Industrial chemical bisphenol A and raw milk: a toxicokinetic study in lactating dairy sheep after repeated dietary and subcutaneous administration

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Abstract

• Background: Dietary intake is the predominant route for human exposure to bisphenol A and one of the food items important for humans is milk, and BPA-polluted animal feed and environments may thus affect human exposure. The aim of our study was to evaluate the BPA exposure and disposition in sheep milk after repeated dietary and subcutaneous administration of a relatively low dose (100 µg/kg of body weight per day) of BPA to a sheep.

• Results: With our toxicokinetic model, we showed that most likely only free BPA passes into the mammary gland and is subsequently conjugated there. The percentage of the dose eliminated with milk was less than 0.1%, regardless of the route of BPA administration.

• Conclusions: It is proven that the BPA is eliminated through the milk of lactating sheep. However, the amounts excreted in the milk that were detected in this study are minimal.

Background

Since the start of the commercial production of bisphenol A (BPA) in the 1950s until the present, the global production and consumption of this substance, regardless of the suspected negative health effects, has continued to rise [1]. With both the wide use of BPA and its leaching from many products and materials [2], it is known to be one of the ubiquitous environmental contaminants [3]. The main route of BPA exposure is thought to be oral ingestion (up to 83% of the total estimated exposure), and in 2013 canned products accounted for about 50% of the

dietary exposure to BPA. Thus, cans and packaging are believed to be the main source of contamination in foods [4]. However, the products from farm animals, being directly exposed to human pollution, could still be, in some cases, an additional risk factor for human exposure.

One such product, of which production is inseparably linked to the environment and depends largely on human activities, is milk. The application of contaminated material on the soil, such as sewage sludge or industrial waste, and atmospheric deposition from nearby industrial activities, have resulted in a broad range of environmental contaminants that enter the milk chain [5]. It is also true that chemicals can enter milk even during the collection and preparation processes of dairy products [6]. For instance, BPA may be introduced during milking from plastic parts of the

milking machines, or also transferred from bulk milk to plastic storage tanks [7]. Finally, BPA can also migrate as an additive from packaging material into the consumable milk. The actual levels of BPA found in commercial milk samples are presented in the review of Mercogliano and Santonicola, and are in the range between not detected (ND) to 521 ppb [5].

To the best of our knowledge, only a few *in vivo* studies are published regarding BPA transfer to milk, with all of them using rodent models, and all report limited excretion of BPA into milk [8-11]. Doerge et al. evaluated the lactational transfer of BPA after repeated oral dosing in rats, and found concentrations of 0.83 +/- 0.26 nM of free BPA and 7.6 +/- 2.8 nM of total BPA 1 hour after the administration of 100 µg/kg of b. w.. They calculated that doses delivered to pups lactationally were 300-fold lower than the dose administered to the dams [11].

The aim of our study was to estimate the transfer of BPA from feed or via subcutaneous administration to milk. To do so, one Slovenian autochthonous dairy sheep, an Istrian Pramenka, and her lamb were used in the study. Time courses of the free, conjugated and total BPA concentrations were followed in the ewe's blood plasma after repeated dietary and subcutaneous administration, as well as BPA transfer in milk. We also aimed to assess lactational transfer of BPA to the suckling lamb by estimating BPA exposure in its blood plasma.

Results

Validation of the analytical methodology used

The validation parameters of the BPA blood plasma and milk analysis are presented in Table 1. The method was linear for BPA standards and matrices, as proved by the determination coefficients (r^2) of ≥0.999 and ≥0.991, respectively. Mean recoveries for free and total BPA in the blood plasma were 82.3 and 49.5%, respectively and in the milk 62.9 and 54.3%, respectively. The total BPA refers to the sum of free and conjugated BPA. The coefficients of variation (CVs) of the concentrations detected and recovery in the fortified samples were from 1.5–24.4% under within-laboratory reproducibility conditions. Limit of detection (LOD) values were 0.05–0.1 µg/L and 0.2–0.4 µg/L for the free and total BPA determination, respectively, and differed according to a more comprehensive chromatographic background in the total BPA extracts.

Table 1: Validation results of BPA determination in blood plasma and milk

Toxicokinetic analysis

BPA levels were checked before conducting both the first and second part of the experiment to provide a baseline for the analysis. The ewe entered the first part of the experiment with 0.05 and <0.4 µg/L of the free and total BPA in the blood plasma, respectively, and with <0.1 and 0.31 µg/L of the free and total BPA in milk, respectively. Just before the start of the subcutaneous administration, the ewe's blood plasma contained 0.15 and 0.72 µg/L of the free and total BPA, respectively, while its milk contained <0.1 and 0.35 µg/L of the free and total BPA, respectively.

