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Pharmacokinetic Analysis of Azelaic Acid Disodium Salt
A Proposed Substrate for Total Parenteral Nutrition

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Summary Azelaic acid was the first dicarboxylic acid proposed as an alternative energy substrate in total parenteral nutrition. In this study, the pharmacokinetics of azelaic acid were investigated in 12 healthy volunteers, 7 receiving a constant infusion (10g over 90 min) and 5 a bolus dose (1g). The 24h urinary excretion and plasma concentration in blood samples taken at regular intervals were assayed by gas-liquid chromatography. Experimental data were analysed by a 2-compartment nonlinear model that describes both tubular secretion and cellular uptake in Michaelis-Menten terms. A high value of urinary excretion (mean 76.9% of infused dose) and a mean clearance of 8.42 L/h were found, suggesting the presence of tubular secretion. Estimating the population mean of the pharmacokinetic model parameters gave a maximal cellular uptake of 0.657 g/h. The model predicts that 90% of the maximal uptake should be reached in the plateau phase of a constant infusion of 2.2 g/h. The presence of extensive and rapid losses through urinary excretion, and the low estimated value of the maximal cellular uptake, indicate that azelaic acid is not suitable as an energy substrate for total parenteral nutrition.

Azelaic or nonandioic acid (molecular weight 188.2 daltons) is a straight saturated medium-chain dicarboxylic acid with 9 carbon atoms which was recently introduced for total parenteral nutrition (TPN) [Mingrone et al. 1989]. This diacid does not exhibit either toxic or teratogenic effects in laboratory animals (Mingrone et al. 1983); after β-oxidation of azelaic acid, a metabolite (pimelic acid) is excreted in urine (Passi et al. 1983).

We recently proposed the use of azelaic acid in the sodium salt form in clinical conditions where the glucose or lipid utilisation by tissues is impaired (Mingrone et al. 1989; Tacchino et al. 1990). In fact, in septic processes a glucose intolerance has been demonstrated, coupled with an increased gluconeogenesis and oxidative defects of the intermediate metabolism (Askanazi et al. 1980; Nanni et al. 1984). An increased lipid fuel dependence has been shown in septic states, thus suggesting an intensive use of long- and medium-chain triglycerides as energetic substrates in TPN. However, in the later stages, a lipoprotein lipase enzyme defect (Robin et al. 1981) intervenes, causing reduced clearance of plasma triglycerides. In these conditions, both lung and cerebral microemboli have been described which result in respiratory distress and nervous tissue damage due to anoxia.

We suggested using azelaic acid in TPN, since it can be administered directly by a peripheral vein and does not require an intermediate hydrolysis step prior to intracellular utilisation. In addition, compared with long-chain fatty acids, dicarboxylic

acids have the advantage of also being oxidised at the peroxisome level (McGarry & Foster 1971a,b; Mortensen et al. 1982) and of being transported through the mitochondrial membranes by a carnitine-independent system. This could be advantageous in clinical conditions where carnitine deficiency is suspected, such as septic processes (Karpati 1975), liver cirrhosis (Rudman et al. 1977), in premature neonates (Schmidt-Sommerfield et al. 1982) and in haemodialysed patients (Bohmer et al. 1978). Calorimetric studies have demonstrated significant oxygen consumption and constant carbon dioxide production during infusion of azelaic acid (Mingrone et al. 1989). In addition, the percentage of calories derived from lipid oxidation at the end of the infusion was greater than the initial value by 120%, thus suggesting good tissue utilisation of azelaic acid. The metabolic effects of azelaic acid analysed by indirect calorimetry were similar to those found for long-chain triglycerides infused in the same amounts (Tacchino et al. 1990).

