Serum Protein Binding of Erythromycin and Erythromycin 2′-Propionate Ester

R. G. WIEGAND and A. H. C. CHUN▲

Abstract □ Human serum and tissue concentrations are reported after oral administration of erythromycin stearate and propionyl erythromycin lauryl sulfate (erythromycin estolate). About 10% of the erythromycin (I) in serum is unbound to serum proteins, whereas about 1.5% of the activity after propionyl erythromycin (erythromycin 2′-propionate ester) (II) is unbound. The half-lives in serum are 1.2 and 1.6 hr., respectively. The area under the total serum concentration curve of II is about fourfold greater than with I, partly because of the longer half-life of the propionyl ester. The threefold difference remaining after adjustment of the areas for the half-lives is the factor by which total II serum levels are higher due to distribution or absorption. Since the percent of free drug in serum is 7 times greater with erythromycin, the higher total levels with the propionyl ester are more than accounted for by distribution alone. No conclusions about the absolute fraction of the dose that is absorbed can be drawn for either drug. Concentrations of both drugs in tissues are more consistent with the free serum levels than with the total concentrations. Since the free levels of antibiotic are a better index of relative effectiveness, earlier reports indicating that higher total serum concentrations of II are due to more complete absorption and better therapeutic efficacy are questioned.

Keyphrases □ Erythromycin—absorption, half-life, tissue levels, serum protein binding, compared to propionyl ester □ Erythromycin 2′-propionate ester—absorption, half-life, tissue levels, protein serum binding, compared to erythromycin □ Serum protein binding—erythromycin and erythromycin 2′-propionate ester

Several forms of erythromycin are given orally. These are erythromycin carbonate, erythromycin ethylsuccinate, propionyl erythromycin lauryl sulfate (erythromycin estolate), enteric-coated erythromycin base, and erythromycin stearate. Of these forms, propionyl erythromycin lauryl sulfate has been shown to produce total serum concentrations that are severalfold higher than those resulting from an equal dose of erythromycin (1, 2). These higher levels have been attributed, perhaps falsely, to better absorption of the propionyl ester (3), and it has been implied that this ester is the preferred drug on the basis of these higher levels (4).

Because erythromycin and propionyl erythromycin are chemically distinct organic compounds, it is not surprising that one gives higher serum levels than the other. Such higher levels in serum are not in themselves sufficient to conclude that one drug is better absorbed, although this is the consensus of the published reports. That such higher levels would be likely to make one drug more clinically effective than the other is also not to be concluded simply from the total serum levels.

Two factors which could account for an incorrect conclusion on efficacy from higher total serum levels would be: (*a*) that the analog giving the higher blood levels is circulating largely in an inactive form, and (*b*) that the analog giving the higher levels is bound to serum proteins to such an extent that the unbound concentration available to exert its action is the same as or lower than the unbound concentration present with the other drug. The first of these possibilities has already been reported. The 2′-esters of erythromycin have been found to be inactive until hydrolyzed (5), and these esters circulate in the blood mainly as esters (6). The second possibility is the topic of this report.

The free or unbound fractions of the total antibiotics in serum are a better measure of the probable therapeutic benefit (7, 8). It is, therefore, useful to reassess the meaning of the serum levels after administration of erythromycin stearate film-coated tablets[1] and propionyl erythromycin lauryl sulfate or erythromycin estolate[2] in terms of what can be deduced about the relative absorption of both drugs and their serum protein binding, as well as tissue levels, serum half-lives, and other pertinent parameters. These data rather seriously alter the previously published conclusions and suggest that other forms of erythromycin, while producing lower total serum concentrations, may be the preferred drugs.