Comparison of the plasma concentration-time profiles

The maximum plasma concentration of free BPA obtained after subcutaneous administration was higher than after dietary administration. In addition, free BPA exposure was prolonged after subcutaneous administration compared to the dietary route of intake. With the dietary route, the maximum plasma concentration (c_{max}) of free BPA was 2.15 µg/L and was obtained very quickly, at 0.33 h. For the subcutaneous route, c_{max} of free BPA was 6.41 µg/L and was obtained after 2 h.

The c_{max} values of BPA-conjugate were similar for both routes of exposure and were 49.64 µg/L (t_{max} = 1 h) for subcutaneous administration and 41.3 µg/L (t_{max} = 0.33 h) for dietary administration.

The same seems to be valid for the c_{max} of total BPA, where c_{max} after subcutaneous administration was 55.6 µg/L (t_{max} = 1 h) and c_{max} after dietary administration was 43.46 µg/L (t_{max} = 0.33 h).

The AUC of free BPA was 33.3 µg h/L for subcutaneous administration and 1.28 µg h/L for dietary administration. However, for conjugated and total BPA the AUC was 274 µg h/L and 307 µg h/L for subcutaneous administration and 409 µg h/L and 410 µg h/L for dietary administration, respectively. Administration by the subcutaneous route led to a higher overall internal exposure to free BPA and lower internal exposure to conjugated/total BPA compared to the dietary route.

Clearance and relative bioavailability of free BPA, BPA-conjugate and total BPA obtained with noncompartmental toxicokinetic (TK) analysis are presented in Table 2.

Table 2. Toxicokinetic parameters of the noncompartmental TK analysis following the first dietary and

subcutaneous BPA administration

The TK parameters obtained with the TK model of the free BPA, BPA-conjugate and total BPA are gathered in Table 3. Blood plasma BPA-conjugate and total BPA concentration time courses were described with a one-compartment model, while a two-compartment model was more suitable for free BPA.

Table 3. Toxicokinetic parameters of the BPA (RSE%) following repeated dietary and subcutaneous administration

Figure 1 shows the plasma free BPA, BPA-conjugate and total BPA concentration-time profiles after repeated dietary and subcutaneous exposure of the ewe during the five days of a daily BPA administration of 100 µg/kg of b. w. and the subsequent three days of no BPA administration. The BPA-conjugate and total BPA concentration-time profiles varied slightly between the two routes, and markedly between the two routes for the free BPA.

[Please insert Figure 1.]

Estimation of the free BPA, BPA-conjugate and total BPA elimination into the milk

Given our model assuming passive transfer (first-order) of BPA into milk, it is more likely that only free BPA is transferred into the mammary gland (Akaike Information Criterion (AIC)=-5.066 for the conjugated and AIC=-5.031 for the total BPA), versus the hypothesis that conjugated BPA is also transported (AIC=-3.900 for the conjugated and AIC=-2.254 for the total BPA). The estimated rate constants of transfer into milk (estimate (RSE%)) were 0.00832 L/h (0.1%) for the free BPA, 0.01839 L/h (3.6 %) for the BPA-conjugate and 0.02669 L/h (2.4%) for the total BPA.

For the first part of the experiment (dietary administration), the percentage of the dose eliminated with milk was 0.0002% (RSE=0.1%) for the free BPA, 0.00045% (RSE=3.6%) for BPA-conjugate and 0.00066% (RSE=24%) for total BPA.

For the second part of the experiment (subcutaneous administration), the percentage of the dose eliminated with milk was 0.00453 % (RSE=0.1%) for the free BPA, 0.01001 % (RSE=3.6%) for BPA-conjugate and 0.01452 % (RSE=2.4%) for total BPA.

Regarding the suckling lamb, which drank milk after his mother was administered with BPA by the dietary or subcutaneous routes, there were only traces of BPA in the samples of its plasma.

Discussion

The purpose of this study was to investigate the toxicokinetics of BPA and to evaluate its elimination into the sheep milk after two different routes (po and sc) of repeated low dose BPA administration.

A comparison of the plasma concentration-time profile for the basic TK parameters of the two administration routes was made using the noncompartmental approach. Regarding the comparison of both routes of BPA administration, our results are similar to those in Guignard et al. [12], where the TK parameters for the same routes of administrations but with higher dose regimens were compared. The formulations for the dietary and as well for subcutaneous route of administration were similar in both studies. In our study, the c_{max} of free BPA for dietary administration was obtained quickly (0.33 h). In their study, mean c_{max} was attained 0-12 h for three ewes and 0.20 h for two others. For the subcutaneous route, c_{max} in our study was obtained after 2 h, in their study it was obtained after 2 h for three ewes and after 1 h for one ewe. In our study, the free BPA c_{max} for the subcutaneous route was three-fold higher than for the dietary route and in their work the free BPA c_{max} for the subcutaneous route was 4.6 ± 1.5-fold higher than for the dietary route. Our study demonstrates a higher cumulative (AUC) internal exposure to free BPA after subcutaneous administration compared to the dietary route, which is in line with the findings of Guignard et al. [12]. In their study the relative bioavailability of BPA for the dietary as compared to subcutaneous route was 3.3 ± 0.3%. In our work, the relative bioavailability of BPA for the dietary as compared to subcutaneous route was 4.5%. Both this earlier work and the current study were also in agreement with regard to the BPA-GLUC concentration time course. Unlike free BPA, the BPA-GLUC concentration time courses are very similar for the two routes of exposure.