The major problem observed during infusion was due to urinary loss of more than 50% of the administered dose (Mingrone et al. 1989). Consequently, the clinical use of azelaic acid in TPN requires a preliminary study of its pharmacokinetics in healthy volunteers. In this connection, in the present work, we studied the pharmacokinetics of azelaic acid using plasma concentration data from both bolus dose and constant infusion, and urinary excretion data from the infusion experiments. By means of a 2-compartment model with nonlinear urinary excretion and nonlinear tissue uptake, and following an approach similar to that in Sheiner et al. (1977), we estimated the pharmacokinetic parameters of the model as mean values of the subject population. Moreover, we predicted the behaviour of other parameters significant for the clinical use of azelaic acid as a function of the infusion rate.

Methods

Subjects and Modality of Administration

For the study, 12 healthy, nonobese volunteers of both sexes, ranging in age from 25 to 40 years (mean ± SD: 33.4 ± 9.2 years), were enrolled. Group I (n = 7) received azelaic acid 10g intravenously in the disodium salt form, dissolved in 500ml of double-distilled water, at an infusion rate of 6.67 g/h over 90 min using a 'Harvard' electric syringe pump. Group II (n = 5) received azelaic acid 1g dissolved in 5ml of double-distilled water injected as a bolus dose in a forearm vein. Blood samples of 5ml were collected without anticoagulant and centrifuged; serum samples were frozen at −20C° until analysis. Times of vein puncture were 30, 60, 90, 120, 150, 180 and 210 min after the start of the infusion, and 10, 20, 30, 40, 50 and 60 min after the bolus. The subjects of group I voided before starting the infusion and the 24h urine of these subjects was collected. The protocol was approved by the ethics committee of the Institutional Review Board of Health of the Science Center of the Catholic University School of Medicine in Rome. Written informed consent was obtained in all cases.

Azelate Solution

A 1 mol/L solution of azelaic acid salified with sodium hydrate (NaOH) was prepared as follows: 1 mol/L azelaic acid + 2 mol/L NaOH → 1 mol/L disodium azelate + 2 mol/L H_2O. Then 1 mol/L azelate was diluted with double-distilled water to achieve the chosen concentrations.

Azelaic Acid Analysis

Azelaic acid concentration measurement in serum and urine samples was performed by gas-liquid chromatography as previously described (Mingrone et al. 1989). Briefly, 1ml of serum or 0.5ml of urine was added to 50μg of suberic acid and 50μg of sebacic acid (Fluka Chemie A.G., Bughs, Switzerland) as internal standards. The samples were acidified to pH ranging from 1 to 2 with 1 mol/L hydrochloric acid and centrifuged at 5000 rpm for 15 min to remove proteins. The pellets were washed twice with small amounts of ethylacetate and the supernatants were collected together in the same glass vial, saturated with sodium chloride and extracted 3 times with 10 vol-

umes of ethylacetate. The combined extracts were dried over anhydrous sodium sulphate and evaporated under a nitrogen stream. The extracted solutes were methylated with diazomethane in diethylether to form dimethyl derivatives. Dicarboxylic acid derivatives were analysed by a Hewlett-Packard model 5890A gas-liquid chromatograph whose inlet system was equipped with a 30m fused silica capillary column ('Supelcowax 10', 0.32mm internal diameter, Supelchem, Milan, Italy). The operative conditions were as reported in Mingrone et al. (1989).

The limit of detection was 10ng and the linearity range of the response was from 0.05 to 100μg. The within- and between-assay variabilities were 2 and 4%, respectively.

