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Pharmacokinetic model for the successive demethylation and biliary secretion of methyl orange in the rat

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Summary

1. A one-compartment pharmacokinetic model was developed in which a drug underwent two successive metabolical reactions (for example, metabolism followed by conjugation) and free drug and both metabolites were excreted.

2. Techniques were described whereby graphical estimates of the first-order rate constants may be derived from cumulative excretion data on the drug and its metabolites. Computer simulation techniques were used to show that the experimental data permit reasonably accurate estimation of the rate constants of the model by graphical and computer methods.

3. Tritiated methyl orange (2 mg) was administered to five groups of six rats with biliary cannulation. The bile produced by each animal was collected at hourly intervals for 6 h and the amounts of methyl orange and its metabolites, 4'sulpho-4-methylaminoazobenzene and 4'sulpho-4-aminoazobenzene, determined by thin layer chromatography and radioactive counting techniques.

4. The data were analysed graphically and with an iterative digital computer programme to yield the first-order rate constants for the successive demethylation steps in the metabolism of methyl orange. The removal of the first methyl group had a rate constant of $0{\cdot}684 \pm 0{\cdot}142\ h^{-1}$ ($\pm$S.D.) and the second methyl group $1{\cdot}00 \pm 0{\cdot}302\ h^{-1}$. The rate constant for biliary excretion of the free methyl orange was $0{\cdot}164 \pm 0{\cdot}042\ h^{-1}$, for the monomethyl derivative $0{\cdot}672 \pm 0{\cdot}461\ h^{-1}$, and for the demethylated metabolite $6{\cdot}413 \pm 3.222\ h^{-1}$.

Introduction

In recent years considerable work has appeared on pharmacokinetics in man (Wagner, 1968). Most studies have considered systems in which drug metabolism is represented as a single first-order process, for example, the acetylation of sulphonamides (Nelson & O'Reilly, 1960; Yamazaki, Aoki & Kamada, 1968). Somewhat more complex systems, in which several metabolites are produced by parallel first-order processes, have been studied by Cummings, King & Martin (1967) with paracetamol, and by Nogami, Hasegawa, Hanano & Imaoka (1968) with sulphonamides. The most common biochemical path for drug metabolism involves two successive reactions: first, metabolical alteration, and then conjugation of the metabolite (Williams, 1959). In this paper a pharmacokinetic model of systems of this type will be described. To illustrate the experimental utility of the model, use will be made of the metabolism and elimination of methyl orange. The

dye, methyl orange, when injected into the rat, undergoes successive demethylation to give 4'sulpho-4-methylaminoazobenzene and 4'sulpho-4-aminoazobenzene. The dye and its metabolites are excreted to a considerable extent in the bile (Barrett, Pitt, Ryan & Wright, 1966).

Methods

Experimental

Metabolic experiments

Tritiated methyl orange was prepared as previously described (Barrett *et al.*, 1966). White male rats (300–400 g) were cannulated to allow collection of bile. Dye (2 mg in aqueous solution) was injected intravenously into a femoral vein and bile collected at hourly intervals up to 6 h after administration. A small dose was used to avoid possible overload of the biliary transport mechanism which occurs with larger doses of dye (Priestly, 1967). In each experiment, six rats were used in order to obtain enough material to permit accurate analyses. The bile collections for each time interval were bulked. Five experiments were carried out. The metabolites and free methyl orange were separated by thin layer chromatography on silica gel with ethyl acetate-ethanol (2:1) solvent. The spots were eluted with methanol and radioactive counting carried out as previously described (Barrett *et al.*, 1966). The amounts of dye and metabolite were calculated as percentage radioactivity recovered setting the initial dose of methyl orange at 100%.

Theory

Derivation of the model

The complexity of models developed for pharmacokinetic purposes is largely dependent on the number of compartments conceived to represent the internal environment of the body (Rescigno & Segre, 1966). There has been considerable argument over the relative merits of one- and two-compartment systems (Riegelman, Loo & Rowland, 1968; Wagner & Metzler, 1969). Here it is assumed that the 'best' model is the simplest giving a good fit of the experimental results which the model is intended to describe. The relationship between the model parameters and events in the biological system described is another question and an attempt will be made to discuss this later.

