# Estimation of the mean AUC of the xenoestrogens daidzein, bisphenol A, and *p-tert*-octylphenol

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## Abstract

Potentially adverse human and environmental effects due to hormone mimicry of environmental estrogens are a matter of current concern. Environmental estrogens belong to the so-called endocrine active compounds (EAC), which alter signalling processes of the endocrine system leading to a broad range of effects during foetal and postnatal development, puberty, adulthood, and aging. A number of synthetic chemicals as well as several plant-derived compounds, so-called phytoestrogens, are known to have weak estrogenic activity.

The present study is part of the risk assessment of the weak environmental estrogens daidzein, *p-tert*-octylphenol, and bisphenol A. The isoflavone daidzein is an important phytoestrogen with respect to dietary exposure (soy beans and soy products). *p-tert*-Octylphenol and bisphenol A are industrial chemicals. The toxicokinetics and the bioavailability of these three substances in female DA/Han rats after oral and single intravenous application were investigated by the use of population models accounting for the differences in the individual metabolism. Furthermore, populations of pregnant and non-pregnant rats are compared.

**Key words**: Bisphenol A, daidzein, *p*-tert-octylphenol, xenoestrogens, phytoestrogens, endocrine active compounds, bioavailability, AUC, population model, EM algorithm, Mann-Whitney-U-test

# 1. Introduction

Human exposure to so-called endocrine active compounds (EAC) in general and environmental estrogens in particular is a matter of current concern (DGPT 1999; Degen and Bolt, 2000[a]; European Community, 1997). EACs may alter the signalling processes of the endocrine system that is responsible for the regulation and the coordination of physiological functions during foetal and postnatal development, puberty adulthood and aging. The signalling processes depend on concentrations of biologically active levels of hormones controlled by biosynthesis, metabolising enzymes, and binding proteins (Barton and Andersen, 1998; National Research Council, 1999).

The particular class of EACs we are investigating are environmental estrogens, which have more or less similar effects as estrogens have. The estrogenic effects are primarily due to the binding to the same receptors although with much lower affinity, but may also arise by other mechanisms (Nilsson *et al.*, 2001 ).

Sources of environmental estrogens are naturally occurring dietary compounds as well as the release of synthetic chemicals: Examples are plant-derived compounds, the so-called phytoestrogens, for instance daidzein and genistein isoflavones that are found in soy beans and soy products, and pesticides like DDT or industrial chemicals like alkylphenols, bisphenol A or PCBs, (Mäkelä *et al.*, 1999). The present study considers the important isoflavone daidzein and the industrial chemicals bisphenol A, used e.g. in the production of plastic coatings in the food packaging industry, and *p-tert*-octylphenol, a model compound for the industrial chemical nonylphenol (technical mixture of a number of different isomers) that belongs to the group of alkylphenols (Certa *et al.*, 1996).

Concerns about endocrine active compounds in general and environmental estrogens in particular are whether effects on the hormonal regulation will lead to potentially *adverse* effects under the actual conditions of exposure.

Observations leading to these concerns have been made in wildlife in certain regions with *high* exposure to environmental estrogens and/or anti-androgens, i.e. decreased fertility of alligators, fishes, and birds as well as feminisation effects and demasculinization of mammals, birds and fishes (Tyler *et al.*, 1998).

***Table 1.*** *Biological Endpoints Proposed to be associated with EACs (Barton and Andersen, 1998)*

| | |
|---|---|
| Cancers (ovarian, breast, testicular) | Altered estrous or menstrual cycling |
| Transplacental carcinogenesis | Steroid receptor binding or inhibition |
| Decreased sperm count and motility | Altered hormone levels |
| Malformations of female reproductive tract and male urogenital tract | Altered cell proliferation and cell differentiation |
| Increased ectopic pregnancies | Altered behaviour |
| Decreased reproductive success | Hyperactivity |
| Decreased weights of uterus or testes | Degraded immune function |
| | Imposex/ hermaphroditism |

At the same time, *beneficial* effects of dietary and pharmaceutical estrogen mimetic compounds are a major focus in medicine (Setchell and Cassidy, 1999).

Sources of exposure to synthetic compounds are mainly food and drinking water except for pharmaceutical xenoestrogens. Human exposure to phyto-estrogens depends much on dietary habits, as the main sources are soybeans and soy products. So in Japan, for instance, the consumption of soybean products is 30 to 50 times higher than that in Western Europe or the United States (Degen and Bolt, 2000[b]; Degen *et al.*, 2002[a]).

Risk assessment for environmental estrogens needs to consider the following aspects:

- Aside from synthetic xenoestrogens humans are mainly exposed to environmental estrogens of natural origin (phytoestrogens).
- A given compound may have agonistic (estrogenic) or anti-agonistic (anti-estrogenic) effects depending on the dose, the target organ and the endocrine state of the whole organism (Mäkelä *et al.*, 1999).
- Endocrine/receptor-mediated effects are not automatically *adverse* effects.

- Exogenous exposure to environmental estrogens has to be considered on the background level of endogenous estrogens.

In assessing the risk of synthetic estrogens it is important to take the exposure to phytoestrogens into account along with information on toxicodynamics (receptor affinity potency), and toxicokinetics (Bolt *et al*., 2001; Bolt and Degen, 2002; Degen *et al*., 2002[c]).

Therefore the present study aims to investigate the toxicokinetics and oral bioavailability of industrial and naturally occurring estrogens.

The major problem that arises with the compounds under investigation, daidzein, bisphenol A, and *p-tert*-octylphenol, are the differences in the data between the observed individuals; that means individual plasma concentration curves differ in scale and functional form from each other. This is probably due to differences in the uptake of the substances, particularly after oral administration, as well as in their metabolism. For the metabolism of these three compounds the so-called enterohepatic circulation seems to play an important role (Figure 1).

