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Journal of Chromatography B, 673 (1995) 81–89

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Determination of erythromycin in gastric juice and blood plasma by liquid chromatography and electrochemical detection

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First received 7 March 1995; revised manuscript received 23 May 1995; accepted 8 June 1995

Abstract

A liquid chromatographic method for determination of the antibiotic erythromycin in biological samples is described. Erythromycin and the internal standard, oleandomycin, were extracted from alkalinized samples with a mixture of 1-hexane and 2-butanol. After evaporation and reconstitution of the sample, separation was performed on a base-deactivated octadecylsilica column. The effects of pH in the mobile phase and of column temperature on the chromatographic performance were studied. Multiple and irregularly shaped peaks were obtained for some chromatographic systems, but by choosing appropriate conditions erythromycin could be eluted as a single symmetric peak. The absolute recovery was above 90% for erythromycin from blood plasma and above 85% from gastric juice. The limits of quantitation were 20 n*M* and 100 n*M*, respectively. Comparison of analytical results for a series of authentic samples with a microbiological assay showed excellent agreement.

1. Introduction

Erythromycin is a macrolide antibiotic used in human and veterinary therapy for treatment of bacterial infections. Several liquid chromatographic (LC) methods for determination of erythromycin in blood plasma or serum have been described in the literature [1–9], but the chromatographic performance has been rather poor for most separation systems. For sample preparation extraction into diethyl ether or *tert.*-butylmethyl ether has generally been employed, with the exception of one assay using solid-phase extraction [3]. A recovery better than 90% was obtained by Khan et al. [9], otherwise recoveries ranging from 55% to 85% are reported [2–8]. In most cases electrochemical detection has been utilized [1–8], as UV detection is not sensitive enough for pharmacokinetic studies and fluorescence detection requires post-column derivatization [9].

The presented LC method for quantitation of erythromycin in blood plasma and gastric juice showed improved recoveries, when using a mixture of hexane and 2-butanol for extraction. Column efficiency was optimized concerning both pH in the mobile phase and column temperature. For a series of plasma and gastric juice samples the method was validated against a microbiological assay. The results corresponded very well indicating that erythromycin does not produce active metabolites, which often hampers

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SSDI 0378-4347(95)00251-0

the selectivity of bioassays and their use in pharmacokinetic studies.

2. Experimental

2.1. Chemicals and reagents

Erythromycin, as base (purity 95%), was supplied as analytical reference substance by Astra Arcus (Södertälje, Sweden) and oleandomycin (internal standard), as phosphate salt (purity 95%), was obtained from Sigma (St. Louis, MO, USA). Acetonitrile and hexane were of HPLC grade from Rathburn (Walkerburn, UK) and 2-butanol was of Fluka quality (Buchs, Switzerland). The buffer substances were of analytical grade (Merck, Darmstadt, Germany). Water was from an ELGA purification system (ELGA, High Wycombe, Bucks, UK).

2.2. Chromatographic system

The LC system consisted of an LKB Model 2248 pump (Bromma, Sweden), a Perkin-Elmer ISS-200 autosampler, refrigerated at 8°C (Überlingen, Germany) and an ESA Coulochem Model 5100A electrochemical detector (Bedford, MA, USA). The detector was equipped with an ESA Model 5020 guard cell (+1.0 V), placed in line before the injector, in order to electrolyze components in the mobile phase, which could contribute to the background current. The ESA Model 5011 dual electrode analytical cell was operated in the oxidative mode with the screen electrode (detector 1) set at +0.65 V and the sample electrode (detector 2) at +0.85 V. ESA carbon filters were installed before the guard and analytical cell to protect these. Data were monitored and processed by a Multichrom Chromatographic Data System (VG Data Systems, Altrincham, UK).

