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Use of Stable Isotopes in the Study of Pharmacokinetics of Drugs by Mass Fragmentography II: Detailed Examination of Pharmacokinetics of a Single Oral Dose of Phenytoin in Humans

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Abstract □ To study the pharmacokinetics of phenytoin in detail, a mass fragmentographic method was applied for the precise, sensitive, and specific analysis of phenytoin, 5-(4-hydroxyphenyl)-5-phenylhydantoin, and 5-(4-hydroxyphenyl)-5-phenylhydantoin glucuronide in plasma and urine after administration of a single oral dose of phenytoin to two healthy volunteers. Salivary phenytoin concentrations also were measured. Phenytoin and 5-(4-hydroxyphenyl)-5-phenylhydantoin were analyzed after the addition of deuterium-labeled internal standards and conversion to volatile methyl derivatives for mass fragmentographic analysis. The lower limit of detection was ~10 ng/ml. The simultaneous pharmacokinetic analysis of the plasma levels and urinary excretion data of phenytoin and its major metabolite, 5-(4-hydroxyphenyl)-5-phenylhydantoin, yielded detailed information about the pharmacokinetics of phenytoin.

Keyphrases □ Mass fragmentography—application to pharmacokinetic study of single oral phenytoin dose using stable isotope labeling, humans □ Phenytoin—pharmacokinetics, application of mass fragmentography after single oral dose using stable isotope labeling, humans □ Pharmacokinetics—phenytoin, application of mass fragmentography after single oral dose using stable isotope labeling, humans

GLC–mass spectrometry coupled with selected-ion monitoring is used increasingly in drug research because of its high sensitivity and specificity. In this technique, stable isotope-labeled carriers serve as ideal internal standards to correct for losses of the compound under study in the initial isolation procedures. The usefulness of mass fragmentography in conjunction with stable isotope labeling has been of recent interest in the measurement of trace amounts of substances in biological materials (1–3). This method was used in these laboratories for the metabolic study of drugs in animals and humans (4–6). The mass fragmentographic technique also has been applied for the sensitive, specific, and reliable determination of plasma testosterone levels in humans (7).

Pharmacokinetic studies represent one field in which the sensitivity and specificity of the mass fragmentographic technique offer an advantage. Sullivan and coworkers (8, 9) successfully used stable isotope-labeled drugs together with mass fragmentography to study steady-state pharmacokinetics. An investigation of the elimination kinetics of drugs was carried out in these laboratories by measuring the nanogram levels of a drug and its major metabolites in human urine using this technique (10).

The present paper describes the use of deuterium-labeled phenytoin (I) and its major metabolite, 5-(4-hydroxyphenyl)-5-phenylhydantoin (II), along with mass fragmentography to follow saliva, plasma, and urinary concentrations of I and II for a reasonable period after a single oral administration of I to humans. A detailed analysis and interpretation of the pharmacokinetic data of I from two healthy volunteers also are presented.

EXPERIMENTAL

Synthesis of Deuterated Internal Standards—*Pentadeuterophenytoin (I-*d_5*)*—Deuterobenzophenone (benzophenone-d_5) was prepared by refluxing a mixture of 9.50 g of hexadeuterobenzene[1] (99.5 atom % D) and 2.44 g of benzoyl chloride[2] for 5 hr with 2.70 g of anhydrous aluminum chloride. The I-d_5 then was synthesized from the benzophenone-d_5 according to the method described by Baty and Robinson (11).

[1] Merck.
[2] Wako Pure Chemical Industries.

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The overall yield was 5.1% based on benzene-d_6, mp 301°, 99.3 atom % D.

5-(4-Hydroxyphenyl)-5-pentadeuterophenylhydantoin (II-d_5)—Condensation of 3.07 g of anisoyl chloride with 9.50 g of hexadeuterobenzene[1] (99.5 atom % D) by refluxing for 5 hr with stirring in the presence of anhydrous aluminum chloride produced 2.68 g of 4-methoxybenzophenone-d_5, mp 151°. This compound (2.63 g) then was refluxed at 120° in 50 ml of nitrobenzene for 4 hr with 6.00 g of aluminum chloride to convert it to the phenol. The reaction mixture was poured into ice-cold water and extracted with dichloroethane. Filtration of the dichloroethane layer, extraction with 5% NaOH, acidification with 5% HCl, and reextraction into dichloroethane followed by drying over sodium sulfate and evaporation under reduced pressure gave 1.01 g of 4-hydroxybenzophenone-d_5, mp 140°.

Compound II-d_5 was obtained when 1.00 g of 4-hydroxybenzophenone-d_5 was subjected to reaction with 13.00 g of acetamide, 2.50 g of ammonium carbonate, and 0.40 g of potassium cyanide in a 50-ml sealed tube at 100° for 48 hr (12). After cooling, the reaction mixture was transferred into water, and the product was extracted with ether under alkaline conditions. Evaporation of the ether and recrystallization from aqueous ethanol gave 0.60 g of II-d_5, mp 320° dec., 98.7 atom % D. The overall yield of this reaction was 2% based on benzene-d_5.

Mass Fragmentography—Mass fragmentographic measurements were made with a gas chromatograph–mass spectrometer[3] equipped with a multiple-ion detector. The electron energy was set at 20 ev, and the trap current was set at 60 μamp. The multiple-ion detector was focused on the ions of the methyl derivatives at *m/e* values of 266 (I) and 271 (I-d_5) for the determination of I and at 296 (II) and 301 (II-d_5) for the determination of II to measure the peak height ratio.

GLC was performed on a glass column (2 m × 3 mm i.d.) packed with 1.5% SE-30 on Chromosorb W (80–100 mesh). The column temperature was 250° for I and 290° for II. The temperature of the flash heater was 280° for I and 300° for II, and the separator was at 280° for I and at 300° for II. The temperature of the ion source was 310°. The helium carrier gas flow rate was ~20 ml/min.

Sample Preparation for Mass Fragmentography—*Phenytoin (I)*—A 0.5–1.0-ml plasma sample was diluted with three volumes of water in a 30-ml centrifuge tube, and 1.0 ml of the internal standard (1.0 μg of I-d_5) was added. After 1.0 ml of 0.5 *N* HCl was added, the plasma sample was extracted with 2 × 5 ml of chloroform. The chloroform phase was collected and dried over anhydrous sodium sulfate. After evaporation of the solvent, the residue of the chloroform extract was dissolved in 0.1 ml of methanol, and 0.2 ml of diazomethane in ether was added. The sample then was allowed to stand for 15 hr at 4°, and the organic solvents were evaporated.