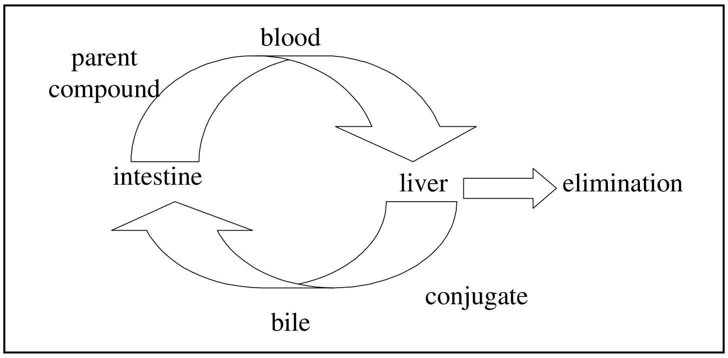


***Figure 1***. *Enterohepatic circulation*

The parent compound is conjugated with glucuronic acid in the liver, transported with the bile in the intestine. Intestinal bacterial enzymes can cleave the conjugate releasing the parent compound (aglycone) that can be reabsorbed and transported to the liver again. Even under the same experimental setting with genetically almost identical experimental animals this leads to great variations in the plasma levels of the investigated compounds.

Thus, the present approach suggests in this situation of few data points per animal with a different number of peaks (usually one or two peaks) of different height at equal doses at different time points to consider first the individual measurements and parameters of interest and to use population models to pool the information in the data together. For this purpose we apply linear hierarchical models using the information from a previous 'naïve' approach, where all the data were pooled together and the AUC was estimated from the complete data set, as prior information. The estimation of the variance and the population mean was performed by an EM algorithm as proposed by Dempster *et al.* (1977). Moreover data sets of pregnant and non-pregnant rats after intravenous application of 10 mg/kg bodyweight daidzein were compared by the use of the Mann-Whitney U-test.

## 2. Data

The present data sets arise from the following study on xenoestrogens at the Institut für Arbeitsphysiologie an der Universität Dortmund (*IfADo*).
First, female non-pregnant DA/Han rats were given the compound of interest either intravenously at a low dosing level or orally at one of two dosing levels. The used estrogen mimetic compounds were daidzein, bisphenol A, and *p-tert*-octylphenol. The plasma concentration was determined at several time-points per animal. Individual samples were available 3 to 7 times per animal. The experimental design is given in table 2. For details see Janning *et al*. (2000) and Upmeier *et al*. (1999; 2000). Note, that each animal is observed at a subset of sampling times. The real sampling times may vary across the planned ones but are usually recorded.

***Table 2.*** *Experimental design of the xenoestrogen study with non-pregnant DA/Han rats. The dose is given in mg/kg body weight, I is the total number of animals observed in that dosing group, sample times in minutes after application of the substance.*

| Substance | Application | Dose | *I* | Sample times |
|---|---|---|---|---|
| daidzein | intravenous | 10 | 8 | 1, 5, 10, 20, 40, 60, 120, 180, 240, 360, 480, 1440, 1920, 2880 |
| | oral | 10 | 7 | 10, 20, 30, 45, 60, 90, 120, 180, 240, 360, 420, 480, 1440, 1920, 2880 |
| | | 100 | 7 | 10, 20, 30, 45, 60, 90, 120, 180, 240, 360, 480, 1440, 1920, 2880 |
| bisphenol A | intravenous | 10 | 9 | 1, 5, 10, 20, 30, 40, 60, 120, 180, 240, 360, 480, 1440, 1920, 2880 |
| | oral | 10 | 3 | 30, 90, 180, 360, 480, 2880 |
| | | 100 | 9 | 10, 20, 30, 45, 90, 120, 180, 240, 360, 480, 1440, 1920, 2880 |
| *p-tert*-octylphenol | intravenous | 5 | 6 | 1, 5, 10, 20, 40, 60, 180, 360, 1440, 2880 |
| | oral | 50 | 6 | 10, 20, 30, 45, 60, 90, 120, 180, 240, 360, 480, 1440, 1920, 2880 |
| | | 200 | 6 | 10, 20, 30, 45, 60, 90, 120, 180, 240, 360, 480, 1440, 1920, 2880 |

The second part of the study was performed with pregnant DA/Han rats. The isoflavone daidzein was given intravenously at a dose of 10 mg/kg bodyweight to totally $I$=21 animals. Plasma concentrations were recorded once per animal (see table 3), at the time of sacrifice. Rat no. 11A was excluded from the analysis as the observation (measured values in plasma and tissue samples) differed unreasonable much from the remaining data.

***Table 3.*** *Experimental design of the xenoestrogen study with pregnant DA/Han rats; j is the index of the sample time,* $n_j$ *is the total number of animals observed in that sampling group, sample times* $t_j$ *in minutes since application of the substance.*

| $j$ | $t_j$ | $n_j$ |
|---|---|---|
| 1 | 5 | 4 (5) |
| 2 | 10 | 4 |
| 3 | 20 | 4 |
| 4 | 40 | 4 |
| 5 | 120 | 4 |

No 11A was dropped out of the analysis

# 3. Models and Methods

The parameters of interest that we aim to estimate from the present study are the area under the concentration-time curve (AUC) and the oral bioavailability, which relates the AUCs of oral and intravenous application accounting, of course, for the doses. A standard method for estimating the AUC is the use of spline functions to approximate the underlying concentration-time curve from data point to data point and subsequent integration of the fitted functions (Glaser, 1985). Hence, a linear approximation is used for the partial function between $t_1$ and $t_J$. The AUC was calculated for every individual separately and pooled together for every dosing group to a population mean AUC applying a linear hierarchical model using the information from the 'naïve' approach as prior information for the estimation of the population mean and its variance as well as for the specification of the prior distribution of the variance of the individual outcomes. In case of intravenous application where the individual differences were not so grave a three-exponential model could be fitted to the pooled data set in an earlier analysis. The calculation was performed applying an EM algorithm as proposed by Dempster *et al*. (1977) and Racine-Poon and Smith (1990) investigating the impact of different variance functions for the error term. The population means of the different dosing and application groups were used to calculate the oral bioavailability of the respective oral dosing group and compound and the results were compared across the different approaches and variance functions.

