liquid Chromatography with Post-Column Ion-Pair Extraction

Kamran Khan[a]; J. Paesen[a]; E. Roets[a]; J. Hoogmartens[a]

[a] Faculteit Farmaceutische Wetenschappen Laboratorium voor Farmaceutische chemie, Katholieke Universiteit Leuven, Leuven, Belgium

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ANALYSIS OF ERYTHROMYCIN A AND ITS METABOLITES IN BIOLOGICAL SAMPLES BY LIQUID CHROMATOGRAPHY WITH POST-COLUMN ION-PAIR EXTRACTION

KAMRAN KHAN, J. PAESEN,
E. ROETS, AND J. HOOGMARTENS*
Katholieke Universiteit Leuven
Faculteit Farmaceutische Wetenschappen
Laboratorium voor Farmaceutische chemie
Van Evenstraat 4
B-3000 Leuven, Belgium

ABSTRACT

The development of a selective and sensitive method for analysis of erythromycin A (EA) and its metabolites in biological fluids (urine and plasma) is reported here. A mobile phase consisting of acetonitrile - 2-methyl-2-propanol - 0.2 M phosphate buffer pH 9.0 - water (3:19:5:73, v/v) at 1.5 ml/min enables the separation of the main component (EA) from all of its potential metabolites on a 250 x 4.6 mm I.D. poly(styrene-divinylbenzene) (PLRP-S 8 μm, 1000 Å) column at 70°C. To improve the sensitivity of the method, a post-column ion-pair extraction with the strongly fluorescent 9, 10-dimethoxy-anthracene-2-sulphonate was used. The ion-pairs were extracted in chloroform for on-line fluorescence detection. Analytical recoveries for erythromycin and its metabolites after extraction of the plasma with tertiary butyl methyl ether were better than 90 %. The calibration curves of EA and its potential metabolites in plasma and urine were linear over the concentration range studied. The detection limits were 12.5 ng/ml plasma and 50 ng/ml urine. The method allows the detection of all the erythromycin metabolites in human plasma and urine.

INTRODUCTION

The major problem in the determination of the macrolide antibiotic erythromycin in biological fluids using liquid chromatography (LC), is the detection of this substance at the low concentration levels present in plasma and urine. Erythromycin is UV transparent at wavelengths above 220 nm, except for a weak chromophore at 280 nm. Below 220 nm, where the molar absorptivity is comparatively higher, additional problems occur, such as interference of species co-extracted from biological samples. Tserng and Wagner [1] determined erythromycin and erythromycin propionate in plasma and whole blood using fluorimetry after addition of the fluorescent ion-pair reagent 2-(stilbyl-4″)-(naphtho-1′, 2′ : 4,5) - 1,2,3 triazole- 2″, 6′ disulfonic acid, sodium salt and extraction. Tsuji [2] used the same detection reagent but in a LC method using a reversed-phase silica-based column (RP-18) and post-column extraction, with considerable loss of efficiency. After administration of erythromycin ethyl-succinate Tsuji was able to detect anhydroerythromycin ethyl succinate, erythromycin A enol ether ethyl succinate and erythralosamine in serum samples. The limit of detection was less than 0.01 µg/ml. Stubbs et al [3] used UV detection at 200 nm and obtained a limit of detection of 0.25 µg/ml and 1 µg/ml for erythromycin in serum and urine, respectively. Detection of metabolites was not reported. Most published LC methods employed electrochemical detection. Chen and Chiou [4] obtained a detection limit for erythromycin A of 5-10 ng/ml in plasma . Anhydroerythromycin A was also detected, but presence of N-demethylerythromycin A in plasma samples was not reported. According to work by Duthu [5], N-demethylerythromycin A was undetectable by the dual coulometric electrode detector used. Nilson et al. [6] reported a method using a polymeric stationary phase and amperometric detection. Only the main compound was detected at plasma concentrations down to 0.2 µmol/l. Stubbs et al. [7] used amperometric detection to detect anhydroerythromycin A, the main degradation product formed during the storage of biological fluids, with a sensitivity limit of 0.1 µg/ml. Recently Kato et al. [8] developed a method for analysis of erythromycin in plasma and whole blood using a reserved-phase Asahipak C-18 polymer-based column with an alkaline mobile phase. The detection limit was 0.1 µg/ml for erythromycin A. So far, no paper has reported the detection of N-

demethylerythromycin in plasma by LC. However, it has been shown that N-demethylerythyromycin A is an important metabolite of erythromycin, formed by demethylation in the liver [9]. Attempts to do quantitative TLC of erythromycin metabolites have been described elsewhere [10].