The analytical column was a base-deactivated octadecylsilica column, Hypersil C_{18} BDS (100 × 4.6 mm I.D., 3 μm) from Shandon (Astmoor, UK) and a guard column, Brownlee CN (15 × 3.2 mm I.D., 7 μm), from Brownlee (CA, USA) was used. The mobile phase (pH 8.0) contained sodium dihydrogen phosphate (2.1 m*M*)–disodiumhydrogen phosphate (27.1 m*M*)–acetonitrile (30:30:40, v/v/v). Prior to use the mobile phase was filtered through a Millipore filter (HA, 0.22 μm) (Milford, MA, USA) and ultrasonicated for degassing. The flow-rate over the analytical column was 1.2 ml/min and the temperature was maintained at 65°C.

2.3. Sample preparation procedure

The pH in gastric juice samples was measured at the event of sampling and when it was below 7, the samples were neutralized in order to avoid decomposition [10]. However, in most cases the pH was found to be about 8, as erythromycin itself enhanced the pH in the stomach.

The frozen blood plasma or gastric juice samples were thawed at room temperature, mixed and centrifuged for 5 min at 1200 *g*. Aliquots of 500 μl of the samples were pipetted into 10-ml centrifuge tubes, mixed with 500 μl phosphate buffer (pH 11; ionic strength $I = 1.0$), and 50 μl of the internal standard solution of oleandomycin in acetonitrile (100 μmol/l). The samples were extracted with 5.0 ml of hexane containing 2-butanol (80:20, v/v) by shaking for 15 min. After centrifugation for 5 min at 1200 *g* the lower aqueous phase was frozen in a dry-ice ethanol bath, and the organic phase was transferred to a 10-ml conical centrifuge tube. The organic phase was evaporated under nitrogen until dry. The extracts were redissolved in aliquots of 300 μl of the mobile phase by vortex-mixing 3 times for 60 s each time and then transferred to glass vials that were placed in the autosampler. Aliquots of 40 μl of the samples were injected onto the LC column. Reference samples were prepared by mixing 50 μl of the standard solution of erythromycin in acetonitrile (100 μmol/l) with 500 μl drug-free blood plasma or gastric juice and were then worked up according to the described procedure.

3. Results and discussion

3.1. Chromatographic conditions

Column efficiency and retention times of oleandomycin and erythromycin were influenced by the choice of separation conditions such as pH in the mobile phase and column temperature. Two kinds of buffer solutions were tested, citrate buffer pH 5 and 6 and phosphate buffer pH 6, 7 and 8, with regard to their effects on retention, separation and peak shape. At pH 5 and 6 the column temperature had a large influence on the peak shape of erythromycin. Multiple peak formation of erythromycin, when using a phosphate buffer at pH 6 in the mobile phase, was investigated as a function of column temperature. Fig. 1 shows chromatograms at column temperatures between 15°C and 75°C. At 15°C the compound eluted as a double peak, but at 65°C and 75°C erythromycin showed a single symmetric peak. A temperature of 65°C was chosen for the assay. In order to demonstrate that both peaks at low temperature derived from erythromycin, eluate fractions from the analytical column were collected at 10°C and reinjected. The fractions represented (1) the front of the small peak before the main peak, i.e. the retention time 4 min 50 s to 5 min 10 s, (2) the main peak, i.e. 6 min to 7 min, and (3) the last part of the main peak, i.e. 6 min 30 s to 7 min. As can be seen in Fig. 2 for all three fractions, two peaks appeared, the ratio between them being the same, which indicates that both peaks were derived from erythromycin. A small peak after the main peak was found to be an impurity. Reinjected fractions of the eluate from that peak showed a single peak. Oleandomycin did not show any peak splitting, but gave symmetric peaks also at low temperature.

Multiple peak formation and changed peak profiles dependent upon eluent composition and column temperature have earlier been described, but mostly for peptide-related molecules [11–13]. This phenomenon is explained by slow kinetics of isomerization and is likely to occur when free rotation around a bond in the molecule is hindered.