EXPERIMENTAL

Microbiological Assays—The tube dilution procedure was similar to that of Rammelkamp (9), using *Streptococcus pyogenes* (ATCC 8668). Serum samples were assayed the same day they were obtained. Zone diameter assays were done by a slight modification of the cylinder-plate technique of Grove and Randall (10), employing *Sarcina lutea* (ATCC 9341). Prior to the zone diameter assay, serum and ultrafiltrate aliquots were extracted in glass-stoppered tubes into 5 ml. of reagent grade anhydrous ethyl ether. A volume of 1.0 *M* K_2HPO_4 buffer (pH 9.2) equal to the sample (usually 2.0 ml.) was added before extraction, and the tubes were shaken 10 min. at 200 c.p.m. and centrifuged. Three milliliters of the ether from serum samples or 4 ml. from ultrafiltrates were taken to dryness and stored at −15°. The samples were reconstituted with the same buffer as the standards when assayed. The ether extraction was at least 96% complete, and no correction for incomplete extraction was made. Tissues were homogenized in four volumes of the assay buffer, and the supernatant solutions were assayed *versus* a rabbit muscle standard curve.

Determination of Serum Binding—*In vitro* studies were done with stored human serum. Erythromycin or propionyl erythromycin, as base or soluble salts, were added to serum as one part standard solution in isotonic pH 7.4 buffer (potassium phosphate and sodium chloride) to 14 parts serum. Ultrafiltrates were obtained at 25° by centrifugation from tubing bags[3] resting in fritted-glass funnels at 2900 r.p.m. for 1.5 hr. in a refrigerated centrifuge[4]. An aliquot of the original serum sample was assayed for total drug, and the fraction unbound was calculated as the ratio of the ultrafiltrate to the serum concentrations.

Serum protein binding by equilibrium dialysis was done using 5 ml. of serum in a tubing bag agitated by a wrist-action shaker in 200 ml. of pH 7.4 isotonic buffer at 25°. Serum was diluted with standard drug solutions as described previously, and the drug was also added to the buffer. Preliminary studies were done to determine that the same equilibrium was approached using an excess

[1] Erythrocin Stearate Filmtab, Abbott Laboratories, North Chicago, Ill.
[2] Ilosone Pulvules, Eli Lilly & Co., Indianapolis, Ind.
[3] Visking.
[4] International PR-2.

Table I—*In Vitro* Determination of Human Serum Protein Binding of Erythromycins

Erythromycin			Propionyl Erythromycin		
Serum, mcg./ml.	Ultrafiltrate or Buffer, mcg./ml.	Percent Unbound	Serum, mcg./ml.	Ultrafiltrate or Buffer, mcg./ml.	Percent Unbound
Ultrafiltration					
0.59	0.086	14.7	0.94	<0.106	<11.2
0.74	0.089	12.0	1.90	<0.077	4.1
0.77	0.169	22.0	2.48	0.119	4.8
0.77	0.162	21.1	2.52	0.104	4.2
0.84	0.158	18.8	2.60	<0.123	<4.7
1.22	0.227	18.6	2.75	<0.118	<4.3
1.62	0.394	24.2	2.98	0.105	3.5
2.87	0.490	17.1	4.05	0.175	4.3
		18.6			<4.3[a,b]
Equilibrium Dialysis					
0.60	0.122	20.3	2.07	0.108	5.2
0.62	0.115	18.4	2.15	0.111	5.2
0.63	0.118	18.6	2.27	0.099	4.4
0.66	0.110	16.2	>3.3	0.087	<2.6
0.68	0.107	15.7	>3.3	0.113	<3.4
0.70	0.130	18.6	>3.3	0.093	<2.8
0.96	0.121	12.6	>3.3	0.113	<3.4
0.99	0.122	12.4			<3.9[b]
1.07	0.108	10.1			
		15.9			

[a] Calculated omitting the value of 11.2. [b] "Less than" values were used at highest value in calculating the mean.

or insufficient drug in the serum. All samples were equilibrated at least 5 hr.

Studies of serum protein binding in fresh serum from subjects at 2 and 4 hr. after administration of 500-mg. oral doses of commercial erythromycin stearate tablets or propionyl erythromycin lauryl sulfate capsules were done by collecting ultrafiltrate from 8-mm. tubing[3] under 15 lb. air pressure (11).