Because of the availability of low dead volume connections and a sandwich-type phase separator [11], causing negligible additional band broadening, it is now possible to develop a selective and sensitive method for analysis of erythromycin A and its metabolites in biological fluids. As described by Tsuji [3], a post-column ion-pair extraction system coupled to a fluorescence detector was used in the method proposed here. In order to separate erythromycin A from its metabolites, the previously developed method for analysis of erythromycin A and its related substances [12] on wide-pore poly(styrene-divinylbenzene) was further investigated.

EXPERIMENTAL

Samples and Sample Solutions

An erythromycin A house standard (EA-HS, 94.7 % pure) was obtained by crystallization of a commercial sample [13]. Reference substances for N-demethylerythromycin A (dMeEA) [14], anhydroerythromycin A (AEA) [15] and erythromycin A enol ether (EAEN) [16] were prepared from EA following described methods. Anhydro-N-demethylerythromycin A (AdMeEA) [13] and N-demethylerythromycin A enol ether (dMeEAEN) [17] were prepared from dMeEA according to the methods used to prepare AEA and EAEN. A commercial sample of josamycin (UCB, Brussels, Belgium) was used as internal standard (IS). The structures of erythromycin A, its metabolites and of josamycin are shown in Figure 1. Sample solutions in methanol-water (1:1) were prepared at a concentration of 1 mg/ml, except for EAEN and dMeEAEN where 0.1 mg/ml solutions were used. These sample solutions were injected during the preliminary LC development work using the UV detector. More dilute solutions (10 μg/ml) were injected when fluorescence detection was used.
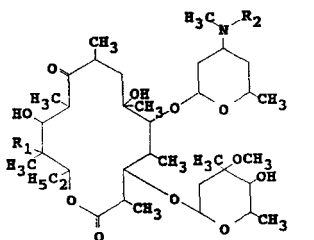
	R_1	R_2
Erythromycin A (EA)	OH	CH_3
Erythromycin B (EB)	H	CH_3
N-demethylery-thromycin A (dMeEA)	OH	H
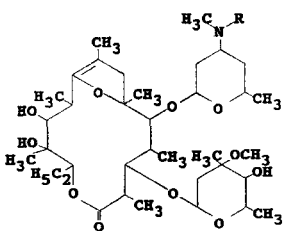
	R
Erythromycin A enol ether (EAEN)	CH_3
N-demethylerythromycin A enol ether (dMeEAEN)	H
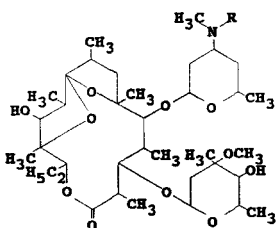
	R
Anhydroerythromycin A (AEA)	CH_3
Anhydro N-demethylerythromycin A (AdMeEA)	H
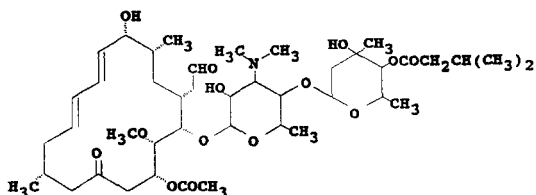
Josamycin

FIGURE 1 : Chemical structures of erythromycin , its metabolites and josamycin.

Solvents and Reagents

2-Methyl-2-propanol 99.5% and dichloromethane were purchased from Janssen Chimica (Beerse, Belgium) and distilled before use. 2-Methoxy-2-methylpropane (Janssen Chimica) was used without purification. Acetonitrile, LC grade S and methanol LC grade, were from Rathburn (Walkerburn, UK). Chloroform was distilled before use (Belgolabo,Overijse, Belgium). Water was distilled twice in glass apparatus. Potassium dihydrogen phosphate, dipotassium hydrogen phosphate, citric acid monohydrate, potassium carbonate and disodium edetate (EDTA) were of pro analysi quality from Janssen Chimica. Sodium 9,10-dimethoxyanthracene-2-sulphonate 97% (DAS), sodium naphthalenesulphonate and bromocresol purple were purchased from Aldrich (Bornem, Belgium). Ammonium 8-anilino-1-naphthalenesulphonate and methyl orange were obtained from Merck (Darmstadt, Germany).

