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Review

Human exposure to bisphenol A by biomonitoring: Methods, results and assessment of environmental exposures

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Abstract

Human exposure to bisphenol A is controversially discussed. This review critically assesses methods for biomonitoring of bisphenol A exposures and reported concentrations of bisphenol A in blood and urine of non-occupationally ("environmentally") exposed humans. From the many methods published to assess bisphenol A concentrations in biological media, mass spectrometry-based methods are considered most appropriate due to high sensitivity, selectivity and precision. In human blood, based on the known toxicokinetics of bisphenol A in humans, the expected very low concentrations of bisphenol A due to rapid biotransformation and the very rapid excretion result in severe limitations in the use of reported blood levels of bisphenol A for exposure assessment. Due to the rapid and complete excretion of orally administered bisphenol A, urine samples are considered as the appropriate body fluid for bisphenol A exposure assessment. In urine samples from several cohorts, bisphenol A (as glucuronide) was present in average concentrations in the range of 1–3 µg/L suggesting that daily human exposure to bisphenol A is below 6 µg per person (<0.1 µg/kg bw/day) for the majority of the population.
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Keywords: Bisphenol A; Human exposure; Biomonitoring; Exposure assessment; Risk assessment

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Introduction

Bisphenol A is primarily used to make polycarbonate plastic and epoxy resins, which are widely used for a variety of applications such as polycarbonate baby feeding bottles and epoxy food-can linings. Although of lesser importance, other uses have been reported, for example as an antioxidant in polyvinylchloride production (EU, 2003). Due to the use of bisphenol A to manufacture products used in many applications, it has been speculated that human exposures to bisphenol A may be widespread and it has been postulated that these exposures may reach high levels (vom Saal and Hughes, 2005; vom Saal et al., 2005; Vandenberg et al., 2007). Bisphenol A is a weak estrogen and has been implicated to cause a variety of effects on reproduction and development in animals, including effects at doses well below those showing adverse effects in routine toxicity studies (vom Saal et al., 2005). Moreover, bisphenol A blood levels determined in humans were statistically associated with human diseases such as ovarian dysfunction or endometrial hyperplasia and recurrent miscarriage (Sugiura-Ogasawara et al., 2005; Takeuchi et al., 2004a; Berkowitz, 2006; Politch, 2006; Hiroi et al., 2004). Therefore, some publications have called for an updated risk assessment (vom Saal and Hughes, 2005; vom Saal et al., 2005).

In risk assessment, exposure and hazard (potential toxicity) are separately assessed. Integration of hazard assessment and exposure with dose–response for toxicity is then translated into risk assessment (Henry, 2003; MacDonald, 2004). The potential toxicity of bisphenol A has been intensively addressed with studies performed along testing guidelines and following good laboratory practices (GLP) available to cover all of the major toxicity endpoints relevant for hazard assessment (EU, 2003; Goodman et al., 2006; Gray et al., 2004; Willhite et al., 2008). The exposure of the general population to bisphenol A has been assessed in a number of studies or assessments by regulatory agencies or in publications (EU, 2003; Kang et al., 2006; Miyamoto and Kotake, 2006; EFSA, 2006; Fujimaki et al., 2004; SCF, 2002; Thomson and Grounds, 2005; Wilson et al., 2007). However, many of the assessments have used data on bisphenol A concentrations in environmental media and in food items, or data on the migration of bisphenol A into food, as a basis for exposure assessment. In combination with food consumption data, this information served as a basis for indirectly estimating human exposures to bisphenol A. Due to the development of highly sensitive analytical techniques, the use of "biomonitoring" is playing a more and more important role in exposure assessment since, when incorporating knowledge on toxicokinetics and biotransformation of the compound of interest and the limitations of analytical methods, biomonitoring gives more exact information on actual exposure to an agent as compared to indirect assessments. Large programs to address population exposures to environmental chemicals using biomonitoring have been started (Angerer et al., 2007; Boogaard, 2007; Calafat et al., 2006; Needham et al., 2007; Pirkle et al., 2005; Yang et al., 2006b).

This review will critically assess the suitability of analytical methodology available for the quantitative determination of bisphenol A in the low concentrations expected in urine and blood samples from non-occupationally exposed humans and the available information on concentrations of bisphenol A in blood and urine from such populations. This information is then translated into an assessment of human exposure to bisphenol A from environmental sources.

Summary of the toxicology of bisphenol A

Due to the focused scientific and public attention to chemicals that may mimic endogenous hormone action and thus interfere with endocrine organ function, many investigations assessing the potential toxicity of bisphenol A have been published in recent years. Due to the high production volumes, the toxicity of bisphenol A has been intensively studied since the 1970s. Bisphenol A is not genotoxic, not carcinogenic and the guideline conforming repeated dose toxicity studies, including studies on reproductive and developmental toxicity covering a wide dose range, showed adverse effects only at doses > 50 mg/kg bw/day (summarized in: EU, 2003; Goodman et al., 2006; Gray et al., 2004). A number of non-guideline and non-GLP studies, often using low numbers of animals per experimental group, have reported effects of bisphenol A administration on a variety of parameters regarding reproductive and developmental toxicity endpoints in much lower dose ranges (2 to 20 µg/kg bw/day), which were claimed to be due to the weak estrogenicity of bisphenol A. The dose ranges in which these effects have been reported have also been claimed to be "environmentally relevant" (Sheehan, 2000; vom Saal et al., 2005) or to result in blood levels of "unconjugated" bisphenol A close to that encountered in humans after environmental exposure (Keri et al., 2007).

The relevance of the low dose effects of bisphenol A in animals for human hazard assessment is unclear, since the other toxicity studies, often using more powerful study designs with larger numbers of animals per dose group, a larger number of dose levels and stringent quality control, were unable to repeat the findings of low-dose effects after bisphenol A exposures (Ashby et al., 1999; Cagen et al., 1999; Ema et al., 2001; Tyl et al., 2002). Moreover, the weak estrogenicity of bisphenol A, as assessed by special protocols, has been known for decades (Bitman and Cecil, 1970; Dodds and Lawson, 1936) and humans are exposed to a variety of natural chemicals with weak estrogenicity (Bolt et al., 2001; Safe, 2004, 2000). Therefore, the studies reporting low-dose effects of bisphenol A were not considered sufficiently robust to serve as pivotal points in risk assessment (EFSA, 2006). To derive a tolerable daily intake (TDI) for bisphenol A, the European Food Safety Authority (EFSA) mainly relied on two rodent multi-generation studies with dose levels covering a wide dose-range including low dose exposures, and on species differences in toxicokinetics (see below). In a three-generation rat study, significant reductions in adult body weight and in pup body and organ weights were observed at a dose of 50 mg bisphenol A/kg bw/day and above (Tyl et al., 2002). In a two-generation study in the mouse, an increased incidence of hepatocyte hypertrophy of mild to minimal severity after doses of 50 mg bisphenol A/kg bw/day

was seen (Tyl et al., 2007). Both studies did not show reproductive and developmental toxicity in doses below 50 mg/kg bw/day.

Therefore, the overall NOAEL (no-observed-adverse-effect-level) of bisphenol A induced effects in both rats and mice was 5 mg/kg bw/day. A tolerable daily intake (TDI) based on this NOAEL using the generally applicable uncertainty factor of 100 (10 for interspecies differences, 10 for inter-individual differences) was set at 0.05 mg/kg bw/day by EFSA's AFC panel (EFSA, 2006) and is considered conservative due to the species differences in bisphenol A toxicokinetics. The United States Environmental Protection Agency (US EPA) has also determined a reference dose (RfD) of 0.05 mg/kg bw/day for bisphenol A, based on a reduction in body weight gain observed in rats after doses of 50 mg/kg bw/day for 103 weeks by the application of a high uncertainty factor of 1000 (due to the use of a lowest-observed-adverse-effect-level, LOAEL) (IRIS, 2002). An oral reference dose of 0.016 mg/kg bw/day has recently been derived based on the NOAEL of the rat and mouse reproductive toxicity studies using US EPA uncertainty factor guidance and application of a 3-fold database uncertainity factor (Willhite et al., 2008).

Possible pathways of bisphenol A exposures in the general population

Due to the low vapor pressure of bisphenol A, inhalation exposures of the general population will likely have only a minor contribution to the overall exposure (EU, 2003). Inhalation of bisphenol A containing household dust is unlikely to result in significant uptake of bisphenol A from the lung since the large particle sizes typically encountered in household dust are not penetrating into the lung. House dust may be trapped in the mucocilliary escalator and may be swallowed resulting in additional oral exposure. A low contribution of bisphenol A present in house dust is indicated in studies determining indoor and outdoor air concentrations of bisphenol A and bisphenol A concentrations in food in a cohort of preschool children (Wilson et al., 2007) for exposure assessment. Bisphenol A concentrations in the house dust samples were up to 707 ng/g. In this study, inhalation of bisphenol A was estimated to result in a daily systemic exposure to bisphenol A, assuming complete absorption from the respiratory tract, of 0.008 to 0.014 µg/person/day, whereas ingestion with food resulted in an daily uptake of 1.7 to 2.7 µg/person/day. Non-occupational dermal exposures to bisphenol A are also considered as infrequent; moreover, systemic bioavailability of bisphenol A after dermal application is limited (<10%) (EU, 2003). Based on these considerations, several organizations concluded that oral exposures with food are the major source for bisphenol A exposure in all age groups of non-occupationally exposed human subjects (EFSA, 2006; EU, 2003; Kang et al., 2006; Wilson et al., 2007). Some additional oral exposure to bisphenol A may also result from the use of bisphenol A-based resins in dentistry, but this is restricted to a short time period after treatment with bisphenol A-based dental sealants or composites (Fung et al., 2000; Joskow et al., 2006; Olea et al., 1996).