Finally the plasma-concentrations of daidzein in pregnant and non-pregnant DA/Han rats after intravenous application in two experimental designs were compared by the use of the Mann-Whitney U-statistic.

## 3.1 Calculation of the individual AUC

The calculation of the $AUC_i$, $i = 1, \ldots, I$, for each individual $i$, was performed as follows:

Given the observations $(t_j, y_j)$, $j = 1, \ldots, J$, of a single individual the concentration-time function is approximated by the function $f(t) = y$, where $t$ is the time since application of the chemical in minutes, $y$ is the plasma concentration in ng/ml and $j$ is the sampling occasion. The function $f(t)$ is given by

$$f(t) = \begin{cases} f_0(t), & t_0 \le t < t_1 \\ f_j(t), & t_j \le t < t_{j+1}, j = 1, \ldots, J-1, \\ f_J(t), & t \ge t_J \end{cases} \tag{1}$$

where $t_0 = \max\{0; t^*\}$ with $t^* = \begin{cases} \min\{t \in IR \mid f_0^*(t) = 0\}, & \text{if } t_0 \le t_1 \\ 0, & \text{otherwise} \end{cases}$

is the lag-time and $f_0^*(t)$ is the linear extrapolation of $f_1$ on $[0; t_1[$.

We assume that $f(t_j) = y_j$, $\forall\ j = 1, \ldots, J$, and $f(t_0) = 0$. Furthermore, we define the partial functions

$$f_0(t) = a_0 t + b_0,\ t_0 \le t < t_1, \tag{2}$$

$$f_j(t) = a_j t + b_j,\ t_j \le t < t_{j+1}, j = 1, \ldots, J-1, \text{ and} \tag{3}$$

$$f_J(t) = y_J \cdot \exp\{y'_J \cdot (t - t_J)/y_J\},\ t \ge t_J, \tag{4}$$

with $y'_J = \lim\limits_{\substack{t \to t_J \\ t < t_J}} \left( f'_{J-1}(t) \right) = a_{J-1}$ if it is reasonable to assume that we are already within the terminal elimination phase at time $t \geq t_J$. In case of $y'_J \geq 0$, $f_J(t) = a_J t + b_J$ with $f_J(t_{J+1}) = 0$. The time point $t_{J+1}$, where the plasma concentration is surely equal to 0, is chosen according to the 'knowledge' about the processes under investigation.

The function $f_0^*(t)$ is the linear extrapolation of $f_1$ on $[0; t_1[$ and is given by

$$f_0^*(t) = a_1 t + b_1 . \tag{5}$$

Thus, $$t^* = \begin{cases} -b_1/a_1 = \left(-y_1 + a_1 t_1\right)/a_1, & \text{if } t_0 \leq t_1 \\ 0, & \text{otherwise} \end{cases} . \tag{6}$$

Hence, the parameters $a_0$ and $b_0$ are given by

$$a_0 = y_1/\left(t_1 - t_0\right) \text{ and} \tag{7}$$

$$b_0 = -a_0 t_0 = -y_1 t_0/\left(t_1 - t_0\right). \tag{8}$$

The parameters $a_j$ and $b_j$, $j = 1, \ldots, J$, are given by

$$a_j = \left(y_{j+1} - y_j\right)/\left(t_{j+1} - t_j\right) \text{ and} \tag{9}$$

$$b_j = y_j - a_j t_j . \tag{10}$$

Hence, we obtain the AUC of each individual by integrating $f(t)$ over $[t_0; \infty)$, that means integrating the partial functions $f_j$, $j = 0, \ldots, J$, and summing up the partial $\text{AUC}_j$.

Integrating $f_0, f_1, \ldots, f_{J-1}$ yields

$$\text{AUC}_j = \int_{t_j}^{t_{j+1}} f_j(t)dt = \frac{a_j}{2}\left(t_{j+1}^2 - t_j^2\right) + b_j\left(t_{j+1} - t_j\right). \tag{11}$$

The terminal partial area under the curve $\text{AUC}_J$ is given by

$$\text{AUC}_J = \int_{t_J}^{\infty} f_J(t)dt = -\frac{y_J^2}{a_{J-1}}, \qquad \text{in case of } y'_J < 0 \text{, and} \tag{12}$$

$$\text{AUC}_J = \frac{a_J}{2}\left(t_{J+1}^2 - t_J^2\right) + b_J\left(t_{J+1} - t_J\right), \text{ in case of } y'_J \geq 0 . \tag{13}$$

So, in general we obtain the AUC by

$$\text{AUC} = \sum_{j=0}^{J} \text{AUC}_j .$$

Considering the AUC for each experimental animal separately the individual $\mathrm{AUC}_i$, $i = 1, \ldots, I$, is denoted by

$$\mathrm{AUC}_i = \sum_{j=0}^{J} \mathrm{AUC}_{ij} \,, \qquad (14)$$

where $i$ is the individual and $j$ is the index of the partial function.