The residue was dissolved in a small amount (0.1–0.2 ml) of acetone and subjected to TLC on Kieselgel 60 F_{254} plates[4] (5 cm × 20 cm × 0.25 mm thick). The plate was developed with benzene–acetone–acetic acid (50:25:1 v/v/v), and the UV-positive zone corresponding to standard 3-methyl-I (monomethyl-I) with R_f 0.77 was scraped off. The methyl derivatives of I and I-d_5 were eluted with 10 ml of acetone, and the solvent was evaporated. The residue was dissolved in 0.1 ml of methanol, and 1 μl of the sample was subjected to GLC–mass spectrometry. The observed peak height ratio of *m/e* 266 to *m/e* 271 was determined in triplicate. Saliva (0.7–1.0 ml) and urine (1.0–10.0 ml) samples were not diluted with water and were processed as for plasma I samples.

5-(4-Hydroxyphenyl)-5-phenylhydantoin (II)—To a 0.5–1.0-ml plasma sample were added three volumes of water and 1.0 ml of the internal standard (1.0 μg of II-d_5). The subsequent procedures were the same as the isolation procedures described for plasma I levels, except that the UV-positive zone corresponding to standard *O*-methyl-3-methyl-II (dimethyl-II) with R_f 0.72 was scraped off. For the mass fragmentographic analysis, 1 μl of the sample was introduced into the gas chromatograph–mass spectrometer. The observed peak height ratio of *m/e* 296 to *m/e* 301 was determined in triplicate. Urine (1.0–10.0 ml) samples were not diluted with water and were processed as for plasma II samples.

5-(4-Hydroxyphenyl)-5-phenylhydantoin Glucuronide (III)—A 0.5–1.0-ml plasma sample was diluted with three volumes of water, and 1.0 ml of the internal standard (1.0 μg of II-d_5) was added in a 30-ml centrifuge tube. The plasma sample was treated with 1.0 ml of 10 *N* HCl for 1 hr on a boiling water bath (90–95°) and neutralized with 3 ml of sodium hydroxide solution. The subsequent procedures were the same as the isolation procedures described for plasma II samples. Urine (1.0 ml) samples were not diluted with water and were processed as described for plasma III samples.

Preparation of Calibration Curve—To each of six standards containing 0.05, 0.1, 0.5, 1.0, 5.0, and 10.0 μg of I or II in 0.1 ml of methanol was added 10 μg of I-d_5 or II-d_5 in 0.1 ml of methanol. After evaporation of the solvent to dryness, the residue was dissolved in 0.1 ml of methanol. To each sample was added 0.2 ml of freshly prepared diazomethane in ether. After standing for 15 hr at 4°, the excess diazomethane and the organic solvents were evaporated to dryness under reduced pressure, and the residue was dissolved in 0.1 ml of methanol. A 1-μl volume of the methanol solution was analyzed by GLC–mass spectrometry. The mass fragmentographic analysis was performed using the methyl derivatives by measuring the molecular ions at *m/e* 266 for monomethyl-I, *m/e* 271 for monomethyl-I-d_5, *m/e* 296 for dimethyl-II, and *m/e* 301 for dimethyl-II-d_5. The peak height ratios of I to I-d_5 and of II to II-d_5 were measured.

Determination of Accuracy—*Phenytoin (I)*—Compound I in amounts of 0.05, 0.5, 2.5, 5.0, and 10.0 μg in 1.0 ml of methanol was mixed with 10 μg of I-d_5 dissolved in 1.0 ml of methanol, and the solution was concentrated to ~20 μl. To the concentrated solution were added 0.5-ml aliquots of pooled plasma, and the sample was allowed to stand for 30 min at room temperature. The sample then was processed as described for the sample preparation for mass fragmentography.

5-(4-Hydroxyphenyl)-5-phenylhydantoin (II)—Compound II in amounts of 0.05, 0.5, 1.0, and 5.0 μg in 1.0 ml of methanol was mixed with 1.0 μg of II-d_5 dissolved in 1.0 ml of methanol, and the solution was concentrated to ~20 μl. To the concentrated solution were added 1.0-ml aliquots of pooled plasma, and the sample was allowed to stand for 30 min at room temperature. One set of plasma samples was treated with 1.0 ml of 10 *N* HCl for 1 hr on a boiling water bath and neutralized with 3 ml of sodium hydroxide solution. The other set was not treated with hydrochloric acid. The samples then were processed as described for the sample preparation for mass fragmentography.

Urine samples (1.0-ml aliquots of human urine) also were used for the accuracy determination. The procedures were essentially the same as those described for the plasma samples.

Drug Administration—Two healthy adult male volunteers, 25 (KY) and 36 (YK) years old, weighed 60 and 65 kg, respectively. They had not taken any drugs during the preceding two months. A single oral dose of I as its sodium salt, prepared by dissolving 250 mg of I and 37.5 mg of sodium hydroxide in 100 ml of water, was given in the morning after an overnight fast. The drug solution container was rinsed with 100 ml of water, and the rinsings also were swallowed. Each subject received 400 ml of water 1 hr before dosing and 100 ml of water at hourly intervals for 4 hr after dosing. The subjects urinated completely just prior to drug intake. No food was permitted for 4 hr after drug administration.

Sample Collection—Ten milliliters of heparinized blood samples was taken just prior to dosing and at 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 24, 48, 72, 96, and 120 hr after dosing. Plasma was separated and kept in a frozen state until analysis. Saliva samples (5 ml) were collected at the time of blood sampling. The saliva pH was measured, and the samples were kept frozen until analysis. Complete collections of urine were made at 0–2, 2–4, 4–6, 6–8, 8–12, 12–36, 36–60, 60–84, 84–108, and 108–132 hr after dosing. The urine volumes and pH were measured, and the samples were kept frozen until analysis. Compound I in saliva and I–III in plasma and urine samples were analyzed in duplicate by mass fragmentography. The initial extraction and isolation procedures were described under *Sample Preparation for Mass Fragmentography*.

RESULTS

Prerequisites for Mass Fragmentographic Analysis—*Preparation of Methyl Derivatives*—In the mass spectra of I and II, relatively abundant molecular ions (*m/e* 252 for I: Σ% 14.3; *m/e* 268 for II: Σ% 33.9)

Table I—Accuracy of Mass Fragmentographic Analysis of I in Plasma

Amount Added, μg	Amount Measured, μg[a]	Relative Error, %
0.0503	0.0502 ± 0.0002	−0.3
0.5026	0.5178 ± 0.0007	+3.0
2.5125	2.4916 ± 0.0062	−0.8
5.0250	4.8802 ± 0.0135	−2.9
10.0500	10.0584 ± 0.0113	−0.1

[a] Mean ± *SD* of triplicate measurements.