Due to the wide range of food contact applications of bisphenol A, many food commodities may contribute to human bisphenol A exposure. Based on conservative assessments of exposure based on food consumption patterns, migration of bisphenol A from food contact materials into food, and measured concentrations of bisphenol A in foods, several expert groups have indirectly estimated daily human exposure to bisphenol A from food depending on age group and age group specific food consumption. For example, the European Union risk assessment report on bisphenol A estimated daily exposures to bisphenol A to range from less than 0.02 µg/kg bw/day to 59 µg/kg bw/day in adults (EU, 2003). The European Food Safety Authority estimated intakes of bisphenol A with food to range from 0.2 µg/kg bw/day (three month old infant fed with breastmilk only) to 13 µg/kg bw/day (six month old infants fed with polycarbonate bottles and commercial food) based on conservative migration values of bisphenol A into food and food consumption patterns (EFSA, 2006). Exposures to bisphenol A based on direct food analysis and food consumption patterns generally resulted in estimates of daily human exposures in the low µg/person/day range (Goodson et al., 2002; Miyakawa et al., 2004; Thomson and Grounds, 2005; Wilson et al., 2007) giving daily doses in the range from 0.005 to 0.37 µg/kg bw/day. Summarizing results from different exposure assessments, an intake of less than 1 µg/kg bw/day was concluded for bisphenol A (Kang et al., 2006).

Biomonitoring in exposure assessment

Biomonitoring directly measures human daily or cumulative exposures to xenobiotics by the determination of blood, urine or tissue concentrations of the chemical or its metabolites (Angerer et al., 2007). Biomonitoring will provide an integrated and reliable measurement of exposures to the xenobiotic from all sources (Calafat et al., 2006; Needham et al., 2007; Pirkle et al., 2005). The reliable estimates of "internal" dose thus generated represent an important part of the exposure assessment and therefore constitute an important contribution to the risk assessment process. If a sufficient number of samples have been analyzed by biomonitoring, an accurate assessment of the range of human exposure to a specific xenobiotic is possible. Often, exposure assessments using biomonitoring are more accurate than indirect exposure assessments ("potential dose") based on concentrations of the xenobiotic in environmental media and food (Angerer et al., 2007). The "indirect" assessments usually have to rely on assumptions on food consumption, extent of migration from food contact materials, and activities and may not well address individual exposure scenarios. However, a transformation of tissue, urine or blood concentrations of a xenobiotic to external exposure estimates requires a detailed knowledge of the biotransformation and toxicokinetics of the xenobiotic, and precise and valid analytical methods including specific sampling and storage protocols with information on the specificity and stability of metabolites monitored as biological markers (Boogaard, 2007; Calafat et al., 2006; Calafat and Needham, 2007; Needham et al., 2007). Moreover, exposure biomonitoring requires knowledge on the

source of the contaminant and possible routes of exposure and estimates of concentrations at the boundary of the human body.

A number of publications have reported blood and urine concentrations of bisphenol A in individuals without occupational exposures to bisphenol A in order to assess "internal" doses. Some studies have also correlated measured "internal" doses to bisphenol A with dietary habits, gender and/or health status. The results from these studies and their relevance for exposure assessment for bisphenol A from environmental sources are put into context with the available information on route-of-exposure dependent toxicokinetics of bisphenol A in humans and the suitability of the analytical methodology applied.

Biotransformation and toxicokinetics of bisphenol A

A detailed knowledge of the biotransformation and the toxicokinetics of a xenobiotic is necessary to develop sampling schedules, to decide if blood or urine samples are more appropriate, to develop analytical procedures to quantitate the most relevant chemical species (parent compound or metabolite), and to be able to translate blood and urine concentrations to a daily exposure estimate (Calafat et al., 2006). For the purpose of estimating exposure, determination of a parent compound in blood or serum is only applicable to xenobiotics with a low extent of biotransformation and a slow clearance from blood unless exposure circumstances are very well known and accounted for in the sampling protocol. Determination of urinary concentrations of a parent xenobiotic can only be applied if the compound is sufficiently water soluble and a larger and constant part of the dose is excreted as unchanged parent compound in urine. In the case of xenobiotics rapidly metabolized in the organism, it is considered more useful to quantitate blood or urinary concentrations of major metabolites since these are present in higher concentrations than the parent compound. In addition, for xenobiotics that are rapidly excreted into urine either as unchanged parent or a metabolite, urinary measurements are most preferred since the xenobiotic will be concentrated in urine. Urine is a widely used matrix for biomonitoring, especially for non-persistent chemicals such as bisphenol A with a biological half-life in humans of less than 6 h (Völkel et al., 2002). Urine samples are also less invasive to collect compared to blood samples, in particular for children.

The toxicokinetics and biotransformation of bisphenol A have been intensively studied in both laboratory animals (i.e. rodents and primates) and in humans. A good database is available for guidance and interpretation of biomonitoring studies since toxicokinetics in humans after controlled oral administration of low doses of bisphenol A are also known (Table 1).

As outlined above, food is the major pathway of general population exposure to bisphenol A due to the migration of bisphenol A from food contact materials into food. Therefore, toxicokinetics after oral administration are considered most relevant for their application to an exposure assessment by biomonitoring and the interpretation of its results. In rodents and in primates, including humans, orally administered bisphenol A is rapidly and efficiently (>95% of dose) absorbed from the gastrointestinal tract (Kurebayashi et al., 2002; Völkel et al., 2002) and undergoes extensive first-pass metabolism in the gut wall (Inoue et al., 2003a) and in the liver (Inoue et al., 2001; Pritchett et al., 2002). During this first-pass metabolism, biotransformation of bisphenol A to bisphenol A-glucuronide is the major pathway of bisphenol A biotransformation in primates and in rats (Fig. 1). In controlled studies in humans, only bisphenol A-glucuronide was identified as a metabolite of bisphenol A present in urine or blood (Völkel et al., 2002); however, the presence of bisphenol A-sulfate has been reported in human urine samples (concentration reached app. 10% of that of bisphenol A-glucuronide) (Ye et al., 2005a) and the potential for formation of bisphenol A-sulfate has been shown with in vitro studies on human hepatocytes (Pritchett et al., 2002). However, bisphenol A-glucuronide is also the major bisphenol A metabolite formed in human hepatocytes (Pritchett et al., 2002; Kuester and Sipes, 2007). Bisphenol A-glucuronide as a major bisphenol A metabolite is sufficiently unique and stable to serve as a biomarker to assess bisphenol A exposure.

Formation of bisphenol A conjugates is considered a deactivation reaction, since both bisphenol A-glucuronide and bisphenol A-sulfate are devoid of estrogenic activity (Matthews et al., 2001; Shimizu et al., 2002; Snyder et al., 2000) whereas bisphenol A has a low affinity to estrogen receptors. After oral administration of low doses of bisphenol A (5 mg/person or 60–80 μg/kg bw, much higher than doses received from "environmental" exposures), first pass metabolism to bisphenol A-glucuronide was essentially complete in humans (Völkel et al., 2002) as predicted by the high metabolic clearance of bisphenol A by glucuronidation in cryopreserved human hepatocytes (Kuester and Sipes, 2007). Parent bisphenol A in plasma with a limit of detection (LOD) of 10 nM (2.3 μg/L) was not detected even a short time after oral application when parent bisphenol A in blood is expected to be at or near the peak level and concentrations of bisphenol A-glucuronide were high (Table 1).

Due to high water solubility, bisphenol A-glucuronide formed during first-pass metabolism in the gut wall and liver is rapidly cleared from blood by the kidneys and excreted with urine in humans with terminal half-lives of less than 6 h after oral administration. In three independent studies in humans covering a dose range from 0.310 μg/kg bw to 80 μg/kg bw, the applied doses of bisphenol A were completely recovered in urine as bisphenol A-glucuronide (Table 1) (Tsukioka et al., 2004; Völkel et al., 2002, 2005) within 42 h and more than 90% of the dose applied was recovered in urine within the first 6 h after administration. The very efficient biotransformation of bisphenol A and the rapid renal excretion of bisphenol A-glucuronide do not indicate a potential for bioaccumulation and do not indicate a need for toxicokinetic studies in humans after repeated oral doses of bisphenol A (Filser et al., 2003) since bioaccumulation has to be considered as highly unlikely due to the rapid clearance.

As in humans, a rapid biotransformation and rapid excretion of orally administered bisphenol A is also observed in primates. Monkeys given a single oral dose of 100 μg ^{14}C-bisphenol A/kg bw excreted 79–86% of the administered radioactivity in urine.

Table 1
Reported concentrations of bisphenol A (BPA) in blood or urine of human subjects after controlled administration of BPA

No. of samples analyzed	Analytical method, sample workup	Concentration ranges reported	Reference
Blood samples from 3 male and 3 female subjects administered 60–80 μg BPA/kg bw	LC/MS-MS, separate assessment of BPA and BPA-glucuronide	Free BPA in blood below LOD of 10 nM (2.3 μg/L), peak concentration of BPA-glucuronide was app. 800 nM, reached 80 min after oral administration	(Völkel et al., 2002)
Urine samples from 3 male and 3 female subjects administered 60–80 μg BPA/kg bw	LC/MS-MS, separate assessment of BPA and BPA-glucuronide	Free BPA below LOD of 6 nM (1.4 μg/L), peak excretion of BPA-glucuronide was reached in urine samples collected within 6 h after administration (total excretion of app. 20 μmol	(Völkel et al., 2002)
Urine samples from 12 male and 13 female subjects consuming 50 μg BPA/person, urine collection for 5 h	GC/MS, separate assessment of BPA and BPA-glucuronide	Peak concentration of total BPA were 81 μg/L of urine (mainly present as BPA-glucuronide), free BPA amounted to 6 μg/L	(Tsukioka et al., 2004)
Urine samples from 3 male subjects administered 310–390 ng BPA/kg bw	LC/MS-MS, separate assessment of BPA and BPA-glucuronide	Free BPA in urine below LOD of 2.5 nM (0.6 μg/L), peak excretion of BPA-glucuronide was reached in urine samples collected within 6 h after administration (total excretion of app. 100 nmol	(Völkel et al., 2005)

The concentrations of parent bisphenol A in blood were at or below the limit of detection (app. 5 nM) (Kurebayashi et al., 2002) and the predominant urinary metabolites of bisphenol A in monkeys were a mono-and a di-glucuronide. In monkeys and in chimpanzees given oral doses of 10 mg bisphenol A/kg bw, peak blood concentrations of parent bisphenol A were 200 to 800 fold lower than those of bisphenol A metabolites (presumably glucuronides and sulfates) (Tominaga et al., 2006).