Calculations—Half-lives were calculated from the average serum concentrations, assuming apparent first-order disappearance of drug. Where serum levels at four or more times were used, a least-squares line was calculated and the disappearance rate (k_a, hr.$^{-1}$) was calculated from its slope. For serum levels at two or three times, the first and last values were used, since this is the same as the least-squares slope.

The serum levels obtained by the tube dilution assay were not directly averaged because they are distributed logarithmically. Instead, each concentration was converted to a tube number (0.01 mcg./ml. = 1, 0.02 mcg./ml. = 2, 0.04 mcg./ml. = 4, *etc.*), and these numbers, which are normally distributed, were averaged. The mean tube number was then converted back to a concentration by the formula, concentration = $0.01 \times 2^{\text{average tube number}-1}$. Concentrations less than the sensitivity were assigned a zero tube number. Areas under the serum level curves were calculated by connecting the average serum levels by straight lines and extrapolating to zero concentration 2 hr. after the last value. Areas are thus expressed as micrograms × hour per milliliter.

Table II—Human Serum Protein Binding by Ultrafiltration in Subjects Given 500-mg. Oral Doses of Erythromycin Stearate or Propionyl Erythromycin Lauryl Sulfate

Hours after Dose	Erythromycin			Propionyl Erythromycin		
	Serum, mcg./ml.	Ultrafiltrate, mcg./ml.	Percent Unbound	Serum mcg./ml.	Ultrafiltrate, mcg./ml.	Percent Unbound
2	1.81	0.311	17.2	2.79	0.047	1.7
	0.79	0.033	10.6	1.87	0.026	1.4
	2.26	0.148	6.6	1.38	0.025	1.8
	1.43	0.163	11.4	1.00	0.007	0.7
	1.67	0.136	8.2	2.10	0.019	0.9
	1.66	0.146	8.8	2.35	0.030	1.3
			10.4			1.3
4	0.71	0.134	18.8	2.18	0.041	1.9
	0.31	0.065	8.1	1.14	0.017	1.5
	1.32	0.092	7.0	1.02	0.022	2.1
	0.83	0.093	11.2	1.02	0.008	0.8
	1.33	0.096	7.2	3.71	0.062	1.7
	0.70	0.043	6.1	1.92	0.035	1.8
			9.7			1.6

Human Serum and Tissue Level Studies—Healthy adult volunteers participated in the serum level studies. The subjects were fasted from midnight until the 1-hr. sample, at which time a standard breakfast was served. Tissue samples and simultaneous blood samples were obtained at surgery 2.5–6.0 hr. after 250-mg. oral doses of erythromycin stearate or propionyl erythromycin lauryl sulfate.

RESULTS

Serum Protein Binding of Erythromycin Stearate and Propionyl Erythromycin Lauryl Sulfate—The *in vitro* determinations of protein binding of erythromycin were done using levels in the range of reported concentrations found in therapeutic use. Data obtained by ultrafiltration and equilibrium dialysis techniques are given in Table I. By both procedures the percent unbound is at least fourfold greater with erythromycin than with propionyl erythromycin. The results from serum samples from subjects given 500-mg. oral doses of the erythromycin stearate and propionyl erythromycin lauryl sulfate are summarized in Table II. Although the average total serum concentrations were greater after propionyl erythromycin, which is consistent with published reports, the ultrafiltrate levels are three to sixfold higher after erythromycin stearate. The differences in unbound concentrations of the two drugs are significant at the $p < 0.005$ level at both 2 and 4 hr. The percent unbound remains essentially constant at 2 and 4 hr. with both drugs, and the threefold variation in percent unbound which occurs with both drugs is primarily a variation between subjects and is not related to the total serum level. The unbound fraction with both drugs is less than what was found *in vitro*.

