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FLUORIMETRIC DETERMINATION OF ERYTHROMYCIN AND ERYTHROMYCIN ETHYLSUCCINATE IN SERUM BY A HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC POST-COLUMN, ON-STREAM DERIVATIZATION AND EXTRACTION METHOD

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SUMMARY

The method described is capable of detecting less than 0.01 μg/ml of erythromycin and/or erythromycin ethylsuccinate in serum. Recoveries of erythromycin and erythromycin ethylsuccinate, when added to the level of 0.6 μg/ml, were 102 and 97% with relative standard deviations of 6.0 and 4.5%, respectively.

Erythromycin ethylsuccinate in serum at 37° was shown to degrade rapidly to erythromycin in a first-order rate. When stored at —20°, however, only 10% of the erythromycin ethylsuccinate in serum was hydrolyzed even after storage for 36 days.

The method was used to analyze erythromycin and erythromycin ethylsuccinate in sera from ten subjects administered with an oral dose of erythromycin ethylsuccinate. In addition to a small amount of anhydroerythromycin ethylsuccinate and 8,9-anhydro-6,9-hemiketal erythromycin ethylsuccinate, at least two other metabolites were detected in sera and one was tentatively identified as erythralosamine.

INTRODUCTION

Erythromycin ethylsuccinate (EES) is a pro-drug esterified at the 2′-position of the desosamine moiety of erythromycin for pediatric use. Esters of erythromycin are involved in considerable controversy concerning their blood levels which are apparently higher than that of erythromycin[1–9]. These claims are based on microbiological assay results, but this method lacks both the specificity and accuracy needed for the determination of esters of erythromycin in the presence of erythromycin itself. The esters of erythromycin must be hydrolyzed to erythromycin in order to produce antimicrobial activity[10] and uncertainty exists over the degree of hydrolysis that occurs during the microbiological analysis[6]. Thus, lack of a suitable method for obtaining definitive quantitative data on the intact pro-drug and its hydrolyzed product, erythromycin, has prevented any logical evaluation of the question of efficacy.

To our knowledge, published work dealing with the *in vivo* quantification of erythromycin and its pro-drug is extremely limited. After an oral dose of erythromycin propionate, Stephens *et al.*[11] reported 20–30% of erythromycin compared with 65–80% of erythromycin propionate in blood. However, the paper chromatographic–

bioautographic procedure used by Stephens *et al.*[11] hydrolyzed approximately 10% of erythromycin propionate during the assay process. In addition, hydrolysis of the ester may occur during the processing of the blood to obtain serum samples. Hence, the precision and accuracy of the data reported may be questionable. Tserng and Wagner[12] developed a sensitive fluorimetric method for the determination of erythromycin and erythromycin propionate in whole blood and plasma[12]. However, their method for the separation of erythromycin and erythromycin propionate relied solely on the solubility characteristics of the drugs and thus metabolites and degradation compounds might not have been separated and could have interfered with the results. The formation of anhydroerythromycin ethylsuccinate and 8,9-anhydro-6,9-hemiketal erythromycin ethylsuccinate from erythromycin ethylsuccinate in simulated gastric fluid has been reported[13].

Gas–liquid chromatographic (GLC) and high-performance liquid chromatographic (HPLC) methods for separation and quantification of erythromycin, its esters, and degradation compounds have been reported[13–15]. However, these methods lack sensitivity sufficient for the detection of serum levels of the antibiotic. This paper describes the development of a sensitive HPLC method for the quantification of serum levels of erythromycin and erythromycin ethylsuccinate.

EXPERIMENTAL

Instrumentation

Several HPLC components were combined to form the analytical system and a flow diagram of the system is shown in Fig. 1.

