Comparison of the toxicokinetics of daidzein and bisphenol A in pregnant and non-pregnant DA/Han rats

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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) and may alter signalling processes of the endocrine system leading to a broad range of effects during fetal 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 and bisphenol A. The isoflavone daidzein is an important phytoestrogen with respect to dietary exposure (soy beans and soy products) whereas bisphenol A is an industrial chemical that occurs at much lower concentrations as a contaminant in food. The toxicokinetics of these compounds in female pregnant and non-pregnant DA/Han rats after single intravenous application were compared by the use of the Mann-Whitney-U-statistic.

Key words: Bisphenol A, daidzein, xenoestrogens, phytoestrogens, endocrine active compounds, Mann-Whitney-U-test

1. Introduction

So-called endocrine active compounds (EAC) in general and environmental estrogens in particular are still a matter of concern (Damstra *et al*., 2002; 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 fetal and postnatal development, puberty, adulthood and aging. The signalling processes depend on concentrations of biologically active levels of hormones which are controlled by biosynthesis, metabolizing 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 steroid estrogens have. The estrogenic effects are primarily due to the binding to the hormone receptors although with much lower affinity, but may also arise by other mechanisms (Nilsson *et al*., 2001).

There is consensus that endocrine active compounds, at sufficient levels, can interfere with the hormonal regulation and lead to potentially *adverse* effects. Observations leading to concerns have been made in wildlife in some regions with *high* exposure to synthetic environmental estrogens and/or anti-androgens: This resulted in decreased fertility of alligators, fishes and birds as well as feminisation effects and demasculinization of mammals, birds and fishes (Tyler *et al*., 1998; Vos *et al*., 2000). Severe adverse health effects occurred also in humans exposed prenatally to high doses of the potent estrogen diethylstilbestrol (Golden *et al*., 1998). However, controversy surrounds the question of risks associated with the actual (low) levels of exposure to environmental agents with much weaker hormonal activity (DGPT, 1999; Safe, 2000; Sharpe and Irvine, 2004).

Sources of human exposure to environmental estrogens are naturally occurring dietary compounds as well as the release of synthetic chemicals: Examples are plant-derived compounds, so-called phytoestrogens, for instance the isoflavones daidzein and genistein that are found in soy beans and soy products. Examples of synthetic agents are pesticides like DDT or industrial chemicals like alkylphenols, bisphenol A or PCBs which give rise to residues in foods (Mäkelä *et al*., 1999; Degen, 2004). The present study considers the isoflavone daidzein and the industrial chemical bisphenol A, used e.g. in the production of plastic coatings in the food packaging industry (EU Commission, 2002).

Human exposure to these prototypical compounds is mainly via food and drinking water. Exposure to phytoestrogens 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[b]). Interestingly, also *beneficial* effects of dietary phytoestrogens have become a major focus in medicine, and these compounds are now popular as alternative hormone replacement therapy (Setchell and Cassidy, 1999). But, a toxicological risk assessment for environmental estrogens has to be unbiased with respect to the origin of compounds. It 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 (DGPT, 1999).

In assessing the risk of synthetic estrogens it is important to take the exposure to phytoestrogens into account along with information on toxico-*dynamics* and toxico*kinetics* (Bolt *et al*., 2001; Bolt and Degen, 2002; Degen *et al*., 2002[c]). In other words, effects of a compound will depend upon its properties (receptor affinity, potency) and the dose, but, also on its fate in the organism, *i.e.* the biologically active fraction in a target tissue upon absorption, distribution, metabolism and elimination. Studies on the toxicokinetics of daidzein and of bisphenol A in adult female rats revealed extensive metabolism (conjugation) by phase-II enzymes for both compounds (Janning *et al.*, 2000; Upmeier *et al*., 2000) and bioavailabilities of a similar order of magnitude (Selinski and Degen, 2003). Yet, the kinetics in non-pregnant rats may differ from those in pregnant animals: On the one hand, phase-II metabolism may be slower (or faster) in pregnant rats resulting in higher (or lower) levels of unconjugated xeno-/phytoestrogen in their blood than in non-pregnant animals. On the other hand, part of a given dose can pass the placenta and distribute into the fetal compartment which may result in lower maternal plasma levels. Thus, along with a direct analysis of the compounds in rat fetuses to investigate transplacental transfer of daidzein and bisphenol A, it was of interest to compare their blood levels in pregnant and non-pregnant rats.