Serum and Tissue Levels in Man Given Erythromycin Stearate and Propionyl Erythromycin Lauryl Sulfate—Serum levels were studied following 125-, 250-, and 500-mg. single oral doses of erythromycin stearate. Serum samples were taken every hour for 6 hr. from groups of four or five subjects on each dose. One, two, or four 125-mg. tablets from the same lot were used. The mean serum concentrations by zone diameter assay and the calculated areas and half-lives are given in Table III. The areas approximately doubled for each doubling of the dose in this dosage range. The half-lives calculated from the values between 2 and 6 hr. are similar for each group, indicating that there is no change in the kinetics of erythromycin elimination over this dose range under these conditions.

Erythromycin stearate, 250-mg. tablets of Lot 750-1329, was given during several studies to a total of 126 subjects, and serum levels were determined by the tube dilution procedure. The mean serum concentrations of erythromycin at 1, 2, 4, and 6 hr. calculated by the tube number method were 0.12, 0.98, 0.28, and 0.09 mcg./ml., respectively. The area calculated from the mean values is 2.33 mcg. × hr./ml., and the half-life is 1.16 hr. This area and half-life are very close to those in Table II calculated from zone diameter data.

Similar calculation from tube dilution serum levels in 98 subjects given 250 mg. of erythromycin activity as propionyl erythromycin lauryl sulfate capsules yields average concentrations at 1, 2, 4, and 6 hr. of 0.65, 3.22, 1.69, and 0.75 mcg./ml., respectively. The calculated area is 10.4 mcg. × hr./ml., and the half-life is 1.72 hr. The half-life using the 4- and 6-hr. data is appreciably longer, 2.15 hr., and is probably due to continued absorption of the relatively insoluble ester salt.

The results of the human tissue and serum level studies are summarized in Table IV. Sufficient samples of muscle were obtained with both drugs to show that neither drug concentrates in muscle relative to serum but that muscle levels are higher with erythromycin stearate than with propionyl erythromycin lauryl sulfate. There are insufficient data to allow firm conclusions for other tissues, although it appears that the tissue–serum ratio varies from tissue to tissue. These data suggest that the propionyl ester of erythromycin does not give high tissue levels in parallel with the high total serum concentrations it produces.

The half-life of erythromycin in man was also determined from the erythromycin stearate data of Griffith and Black (4), using the

Table III—Mean Serum Erythromycin Concentrations after 125-, 250-, and 500-mg. Oral Doses of Erythromycin Stearate in Man

Number of Subjects	Dose, mg.	Serum Concentration, mcg./ml. 1 hr.	2 hr.	3 hr.	4 hr.	5 hr.	6 hr.	Area, mcg. × hr./ml.	$t_{1/2}$, hr.
4	125	0.094	0.593	0.389	0.225	0.076	0.058	1.44	1.10
5	250	0.037	1.108	0.555	0.324	0.173	0.098	2.30	1.15
5	500	0.001	2.057	1.382	0.830	0.417	0.253	5.54	1.29
								Mean	1.18

average serum levels at 4 and 8 hr. calculated by the tube number procedure. The half-life is 1.19 hr., which is in good agreement with the above data. Similar calculation of the half-life of propionyl erythromycin from the same publication gives a value of 1.57 hr. The calculated areas under the serum curves are 1.08 and 3.52 mcg. × hr./ml., respectively, after oral doses of 250 mg. of erythromycin activity of both drugs.

Partition Coefficients -Apparent partition coefficients (organic concentration/aqueous concentration) between carbon tetrachloride and pH 7.4 buffer were 8.8 for erythromycin and 56.4 for propionyl erythromycin. Using cyclohexane, the values were 0.06 and 2.93 for erythromycin and its propionyl ester, respectively.

DISCUSSION

It is not possible to conclude that one drug is better absorbed than another drug by comparing the magnitude of the serum levels obtained, because the two drugs may distribute differently in the body. The difference in serum levels produced by equal doses of erythromycin given as the stearate salt or the propionyl ester lauryl sulfate (Table II) is adequately explained by the greater serum protein binding of the propionyl ester. The unbound concentrations of propionyl erythromycin are actually significantly lower than the unbound erythromycin levels, while the total levels are higher.