Most of the studies examining the toxicokinetics of bisphenol A in rodents used rats as experimental models. As in humans, the major metabolite of bisphenol A in rats is bisphenol A-glucuronide, which is formed in the liver and the intestinal wall during first-pass metabolism after oral administration. However, major differences in the elimination of chemicals from the liver due to differences in the molecular weight thresholds for biliary elimination between rodents and humans exist. Chemicals with molecular weight > 350 D are preferentially eliminated with bile from rodent liver, whereas this threshold for biliary elimination is 500–600 D in humans. This results in major species differences between rodents and humans in the elimination of bisphenol A-glucuronide (molecular weight 403 D) from the liver and thus in major species differences in bisphenol A toxicokinetics. In contrast to humans, due to elimination of bisphenol A-glucuronide with bile and reabsorption of bisphenol A from the intestine after cleavage of bisphenol A-glucuronide by glucuronidase in intestinal bacteria, bisphenol A undergoes enterohepatic circulation in rats. Thus, urinary excretion of bisphenol A and its metabolites accounts for only 10 to 40% of applied dose. A significant part of the administered dose is excreted with feces, in part as parent bisphenol A (Kurebayashi et al., 2005; Sakamoto et al., 2002). The apparent half-life of elimination of ^{14}C-bisphenol A derived radioactivity from the blood of rats given ^{14}C-bisphenol A has been reported to range from 20 to 80 h. Rodents also excrete parent bisphenol A with urine (Domoradzki et al., 2004; Kurebayashi et al., 2003, 2005; Pottenger et al., 2000).

Using subcellular fractions from the liver of both mice and rats, a number of bisphenol A metabolites were formed by

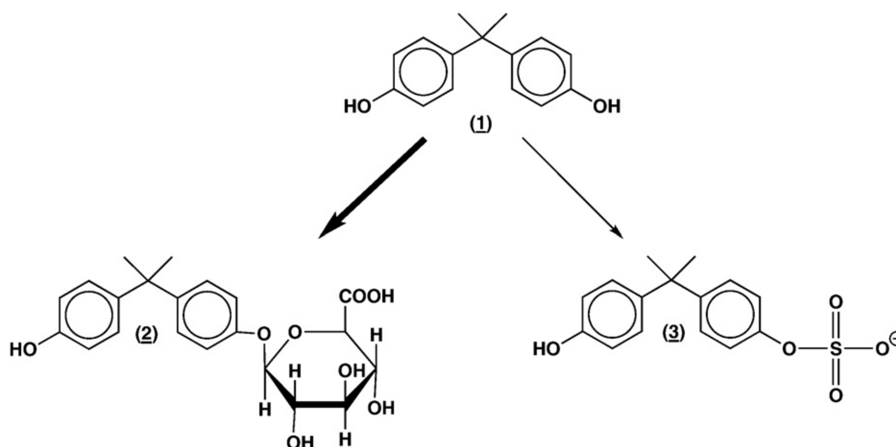

Fig. 1. Biotransformation of bisphenol A (*1*) in rodents and humans to bisphenol A-glucuronide (*2*) and bisphenol A-sulfate (*3*). Biotransformation of *1* to *2* and *3* occurs very efficiently in humans and the highly water soluble conjugates *2* and *3* are rapidly excreted with urine.

oxidative biotransformation (Jaeg et al., 2004; Yoshihara et al., 2004). Some of the metabolites were reported to have a higher affinity to the estrogen receptor as compared to bisphenol A. However, there is no evidence that such metabolites are formed to a significant extent in rats or in primates in vivo, in particular after oral administration, or in intact hepatocytes from rodents or humans (Pritchett et al., 2002), likely due to the effective glucuronidation of bisphenol A (Domoradzki et al., 2003; Kurebayashi et al., 2002, 2003, 2005; Pottenger et al., 2000; Snyder et al., 2000; Völkel et al., 2002, 2005).

The outlined results on biotransformation and toxicokinetics of bisphenol A have a major influence on the biomonitoring of bisphenol A and its metabolites for assessment of human exposures from diffuse environmental sources. Due to the rapid first-pass biotransformation of bisphenol A after oral uptake, only very low levels of parent bisphenol A will reach systemic circulation and only very low blood concentrations of parent bisphenol A are expected (Filser et al., 2003; Teeguarden and Barton, 2004; Teeguarden et al., 2005) requiring the development of extremely sensitive methods to determine parent bisphenol A in human blood. In addition, due to the rapid excretion, blood concentrations of bisphenol A or bisphenol A-glucuronide determined at one time point are very difficult to translate into an assessment of external exposure since they are heavily dependent on the time of blood sampling after bisphenol A consumption with meals. Unless this is taken into account in sampling design, bisphenol A or bisphenol A-glucuronide blood concentrations cannot be used for estimation of average exposures.

Therefore, a conclusive exposure assessment of bisphenol A by biomonitoring will need to directly quantitate the major metabolite of bisphenol A, bisphenol A-glucuronide, or include a sample preparation demonstrated to completely cleave bisphenol A-glucuronide to bisphenol A. Since most of orally consumed bisphenol A is excreted with urine within less than 24 h after consumption, and the expected very low blood concentrations and demonstrated rapid clearance of bisphenol A-glucuronide from human blood, urine is the preferred body fluid for exposure estimation of bisphenol A in humans.

Analytical methods for analysis of bisphenol A and bisphenol A metabolites in biological media

Biomonitoring will only provide conclusive results when adequate sampling and storage protocols and validated analytical methods are applied (Hoppin et al., 2006). Due to the low concentrations of analytes encountered in biomonitoring of environmental exposures, the complex matrices analyzed with many potential interfering signals, often complex sample workup procedures, and low stability of some analytes (such as bisphenol A conjugates), a considerable investment in quality assurance and method validation is required to produce correct results. One of the most important points in this context is the use of an appropriate internal standard to compensate for possible loss of analyte during sample processing and for variations in instrument performance. Usually, stable isotope (^{2}H or ^{13}C with regard to bisphenol A) labeled standards are most appropriate for this purpose since their physicochemical characteristics are practically identical to that of the unlabeled analyte. However, such standards can only be used when applying mass spectrometers as detectors. Instrumental analytics other than mass spectrometric detection that use internal standards will require additional quality assurance, especially in complex matrices such as urine or blood/serum; whereas methods without inclusion of internal standards may give the necessary accuracy only when very elaborate validation and quality assurance is applied.

A number of methods to quantitate low concentrations of bisphenol A in biological samples have been published (Tables 2 and 3). The analytical methods developed include ELISAs (enzyme-linked immunosorbent assays), single trace chromatographic separations such as HPLC (high performance liquid chromatography) with fluorescence detection (both with and without fluorophore derivatization), and HPLC with electrochemical detection. Methods using single chromatographic traces for bisphenol A detection may not be sufficiently specific, especially in the low concentration ranges encountered in biomonitoring of non-occupationally exposed humans. More specific methods for bisphenol A quantitation based on mass spectrometry using both single and triple quadrupole instruments have also been published. Mass spectrometric methods may monitor intensity of several fragments or transitions during the chromatographic separation (quantifier and qualifier) and therefore have a higher specificity. In addition to the widely varying analytical instrumentation, the published studies used different sample workup procedures. These include simple dilution of aqueous samples with polar organic solvents, extraction of bisphenol A from diluted blood or urine into ethyl acetate or ether, solid phase extractions, and chromatography involving column switching. Some studies included treatment with glucuronidase and/or sulfatase to cleave bisphenol A-glucuronide and bisphenol A-sulfate, the expected major metabolites of bisphenol A, some applied specific methods to quantitate bisphenol A-glucuronide while others only determined parent bisphenol A (Tables 2 and 3). Since, based on kinetics of bisphenol A in humans, metabolites are expected to be present in much higher concentrations than bisphenol A, a demonstration of an efficient cleavage of bisphenol A conjugates is considered important.

A general problem with the interpretation of the results on bisphenol A blood and urine levels is background contamination of samples with bisphenol A, which may interfere with quantitation at low concentrations. Studies have reported contamination of reagents or solvents with bisphenol A or leaching of bisphenol A from the materials used for sampling and sample storage, processing, and analysis (Fujimaki et al., 2004; Völkel et al., 2002, 2005; Sajiki, 2001; Sajiki et al., 1999). For example, trace amounts of approximately 0.01 μg bisphenol A/L were eluted from filtration columns, sorbent beds and frits by methanol and similar concentrations of bisphenol A were present in tap water and Milli-Q-purified water. Leaching of bisphenol A from plastic storage containers has also been reported with a leaching rate 40 fold higher into sheep plasma as compared to water. Due to this leaching, fetal bovine serum

Table 2
Characteristics of biomonitoring methods developed to analyze bisphenol A (BPA) and its metabolites in human blood. LOD, limit of detection; LOQ, limit of quantitation; CV, coefficient of variation; RSD, relative standard deviation

