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Pharmacokinetic Analysis on the Disappearance of Ethoxybenzamide from Plasma. Statistical Treatment of Data of Two Compartmental Model by a Digital Computer[1]

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The disappearance of ethoxybenzamide from a rabbit plasma after an intravenous injection was studied.

1. The distribute phase after the injection could not be neglected, and the two compartmental model was applied to analize the time course of plasma concentration.

2. The iterative least square method with a digital computer was proposed to obtain pharmacokinetic constants and their statistical values.

3. The present method gave the unique parameters independent of personal error for drawing straight lines in the semilogarithmic plots of plasma concentration *vs.* time, and it could test the adoptability of data for the model.

The phenomenon that the biological half life of salicylic acid increases when a certain amount or more are administered, was analyzed by a non-linear pharmacokinetics.[3] Since the non-linear process is important to determine a dose schedule as well as it gives a mechanistic interesting point, the process has been reported for other drug recently.[4] The pharmacokinetic behaviour of salicylamide[5] is also known to have the non-linear process, but as for ethoxybenzamide which is a homologous antipyretics, little is known about the behaviour. Since deethylation is the first metabolic step giving salicylamide, the pharmacokinetic pattern of ethoxybenzamide will be more complicated than the latter and be very interesting. The authors intended to study the pharmacokinetic behaviour of ethoxybenzamide, and found that when it was administered intravenously, the initial decrease of the plasma concentration was rather rapid and the distribute phase could not be neglected. The general and simple model to analyze the time course of plasma concentration with a distribute phase is a two compartmental model as shown in Chart 1.[6]

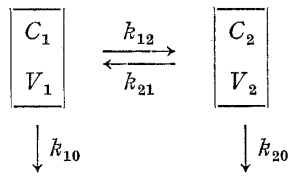

Chart 1

Compartment 1 can be assumed as the plasma and other fluids and tissues which, in effect, rapidly equilibrate with the drug and Compartment 2 is the compartment which has a significant barrier for the distribution of the drug from Compartment 1. V and C are the distribution volume and the concentration of each compartment, respectively; k_{12} and k_{21} are the first order distribution rate constants from the compartment to the other compartment and k_{10} and k_{20} are the first order elimination rate constants from the corresponding compartment with metabolism and excretion. The above two compartmental model gives the following general equations, which describes the plasma concentration of a drug.[6]

1) This forms Part XIV of "Studies on Absorption and Excretion of Drug," by H. Nogami. Part XIII: H. Nogami, J. Hasegawa, M. Hanano and T. Fuwa, *Chem. Pharm. Bull.* (Tokyo), **16**, 2101 (1968).
2) Location: *Bunkyo-ku, Hongo, Tokyo.*
3) G. Levy, *J. Pharm. Sci.*, **54**, 959 (1965).
4) J. Shibasaki, T. Koizumi and T. Tanaka, *Chem. Pharm. Bull.* (Tokyo), **16**, 1661 (1968).
5) G. Levy and T. Matsuzawa, *J. Pharmacol. Exptl. Therap.*, **156**, 285 (1967).
6) D.S. Riggs, "Mathematical Approach to Physiological Problems," Williams and Wilkins, Baltimore, Md., 1963; J.G. Wagner and J.I. Northam, *J. Pharm. Sci.*, **56**, 529 (1967).

$$C_1=A\exp(-\alpha t)+B\exp(-\beta t) \tag{1}$$

$$A=\frac{\text{Dose}}{V_1}\cdot\frac{(k_{12}+k_{10}-k_{21}-k_{20})+Z}{2Z} \tag{2}$$

$$B=\frac{\text{Dose}}{V_1}\cdot\frac{Z-(k_{12}+k_{10}-k_{21}-k_{20})}{2Z} \tag{3}$$