The model (Fig. 1) presents the metabolism of methyl orange; it could also be applied to other demethylation systems (McMahon, Culp & Marshall, 1965) and to metabolical systems in which metabolism is followed by conjugation. The simplest

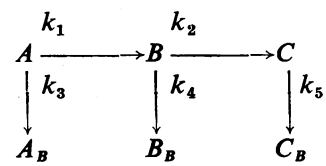

FIG. 1. One-compartment model for two successive metabolic steps. A represents the amount of methyl orange in the compartment, B and C the amounts of metabolites successively produced from A in the same compartment. A_B, B_B and C_B represent the amounts of methyl orange and metabolites excreted in the bile. k_1 and k_2 are first order rate constants of the first and second demethylation, respectively. k_3, k_4 and k_5 are first order rate constants for the biliary excretion of methyl orange and metabolites.

model for these processes assumes that the body (or metabolic system) can be considered as one compartment of constant volume in which drug is absorbed instantaneously (i.v. injection) and distributed evenly throughout the whole course of elimination. A represents the amount of methyl orange in the central compartment, B and C the amounts of metabolites successively produced from A in the same compartment. A_B, B_B and C_B represent the amounts of methyl orange and metabolites excreted from the body compartment via the bile. All metabolical and excretory reactions are assumed to be simple first-order reactions; k_1 and k_2 are assigned as rate constants for the first and second demethylations.

The following system of differential equations describes the model :

$$\frac{dA}{dt} = -(k_1+k_3)A = -KA \qquad (1) \text{ where } K=k_1+k_3$$

$$\frac{dB}{dt} = k_1A-(k_2+k_4)B \qquad (2)$$

$$\frac{dC}{dt} = k_2B-k_5C \qquad (3)$$

$$\frac{dA_B}{dt} = k_3A \qquad (4)$$

$$\frac{dB_B}{dt} = k_4B \qquad (5)$$

$$\frac{dC_B}{dt} = k_5C \qquad (6)$$

If equation (1) is integrated and the constant of integration evaluated for the condition when $t=0$, $A=A_o$ equation 7 is obtained. A_o is the amount of free drug initially in the central compartment.

$$A=A_oe^{-Kt} \qquad (7)$$

The value of A (equation 7) is substituted into equation 2 and the result integrated by standard methods (Thomas, 1957) equation 8 results.

$$B= \frac{k_1A_o}{k_2+k_4-K}\left(e^{-Kt}-e^{-(k_2+k_4)t}\right) \qquad (8)$$

To obtain a value for C, equation 8 is substituted into equation 3 and the equation integrated as with (8) to yield:

$$C= \frac{k_1k_2A_o}{k_2+k_4-K}\left[\frac{e^{-(k_2+k_4)t}}{k_2+k_4-k_5}-\frac{e^{-Kt}}{K-k_5}+\frac{(k_2+k_4-K)e^{-k_5t}}{(K-k_5)(k_2+k_4-k_5)}\right] \qquad (9)$$

If the values of A, B and C (equations 8, 9 and 10), respectively, are substituted into equations 4, 5 and 6, then these equations may be integrated by standard methods to give equations 10, 11 and 12, which describe the cumulative excretion of A, B and C into the external compartment.

$$A_B= \frac{k_3A_o}{K}(1-e^{-Kt}) \qquad (10)$$

$$B_B= \frac{k_1k_4A_o}{k_2+k_4-K}\left[\frac{1}{K}(1-e^{-Kt})-\frac{1}{(k_2+k_4)}\quad(1-e^{-(k_2+k_4)t})\right] \qquad (11)$$

$$C_B = \frac{k_1 k_2 k_5 A_o}{k_2+k_4-K}\left[\frac{(1-e^{-(k_3+k_4)t})}{(k_2+k_4)(k_2+k_4-k_5)}+\frac{(1-e^{-Kt})}{K(k_5-K)}-\frac{(k_2+k_4-K)(1-e^{-k_5 t})}{k_5(k_5-K)(k_2+k_4-k_5)}\right] \quad (12)$$

Analysis of data

The experimental data from which rate constants may be derived consist of either body compartment levels (A, B and C) or cumulative excretion measurements (A_B, B_B and C_B). In this paper a method is developed to obtain the rate constants of the model from cumulative excretion data.

Estimates of the rate parameters may be obtained by graphical analysis of the data. If the value of A in equation 7 is substituted into equation 4 we obtain:

$$\frac{dA_B}{dt} = k_3 A_o e^{-Kt} \quad (13)$$

Taking logarithms this yields:

$$\log \frac{dA_B}{dt} = \log k_3 A_o - \left(\frac{K}{23{\cdot}03}\right)t \quad (14)$$

Therefore, if the log rate of excretion of A_B (free drug) is plotted versus time, a straight line should result with slope $K/2{\cdot}303$. A plot to illustrate this is shown in Fig. 2(a). The data are plotted as log rate of biliary secretion of the respective compounds against the mid point of each collection period (Wagner, 1963). Cummings, Martin & Park (1967) proposed an identical method of plotting in their treatment of the one-compartment model for drug elimination. K, the rate constant for elimination for free drug, may be partitioned into its component constants k_1 and k_3 if the total amounts of drug and metabolites can be estimated. Inspection of the model (Fig. 1) indicates that the path followed by A is totally determined by the relative values of k_1 and k_3. At infinite time, following Nelson & O'Reilly (1960), we may write:

$$f = \frac{\overset{\infty}{A_B}}{\overset{\infty}{A_B}+(\overset{\infty}{B_B}+\overset{\infty}{C_B})} = \frac{k_3}{k_1+k_3} = \frac{k_3}{K} \quad (15)$$

so $k_3 = fK$ and $k_1 = K-k_3$

where f is the fraction of drug excreted unchanged.