The comparison of our study to Guignard et al. [12] is important to ensure our data coincided well with theirs, as a limitation of our work was the use of only one animal and a very low dose of BPA, which resulted in even lower measured concentrations in plasma. The similarity of the results from

both studies thus indicates the credibility of our data. This is important, as these data were the base for our TK model, which we used to evaluate the elimination of BPA into the sheep milk.

Sampling of the milk was possible only at a couple of sampling points, and thus it was not possible to make time-concentration profiles for it. However, our TK model enabled us to estimate the percentage of the dose eliminated with milk, which was less than 0.1% for free BPA, conjugated-BPA and total BPA, regardless of the route of administration. This result is comparable with the results of Snyder et al., where they found only a small fraction of the 14^{C} labelled BPA (0.63+/- 0.13 µg/equiv/mL) 8 hours after dosing [8]. Regarding free, conjugated and total BPA, it is already indirectly proven in rats that free BPA is transferred into the mammary gland to a greater extent than bisphenol A glucuronide (BPA-GLUC) [11, 13]. Given our TK models, the same was true in our study for the ewe. In the first model we were assuming passive transfer (first-order) of free BPA into milk, and in the second we were assuming that conjugated BPA would also be transported. Based on the lower value of the Akaike information criterion (AIC), with the first model it is more likely that only free BPA is transferred into the mammary gland. Nevertheless, it was reported that the major molecular species in the milk of rats after oral administration of 14^{C}-BPA was BPA-GLUC [8]. The concentrations measured in milk six hours after BPA administration (dietary and subcutaneous) in our study show the same result. Six hours after dietary administration the concentration of free BPA was 0.05 µg/L and the concentration of BPA-GLUC was 0.78 µg/L. Similarly, the concentrations of free BPA and BPA-GLUC after subcutaneous administration were 0.87 and 1.89 µg/L, respectively. Regarding the BPA-GLUC in the milk, we hypothesise that free BPA is passively transferred into the mammary gland, and subsequently conjugated in its glucuronidated form by the uridine 5'-diphospho-glucuronosyltransferase (UDP-glucuronosyltransferases) in the mammary gland. Only a few studies have evaluated UDP-glucuronosyltransferases (UGTs) presence in breast tissue. Expression of UGT2B10, UGT2B11, UGT2B15 and UGT2B UGT1A10 and UGT2B7, and UGT2B11 enzymes have been proven in humans, and the results of Street et al. confirm the capability of glucuronidation of BPA in human breast tissue, although with glucuronidation activities that are much lower (by more than

100,000-fold) compared with those seen in the liver [14]. There are currently no (to the best of our knowledge) known studies that have evaluated the presence of UDP-glucuronosyltransferases in the ewe mammary gland, although it seems reasonable to assume that the mammary glands of all mammals are equipped with similar detoxifying mechanisms.

The above mentioned concentrations of free BPA measured in this study are well within the range with the concentrations found in raw milk measured in a recent Italian monitoring study, where the concentrations of only free BPA ranged from 0.081 – 2.492 µg/L [15]. However, the concentrations measured in commercial milk samples were generally higher (from 14.0 to 521.0 µg/L) [5], meaning that the BPA load in consumption milk is greater at the end of the production line.

Conclusion

Considering the widespread consumption of milk and dairy products, the origins of milk contamination with BPA should be well investigated. To the best of our knowledge, this is the first study in which BPA elimination in milk was evaluated in sheep. Our study carried out in an animal model relevant to dairy cattle shows that BPA and BPA-GLUC are detected in the milk sample obtained from the ewe administered by two different routes of administration. We estimated that the percentage of the eliminated BPA in the milk is less than 0.1% of the administered dose for both dietary and for subcutaneous routes.

Methods

Chemicals

Bisphenol A ≥ 99% purity (Merck, Sigma-Aldrich, Darmstadt, Germany) was dissolved in absolute ethanol and corn oil for the dietary (po) and subcutaneous (sc) routes of administration, respectively. The volume administered to the sheep was adjusted to the body weight recorded on the day of the administration. For the dietary administration, approximately 1 mL of BPA solution in absolute ethanol was applied onto the pellet ration to obtain the single dose of 100 µg/kg body weight, and applied with the morning feed of pellets (400 g). For the sc administration, the injection of BPA solution was performed in the shoulder area (2.9 mL) at the same dose. Both solutions were stored at the ambient temperature in sealed amber glass bottles for the entire duration of use. All materials used for the

solution preparation, sample processing and assays were either made of glass or of BPA-free plastics.