The greater serum protein binding of the propionyl ester of erythromycin is not unexpected. Douglas *et al.* (12) showed that in a homologous series of compounds, the more lipid-soluble drugs are bound to a greater extent. The apparent partition coefficients between pH 7.4 buffer and carbon tetrachloride or cyclohexane are considerably greater with the propionyl ester, supporting its greater binding. The bound drug is not inactive, however. It acts as a reservoir, replacing free drug lost by metabolism, distribution to tissue, or excretion. But the magnitude of this reservoir effect is small since at the levels in Table II and assuming 3 l. of serum, drug bound to serum proteins accounts for only about 1% of the dose. By far, the largest fraction of drug in the body is in tissues. Since the free levels of drug in tissue water are probably in equilibrium with the unbound level in serum (13), this drug in tissue is mostly bound and must constitute a much larger reservoir than the drug bound to serum proteins.

Table IV—Human Serum and Tissue Concentrations (Total Levels) after Administration of Erythromycin Stearate and Propionyl Erythromycin Lauryl Sulfate

Tissue	Erythromycin Concentration— Serum, mcg./ml.	Tissue, mcg./g.	Propionyl Erythromycin Concentration— Serum, mcg./ml.	Tissue, mcg./g.
Muscle	1.67	0.49	1.18	<0.17
	1.32	0.55	0.44	<0.17
	0.98	0.52	0.40	<0.17
	0.92	0.58	0.32	<0.17
	0.54	0.22	0.24	<0.17
	0.45	0.32	0.15	<0.17
	0.08	<0.17		
Colon	0.36	1.23	2.47	0.68
	0.50	0.23	0.63	0.21
Prostate	<0.04	>5.0	0.15	<0.17
Salivary	0.62	2.35	0.16	1.65
Gallbladder	1.65	>5.0	1.29	2.63
	0.73	0.45	1.14	0.85
Urinary	—	—	1.85	0.64
Omentum	—	—	1.12	0.18
Kidney	—	—	1.12	1.25
Testicle	0.57	>5.0	—	—
	0.50	0.63	—	—

The tissue levels in humans given in Table IV suggest that there are large differences between the levels of drug in various tissues. For instance, salivary tissue gives considerably higher levels than serum, whereas muscle does not. The tissue levels are high enough, however, to account for the majority of drug in the body.

The free levels in tissue water are in equilibrium with free levels in serum, and the concentrations of unbound drug are six to eightfold higher with erythromycin as compared to the propionyl erythromycin ester (Table II). Therefore, it is not surprising that the total tissue levels in Table IV are greater after erythromycin stearate was ingested than after the ingestion of propionyl erythromycin lauryl sulfate.

The determination of total (bound and unbound) drug in serum as a measure of antibacterial activity is considered misleading by several authors (7, 8, 12). That the unbound drug is a better measure of activity is logical because the protein bound drug is not diffusable and is not in solution in the same sense as free drug. It cannot, therefore, contribute to the concentration gradient that causes the antibiotic to penetrate into the tissues or the bacterial cell.

The half-life of erythromycin serum levels in man averages 1.18 hr. from the data reported here. After intravenous administration, Spitzy and Hitzenberger (14) reported a value of 1.02 hr. Propionyl erythromycin has a slightly longer half-life of 1.6-1.7 hr. The area under a serum curve is independent of the rate or the kinetics of absorption. It is a function only of the dose, the apparent relative volume of distribution, and the disappearance rate, according to the equation: area = dose/$V_d' - k_d$ (15, 16). With the areas and half-lives reported here at equal dose, the volumes of distribution differ by a factor of 3.0. From the Griffith and Black (4) data the ratio of volumes of distribution is 2.5. This ratio is the factor by which propionyl erythromycin serum levels are expected to be higher than with erythromycin, after adjustment for the difference in half-lives. Because erythromycin gives six to eightfold higher unbound serum levels, the difference in apparent volumes of distribution is more than accounted for. Erythromycin, then, is either better absorbed or more favorably distributed. The available data do not allow a distinction between these two possibilities. Based on these considerations, it is logical that the propionyl ester has not been demonstrated to be therapeutically superior, although the higher total serum levels have been suggested to cause this effect.