The retention times for oleandomycin and erythromycin increased 2.5 and 4 times, respectively, when changing pH in the mobile phase from 6 to 8. A pH of 8.0 was chosen as peak shape and column efficiency, expressed as plate number (N), improved with increasing pH. In-

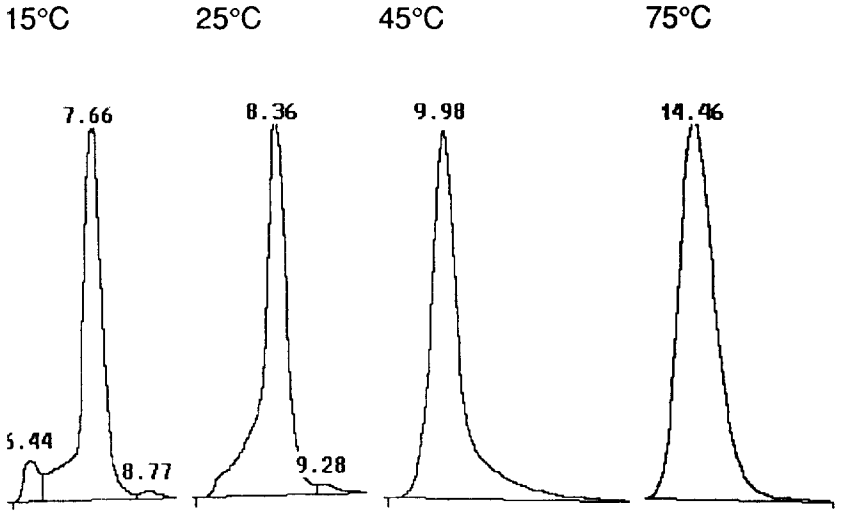

Fig. 1. Chromatograms of erythromycin, using a pH of 6 in the mobile phase, at the column temperatures 15, 25, 45 and 75°C. The retention times of the peaks are indicated.