Substitution of the value for B (equation 8) into equation (5), yields a rate equation for the excretion of B_B (equation 16).

$$\frac{dB_B}{dt} = \frac{k_1 k_4 A_o}{k_2+k_4-K}(e^{-Kt}-e^{-(K_3+K_4)t}) \quad (16)$$

When $\log dB_B/dt$ is plotted versus time, the resultant curve will rise to a maximum, associated with the build up of B in the body compartment, and thereafter decline. K is already known from the plot described by equation 14 and the problem is to obtain an estimate of k_2+k_4. As t becomes large the curve will tend to become linear and the slope of the linear portion will depend on the relative values of K and

k_2+k_4 (Cummings, Martin & Park, 1967). If $K>k_2+k_4$, when t is large e^{-Kt} will tend towards zero and the terminal slope will give k_2+k_4. Equation 16 becomes:

$$\log \frac{dB_B}{dt} = \log \frac{k_1k_4A_o}{K-(k_2+k_4)} - \frac{k_2+k_4}{2{\cdot}303}\, t \qquad (17)$$

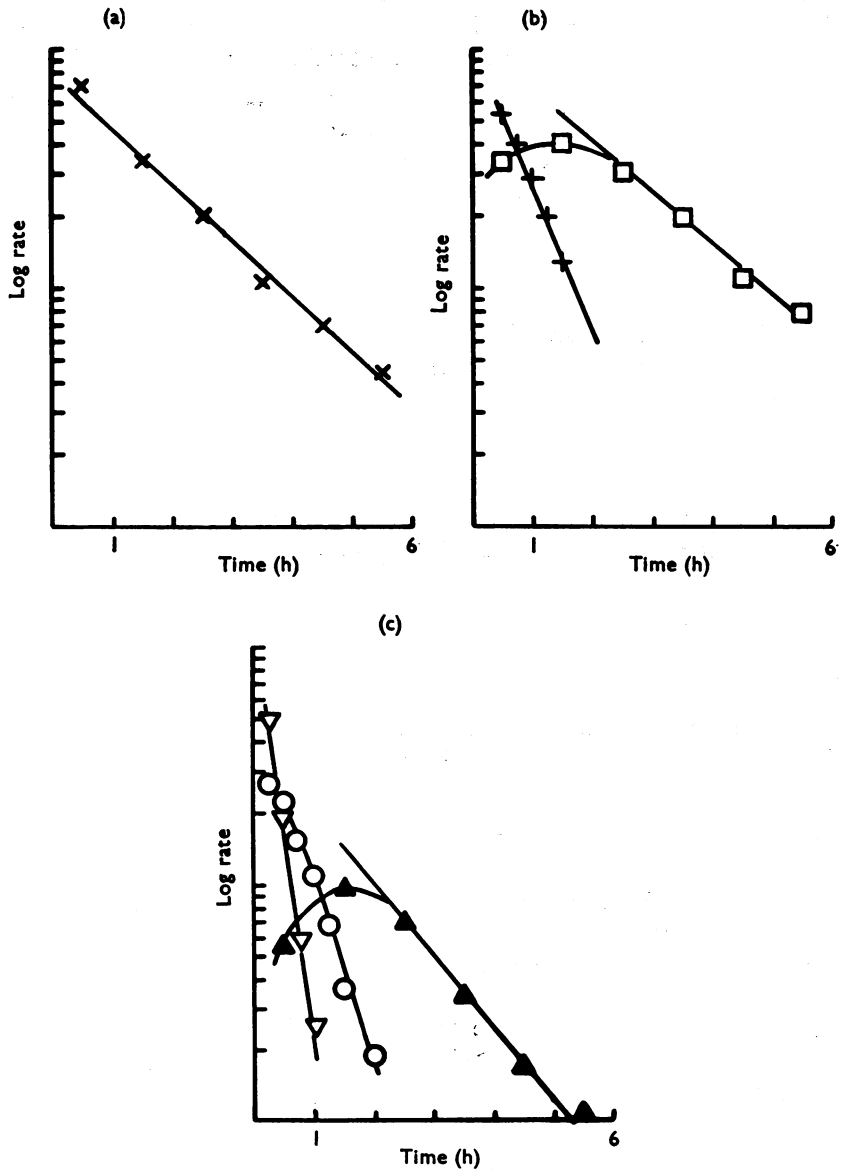

FIG. 2. Graphical analysis of data from rat group 1. (a), Plot of log rate (% dose/h) of excretion of free methyl orange (A_B); slope gives estimate of K (×). (b), Plot of log rate of excretion of monodemethylated metabolite (B_B) (□). Primary linear slope gives K estimate, slope of residuals line (+) gives k_2+k_4. (c), Plot of log rate of excretion of demethylated metabolite (C_B) (▲) gives K, and the estimates of k_2+k_4 and k_5 are obtained from the peeled residuals from the primary curve (k_2+k_4, ○ and k_5 ▽). All plots are constructed using the mid point of the collection interval on the time axis.