[3] Shimadzu LKB 9000.
[4] Merck, Darmstadt, West Germany.

Table II—Accuracy of Mass Fragmentographic Analysis of II in Plasma and Urine [a]

Amount Added, μg	Blood: Amount Measured, μg [b]	Blood: Relative Error, %	Urine: Amount Measured, μg [b]	Urine: Relative Error, %
0.0506	0.0526 ± 0.0002	+4.0	0.0530 ± 0.0005	+4.7
	(0.0482 ± 0.0003)	(−4.7)	(0.0528 ± 0.0001)	(+4.3)
0.1012	0.1053 ± 0.0039	+4.1	0.1043 ± 0.0034	+3.1
	(0.0994 ± 0.0004)	(−1.8)	(0.0974 ± 0.0043)	(−3.8)
0.5060	0.5187 ± 0.0029	+2.5	0.4911 ± 0.0020	−2.9
	(0.5236 ± 0.0013)	(+3.5)	(0.5103 ± 0.0008)	(+0.8)
1.0120	1.0039 ± 0.0045	−0.8	1.0286 ± 0.0013	+1.6
	(1.0322 ± 0.0128)	(+2.5)	(1.0152 ± 0.0035)	(+0.3)
5.0600	5.0499 ± 0.0216	−0.2	5.0523 ± 0.0096	−0.2
	(5.1858 ± 0.0132)	(+2.5)	(5.0687 ± 0.0042)	(+0.2)

[a] Figures in parentheses indicate accuracy determinations with hydrochloric acid treatment. [b] Mean ± *SD* of triplicate measurements.

Table III—Plasma Concentrations of I–III after Single Oral 250-mg Dose of I

Hours	Plasma Concentration, μg/ml [a]: Subject YK I	Subject YK II	Subject YK III	Subject KY I	Subject KY II	Subject KY III
1	3.9216	0.0318	0.7236	2.1200	0.0484	0.5095
2	4.0674	0.0411	0.9753	4.1870	0.0943	0.9753
3	3.9552	0.0477	1.1718	4.7260	0.0993	1.3961
4	4.3498	0.0545	1.3872	5.0336	0.1173	1.5844
5	4.0390	0.0548	1.4065	4.7968	0.1077	1.5462
6	4.0226	0.0594	1.5175	5.2586	0.1145	1.7553
7	3.7546	0.0577	1.6076	4.8334	0.1121	1.7408
8	3.5358	0.0585	1.6248	4.8854	0.1146	1.8967
10	3.8494	0.0602	1.5952	4.4282	0.1200	2.1725
12	3.3232	0.0454	1.5404	3.7540	0.1075	1.9620
24	2.0792	0.0387	1.1915	2.1618	0.0859	1.6122
48	0.4654	0.0121	0.3598	0.6211	0.0344	0.7483
72	0.0772	0	0.0720	0.1294	0.0145	0.2385
96	0.0178	0	0.0114	0.0232	0	0.0359
120	0	0	0	0	0	0

[a] Mean of triplicate measurements.

were observed. However, without derivative formation, both I and II did not possess good GLC properties. On the other hand, the methyl derivatives were suitable for GLC analysis, and molecular ions of monomethyl-I (*m/e* 266) and dimethyl-II (*m/e* 296) were prominent in the mass spectra. The abundance of the molecular ions was Σ% 19.7 for monomethyl-I and Σ% 26.5 for dimethyl-II, providing good potential for use in mass fragmentography.

Selective-ion recordings of I and II showed that no contaminating by-products with retention times close to those of the methyl derivatives of I and II were seen in the mass fragmentograms. After the initial extraction and purification procedures, there was no interference for both I and II in the molecular ion peaks from other materials in the saliva, plasma, or urine extract at these masses.

Sensitivity—The sensitivity of the procedure described here was judged based on the signal to noise ratio. The lower limit of detection of the mass spectrometer was 100 pg for I and II.

Calibration Curve—Compounds I-d_5 and II-d_5 were used as internal standards. Known mixtures of I and I-d_5 and of II and II-d_5 were prepared so that the sample size (1 μl) injected into the gas chromatograph–mass spectrometer covered the I or II range of 0.5–100 ng with a fixed amount (100 ng) of I-d_5 or II-d_5. Each mixture then was analyzed as the methyl derivative, using the *m/e* settings for the molecular ions at 266 (I), 271 (I-d_5), 296 (II), and 301 (II-d_5). In constructing calibration curves, corrections were made for the contributions of the molecular ion to the *m/e* values 5 mass units higher (I → I-d_5 and II → II-d_5) and 5 mass units lower (I-d_5 → I and II-d_5 → II). There was a good correlation between the mixed molar ratio and the observed peak height ratio. Least-squares analysis of the observed ratio gave a regression line with a slope of 1.000.

Table IV—Saliva to Plasma Ratio of I

Hours	Subject YK	Subject KY
4	0.070	0.088
5	0.075	0.091
6	0.070	0.081
7	0.072	—
8	0.065	0.075
10	0.067	0.075
12	—	0.080
24	0.055	0.076
48	0.085	0.069
72	0.130	—
96	0.220	0.073

Accuracy—The accuracy of measurements was determined for I and II added to aliquots of pooled plasma or urine. The plasma or urine samples contained 10 (I-d_5) or 1.0 (II-d_5) μg of the internal standard and different amounts of I (0.05–10.0 μg) or II (0.05–5.0 μg). The amount of I or II then was measured by the present method. The amounts of I and II added were in good agreement with the amounts of I and II measured (Tables I and II). The relative error was <3% for I and <5% for II. In the subsequent experiment, hydrochloric acid was used for the hydrolysis of III. Treatment of plasma or urine samples with hydrochloric acid did not affect the measurement of II in these samples (Table II).

Pharmacokinetics of Phenytoin—A single oral dose of 250 mg of I as its sodium salt dissolved in water was administered to two male volunteers, 25 (KY) and 36 (YK) years old. Time courses of I–III concentrations in plasma, saliva, and urine then were followed by the present method.