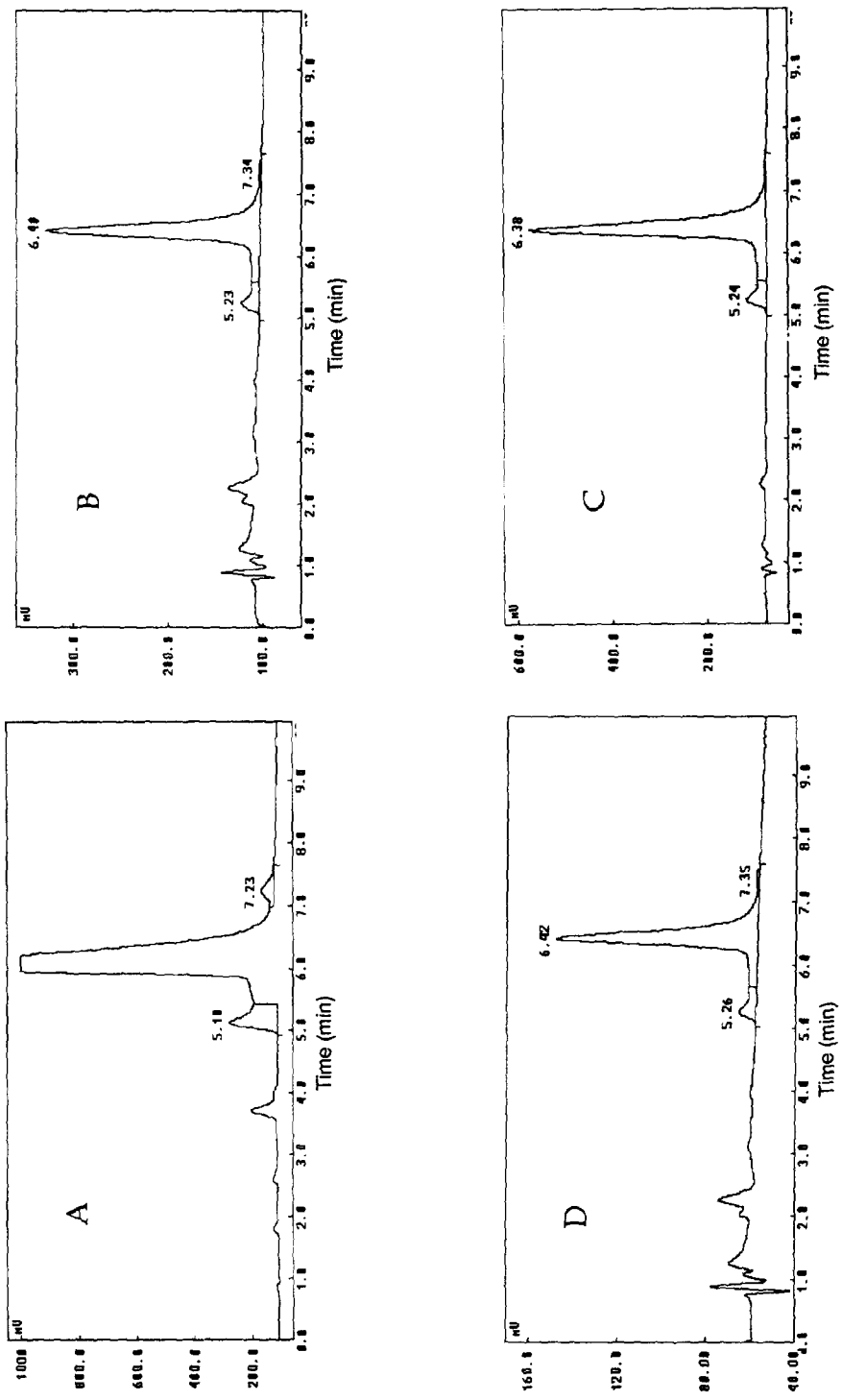

Fig. 2. Chromatograms of erythromycin, using a pH of 6 in the mobile phase, at the column temperature 10°C. (A) Standard solution, (B) reinjection of fraction 4 min 50 s–5 min 10 s, (C) reinjection of fraction 6 min–7 min, and (D) reinjection of fraction 6 min 30 s–7 min.

creasing the column temperature from 25°C to 75°C resulted in a doubling of the retention times for both erythromycin and oleandomycin and a five-fold increase in column efficiency for erythromycin. The base-deactivated column, Hypersil C_{18}, was stable for one to two months in continuous use.

3.2. Quantitation and accuracy

The ratios of the peak height of the analyte to that of the internal standard, in reference samples of plasma or gastric juice spiked with erythromycin, were measured and used for calculation of unknown samples.

Absolute recovery of erythromycin was determined by comparing extracted plasma or gastric juice samples containing known amounts of erythromycin with direct injections of reference solutions containing equal concentrations of the substance. The absolute recovery was above 90% for erythromycin from blood plasma and above 85% from gastric juice.

Within-day precision was assessed using spiked samples at different concentration levels. The coefficients of variation ($n = 8$) were 1.2%, 2.4%, 3.9% and 7.6% for gastric juice samples containing 54.5 μM, 12.4 μM, 0.65 μM and 0.11 μM, respectively and 2.8%, 3.7%, 2.3% ($n = 7$) and 13% ($n = 5$) for plasma samples containing 11.2 μM, 5.59 μM, 0.280 μM and 0.020 μM, respectively. Between-day precision was studied using quality control samples in blood plasma containing 1.40 μM of erythromycin. Analyses, independently performed 17 times within a 3-months period, gave a coefficient of variation of 2.0%.

Calibration curves with 8 different concentrations of erythromycin in plasma and gastric juice, obtained by plotting the peak-height ratio of erythromycin to that of the internal standard, were linear over the concentration ranges studied. For plasma the range was 22–27000 nM and for gastric juice 100–60000 nM ($r^2 = 0.999$ and 0.998, respectively). The limit of quantitation was 20 nM in plasma and 100 nM in gastric juice, when using 0.5 ml of sample.