$$Z=\sqrt{(k_{12}+k_{10}-k_{21}-k_{20})^2+4k_{12}k_{21}} \tag{4}$$

$$\alpha=(1/2)\{(k_{12}+k_{21}+k_{10}+k_{20})+Z\} \tag{5}$$

$$\beta=(1/2)\{(k_{12}+k_{21}+k_{10}+k_{20})-Z\} \tag{6}$$

Although the four parameters in Eq. (1) can be determined geometrically, such a method gives biased values and can not give any statistical information. Although pharmacokinetic parameters are obtained with biological experiments, very little are reported with a reasonable statistical consideration. The authors' opinion is that the statistical approach for pharmacokinetic data is very important to know the confidence of parameters and to compare the parameters reported for different sex or species, or from different laboratories, therefore it was intended to obtain statistical parameters for ethoxybenzamide as the first step for the study of its pharmacokinetic behaviour. The present report is to give the statistical method to obtain the four parameters of two compartmental model, using a digital computer.

Analysis of Data

It is well known statistically that the best parameters should be determined by the least square method. The general principle and procedure of the method to obtain parameters is written in the text of Deming[7] and also reported by Berman, *et al.*[8] Krüger-Thiemer[9] used already the method to obtain two parameters in the case of protein binding of drug and Wiegand[10] used it also to obtain three parameters in the case of blood concentration analysis of an orally administered drug with a digital computer. The present paper concerns with the case of a drug distribution and elimination which are described with four parameters. For the convenience sake of writing the paper, the least square method for four parameters will be written here according to Deming.[7]

When the observed values of X_i[11] and Y_i are fitted on the curve of the equation, $y=f(x, a, b, c, d)$, the parameters, a, b, c, and d should give the minimum value of the weighted sum of squares of differences between Y_i and $f(X_i, a, b, c, d)$, *i.e.*,

$$\sum\omega_i\{Y_i-f(X_i, a, b, c, d)\}^2=\sum\omega_i F_i^2=\text{minimum}$$

where ω is a weighting value for each point and F is given with Eq. (8). When a_0, b_0, c_0 and d_0 are assumed as the approximate values of a, b, c and d, respectively, the best parameters are given;

$$a=a_0-A \quad (7-\text{a}) \qquad b=b_0-B \quad (7-\text{b})$$

$$c=c_0-C \quad (7-\text{c}) \qquad d=d_0-D \quad (7-\text{d})$$

where A, B, C and D are the residual values. Using the Taylor's expansion,

$$F=Y-f(X, a, b, c, d)$$
$$=F_0-F_aA-F_bB-F_cC-F_dD \tag{8}[12]$$

7) W.E. Deming, "Statistical Adjustment of Data," John Wiley and Sons, Inc., New York, N.Y., 1946.
8) M. Berman, E. Shahn and M.F. Weiss, *Biophysic. J.*, **2**, 275 (1962).
9) E. Krüger-Thiemer and B. Schlender, *Arzneim.-Forsch.*, **13**, 894 (1963).
10) R.G. Wiegand and P.G. Sanders, *J. Pharmacol. Exptl. Therap.*, **146**, 271 (1964).
11) Supposed to have no error.
12) Suffixes i for Y and X are omitted, and some abbreviations are used; $F_0=Y-f(X, a_0, b_0, c_0, d_0)$ and $F_a=(\partial F/\partial a)$, $F_b=(\partial F/\partial b)$, ... , for X, a_0, b_0, c_0 and d_0.

Based on the least square principle, the following simultaneous equation (normal equation) are given for the unknown values of A, B, C and D.

$$\left.\begin{aligned}
&[\omega F_a F_a]A+[\omega F_a F_b]B+[\omega F_a F_c]C+[\omega F_a F_d]D=[\omega F_a F_0]\\
&[\omega F_a F_b]A+[\omega F_b F_b]B+[\omega F_b F_c]C+[\omega F_b F_d]D=[\omega F_b F_0]\\
&[\omega F_a F_c]A+[\omega F_b F_c]B+[\omega F_c F_c]C+[\omega F_c F_d]D=[\omega F_c F_0]\\
&[\omega F_a F_d]A+[\omega F_b F_d]B+[\omega F_c F_d]C+[\omega F_d F_d]D=[\omega F_d F_0]
\end{aligned}\right\} \quad (9)^{13)}$$