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 intravenously at a low dosing level of 10 mg/kg bodyweight. The used estrogen mimetic compounds were daidzein and bisphenol A. The plasma concentration was determined at several time-points per animal. Individual samples were available 3 to 6 times per animal. The experimental design is given in table 2. For details see Janning *et al.* (2000) and Upmeier *et al.* (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, j* is the index of the sampling times* t_{j*} *in minutes since intravenous application of 10 mg/kg bodyweight daidzein or bisphenol A,* n_{j*} *is the respective number of observations.*

Substance	j^*	t_{j*}	n_{j*}
Daidzein	1	1	2
	2	5	3
	3	10	3
	4	20	2
	5	40	3
	6	60	2
	7	120	2
	8	180	3
	9	240	3
	10	360	4
	11	480	4
	12	1440	5
	13	1920	1
	14	2880	1
Bisphenol A	1	1	2
	2	5	3
	3	10	3
	4	20	2
	5	30	1
	6	40	3
	7	60	3
	8	120	3
	9	180	3
	10	240	3
	11	360	3
	12	480	3
	13	1440	2
	14	1920	4
	15	2880	2

Second, pregnant DA/Han rats were given the compound of interest, daidzein or bisphenol A, intravenously at a low dosing level of 10 mg/kg bodyweight. The plasma and further tissue concentrations were determined once per animal at sacrifice. Plasma concentrations of a few animals were observed at an early point of time. For these animals we were able to ascertain that the dose was indeed given intravenously and not paravenously. Such injection errors can occur because of the heavy pigmentation of the DA/Han rats and result in much lower blood levels.

The experimental design is given in table 3. For details see Degen *et al.* (2002^a) and Bolt *et al.* (2002) or Moors *et al.* (2003, 2004, and manuscript in preparation).

The real sampling times may vary across the planned ones but are usually recorded.

Table 3. *Experimental design of the xenoestrogen study with pregnant DA/Han rats, j^{**} is the index of the sampling times $t_{j^{**}}$ in minutes since intravenous application of 10 mg/kg bodyweight daidzein or bisphenol A, $n_{j^{**}}$ is the respective number of observations.*

Substance	j^{**}	$t_{j^{**}}$	$n_{j^{**}}$
Daidzein	1	5	4
	2	10	4
	3	20	4
	4	40	4
	5	120	4
Bisphenol A	1	5	11
	2	10	10
	3	20	6
	4	30	6
	5	40	5
	6	120	4
	7	360	4

3. Models and Methods

The main interest of this analysis is focused on the impact of a pregnancy on the toxicokinetic behaviour of estrogen mimetic compounds. Here, we have to deal with the difficulty of different experimental designs: Short concentration-time curves with very few observations at each time-point, in case of non-pregnant rats, and a destructive design with many variables observed at a subset of sampling times, in case of the pregnant rats. Moreover, the tissue data recorded from the pregnant animals after administration of either daidzein or bisphenol A may be used to obtain information about differences between the toxicokinetics of the two compounds.

3.1 Comparison of the toxicokinetics of pregnant and non-pregnant rats

Comparing the toxicokinetics of the pregnant and non-pregnant rats after intravenous application of either daidzein or bisphenol A we had to take the different experimental design into account. Individual concentration-time curves were only available for the non-pregnant rats. In case of the pregnant rats each individual was observed just one time for daidzein or two times at most for some animals after application of bisphenol A. Furthermore samples were taken just on a subset of the sampling times we had for the non-pregnant rats. The last samples from the pregnant rats were taken after 120 minutes in case of daidzein and after 360 minutes in case of bisphenol A instead of 2880 minutes in case of non-pregnant animals.