Plasma Concentration—Plasma was analyzed for I–III at various times for 120 hr after the administration of I (Table III). In Subject YK, there was a relatively rapid rise in the plasma I concentration to 3.9 μg/ml at 1 hr, giving a peak level of 4.3 μg/ml at 4 hr. In Subject KY, the plasma I concentration became 5.0 μg/ml at 4 hr, and this plasma level was maintained until 10 hr after the administration of I. Semilogarithmic plots of the plasma concentrations *versus* time indicated that, after absorption, a distribution phase was not visible and that the terminal decline phase was monoexponential at time points later than 48 hr for Subjects YK and KY.

Plasma III concentrations were the highest (1.6 μg/ml) at 8 hr in Subject YK, and the plasma decline of III thereafter was parallel to that of I. In Subject KY, a higher peak plasma III concentration of 2.2 μg/ml was obtained at 10 hr, and the plasma III levels exceeded the plasma I levels at 48 hr and later.

There was an excellent parallelism in the time course of the plasma

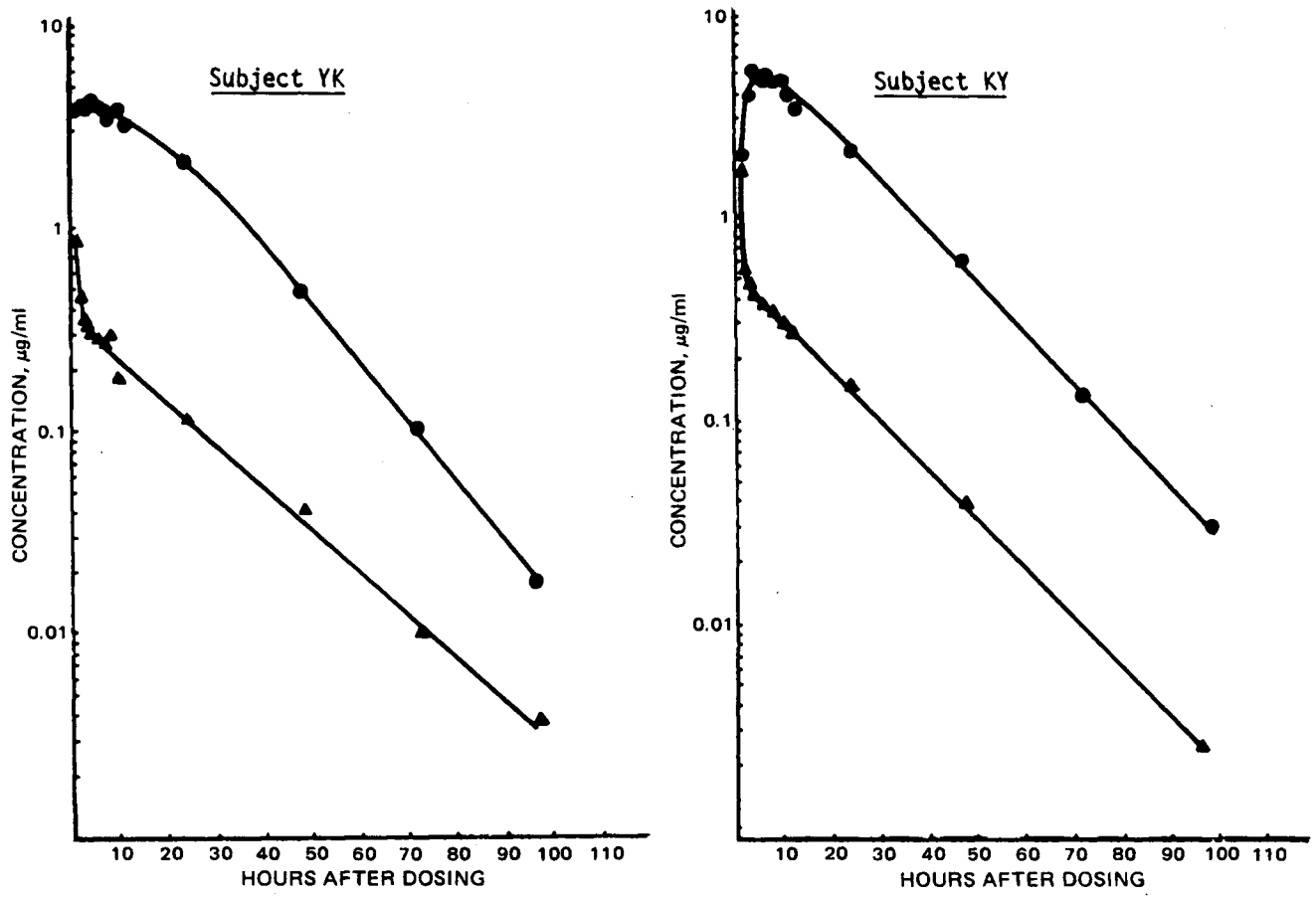


Figure 1—*Semilogarithmic plot of plasma* (●) *and saliva* (▲) *concentrations of I* versus *time after a single oral 250-mg dose of I.*

concentrations of II to that of III in both subjects. However, the levels of II were much lower in Subject YK than in Subject KY. At each time point, the plasma level of II was about 0.033 of the plasma level of III in Subject YK; in Subject KY, the level of II was ~0.1 of the level of III.

Saliva Concentration—Saliva samples were obtained at each time point of blood sampling. The results of measurements of saliva I concentrations are shown in Fig. 1, together with the time course of plasma I concentrations for comparison. Both subjects showed a rapid drop in saliva concentrations of I during the first 3 hr after administration of I; thereafter, the log concentration–time curves could be computed as straight lines. There was a close similarity in the decline of the slope of the saliva elimination curve to that of the plasma elimination curve for I. The slopes of the saliva and plasma elimination curves coincided with each other, especially in Subject KY, with the saliva to plasma ratios after 3 hr being consistent (Table IV). In Subject YK, there was only a slight fluctuation in the saliva to plasma ratio at each time point between 4 and 48 hr, while the ratio became higher at 72 and 92 hr.

Urinary Excretion—Urine samples were collected at appropriate time periods until 132 hr after the administration of I. The urinary pH and flow rate at each urine collection period are presented in Table V. These urine samples were analyzed for I–III, and the urinary excretion data are summarized in Tables VI–VIII. During the first 12 hr, fluctuations in the elimination rate were largely reflected in the excretion rate plots of I–III. After this period, plots of urinary excretion rates against the midpoints of urine collection periods were not scattered so much.

Subject YK excreted a much higher percentage of administered I as III (81%) than did Subject KY (56%) up to 132 hr. The excretion of unconjugated II and unmetabolized I was very small in both subjects, with the percentage being ~1% of the administered I dose. The concentration of conjugated II in plasma and urine was calculated by subtracting the unconjugated II from the total II measured after acid hydrolysis. However, a minor metabolite of I, 5-(3,4-dihydroxy-1,5-cyclohexadien-1-yl)-5-phenylhydantoin, is partially converted to II (13, 14). Therefore, in the present assay, the total plasma or urine II includes these minor pathways.