Equipment

Figure 2 shows a schematic diagram of the assembly of the analytical equipment used for on-line ion-pair extraction, phase separation and fluorescence detection.

The mobile phase was delivered by a Merck-Hitachi, L-6200 intelligent pump. For injection of the samples a Valco injector model CV-6-UHPa-N60 (Houston, TX, USA) equipped with a fixed loop of 100 μl was applied. The stationary phase, PLRP-S 8μm, 1000 Å (Polymer Labs, Church Stretton, Shropshire, UK) was packed in a 250 mm x 4.6 mm I.D. stainless steel column and kept at 70°C using a water bath. The detector used in the development of the mobile phase was a Waters Model 420 UV detector (Milford, MA, USA) set at 215 nm. In the system with ion-pair extraction finally used, a Merck-Hitachi F-1050 fluorescence spectrofluorometer with variable excitation and emission wavelengths setting was used. Chromatograms were recorded on a Hewlett-Packard HP 3396A integrator (Avondale, PA, USA). For delivery of the reagent solution, a Milton Roy minipump (Laboratory Data Control, Riviera Beach, FL, USA) was used, equipped with a pulse dampener and a Bourdon manometer as described previously [18]. A 250 x 4.6 mm stainless steel column packed with glass beads was used as an
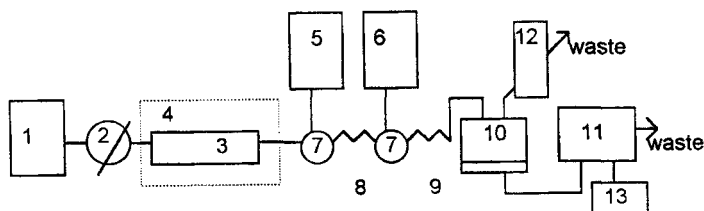
FIGURE 2 : Scheme of the assembly of the equipment used for on-line ion-pair extraction and fluorescence detection. 1: eluent pump, 2: injector, 3: column, 4: heating device, 5: reagent pump, 6: extraction solvent pump, 7: T-piece, 8: reaction coil, 9: extraction coil, 10: phase separator, 11: fluorescence detector, 12: back pressure regulator, 13: integrator.

additional pulse dampener. A second Merck Hitachi, L-6200 intelligent pump delivered the organic extraction solvent. Mixing and extraction coils were of stainless steel capillary coiled to a helix with a diameter of 45 mm. A sandwich-type phase separator (Vrije Universiteit, Amsterdam, The Netherlands) with a 40 μ l groove volume [11] was connected to a SSI back pressure regulator (State College, PA, U.S.A) for adjustment of the flow of organic solvent through the fluorescence detector.

Urine and Plasma Samples

For urine, no extraction procedure was needed. Urine was centrifuged at 2500 g for 5 min and an aliquot of the supernatant (100 μl) was injected directly on the system. For spiking experiments with plasma, rabbit blood (1.0 ml) was collected in a tube containing 0.5 ml of 10 % m/v EDTA solution and centrifuged to separate the plasma. Plasma (1.0 ml) was spiked with EA and metabolites. 250 μl of internal standard solution (IS) (0.004 mg of josamycin/ml methanol-water 1:1) and 60 μl of saturated solution of K_2CO_3 were added. Extraction was done with 5 ml of 2-methoxy-2-methylpropane and 4 ml of the ether layer was evaporated under reduced pressure. The residue was reconstituted with 200 μl of methanol-water (1:1) and an aliquot of 100 μl was injected. Real human blood samples (200

µl) were collected, after pricking the index finger, into a tube containing 100 µl of 10 % m/v EDTA solution. The tube was weighed before and after taking blood. IS (50 µl) (0.02 mg/ml methanol-water 1:1) was added and the mixture was centrifuged to separate the plasma. To 200 µl of plasma, 20 µl of saturated K_2CO_3 solution and 1.0 ml of 2-methoxy-2-methylpropane were added. After centrifugation, 0.8 ml of the ether layer was evaporated and the residue was reconstituted with 200 µl of methanol-water (1:1). Here too, a 100 µl aliquot was injected.