Thus, observations from both populations, pregnant and non-pregnant rats, 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).

$$W = \sum_{j=1}^{n} R(Y_j) = U + n \cdot (n+1)/n \tag{1}$$

where $R(Y_j)$ is the rank of Y_j in the combined $X_1, \ldots, X_m, Y_1, \ldots, Y_n$ sample and

$$U = \sum_{i=1}^{m} \sum_{j=1}^{n} \phi(X_i, Y_j) \tag{2}$$

with $\phi(X_i, Y_j) = \begin{cases} 1, & \text{if } X_i < Y_j \\ 0, & \text{if } X_i > Y_j \end{cases}$ in case of no ties and (3)

$$\phi(X_i, Y_j) = \begin{cases} 1, & \text{if } X_i < Y_j \\ \frac{1}{2} & \text{if } X_i = Y_j \\ 0, & \text{if } X_i > Y_j \end{cases} \text{ in case of ties.} \tag{4}$$

In case of large samples

$$W^{*} = \frac{W - E_0(W)}{\sqrt{\mathrm{var}_0(W)}} \tag{5}$$

is asymptotically normally distributed with mean

$$E_0(W) = n(m+n+1)/2 \tag{6}$$

and variance

$$\mathrm{var}_0(W) = m\,n(m+n+1)/12 \tag{7}$$

when the null hypothesis is true.

In case of ties, the null mean of W is unaffected, but the null variance is reduced to

$$\mathrm{var}_0(W) = \frac{mn(m+n+1)}{12} - \left\{ \frac{mn}{12(m+n)(m+n-1)} \cdot \sum_{k=1}^{K} (t_k - 1) t_k (t_k + 1) \right\}, \tag{8}$$

where K denotes the number of tied groups and t_k is the size of the kth tied group. An untied observation is considered to be a tied group of size 1.

Reject H_0 if

a. One-Sided Upper-Tail-Test

$$W^{*} \geq z_{\alpha}; \quad \text{otherwise do not reject,} \tag{9}$$

b. One-Sided Lower-Tail-Test

$$W^{*} \leq -z_{\alpha}; \quad \text{otherwise do not reject,} \tag{10}$$

c. Two-Sided Test

$$\left| W^{*} \right| \geq z_{\alpha/2}; \quad \text{otherwise do not reject} \tag{11}$$

For the present analysis the one-sided tests were applied.

3.2 Comparison of the toxicokinetics of pregnant DA/Han rats after application of different xenoestrogenes

For comparison of the toxicokinetic behaviour of the two chemicals we calculated the ratios of the different tissue concentrations, their medians and the probability of the Wilcoxon rank sum test statistic assuming that there are no differences in the toxicokinetic behaviour of daidzein and bisphenol A in pregnant DA/Han rats.

4. Results

A chemical’s distribution among tissues depends on the blood flow, the free (non-protein-bound) fraction and its tissue:blood partition coefficient. Csanady *et al.* (2002) measured higher fat:blood partition coefficients for bisphenol A (3.3) than for daidzein (0.3) in *in vitro* studies, but, all partition coefficients in non-adipose tissues (liver, kidney, placenta, muscle, etc.) were similar for both compounds (average values: BPA 1.4, DA 1.2). Despite the more lipophilic nature of bisphenol A the two compounds daidzein and bisphenol A seem to behave rather similar *in vivo* comparing pregnant and non-pregnant DA/Han rats.

4.1 Comparison of pregnant and non-pregnant DA/Han rats

Comparing the plasma concentrations of pregnant and non-pregnant DA/Han rats reveals that the maternal blood levels are not consistently higher or lower over the complete duration of the experiment for both chemicals. The levels for daidzein and bisphenol A aglycones are depicted below (at different concentration and time scales to show all data points; see figures 1-3 for daidzein and 4-6 for bisphenol A).