Pharmacokinetic Analysis

Data were analysed by a 2-compartment nonlinear model, compartments 1 and 2 being the central and tissue compartments, respectively. We assumed that the fraction of azelaic acid bound by plasma proteins and the uptake by blood cells were negligible. The flow between the compartments was considered to be due to passive diffusion. Renal excretion was represented by glomerular filtration plus a Michaelis-Menten term that describes active secretion (van Ginneken & Russel 1989). Cellular uptake of azelaic acid was also represented by Michaelis-Menten kinetics and was assumed not to be affected by its subsequent metabolism. Moreover, the Michaelis-Menten constants for both secretion and cellular uptake were assumed to be the same. A block diagram of the model ('the original model') is shown in figure 1. The model equations are as follows:

$$V_1 \frac{dc_1}{dt} = -rc_1 - \frac{T_m c_1}{Km + c_1} - kc_1 + kc_2 + I \qquad \text{(Eq. 1)}$$

$$V_2 \frac{dc_2}{dt} = -kc_2 + kc_1 - \frac{T'_m c_2}{Km + c_2} \qquad \text{(Eq. 2)}$$

where c_1 and c_2 are the concentrations (mg/L) in the 2 compartments; I is the infusion rate (μg/min); V_1 and V_2 are the compartment volumes (ml); k is the rate of exchange between compartments (ml/min); r is the glomerular filtration rate (ml/min); T_m and T'_m are the maximal transport rates (μg/min) of the carrier system for the renal secretion and for the tissue uptake, respectively; and Km is the apparent Michaelis-Menten constant (μg/ml). In the case of infusion, equations 1 and 2 have the initial conditions $c_1(0) = 0$ and $c_2(0) = 0$. When azelaic acid is given as an intravenous bolus, the

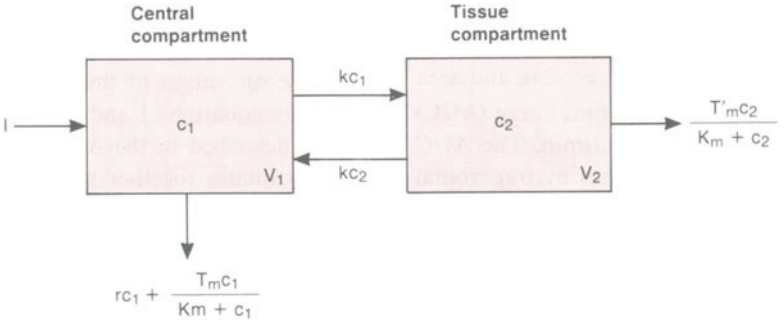

Fig. 1. Diagram of the 2-compartment model used for pharmacokinetic analysis of azelaic acid. *Key:* I = infusion rate; c_1, c_2 = concentration in each compartment; V_1, V_2 = volume of each compartment; k = rate of exchange between compartments; r = glomerular filtration rate; T_m (T'_m) = maximal transport rates of the carrier system for renal secretion (tissue uptake); Km = Michaelis-Menten constant.

input I is set to zero in equation 1, and the initial condition $c_1(0)$ is set to U/V_1, where U (μg) is the dose administered as a bolus. The amount of drug excreted in the 24h urine is given by:

$$Q = \int_0^{24h} \left(rc_1 + \frac{T_m c_1}{Km + =c_1} \right) dt \qquad \text{(Eq. 3)}$$

The measured plasma azelaic acid concentration is considered as the measured value of c_1 and the measured amount of azelaic acid excreted in the 24h urine is the measured value of Q. The values of the model parameters, V_1, V_2, k, r, T_m, T'_m and Km, were estimated as mean values of the subject population. These estimates were obtained, according to the procedure described in the Appendix, from the combined experimental data collected from all the subjects examined.

Two pharmacokinetic models, alternative to the original model, were considered for comparison. The first (model A) is a 2-compartment model in which the Michaelis-Menten term in equation (2) was substituted by a linear term. The second (model B) is a 1-compartment model in which, as in the original model, both renal secretion and cellular uptake were represented as saturable mechanisms.