3.3. Stability

The stability on storage was studied with spiked plasma samples (1400 nM). Erythromycin was stable for at least 4 months at −20°C and at room temperature for at least 6 days. Processed samples were stable in the refrigerated autosampler for more than 24 h and at −70°C for at least 13 days.

3.4. Detection

The response of the electrochemical cell could occasionally change slowly during a series of analyses, so it was important to have an internal standard of similar properties as the analyte. If the response of the electrochemical cell decreased significantly, the cell had to be cleaned. This was done by flushing with 6 M nitric acid and 1 M sodium hydroxide followed by rinsing with deionized water.

3.5. Ruggedness

Factors, that could influence the extraction recovery and the ruggedness of the method, were investigated by experimental design [14]. A statistical full factorial design was set up and the four factors studied were (1) plasma volume, (2) extraction time, (3) volume of organic extraction phase and (4) buffer volume influencing pH at time of extraction. Each factor was studied at two levels: (1) 200 and 600 μl, (2) 10 and 20 min, (3) 4.5 and 5.5 ml and (4) 450 and 550 μl, respectively. The model comprised 16 (2^4) experiments and additional four centerpoints, i.e a plasma volume of 400 μl, an extraction time of 15 min, an extraction volume of 5 ml and a buffer volume of 500 μl. Statistical analysis of variance (ANOVA) was performed to evaluate any effect of the experimental variables. No statistically significant effect on the recovery of erythromycin was found and the coefficient of variation was only 4.7% for all the 20 experiments. The corresponding value for the ratio of erythromycin to the internal standard was 3.9%, when varying any of the four factors, and the

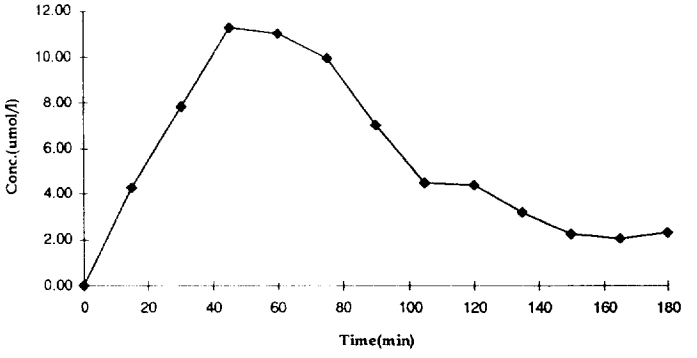

Fig. 3. Median gastric juice concentration after i.v. administration of 1 g of erythromycin ($n = 9$).

method was proved rugged within the intervals studied.

3.6. Application

After intravenous administration of 1 g erythromycin, concentrations in gastric juice were obtained, showing that systemically available erythromycin penetrates the gastric mucosa. Plots giving median concentrations of erythromycin versus time in gastric juice and blood plasma samples are shown in Figs. 3 and 4, respectively. Samples contaminated with bile were excluded from the study, as they gave much enhanced levels, indicating secretion of erythromycin via bile. Chromatograms of a human gastric juice sample containing 13.2 μM of erythromycin and a human plasma sample containing 24.2 μM of erythromycin are shown in Figs. 5A and 6A, respectively. Chromatograms of drug-free human gastric juice and blood plasma samples are shown in Figs. 5B and 6B, respectively.

3.7. Comparison with bioassay

The method was compared with a bioassay, a microbiological agar diffusion assay, using *Micrococcus luteus* (ATCC 9341) as indicator organism [15]. Results from the analyses of 23 authentic plasma samples, ranging from 3.51 μM to 24.2 μM of erythromycin and 135 gastric juice samples, ranging from 0.37 μM to 27.1 μM, were compared. The estimated ratio of the methods