The sigma-minus plots shown in Fig. 2 apparently were much less sensitive to fluctuations in the elimination rate of I. Figure 2 shows that the slopes of the terminal monoexponential phase in the plots of the log amount remaining to be excreted against time for I–III were parallel to each other in both subjects. This finding indicates that the slopes of the terminal straight lines for the metabolites (II and III) reflect the elimination rate constant for the substrate (I). The half-lives of I calculated

Table V—Urinary pH and Flow Rate in Subjects KY and YK

Urine Collection Time, hr	Urine Collection Period, hr	pH		Urine Volume, ml		Flow Rate, ml/min	
		Subject KY	Subject YK	Subject KY	Subject YK	Subject KY	Subject YK
2	2	5.0	5.4	694	760	5.8	6.3
4	2	5.2	5.6	366	235	3.1	2.0
6	2	6.4	5.6	189	160	1.6	1.3
8	2	5.6	5.6	267	229	2.2	1.9
12	4	6.8	6.6	795	336	3.3	1.4
36	24	5.8	6.2	1130	2175	0.8	1.5
60	24	5.4	5.8	1850	2000	1.3	1.4
84	24	6.2	6.6	2750	2255	1.9	1.6
108	24	6.0	6.4	1800	1675	1.3	1.2
132	24	6.2	6.4	2290	2160	1.6	1.5

Table VI—Urinary Excretion Data for I in Subjects YK and KY

t, hr	Δt, hr	Midpoint t, hr	Subject YK ΔX, μg	Subject YK $\Delta X/\Delta t$, μg/hr	Subject YK ΣX, μg	Subject YK $X^\infty - X$, μg	Subject KY ΔX, μg	Subject KY $\Delta X/\Delta t$, μg/hr	Subject KY ΣX, μg	Subject KY $X^\infty - X$, μg
2	2	1	344.97	172.49	344.97	1865.60	342.08	171.04	342.08	1711.79
4	2	3	221.51	110.76	566.48	1644.09	415.87	207.94	757.95	1295.92
6	2	5	130.88	65.44	697.36	1513.21	181.63	90.82	939.58	1114.29
8	2	7	155.07	92.54	882.43	1328.14	264.50	132.25	10,204.08	849.79
12	4	10	232.71	58.18	1115.14	1095.43	327.56	81.89	1,531.64	522.23
36	24	24	888.36	37.02	2003.50	207.07	329.42	13.73	1,861.06	192.81
60	24	48	170.92	7.12	2174.42	36.15	152.37	6.35	2,013.43	40.44
84	24	72	30.62	1.28	2205.04	5.53	30.00	1.25	2,043.43	10.44
108	24	96	4.17	0.17	2209.21	1.36	10.44	0.44	2,053.87	—
132	24	120	1.36	0.06	2210.57	—	—	—	—	—

Table VII—Urinary Excretion Data for II in Subjects YK and KY

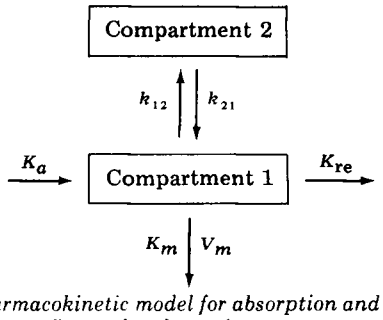
t, hr	Δt, hr	Midpoint t, hr	Subject YK ΔX, μg	Subject YK $\Delta X/\Delta t$, μg/hr	Subject YK ΣX, μg	Subject YK $X^\infty - X$, μg	Subject KY ΔX, μg	Subject KY $\Delta X/\Delta t$, μg/hr	Subject KY ΣX, μg	Subject KY $X^\infty - X$, μg
2	2	1	59.98	29.99	59.98	2379.06	84.10	42.05	84.10	3202.81
4	2	3	107.83	53.92	167.81	2271.23	184.54	92.27	268.64	3018.27
6	2	5	112.84	56.42	280.65	2158.39	152.66	76.33	421.30	2865.61
8	2	7	162.01	81.01	442.66	1996.38	176.09	88.05	597.39	2689.52
12	4	10	295.49	73.87	738.15	1700.89	162.09	40.52	759.48	2527.43
36	24	24	1145.01	47.71	1883.16	555.88	1689.58	70.40	2449.06	837.85
60	24	48	456.84	19.04	2340.00	99.04	662.60	27.61	3111.66	175.25
84	24	72	76.26	3.18	2416.26	22.78	136.62	5.69	3248.28	38.63
108	24	96	19.93	0.83	2436.19	2.85	38.63	1.61	3286.91	—
132	24	120	2.85	0.12	2439.04	—	—	—	—	—

Table VIII—Urinary Excretion Data for III in Subjects YK and KY

t, hr	Δt, hr	Midpoint t, hr	Subject YK ΔX, mg	Subject YK $\Delta X/\Delta t$, mg/hr	Subject YK ΣX, mg	Subject YK $X^\infty - X$, mg	Subject KY ΔX, mg	Subject KY $\Delta X/\Delta t$, mg/hr	Subject KY ΣX, mg	Subject KY $X^\infty - X$, mg
2	2	1	5.723	2.862	5.723	209.700	2.494	1.247	2.494	145.300
4	2	3	10.840	5.420	16.563	198.860	7.698	3.849	10.192	137.602
6	2	5	10.914	5.457	27.477	187.946	8.378	4.189	18.570	129.224
8	2	7	14.555	7.278	42.032	173.391	11.377	5.689	29.947	117.847
12	4	10	26.711	6.678	68.743	146.680	10.741	2.685	40.688	107.106
36	24	24	106.058	4.419	174.801	40.622	70.696	2.946	111.384	36.410
60	24	48	32.642	1.360	207.443	7.980	26.954	1.123	138.338	9.456
84	24	72	6.422	0.268	213.865	1.558	7.375	0.307	145.713	2.081
108	24	96	1.174	0.049	215.039	0.384	1.476	0.062	147.189	0.605
132	24	120	0.384	0.016	215.423	—	0.605	0.025	147.749	—

from these slopes were 9.4 hr for Subject YK and 11.5 hr for Subject KY.