The better parameters can be obtained with the roots of the normal equation (Eq. (9)), A, B, C and D, using Eq. (7). Since the above procedure usually does not give the best parameters, the iteration of the procedure is necessary, assuming the better parameters as the new approximate parameters. The statistical values are given with the following equations.

$$\text{Sum of squares } (S)=[F_0F_0]-[F_aF_0]A-[F_bF_0]B-[F_cF_0]C-[F_dF_0]D$$

$$\sigma^2=S/(n-4) \qquad n\text{: number of data}$$

$$(\text{estimated standard error of } a)^2=c_{11}\sigma^2$$

$$(\text{estimated standard error of } b)^2=c_{22}\sigma^2$$

$$(\text{estimated standard error of } c)^2=c_{33}\sigma^2$$

$$(\text{estimated standard error of } d)^2=c_{44}\sigma^2$$

where c's are the elements of the inverse matrix for the matrix of the coefficients of the unknowns in Eq. (9). The limits of 95% confidence for each parameter can be given by the following equation.

$$\text{confidence limit}=\text{standard error}\times t_{95}$$

where t_{95} is the Student's t value of 95% confidence for a given degree of freedom. And the estimated standard error of y at each time is given by Eq. (10).

$$\begin{aligned}(\text{estimated standared error of } y)^2&=\sigma_y^2\\ &=\sigma^2(c_{11}F_a^2+c_{22}F_b^2+c_{33}F_c^2+c_{44}F_d^2+2c_{12}F_aF_b+2c_{13}F_aF_c\\ &\quad+2c_{14}F_aF_d+2c_{23}F_bF_c+2c_{24}F_bF_d+2c_{34}F_cF_d)\end{aligned} \quad (10)$$

Experimental

Ethoxybenzamide is so insoluble in water that it was solved in propyleneglycol to have 50 mg/ml solution. The indicated amounts of ethoxybenzamide was administered intravenously through an ear vein (*v. auricularis*) in solution to male rabbits of which body weights were 3.0—3.5 kg. Immediately after the administration, 1 ml of blood was collected from the other ear vein as the zero time sample, after which blood collections were made at given times.

Analytical Methods——After about 1 ml of blood was centrifuged at 3,000 rpm, 0.5 ml of plasma was taken and added to the mixture of 2 ml isotonic NaCl solution, and 3.5 ml deionized water. Ethoxybenzamide was extracted from the solution to 20 ml of an acidic ether which had 8 mg/ml of oxalic acid with a vigorous shaking. Ether was removed at about 35° under vacuum from the 10 ml ethereal layer and the residue was dissolved again into 10 ml of 0.1M phosphate buffer solution of pH 7.0. After the phosphate buffer solution was centrifuged at 15,000 rpm of Kubota KR-6P centrifuge, the transparent solution was used for the ethoxybenzamide determination. Ethoxybenzamide was found to have the maximum fluorescence at 355 mμ excited at 295 mμ, where salicylamide, the metabolite of ethoxybenzamide, had the slight fluorescence. Since salicylamide had the maximum fluorescence at 418 mμ excited at 295 mμ, where ethoxybenzamide had also fluorescence, the latter was determined from the intensities of fluorescence at 355 mμ and 418 mμ excited at 295 mμ, as the solution of the simultaneous equation for the intensity of ethoxy-

13) The symbol [ ] means that the terms in the parenthesis are summed up for i.

benzamide and salicylamide at each wave length. Hitachi 203 fluorescence spectrophotometer was used for fluorometry.

Materials——Ethoxybenzamide was a kind gift from Takeda Chemical Industries Ltd. (mp 130°), and was used without further purification. Ether of special grade was distilled and water was deionized with Amberlite IR-120 and IRA-410. Other chemicals were all of special grade.

Computation Flow——The initial approximate values of parameters are necessary for this method. To obtain them, the equivalent method to the usual geometrical method is programmed for the computer. The number of datum after which the semi-logarithmic plots of plasma concentration *v.s.* time can be assumed straight is given as one of the input data. B and β are calculated with a simple linear regression equation ($\ln x = \ln B - \beta t$) and afterwords, A and α are calculated with the same method. Hitachi 5020E in the computer center of the University of Tokyo was used and the sweep out method of the double precision programmed as the subroutine (Fl/HC/DINV) in the computer center was used to solve the normal equation and to obtain the inverse matrix. Computation flow is shown in Chart 2.