Animal husbandry

All animal procedures were carried out in accordance with ethical standards and approved by the Administration of the Republic of Slovenia for Food Safety, Veterinary Sector and Plant Protection with permission no. U34401-3/2015/8. The study was performed on one stable, healthy lactating Istrian Pramenka sheep with a single suckling lamb in a sheepfold at the Centre for Sustainable Recultivation at Vremščica belonging to the Veterinary Faculty of the University of Ljubljana, Slovenia. The ewe was six years old and weighed 59 kg, while the suckling lamb was four weeks old and weighed 12 kg. The ewe and lamb were kept under natural temperature and photoperiodic conditions, with free access to water, hay and salt. In addition, the sheep was fed twice a day with 400 g plant based pellets (SchafKorn Lac, Unser Lagerhaus Warenhandels Ges., Austria). Eventual contamination of the experimental environment was checked by preliminary testing of drinking water and pellets by HPLC analysis, which revealed the slight presence of BPA of 0.02 µg/L and 5 µg/kg in these two matrices, respectively. The sheep and its lamb were, at both periods of the study, penned individually the day before the first administration until three days after the last administration. The lamb was kept with its mother, except on sampling days, when they were separated for a few hours before sampling time to collect enough milk for analysis. The animals were clinically healthy, as indicated by medical (temperature, breathing and rumination frequency, pulse rate), haematological, biochemical and faecal examinations. Fourteen days after the second experimental period, the sheep and its lamb were released in their original herd.

A sheep was chosen for this study due to its physiological similarities with cows, but easier manipulation. However, cows are mainly used in milk production in Europe (accounting for 96.9% of the total milk produced) [16].

Experimental design

The experiment was divided into two periods, the first being the dietary administration period and the second being the subcutaneous administration period. The same ewe was used for both exposure routes, thus a 13 days wash-out period was permitted to ensure that BPA was removed from the body

of the ewe before the start of the second period. Regarding the administration of BPA, in the first period the ewe received BPA in its diet (100 µg/day/kg of body weight) for five consecutive days (dietary route of administration). The ewe ingested all pellets within 2-9 minutes. During the second period, the same ewe was injected in the shoulder area with 100 µg/kg of b. w. of BPA subcutaneously per day for five consecutive days (subcutaneous route of administration).

On the first day of the dietary period of the experiment, the ewe`s blood samples were taken at time 0 (before the first administration) and 0.083, 0.16, 0.33, 0.5, 1, 2, 4, 6, 8, 10 and 24 hours after the first administration. The blood samples were then taken every day for the next seven days (trough concentrations). The sampling time started when the ewe ingested the whole portion of pellets. Similar sampling intervals were used in the second subcutaneous period of the experiment, with the exception of the first blood sampling (0.083 h after the sc administration), which was not taken. Blood samples from the suckling lamb were collected on the first day 10 hours after BPA administration to the ewe, and on every following day before the next administration to the ewe. Jugular vein blood samples were collected in heparinised glass vacuum tubes, cooled to 4 °C and transported to the laboratory where blood plasma was separated by centrifugation at 2640×g for 15 min. The plasma was transferred and stored in polypropylene (PP) tubes. Plasma samples were kept frozen at -20 °C until analysis.

A diagram illustrating the design of the study, including the two experimental periods, BPA administration and blood sampling schedule in the ewe, is provided below (Figure 2).

[Please insert Figure 2.]

Milk sampling was done in both periods. On the first day of the experiment milk was collected six and 10 hours after the first BPA administration and every next day just before the following administration. Before the first administration in each period the ewe was milked and then the lamb was separated from the ewe during next six hours to allow estimation of the amount of BPA excreted in milk. The sampling period continued from the 5^{th} until the 8^{th} day of both periods, when there was no BPA administration. Milk was collected in polypropylene (PP) containers and stored at -20 °C until analysis.

Blind samples of blood plasma from the sheep and suckling lamb and milk from the sheep were taken just before the start of both periods, to provide a baseline for the analysis.