Analytical method	Sample workup	Biological samples analyzed	Analyte determined	Analytical performance	Suitability to determine BPA in human blood	Reference
ELISA (enzyme-linked immunosorbent assay)	None	Serum	BPA, no assessment of BPA-glucuronide	LOD of 0.3 µg BPA/L, deviations of app. 20% when data for spiked samples were confirmed by instrumental analytics	− −	(Kim et al., 2007; Kodaira et al., 2000; Ohkuma et al., 2002)
GC/MS with electron impact ionization	Solid phase extraction	Umbilical cord blood	BPA, no assessment of BPA-glucuronide	LOD of 0.05 µg BPA/L, recovery between 65 and 120 for spiked samples, CV < 15%	−	(Tan and Mohd, 2003)
GC/MS with electron impact ionization	Solvent extraction with ethyl acetate, derivatization to trimethylsilyl ether	Plasma, placenta tissue	BPA, no assessment of BPA-glucuronide	LOD of 0.01 µg BPA/L, LOQ of 0.1 µg BPA BPA/L, no data on precision and extraction efficiency	−	(Schönfelder et al., 2002)
GC/MS with EI ionization, thermal desorption	Glucuronidase treatment, sorptive extraction after derivatization	Plasma	Total BPA	LOD, 0.1 µg BPA/L, LOQ, 0.5 µg/L, recovery of 99%, RSD 9.6%	+	(Kawaguchi et al., 2004)
HPLC with electrochemical detection	Solid phase extraction	Serum	BPA, no assessment of BPA-glucuronide	LOD, 0.1 to 0.2 µg BPA/L, CV of 2.9%, recovery >93%	−	(Sajiki et al., 1999)
HPLC with multi-electrode electrochemical detection	Solid phase extraction	Serum	BPA, no assessment of BPA-glucuronide	LOD of 0.01 µg BPA/L, LOQ of 0.05 µg BPA/L, recovery of 79–87%, RSD between 5 and 14%	−	(Inoue et al., 2000)
HPLC with fluorescence detection	Solid phase extraction	Serum	BPA, no assessment of BPA-glucuronide	LOD of 5 µg BPA/L, recovery of 90%, no information on precision	−	(Fung et al., 2000)
HPLC with column switching and fluorophore labelling	Extraction with chloroform after acid treatment	Serum, ascites fluid	BPA, no assessment of BPA-glucuronide	LOD of 0.04 µg BPA/L, LOQ of 0.1 µg BPA/L, RSD between 4.2 and 8%, recovery of 78.6%	−	(Kuroda et al., 2003)
HPLC with electrochemical detection and column switching	With and without glucuronidase treatment, solid phase extraction	Serum	BPA, BPA-glucuronide, and total BPA	LOD 0.2 µg BPA/L, recovery of >90%, RSD <5%	+	(Fukata et al., 2006)
LC/MS, isocratic elution with electrospray ionization and negative ion detection	Solid phase extraction	Serum	BPA, no assessment of BPA-glucuronide	LOD of 0.1 µg BPA/L, CV of 7%, recovery >93%	−	(Sajiki et al., 1999)
LC/MS, electrospray ionization	Solid phase extraction, glucuronidase treatment	Plasma	Total BPA	LOD of 0.1 to 1 µg BPA/L, RSD of 0.3%,	+	(Inoue et al., 2001)
LC/MS-MS after electrospray ionization and negative ion detection	With and without glucuronidase treatment	Serum	BPA, BPA-glucuronide, and total BPA	LOD 0.5 µg BPA/L, LOQ of 3.4 µg BPA/L	±	(Völkel et al., 2005)

Table 3
Characteristics of biomonitoring methods developed to analyze bisphenol A (BPA) and its metabolites in human urine

Analytical method	Sample workup	Analyte determined	Analytical performance	Suitability to determine BPA in human urine	Reference
ELISA (enzyme-linked immunosorbent assay)	None	"Total immunoreactive" BPA, total BPA	Not given	−(crossreactivity of antibodies)	(Kodaira et al., 2000)
GC/MS	Glucuronidase treatment, solid phase extraction, derivatization	Total BPA	LOD, 3 μg BPA/L, LOQ of 7 μg BPA/L, recovery of app. 100%, CV between 3 and 7%	−(low sensitivity)	(Moors et al., 2007)
GC/MS-MS	Glucuronidase treatment, solvent extraction followed by solid phase extraction	Total BPA	LOD, 0.38 μg BPA/L, recovery of 87%, CV 9%	+	(Arakawa et al., 2004)
GC/MS with chemical ionization and electrophore derivatization, negative ion detection	Glucuronidase treatment and two step solid phase extraction	Total BPA	LOQ, 0.1 μg BPA/L, recovery of 83%, RSD 7.4%	+	(Tsukioka et al., 2003)
GC/MS with chemical ionization and electrophore derivatization, negative ion detection, automated sample preparation	Glucuronidase treatment and extractive derivatization	Total BPA	LOD, 0.1 μg BPA/L, LOQ of 0.3 μg BPA/L, recoveries of 95 to 116%, RSD 6–10%	++(automated)	(Brock et al., 2001; Calafat et al., 2005; Kuklenyik et al., 2003)
GC/MS with EI ionization, thermal desorption	Glucuronidase treatment, sorptive extraction after derivatization	Total BPA	LOD, 0.02 μg BPA/L, LOQ 0.1 μg BPA/L, recovery of 89 to 95%, RSD 6.3%	++	(Kawaguchi et al., 2004; Kawaguchi et al., 2005)
GC/MS after silylation	Glucuronidase treatment, solvent extraction, and solid phase clean-up	Total BPA	LOQ of 0.1 μg BPA/L, no further information	−	(Tsukioka et al., 2004)
HPLC with fluorescence detection	With and without glucuronidase treatment, solvent extraction	Total BPA, BPA, BPA-glucuronide	LODs from 0.012 to 0.34 μg BPA/L	+	(Kim et al., 2003; Matsumoto et al., 2003; Yang et al., 2003)
HPLC with electrochemical detection	Glucuronidase treatment followed by protein precipitation	Total BPA	LOD, 0.25 μg BPA/L, recovery of 92 to 103%, RSD <5%	+	(Fukata et al., 2006; Hanaoka et al., 2002)
HPLC with fluorescence detection	Combination of sol-gel columns, with and without enzymatic cleavage of conjugates	Total BPA, BPA, BPA-glucuronide	LOD, 0.2 μg BPA/L, RSD 3.4%, recovery of 78	+	(Schöringhumer and Cichna-Markl, 2007)
HPLC	Fluorophore derivatization with *p*-nitrobenzoyl chloride; acid hydrolysis	Total BPA	LOD, 2.7 μg BPA/L, RSD of 3.9%, recovery of 96%	−	(Mao et al., 2004)
HPLC with electrochemical detector and column switching	Determination with and without pretreatment with glucuronidase	Total BPA, BPA, BPA-glucuronide	LOQ of 0.2 μg BPA/L, recovery of 96%, RSD <3%	+	(Ouchi and Watanabe, 2002)
HPLC with electrochemical detector	Glucuronidase treatment and solid phase clean-up	Total BPA	LOD, 0.5 μg BPA/L	+	(Liu et al., 2005)
LC/MS with electrospray ionization and negative ion detection	Glucuronidase treatment and automated size exclusion flow extraction	Total BPA	LOD and LOQ of 0.1 μg/BPA/L, recovery from 98 to 107%, RSD 4.1 to 7.4%	++	(Inoue et al., 2003b)
LC/MS-MS with electrospray ionization and negative ion detection	With and without glucuronidase treatment	Total BPA, BPA, BPA-glucuronide	LOD, 1.14 μg BPA/L, accuracy of 92 to 121%	+	(Völkel et al., 2005)
LC/MS-MS with atmospheric pressure chemical ionization and negative ion detection	Automated column switching for online SPE clean-up, with or without glucuronidase and sulfatase treatment	Total BPA, BPA, BPA-glucuronide	LOD, 0.3 to 0.4 μg BPA/L, CV of 8 to 17%	++(automated)	(Ye et al., 2005a)
LC/MS-MS with atmospheric pressure ionization and negative ion detection	Dilution with organic solvent	BPA-glucuronide	LOD 25 nmol/L (10 μg/L)	−(low sensitivity)	(Völkel et al., 2005)
LC/MS-MS with atmospheric pressure ionization and negative ion detection and column switching	Dilution with organic solvent	BPA-glucuronide	LOD 1 nmol/L (0.4 μg/L, recovery >90%, RSD <7%	+	(Fukata et al., 2006)

contained up to 236 μg bisphenol A/L after storage in plastic bottles for several days at −20 °C (Sajiki et al., 1999). The background may interfere with the analytical quantitation of bisphenol A in low concentrations suggesting higher bisphenol A concentrations than actually present. Some authors have taken specific measures to prevent background contamination such as using an "internal derivatization" (Schönfelder et al., 2002), specific purification procedures for solvents and/or materials (Sajiki et al., 1999; Völkel et al., 2005), and use of glass instead of plastics for most of the sample workup. When developing a LC/MS-MS (liquid chromatography/mass spectrometry-mass spectrometry) method to quantify bisphenol A, (Völkel et al., 2005) reported interference from bisphenol A released from other sources giving a high background under certain analytical conditions, probably due to leaching effects in the HPLC capillary system. Moreover, when analytes are ubiquitously present in low concentrations and no real matrix blanks are available, calibration in the low concentration range (bisphenol A concentrations in many biological samples are close to the limit of detection/quantitation) is difficult against environmental background and instrument noise levels.

Another concern of particular importance for the determination of parent bisphenol A levels and conclusions on possible health significance of the results is the stability of bisphenol A conjugates in biological samples during sample storage and processing. It has been shown that bisphenol A conjugates will spontaneously hydrolyze to form parent bisphenol A in human urine (and likely serum) if stored at ambient temperatures for only short periods of time (Schöringhumer and Cichna-Markl, 2007; Ye et al., 2007). Hydrolysis of conjugates in rat urine has also been demonstrated for various sample storage and processing conditions indicating a need for rapid freezing of samples immediately after collection. Moreover, some sample processing conditions have been shown to degrade bisphenol A conjugates to products other than bisphenol A (Waechter et al., 2007).

Enzyme-linked immunosorbent assays (ELISAs)

ELISAs are simple, rapid, and cost-efficient. Several ELISAs to detect and quantitate bisphenol A in environmental media have been developed (Kodaira et al., 2000; Nishi et al., 2003; Ohkuma et al., 2002) and applied to determine bisphenol A concentrations in blood of humans. Limits of detection of the ELISAs for the determination of bisphenol A in biological samples such as serum are reported to be low (Table 2). However, one of the ELISAs was developed for determination of BPA in urine (Kodaira et al., 2000).