Pharmacokinetic Parameters—Linear regression of $(t_{n+1} - t_n)/(\ln C_n - \ln C_{n+1})$ on $(C_nC_{n+1})^{1/2}$ was used to differentiate between first-order and Michaelis–Menten elimination kinetics (15), where C_n and C_{n+1} are the plasma concentrations at time points t_n and t_{n+1}, respectively. The regression line of the plasma data (Table III) beginning at 12 hr after administration of I gave an apparent straight line whose slope was not equal to zero. Therefore, preliminary estimates of the maximum rate of the process (V_m) and the Michaelis–Menten constant (K_m) were obtained from the y-axis intercept and the slope of the line (16). Since it already was reported that I follows a two-compartment open model after intravenous administration (17), the observed set of plasma data in the present study was fitted to a two-compartment open model with Michaelis–Menten elimination kinetics (Scheme I).



Scheme I—Pharmacokinetic model for absorption and elimination of I. Key: K_a, *apparent first-order absorption rate constant;* k_{12}, *apparent first-order distribution rate constant from Compartment 1 to Compartment 2;* k_{21}, *apparent first-order distribution rate constant from Compartment 2 to Compartment 1;* K_{re}, *apparent first-order rate constant for renal excretion of I;* V_m, *maximum rate of metabolism of I; and* K_m, *Michaelis–Menten constant.*

The differential equations for the model shown in Scheme I are:

$$\frac{dC_1}{dt} = K_aC_0e^{-K_at} - \left(k_{12} + \frac{V_m}{K_m + C_1}\right)C_1 + k_{21}C_2 - K_{re}C_1 \quad \text{(Eq. 1)}$$

$$\frac{dC_2}{dt} = k_{12}C_1 - k_{21}C_2 \quad \text{(Eq. 2)}$$

where C_0 is the fraction absorbed (F) times the administered dose (D) divided by the volume of distribution (V_d), C_1 is the drug concentration in Compartment 1, C_2 is the drug concentration in Compartment 2, t is time, K_a is the first-order absorption rate constant, k_{12} is the first-order distribution rate constant from Compartment 1 to Compartment 2, k_{21} is the first-order distribution rate constant from Compartment 2 to Compartment 1, and K_{re} is the first-order rate constant for renal excretion of I.

Equations 1 and 2 were numerically integrated by a computer[5] using

[5] CMB model 2001-32 personal computer.

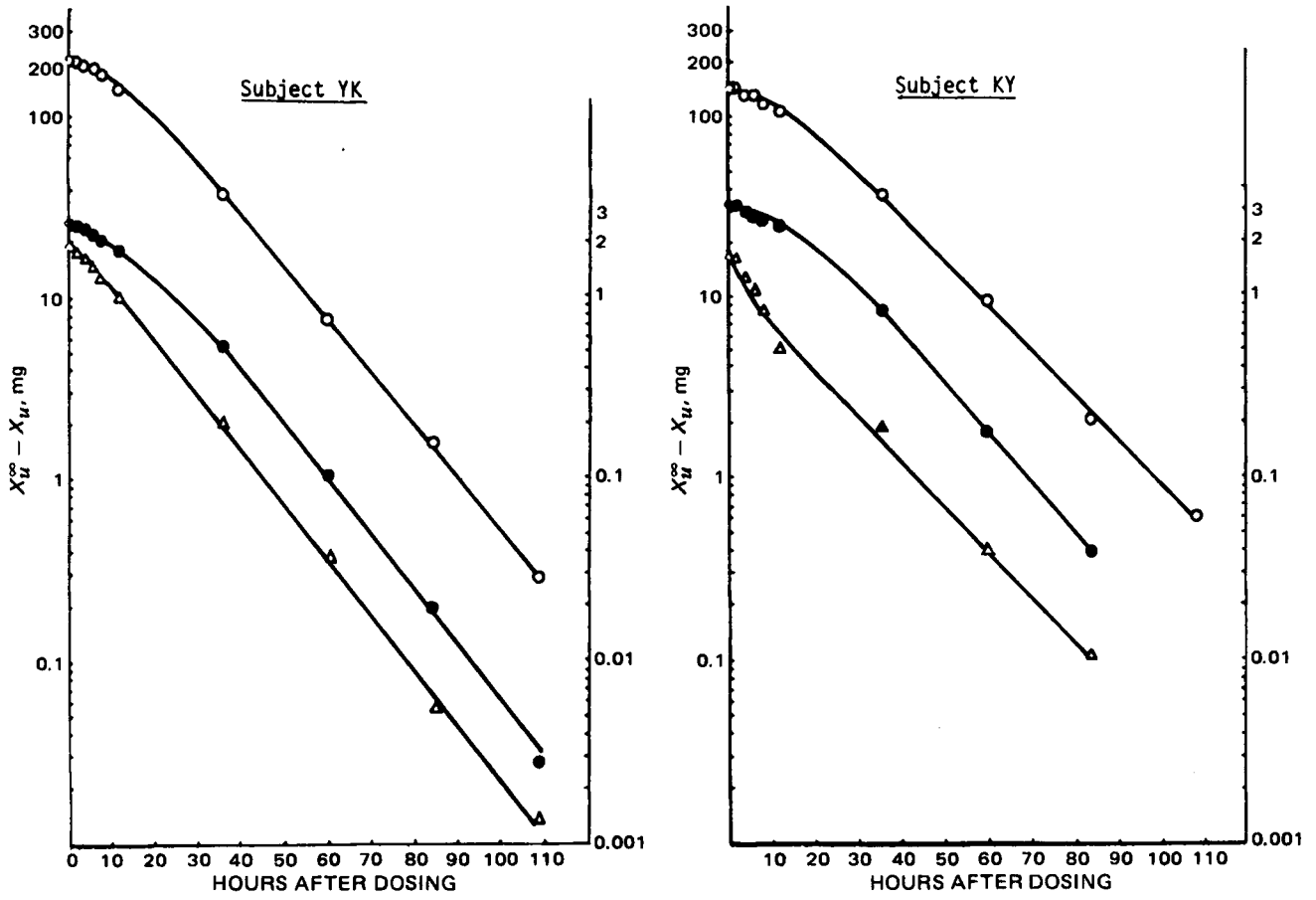


Figure 2—*Semilogarithmic plot of amounts of I–III remaining to be excreted* ($X_u^\infty - X_u$) versus *time for the determination of the elimination rate constant from urinary excretion data. Key:* △, *I (right axis);* ●, *II (right axis); and* ○, *III (left axis).*

the Runge–Kutta method, and the values for the pharmacokinetic parameters (K_a, V_m, K_m, k_{12}, k_{21}, and V_d) were obtained by a least-squares estimation using the steepest descent method. The estimate of the elimination half-life ($t_{1/2}$) was obtained from $t_{1/2} = 0.693/(V_m/K_m)$, without consideration of the minor contribution of K_{re}. The total plasma clearance (Cl_{pl}) was expressed as the amount absorbed divided by the area under the plasma concentration curve, assuming 100% absorption (18). The area under the plasma concentration–time curve (AUC) from t = 0–120 (infinity) was measured by the trapezoidal rule.