In case of intravenous application it was possible to apply a three-exponential model to estimate the AUC from the pooled data set. The concentration-time curve was given by

$$f_{intravenous}(t) = A\exp\{-\alpha t\} + B\exp\{-\beta t\} + C\exp\{-\gamma t\}, \qquad (15)$$

and so,

$$\begin{aligned} AUC_{intravenous} &= \int_0^{\infty} f_{intravenous}(t)dt = -\frac{A}{\alpha}\exp\{-\alpha t\} - \frac{B}{\beta}\exp\{-\beta t\} - \frac{C}{\gamma}\exp\{-\gamma t\}\Big|_0^{\infty} \\ &= \frac{A}{\alpha} + \frac{B}{\beta} + \frac{C}{\gamma}. \qquad (16) \end{aligned}$$

## 3.2 Oral bioavailability

The parameter of interest is the oral bioavailability, which is given by

$$F_{oral,\,dose} = \frac{\mathrm{AUC}_{oral} \cdot \mathrm{dose}_{intravenous}}{\mathrm{AUC}_{intravenous} \cdot \mathrm{dose}_{oral}}, \qquad (17)$$

with '$\mathrm{AUC}_{oral}$' denoting the mean AUC from the experiment with oral application of the respective '$\mathrm{dose}_{oral}$' and '$\mathrm{AUC}_{intravenous}$' and '$\mathrm{dose}_{intravenous}$' denoting the respective quantities from the experiment with intravenous application of the chemical.

The problem is to obtain a 'mean AUC' especially in case of oral application. Hence, population models are applied to cope with this difficulty.

## 3.3 Population model

According to the approach of Lindley and Smith (1972) and Racine-Poon and Smith (1990) the following linear hierarchical model is assumed for the log-transformed data.

| | | |
|---|---|---|
| $\ln(\text{AUC}), \sigma^2 \vert$ | $\ln(\text{AUC}_i) \sim N(\ln(\text{AUC}), \sigma^2 g(\ln(\text{AUC}_i)))$, | $i = 1, \ldots, I$ |
| $\ln(\text{AUC}_{a\ priori}), \tau^2 \vert$ | $\ln(\text{AUC}) \sim N(\ln(\text{AUC}_{a\ priori}), \tau^2)$ | |
| $r, \lambda \vert$ | $1/\sigma^2 \sim Ga(r, \lambda)$ | |

$\text{AUC}_{a\ priori}$ denotes the prior information obtained by the naïve approach and the function $g$ allows for a functional relationship between observations and variance term. Usually it is reasonable to assume that the variance is increasing with increasing observations. In the present analysis we choose $g(x) = x^2$ but investigated also other functional relationships. The variance $\tau^2$ was determined using

$$\hat{\tau}^2 == \sum_{i=1}^{I} \left(\ln(\text{AUC}_i) - \ln(\text{AUC}_{a\ priori})\right)^2 \Big/ I - 1 . \tag{18}$$

The parameters $r$ and $\lambda$ of the Gamma distribution, denoted by $Ga(r, \lambda)$, are chosen considering the features of several Gamma distributions with $r$ and $\lambda$ determined by

$$E(1/\sigma^2) = r/\lambda \approx 1/\hat{\tau}^2 , \tag{19}$$

assuming that the individuals show a similar variation across the population mean as across the *a priori* estimate.

## 3.4 EM Algorithm

The estimation of the unknown population mean ln(AUC) as well as of the hyperparameter $\sigma^2$ was performed by the use of an EM-type algorithm as proposed by Dempster *et al.* (1977). This algorithm computes iteratively the conditional expectation of the parameter − ln(AUC), for instance − given the observations and the current estimates of the hyperparameters (E-step) and the maximum of the posterior distribution of the hyperparameters – $\sigma^{-2}$, for instance – given the observations and the current estimates of the parameters. The algorithm may be used for both: estimation within a maximum likelihood and within a Bayesian framework. For more complex hierarchical models, i.e. three or four stages, see also Selinski (2001). In the present case the algorithm can be easily implemented by computing the *l*th iteration step:

**E-step**: Given $\sigma^{2^{(l-1)}}$, $l \in IN$, $l > 1$, yields

$$\ln(\mathrm{AUC})^{(l)} = \left[\sum_{i=\hat{}1}^{I}\left(\sigma^{2^{(l-1)}} g\left(\ln(\mathrm{AUC}_i)\right)\right)^{-1} + \left(\hat{\tau}^2\right)^{-1}\right] \cdot \left[\sum_{i=\hat{}1}^{I}\left(\sigma^{2^{(l-1)}} g\left(\ln(\mathrm{AUC}_i)\right)\right)^{-1} \cdot \ln(\mathrm{AUC}_i) + \left(\hat{\tau}^2\right)^{-1} \ln(\mathrm{AUC}_{a\ priori})\right] \quad (20)$$

and

**M-step**: Given $\ln(\mathrm{AUC})^{(l)}$, $l \in IN$, $l \geq 1$, we obtain

$$\sigma^{2^{(l)}} = \frac{\sum_{i=1}^{I}\left(\ln(\mathrm{AUC}_i) - \ln(\mathrm{AUC})^{(l)}\right)^2 / g\left(\ln(\mathrm{AUC}_i)\right) + 2\lambda}{I + 2\cdot(r-1) - 1}. \quad (21)$$

A reasonable starting value is given by

$$\sigma^{2^{(0)}} = \frac{\sum_{i=1}^{I}\left(\ln(\mathrm{AUC}_i) - \ln(\mathrm{AUC}_\bullet)\right)^2 / g\left(\ln(\mathrm{AUC}_i)\right) + 2\lambda}{I + 2\cdot(r-1) - 2} \quad (22)$$

with $\ln(\mathrm{AUC}_\bullet) = \sum_{i=1}^{I} \ln(\mathrm{AUC}_i)/I$.

## 3.5 Comparison of the toxicokinetics of pregnant and non-pregnant rats

Comparing the toxicokinetics of the pregnant and non-pregnant rats after intravenous application of daidzein we had to take the different experimental design into account. Individual concentration-time curves were only available for the non-pregnant rats (Janning *et al.*, 2000). In case of the pregnant animals each individual was observed just one time (at sacrifice) due to a destructive design (Degen *et al.*, 2002b). Furthermore samples were taken just on a subset of the sampling times we had for the non-pregnant rats with the last samples taken from the pregnant rats 120 minutes after application of daidzein instead of 2888 minutes.