When applying ELISAs to complex matrices containing only low analyte concentrations, cross-reactivity may reduce the precision of the ELISA. Three ELISAs developed to quantitate bisphenol A were checked for cross-reactivity with structurally related compounds such as bisphenol B and some other xenobiotics. With the compounds used (e.g., phthalates, other bisphenols, some endogenous hormones), no significant cross-reactivity was observed except for other bisphenol derivatives, but the compounds tested were limited in number and did not include phytoestrogens such as resveratrol having a very similar chemical structure and identical molecular weight. In addition, the antibodies have been reported to cross-react with equol (Fukata et al., 2006), which is a very common phytoestrogen that has been reported to be present throughout the human population at levels comparable to or well above levels of bisphenol A reliably measured in urine (Valentin-Blasini et al., 2005). The cross-reactivity of the available antibody with bisphenol A conjugates was indirectly assessed for only one of the ELISAs developed. A good correlation of the bisphenol A concentrations determined in human urine samples by HPLC-FLD (high performance liquid chromatography-fluorescence detector) after enzymatic cleavage of glucuronides with the bisphenol A concentrations indicated by ELISA without prior enzymatic hydrolysis suggests a cross-reactivity of the "specific" bisphenol antibodies with bisphenol A-glucuronide. This observation indicates that this ELISA assay may quantitate both bisphenol A and bisphenol A conjugates (Kodaira et al., 2000). Actually, the authors refer to the bisphenol A concentrations given by their procedure as "total immunoreactive bisphenol A". The cross-reactivity makes designation of the signal to bisphenol A difficult and the method is thus unsuitable for exposure monitoring.

Gas chromatography/mass spectrometry (GC/MS)

GC/MS is frequently applied to determine the concentrations of bisphenol A in environmental samples. Determination of bisphenol A by GC/MS requires derivatization since bisphenol A has insufficient volatility for direct analysis by gas chromatography and shows poor chromatographic properties in GC. Therefore, time consuming sample preparation protocols using different extraction and preconcentration procedures are required. For determination of bisphenol A conjugates, which are expected to be present as major metabolites of bisphenol A in blood or urine, a complete enzymatic cleavage of the conjugates is required prior to solvent extraction since volatile derivatives of bisphenol A-glucuronide and bisphenol A-sulfate are difficult to obtain.

Two of the published GC/MS methods were directly developed to assess the concentrations of bisphenol A in human serum and tissue (Schönfelder et al., 2002, Tan and Mohd, 2003). To define the many potential problems with the analyses of low concentrations of bisphenol A in biological samples, the methods are described in more detail. Sample workup includes direct extraction of plasma or homogenized tissue samples (after dilution with water) using ethyl acetate (Schönfelder et al., 2002) or direct addition of cord blood to a SPE (solid phase extraction) column with elution of bisphenol A by organic solvents. Internal standards (bisphenol C) are added to the extract or to the eluate from the SPE-column (mixture of three deuterium labeled polycyclic aromatic hydrocarbons) (Schönfelder et al., 2002, Tan and Mohd, 2003). The addition of the standard after extraction does not compensate for a potentially incomplete extraction and due to the different physiochemical properties of the internal standards, may not adequately compensate for analyte loss or variations in detector sensitivity. Appropriate internal standards, d_{14}-bisphenol A and $^{13}C_{12}$-bisphenol A, are commercially available at modest price

and can easily be added to blood or serum. It is not clear in the publications why this compound was not used. In both methods, the concentrated extracts are transformed to volatile trimethylsilyl ethers. As a major deficiency, both sample workups do not include a step involving glucuronidase treatment. Due to the absence of a glucuronidase step and information on the fate of bisphenol A-glucuronide, it is unclear if both methods determine total or only free bisphenol A. The many critical points listed suggest, at best, only a limited reliability of the assays.

Several GC/MS methods to assess urinary excretion of bisphenol A in "unexposed" human subjects are available (Table 3). GC/MS (both with a conventional quadrupole and a high resolution instrument using negative ion detection after derivatization of bisphenol A with pentafluorobenzyl bromide represents a sensitive procedure. Urine samples of only 1 ml volume are required. After glucuronidase treatment, urine samples are acidified and bisphenol A is concentrated using SPE-extraction. The authors also report that modification of the procedure to include trimethylsilylation of bisphenol A gave a lower detection limit, but results in additional peaks interfering with quantitation (Brock et al., 2001), further questioning the validity of the above mentioned methods (Schönfelder et al., 2002; Tan and Mohd, 2003) which also used trimethylsilylation.

Negative ion detection after chemical ionization and pentafluorobenzylation using a modified sample workup was reported to have a similar LOD (0.1 µg bisphenol A/L) for bisphenol A in urine, and also requires only small volumes of urine (1 to 2 mL) (Tsukioka et al., 2003) (Table 3). The method included glucuronidase treatment, concentration of bisphenol A by solid phase extraction using C18-modified SPE followed by pentafluorobenzylation. The pentafluorobenzyl derivatives generated are subjected to a second solid phase extraction and are then analyzed by GC/MS. An internal standard (apparently $^{13}C_{12}$-bisphenol A) is included to compensate for possible analyte loss (Tsukioka et al., 2003). A similar method involving sample preparation by glucuronidase treatment and solid phase extraction, but derivatization with bis-trimethylsilyl trifluoroacetamide, a more time consuming workup with solvent extraction, a second solid phase extraction and electron impact ionization has also been described. This method requires large sample volumes (100 mL of urine).

A method developed to analyze bisphenol A together with several isoflavones and their metabolites applied pretreatment with glucuronidase/sulfatase from E. coli, concentration of analytes by SPE ($^{13}C_{12}$-bisphenol A as internal standard), and transformation of bisphenol A to the volatile bis-tert-butyldimethylsilyl ether with *N*-(tert-butyldimethylsilyl)-*N*-methyltrifluoroacetamide. The method has a LOD and LOQ (limit of quantitation) one order of magnitude higher than the other GC/MS methods (Moors et al., 2007) (Table 3).

Due to the time consuming sample preparation by manual solid phase extractions, automated methods have been developed. These procedures are aimed at the quantitation of trace amounts of bisphenol A simultaneously with other phenols such as octyl and nonyl phenol or pentachlorophenol and for phenolic metabolites of environmental chemicals such as dichlorobenzene. After addition of stable isotope labeled standards and glucuronidase treatment, the samples are transferred to a preconditioned SPE-cartridge, subjected to on-column derivatization with pentafluorobenzyl bromide and eluted from the column. The concentrations of bisphenol A are determined by chemical ionization and negative ion detection. Due to the rapid and automated sample preparation, this procedure may be used to analyze larger numbers of samples (Calafat et al., 2005).

Another rapid method with high sample capacity for the determination of bisphenol A by GC/MS may use glucuronidase treatment, followed by derivatization with acetic acid anhydride to give bisphenol A diacetate, which is extracted using a specially coated stirring bar from the aqueous solution (stir bar sorptive extraction). The bisphenol A diacetate adsorbed on the stirring bar is released by thermodesorption, cryofocused, and quantified by electron impact mass spectrometry using single ion monitoring ($^{13}C_{12}$-bisphenol A as internal standard). The method (Table 2) has been validated for the determination of bisphenol A in human plasma and urine and for the simultaneous determination of bisphenol A and some other phenols in human urine (Kawaguchi et al., 2004, 2005).

One of the methods developed for bisphenol A in urine applied GC/MS-MS which gives a higher selectivity when analyzing complex matrices. The sample workup is again complex involving addition of d_{16}-bisphenol A as internal standard, enzymatic cleavage of bisphenol A conjugates, a combination of solvent and SPE-extractions and, finally, derivatization of bisphenol A to the bis-trimethylsilyl ether (Arakawa et al., 2004).

HPLC with detectors other than mass spectrometry

Determination of bisphenol A by HPLC has the advantage that derivatization is not required and therefore sample workup may be simpler. Conventional HPLC with wide bore columns, however, has the disadvantage of lower separation efficiency as compared to capillary gas chromatography. Therefore, overlapping signals may be present generating difficulties in quantitation. Selective detection methods may thus be required. To our knowledge, application of HPLC-UV methods to human blood or urine samples to quantify bisphenol A have not been published, most probably due to the low sensitivities of UV detectors for compounds without specific chromophores such as bisphenol A, and the poor selectivity of HPLC-UV methods for specific analytes in complex matrices such as serum, plasma or urine. One publication applied HPLC with UV-detection to analyze bisphenol A in human saliva samples, but the method suffers from a poor peak shape for bisphenol A, and an elution of bisphenol A close to the solvent front and not well separated from other components generating problems in analyte identification and quantitation (Olea et al., 1996).

A higher sensitivity and some improvement in selectivity may be achieved by fluorescence detection since bisphenol A, after excitation at 275 nm, shows fluorescence at 300–320 nm. Two methods applying fluorescence detection, one for urine and one for plasma, have been described. The method to determine bisphenol A in urine may include glucuronidase treatment of urine, extraction at low pH using ethyl acetate containing the internal standard bisphenol B and concentration of the extract.

To analyze serum for bisphenol A by HPLC with fluroescence detection, serum samples are reported to be directly applied to SPE-cartridges and eluted with acetonitrile, but the detection limit is comparatively high (Table 2) and glucuronidase is not included in the sample workup (Fung et al., 2000).

Use of HPLC with fluorescence detection has been shown to be sensitive and precise when combined with a specific sample workup based on sol-gel columns. A combination of two sol-gel columns, one immunoaffinity column containing anti-bisphenol A antibodies, and an enzyme column containing arylsulfatase and glucuronidase, permits the determination of urinary total bisphenol A with high sensitivity. When only the immunoaffinity column is applied, parent bisphenol A may be selectively determined without interference from bisphenol A-glucuronide (Schöringhumer and Cichna-Markl, 2007).

HPLC-FLD after fluorophore derivatization

Two methods intended for biomonitoring of bisphenol A applied fluorophore labeling of bisphenol A to obtain low detection limits (Tables 2 and 3); 4-(4,5-diphenyl-1H-imidazol-2-yl)benzoyl chloride (DIB-Cl) and *p*-nitrobenzyl chloride were used as derivatization agents. Prior to derivatization of bisphenol A with DIB-Cl, the serum samples are acidified and extracted into chloroform. The chloroform phase is then treated with DIB-Cl, filtered and analyzed by a column-switching HPLC system (Kuroda et al., 2003). Fluorophore derivatization with *p*-nitrobenzoyl chloride for determination of bisphenol A in urine involves acid treatment of urine to cleave conjugates followed by solid phase extraction and derivatization (Mao et al., 2004). The reported detection limit for bisphenol A is well above those of other methods. Both methods do not apply internal standards and acid treatment has been shown to cause decomposition of bisphenol A-glucuronide to products other than bisphenol A (Waechter et al., 2007).