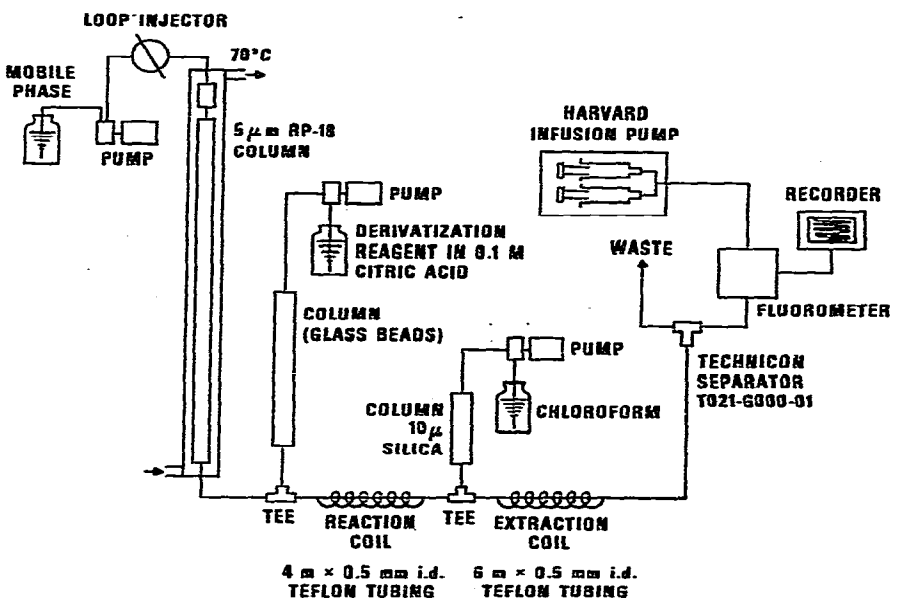

Fig. 1. Schematic diagram of HPLC fluorimetric system for the determination of erythromycin and erythromycin ethylsuccinate.

A mobile phase was pumped at a flow-rate of 0.9 ml/min (1250 p.s.i.) through a 300-μl fixed-loop Rheodyne Model 7120 injector (Rheodyne, Berkeley, Calif., U.S.A.) into a water-jacketed RP-18 column using an LDC Model 19-60066-002 high-pressure mini-pump (Laboratory Data Control, Riviera Beach, Fla., U.S.A.). A 5-μm particle size reversed-phase column of LiChrosorb RP-18 (250 × 4.6 mm I.D.) (Brownlee Labs., Santa Clara, Calif., U.S.A.) and a 50 × 2.1 mm I.D. stainless-steel pre-column packed with μBondapak C_{18} (Waters Assoc., Milford, Mass., U.S.A.) were maintained at 70 ± 0.2° by connecting the water-jacket to a Lauda K-2/R controlled-temperature circulating water-bath (Brinkmann, Lauda, G.F.R.). Both the pre-column and the analytical column were placed inside the water-jacket and were mounted vertically with the inlet side up to prevent possible channeling due to the dissolution of silica gel under the chromatographic conditions used. The pre-column may need replacement after 1–2 weeks of continuous use owing to sudden pressure build-up; however, the analytical column has proved to be stable over several months of use.

A derivatization reagent was pumped at a flow-rate of 0.9 ml/min through a 300 × 3.9 mm I.D. stainless-steel column packed with 180–210-mesh glass beads (Cat. No. 05483, Applied Science Labs., State College, Pa., U.S.A.) using an LDC high-pressure mini-pump. The glass beads-packed column was used to give a head pressure of about 250 p.s.i. for a pulseless flow of the reagent. The reagent was mixed with the column effluent through a 1/16-in. Swagelok Union Tee (Part No. SS-100-3). A 4 m × 0.5 mm I.D. PTFE tube (Part No. 24005, Durrum, Palo Alto, Calif., U.S.A.) at room temperature served as a reaction coil.

Chloroform (distilled in glass; Burdick & Jackson, Muskegon, Mich., U.S.A.) was pumped through a 50 × 2.1 mm I.D. stainless-steel column packed with μPorasil (Waters Assoc.) at a flow-rate of 1.3 ml/min using an LDC low-pressure mini-pump. Chloroform is mixed through a 1/16-in. Swagelok Union Tee to extract the reacted erythromycins. A 6 m × 0.15 mm I.D. PTFE tube (Durrum) served as the extraction coil.