When $k_2+k_4>K$, equation 16 becomes:

$$\log \frac{dB_B}{dt} = \log \frac{k_1k_4A_0}{k_2+k_4-K} - \frac{K}{2{\cdot}303}\,t \tag{18}$$

and the slope of the terminal part of the line will give K (Fig. 2b). In the second situation an estimate of k_2+k_4 may be obtained in two ways. The linear portion of the curve may be projected back to intersect the ordinate, the residuals between this line and the curve are plotted and k_2+k_4 estimated from the slope of the residuals curve (Fig. 2b). At infinite time, the total amounts of metabolites excreted may be denoted by $\overset{\infty}{B}_B$ and $\overset{\infty}{C}_B$ and the ratio (r) between these amounts is obtained by dividing equation 12 by equation 13. At infinite time, the exponents all approach zero and after simplification, equation 19 is obtained, thus

$$\frac{\overset{\infty}{B}_B}{\overset{\infty}{C}_B} = \frac{k_4}{k_2} = r \tag{19}$$

This equation permits k_2 and k_4 to be calculated from the estimated value of k_2+k_4. Equation 19 may be used to determine k_2 and k_4 by another method using the value of the intercept of equation 18. If equation 19 is cross multiplied and k_2 added to both sides equation 20 results:

$$k_2+k_4=k_2(1+r) \tag{20}$$

If this value is substituted into the intercept of equation 18 and rk_2 (from equation 19) is substituted for k_4 in the same equation then the intercept becomes:

$$\log \frac{rk_1k_2A_0}{K-k_2(1+r)}$$

and k_2 (and k_4) may be calculated.

If the value for C (equation 9) is substituted into equation 6, equation 21 is obtained and an estimate of the value of k_5 may be made:

$$\frac{dC_B}{dt} = \frac{k_1k_2k_5A_0}{k_2+k_4-K}\left[\frac{e^{-(k_2+k_4)t}}{k_2+k_4-k_5} - \frac{e^{-Kt}}{K-k_5} + \frac{(k_2+k_4-K)e^{-k_5t}}{(K-k_5)(k_2+k_4-k_5)}\right] \tag{21}$$

All the constants in this equation are known except k_5. A semi-log plot of the rate of C_B excretion versus time will give a curve with the terminal portion tending towards linearity with a slope related to the smallest of the three exponents in equation 22. The lowest value is obtained from the linear terminal line and the other values derived by progressive 'peeling' of the curve, the largest exponent being extracted last. If $k_2+k_4>K>k_5$ then at large values of t the equation will have the form:

$$\log \frac{dC_B}{dt} = \log \frac{k_1k_2k_5A_0}{(K-k_5)(k_2+k_4-k_5)} - \frac{k_5}{2{\cdot}303}\,t \tag{22}$$

and the terminal portion of the curve will be linear and the slope will yield k_5. If either k_2+k_4 or K is less than k_5, then k_5 may be estimated by an appropriate peeling of the curve. This procedure is illustrated in Fig. 2c. The peeling of tri-exponential curves is an inaccurate procedure but it must be remembered that the values

obtained in this way are used as input for an iterative digital computer programme intended to produce refined parameter values. In the case $k_5 > (k_2 + k_4) > K$, curve peeling may fail to give an estimate of k_5 but the equation of the linear portion of the rate curve will be (from equation 21),

$$\log \frac{dC_B}{dt} = \log \frac{k_1 k_2 k_5 A_o}{(k_2 + k_4 - K)(k_5 - K)} - \frac{Kt}{2{\cdot}303} \qquad (23)$$

and k_5 may be calculated from the value of the ordinate intercept since k_1, k_2, k_3, k_4 and A_o are known.

Graphical estimation of methyl orange parameters. Cumulative excretion curves were constructed for the data of each group of rats and estimates were made for A_o. The dose input into the model system was not 100% of the dye injected; some of the dye is excreted in the urine and some disappears to unknown sites of loss. The model accounts for that part of the dose emerging in the bile as radioactive dye and metabolites. The dose input (A_o) to the model compartment is estimated as total recovery of dye and metabolites in the bile. This method is analogous to the estimation of dose absorbed in the treatment of orally absorbed drug (Cummings, King & Martin, 1967), where the amount of dose absorbed into the body system is taken to equal the total drug recovered in the urine at infinite time. Unfortunately, it is not possible to determine the amount of dye and metabolites excreted in the bile at infinite time because bile collection cannot be carried on for a sufficiently long period of time (about ten half lives). The total amount of dye and each metabolite collected ($\overset{\infty}{A}_B$, $\overset{\infty}{B}_B$ and $\overset{\infty}{C}_B$) were estimated by extrapolation of the cumulative excretion curve for each component. These extrapolated estimates were combined to give an estimate of total dye output which was used as A_o in the computer input and refined along with the other parameters by the computer programme. The values of f and r were estimated along with A_o (Table 1). The rate constants were estimated by graphical analysis as explained in the previous section. The method used is illustrated in Fig. 2 for rat group 1 and the parameter estimates for each rat group are shown in Table 1.