Renal clearance (Cl_{re}) for I and II was obtained from the slope of the straight line resulting from a plot of the average excretion rate ($\Delta X_u/\Delta t$) *versus* the plasma concentration at the midpoint of the urine collection period (19). The rate constant for renal excretion of I (K_{re}) was determined by employing $K_{re} = Cl_{re}/V_d$. These pharmacokinetic parameters are listed in Table IX. There were no large differences in V_m, K_m, $t_{1/2}$, Cl_{pl}, and V_d between the two subjects. On the other hand, the values for K_a, K_{re}, AUC, and Cl_{re} were considerably different, except that only a small difference was observed for the AUC value for I.

Table IX—Pharmacokinetic Parameters Derived after Single Oral 250-mg Dose of I

Subject	K_a, hr^{-1}	V_m, μg/ml/hr	K_m, μg/ml	$t_{1/2}$, hr	k_{12}, hr^{-1}
YK	0.80	0.87	12.3	9.8	0.09
KY	0.52	0.81	11.7	10.0	0.06

Subject	k_{21}, hr^{-1}	K_{re}, hr^{-1}	Cl_{pl}, liters/hr	V_d, liters/kg
YK	0.57	0.00041	2.18	0.64
KY	0.78	0.00020	1.91	0.60

	AUC, (μg hr)/ml			Cl_{re}, ml/hr		
Subject	I	II	III	I	II	III
YK	114.80	1.84	57.05	17.2	1359.3	3737.0
KY	131.00	4.55	83.80	7.1	811.5	1684.7

DISCUSSION

The usefulness of a mass fragmentographic technique in conjunction with stable isotope labeling in the pharmacokinetic study of I was a major interest in the present study. Various analytical techniques such as GLC, radioimmunoassay, enzyme immunoassay, liquid chromatography, and spectrophotometry are used frequently for monitoring plasma levels of I during chronic therapy, and comparisons of these analytical methods have been reported (20, 21). The pharmacokinetics of I have been examined by GLC (22–24), colorimetry (25, 26), radioimmunoassay (17), and a radioisotope tracer technique (27, 28). However, the sensitivity or specificity of these methods often does not provide reliable and precise determination of I in biological samples. In addition, no detailed information is available regarding the simultaneous pharmacokinetic analysis of plasma levels and urinary excretion data of I together with its major metabolite II. The present single-dose study in two healthy male volunteers was designed to give information about the precise and detailed pharmacokinetics of I determined from both blood level curves and urinary excretion data for I and II.

The metabolic fate of I has been studied extensively (29–31). Butler (32) showed that I is *para*-hydroxylated in the liver to yield II. This metabolite is conjugated with glucuronic acid, is present in plasma largely as the conjugate, and is excreted in the urine. The colorimetric method described by Dill *et al.* (33) and GLC measurement using derivatization with either trimethylsilyl groups (34) or flash methylation (35) are not sensitive enough to detect unconjugated II in plasma.

A mass fragmentographic method using deuterium-labeled internal standards was described recently (11, 36) for the sensitive quantitative determination of I and II in human plasma. The lower limit of detection was ~0.01 μg/ml for I and II using an extractive alkylation technique (36). Rane *et al.* (37) studied the fate of transplacentally transferred I in newborn infants by a mass fragmentographic method. The molecular ions

Table X—Ratio of II to I in Plasma

t, hr	Subject YK	Subject KY
4	0.013	0.023
12	0.014	0.029
24	0.019	0.040
48	0.026	0.055

were used for the detection of the monomethyl derivatives of I and the internal standard (I-d_{10}). The analytical procedure permitted precise determinations of I in plasma in concentrations down to 0.01 μg/ml. The high sensitivity generally can be obtained by introducing appropriate volatile derivatives for mass fragmentographic analysis.

The mass fragmentographic method described here was satisfactory for the sensitive and specific measurement of I in saliva, plasma, and urine and I and II in plasma and urine. Compound I and its internal standard (I-d_5) were analyzed as the monomethyl derivatives, and II and its internal standard (II-d_5) were converted to the dimethyl derivatives by simple derivatization with diazomethane. The lower limit of detection in this study was ~0.01 μg/ml for both I and II, which was comparable to that reported previously (11, 36, 37).

In the present study, I was administered as the sodium salt dissolved in water, and relatively high saliva I concentrations were observed during the first 3 hr after drug administration (Fig. 1). This result must have been due to residual I in the mouth, although care was taken to avoid this influence by administration of 100 ml of water immediately after drug intake. After this period, the saliva I concentration declined in parallel with the plasma I concentration. Several recent studies (38–40) demonstrated a significant linear correlation between salivary concentrations of I and the plasma total and plasma free concentrations. The data in Table III substantiate the close relationship between the whole salivary and the plasma free I concentrations.

Pharmacokinetic studies in humans usually have been performed with drugs that are present unchanged in the blood or are excreted unchanged in the urine. However, it sometimes is important to carry out simultaneous analysis of blood levels and urinary excretion of both the unchanged drug and its metabolites to determine correct pharmacokinetic parameters, especially when interindividual variations in the pharmacokinetics of a drug are to be investigated. The analysis and interpretation of pharmacokinetic data presented here give some insight into interindividual differences in the metabolic disposition of I.

The shape and peak concentrations of the plasma concentration curves for unchanged I did not differ considerably between Subjects YK and KY. Furthermore, the slopes of the sigma-minus plots for unchanged I (Fig. 2) were very similar in the two subjects. Although there was an approximately twofold difference in the rate constant for renal excretion of I (K_{re}) between Subjects YK and KY, this difference contributed little to the difference in the overall elimination of I since extensive metabolism of I was the predominant route of its elimination. Consequently, it may be stated from these findings that the pharmacokinetic behavior of I in Subjects YK and KY was practically the same.

However, the precise analysis of plasma concentration–time data for I–III revealed that the kinetics of urinary excretion characterized by renal clearance values (Cl_{re}) not only for I but also for II and III differed considerably between the two subjects (Table IX), although both subjects gave normal creatinine clearances of 95.2 (YK) and 105.2 (KY) ml/min. This difference was reflected by the difference in *AUC*. Bochner *et al.* (22) suggested that I is actively secreted in the renal tubules, a process that may differ between subjects. Such a mechanism may explain the difference in renal clearance observed in this study.