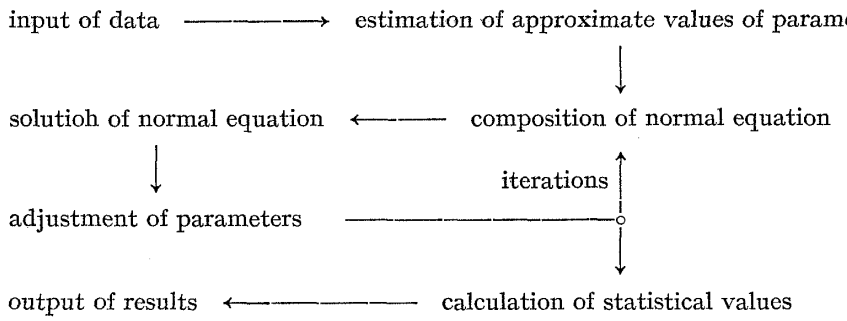

Chart 2

All input data are as the followng.

1. Experimental run number 2. Dose 3. Number of data 4. M (the number of the datum after which the semilogarithmic plots of plasma concentration *v.s.* time can be assumed straight) 5. Number of iterations 6. Plasma concentration 7. Time

Results

The data of the ethoxybenzamide elimination from plasma are shown in Table I, and the semilogarimic plots of time course are shown in Fig. 1. Since Fig. 1 is not straight, the

Table I. Time Course of Ethoxybenzamide Disappearance from Rabbit Plasma[a)]

Time (min)	Plasma concn. (μmole/ml)	Calcd. plasma concn.[b)] (μmole/ml)
0	0.1800	0.180
2.5	0.1088	0.109
5	0.0949	0.0941
10	0.0858	0.0821
15	0.0703	0.0727
20	0.0629	0.0644
30	0.0478	0.0506
40	0.0388	0.0397
50	0.0307	0.0312
60	0.0261	0.0245
70	0.0196	0.0192
80	0.0191	0.0151

a) Dose is 50 mg. (302.7 μmole).
b) Calculated with Eq. (1), using the final values in Table II.

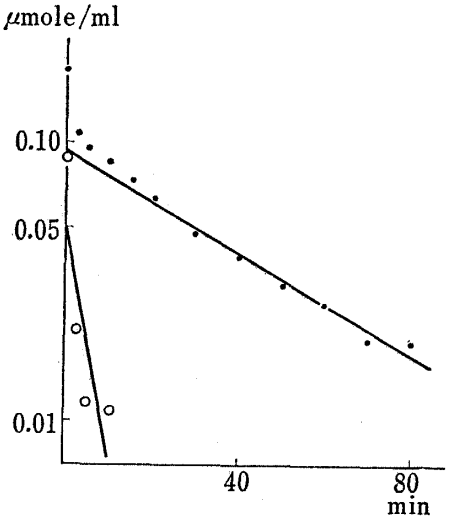

Fig. 1. Semi Logarithmic Plots of Disappearance of Ethoxybenzamide from Plasma

Lines were obtained from a simple linear regression equation.
●: observed values
○: the difference between the observed values and the values of $B \exp(-\beta t)$

elimination can not be analyzed with a single compartmental model. Taking account of the following that the semilogarithmic plots for the later time can be assumed linear, the elimination was analyzed with the two compartmental model as shown in Chart 1. Assuming that after the fifth point the semilogarithmic points are linear and putting 5 as M into one of the input data (see text), the four parameters of A, B, α and β in Eq. 1 were tried to be obtained statistically with a digital computer, treating them independent. The four parameters were converged well sufficiently after eight or nine iterations as shown in Table II. And the final values were shown in Table III, with the standard errores.