The chloroform layer was separated from the aqueous layer with a PTFE-lined inserted, hydrophobically treated separator (Cat. No. T 021-G000-01, Technicon, Tarrytown, N.Y., U.S.A.) and was withdrawn through the Varian Autograph Fluorichrom detector (Varian, Palo Alto, Calif., U.S.A.) at a flow-rate of 0.76 ml/min by a Harvard Infusion/Withdrawal pump (Harvard Apparatus, Millis, Mass., U.S.A.) using 2–50-ml disposable syringes. CS7-54 and CS7-60 excitation filters and CS3-72 and CS4-76 emission filters were used for the maximal excitation at 360 nm and for the optimal emission above 440 nm. The attenuation setting used was 50× and a Hewlett-Packard Model 7123A strip-chart recorder was used. A Chromatopac EIA data processor (Shimadzu Seisakusho, Kyoto, Japan) was used to quantitate the data. Use of the Harvard Infusion/Withdrawal pump is essential for a pulseless flow of chloroform in order to obtain a stable baseline; an extremely noisy baseline was obtained when a Technicon proportioning pump was used. Use of a Technicon separator (T021-G000-01), with its low dead volume and efficient separation of chloroform, is a key to successful operation.

Mobile phase

The chromatographic conditions used were similar to those reported by Tsuji

and Goetz[13]. The mobile phase was acetonitrile–0.2 *M* ammonium acetate–water (65–60:10:25–30), pH 7.0. The acetonitrile used was UV-grade, distilled in glass, obtained from Burdick & Jackson. The 0.2 *M* ammonium acetate solution was prepared by weighing 15.5 g of analytical-reagent grade ammonium acetate into a 1-l measuring cylinder and adding water to volume. The amounts of the solvent in the mobile phase may have to be modified in order to obtain the maximal performance of the column.

The mobile phase was filtered through a Fluoropore filter (FHL PO4700, Millipore, Bedford, Mass., U.S.A.). After the analysis, the column was thoroughly rinsed with methanol and stored in methanol until used again.

Derivatization reagent

The reagent was prepared by weighing 250 mg of disodium 2-(stilbyl-4″)-naphtho-1′,2′:4,5)-1,2,3-triazole 2″-6′-disulfonate (Tinopal GS, Ciba-Geigy, Ardsley, N.Y., U.S.A.) into a 1-l measuring cylinder, dissolving it in 100 ml of 0.1 *M* citric acid (pH about 1.8) and diluting to volume with water. This reagent solution was washed three times with chloroform in a separator funnel to remove chloroform-soluble materials. The washed reagent was then filtered through a 0.45-μm pore size Millipore filter (Cat. No. HAWP 047 SO).

Erythromycin reference standard solution

About 7 mg of U.S.P. Erythromycin Reference Standard were accurately weighed into a 100-ml volumetric flask. Just prior to the analysis, the mobile phase was added to the required volume and sonciated to facilitate dissolution. A 4-ml volume of this solution was pipetted into a 100-ml volumetric flask and the mobile phase was added to volume. The solution was then injected into the column.

Erythromycin ethylsuccinate reference standard solution

About 7 mg of U.S.P. EES Reference Standard powder were accurately weighed into a 100-ml volumetric flask. Just prior to the analysis, the mobile phase was added to the required volume and sonciated to facilitate dissolution. A 4-ml of this EES solution was pipetted into a 100-ml volumetric flask and the mobile phase was added to volume. The solution was then injected into the column.

Dosing of erythromycin ethylsuccinate

Subjects fasted overnight, 5 ml of EES liquid (200 mg per 5 ml) were dosed orally and approximately 10 ml of blood were withdrawn 0.25, 0.5, 1.0, 1.5, 3.0, 4.5 and 6.0 h after the dose. A 0-h sample was collected just prior to administration of the drug.

The blood samples collected were allowed to stand for approximately 10 min in order to coagulate and were then centrifuged for 5 min at 1700 *g* to obtain serum samples. The serum samples were placed into screw-capped vials in two equal amounts and were immediately frozen at −20°. The frozen samples were packed under dry-ice and transported immediately to the assay laboratories. Upon receipt of the samples, they were immediately placed in a liquid-nitrogen atmosphere until analyzed.