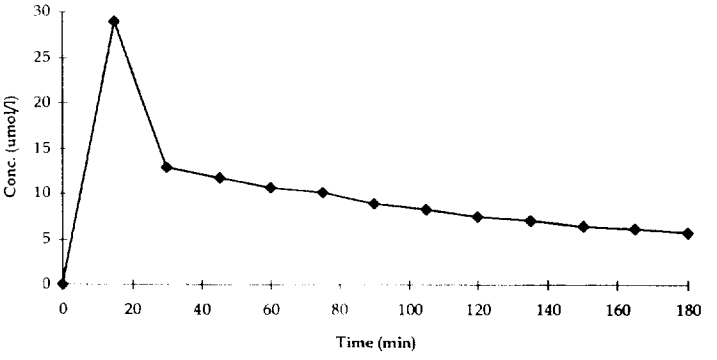

Fig. 4. Median blood plasma concentration after i.v. administration of 1 g of erythromycin ($n = 2$).

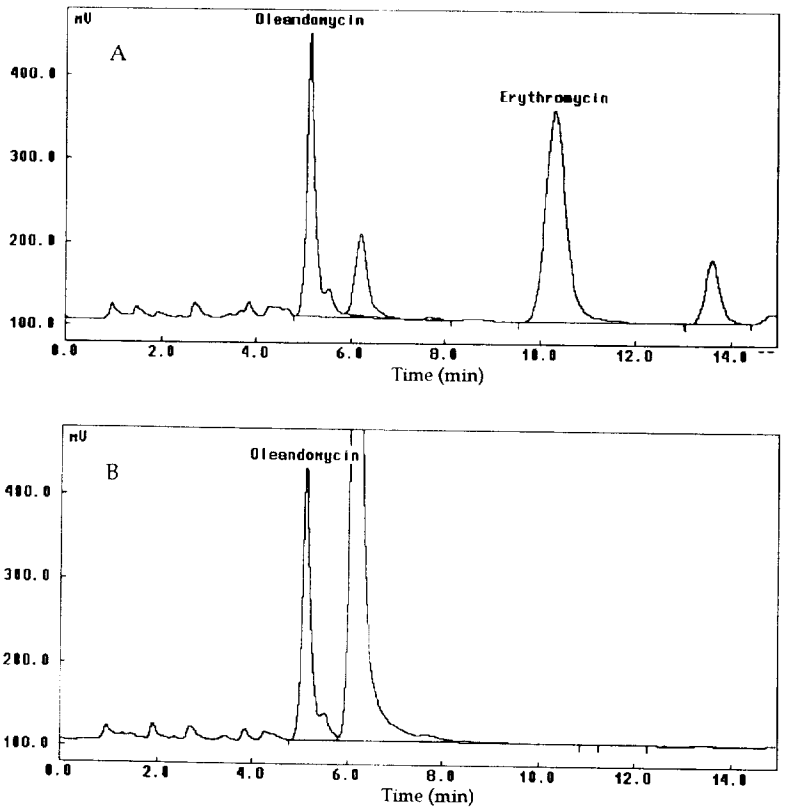

Fig. 5. Chromatograms of (A) a human gastric juice sample containing 13.2 μM of erythromycin and 13.1 μM of oleandomycin and (B) a pre-dose gastric juice sample containing 13.1 μM of oleandomycin. Separation column: Hypersil C_{18} BDS (100x4.6 mm I.D., 3 μm). Mobile phase: 40% acetonitrile in phosphate buffer, pH 8.0. Column temperature: 65°C.

and the 95% confidence interval (C.I.), calculated using t distribution, were 98.1 (C.I. 0.951, 1.012) for the plasma samples and 1.053 (C.I. 1.019, 1.089) for the gastric juice samples. The assumption was made that the logarithmic transformation of the values follows a bivariate normal distribution. Plots over the results from the LC method versus the ones from the bioassay are shown in Fig. 7.

4. Conclusions

The described method, for determination of the macrolide antibiotic erythromycin in blood plasma and gastric juice, yielded improved recovery and good precision. Results obtained with the proposed method showed excellent correlation with results obtained with a microbiological assay employing *Micrococcus luteus*. The presence of erythromycin in gastric juice after i.v. administration indicates that erythromycin penetrates through the gastric mucosa.