Results

The mean (± SD) 24h urinary excretion of azelaic acid in the 7 subjects who received an infusion was 7.69 ± 0.89g, which corresponds to 76.9% of the administered dose. The mean ratio between azelaic acid excreted with urine over 24h and area under the plasma concentration-time curve (AUC) was equal to 140.41 ± 24.66 ml/min. The AUC was calculated from 0. to 210 min by trapezoidal approximation. To this value we added the AUC from 210 min to 24h, which was calculated by extrapolating the concentration profile using an exponential decay with time constant determined from the data at 180 and 210 min. The resulting value of the above ratio was larger than the normal glomerular filtration rate (GFR) value.

Figure 2 shows 3 curves of plasma azelaic acid concentration plotted on a semilogarithmic scale

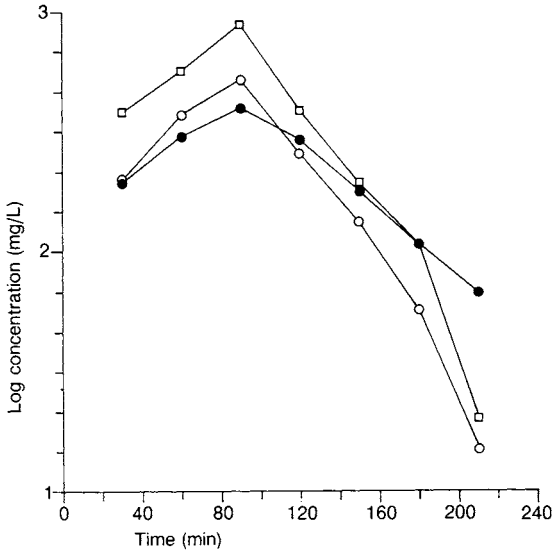

Fig. 2. Plasma drug concentrations in 3 of 7 subjects infused with azelaic acid 10g over 90 min.

from 3 group I subjects. As shown in 2 of the 3 curves, in most cases, the morphology of the descending branch of the constant infusion presented a slope that increased as the concentration decreased. The peak value of plasma azelaic acid concentration was reached at the end of the infusion, and showed a high interindividual variability. The average peak value for group I was 623 ± 195 mg/L.

Data from group I (plasma and 24h urinary concentrations of azelaic acid) and group II (plasma concentrations) were used to estimate the population mean values of the pharmacokinetic parameters in equations 1 and 2, according to the procedure described in the Appendix. Table I shows these estimates together with the standard errors (SE) of the estimates. Inspection of the residual plot did not show evident deviations from a random pattern. Figure 3 depicts the concentration profiles predicted by the model using the estimated values of the pharmacokinetic parameters, together with all the individual experimental data on plasma concentration. The amount of azelaic acid in the 24h urine predicted by the model was 7.70g, with 14.7% being excreted by tubular secretion.

The 2 alternative pharmacokinetic models (A and B, see Methods) were inferior to the proposed original model. The optimal value of the objective function (equation A8 in the Appendix) was equal to 1174 for model A and to 1187 for model B, whereas a value of 1065 was obtained with the original model. This difference was mainly due to the poorer fit of the bolus data.

Simulation of the original pharmacokinetic model with the parameters of table I allows the prediction, for an 'average' subject, of other parameters of clinical interest as a function of the infusion rate. Figure 4 shows the urinary azelaic acid excretion rate and the rate of uptake by tissue cells in plateau conditions. The rate of tissue uptake of azelaic acid normalised to the infusion rate (uptake fraction) is shown in the same figure. It can be noted that tissue uptake rapidly approaches saturation when the infusion rate is increased, reaching 90% of the maximal uptake (T'_m = 0.657 g/h) at an infusion rate of 2.2 g/h.

Table I. Population mean (±SE) of pharmacokinetic parameters of azelaic acid estimated from a 2-compartment model

Parameter (units)	Estimate of mean	SE
V_1 (ml)	3.74×10^3	0.20×10^3
V_2 (ml)	5.93×10^3	0.18×10^3
k (ml/min)	3.71×10^2	0.12×10^2
r (ml/min)	1.09×10^2	0.02×10^2
T_m (μg/min)	5.32×10^3	0.61×10^3
T'_m (μg/min)	10.95×10^3	0.50×10^3
Km (μg/ml)	1.91×10	0.18×10

Abbreviations: V_1 (V_2) = volume of the central (tissue) compartment; k = rate of exchange between compartments; r = glomerular filtration rate; T_m (T'_m) = maximal transport rates of the carrier system for renal secretion (tissue uptake); Km = apparent Michaelis-Menten constant.