RESULTS AND DISCUSSION

Development of Mobile Phase

The previously described LC method [11] for analysis of erythromycin A and related substances on wide-pore poly(styrene-divinylbenzene) (PLRP-S 8µm, 1000 Å) was taken as a starting point. This method used as mobile phase a mixture of 2-methyl-2-propanol - acetonitrile - 0.2 M potassium phosphate buffer pH 9.0 - water (165:30:50:755, v/v) , at a flow rate of 2.0 ml/min. This mobile phase was investigated for its selectivity towards EA and its potential metabolites (dMeEA, AEA, EAEN, AdMeEA and dMeEAEN). The pH and the amount of 2-methyl-2-propanol (t-BuOH) were the parameters most influencing the selectivity. As can be seen in Figure 3, AdMeEA and EA were not separated at a pH < 8.0. Good selectivity was obtained at higher pH. Finally pH 9.0 was adapted. As we wanted EAEN to be eluted within 40 min, the initial amount of 2-methyl-2-propanol had to be adapted. As shown in Figure 4, the capacity factor of EAEN decreased significantly with increasing the amount of 2-methyl-2-propanol. An amount of 19 % gave good separation and a sufficiently short analysis time. The flow rate was decreased to 1.5 ml/min, in view of the use of an on-line phase separator. Smaller volumes of aqueous phase are easier to separate and the volume of organic phase consequently can be reduced. A typical chromatogram of a mixture of reference compounds of EA and its metabolites recorded at 215 nm, is shown in Figure 5.
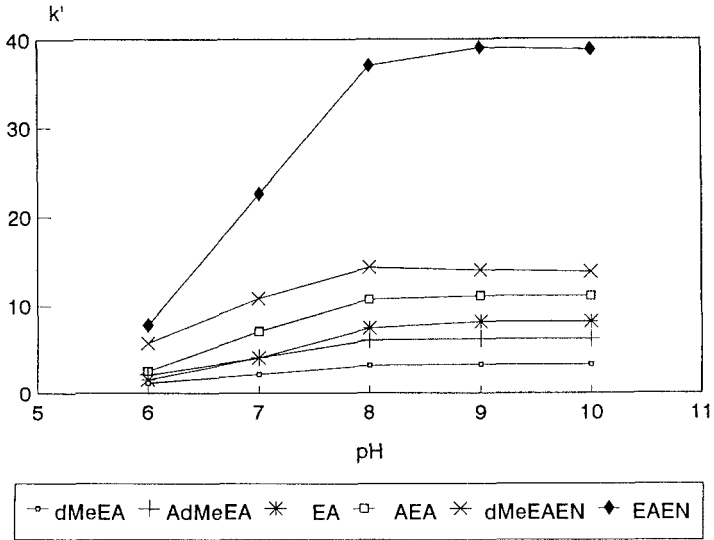
FIGURE 3 : Effect of the pH of mobile phase on the mass distribution ratios (k') of erythromycin A and its metabolites.
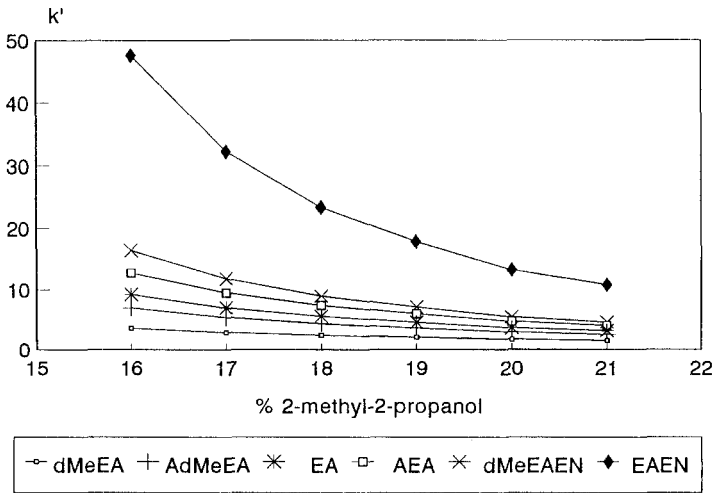