Table II. Adjustment of Parameters with Iterations

Number of iterations	A	B	α	β
0	0.50121405 E−01	0.92539787 E−01	0.18893519 E+00	0.21068294 E−01
1	0.54504016 E−01	0.12055476 E+00	0.53361228 E+00	0.27189456 E−01
2	0.75203456 E−01	0.10464792 E+00	0.77162483 E+00	0.24475213 E−01
3	0.75518443 E−01	0.10445968 E+00	0.77991592 E+00	0.24174343 E−01
4	0.75538922 E−01	0.10444008 E+00	0.77965287 E+00	0.24164317 E−01
5	0.75540093 E−01	0.10443886 E+00	0.77960790 E+00	0.24163775 E−01
6	0.75540179 E−01	0.10443877 E+00	0.77960362 E+00	0.24163738 E−01
7	0.75540186 E−01	0.10443876 E+00	0.77960326 E+00	0.24163735 E−01
8	0.75540186 E−01	0.10443876 E+00	0.77960323 E+00	0.24163735 E−01
9	0.75540187 E−01	0.10443876 E+00	0.77960322 E+00	0.24163735 E−01
10	0.75540187 E−01	0.10443876 E+00	0.77960322 E+00	0.24163735 E−01

A, B, α and β are hybrid parameters as in Eq. (2)—(6), and truely important parameters for pharmacokinetics are k_{12}, k_{21}, etc. which are described with A, B, α and β as the following.

$$V_1=\frac{\text{Dose}}{A+B} \tag{11}$$

$$k_{21}+k_{20}=\frac{A\beta+B\alpha}{A+B} \tag{12}$$

$$k_{12}+k_{10}=\frac{A\alpha+B\beta}{A+B} \tag{13}$$

$$k_{10}k_{20}+k_{10}k_{21}+k_{12}k_{20}=\alpha\beta \tag{14}$$

$$k_{12}+k_{21}+k_{10}+k_{20}=\alpha+\beta \tag{15}$$

The above new parameters, however can not be obtained from Eq. (11)—(15), except in the following two cases.

Case 1. k_{20} can be neglected.

$$k_{21}=\frac{A\beta+B\alpha}{A+B}$$

$$k_{el}=\alpha\beta/k_{21}$$

$$k_{12}=(\alpha+\beta)-k_{21}-k_{el}$$

where the symbol of k_{el} was used instead of k_{10} to describe the physiological meaning of elimination.

Case 2. k_{10} can be neglected.

$$k_{12}=\frac{A\alpha+B\beta}{A+B}$$

$$k_{el}=\alpha\beta/k_{12}$$

$$k_{21}=(\alpha+\beta)-k_{12}-k_{el}$$

where the symbol of k_{el} was used instead of k_{20} to describe the physiological meaning of elimination. The new parameters calculated with the above equations corresponding each case in the computer were used as the initial approximate values to obtain the statistical values, solving the normal equation witn the same computing process as for A, B, α and β. As the results, the statistical values of the pharmacokinetic constants k_{12}, k_{21}, k_{el} and V_1[14] which are essentially important for the two compartmental model analysis were obtained, after long computing process of the least square method which would not be possible without a digital computer technique. The values are listed in Table III.

Table III. Best Parameters for the Two Compartmental Model with Their Standard Errores

	(1)[a]	S.E.[c]	(2)[b]	S.E.[c]
A[d]	0.0755	0.0036	0.0813	0.0127
B[d]	0.104	0.003	0.0978	0.0045
α[e]	0.780	0.125	0.589	0.174
β[e]	0.0242	0.0010	0.0222	0.0010
	Case 1		Case 1	
k_{12}[e]	0.301	0.046	0.240	0.080
k_{21}[e]	0.463	0.080	0.332	0.099
k_{el}[e]	0.0407	0.0012	0.0393	0.0027
V_1[f]	1680	20	1690	110
V_2[f]	1090	250	1220	560
	Case 2		Case 2	
k_{12}[e]	0.341	0.047	0.280	0.082
k_{21}[e]	0.407	0.077	0.285	0.096
k_{el}[e]	0.0552	0.0042	0.0467	0.0055
V_1[f]	1680	20	1690	110
V_2[f]	1410	330	1660	750

a) σ of observed concentration is supposed to be constant.
b) σ of observed concentration is supposed to be proportional to the cocentcentration.
c) standard error
d) μmole/ml
e) min^{-1}
f) ml