Electrochemical detection (ED) may be used for the sensitive and selective determination of phenols such as bisphenol A by HPLC. Selectivity of the analysis may be increased by coulometric arrays, thus removing some potentially interfering compounds and by using specific modes of electrochemical detection. Several methods for the determination of bisphenol A in serum and in urine by ED are described in the literature. A comparatively high selectivity of this procedure due to the specific response of bisphenol A in the ED is an advantage of this detector, a limitation of the detection method is the frequent need to use a constant solvent composition resulting in reduced separation efficiency.

Application of a HPLC method with multi-electrode electrochemical detection (HPLC-ED) including a coulometric array of 4 electrochemical sensors to analyze bisphenol A in human serum is very sensitive (Inoue et al., 2000). For sample preparation, serum samples are treated with acid to precipitate proteins and then subjected to SPE-extraction. Internal standards and glucuronidase treatment are not included in the sample workup.

Another HPLC-ED method for the detection of bisphenol A in serum applied solid phase extraction to concentrate bisphenol A from serum involving removal of polar and non-polar lipids from 10-fold diluted serum samples (Table 2). The accuracy of the procedure was confirmed by analysis of samples by HPLC-MS. Again, internal standards and enzymatic cleavage of bisphenol A conjugates is not included in the sample workup (Sajiki et al., 1999).

One method using HPLC-ED was developed to determine bisphenol A both in urine and in serum. The method performs sample workup both with and without glucuronidase. Bisphenol A is extracted by SPE and analyzed by electrochemical detection using column switching (Table 3) (Fukata et al., 2006). The method requires only 0.5 ml urine or serum.

Two methods using electrochemical detection were developed to assay bisphenol A in urine (Table 3). One of the methods is intended to determine bisphenol A-glucuronide and parent bisphenol A excretion and involves direct extraction of bisphenol A from urine into diethyl ether followed by analysis. To determine total bisphenol A, enzymatic cleavage of bisphenol A-glucuronide is performed followed by extraction of released bisphenol A into diethyl ether. The ether extracts are concentrated and analyzed by HPLC with automated column switching and an array of 8 coulometric electrodes using the redox mode (Ouchi and Watanabe, 2002).

Another ED-based method was developed to determine bisphenol A in addition to the phytoestrogens daidzein, genistein, and enterolactone. Separation was performed using conventional HPLC, sample workup included glucuronidase treatment, solid phase extraction and gradient elution (Liu et al., 2005).

Liquid chromatography/mass spectrometry (LC/MS)

LC/MS combines high selectivity, especially when using MS-MS, and sensitivity with reduced need for sample processing since volatile derivatives are not needed for chromatography. Recently, several methods based on LC/MS for the determination of bisphenol A and its metabolites were published (Inoue et al., 2002, 2003b; Völkel et al., 2002; Ye et al., 2005a,b) (Tables 2 and 3). Most of these reported methods focused on the quantitation of parent bisphenol A and therefore included an enzymatic cleavage step to transform bisphenol A-glucuronide and bisphenol A-sulfate to bisphenol A, which was then analyzed after further sample processing, usually by solid phase extraction and without further derivatization.

Applying a single quadrupole instrument and using selected ion monitoring for only one representative fragment of bisphenol A, a method was designed to quantitate both bisphenol A and hydroxylated bisphenol A diglycidyl ethers in human plasma or serum. The sample preparation procedure included glucuronidase cleavage and automated solid phase extraction. Apparently, a stable isotope labeled standard was not used (Inoue et al., 2001).

A single quadrupole instrument and isocratic elution was also used in one method to quantitate bisphenol A in human serum (Table 2). The applied method did not include glucuronidase cleavage of bisphenol A conjugates and no stable isotope labeled internal standard, only m/z 227 $[M\text{-}H]^-$ as the pseudomolecular ion in the negative ion mode was monitored for bisphenol A quantitation (Sajiki et al., 1999).

To analyze larger numbers of samples for population exposure monitoring to phenols or phenolic metabolites of xenobiotics, an

online solid phase extraction system combined with LC/MS-MS was developed. The method permits the simultaneous quantitation of bisphenol A, 2,5-dichlorophenol, and 2-hydroxy-4-methoxybenzophenone in a single run. The automated method includes stable isotope labeled standards ($^{13}C_{12}$-bisphenol A), optimized glucuronidase cleavage including an internal standard to check for completeness of enzymatic hydrolysis, and automated sample concentration using trap columns. Bisphenol A is quantified after atmospheric pressure ionization in the negative ion mode (Ye et al., 2005a). An expansion of the method to detect nine environmental phenols including bisphenol A in a single separation with similar precision and detection limit for bisphenol A has also been developed (Ye et al., 2005b).

Sample preparation by size-exclusion flow extraction using an automated column switching system combined with LC/MS-MS analysis resulted in a low detection limit for bisphenol A in urine (Table 3) (Inoue et al., 2003b). The method includes $^{13}C_{12}$-bisphenol A as internal standard and glucuronidase cleavage, MS-MS is performed after electrospray ionization with negative ion detection and single ion monitoring.

Only investment of considerable time to adjust sample preparation and analytical methodology resulted in a reduction of bisphenol A background to below the LOD of 1 μg/L during the development of a LC/MS-MS based method for determination of bisphenol A in human urine or blood (Völkel et al., 2005). The method finally used relied on a stable isotope labeled standard (d_{16}-bisphenol A) and involved glucuronidase pretreatment.

Only one sufficiently sensitive procedure to directly analyze bisphenol A-glucuronide in human urine samples in environmentally relevant concentrations has been developed. A direct analysis of the glucuronide may facilitate sample workup since the glucuronidase step is not required. The procedure uses dilution of the urine sample as the only sample workup and applied column switching. Bisphenol A-glucuronide quantitation is based on monitoring the transition of m/z 403 to m/z 113 after electrospray ionization using negative ion detection. Unfortunately, no internal standard ($^{13}C_{12}$- or d_{14}-bisphenol A-glucuronide) is included in the method to compensate for variations in instrument performance or enzymatic cleavage efficiency (Fukata et al., 2006).

Another LC/MS-MS method to determine bisphenol A-glucuronide used dilution of urine samples with organic solvents followed by single column LC and MS-MS quantitation using electrospray ionization and negative ion detection, d_{14}-bisphenol A-glucuronide served as internal standard. The method had a high recovery and precision and was successfully applied to quantitate bisphenol A-glucuronide in urine samples from individuals administered 25 μg bisphenol A/person, but LOD and LOQ were unsatisfactory for analyses of samples from non-intentionally exposed individuals due to interfering peaks (Völkel et al., 2005).

Summary of analytical methodology

A wide variety of methods have been developed to determine low concentrations of bisphenol A and its biologically relevant metabolites bisphenol A-glucuronide and bisphenol A-sulfate in biological fluids (Tables 2 and 3). The low concentrations of bisphenol A and its metabolites observed result in a need for precise and robust methods to clearly correlate a signal to the target analyte. This requirement is best fulfilled by the mass spectrometry based methods since detection is highly selective when using MS-MS, or the chromatographic separation is very effective and reproducible in the case of GC/MS. Moreover, mass spectrometry based methods can rely on stable isotope labeled standards, and in addition, have a sufficiently low LOD and LOQ for quantitation of bisphenol A metabolites in urine samples of environmentally exposed humans. As compared to GC/MS, an advantage of MS coupled to liquid chromatography may be the higher throughput due to absence of a derivatization step to generate volatile bisphenol A derivatives. However, the most recently developed GC-and LC/MS methods are usually relying on automated sample preparation thus generating the necessary high throughput for analysis of a larger number of samples.

Of the other analytical methods for bisphenol A, only HPLC coupled to an electrochemical detector has the necessary sensitivity and, using coulometric arrays, also gives good selectivity. Fluorescent based procedures may suffer from interference from other fluorescent compounds in urine since separation efficiency of the reported method is less as compared to GC and the needed excitation of bisphenol A in the low UV-range may reduce selectivity.

For determination of bisphenol A in human blood, a suitable method needs to be extremely sensitive and specific, contain a stable isotope labeled internal standard added directly to the blood or serum sample and requires glucuronidase cleavage. Moreover, method validation needs to address the issue of bisphenol A leaching into protein-rich samples. Therefore, most of the available methods are not well suited for determination of bisphenol A in blood of non-occupationally exposed humans (Table 2).

Regarding determination of bisphenol A in urine of non-occupationally exposed humans, a number of sensitive methods are available. Due to the strict quality control and the capacity for analysis of larger numbers of samples, automated methods such as used in NHANES are considered most suitable (Table 3).

While ELISAs are simple, cost efficient and do not require expensive equipment, the problems with standardization and cross reactivity with other components commonly found in urine or blood samples severely hampers the application of these methods to quantify the expected low bisphenol A concentrations in human blood or urine samples. A comparison of results from three ELISA kits commercially available and instrumental analytics (HPLC with electrochemical detection and LC/MS-MS) in 52 matched human urine and serum samples was performed. The LC/MS-MS method, which positively identifies bisphenol A and bisphenol A-glucuronide, gave results that correlated very well with those of the LC/ECD method. However, the three ELISA kits not only had poor correlation with the reliable LC-based methods, but they also had poor correlation with each other. Other studies have also demonstrated a wide overestimation of the concentrations of bisphenol A present in human semen or serum from monkeys given bisphenol A by the ELISAs (Inoue et al., 2002; Tominaga et al., 2006) and a

much lower sensitivity (detection limit of 12.5 μg bisphenol A/L) when used to determine bisphenol A in serum of primates administered bisphenol A (Tominaga et al., 2006). Recently, development of a modified competitive ELISA method to determine bisphenol A in low concentrations has been reported, but data on the performance of this method when analyzing bisphenol A concentrations in biological samples are not available (Kim et al., 2007).

Therefore, the possible issue of cross-reactivity of the ELISAs with other components in serum and the discrepancies reported between the ELISA results and concentrations of bisphenol A in the same samples determined by instrumental analytics raises serious questions about the accuracy of blood concentrations of bisphenol A measured by ELISA. From this set of data, it can be concluded that the ELISA kits not only produce inaccurately high values for bisphenol A, but that they may not be measuring bisphenol A at all, in particular at the very low part per billion concentrations that might be present in biological samples from "unexposed" humans. As concluded "ELISA is not suitable for bisphenol A measurement in human samples" (Fukata et al., 2006).