FIGURE 4 : Influence of the concentration of 2-methyl-2-propanol (t-BuOH) on the mass distribution ratios (k') of erythromycin A and its metabolites.
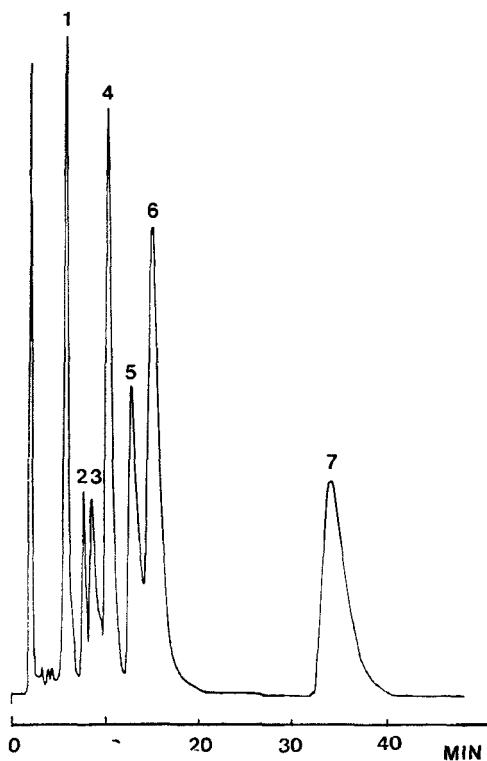
FIGURE 5 : Typical chromatogram of a mixture of reference compounds of erythromycin A and its metabolites. Column: PLRP-S (8 μm 1000Å) at 70 °C. Mobile phase: acetonitrile - 2-methyl-2-propanol - 0.2 M phosphate buffer pH 9.0 - water (3:19:5:73, v/v) at 1.5 ml/min. Detection: UV 215 nm. 1. dMeEA, 2. Impurity of 6, 3. AdMeEA, 4. EA, 5. AEA, 6. dMeEAEN, 7. EAEN.

Ion-pairing Reagent and Extraction Solvent

In acidic medium, compounds containing tertiary or secondary amino groups easily form ion-pairs with strongly UV-absorbing or fluorescent counter-ions. By adding a non-miscible organic solvent after the chromatographic separation, these ion-pairs can be extracted and subsequently detected. In our study, a number of reagents described in the literature was evaluated as counter-ion for erythromycin

and their sensitivity was compared. The reagents tested were dissolved in 0.1 M citric acid solution at a concentration of 0.5×10^{-5} M. Chloroform was used as extraction solvent. With methyl orange and bromocresol purple [19], the limit of detection was 1 μg, which is not sensitive enough for detecting serum levels of erythromycin. Using sodium naphthalenesulphonate [1], the ion-pair could not be extracted. With two other fluorescent dyes, ammonium 8-anilino-1-naphthalenesulphonate [20] and DAS [21], erythromycin could be detected down to 10 ng and 5 ng respectively. The fluorescent reagent used by Tsuji [2] was not available for evaluation. DAS was chosen as the fluorescent reagent for further work. Maximum signal height for DAS was obtained at excitation and emission wavelengths of 365 nm and 450 nm, respectively. On replacing chloroform by dichloromethane as the extraction solvent, the signal height decreased by 20 %. Hexane was too apolar as extraction solvent. Moreover, organic solvents with high density are preferred because of the ease of separating the layers in the sandwich phase separator.