BPA and total sample analysis

A stock solution of BPA of 200 µg/mL was prepared in acetonitrile, while the intermediate and working standard solutions ranging from 2,000 to 1.0 ng/mL were further prepared in a mixture of acetonitrile and water at a ratio of 35 : 65 (v/v). Working standard solutions ranging from 50,000 to 50 ng/mL for fortification of the total BPA samples were prepared in water with a small portion (≤ 20%, v/v) of ethanol or acetonitrile. All solutions were prepared using high purity deionised water obtained using a PureLab Option and PureLab Classic water purification system (Elga, Woodridge, Illinois, USA). The acetonitrile and methanol used were of HPLC gradient grade purity and purchased from J.T. Baker (Center Valley, PA, USA). Only high quality glass or PP labware were used for the sample analysis. Samples of the sheep blood plasma and milk were tested for the presence of both free (unconjugated) and total BPA (free and conjugated), of which the latter was determined indirectly by conversion of the BPA-GLUC to free BPA. Sample aliquots of 1.5 and 5 mL were taken for the analysis of free BPA in the blood plasma and milk, respectively, while aliquots of 1.0 and 2.5 mL were taken to determine the total BPA and were diluted by 1.1 M Na-acetate buffer solution with pH values of 5.3 and 5.1 and volumes of 1.0 and 2.5 mL for the blood plasma and milk, respectively. Forty and 70 µL of ß-glucuronidase from *Helix pomatia*, type HP-2, ≥100,000 units/mL including also ≤7,500 sulfatase units/mL (Merck, Sigma-Aldrich, Darmstadt, Germany) were added to each sample of the blood plasma and milk, respectively. Samples were then incubated in a shaking water bath at 37 °C for 4 h. The blood plasma and milk samples were further extracted by 6 and 10 mL of acetonitrile, respectively and ultrasonicated before being evaporated to dryness at 40–42 °C under a stream of N_2 using an N-evap 111 evaporator (Organomation Associates, Berlin, MA, USA). A further clean-up procedure included solid phase extraction (SPE) by the use of molecularly imprinted polymer (MIP) columns AFFINIMIP® SPE Bisphenols, 6 mL, 100 mg (AFFINISEP, Petit-Couronne, France), while the additional use of a Chromabond HR-X phase, with 6 mL columns, 200 mg, and 85 µm particle size

(Macherey-Nagel, Düren, Germany) was previously utilised for all deconjugated sample extracts, as described by Deceuninck et al. [17]. Final SPE extracts were re-dissolved in acetonitrile/H_2O (35/65, v/v) as follows: both free BPA blood plasma and milk samples in 0.5 mL, and total BPA blood plasma and milk samples in 1.0 and 0.5 mL, respectively. Fifty µL of the final extract were taken for the high-performance liquid chromatography (HPLC) analysis.

HPLC measurements were performed using a Varian ProStar HPLC system (Varian Analytical Instruments, Walnut Creek, CA, USA), comprised of a tertiary pump (240 model), automatic injector (410 model), fluorescence detector (363 model), degasser and Galaxie 1.7.4.5 analytical software. Chromatographic separation was performed at room temperature by the gradient binary pumping of water and acetonitrile at a flow rate of 1 mL/min through a Hypersil Gold C18 analytical column, 150 x 4.6 mm, with a particle size of 3 µm, which was protected with Hypersil GOLD 3µ Drop in the guards (Thermo Scientific, Waltham, MA, USA). The mobile phase gradient was as follows: 0–2 min, 35% (v/v) of acetonitrile, gradient to 12 min, 35–50% (v/v) of acetonitrile, held to 20 min, gradient to 20.5 min, 50–35% (v/v) of acetonitrile, held to 21 min. The excitation and emission wavelengths of the fluorescence spectrophotometry analysis were set at 230 and 315 nm, respectively [18]. The results were evaluated in accordance with the external standard method using a standard calibration curve as a function of chromatographic peak areas and standard concentrations. Each sample series consisted of a matrix sample, obtained before the first periodic BPA administration (a baseline sample), five to seven animal study samples in duplicate and two baseline matrix samples fortified with BPA to control the recovery rate. The measured sample concentrations were corrected for the possible baseline matrix response and for the mean recovery of the respective series and then used as final results.

Validation of the analytical methodology used was performed to demonstrate its fitness for the stated purpose. Linearity was determined by the least-squares method to calculate regression and correlation parameters for six to seven standard concentration points per calibration curve (range 1.0–100 ng/mL), and for both matrices as a correlation between measured and added concentrations (ranges 0.25–10 µg/L and 1.0–50 µg/L for free and total BPA in blood plasma, respectively, 0.5–15

µg/L for both free and total BPA in milk). Mean recovery was evaluated by analysis of four to six fortified blank materials at two concentration levels at separate time points (blood plasma: free BPA 2 and 10 µg/L, total BPA 25 and 50 µg/L; milk: free BPA 2 and 5 µg/L, total BPA 5 and 10 µg/L). The within-laboratory reproducibility of the method was evaluated as the CV of the determined and recovery values. The LOD value was estimated as the BPA concentration in the retention time window where the analyte was to be expected, which corresponded to 3 × noise and was corrected for the blank matrix response.