Reported concentrations of bisphenol A in human blood in non-intentionally exposed individuals

Despite the many limitations discussed above regarding conclusions on human exposures to bisphenol A based on blood levels of bisphenol A, a number of studies report concentrations of bisphenol A in blood of the general population and possible effects of gender and hormonal status on these blood levels (Table 4). However, the reported concentrations of bisphenol A in blood using different methods are inconsistent (Table 4).

Applying ELISAs, bisphenol A is reported to be detected in most of the samples analyzed and blood concentrations up to 10 μg/L in plasma or serum, with means or medians of 2 to 3 μg/L are reported. Only one study using ELISAs reports that the bisphenol A concentrations in most of the samples analyzed were below the LOD (Ohkuma et al., 2002). The limitations of the ELISA technique become particularly important in regard to the few limited human studies that have been conducted on bisphenol A (Hiroi et al., 2004; Takeuchi and Tsutsumi, 2002; Takeuchi et al., 2004b; Tsutsumi, 2005). Of particular note is the study of Sugiura-Ogasawara et al. (2005), which attempted to examine an association between miscarriage and bisphenol A exposure, using the ELISA technique to measure bisphenol A in blood samples. Considering the significant limitations of the ELISA technique and that it may not measure bisphenol A at all, the reported association between serial miscarriage and bisphenol A exposure is not supported by the evidence.

One study using GC/MS to determine bisphenol A in maternal and fetal blood and in placental tissue reported up to 9.2 μg/L in plasma and up to 105 μg/kg in placental tissue (Schönfelder et al., 2002). Another study also using GC/MS reported detection of bisphenol A in 158 out of 180 cord blood samples in concentrations up to 4 μg/L (Tan and Mohd, 2003). The high concentrations of bisphenol A reported are not consistent with the other available data and bisphenol A toxicokinetics, this discrepancy may be due to problems with sample storage and preparation, or analytical issues as discussed above.

In contrast to the two studies using GC/MS, application of HPLC-based methods using mass spectrometry or electrochemical detection to quantitate bisphenol A indicates that many of the analyzed blood samples did not contain concentrations of bisphenol A, either in the form of parent or conjugates, above detection limits between 0.2 and 5 μg/L. When detected, bisphenol A concentrations were <1 μg/L, often close or at the LOD. The toxicokinetics of bisphenol A in humans and PBPK-modeling support the validity of these studies since, due to the efficient first-pass metabolism, parent bisphenol A concentrations in blood are, as demonstrated, expected to be very low. For comparison, reported blood concentrations of parent bisphenol A in the studies using GC/MS (Schönfelder et al., 2002; Tan and Mohd, 2003) (Table 4) are much higher than the detection limits for bisphenol A in blood in a study to determine toxicokinetics of bisphenol A in humans (Völkel et al., 2002) given doses of 60–80 μg/kg bw (5 mg/person). In this study, parent bisphenol A concentrations in blood were below 2.3 μg/L even a short time after oral application when peak concentrations of bisphenol A-glucuronide were present in blood.

In addition, simulations of bisphenol A blood levels using physiologically-based pharmacokinetic models based on experimental toxicokinetics predict that the levels of parent bisphenol A in blood at reasonably expected exposure levels will be very low at all times (shortly after a meal, peak blood level of 40 pmol or 9 ng/L at a daily dose of 1 μg/kg bw). Even this predicted level is an upper bound estimate based on the assumption that parent bisphenol A was actually present at the limit of detection at the highest dose tested in the controlled human studies (Filser et al., 2003; Teeguarden and Barton, 2004; Teeguarden et al., 2005). This predicted concentration of bisphenol A is well below the limit of detection for all analytical methods developed so far (see above). The low concentrations of parent bisphenol A reported in human blood in some studies are therefore likely to be due to artifacts caused by leaching of bisphenol A from materials as demonstrated, or by hydrolysis of BPA-glucuronide due to inadequate storage or sampling (Sajiki et al., 1999; Völkel et al., 2002).

Reported concentrations of bisphenol A in human urine after controlled exposure and in the general population

The more recent studies analyzing bisphenol A concentrations in human urine often applied sensitive and selective mass spectrometry and advanced chromatographic procedures such as column switching. Specific procedures to avoid background contamination of bisphenol A were also used in some studies and most studies determining urinary concentrations of bisphenol A in humans included enzymatic cleavage of bisphenol A-glucuronide in the protocols; only one study used acid to cleave bisphenol A-glucuronide (Table 5). Since decomposition of bisphenol A-glucuronide using acid has been reported, the results from this single study should be viewed with care. Leaching of bisphenol A from storage containers into urine seems not to be a major issue since bisphenol A is only slowly

Table 4
Reported concentrations of bisphenol A (BPA) in blood of unintentionally exposed individuals

No. of samples analyzed	Analytical method, sample workup	Concentration ranges reported	Reference
Blood samples from 30 healthy pre-menopausal women, 37 women with early pregnancy, 37 late pregnancy, 32 umbilical cord blood samples, 36 ovarian follicular fluid samples	ELISA	2.0±0.8 μg BPA/L (non-pregnant); 1.5±1.2 μg BPA/L (early pregnancy); 2.4±0.8 μg BPA/L (follicular fluid); 2.2±1.8 μg BPA/L (fetal serum); 8.3±8.9 μg BPA/L (amniotic fluid)	(Ikezuki et al., 2002)
100 samples analyzed, no details on sample workup	ELISA	Many<0.3 μg BPA/L, up to 1 μg BPA/L (no details given)	(Ohkuma et al., 2002)
14 healthy women, 16 women with polycystic ovary syndrome (PCOS) and 11 healthy men	ELISA	0.64±0.1 μg BPA/L (normal women); 1.49±0.11 μg BPA/L (healthy men); 1.04±0.1 μg BPA/L (women with PCOS)	(Takeuchi and Tsutsumi, 2002)
Serum from 11 premenopausal women, 10 women with simple endometrial hyperplasia, 9 women with complex endometrial hyperplasia and 7 women with endometrial cancer	ELISA	2.5±1.5 μg BPA/L in premenopausual women 2.9±2.0 μg BPA/L in women with simple endometrial hyperplasia 1.4±0.4 μg BPA/L in women with complex endometrial hyperplasia 1.4±0.5 μg BPA/L in women with endometrial cancer	(Hiroi et al., 2004)
Blood samples (n=73) from women with different endocrine status	ELISA	1.17±0.16 μg BPA/L to 0.71±0.09 μg BPA/L in obese rep. non-obese women (mean+SD)	(Takeuchi et al., 2004a)
Blood samples from healthy women (n=32) and women with recurrent miscarriage (n=45)	ELISA	2.59±5.23 μg BPA/L in “patients” and 0.77±0.38 μg BPA/L in controls (mean+SD)	(Sugiura-Ogasawara et al., 2005)
248 samples of maternal serum and amniotic fluid	ELISA	0.64 to 6.63 μg BPA/L in maternal serum (90th percentile) <LOD to 0.81 μg BPA/L in amniotic fluid	(Yamada et al., 2002)
Randomly collected from 21 male and 31 female subjects, age 22–51 years	ELISA with three different kits, same samples analyzed by HPLC with electrochemical detection	Two of the ELISA kits indicated BPA-concentrations of 0.66±0.29 resp. 0.71±0.49 μg/L, LC with electrochemical detection all samples <LOD of 0.2 μg/L, BPA below LOD in all samples with 3rd ELISA	(Fukata et al., 2006)
Serum from 12 females and 9 males	Apparently by HPLC with electrochemical detection and solid-phase extraction	0.33±0.54 μg BPA/L (range of 0–1.6 μg BPA/L) in females and 0.59±0.21 μg BPA/L (range of 0.38–1.0 μg BPA/L) in males	(Sajiki et al., 1999)
Serum samples from 18 men and 22 women after application of dental sealant containing BPA, saliva concentrations of BPA up to 0.1 ppm	HPLC with fluorescence detection and solid phase extraction	None above detection limit of 5 ppb (5 μg BPA/L)	(Fung et al., 2000)
Apparently only five blood samples from “healthy” individuals analyzed	HPLC with multi-electrode electrochemical detection, solid phase extraction, no glucuronidase	0.32 μg BPA/L, mean of 5 samples	(Inoue et al., 2000)
Serum from nine pregnant females, 21 serum samples from sterile females	HPLC after fluorophore derivatization	Range 0.21 to 0.79 μg BPA/L, mean 0.46±0.21 in pregnant females; range of 0.22 to 0.87 μg BPA/L, mean 0.46±0.20 in sterile females	(Kuroda et al., 2003)
37 maternal and fetal plasma samples, placenta	GC/MS after derivatization by silylation, solvent extraction with ethyl acetate	Median BPA conc. in maternal plasma 3.1 μg BPA/L (range from 0.3 to 18.9 μg/L); 2.3 μg BPA/L in fetal plasma (range from 0.2 to 9.2 μg BPA/L);median of 12.7 μg BPA/kg of placenta tissue (range from 1 to 104.9 μg BPA/kg)	(Schönfelder et al., 2002)
Umbilical cord blood samples from 180 females	GC/MS after derivatization by silylation, solid phase extraction	BPA detected in 88% of samples above LOD of 0.05 μg/L, range up to 4 μg/L	(Tan and Mohd, 2003)
Umbilical cord blood samples from 20 females	Not given	4.43±5.04 μg BPA/g wet weight (mean+SD)	(Todaka and Mori, 2002)
Plasma samples from 3 “healthy” subjects, no further information	GC/MS with EI ionization, thermal desorption; with and without glucuronidase treatment,	None above LOD of 0.1 μg BPA/L	(Kawaguchi et al., 2004)
Apparently only 3 blood samples analyzed	LC/MS, electrospray ionization, glucuronidase treatment, solid phase extraction	0.1 to 1 μg BPA/L	(Inoue et al., 2001)
Randomly collected blood samples from 7 males and 12 females	LC/MS-MS with and without glucuronidase treatment	All samples below LOD of 0.5 μg BPA/L	(Völkel et al., 2005)