Influence of Concentration and Flow Rate of DAS Reagent

The derivatization reagent DAS was dissolved in 0.1 M citric acid. The influence on the signal area of the concentration of DAS in the 0.1 M citric acid solution was investigated. The peak area of erythromycin after injection of 1 μg on column was recorded at every condition. Figure 6 shows that on decreasing the DAS concentration from 10^{-4} M to 0.5×10^{-5} M, there was an increase in the peak area, which can be explained by the fact that at higher reagent concentration, the blank fluorescence signal was increased, which masks the sensitivity for detection of the ion-pair. The concentration of the citric acid solution had no effect on the signal area in the concentration range studied (0.025 M to 0.2 M). The flow rate of the reagent solution was investigated in the range 0.5 to 0.9 ml/min. 0.7 ml/min was chosen because flow rates lower than this were less sensitive for the detection of ion-pairs and flow rates higher than 0.7 ml/min would only increase the flow rate of extraction solvent required, and hence the total flow rate, which makes the operation of the sandwhich phase separator less efficient.
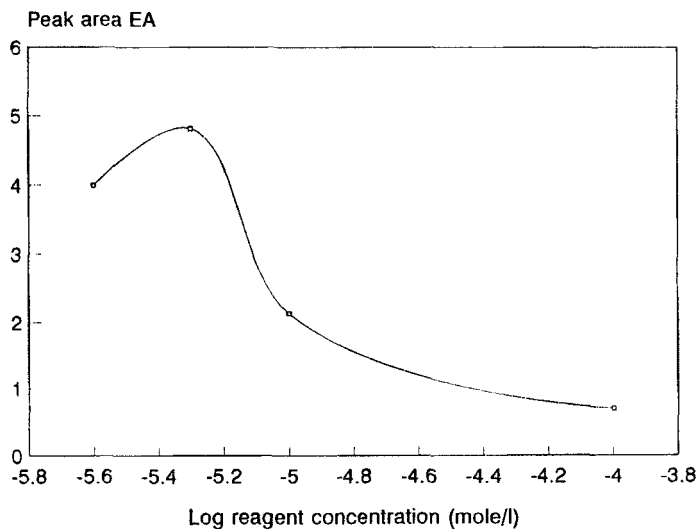
Peak area EA

Log reagent concentration (mole/l)

FIGURE 6 : Effect of concentration of fluorescence reagent on peak area (EA).

Influence of Dimensions of Reaction and Extraction Coils

The influence of the internal diameter of the extraction coil on the extraction was evaluated using a reaction coil of 1 m x 0.02 inch. Changing the internal diameter from 0.01 to 0.02 inch had little effect on the resolution, but the peak area increased with a factor 1.7. The repeatability also improved much, because of fewer problems associated with the separation of the organic layer in the phase separator. A further increase in internal diameter (0.02 to 0.03 inch) was not suitable because the resolution decreased and the peak area decreased with a factor 0.9. The length of the reaction coil was investigated for 1 and 1.5 m. No change in signal area was seen. Finally a uniform combination of 1.5 m x 0.02 inch I.D. was chosen for both reaction and extraction coils.

Influence of Total Flow Rate of Extraction Solvent and Flow Rate Through Detector

The effect of different total flow rates of chloroform (0.75, 1.0, 1.25, 1.5, and 1.75 ml/min) on the peak area of EA (amount injected, 1 µg) was recorded. The

flow rate of chloroform through the detector was kept constant at 0.5 ml/min. This resulted in different separation effeciencies (i.e. total flow rate of chloroform/flow rate of chloroform through detector). It was seen (Figure 7) that decreasing the total flow rate of chloroform caused an increase in the peak area due to the concentration effect. A flow rate of 1.5 ml/min was finally chosen because the risk of water leakage towards the detector excluded the use of lower flow rates of chloroform. Variation of the flow rate of chloroform through the detector (0.1, 0.2, 0.3, 0.4, 0.5 and 0.75 ml/min) did not influence the peak area. Resolution and number of theoretical plates were found to be optimal at 0.5 ml/min.

Linearity, Repeatability and Limit of Detection for Urine Samples

Calibration curves were constructed by spiking human urine to yield five different concentrations of 2.0 to 26.0 µg of EA per ml and 1.0 to 12.0 µg of metabolite per ml. Each concentration was injected three times. Calibration curves were linear and yielded the following equations, with y = peak area, x = concentration in µg/ml and r = correlation coefficient. For EA, $y = 6767 x + 358$, r = 0.9974; for dMeEA, $y = 5697 x + 164$, r = 0.9963; for AdMeEA, $y = 11882 x - 17$, r = 0.9981; for AEA, $y = 10582 x - 167$, r = 0.9991; for dMeEAEN, $y = 12637 x - 37$, r = 0.9988 and for EAEN, $y = 7887 x + 277$, r = 0.9969.

The repeatability was tested by injecting six times a urine sample spiked with EA (20 µg/ml, amount injected 2 µg). The relative standard deviation (RSD) value on the peak area of EA was 0.9 %.

The limit of detection was found to be 4 ng on column at a signal-to-noise ratio of 3. Figure 8 shows a chromatogram of blank urine and urine spiked with EA and its metabolites.

Recovery, Linearity and Limit of Detection for Plasma Samples

Rabbit plasma was utilized to investigate the recovery, linearity and sensitivity of the method. The developed method was found to be applicable on human plasma.