Computational techniques

The digital computer programme (NONLIN, kindly supplied by Dr. Carl Metzler, The Upjohn Co., Kalamazoo, Michigan) was modified to run on the IBM 360-65 computer in the Computer Centre, University of Manitoba. A subroutine was included in the programme to define the parameters of the model (the rate constants and A_o, the dose input) and the equations for cumulative excretion of dye

TABLE 1. *Initial parameter estimates for the metabolism and biliary secretion of methyl orange by the rat*

Rat group	k_1 h^{-1}	k_2 h^{-1}	k_3 h^{-1}	k_4 h^{-1}	k_5 h^{-1}	A_o % Dose excreted	f	r
1	0·52	1·55	0·18	0·35	4·00	60·0	0·26	0·21
2	0·68	1·25	0·18	0·45	4·00	60·0	0·26	0·21
3	0·56	1·35	0·16	0·35	4·00	60·0	0·26	0·21
4	0·57	1·54	0·14	1·50	6·40	50·0	0·20	0·98
5	0·67	1·24	0·17	1·40	6·80	50·0	0·20	0·98

Estimated rate constants were obtained by graphical analysis of biliary secretion curves, and f and r by extrapolated estimates of A_B, B_B and C_B.

and metabolites (A_B, B_B and C_B, equation 10–12). The programme is intended to estimate the parameters of a system of non-linear functions when the data are observations of the functions. To operate the programme, estimated values of the parameters (Table 1) and the cumulative excretion data A_B, B_B and C_B at the stated time intervals were used as input. The computer uses the equations 10–12 and the parameter values to estimate values of A_B, B_B and C_B at the stated time intervals. An iterative process of non-linear regression is carried out (Hartley & Booker, 1965). The parameter values are varied until the sums of squares of the residuals between the observed and calculated points are at a minimum. When this minimum is obtained, the programme is considered to have obtained a 'best fit' in terms of correspondence between theoretical and observed values for the data. Parameter values for the line of best fit are printed together with statistical information of their errors. The standard deviations of the parameters are computed in the programme by a method modified from that of Edwards Deming (1946).

Results

Simulation studies

Before any but the simplest pharmacokinetic scheme is applied to experimental data, two matters should be examined. The reliability of the parameter estimation method should be tested over a wide range of experimental possibilities. Further, when experimental data are obtained, the collection times or analytical limitations often restrict the number of points that are available to define a curve. The model

TABLE 2. *Computer simulation studies on the model for methyl orange demethylation and biliary secretion*

Simulation set	k_1 h^{-1}	k_2 h^{-1}	k_3 h^{-1}	k_4 h^{-1}	k_5 h^{-1}	K h^{-1}	A_0 % Dose excreted
(a) Assigned values							
1	0·40	1·00	0·30	0·40	0·50	0·70	100
2	1·00	0·40	0·30	0·40	0·50	1·30	100
3	0·30	0·60	0·80	1·00	1·20	1·10	100
4	0·60	0·30	0·80	1·00	1·20	1·40	100
5	0·30	0·60	1·20	0·40	0·60	1·50	100
6	0·60	0·30	1·20	0·40	0·60	1·80	100
7	1·00	2·00	0·30	0·50	0·80	1·30	100
8	0·50	0·92	0·16	0·45	4·00	0·66	100
(b) Graphical values							
1	0·40	0·97	0·30	0·38	0·30	0·70	101·2
2	0·99	0·75	0·29	0·75	0·44	1·28	95
3	0·30	0·74	0·80	1·15	0·91	1·10	103·5
4	0·57	0·37	0·83	1·50	1·53	1·40	96·5
5	0·30	0·84	1·18	0·60	0·95	1·48	100·5
6	0·63	0·70	1·21	0·92	0·83	1·84	101
7	1·26	1·88	0·40	0·49	1·23	1·66	102
8	0·49	0·86	0·16	0·41	4·52	0·65	105
(c) Computer values							
1	0·40±0·004	0·89±0·030	0·31±0·003	0·36±0·006	0·53±0·02	0·71	99·8±0·40
2	1·01±0·054	0·39±0·020	0·31±0·020	0·41±0·02	0·58±0·04	1·32	97·9±0·58
3	0·31±0·002	0·59±0·017	0·80±0·004	0·94±0·02	1·29±0·20	1·11	100·7±0·12
4	0·59±0·001	0·31±0·004	0·80±0·003	1·09±0·03	1·40±0·04	1·39	99·4±0·10
5	0·30±0·002	0·63±0·023	1·20±0·004	0·42±0·001	0·60±0·03	1·50	99·8±0·07
6	0·60±0·016	0·30±0·020	1·21±0·018	0·41±0·003	0·65±0·09	1·81	99·7±0·14
7	0·98±0·020	2·29±0·037	0·29±0·004	0·57±0·02	0·76±0·01	1·27	100·4±0·37
8	0·50±0·020	0·92±0·092	0·16±0·01	0·44±0·06	4·16±0·56	0·66	100·0±0·18