Propionyl erythromycin is known to circulate in the body primarily as the intact ester (6). The relatively small fraction that circulates as the free base probably accounts for most of the unbound drug. The propionyl erythromycin itself is probably bound to serum proteins to a greater degree than these data suggest. Even in the *in vitro* experiments where presumably only the ester was present, hydrolysis of the ester to the base would tend to increase artificially the amount of unbound activity.

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▲ To whom inquiries should be directed.

DRUG STANDARDS

NMR Stability Assay for Amyl Nitrite Ampuls

R. E. SCHIRMER▲, R. E. ZEMER, and G. G. COOKE

Abstract □ The percentage of amyl nitrite in the contents of an amyl nitrite ampul is determined from the ratio of the area under the $-CH_2-ONO$ line to the area under the $-CH_3$ lines in the NMR spectrum of the sample. The relative standard deviation of the method is 1.4%, and the accuracy is better than 2%.

Keyphrases □ Amyl nitrite ampuls—NMR stability assay □ NMR spectroscopy—stability assay, amyl nitrite ampuls

Amyl nitrite is a highly volatile vasodilator which is used as an inhalant. Many analytical procedures for amyl nitrite have been reported in the literature, including potentiometric determination (1), GC determination (2), colorimetric determinations based on diazotization and coupling of sulfanilic acid with *N*-(1-naphthyl)ethylenediamine (3, 4) and on reaction with ferrous sulfate in sulfuric acid (5), and others (6, 7). NF XIII specified a nitrometric procedure in the past (8) but recently described a GC method (9). No direct spectroscopic method of analysis has been reported for this compound, although its IR (10), UV (11, 12), and 30-MHz. NMR spectra have been studied (13).

The reported methods are not generally well suited for use as stability assays for amyl nitrite ampuls, because most of them are lengthy and special problems are encountered due to the instability and volatility of the compound. In addition, many of the reported methods do not distinguish amyl nitrite from one or more of its many degradation products. The decomposition of amyl nitrite in ampuls has been shown to produce N_2, N_2O, NO, CO, CO_2, and at least 12 liquid components including water, amyl alcohol, isovaleric acid, isovaleraldehyde, amyl isovalerate, and amyl nitrate (14–18). In this paper, an NMR stability assay which is very rapid and which avoids most of the difficulties associated with previous methods is reported.

EXPERIMENTAL

A filled ampul was cleaned on the outside, dried, and weighed. The ampul was then placed in a 10-ml. conical flask with about 0.3 ml. of 0.5% tetramethylsilane in deuterochloroform; it was cracked with a glass rod, and the contents of the flask were swirled briefly. The chloroform solution was placed in a precision NMR tube, the tube was capped, and the 60-MHz. NMR was recorded on an NMR spectrometer[1]. The spectrum was integrated six times, and the ratio of the integral of the $-CH_2ONO$ signal (triplet at 4.70 p.p.m.) to the integral of the methyl proton signals (0.90–1.00 p.p.m.) was computed for each repetition. The percent of amyl nitrite in the ampul is given by:

$$\% \text{ amyl nitrite} = 3 \times \text{mean ratio} \times 100 \qquad \text{(Eq. 1)}$$

The glass from the cracked ampul was washed, dried, and weighed to determine the original fill weight of the ampul.

DISCUSSION

The decomposition of amyl nitrite does not alter the number of methyl groups present, but it does reduce the number of $-CH_2-ONO$ groups (14–18). This allows the degradation of the compound

[1] Varian A-60.