Peak area EA

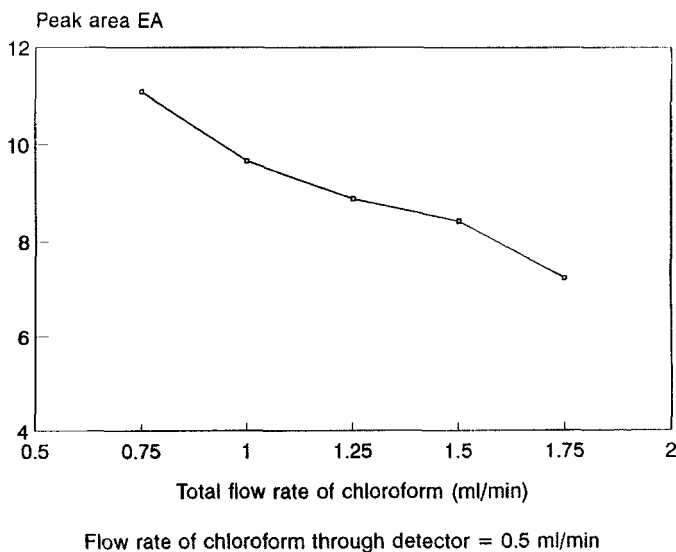Flow rate of chloroform through detector = 0.5 ml/min

FIGURE 7 : Influence of total flow of extraction solvent on peak area.

Other macrolides such as troleandomycin, midecamycin and josamycin were chromatographed in order to find a suitable internal standard. Troleandomycin and midecamycin could not be used because they were co-eluted with some metabolite of EA. Josamycin was chosen because it was eluted in the area between dMeEAEN and EAEN.

Plasma samples, spiked to a final concentration of 10 µg/ml with EA, dMeEA or AEA were taken through the sample preparation procedure described under experimental. The absolute recovery of EA and its metabolites was better than 90 % and for josamycin (IS) it was about 84 %.

The calibration curves for EA and some of its metabolites in plasma were linear over the range of concentration studied (0.25 to 2.0 µg/ml). The following data were obtained (number of analysis n = 9). For EA, $y = 3x + 0.12$, $r = 0.9847$; for dMeEA, $y = 2.5x + 0.19$, $r = 0.9872$ and for AEA, $y = 4.7x + 0.17$, $r = 0.9818$, where y = ratio peak area/peak area of the IS, x = amount injected in micrograms and r = correlation coefficient.
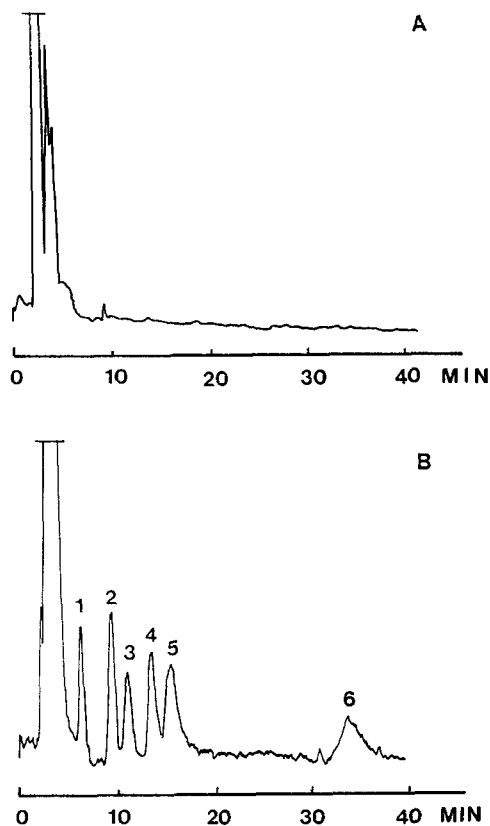
FIGURE 8 : Typical Chromatograms of blank urine (A) and urine spiked with 25 ng each of EA and its metabolites (B). 1. dMeEA, 2. AdMeEA, 3. EA, 4. AEA, 5. dMeEAEN, 6. EAEN.

The limit of detection in plasma was 5 ng on column at a signal-to-noise ratio of 3. Typical chromatograms obtained with blank rabbit plasma and plasma spiked with EA and some of its metabolites are shown in Figure 9.