Assigned values (a) are the values used as input to generate theoretical model curves. Graphical values (b) are parameter values obtained by graphical analysis of the model curves and were used as computer input to obtain computer refined or computer values (c) which are shown ± standard deviation of the parameter.

may be tested in simulated situations to determine if it can furnish useful information (that is, values of parameters) under the conditions imposed by experimental necessity.

The factors above may be evaluated by simulation studies in which parameters are substituted in the equations of the model to generate artificial data. The data thus generated may be subjected to graphical analysis to determine the accuracy of parameter estimation. These estimates may be used as computer input data to determine if the iterative programme is able to converge on the correct parameter value (Wagner, 1968). By this means, the reliability of the estimation method may be evaluated. It is possible to obtain simulated data for any number of data points. In any experimental situation, only a limited amount of data can be collected and it is useful to know the minimum number of data points required to yield useful information. Simulation studies can be used to establish such guidelines in experimental design. In the work described here the data points were limited by the necessities of the analytical method to six collections made at hourly intervals. From each collection of bile, three data points (A_B, B_B and C_B) were obtained. Simulations were designed to determine if such data would give useful measures of parameter values. For this purpose, possible parameter values were selected and are shown as 'assigned values' (Table 2a). From the 'assigned values' and equations 10–12, artificial data for A_B, B_B and C_B were generated for $t=1, 2, 3, 4, 5$ and 6 h corresponding to the experimental collection points. These data were plotted and a curve drawn. 'Experimental' points were selected along the curves with errors of up to $\pm 5\%$ off the line in order to simulate more closely practical conditions. The 'experimental' data thus obtained, were processed by graphical analysis as described earlier. The parameters estimated by graphical methods are shown in Table 2b. These values illustrate the hazards of graphical analysis. The constant K (k_1+k_3) is obtained from the slope of a single exponential and generally the error in its determination is quite small (except set 7 where random errors introduced quite a large error in k_1 and k_3). k_2+k_4 was frequently an overestimate particularly in those sets where $k_2+k_4>K$ and the constant was evaluated by peeling a curve with few experimental points to define it. The greatest error tends to arise in situations where $K \approx k_2+k_4$ ($\approx k_5$) (set 3), assigned values K 1·1 (1·1), k_2+k_4 1·6 (1·89) and k_5 1·2 (0·91) (the assigned values are followed by the graphical values in brackets), or set 4, K 1·4 (1·40), k_2+k_4 1·3 (1·87) and k_5 1·2 (1·53). As pointed out by Riggs (1963), it is very difficult to separate terms with similar exponents by graphical methods.

TABLE 3. *Pharmacokinetic parameters for the demethylation and biliary secretion of methyl orange in the rat*

Rat group	k_1 h^{-1}	k_2 h^{-1}	k_3 h^{-1}	k_4 h^{-1}	k_5 h^{-1}	A_0 % Dose excreted
1	0·503±0·047	0·924±0·190	0·164±0·014	0·439±0·086	4·158±1·329	59·584±1·022
2	0·749±0·130	1·029±0·300	0·165±0·027	0·493±0·132	2·542±0·939	59·586±1·028
3	0·728±0·079	0·674±0·086	0·140±0·013	0·287±0·037	7·806±0·939	57·392±0·544
4	0·579±0·094	1·489±0·737	0·142±0·025	1·458±0·702	6·739±3·720	49·905±1·620
5	0·861±0·274	0·882±0·402	0·207±0·059	0·684±0·302	10·819±5·245	52·948±1·351
Average	0·684±0·142	1·000±0·302	0·164±0·024	0·672±0·461	6·413±3·222	55·90 ±4·30

k_1 and k_2 are the rate constants of the first and second demethylation, respectively. k_3, k_4, k_5 are the rate constants for the biliary secretion of methyl orange and its metabolites. A_0 is the estimated total amount of dye finally excreted in the bile. Parameter values are quoted ±S.D. and the average value ±S.D.M.