Extraction of erythromycin and erythromycin ethylsuccinate from serum

Just prior to the assay, the frozen serum samples were thawed quickly by

placing the vials in a warm water-bath and shaking them vigorously. Approximately 1–3-ml amounts of serum samples were pipetted into a 15-ml ground-glass stoppered conical centrifuge tube, 5 ml of diethyl ether were added and the tube was stoppered and shaken vigorously for 5 min on an Eberbach reciplicating shaker to extract the erythromycin ethylsuccinate. The mixture was then centrifuged for 1 min at 1500 *g* and the ether layer was transferred into a 15-ml conical centrifuge tube using a disposable Pasteur capillary pipette. The tube containing diethyl ether was immediately placed under a stream of dry nitrogen.

The pH of the serum in the centrifuge tubes was adjusted to 10 by the addition of saturated sodium carbonate solution (approximately 3 drops) and 5 ml of diethyl ether were then added to extract erythromycin and the erythromycin ethylsuccinate remaining in the serum. The mixture was shaken vigorously for 5 min on an Eberbach shaker and centrifuged for 1 min at 1500 *g*. The ether layer was then pooled into the centrifuge tube and again placed under a stream of dry nitrogen.

To the centrifuge tube containing the serum, an additional 5 ml of diethyl ether were added and the tube was shaken vigorously for 5 min. The tube was then centrifuged and the ether layer was pooled and dried. The procedure was repeated.

Upon drying the diethyl ether in the centrifuge tube, a small amount of diethyl ether was added to rinse the inner wall of the tube to concentrate the residue at the end of the conical centrifuge tube. The tube was placed under a stream of dry nitrogen to dry the contents completely.

HPLC analysis

Just prior to the analysis, 500 μl of the mobile phase were added to the centrifuge tube using an Eppendorf pipette. The tube was sonicated and vortexed three times to facilitate rapid dissolution of the sample. The tube was capped and centrifuged for 5 min at 2000 *g*. A glass syringe was used to obtain approximately 400 μl of clear aqueous sample and the sample was injected into the HPLC column using a 300-μl loop injector.

Calculation

The following equations were used to calculate the amounts of erythromycin ethylsuccinate, [EES], and erythromycin, [E], in serum:

$$[\text{EES}]\ (\mu\text{g/ml}) = \frac{A}{A_t} \cdot \frac{W_{At}}{V} \cdot F_1 F_2 \tag{1}$$

$$[\text{E}]\quad (\mu\text{g/ml}) = \frac{E}{E_t} \cdot \frac{W_{Et}}{V} \cdot F_1 F_3 \tag{2}$$

The total microbiological equivalence was calculated by assuming 100% conversion of EES into E, using the equation

$$\text{Total microbiological equivalence } (\mu\text{g/ml}) = [\text{E}] + [\text{EES}]F_4 \tag{3}$$

where

A = peak area of EES in a sample,
A_t = peak area of EES in the U.S.P. EES Reference Standard;

E = peak area of E in a sample;
E_t = peak area of E in the U.S.P. E Reference Standard;
V = volume of serum sample pipetted (ml);
W_{At} = weight of the U.S.P. EES Reference Standard (mg);
W_{Et} = weight of the U.S.P. Reference Standard (mg);
F_1 = dilution factor;
F_2 = purity of the U.S.P. EES Reference Standard (0.91);
F_3 = purity of the U.S.P. E Reference Standard (0.99);
F_4 = ratio of molecular weights (E/EES) (734/872 = 0.852).

The amounts of the metabolites that eluted slightly after the erythromycin peak were calculated by assuming their specific fluorescence to be identical with that of erythromycin.

RESULTS AND DISCUSSION

The fluorimetric detection of erythromycin and its esters with naphthotriazole disulfonate used in this HPLC post-column derivatization technique is a modified form of that of Tserng and Wagner[12]. Naphthotriazole disulfonate forms an ion pair with a molecule protonated at low pH. Erythromycin and its esters contain one secondary amine in the desosamine moiety and can be readily protonated. The method has a potentially wide application in the analysis of compounds containing various forms of amine groups.

Both naphthotriazole disulfonate and its ion-paired compounds have identical maximal excitation (360 nm) and emission (>440 nm) characteristics; therefore, they must be separated if quantification is required. The separation of the ion-paired compounds from the reagent was accomplished by an on-stream extraction with chloroform, leaving an excess of the reagent in the aqueous layer.