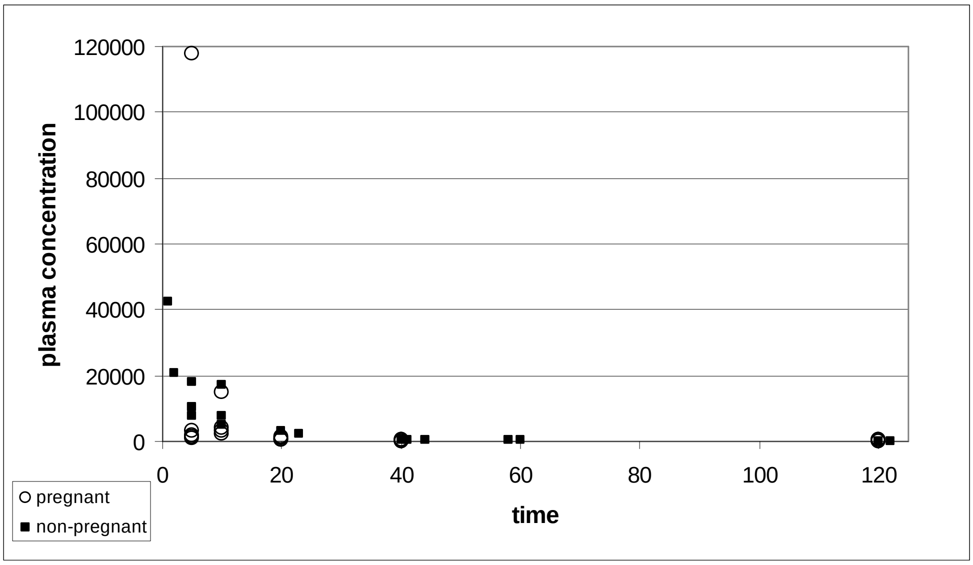

Figure 1. *Plasma concentration in ng/ml in pregnant and non-pregnant DA/Han rats, time in minutes since application of daidzein (overview).*

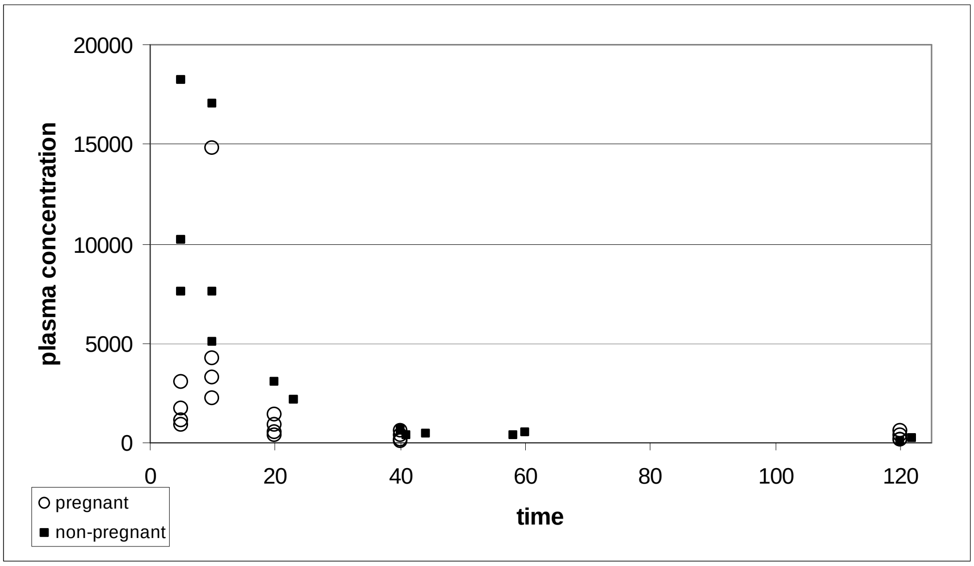

Figure 2. *Plasma concentration in ng/ml in pregnant and non-pregnant DA/Han rats, time in minutes since application of daidzein (zoomA).*