Inspection of the assigned and graphical parameter values (Table 2a and b) reveals that if the graphical method of parameter determination is used the values obtained are markedly in error (especially for k_2, k_4 and k_5). In this study, it is intended that the graphical values be used as input into a computer programme to

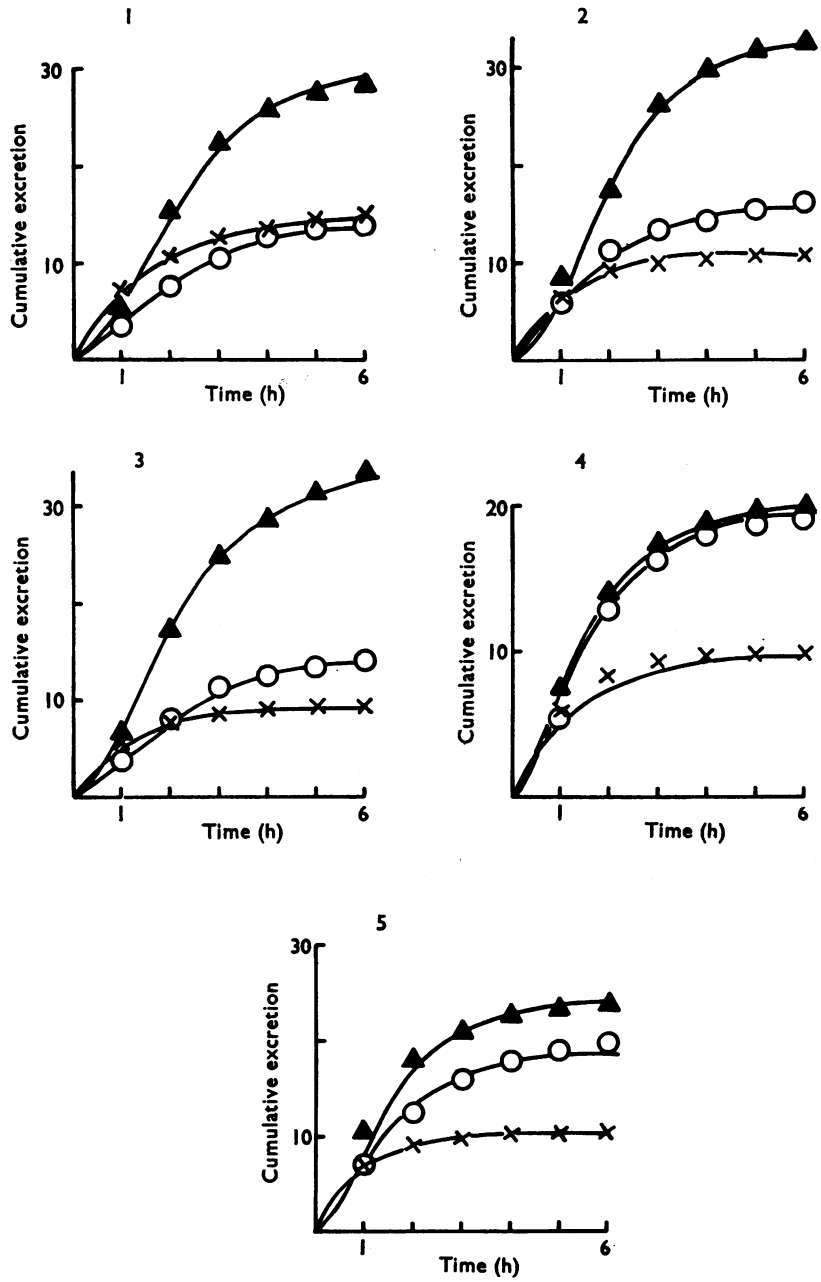

FIG. 3. Cumulative excretion plots for methyl orange and its metabolites in the rat. Each graph (1–5) represents a single group of rats. Cumulative excretion is the cumulative percentage of initial dose recovered in the bile as methyl orange (×), the monomethyl metabolite (○) and the demethylated metabolite (▲). The solid line is the computer generated line of best fit.

determine refined parameter values by an iterative procedure. The computer values are shown in Table 2c. In each case the computer analysis has produced parameter values very close to the assigned value. With K the results are excellent; for example in set 7, K 1·30, 1·66, 1·26; assigned, graphical and computer values. Values of k_2+k_4 were improved even more; in set 2, 0·8 gave 1·5 on graphical solution to give 0·794 as the computer value. The data in Table 1 confirm that computer analysis will give reliable estimates of parameters.