The fluorimetric method used can be made more sensitive, *e.g.*, by use of a higher detector sensitivity, a smaller amount of chloroform for extraction or a larger loop size for sample injection. However, the present detection sensitivity of less than 0.01 μg/ml of erythromycin and/or erythromycin ethylsuccinate in serum, which is equivalent to, if not better than, that of the microbiological assay method[16], has been judged sufficient for monitoring the serum levels of E and EES.

The use of two long PTFE tube, two connecting T-pieces and a separator for continuous fluorimetric analysis resulted in an approximately 40% decrease in the number of theoretical plates. In spite of this decrease, the efficiency obtained by 1270 theoretical plates for a 25-cm column was sufficient for the separation and quantification of erythromycin, erythromycin ethylsuccinate and their metabolites and degradation compounds in human sera.

Standard graphs

The standard curves for the fluorimetric HPLC determination of erythromycin and erythromycin ethylsuccinate are shown in Fig. 2. The standard curve of erythromycin ethylsuccinate has a greater slope than that of erythromycin; although the slopes for the two compounds at concentrations below 0.5 μg/ml are virtually identical. This observation agrees with the results of Tserng and Wagner[12], who found

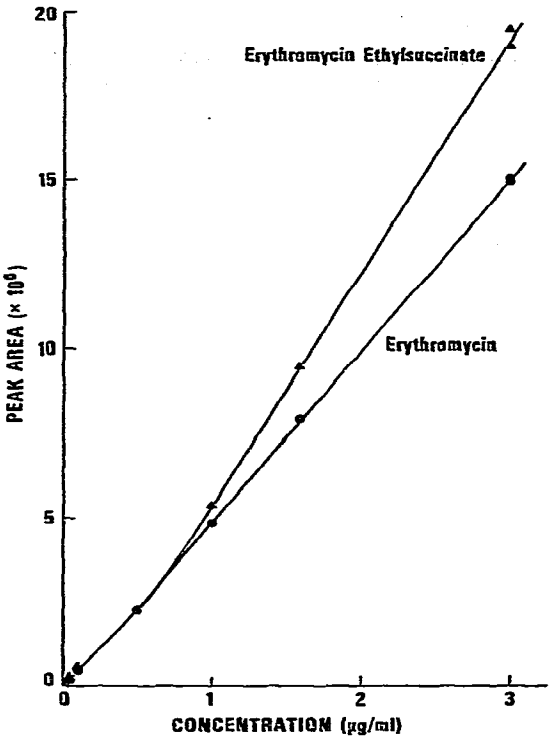

Fig. 2. Standards curves for the HPLC fluorimetric determination of erythromycin and erythromycin ethylsuccinate.

that the propionyl ester of erythromycin has a greater specific fluorescence than its free base.

The concentration of naphthotriazole disulfonate used (0.25 mg/ml) is much lower than that used by Tserng and Wagner[12]. This low reagent concentration minimized the blank reading and stabilized the baseline noise, but it was enough to give a linear response beyond 60 μg/ml of erythromycin and/or erythromycin ethylsuccinate.

Precision and recovery of erythromycin and erythromycin ethylsuccinate from serum

Erythromycin or erythromycin ethylsuccinate was added to human serum at a concentration of approximately 0.6 μg/ml. As the ion-pairing derivatization method is selective, a simple extraction of serum with diethyl ether was sufficient to eliminate interference from the human serum from fasting subjects. In order to minimize hydrolysis and to extract erythromycin ethylsuccinate, the pH should remain unadjusted in the initial diethyl ether extraction. Then the pH of the serum was adjusted to 10 to extract erythromycin and the remaining erythromycin ethylsuccinate.

As can be seen in Tables I and II, the recoveries of erythromycin and erythromycin ethylsuccinate from serum, when added at a concentration of 0.6 μg/ml, were 102 and 96%, respectively. The relative standard deviations of the assay method were 6.0 and 4.5% for erythromycin and erythromycin ethylsuccinate, respectively.