Thus, observations from both populations were compared separately for each sampling time $t_j$, $j = 1, \ldots, J$, by the use of the Mann-Whitney U-test which is equal to the Wilcoxon rank sum test (see Büning and Trenkler, 1994; Hollander and Wolfe, 1999).

# 4. Results

The individual $AUC_i$ were estimated for all dosing groups and substances according to eqs. (6)-(14) where $t_{J+1}$ was set to 5000 if necessary. The results are given in tables 8-10 in the appendix. Estimates using (13) instead of (12) for the terminal partial $AUC_{iJ}$ are printed bolt. The estimation of the respective population means and variance $\sigma^2$ was performed by the use of the EM algorithm given by (20) and (21) where $\hat{\tau}^2$ and the parameters $r$ and $\lambda$ of the gamma distribution were determined according to (18) and (19) (see table 5). The function $g$ was chosen as $g(x) = x^2$. Results for $g(x) = 1$, $g(x) = x$ and $g(x) = 2x$ are given in tables 11-21 in the appendix. The oral bioavailability was calculated according to (17) using both population means of the oral and the intravenous application group (F), the population mean of the oral administration experiment and the AUC due to the analysis with the three-exponential function (15) and (16) ($F_{3\text{-}exponential}$) and by the use of both prior AUCs ($F_{3\text{-}exponential}$, *a priori* line) (see table 4).

***Table 4.*** *Estimates of the population means of the AUC and the bioavailability; the italic quantities are the estimates from the naïve (a priori) approach; i.v. denotes the intravenous administration route.*

| substance | dose, application | AUC | F | $F_{3\text{-exponential}}$ |
|---|---|---|---|---|
| daidzein | 10 mg/kg, i.v. | 501423.75 | — | — |
| | *a priori* | *501263.49* | — | — |
| | 10 mg/kg, oral | 45442.08 | 9.06 % | 9.07 % |
| | *a priori* | *48622.56* | — | *9.7 %* |
| | 100 mg/kg, oral | 89884.37 | 1.79 % | 1.79 % |
| | *a priori* | *110277.97* | — | *2.2 %* |
| bisphenol A | 10 mg/kg, i.v. | 858089.98 | — | — |
| | *a priori* | *1128319.31* | — | — |
| | 10 mg/kg, oral | 173923.49 | 20.27 % | 15.41 % |
| | *a priori* | *185044.37* | — | *16.4 %* |
| | 100 mg/kg, oral | 430303.18 | 5.01 % | 3.81 % |
| | *a priori* | *631858.81* | — | *5.6 %* |
| *p-tert-* | 5 mg/kg, i.v. | 129474.05 | — | — |
| | *a priori* | *136661.83* | — | — |
| octylphenol | 50 mg/kg, oral | 160625.32 | 12.41 % | 11.75 % |
| | *a priori* | *168094.05* | — | *12.3 %* |
| | 200 mg/kg, oral | 444345.49 | 8.58 % | 8.13 % |
| | *a priori* | *459183.74* | — | *8.4 %* |

***Table 5.*** *Estimates of $\tau^2$ and $\sigma^2$ and chosen parameters r and $\lambda$ of the gamma prior of $1/\sigma^2$; i.v. denotes the intravenous administration route.*

| substance | dose, application | $\hat{\tau}^2$ | $\tilde{\sigma}^2$ | $r$ | $\lambda$ |
|---|---|---|---|---|---|
| daidzein | 10 mg/kg, i.v. | 0.25 | 0.19 | 8 | 2 |
| | 10 mg/kg, oral | 0.90 | 0.28 | 2 | 1.81 |
| | 100 mg/kg, oral | 1.59 | 0.49 | 2 | 3.19 |
| bisphenol A | 10 mg/kg, i.v. | 2.18 | 0.50 | 1 | 2 |
| | 10 mg/kg, oral | 6.98 | 7.02 | 1 | 7 |
| | 100 mg/kg, oral | 3.90 | 1.00 | 1 | 4 |

| | | | | | |
|---|---|---|---|---|---|
| *p-tert*-octylphenol | 5 mg/kg, i.v. | 0.99 | 0.57 | 2 | 2 |
| | 50 mg/kg, oral | 0.72 | 0.44 | 3 | 2 |
| | 200 mg/kg, oral | 0.64 | 0.44 | 3 | 2 |

Comparing the estimates from the population approach, the combined population and three-exponential approach and the prior estimates reveals minor differences for the substances daidzein and *p-tert*-octylphenol whereas these differences are much more prominent in case of bisphenol A.

EM algorithm converged very fast (2-4 iteration steps) and was easily implemented even in EXCEL.

The estimated $\sigma^2$ differed usually from its respective expectation (see tables 11-21, appendix) where the specification of $g$ seems to have a minor impact on the estimate except for denying a dependence of the individual variance on the magnitude of the observations, that means $g(x) = 1$.

In case of daidzein the choice of $g$ also seems to have a small impact on the estimation of the AUC and the bioavailability (tables 11-14), but, for the industrial chemicals bisphenol A and *p-tert*-octylphenol the differences between the estimated AUC and oral bioavailability are considerable (tables 18-21).

The impact of the choice of $r$ and $\lambda$ were investigated for the daidzein data set (100 mg/kg, oral administration), see tables 13-17 (appendix), and revealed a negligible impact on the estimates of the AUC and $\sigma^2$.

Comparing the plasma concentration of the pregnant and non-pregnant DA/Han rats reveals that the plasma levels are not consistently higher or lower in pregnant rats over the complete duration of the experiment (see table 6).