Toxicokinetic analysis

Each entity (free, conjugated, and total) plasma concentration time course until the second BPA administration was first analysed using a noncompartmental approach to obtain the estimates of the area under the concentration–time curve extrapolated to infinity (AUC), maximum concentration in plasma and time when it occurs (c_{max} and t_{max}, respectively). AUC was calculated using the linear trapezoidal method and extrapolated to infinity by addition of the term C_{last}/λ_z, where C_{last} is the last quantified concentration measurement and λ_z is the terminal slope of the concentration profile in the semi-log plot calculated by linear regression. t_{max} and c_{max} were reported as observed. AUCvalues were used to estimate clearance (CL) as CL = Dose/AUC_{sc} and relative bioavailability after dietary administration (F_r) as $F_r = AUC_{po}/AUC_{sc}$. The indexes po and sc refer to the route of administration (dietary and subcutaneous, respectively) and Dose is the single BPA dose (100 µg/kg of b. w.). Note that CL can be estimated only after intravenous administration. Our estimate of CL is therefore apparent clearance, i.e. assuming complete bioavailability after subcutaneous administration. Subsequently, all TK data after both routes of administration were simultaneously fitted to a one- and two-compartment model with first-order absorption and elimination. The estimated parameters were clearance (CL), volume of the central and peripheral compartment (V_c and V_p, respectively), distribution clearance (Q), absorption rate constants after subcutaneous and dietary administration ($k_{a\ sc}$ and $k_{a\ po}$, respectively) and relative bioavailability (F_r). Parameter fitting was performed using ADAPT II software [19] with the maximum likelihood method and a proportional variance model, V_i =

($\square \times Y_i)^2$, where V_i is the variance of the i-th data point and Y_i is the value predicted by the model. The AIC value was used to select the model.

Permeation of free, conjugated and total BPA into milk was modelled as a first order process $dA_m/dt = k_m \times C_p(t)$, where dA_m/dt is the transfer rate in µg/h, $C_p(t)$ is the BPA plasma concentration at time t, and k_m is the transfer rate constant. k_m was estimated by simultaneous fitting of the amounts excreted into milk up to six hours after the first subcutaneous and dietary administration, with TK parameters for the plasma data fixed to previously estimated values. The amounts excreted in milk up to 6 h were approximated by multiplication of the concentration in milk at 6 h by 0.25 L, i.e. assuming an average milk yield of 1 L/day. We tested the hypothesis that only free BPA is transferred into milk and subsequently conjugated in the mammary gland, i.e. fixing the TK parameters to the values estimated for the free BPA versus the hypothesis that conjugated BPA is also transferred, i.e. fixing the TK parameters to the values obtained for the conjugated and total BPA.

Abbreviations

AIC: Akaike Information Criterion

AUC: area under the concentration–time curve extrapolated to infinity

BPA: bisphenol A

BPA-GLUC: bisphenol A glucuronide

CL: clearance

C_{last}: last quantified concentration measurement

c_{max}: maximum plasma concentration

CV: coefficient of variation

F_r: relative bioavailability

HPLC: high-performance liquid chromatography

$k_{a\ po}$: absorption rate constant after dietary administration

$k_{a\ sc}$: absorption rate constant after subcutaneous administration

LOD: limit of detection

po: dietary

PP: polypropylene

Q: distribution clearance

RSE: relative standard error

sc: subcutaneous

TK: toxicokinetic

t_{max}: the time at which the cmax is observed

UDP-glucuronosyltransferase: Uridine 5'-diphospho-glucuronosyltransferase

UGT: UDP-glucuronosyltransferase

Vc: volume of central compartment

V_p: volume of peripheral compartment

λ_z: terminal slope of the concentration profile in the semi-log plot calculated by linear regression

Declarations

Ethics approval and consent to participate

All animal procedures were carried out in accordance with ethical standards and were approved by the Administration of the Republic of Slovenia for Food Safety, Veterinary Sector and Plant Protection with permission no. U34401-3/2015/8.

Consent for publication

Not applicable.

Availability of data and materials

The datasets analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

MP, VCF, AŠ and SŠ conceived the study, participated in the design and coordination. SŠ and VCF performed the analysis of the samples. IG and SŠ performed the toxicokinetic analysis. SŠ, VCF and IG participated in the writing of the manuscript. All authors have read and approved the final manuscript.

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Tables

Table 1: Validation results of BPA determination in blood plasma and milk

Parameter		Free BPA				Total BPA			
		Blood plasma		Milk		Blood plasma		Milk	
Linearity									
Standards	Range (ng/mL)	1.0–100							
	Correlation (r2)	0.9993–0.9999							
Matrix	Range (µg/L)	0.25–10		0.5–15		1.0–50		0.5–-15	
	Correlation (r2)	0.9956		0.9984		0.9982		0.9908	
Recovery and precision	Added concentration (µg/L)	2	10	2	5	25	50	5	10
	Recovery (s.d.) (%)	76.13 (2.26)	88.37 (1.29)	56.76 (13.83)	69.08 (11.90)	57.11 (13.64)	41.96 (2.99)	57.67 (8.53)	51.04 (10.61)
	CV (%)	2.97	1.46	24.36	17.23	23.88	7.13	14.79	20.78
LOD (µg/L)		0.05		0.1		0.4		0.2	

Table 2. Toxicokinetic parameters of the noncompartmental TK analysis following the first dietary and subcutaneous BPA administration

Parameter		Free BPA	BPA-conjugate	Total BPA
Subcutaneous administration	CL (L/h/kg)	3.0055	0.3650	0.3258
Dietary administration	Fr (%)	3.8	149	134