TABLE I

PRECISION AND RECOVERY OF ERYTHROMYCIN ADDED TO HUMAN SERUM

Serum sample	*Erythromycin (μg/ml)*	
	Added	*Recovered*
1	0.6046	0.6008
2	0.6046	0.5952
3	0.6046	0.6260
4	0.6046	0.6683
5	0.6046	0.6643
6	0.6046	0.5702
7	0.6046	0.5987
		Average: 0.6176
		Relative standard deviation: 5.99%
		Recovery: 102.2%

TABLE II

PRECISION AND RECOVERY OF ERYTHROMYCIN ETHYLSUCCINATE ADDED TO HUMAN SERUM

Serum sample	*Erythromycin ethylsuccinate (μg/ml)*	
	Added	*Recovered*
1	0.5813	0.5503
2	0.5813	0.5582
3	0.5813	0.5922
4	0.5813	0.5318
5	0.5813	0.5702
6	0.5813	0.5257
7	0.5813	0.5845
		Average: 0.5590
		Relative standard deviation: 4.50%
		Recovery: 96.2%

The hydrolysis of erythromycin ethylsuccinate, which was a problem encountered by Stephens *et al.*[11] in the analysis of erythromycin propionate, was not a problem in this HPLC analysis (Table III). The erythromycin ethylsuccinate used in this study contained approximately 2.6% of free erythromycin base.

TABLE III

STABILITY OF ERYTHROMYCIN ETHYLSUCCINATE IN HUMAN SERUM STORED AT −20°

Time (days)	*Relative concentration (%)*	
	Erythromycin	*Erythromycin ethylsuccinate*
0	2.2	97.8
0.17	4.5	95.5
1	4.0	96.0
13	11.3	88.7
22	10.1	89.9
36	9.4	90.6

Stability of erythromycin ethylsuccinate

Erythromycin ethylsuccinate has been shown[13] to be unstable and rapidly hydrolyzed to erythromycin in phosphate buffer of pH 7.0 at 37°. The hydrolysis is a first-order reaction and can be expressed as the linear regression equation of ln $y = -1.87x + 4.57$ with a correlation coefficient of 0.998. The rate of hydrolysis of erythromycin ethylsuccinate in human serum at 37° is approximately three times slower and follows the linear regression ln $y = -0.87x + 4.81$ with a correlation coefficient of 0.9999 (Fig. 3).

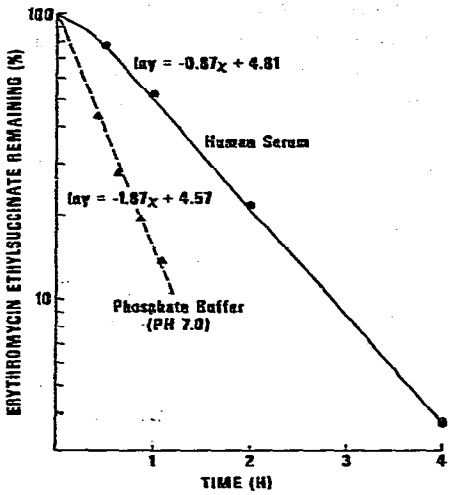

Fig. 3. Stability of erythromycin ethylsuccinate in pH 7.0 phosphate buffer and in human serum at 37°.

The instability of erythromycin ethylsuccinate in serum at 37° indicated above may be a problem in bioavailability studies owing to the possible hydrolysis that might occur during the operations for obtaining serum from a blood sample. Approximately 10% hydrolysis of erythromycin ethylsuccinate could take place during the 10–15 min required for the coagulation and centrifugation operations to obtain the serum. Therefore, in order to completely eliminate the possibility of hydrolysis, whole blood must be analyzed immediately upon withdrawal.

When the serum was stored frozen at −20°, however, erythromycin ethylsuccinate was stable (Table III). Approximately 2% hydrolysis of erythromycin ethylsuccinate was shown to take place during the 4 h of the freezing process, but no further hydrolysis was apparent after storage for 24 h. The erythromycin ethylsuccinate used in this study contained approximately 2.6% of free erythromycin base. The hydrolysis proceeded very slowly to approximately 10% after storage for 13 days at −20°. However, no further hydrolysis was detected in serum, even after storage for 36 days. Therefore, the storage and transport of the serum sample when frozen would pose a limited problem with respect to the stability of erythromycin ethylsuccinate. The serum samples analyzed in this study were frozen at −20° and transported in dry-ice. Upon the receipt of the sample, they were immediately placed in a

liquid nitrogen atmosphere and stored until taken for analysis. All of the samples were analyzed within 1 month after the dose.