***Table 6.*** *Sampling times in minutes and plasma concentration of daidzein (aglycone) in ng/ml in pregnant and non-pregnant DA/Han rats after i.v. administration of 10 mg/kg daidzein.*

| Non-pregnant | | | Pregnant | | |
|---|---|---|---|---|---|
| Rat no. | time | concentration | Rat no. | time | concentration |
| 63 | 1 | 42358 | | | |
| 16 | 2 | 20575 | | | |
| 8 | 5 | 18248 | 9A | 5 | 3020 |
| 23 | 5 | 7602 | (11A | 5 | 117880) |
| 71 | 5 | 10172 | 12A | 5 | 1712 |
| | | | 16A | 5 | 1140 |
| | | | 22A | 5 | 875 |
| 12 | 10 | 17033 | 2B | 10 | 14780 |
| 68 | 10 | 5057 | 4B | 10 | 3290 |
| 69 | 10 | 7592 | 6B | 10 | 4240 |
| | | | 14B | 10 | 2240 |
| 63 | 20 | 3071 | 36E | 20 | 1422 |
| 16 | 23 | 2143 | 37E | 20 | 404 |
| | | | 38E | 20 | 511 |
| | | | 39E | 20 | 897 |
| 71 | 40 | 674 | 7C | 40 | 345 |
| 23 | 41 | 390 | 8C | 40 | 123 |
| 8 | 44 | 447 | 10C | 40 | 45 |
| | | | 15C | 40 | 624 |
| 12 | 58 | 405 | | | |
| 68 | 60 | 511 | | | |
| 63 | 120 | 45 | 1D | 120 | 173 |
| 16 | 122 | 191 | 3D | 120 | 359 |
| | | | 5D | 120 | 148 |
| | | | 13D | 120 | 593 |
| 23 | 179 | 121 | | | |
| 71 | 180 | 105 | | | |
| 8 | 191 | 115 | | | |
| 68 | 240 | 23 | | | |
| 69 | 240 | 107 | | | |
| 12 | 242 | 93 | | | |
| 12 | 360 | 15 | | | |
| 63 | 360 | 25 | | | |
| 69 | 360 | 8 | | | |
| 71 | 360 | 108 | | | |
| 16 | 478 | 47 | | | |
| 12 | 480 | 0 | | | |
| 69 | 480 | 5 | | | |
| 71 | 480 | 72 | | | |
| 63 | 1440 | 0 | | | |
| 69 | 1440 | 11 | | | |
| 71 | 1440 | 106 | | | |
| 8 | 1442 | 0 | | | |
| 23 | 1444 | 27 | | | |
| 8 | 1926 | 0 | | | |
| 16 | 2888 | 37 | | | |

The plasma concentration seems to be lower in pregnant rats that in non-pregnant individuals at least within the first half hour after intravenous application of daidzein. The Mann-Whitney U-test reveals notable differences after $t$ = 5 minutes ($p < 0.05$) and after $t$ = 20 minutes ($p < 0.1$). No differences are detectable for $t$ = 10, 40, and 120 minutes, respectively, (table 7) although the explorative analysis suggests that the plasma concentrations are higher in pregnant than in non-pregnant rats 120 minutes after administration of daidzein.

***Table 7.*** *Results of the Mann-Whitney U-test of differences between pregnant and non-pregnant DA/Han rats after i.v. administration of 10 mg/kg daidzein.*

| non-pregnant | | | pregnant | | | Mann-Whitney U-test |
|---|---|---|---|---|---|---|
| rat no. | time | concentration | rat no. | time | concentration | reject $H_0$, $\alpha =$ |
| 23 | 5 | 7602 | 22A | 5 | 875 | reject $H_0$, $\alpha = 0.05$ |
| 71 | 5 | 10172 | 16A | 5 | 1140 | |
| 8 | 5 | 18248 | 12A | 5 | 1712 | |
| | | | 9A | 5 | 3020 | |
| 68 | 10 | 5057 | 14B | 10 | 2240 | not reject $H_0$ |
| 69 | 10 | 7592 | 4B | 10 | 3290 | $\alpha = 0.1/\ 0.05$ |
| 12 | 10 | 17033 | 6B | 10 | 4240 | |
| | | | 2B | 10 | 14780 | |
| 16 | 23 | 2143 | 37E | 20 | 404 | reject $H_0$, $\alpha = 0.1$ |
| 63 | 20 | 3071 | 38E | 20 | 511 | |
| | | | 39E | 20 | 897 | |
| | | | 36E | 20 | 1422 | |
| 23 | 41 | 390 | 10C | 40 | 45 | not reject $H_0$ |
| 8 | 44 | 447 | 8C | 40 | 123 | $\alpha = 0.1/\ 0.05$ |
| 71 | 40 | 674 | 7C | 40 | 345 | |
| | | | 15C | 40 | 624 | |
| 63 | 120 | 45 | 5D | 120 | 148 | not reject $H_0$ |
| 16 | 122 | 191 | 1D | 120 | 173 | $\alpha = 0.1/\ 0.05$ |
| | | | 3D | 120 | 359 | |
| | | | 13D | 120 | 593 | |

# Discussion

Dealing with a data set of the presented structure where individuals show a quite diverse behaviour with respect to the processes under investigation the statistical analysis should account for these individual differences by the use of the population approach. Since the last two decades a broad literature is dedicated to this problem, see for instance Racine *et al.* (1986) for applications in the pharmaceutical industry, Sheiner and Beal (1980; 1981; 1983) for the NONMEM approach, Steimer *et al*. (1984), Davidian and Giltinan (1993[a,b]) Gelman *et al*. (1996), Bois *et al.* (1996). Recently, the major focus are the application of MCMC methods, particularly the Gibbs sampler (Wakefield, 1996) and *physiologically based pharmacokinetic* (PBPK) modelling (Bois, 1999) – an approach that aims to model the most relevant processes with respect to the subject under investigation.

Generally, a population approach should be applied in cases where individual differences lead to unreliable 'naïve' summary statistics even if difficulties arise with the estimation of the individual parameters as in the present case with the estimation of the $AUC_i$. Past experience has shown that the EM estimation of the population mean is a minor problem even in more complex situations (cf. Selinski *et al*., 2000; Selinski, 2001).