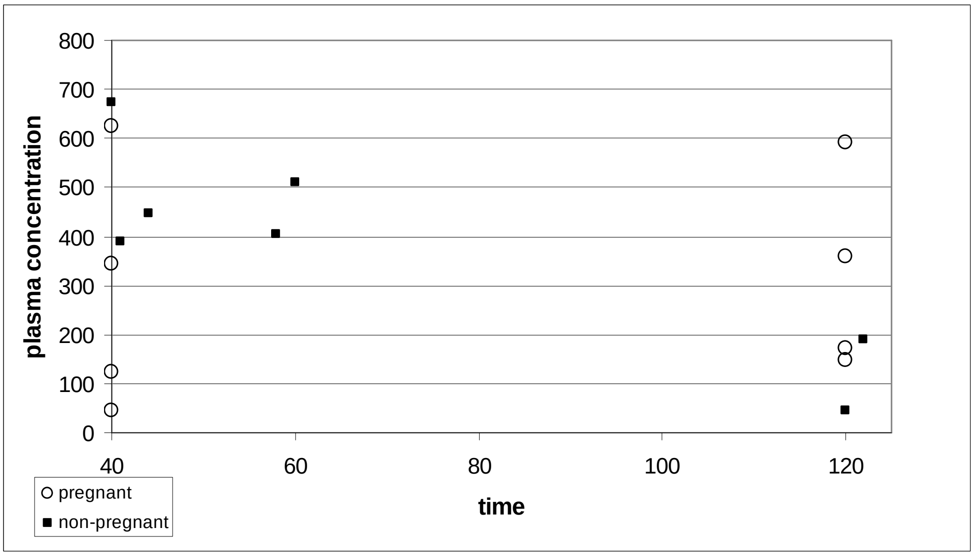

Figure 3. *Plasma concentration in ng/ml in pregnant and non-pregnant DA/Han rats, time in minutes since application of daidzein (zoomB).*

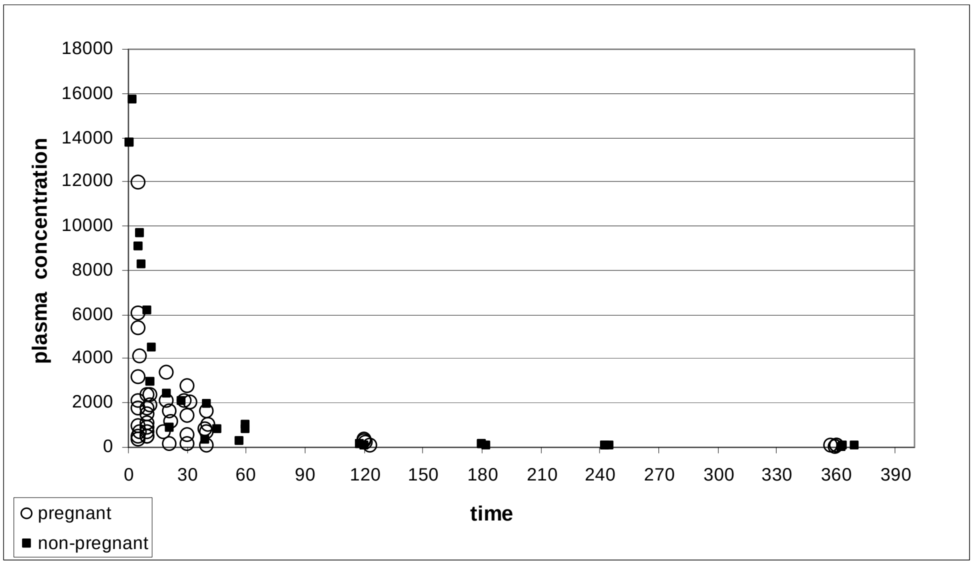

Figure 4. *Plasma concentration in ng/ml in pregnant and non-pregnant DA/Han rats, time in minutes since application of bisphenol A (overview).*

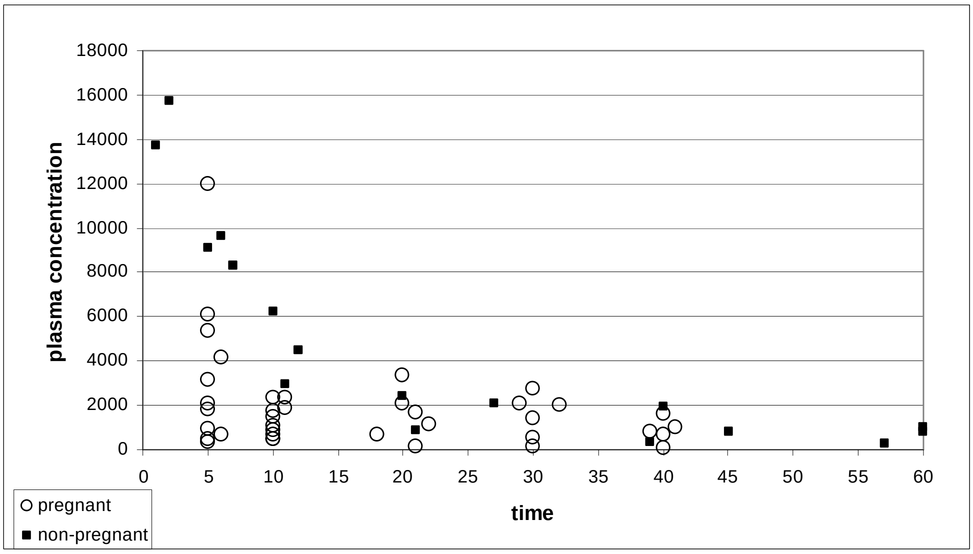

Figure 5. *Plasma concentration in ng/ml in pregnant and non-pregnant DA/Han rats, time in minutes since application of bisphenol A (zoomA).*