It is well known that the clearance of I decreases as the dose of I is increased (41–43). Therefore, it is not unreasonable to assume that the ratio of II to I in plasma could serve as an indicator to predict when one will show these dose-dependent kinetics, regardless of the mechanisms involved. Along this line, Hoppel *et al.* (44) used a mass fragmentographic method to determine II and III in the plasma of patients treated with I. However, no apparent relationship was found in the ratio of the plasma concentrations of I and II at similar doses. Comparison of the ratio of II to I in plasma in the present single-dose study revealed that the ratio tended to increase with the time after administration of I and that the ratio was about two times higher in Subject KY than in Subject YK at each time point (Table X). When the ratio of II to I was compared by calculating the ratio of the *AUC* values for II and I, there also was an approximately twofold difference between the two subjects (KY = 0.035 and YK = 0.016). This in part explains the interindividual differences in the ratios between I and unconjugated II in plasma at a constant dose reported by Hoppel *et al.* (44).

In summary, the mass fragmentographic method described in this study adequately meets the criterion of reliability for the precise, sensitive, and specific determination of I and its main metabolite (II) in biological samples. The pharmacokinetics of I were examined in detail based on the analysis of blood level and urinary excretion data from a single oral dose study in two healthy volunteers.

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Correlation of *In Vivo* Bioavailability of Erythromycin Stearate Tablets with *In Vitro* Tests

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Abstract □ Correlations between the bioavailability parameters for erythromycin stearate tablets from five manufacturers and *in vitro* tests of these tablets were examined using forward (stepwise), multiple linear regression analysis. Bioavailability parameters were determined in clinical studies employing a balanced, incompete block design. *In vitro* tests used disintegration, dissolution, and dissolution/dialysis as the independent variables in regression equations. Significant correlations were found between linear combinations of these parameters and the time to peak and the peak serum levels. The inclusion of an *in vitro* disintegration test to describe peak serum levels of erythromycin is noteworthy since it has been suggested that disintegration tests are of less value than dissolution tests in predicting tablet performance *in vivo*. The multiple regression techniques employed in the present investigation may be useful for selection of appropriate physicochemical tests for continued monitoring of the bioavailability of erythromycin stearate tablets.

Keyphrases □ Erythromycin stearate—correlation of bioavailability of tablets with *in vitro* tests □ Antibiotics—erythromycin stearate, correlation of bioavailability of tablets with *in vitro* tests □ Multiple regression analysis—correlation of bioavailability of erythromycin stearate tablets with *in vitro* tests

Erythromycin stearate is erratically absorbed from the GI tract (1) and is subject to degradation by acids (2). Food and the volume of fluid taken with erythromycin stearate tablets affect the serum erythromycin levels in humans (3). The bioavailability variations, coupled with the numerous erythromycin stearate products marketed, create a potential bioequivalence problem. The difficulties encountered with clinical studies in terms of cost and human safety provide an impetus for prediction of product bioavailability from *in vitro* tests.

BACKGROUND

Bivariate correlations of *in vivo* with *in vitro* results have been reported for a variety of drugs. Dissolution rates of three brands of prednisone tablets indicated the clinical efficacy of the tablets (4). Chloramphenicol bioavailability was correlated with *in vitro* dissolution tests by Ogata *et al.* (5). Aguiar *et al.* (6) reported correlations of both the deaggregation and dissolution rates of four commercial lots of chloramphenicol capsules with human plasma levels.

An *in vitro* dissolution rate test that quantitatively predicted the GI absorption of aspirin from three different dosage forms was developed by Levy *et al.* (7). A linear correlation between salicylate excretion following oral administration of two aspirin tablets and the rate of tablet dissolution also was shown (8). In addition, a linear correlation was found between *in vivo* aspirin absorption and the *in vitro* dissolution rate using a rotating-flask apparatus (9). Similar linear correlations also were demonstrated for salicylamide tablets and suspensions (10).

Bergan *et al.* (11) compared the *in vitro* dissolution rates of two tetracycline and seven oxytetracycline products with their absorption characteristics in healthy human volunteers. Correlations were significant for some of these tetracycline products but not for others. Smolen and Weigand (12) developed a flow-through dissolution apparatus which could be optimized through feedback control to simulate *in vivo* drug release properties. They found that systematic adjustment of dissolution conditions resulted in improved *in vitro* to *in vivo* correlations.

These studies, with the exception of the chloramphenicol deaggregation studies, used *in vitro* dissolution results to predict drug absorption *in vivo*. However, bioavailability is a complex parameter which is influenced by the physicochemical properties of the drug, the dosage form, and the physiological conditions of the system. Thus, dissolution testing should be complemented by other *in vitro* tests to increase the prediction ability of *in vivo* drug absorption from *in vitro* results. Data on dissolution, dialysis, disintegration, and other physical tests are presented, and the usefulness of multiple linear regression analysis in the correlation between *in vivo* bioavailability and *in vitro* parameters for five commercially available erythromycin stearate tablets is demonstrated.

EXPERIMENTAL

***In Vitro* Studies**—*In vitro* tests were performed on selected lots of 250-mg film-coated erythromycin stearate tablets from five manufacturers. All tablets were from the same lots as those used in the clinical studies (13). Tablet hardness was measured with a motorized pendulum tablet hardness tester[1]. When the tablet was harder than 16 kg, a spring-type hand-held tester was used[2]. Weight variation was determined by weighing 10 tablets from each lot.

Four disintegration methods were investigated using the USP XIX apparatus at 37 ± 2°. In Method 1, tablets were run for 30 min in a 0.2% aqueous NaCl solution adjusted to pH 3 with dilute hydrochloric acid. These tablets then were placed in a pH 7.35 phosphate buffer solution containing 6.8 g of monobasic potassium phosphate and 190 ml of 0.2 *N* NaOH adjusted to 1000 ml with water. Method 2 involved placing the tablets in USP XIX simulated gastric fluid test solution (pH 1.22) for 30 min and then removing the tablets and placing them in USP XIX simulated intestinal fluid test solution (pH 7.55) for the time required to bring about disintegration. Method 3 was the same as Method 2, except that no enzymes were used in the simulated gastric and intestinal fluids. Method 4 also was the same as Method 2, except that the time in the gastric fluid was increased to 1 hr.

Dissolution studies on the five erythromycin stearate lots were per-

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