Legend:

CL = clearance

F_r = relative bioavailability, dietary vs. subcutaneous

Table 3. Toxicokinetic parameters of the BPA (RSE%) following repeated dietary and subcutaneous administration

Parameter	Free BPA	BPA-conjugate	Total BPA
CL (L/h/kg)	3.12 (7.2%)	0.388 (9.9%)	0.343 (9.1%)
V_c (L/kg)	2.45 (23.4%)	2.17 (13.3%)	1.89 (12.4%)
$k_{a\ sc}$ (h^{-1})	0.455 (16.1%)	2.57 (29.1%)	2.59 (27.1%)
Q (L/h/kg)	0.425 (39.4%)	/	/
V_p (L/kg)	5.75 (27.4%)	/	/
$k_{a\ po}$ (h^{-1})	6.39 (67.1%)	3.24 (19.2%)	3.44 (20.0%)
Fr (%)	4.52 (29.9%)	115 (12.9%)	101 (12.4%)

Legend:

CL = clearance

V_c = volume of central compartment

$k_{a\ sc}$ = absorption rate constant after subcutaneous administration

Q = distribution clearance

V_p = volume of peripheral compartment

$k_{a\ po}$ = absorption rate constant after dietary administration

F_r = relative bioavailability, dietary vs. subcutaneous

RSE = relative standard error, it was calculated by dividing standard error by mean value (%)