Application to Real Urine and Plasma Samples

To test the clinical applicability of this method, plasma and urine samples from several healthy male volunteers receiving one oral dose of enrobed microgranules
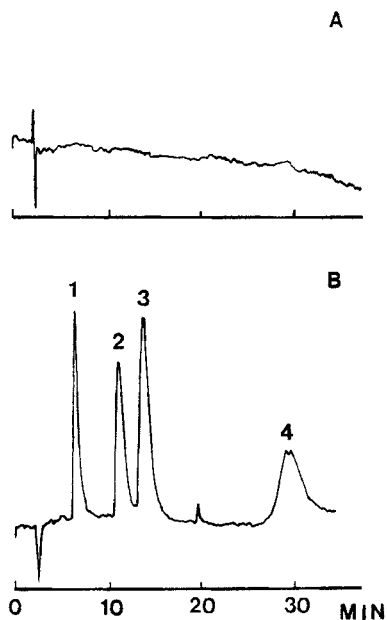
A

B

1
2
3
4

0   10   20   30   M I N

FIGURE 9 : Typical chromatograms obtained with blank rabbit plasma (A) and rabbit plasma spiked with 100 ng each of EA, AEA and dMeEA (B). 1. dMeEA, 2. EA, 3. AEA and 4. IS.

containing 250 mg of erythromycin were analyzed. The chromatograms for urine and plasma samples from one healthy male volunteer taken 3h after administration are shown in Figure 10. In the urine sample, unchanged EA was present in high concentration. The main metabolite present was AEA, small amounts of dMeEA and AdMeEA were also present. EB which is present in commercial erythromycin was also detected. EB, unlike EA, is stable in acid conditions. In the plasma sample shown, the major metabolite dMeEA was also the major component. However, in plasma samples from other volunteers, EA was the major component. This is an indication for unequal metabolism of erythromycin in different organisms. Therefore, it is important to utilize a method which allows the detection of N-demethyl derivatives of erythromycin.
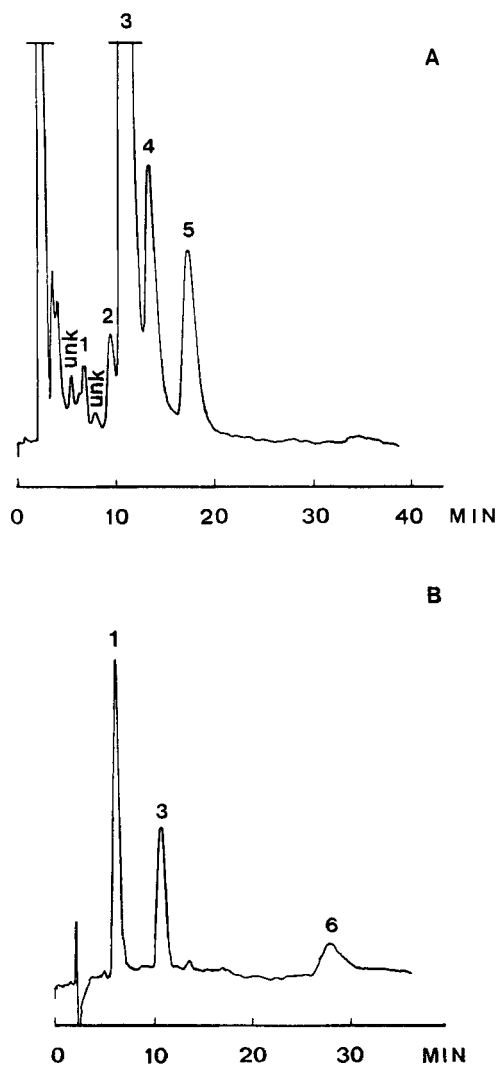
FIGURE 10 : Typical chromatograms of samples taken from a healthy male 3 h after oral administration of 250 mg of erythromycin: urine (A) and plasma (B). 1. dMeEA, 2. AdMeEA, 3. EA, 4. AEA, 5. EB, 6. IS, unk = of unknown identity.

CONCLUSION

The method described here is the only method reported in the literature which enables the separation and quantitation of EA from all of its potential metabolites present in biological samples. This is a suitable method for pharmacokinetic studies of erythromycin.

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