Acknowledgements

We thank Mr. Lennart Magni, Astra Arcus AB, for the microbiological assays and Dr. Bengt-Arne Persson for helpful reading of the manuscript.

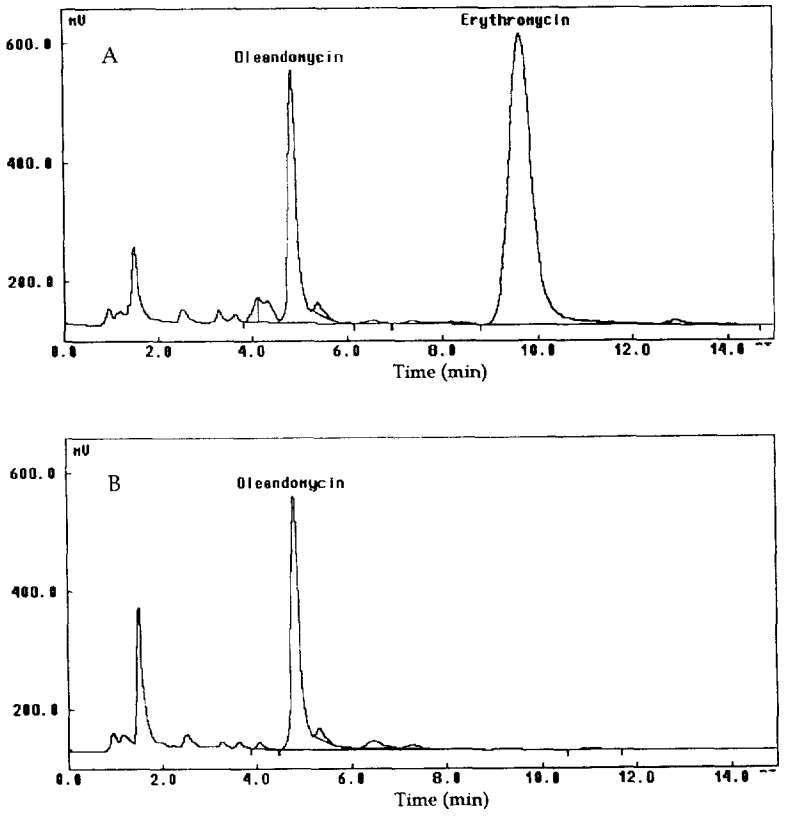

Fig. 6. Chromatograms of (A) a human plasma sample containing 24.2 μM of erythromycin and 13.3 μM of oleandomycin, and (B) a pre-dose plasma sample containing 13.3 μM of oleandomycin. Separation column: Hypersil C_{18} BDS (100x4.6 mm I.D., 3 μm). Mobile phase: 40% acetonitrile in phosphate buffer, pH 8.0. Column temperature: 65°C.

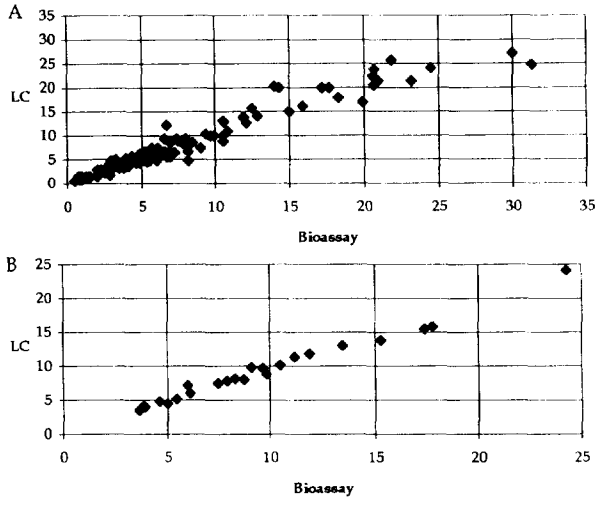

Fig. 7. Plots of the results, in μM, from the LC method versus the ones from the bioassay: (A) in gastric juice, and (B) in blood plasma samples.

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