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# Appendix

***Table 8:*** *Individual $AUC_i$ after oral administration of 10 mg/kg body weight daidzein, with values printed bolt, if the terminal partial area under the curve was determined by eq. (13).*

| dose, application | $i$ | rat no. | $AUC_i$ |
|---|---|---|---|
| 10 mg/kg, i.v. | 1 | 8 | **523412** |
| | 2 | 12 | 556765 |
| | 3 | 16 | 848160 |
| | 4 | 23 | 301540.48 |
| | 5 | 63 | 630454.5 |
| | 6 | 68 | 212740.12 |
| | 7 | 69 | **958285** |
| | 8 | 71 | **573855** |
| 10 mg/kg, oral | 1 | 4 | **112321** |
| | 2 | 9 | **26368** |
| | 3 | 10 | **13534.5** |
| | 4 | 14 | 25239.4 |
| | 5 | 15 | **42447** |
| | 6 | 18 | 82529.91 |
| | 7 | 19 | 207489.82 |
| | 8 | 20 | 27624.5 |
| | 9 | 31 | 35769.17 |
| | 10 | 64 | 148986.52 |
| | 11 | 70 | 43504.35 |
| | 12 | 73 | 9516.57 |
| 100 mg/kg, oral | 1 | 21 | 35869.82 |
| | 2 | 22 | 133553.18 |
| | 3 | 26 | **201136.5** |
| | 4 | 28 | 59290.5 |
| | 5 | 29 | **154682.5** |
| | 6 | 30 | 94875.13 |
| | 7 | 33 | **72256.5** |
| | 8 | 48 | 21652 |
| | 9 | 49 | **9608.5** |
| | 10 | 65 | 71670 |
| | 11 | 66 | 246981.43 |
| | 12 | 67 | 10038.79 |

***Table 9:*** *Individual $AUC_i$ after oral administration of 10 mg/kg body weight daidzein, with values printed bolt, if the terminal partial area under the curve was determined by eq. (13).*

| dose, application | *i* | rat no. | $AUC_i$ |
|---|---|---|---|
| 10 mg/kg, i.v. | 1 | 25 | 364983 |
| | 2 | 34 | 365386.21 |
| | 3 | 35 | 370711.53 |
| | 4 | 36 | 254084.5 |
| | 5 | 42 | 313202.31 |
| | 6 | 44 | 235183.5 |
| | 7 | 46 | 286271.5 |
| | 8 | 47 | 150710.09 |
| | 9 | 55 | 345622.47 |
| 10 mg/kg, oral | 1 | 38 | **52756.54** |
| | 2 | 39 | **11610** |
| | 3 | 40 | **21033** |
| 100 mg/kg, oral | 1 | 50 | 188663.57 |
| | 2 | 51 | 44280.00 |
| | 3 | 52 | **65375.00** |
| | 4 | 53 | 109391.54 |
| | 5 | 54 | 78268.26 |
| | 6 | 57 | 216021.39 |
| | 7 | 58 | 102500.00 |
| | 8 | 59 | 130462.00 |
| | 9 | 60 | **107163.15** |

***Table 10.*** *Individual $AUC_i$ after oral administration of 10 mg/kg body weight daidzein, with values printed bolt, if the terminal partial area under the curve was determined by eq. (13).*

| dose, application | *i* | rat no. | $AUC_i$ |
|---|---|---|---|
| 5 mg/kg, i.v. | 1 | 4 | 70231.75 |
| | 2 | 33 | 20791.43 |
| | 3 | 35 | **117140.00** |
| | 4 | 36 | 143213 |
| | 5 | 6 | **51307.00** |
| | 6 | 34 | 133596.08 |
| 50 mg/kg, oral | 1 | 11 | 188755.82 |
| | 2 | 12 | 113551.04 |
| | 3 | 13 | 76384.50 |
| | 4 | 14 | 105192.86 |
| | 5 | 15 | 39814.07 |
| | 6 | 16 | **82422.50** |

| | | | |
|---|---|---|---|
| 200 mg/kg, oral | 1 | 21 | 125646.27 |
| | 2 | 22 | 462298.04 |
| | 3 | 23 | 378220.32 |
| | 4 | 24 | 224509.86 |
| | 5 | 25 | 254417.00 |
| | 6 | 26 | 211032.01 |

***Table 11.*** *Daidzein 10 mg/kg, intravenous administration, non-pregnant DA/Han rats, AUC,* $\tilde{\sigma}^2$ *and oral bioavailability for different specifications of g.*

| | $g(x)$= | | | |
|---|---|---|---|---|
| | 1 | $x$ | $2x$ | $x^2$ |
| AUC | 519927.68 | 506445.68 | 504597.57 | 501423.75 |
| $\tilde{\sigma}^2$ | 0.2100 | 0.1913 | 0.1909 | 0.1905 |

***Table 12.*** *Daidzein 10 mg/kg, oral administration, non-pregnant DA/Han rats, AUC,* $\tilde{\sigma}^2$ *and oral bioavailability for different specifications of g.*

| | $g(x)$= | | | |
|---|---|---|---|---|
| | 1 | $x$ | $2x$ | $x^2$ |
| AUC | 43564.65 | 41953.80 | 43060.99 | 45442.08 |
| $\tilde{\sigma}^2$ | 0.3416 | 0.2851 | 0.2821 | 0.2798 |
| F | 8.38% | 8.28% | 8.53% | 9.06% |
| $F_{3\text{-exponential}}$ | 8.69% | 8.37% | 8.59% | 9.07% |

***Table 13.*** *Daidzein 100 mg/kg, oral administration, non-pregnant DA/Han rats, AUC,* $\tilde{\sigma}^2$ *and oral bioavailability for different specifications of g.*

| | $g(x)$= | | | |
|---|---|---|---|---|
| | 1 | $x$ | $2x$ | $x^2$ |
| AUC | 61215.30 | 63348.00 | 69949.10 | 89884.37 |
| $\tilde{\sigma}^2$ | 0.5750 | 0.4984 | 0.4947 | 0.4916 |
| F | 1.18% | 1.25% | 1.39% | 1.79% |
| $F_{3\text{-exponential}}$ | 1.22% | 1.26% | 1.40% | 1.79% |