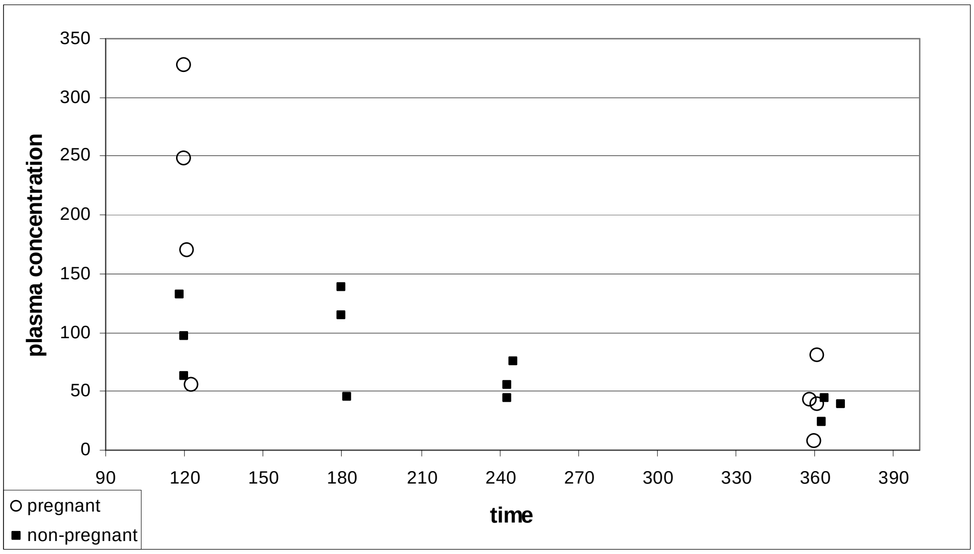

Figure 6. *Plasma concentration in ng/ml in pregnant and non-pregnant DA/Han rats, time in minutes since application of bisphenol A (zoomB).*

The results of the Wilcoxon rank sum test for differences in the plasma levels in pregnant and non-pregnant rats after intravenous application of 10 mg/kg bodyweight daidzein are given in table 4. Comparing the plasma levels it seems as if the concentration of daidzein aglycone is lower in pregnant rats than in non-pregnant rats in the beginning of the experiment. Differences at an α level of significance of 0.05 and 0.1, respectively, are detected at sampling times of 5 and 20 minutes. For t_j = 10, 40 and 120 minutes there were no clear difference though the plasma levels seem to be higher in pregnant rats after 120 minutes.

Table 4. *Results of the Wilcoxon rank sum test, sampling times in minutes and plasma concentration of daidzein aglycone in ng/ml.*

Non-pregnant			Pregnant			Wilcoxon rank-sum test
Rat No.	t in min	Concentration	Rat No	t in min	Concentration	
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	Do not reject H_0 $\alpha = 0.1/\ 0.05$
69	10	7592	4B	10	3290	
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	Do not reject H_0 $\alpha = 0.1/\ 0.05$
8	44	447	8C	40	123	
71	40	674	7C	40	345	
			15C	40	624	
63	120	45	5D	120	148	Do not reject H_0 $\alpha = 0.1/\ 0.05$
16	122	191	1D	120	173	
			3D	120	359	
			13D	120	593	

The results for bisphenol A are quite similar (see table 5).

Comparing the plasma levels it seems as if the concentration of bisphenol A aglycone is lower in pregnant rats than in non-pregnant rats in the beginning of the experiment. Differences at an α level of significance of 0.05 are detected at sampling times of 5 and 10 minutes. For t_j = 20, 40, 120 and 360 minutes there were no clear difference though the plasma levels seem to be higher in pregnant rats after 120 minutes.