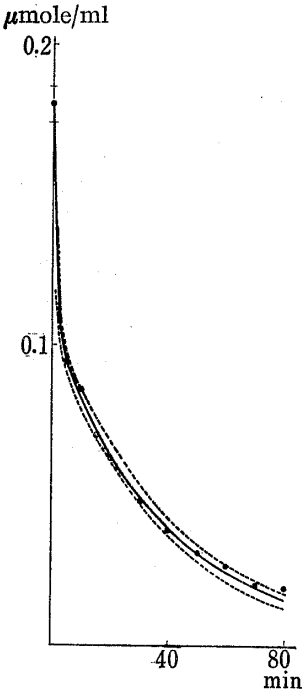

Fig. 2. Disappearance of Ethoxybenzamide from Plasma

——: calculated
I, ------: limits of 95% confidence
●: observed values

Since the standard error of each point (σ_y) can be calculated with Eq. (10), the limits of 95% confidence can be given with Eq. (16).

$$\text{confidence limit} = \sigma_y \times t_{95} \tag{16}$$

The computer programm was also designed to give the limits with the calculated standard errores of parameters and the elements of the inverse matrix of the normal equation. The calculated and observed values are shown in Fig. 2 with 95% confidence limits.

Discussion

In the present method the only one personal estimation is used to determine M, the number of the datum after which the semilogarithmic plots can be assumed straight. In

14) $V_2 = k_{12}V_1/k_{21}$ The variance of V_2 ($\sigma_{V_2}^2$) can be calculated with the following equation.
$$\sigma_{V2}^2 = (\partial V_2/\partial k_{12})^2\sigma_{k12}^2 + (\partial V_2/\partial V_1)^2\sigma_{V1}^2 + (\partial V_2/\partial k_{21})^2\sigma_{k21}^2$$
$$= (V_1/k_{21})^2 \text{ (standard error of } k_{12})^2 + (k_{12}/k_{21})^2 \text{ (standard error of } V_1)^2$$
$$+ (k_{12}V_1/k_{21}^2)^2 \text{ (standard error of } k_{21})^2 \quad \text{(standard error of } V_2) = \sigma_{V2}.$$

order to know the effect of M on the final converged values of parameters, the different values of M were put into the data. Since the value of 5 was used in the results section, the values of 3 and 6 were checked. The calculated results were written in Table IV. They gave the same converged values. On the other hand, the first approximate values which were obtained with the equivalent method to the usual geometrical one and are listed in the column of the iteration number zero in Table IV, are rather different each other and different from the final results. This means that the geometrical method gives rather arbitrary results but the present method can give the definite results which are independent of a personal estimation.

Table IV. Effect of the Number of M[a] on the Parameters

M		Iteration No. 0	Converged
3	A[b]	0.080147184	0.075540187
	B[b]	0.099852810	0.10443876
	α[c]	0.68725491	0.77960322
	β[c]	0.022370912	0.024163735
5	A[b]	0.050121405	0.075540187
	B[b]	0.092539787	0.10443876
	α[c]	0.18893519	0.77960322
	β[c]	0.021068294	0.024163735
6	A[b]	0.052366527	0.075540187
	B[b]	0.089661037	0.10443876
	α[c]	0.16767883	0.77960322
	β[c]	0.020553835	0.024163735

a) the number of the datum after which the semilogarithmic plots can be assumed straight.
b) μmole/ml c) min^{-1}

The assumption that the variance of y (observed concentration) is constant was adopted for the calculation in the results section, but on the other hand, there is another probable assumption that the variance of y is proportional to y^2 (the standard error of y is proportional to y). The results which came from the latter assumption were calculated with the simple modification of the programm, putting $1/y^2$ into ω of Eq. (9) and are written in Table III.

Since the results are somewhat different from those of the former assumption, the firm standpoint for the variance is necessary for the calculation. As for the present study the authors stand on the same variance assumption according to the experimental procedure. If proper dilutions of sample, different standard curves or different sensitivities for the determination in one experimental run are taken, depending on the sample concentration, the other assumption would be recommended.