Discussion

Experimental data showed a high urinary excretion of azelaic acid. The high value of the ratio between drug excreted with urine over 24h and plasma AUC (this ratio can be interpreted as a time-average renal clearance) suggests the presence of a tubular secretion mechanism. This agrees with Ullrich et al. (1984, 1987), who demonstrated that 3 different anion transport systems exist at the contraluminal cell side of the proximal renal tubule of the rat kidney. One of these transport systems is a sodium-dependent dicarboxylate transporter, which accepts dicarboxylic acids with chain lengths between 6.5 and 10Å. Another transport system, which shows a high specificity for *p*-aminohippurate, accepts dicarboxylates with chain lengths over 7.5Å. Since azelaic acid has a chain length of 9Å, it can be accepted by both transport systems. These findings explain the presence in equation 1 of a term that represents tubular secretion.

Azelaic acid salts, having acid dissociation constants equal to $K_1 = 2.96 \times 10^{-5}$ mol/L and $K_2 = 4.60 \times 10^{-6}$ mol/L, are mostly dissociated in ionic form at physiological pH, and thus are easily soluble in plasma and interstitial fluid. Because cellular membranes are permeable only to liposoluble molecules, it is likely that an active dicarboxylate transport system exists at the cellular membrane level. An active transport system has been recently described by Saint-Macary and Foucher (1985) in the inner mitochondrial membranes; it is involved in the dicarboxylate-dicarboxylate and dicarboxylate-phosphate exchanges. Recent reports also suggest the presence of an active transport system at the cellular membrane level for monocarboxylic acids (Tremmel 1988). The above facts justified the use of a term representing a saturable membrane transport in equation 2 of the model, a choice confirmed by the poor fit obtained with the alternative model A. For simplicity, we assumed the same value of the Michaelis-Menten constant for both the active tubular secretion and the cellular uptake of azelaic acid. This is in agreement with the view that a unique carrier is involved in both mechanisms.

We assumed that azelaic acid is almost completely free in serum, in view of the results from Tonsgard et al. (1988) showing that the affinity for albumin of the superior homologue of azelaic acid (sebacic acid) is markedly smaller than that of

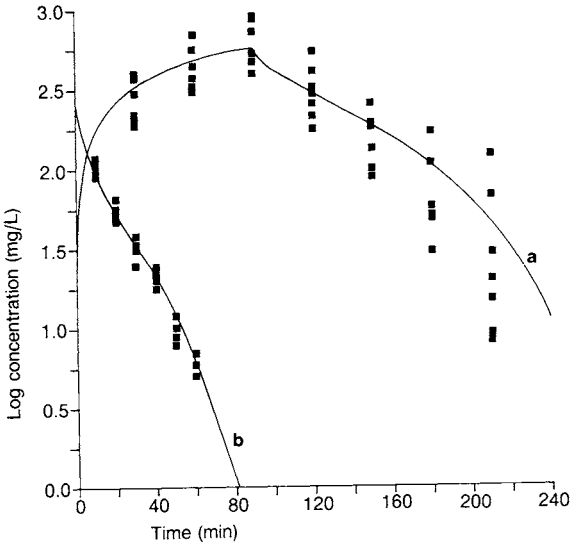

Fig. 3. Model prediction of plasma azelaic acid concentration *vs* time (—) and experimental data of plasma concentration (■). Curve **a**: constant infusion of azelaic acid 10g over 90 min; curve **b**: bolus dose of azelaic acid 1g.

monocarboxylic fatty acids. Thus it is likely that, in our experimental conditions, the albumin binding sites are saturated by fatty acids as well as by other substances (e.g. bilirubin and other biliary pigments, bile salts, steroid hormones).