The main interest of this study is focussed on k_1 and k_2, the rate constants for the removal of the first and second methyl groups respectively, but the biliary excretion constants are also of interest. The first two of these k_3 and k_4 are obtained from K and k_2+k_4 and as seen above good values can be obtained for these parameters and hence for k_3 and k_4. The last excretory parameter k_5 is the most difficult to obtain graphically except in the special case where k_2+k_4 and K both $\gg k_5$. Elsewhere the estimation of k_5 involves the peeling of a triexponential curve and when k_5 is large the resultant estimation is inaccurate. Inspection of assigned and computer values for k_5 (Table 2a and c) reveals that significant errors can occur in k_5; for example set 4 a and c, k_5 1·2 and 1·4. Not only is graphical estimation difficult (especially in set 4 where $K \approx k_2+k_4 \approx k_5$, 1·4, 1·3 and 1·2) but in the computer programme k_5 occurs in only one equation (12) to be iterated whereas all other parameters appear in either two or three equations. Computer solution for k_5 is dependent only on values for C_B and hence more likely to be in error than the other values. A_o was estimated from the simulated cumulative excretion curves by projecting the curves until they became asymptotic and the estimate used as input. In each case the output very closely approximated the correct value. Set 8 was carried out with values approximating to those actually found for methyl orange and gave good values (Table 2) even with the high value of k_5.

Metabolism and excretion of methyl orange

The graphical estimates for the parameters of methyl orange metabolism and excretion (Table 1) were used as input in the computer programme and refined parameter values obtained as output. The parameters together with their standard deviations are shown in Table 3. The experimental points and computer estimated lines of best fit are shown in Fig. 3. The calculated curves gave a good to excellent fit of the experimental data and confirm the utility of computer analysis in pharmacokinetics.

Discussion

In the model the quantity A_o is taken as the amount of material input to the system at time zero (that is the dose by intravenous injection). If the model is followed exactly then A_o should be 100% but here it averages 56%. Many dyes (including methyl orange) are rapidly taken up by the liver (Priestly, 1967; Priestly & O'Reilly, 1966). This process is extremely rapid and where it has been estimated has a rate constant of the order of 5 h^{-1} (Priestly, 1967). On this basis, the single compartment in the model approximates to the liver. The dye is absorbed from the blood into the liver so rapidly that the rate of uptake may be neglected in formulating the model. This approach is analogous to the pharmacokinetic practice of neglecting a rapid absorptive process in the determination of elimination rate constants after oral administration of drug (Cummings, King & Martin, 1967).

The methyl orange not excreted in the bile is considered to follow an extrahepatic route of elimination.

The model proposed for methyl orange excretion in the bile indicates that a plot of log rate of methyl orange excretion versus time is linear. The experimental results support this conclusion. Many other dyes and other compounds show a more complex behaviour in which dye excretion in the bile is best described by a biphasic log rate plot (Priestly & O'Reilly, 1966). Fortunately, methyl orange does not exhibit this behaviour which requires for interpretation a more complex model with at least two compartments. Further research should reveal the origin of the different patterns of biliary secretion kinetics with different compounds.

The rate constant (k_1) for the removal of the first methyl group from methyl orange was generally somewhat less than that for the second methyl group (k_2). Taking into account the higher error of k_2, and the fact that in group 3 (Table 3), k_2 was actually a little less than k_1, it may be concluded that there is not a large difference in the two rate constants. This is in contrast to the finding of McMahon, Culp & Marshall (1965) that with the demethylation of α-(±)-acetylmethadol the first methyl group is removed at a rate approximately three times that of the second.

The model proposed permits the determination of pharmacokinetic constants which will generate curves to fit the experimental data. Inspection of Fig. 3 indicates that the proposed model gives an excellent fit to the experimental data but it cannot be claimed that other models would not give an equally good fit. There are two ways in which the problem of the relation between the pharmacokinetic model and experimental reality may be explored. One is to study other models to determine their ability to fit the data and the other approach is to demonstrate a relationship between the pharmacokinetic constants and the metabolic or excretory paths they are intended to describe. Interpretation of the pharmacokinetic constants in terms of real biological events in the complex intact animal is a difficult and hazardous undertaking. For example, k_1 and k_2 are assigned in the model as first order rate constants to represent the chemical reactions involved in biological demethylation. The actual demethylation reactions are undoubtedly more complex than a simple first order process. At higher doses than were used in the experiments described here, methyl orange displays non-first order kinetics due to overload of biliary transport mechanisms (O'Reilly, unpublished results). Other examples of non-first order behaviour have been attributed to metabolic enzyme saturation and other causes (Wagner, 1968). Nelson (1962) has suggested that pharmacokinetic rate constants may be related to some rate limiting step in the overall metabolic process. It is hoped that further work with structurally related compounds and comparisons of *in vivo* and *in vitro* systems will throw light on this problem. At this stage, pharmacokinetic constants should be regarded as tentative guides to be used for comparative work rather than as absolute values of biological systems.

The biliary elimination rate constants for methyl orange and its metabolites raise an interesting question. For methyl orange (Table 3) k_3 averages 0·164, for the monomethyl derivative 0·672 and for the demethylated end product 6·413 h^{-1}, corresponding to half lives for biliary excretion of 4·23, 1·03 and 0·11 h, respectively. Even taking into account the large error possible in the measurement of k_5, the successive removal of methyl groups results in much faster biliary excretion of the compounds.

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