More than thirty sets of data[15] were obtained for several different rabbits and doses, and most of them were found to follow the two compartmental model, but there were some exceptional examples. One example is shown in Fig. 3. Although it did not evidently follow the model, the calculation with the present method was tried. In the case of the constant variance, the results did not converge. On the other hand, the converged parameters were obtained in the case of the proportional variance. But even in the latter case one of the converged parameters which must be positive was negative as shown in Table V. It means in any case that the computer can tell the adoptability of the two compartmental model.[16] Another example is shown in Fig. 4. It was not impossible to draw straight lines both in the latter and earlier period though rather arbitrarily, and seemed to follow the two compartmental

15) They will be published.
16) It may be the explanation that the data were of the first experiment and the experience for the technique was not enough.

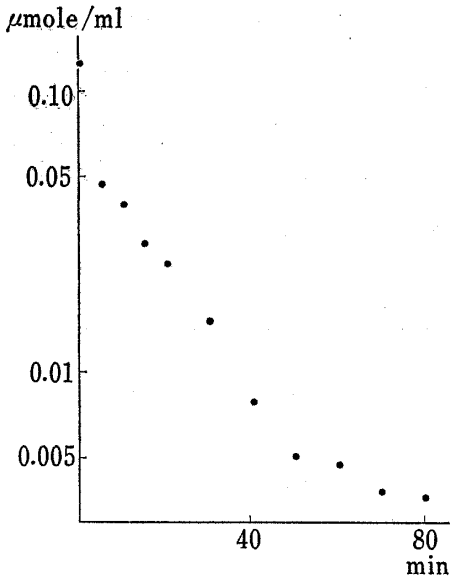

Fig. 3. Semi Logarithmic Plots of Disappearance of Ethoxybenzamide from Plasma

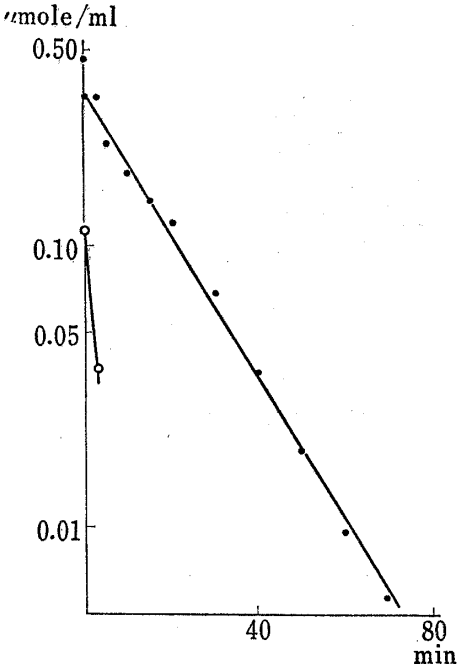

Fig. 4. Semi Logarithmic Plots of Disappearance of Ethoxybenzamide from Plasma

Lines were obtained from a simple linear regression equation.
●: observed values
○: the difference between the observed values and the values of $B \exp(-\beta t)$

TABLE V. Calculated Parameters from the Data in Fig. 3 and 4

	Fig. 3[a,f]	S.E.[c]	Fig. 4[b,f]	S.E.[c]
A[d]	0.00223	0.00436	0.119	0.044
B[d]	0.0772	0.0099	0.344	0.020
α[e]	−0.00472	0.0243	0.383	1.16
β[e]	0.0670	0.0154	0.0582	0.0013

a) Dose is 50 mg (302.7 μmole). *b*) Dose is 200 mg (1211 μmole).
c) standard error *d*) μmole/ml *e*) min^{-1}
f) σ of observed concentration is supposed to be proportional to the concentration.

model. But the constant variance assumption did not give any converged results, while the other gave the converged parameters in Table V. But, as seen easily from the large standard error, it was found that the data could not or not well enough be fitted to the equation for the model.

Then, it was concluded that the present statistical method can give the essential parameters for the two compartmental model with the standard errores and can test the adoptability of the data for the model.

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