Table 5. *Results of the Wilcoxon rank sum test, sampling times in minutes and plasma concentration of bisphenol A aglycone in ng/ml.*

Non-pregnant			Pregnant			Wilcoxon rank-sum test
Rat No.	t in min	Concentration	Rat No.	t in min	Concentration	
36	7	8271	18	5	327	Reject H_0, $\alpha = 0.05$
34	5	9085	64	5	444	
35	6	9643	21	6	664	
			27	5	918	
			53	5	1774	
			59	5	2098	
			67	5	3138	
			65	6	4118	
			25	5	5374	
			58	5	6066	
			62	5	11970	
47	11	2928	20	10	462	Reject H_0, $\alpha = 0.05$
46	12	4496	31	10	499	
25	10	6209	33	10	697	
			29	10	852	
			34	10	1093	
			60	10	1500	
			55	10	1760	
			66	11	1896	
			57	11	2332	
			54	10	2367	
55	21	887	47	21	140	Do not reject H_0 $\alpha = 0.05/0.1$
44	20	2403	53	18	640	
			51	22	1129	
			40	21	1643	
			46	20	2058	
			62	20	3350	
36	39	358	19	40	63	Do not reject H_0 $\alpha = 0.05/0.1$
34	45	795	35	40	684	
35	40	1916	32	39	810	
			30	41	1017	
			28	40	1607	
44	120	63	17	123	54.9	Do not reject H_0, $\alpha = 0.05/0.1$
42	120	97	23	121	170.2	
55	118	132	26	120	248.4	
			24	120	327.3	
46	363	24	44	360	8.1	Do not reject H_0 $\alpha = 0.05/0.1$
47	370	39	43	361	39.1	
25	364	44	42	358	43.0	
			45	361	81.1	

4.2 Comparison of the tissue concentration ratios in pregnant rats

Ratios of the different tissue concentrations were calculated for each animal and compared for the two experimental groups of pregnant DA/Han rats which received an intravenous application of 10 mg/kg bodyweight daidzein or bisphenol A.

Table 6. *Medians of the ratios of tissue concentration after application of either daidzein or bisphenol A, where agly. denotes aglycone, mat. maternal, fet. fetal, and fetus denotes the homogenate without liver.*

	time in min	plasma agly.: plasma total	mat. liver: plasma total	uterus: plasma total	placenta: plasma total	kidney: plasma total	fet. liver: plasma total
Daidzein	5	0.97	1.53	0.14	0.13	1.44	0.06
	10	0.56	3.21	0.51	0.32	1.46	0.12
	20	0.44	3.70	0.56	0.34	1.92	0.13
	40	0.20	3.58	0.88	0.54	1.97	0.14
	120	0.39	2.85	0.81	0.36	1.49	0.15
BPA	5	0.75	3.26	0.87	0.56	3.29	0.48
	10	0.71	3.71	0.91	0.78	2.75	0.77
	20	0.48	2.89	0.98	0.92	2.39	1.27
	30	0.42	2.76	2.45	1.25	1.93	1.27
	40	0.45	5.26	1.03	1.02	1.24	0.84
	120	0.28	6.98	1.23	1.12	1.22	1.44
	360	0.05	5.72	1.96	1.29	1.18	1.85
	time in min	fetus: plasma total	placenta: uterus	fet. liver: placenta	fetus: placenta	fet. liver: mat. liver	fet. liver: fetus
Daidzein	5	0.08	0.40	0.45	0.38	0.03	1.07
	10	0.10	0.60	0.41	0.32	0.04	1.26
	20	0.11	0.63	0.37	0.31	0.04	1.15
	40	0.13	0.61	0.24	0.21	0.04	1.07
	120		0.42	0.19		0.03	
BPA	5	0.23	0.66	1.06	0.56	0.14	2.18
	10	0.42	1.02	0.96	0.49	0.20	1.91
	20	0.71	0.56	0.93	0.63	0.34	1.68
	30	0.92	0.59	1.04	0.73	0.42	1.19
	40	0.61	0.96	0.59	0.52	0.15	1.21
	120	0.65	0.77	1.28	0.47	0.18	2.01
	360	1.52	0.75	1.29	1.23	0.33	1.09