Figures

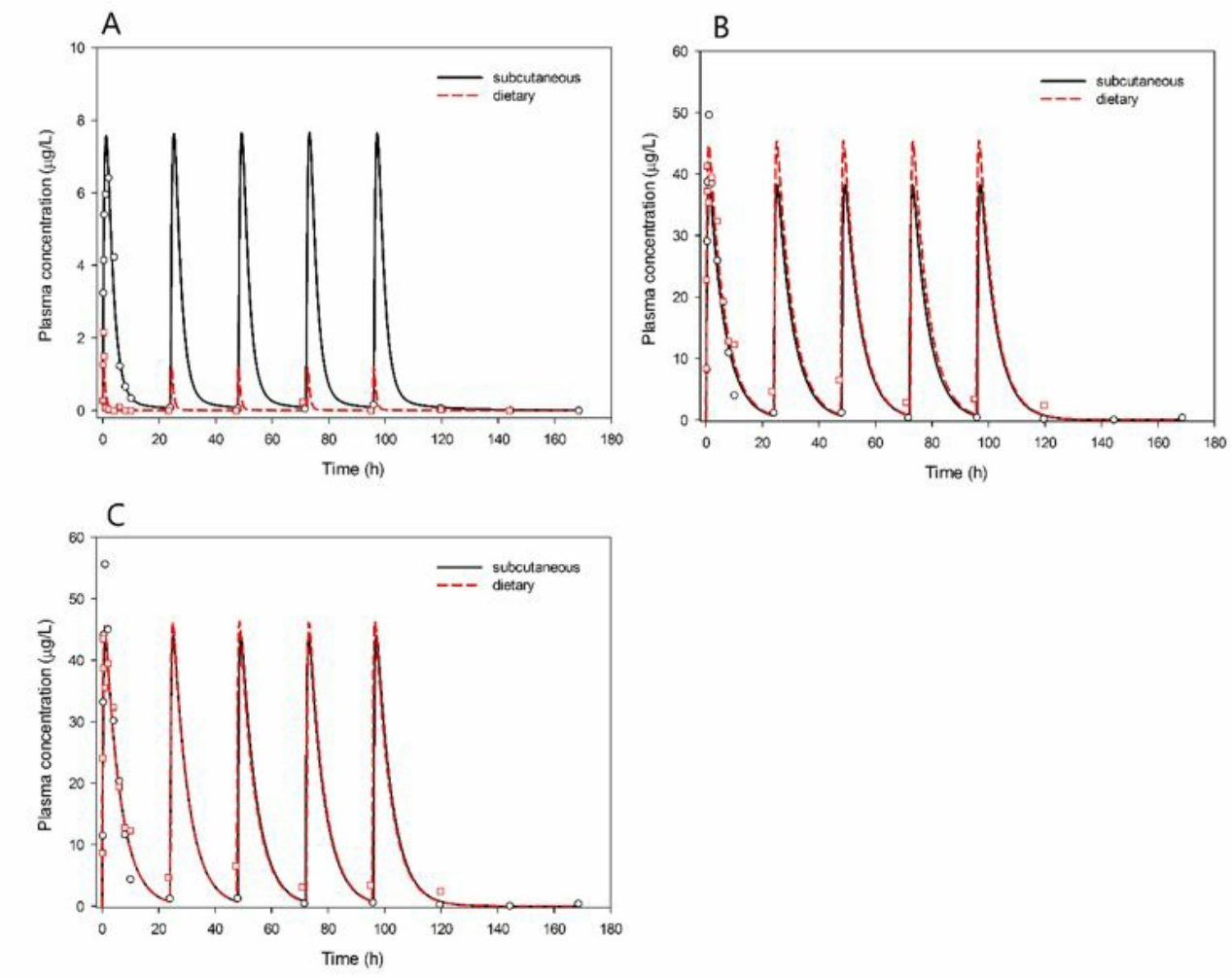

Figure 1

Time course of free (A), conjugated (B) and total (C) BPA plasma concentration. The ewe was administered 100 µg/kg/day of BPA sc or po for five days, one time per day in the morning. The administration was stopped the sixth day of the experiment, while the sampling continued until the eighth day. Blood samples were collected at 0.08 – 0.17 – 0.33 – 0.5 – 1 – 2 – 4 – 6 – 8 – 10 – 23.33 – 46.58 – 69.35 – 92.35 – 116.1 – 140.1 – 164.1 hours after the first dietary administration and 0.17 – 0.33 – 0.5 – 1 – 2 – 4 – 6 – 8 – 10 – 23.92 – 47.84 – 71.34 – 95.17 – 118.67 – 143 – 167.5 hours after the first subcutaneous administration.

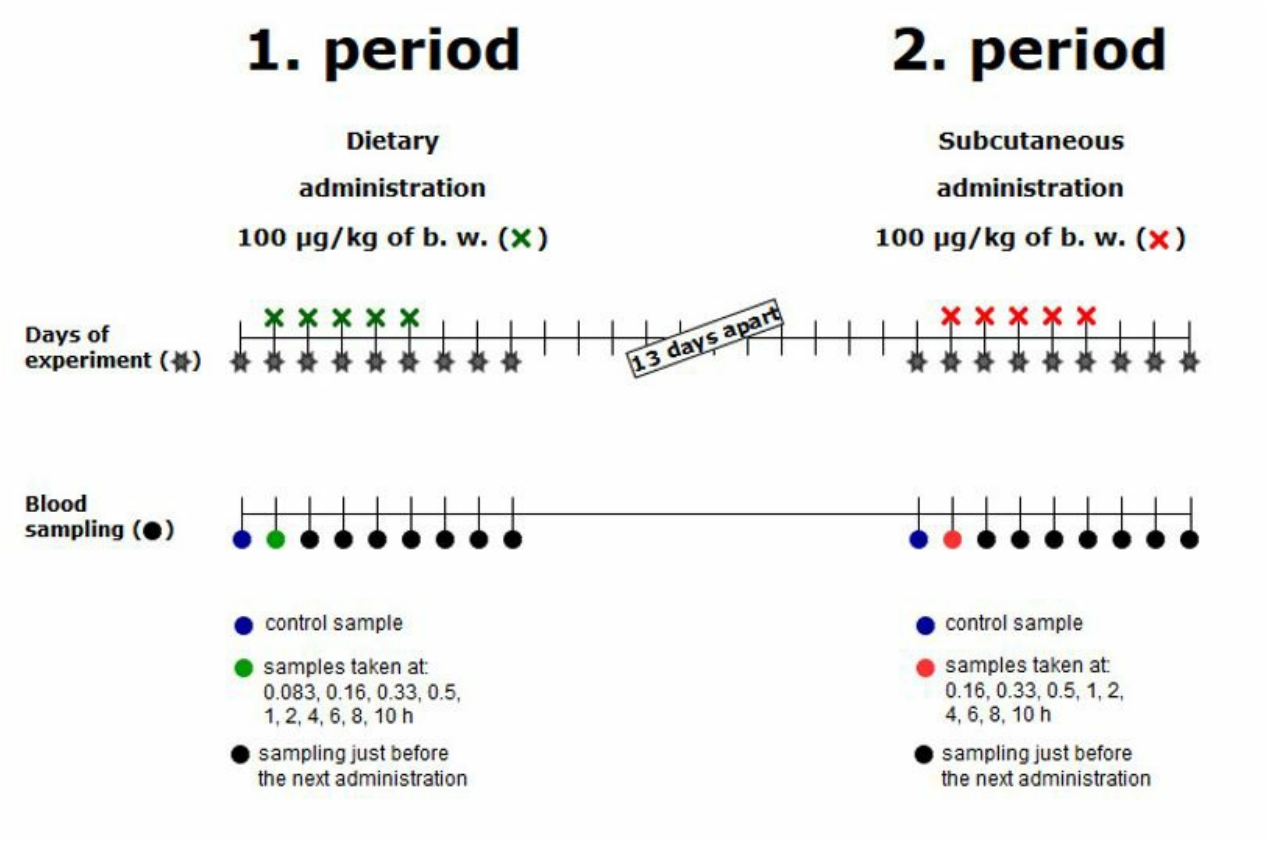

Figure 2

Study design with two experimental periods, BPA administration and blood sampling schedule for the ewe.

Supplementary Files

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