Preliminary fitting experiments on infusion data, attempting to determine for each subject the parameters of the simpler 1-compartment model (model B), gave unreliable estimates possibly due to the limited number of data available for each subject. On the other hand, it is known that models with Michaelis-Menten terms can produce parameter estimates with high variance (Carson et al. 1983). Consequently we estimated population mean values of the parameters from the entire population of available subjects, following the procedure reported in the Appendix. This procedure is a modification of the approach proposed by Sheiner et al. (1977) and implemented in the NONMEM program (Beal & Sheiner 1985) [for an application, see Grevel et al. 1989]. The high number of parameters in the original model and the possible presence of parameter correlations, compared with the number of available subjects, led us to exclude from the present study the estimation of interindividual variability in pharmacokinetic parameters.

The estimated volume of the central compartment, which slightly exceeded the average plasma volume, suggested some degree of fixation of azelaic acid in the blood cells and/or on the plasma proteins. The sum of the volumes of the 2 compartments was near to the 'inulin space' (10.7L for a 70kg man, see table 2 in Bischoff 1975). The value of k appears to be high: in fact the simulation of the pharmacokinetic model with the parameters of table I showed a small difference between the predicted azelaic acid plateau levels in the 2 compartments (about 30 mg/L for infusion rates greater than 3 g/h). This agrees with the view that the second compartment corresponds to the rapidly equilibrating interstitial water (Bischoff 1975). The estimated GFR was within the physiological range. The T_m value indicates that the urinary loss is principally due to glomerular filtration. The estimated Km value (0.10 mmol/L) is approximately equal to the inhibitory constant (0.08 mmol/L) of azelaic acid competing with the contraluminal *p*-

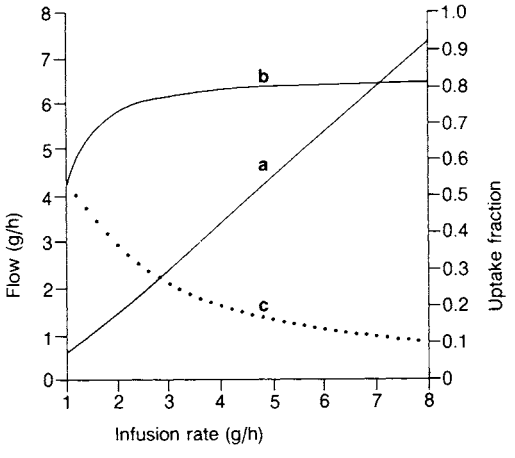

Fig. 4. Model prediction of (**a**) urinary excretion rate and (**b**) rate of tissue uptake of azelaic acid in steady-state *vs* infusion rate. Ordinates of curve **b** are amplified by a factor 10. Curve **c** represents the ratio between rate of tissue uptake and infusion rate (uptake fraction).

aminohippurate transport in the rat kidney (Ullrich et al. 1987). The T'_m value demonstrates a higher transport capacity of the tissue cells than of the proximal renal tubules. The model predicts that the saturation of the carrier for cellular uptake of azelaic acid occurs at about an infusion rate of 2.2 g/h (fig. 4). Higher infusion rates do not result in higher tissue utilisation, whereas urinary excretion increases proportionally with the increase in infusion rate.

In conclusion, at least on the basis of the present data, the maximal amount of azelaic acid available for cellular utilisation should be about 0.66 g/h for an 'average' subject, which corresponds to a daily energy production of 78.7 kcal (Mingrone et al. 1989). Although longer infusion times could reveal a larger distribution volume and a greater tissue utilisation of the agent, it seems unlikely that a tissue uptake suitable for TPN can be found. However, the present pharmacokinetic analysis should be valuable for the use of azelaic acid in other clinical conditions. Furthermore, the present mathematical model could be applied to the pharmacokinetic analysis of other dicarboxylic acids, such as sebacic acid, for which a urinary excretion of about 16% has been found (Mingrone et al. 1990) and which is therefore more promising as a cellular fuel substrate.