Total microbiological equivalence

The HPLC method is capable of separating and quantifying erythromycin ethylsuccinate and free erythromycin base. In addition, their metabolites and degradation compounds can also be detected. In order to correlate the HPLC data with those of the microbiological cylinder cup–agar diffusion assay method[16], the amount of erythromycin ethylsuccinate determined by the HPLC method must be expressed in terms of the total microbiological equivalence. Therefore, the following assumption was made for the calculation: 100% conversion of erythromycin ethylsuccinate to erythromycin takes place during the microbiological analysis. As some doubts have been expressed[1,6] about this assumption, the following experiment was performed in order to verify the assumption.

A serum spiked with erythromycin ethylsuccinate at 0.6 μg/ml and a serum sample taken 30 min after an oral dose of EES liquid were incubated overnight at 37° and assayed by the HPLC method. The chromatograms of the samples after overnight incubation showed only the erythromycin peak and no trace of erythromycin ethylsuccinate. The conversion efficiency was calculated by dividing the total microbiological equivalence in the pre-incubated serum sample by the amount of erythromycin found in the incubated serum sample.

Conversion efficiencies of 92.0 and 97.3% were obtained from the spiked serum and from the serum of an orally dosed subject, respectively. These values are very close to the theoretical conversion of 100%, and therefore the total microbiological equivalence was calculated from the HPLC data using a conversion factor of 100%.

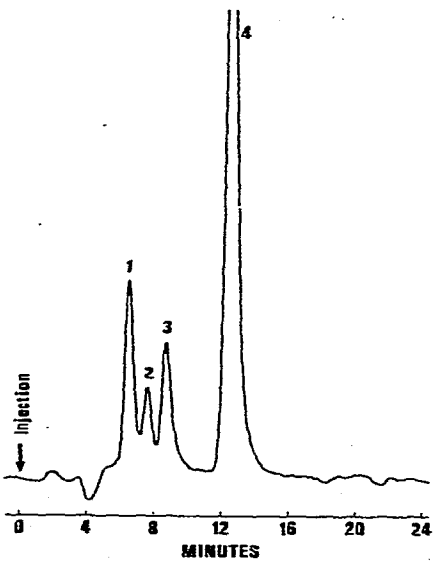

Fig. 4. HPLC results for the detection of erythromycin and erythromycin ethylsuccinate in serum from the subject 9 taken 0.25 h after an oral dose of EES liquid (200 mg per 5 ml). Peaks: 1 = erythromycin; 2 = erythralosamine; 3 = 4″-acetylerythromycin or anhydroerythromycin; 4 = erythromycin ethylsuccinate.

Excellent correlation between the HPLC and the microbiological assay methods for the quantification of erythromycin has previously been reported[15].

Serum of subjects dosed with erythromycin ethylsuccinate

The method was used to examine sera from ten fasting subjects administered with an oral dose of erythromycin ethylsuccinate. A typical chromatogram of a serum sample taken at 0.25 h after an oral dose is shown in Fig. 4. Examination of the chromatogram indicates that, in addition to a small amount of anhydroerythromycin ethylsuccinate and 8,9-anhydro-6,9-hemiketal erythromycin ethylsuccinate, at least two other metabolite peaks with slightly slower retention times than the erythromycin peak were present in the serum. The 0-h serum of the same subject contained no detectable peaks (Fig. 5).

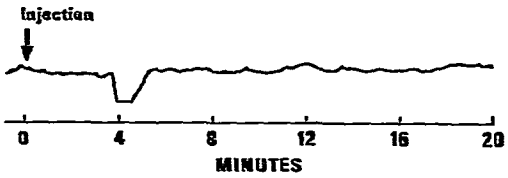

Fig. 5. HPLC results on 0-h serum from subject 9.

From the relative retention data, the above metabolite has been tentatively identified as erythralosamine. The other may be 4″-acetylerythromycin or anhydroerythromycin. Erythralosamine has been identified as one of the degradation compounds formed during the inactivation of erythromycin A by a pseudomonas[17]. Desosamine, another microbial degradation compound[17], elutes much earlier than the erythromycin A peak, and des-N-methylerythromycin, formed by enzymes in the tissue of a rabbit[18], elutes slightly earlier than the erythromycin A peak.

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