***Table 14.*** *Daidzein 100 mg/kg, oral administration, non-pregnant DA/Han rats, AUC,* $\tilde{\sigma}^2$ *and oral bioavailability for different specifications of g, r =2,* $\lambda$ *=0.8.*

| | $g(x)=$ | | | |
|---|---|---|---|---|
| | 1 | $x$ | $2x$ | $x^2$ |
| AUC | 60650.73 | 57633.34 | 60574.08 | 72938.40 |
| $\tilde{\sigma}^2$ | 0.2695 | 0.1700 | 0.1650 | 0.1609 |
| F | 1.17% | 1.14% | 1.20% | 1.45% |
| $F_{3\text{-exponential}}$ | 1.21% | 1.15% | 1.21% | 1.46% |

***Table 15.*** *Daidzein 100 mg/kg, oral administration, non-pregnant DA/Han rats, AUC,* $\tilde{\sigma}^2$ *and oral bioavailability for different specifications of g, r =0.5,* $\lambda$ *=0.8.*

| | $g(x)=$ | | | |
|---|---|---|---|---|
| | 1 | $x$ | $2x$ | $x^2$ |
| AUC | 60650.73 | 57633.34 | 60574.08 | 72938.40 |
| $\tilde{\sigma}^2$ | 0.2695 | 0.1700 | 0.1650 | 0.1610 |
| F | 1.17% | 1.14% | 1.20% | 1.45% |
| $F_{3\text{-exponential}}$ | 1.21% | 1.15% | 1.21% | 1.46% |

***Table 16.*** *Daidzein 100 mg/kg, oral administration, non-pregnant DA/Han rats, AUC,* $\tilde{\sigma}^2$ *and oral bioavailability for different specifications of g, r =6,* $\lambda$ *=10.*

| | $g(x)=$ | | | |
|---|---|---|---|---|
| | 1 | $x$ | $2x$ | $x^2$ |
| AUC | 61988.60 | 69572.92 | 78419.36 | 97813.86 |
| $\tilde{\sigma}^2$ | 1.0045 | 0.9572 | 0.9549 | 0.9529 |
| F | 1.19% | 1.37% | 1.55% | 1.95% |
| $F_{3\text{-exponential}}$ | 1.24% | 1.39% | 1.56% | 1.95% |

***Table 17.*** *Daidzein 100 mg/kg, oral administration, non-pregnant DA/Han rats, AUC,* $\tilde{\sigma}^2$ *and oral bioavailability for different specifications of g,* $r = \lambda = 1$.

| | $g(x)$= | | | |
|---|---|---|---|---|
| | 1 | $x$ | $2x$ | $x^2$ |
| AUC | 60672.89 | 58036.35 | 61307.04 | 74861.06 |
| $\tilde{\sigma}^2$ | 0.2813 | 0.1909 | 0.1863 | 0.1827 |
| F | 1.17% | 1.15% | 1.21% | 1.49% |
| $F_{3\text{-exponential}}$ | 1.21% | 1.16% | 1.22% | 1.49% |

***Table 18.*** *Bisphenol A 10 mg/kg, oral administration, non-pregnant DA/Han rats, AUC,* $\tilde{\sigma}^2$ *and oral bioavailability for different specifications of g.*

| | $g(x)$= | | | |
|---|---|---|---|---|
| | 1 | $x$ | $2x$ | $x^2$ |
| AUC | 40060.49 | 114934.68 | 141213.86 | 173923.49 |
| $\tilde{\sigma}^2$ | 7.3375 | 7.1449 | 7.0898 | 7.0217 |
| $F_{3\text{-exponential}}$ | 3.55% | 10.19% | 12.52% | 15.41% |

***Table 19.*** *Bisphenol A 100 mg/kg, oral administration, non-pregnant DA/Han rats, AUC,* $\tilde{\sigma}^2$ *and oral bioavailability for different specifications of g.*

| | $g(x)$= | | | |
|---|---|---|---|---|
| | 1 | $x$ | $2x$ | $x^2$ |
| AUC | 109675.34 | 160631.78 | 210849.99 | 430303.18 |
| $\tilde{\sigma}^2$ | 1.0277 | 1.0044 | 1.0039 | 1.0021 |
| $F_{3\text{-exponential}}$ | 0.97% | 1.42% | 1.87% | 3.81% |

***Table 20.*** *p-tert-Octylphenol 50 mg/kg, oral administration, non-pregnant DA/Han rats, AUC, $\tilde{\sigma}^2$ and oral bioavailability for different specifications of g.*

| | $g(x)$= | | | |
|---|---|---|---|---|
| | 1 | $x$ | $2x$ | $x^2$ |
| AUC | 96583.88 | 125733.89 | 139194.98 | 160625.32 |
| $\tilde{\sigma}^2$ | 0.4694 | 0.4476 | 0.4464 | 0.4449 |
| $F_{3\text{-exponential}}$ | 7.07% | 9.20% | 10.19% | 11.75% |

***Table 21.*** *p-tert-Octylphenol 200 mg/kg, oral administration, non-pregnant DA/Han rats, AUC, $\tilde{\sigma}^2$ and oral bioavailability for different specifications of g.*

| | $g(x)$= | | | |
|---|---|---|---|---|
| | 1 | $x$ | $2x$ | $x^2$ |
| AUC | 270286.92 | 358344.02 | 392880.33 | 444345.49 |
| $\tilde{\sigma}^2$ | 0.4646 | 0.4471 | 0.4461 | 0.4448 |
| $F_{3\text{-exponential}}$ | 4.94% | 6.56% | 7.19% | 8.13% |