Appendix

Let $\theta = (V_1\ V_2\ k\ r\ T_m\ T'_m\ K_m)^T$ be the vector of parameters in the original model. The parameter values are expected to show individual variability when subjects are randomly chosen in a population of subjects. Thus θ will be assumed as a (vector) random variable. Let $\bar{\theta}$ be the mean value of θ. Considering n subjects, let $\theta^{(j)}$, j = 1,. . . ,n, be the parameter vector of the jth subject. We assume that the random variables $\theta^{(j)}$ are mutually independent and identically distributed, i.e. that they constitute a random sample.

Let $n = n_1 + n_2$, where n_1 and n_2 are the number of subjects in groups I and II, respectively. The measured plasma azelaic acid concentration at time t_i for the jth subject of group I (j = 1, . . . ,n_1) will be a random variable that can be expressed as:

$$z_i^{(j)} = f_i(\theta^{(j)}) + \epsilon_i^{(j)} \qquad i = 1, \ldots, N_1 \qquad \text{(Eq. A1)}$$

where $f_i(\theta^{(j)})$ denotes the model solution in the case of infusion for concentration c_1 at time t_i, $\epsilon_i^{(j)}$ denotes the (zero mean) measurement error at the same time, and N_1 is the number of measurement times. The measured urinary amount of azelaic acid for the jth subject is expressed as:

$$w^{(j)} = Q(\theta^{(j)}) + \eta^{(j)} \qquad \text{(Eq. A2)}$$

where $Q(\theta^{(j)})$ is given by equation 3 and $\eta^{(j)}$ is the (zero mean) measurement error.

Assuming that the common covariance of the vectors $\theta^{(j)}$ is small enough to allow a linearisation of function f_i in equation A1 and function Q in equation A2 around $\bar{\theta}$, we obtain:

$$z_i^{(j)} = f_i(\bar{\theta}) + v_i^{(j)} \qquad i = 1, \ldots, N_1 \qquad \text{(Eq. A3)}$$

and

$$w^{(j)} = Q(\bar{\theta}) + u^{(j)} \qquad \text{(Eq. A4)}$$

with

$$v_i^{(j)} = \sum_{k=1}^{p} \frac{\partial f_i}{\partial \theta_k}\bigg|_{\bar{\theta}} (\theta_k^{(j)} - \bar{\theta}_k) + \epsilon_i^{(j)}$$

$$u^{(j)} = \sum_{k=1}^{p} \frac{\partial Q}{\partial \theta_k}\bigg|_{\bar{\theta}} (\theta_k^{(j)} - \bar{\theta}_k) + \eta^{(j)}$$

where p = 7 is the dimension of the parameter vector and k denotes its kth component. Defining the vectors:

$$z^{(j)} = [z_1^{(j)} \ldots z_{N_1}^{(j)}\ w^{(j)}]^T$$

$$F(\bar{\theta}) = [f_1(\bar{\theta}) \ldots f_{N_1}(\bar{\theta})\ Q(\bar{\theta})]^T$$

$$v^{(j)} = [v_1^{(j)} \ldots v_{N_1}^{(j)}\ u^{(j)}]^T$$

equations A3 and A4 can be concisely written as:

$$z^{(j)} = F(\bar{\theta}) + v^{(j)} \qquad j = 1, \ldots, n_1 \qquad \text{(Eq. A5)}$$