Changes in ratios between plasma and tissues over time reflect the decline in blood levels of the xeno-/phytoestrogen and its distribution to other maternal and fetal compartments. For instance, the ratios are highest for maternal liver due to an efficient hepatic extraction of the compounds from the circulation, followed by kidney, the main organ for their excretion. The ratios for plasma / fetal liver are lower and similar to those of other tissues (*e.g.* uterus, placenta); but they are clearly higher for bisphenol A than for daidzein. This is probably due to the higher lipophilicity of bisphenol A which is thought to facilitate a rapid distribution to uterus, placenta and fetus. Differences are detected mainly at early sampling times at in the plasma ratios. The fetus-plasma or placenta-plasma ratios show differences during up to sampling times of 40 or 120 minutes, respectively.

Table 7*. P-values based of the Wilcoxon rank-sum test for differences between the tissue ratios in daidzein or bisphenol A treated pregnant DA/Han rats, where agly. denotes aglycone, mat. maternal, fet. fetal, and fetus denotes the homogenate without liver.*

time	plasma agly.:	mat. liver:	uterus:	placenta:	kidney:	fet. liver:
	plasma total	plasma total	plasma total	plasma total	plasma total	plasma total
5	0.0616	0.0128	0.0054	0.0066	0.0281	0.0404
10	0.0897	0.6434	0.0206	0.0055	0.0087	0.0105
20	0.5224	0.2568	0.0233	0.089	0.4497	0.0143
40	0.1416	0.4624	0.6242	0.05	0.4624	0.0143
120	0.7728	0.0209	0.1489	0.0433	0.5637	0.1573

time	fetus:	placenta:	fet. liver:	fetus:	fet. liver:	fet. liver:
	plasma total	uterus	placenta	placenta	mat. liver	fetus
5	0.0676	0.7341	0.3798	0.4334	0.079	0.2752
10	0.0055	0.217	0.0881	0.0641	0.0105	0.3938
20	0.0201	0.8501	0.0881	0.0201	0.0143	0.2938
40	0.0143	0.05	0.1416	0.2207	0.0143	0.6242
120		0.1489	0.1573		0.1573	

5. Discussion

As mentioned already above (p.3-4), it was the primary goal of our toxicokinetic studies in pregnant animals to obtain experimental data on the transplacental transfer of two prototypical environmental estrogens. Both daidzein and bisphenol A apparently cross the placenta, and a small part of the maternal dose will reach the fetus (Degen *et al.*, 2002[a]; Bolt *et al.*, 2002). Secondly, it was of interest to compare *i)* the kinetics of these agents with each other and *ii)* with that of the test agents in non-pregnant animals.

Studying the differences in the toxicokinetic behaviour due to pregnancy of the investigated organism we have to take into account the sparse data situation. As we deal with dynamic processes we have to consider that a tendency towards higher concentrations immediately after the application of a compound may change after some time. Thus, the different sampling times have to be investigated separately at first. If there seems to be just a shift in the concentration-time curve a global test may be applied summarizing the single Mann-Whitney U-tests.

In the present data sets pregnant DA/Han rats seem to have lower plasma concentrations of daidzein or bisphenol A, respectively, than the non-pregnant animals only in the first (early) phase after intravenous application of the compound. At – and for bisphenol A after – 120 minutes the situation seems to be reverse though it was not possible to confirm higher plasma concentrations in pregnant rats at a significance level of 0.05 or 0.1. With intravenous application, early time points of the concentration-time curves reflect mainly distribution and metabolism in the organisms, the later also elimination of metabolites. Major differences in these kinetic processes were not seen between pregnant and non-pregnant rats. Thus, we can conclude that metabolic inactivation by phase-II enzymes and subsequent elimination of our test agents is similarly efficient in both groups.

With respect to the other point of interest, *i.e.* a comparison between bisphenol A and daidzein, we conclude that, at the same dose, elimination of the phytoestrogen is apparently more rapid than that of bisphenol A in both pregnant and non-pregnant rats. Neither this factor nor the higher ratio for plasma/fetus concentrations for bisphenol A than for daidzein will, however, strongly impact on internal levels in light of the pronounced differences in exposure to these environmental estrogens. Human dietary intake estimates for bisphenol A are ≤ 10 µg / day, but 1 – 50 mg for daidzein and the related isoflavone genistein (Degen, 2004).

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