An expression similar to equation A5 can be written for the vector $\tilde{z}^{(j)}$ of measured plasma concentrations in the subjects of group II (N_2 measurement times), obtaining

$$\tilde{z}^{(j)} = \tilde{F}(\bar{\theta}) + \tilde{v}^{(j)} \qquad j = 1, \ldots, n_2 \qquad \text{(Eq. A6)}$$

The random vectors $v^{(j)}$ and $\tilde{v}^{(j)}$ have zero mean, with $E[v^{(j)}(v^{(l)})^T] = \Psi_v$ and $E[\tilde{v}^{(j)}(\tilde{v}^{(l)})^T] = \Psi_{\tilde{v}}$ if $j = l$. The above covariances are zero if $j \neq l$, and the same holds for $E[v^{(j)}(\tilde{v}^{(l)})^T]$ for any j and l.

Estimates of mean and covariance of θ could be obtained following the method of Sheiner et al. (1977); but the limited number of subjects available in the present study would make the estimate of the covariance quite unreliable. Thus, only the estimate of $\bar{\theta}$ was pursued, by means of least squares weighted with an estimate of Ψ_v and $\Psi_{\tilde{v}}$. These matrices, which depend on mean and covariance of θ and therefore are unknown, can be estimated from the data as follows (Kshirsagar 1972). First, define the $(N_1 + 1)$-vector μ, with the first N_1 entries given by $(1/n_1)\sum_{j=1}^{n_1} z_i^{(j)}$ and the last entry equal to $(1/n_1)\sum_{j=1}^{n_1} w^{(j)}$. Then define the $(N_1 + 1 \times n_1)$ matrix:

$$D = [z^{(1)} - \mu \mid \ldots\ldots \mid z^{(n_1)} - \mu]$$

so that the sample estimate $\hat{\Psi}_v$ of Ψ_v is given by:

$$\Psi_v = \frac{1}{n_1 - 1} DD^T \qquad \text{(Eq. A7)}$$

Note that a necessary condition for the nonsingularity of $\hat{\Psi}_v$ is that $n_1 > N_1 + 1$. From the data $\tilde{z}^{(j)}$ the sample estimate $\hat{\Psi}_{\tilde{v}}$ of $\Psi_{\tilde{v}}$ can be similarly obtained. In this case, in the absence of the urinary excretion data, it is required that $n_2 > N_2$. Since in our sample it is $n_1 = 7$ and $n_2 = 5$, we have selected $N_1 = 5$ measurement times from infusion experiments (times 30, 90, 120, 150 and 210 minutes), and $N_2 = 4$ measurement times from bolus experiments (times 10, 30, 40 and 60 minutes). The estimate of the vector $\bar{\theta}$ was determined by minimisation of the index:

$$J(\bar{\theta}) = \frac{1}{2}\sum_{j=1}^{n_1} [z^{(j)} - F(\bar{\theta})]^T \hat{\Psi}_v^{-1} [z^{(j)} - F(\bar{\theta})] + \frac{1}{2}\sum_{j=1}^{n_2} [\tilde{z}^{(j)} - \tilde{F}(\bar{\theta})]^T \hat{\Psi}_{\tilde{v}}^{-1} [\tilde{z}^{(j)} - \tilde{F}(\bar{\theta})] \qquad \text{(Eq. A8)}$$

Minimisation was performed by a quasi-Newton method implemented on a Digital VAX 780. For solving the differential original model in order to compute $F(\bar{\theta})$and $\tilde{F}(\theta)$, a Runge-Kutta routine of the NAG Library was used.

It can be noted that, if the distributions of θ and of the measurement errors are assumed to be Gaussian, the index (equation A8) approximates the negative log-likelihood function except for an additive constant. Thus, the covariance matrix of the estimate of $\bar{\theta}$ can be approximately evaluated as the inverse Hessian matrix of the index, computed at the minimum (Chambers 1973). The Hessian was computed by finite-difference approximation, and the square roots of the diagonal elements of its inverse gave the standard error of the estimate of $\bar{\theta}$ reported in table I.

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