Table 5
Reported concentrations of bisphenol A (BPA) in urine of unintentionally exposed individuals

No. of samples analyzed	Analytical method sample workup	Concentration ranges reported	Reference
Randomly collected and pooled urine samples, five pools analyzed	GC/MS with chemical ionization and negative ion detection, with and without glucuronidase	Free BPA <0.12 μg/L; total BPA concentrations from 0.11 to 0.51 μg/L	(Brock et al., 2001)
Males (n=42) serving as controls in a study to determine excretion of BPA and BPA-diglycidyl ether metabolites in epoxy resin workers	**HPLC with electrochemical detection, glucuronidase treatment**	**BPA concentrations given as median of 0.52 μmol/mol creatinine (range from <LOD to 11 μmol BPA/mol creatinine); app. 0.2 to 0.4 μg BPA/L**	(Hanaoka et al., 2002)
Morning spot urine samples from 48 female students	HPLC with electrochemical detector and column switching, determination with and without pretreatment with glucuronidase	BPA below LOD except for one sample with 0.2 μg BPA/L; BPA-glucuronide detected in all samples with concentrations from 0.2 to 19.1 μg BPA-gluc/L (median of 1.2 μg/L), equivalent to 0.7 μg BPA/L	(Ouchi and Watanabe, 2002)
Urine samples pooled over 24 h from 5 male and 3 female adults; spot urine samples from 7 males	GC/MS with electron impact detection after glucuronidase treatment	In the pooled 24 h samples, BPA was present below the LOD in 4 samples, BPA-concentrations in the other 4 samples above LOD, but below LOQ, approximate mean of 4.8 μg/L; BPA detected in 5 of the 7 spot urine samples, conc. between 8 and 55 μg/L	(Moors et al., 2007)
Urine samples from controls and male individuals employed in epoxy resin spray painting handling BPA bisglycidyl ether, levels and duration of exposure not known	HPLC with electrochemical detection, glucuronidase treatment	Median concentration in controls 0.2 μg BPA/L, in “exposed” 0.4 μg BPA/L	(Kim et al., 2003)
Urine samples from “normal” adults, 15 males and 15 females	**HPLC with fluorescence detection, purified glucuronidase used for cleavage of conjugates**	**“Total” BPA in males was 2.82+0.73 μg BPA/L in males and 2.76+0.54 μg BPA/L; differences in relative concentrations of sulfate and glucuronide conjugates in males and females**	(Kim et al., 2003)
30 urine samples from individuals painting houses and 6 unexposed individuals used as controls	GC/MS with chemical ionization and electrophore derivatization; glucuronidase treatment	Quantitative evaluation of BPA levels in urine of unexposed controls not detailed, range for BPA given as 0.4–22 μg/L, based on graphic presentation estimated as below 2 μg/L, BPA detected in 96% of analyzed samples	(Kuklenyik et al., 2003)
Morning spot urine from 46 male and 4 female students	HPLC with fluorescence detection, glucuronidase treatment	Up to 30 μg BPA/g creatinine /app. 18 μg BPA/L, 39% of samples collected in 1999 were below LOD of 1.7 μg BPA/g creatinine (app. 1.2 μg BPA/L)	(Matsumoto et al., 2003)
Randomly collected urine samples (n=6), no further information	GC/MS with chemical ionization and negative ion detetction after electrophore derivatization, glucuronidase included	Range of 0.2 to 3.8 μg BPA/L, “average” of 1.6 μg BPA/L	(Tsukioka et al., 2003)
Morning spot urine from 34 adult males and 39 adult females,	HPLC with fluorescence detection; glucuronidase treatment,	Geometric mean of 9.54+8.32 μg BPA/L, range from 0.68–586 μg BPA/L	(Yang et al., 2003)
Samples from 5 health adults, on 5 consecutive days, in addition, 2 h urine samples from 36 male subjects	**GC/MS-MS, glucuronidase treatment**	**<0.58 to 13 μg BPA/day (median of 1.3 μg/day); <0.21 μg BPA/day to 14 μg BPA/day for the 36 other subjects (median of 1.2 μg BPA/day**	(Arakawa et al., 2004)
Spot urine samples from 56 pregnant women	**GC/MS-MS, glucuronidase treatment, apparently as developed by** (Arakawa et al., 2004)	**<1.1 to 5.4 μg BPA/L with a mean value of <1.1 μg BPA/L, BPA detected in 17 samples above LOD of 1.1 μg/L**	(Fujimaki et al., 2004)
Urine samples from 3 “healthy” subjects, no further information	GC/MS with EI ionization, thermal desorption; with and without glucuronidase treatment	0.45, 0.21 and 0.41 μg BPA/L when using glucuronidase, no parent BPA detected	(Kawaguchi et al., 2004)
10 healthy male and 10 healthy female subjects, no information on urine collection	HPLC after fluorophore derivatization; acid hydrolysis to cleave conjugates	Range from <LOD to 3.95 μg BPA/L, mean 1.22+1.38 μg BPA/L; very high concentrations of endogenous hormones suggest systematic error, BPA concentrations may actually be μg	(Mao et al., 2004)
Urine samples from 46 male and 23 female subjects, (2 collections combined); and collection and pooling of all urine excreted over 24 h by 11 male and 11 female subjects	**GC/MS with glucuronidase treatment**	**Average for BPA (present as glucuronide) was 0.81 μg/L (0.14–5.47 μg/L), urinary excretion over 24 h of 1.68 μg BPA/day (0.48–4.5 μg BPA/day**	(Tsukioka et al., 2004)
Spot urine samples from 394 adults in the US, collected at different times of the day	**GC/MS with chemical ionization and electrophore derivatization; glucuronidase treatment**	**Geometric mean of 1.21 μg BPA/L for urban and of 1.56 μg BPA/L for rural residents**	(Calafat et al., 2005)

Table 5 (*continued*)

No. of samples analyzed	Analytical method sample workup	Concentration ranges reported	Reference
Urine samples from 5 "healthy" subjects, no further information	GC/MS with EI ionization, thermal desorption; glucuronidase treatment	BPA concentration in one sample < LOD of 0.1 μg/L, 4 samples at 2.08, 5.41, 0.93 and 1.84 μg/L	(Kawaguchi et al., 2005)
Urine samples from 2 studies on phytoestrogen exposures, one study in adults (10 males and 14 females, mean age of 52.5 years; the other samples (n=9) were apparently taken from the study on pubertal development given below	HPLC with electrochemical detector after glucuronidase treatment and solid phase cleanup	Median of 0.47 μg BPA/L (range of <LOD of 0.5 μg/L to 2.24) in adults and 2.4 (range from 0.04 to 16.6) in girls; BPA detected in 52% of the samples from adults and 89% from girls	(Liu et al., 2005)
Randomly collected urine samples from 7 males and 12 females without known BPA exposure	LC/MS-MS with and without glucuronidase treatment	All samples below LOD of 1.14 μg BPA/L	(Völkel et al., 2005)
Randomly collected urine samples from 30 adult individuals without known BPA exposure	**LC/MS-MS with column switching, separate analysis for free BPA without enzymatic hydrolysis, after glucuronidase and after sulfatase treatment**	**Means for free BPA were <LOD (range of < LOD of 0.3 μg/L to 0.6 μg/L), for BPA-glucuronide 3.1 μg/L LOD (range of <LOD of 0.3 μg/L to 19 μg/L), BPA-sulfate 0.5 μg/ LOD (range of <LOD of 0.3 μg/L to 1.8 μg/L), total BPA 3.2 μg/L LOD (range of <LOD of 0.3 μg/L to 19.8 μg/L), parent BPA detected in 10% of samples**	(Ye et al., 2005a)
Randomly collected urine samples from 30 adult individuals without known BPA exposure	**LC/MS-MS with column switching, peak focusing**	**Mean for total BPA 3.5 μg/L, BPA detectable in 26 out of 30 samples, 95th percentile of 11.5 μg/L**	(Ye et al., 2005b)
Randomly collected urine samples from 15 adult subjects without known BPA exposure	**LC/MS-MS with column switching, peak focusing**	**Mean urinary BPA concentrations were 2.4 μg BPA/L, median of 0.9 μg BPA/L**	(Ye et al., 2007)
Randomly collected urine samples from 21 male and 31 female subjects, age 22–51 years	**HPLC with electrochemical detection for total and free BPA; BPA-glucuronide concentrations in some samples confirmed by LC/MS-MS**	**Only two samples showed free BPA (0.24 and 0.35 μg/L); mean of total BPA was 1.92 ± 1.99 μg/L. ELISA kits gave total BPA concentrations of 15.9 ± 9.9; 16.7 ± 19.5; and 18.6 ± 23.7 μg/L**	(Fukata et al., 2006)
Urine samples from 15 healthy military personnel before application of dental sealants	High resolution GC/MS after electrophore derivatization, glucuronidase treatment. Sample workup as described in Brock	Mean urinary BPA concentrations were 2.41 ± 1.24 μg BPA/L, median of 2.35 μg BPA/L	(Joskow et al., 2006)
Morning spot urine from 172 Korean individuals, 51% males	HPLC with fluorescence detection, with or without glucuronidase	6.88 ± 3.72 μg BPA/L in males and 5.01 ± 3.16 μg BPA in females (geometric mean ± SD)	(Yang et al., 2006a)
Urine samples from 12 controls and 10 dialysis patients, no details when samples were collected	HPLC with fluorescence detection and combination of sol-gel columns, with and without enzymatic cleavage of conjugates	Unconjugated BPA concentrations were 0.3 μg BPA/L (median) and total BPA concentrations were 1.1 μg/L in healthy adults; unconjugated BPA concentrations of 0.2 μg/L (median) and total BPA concentrations of 1.2 μg/L were found in dialysis patients	(Schöringhumer and Cichna-Markl, 2007)
Spot and early morning urine samples from 90 girls (aged 6 to 9) enrolled in longitudinal study on pubertal development	**LC/MS-MS with automated sample preparation, glucuronidase cleavage**	**Geometric mean BPA concentrations were 2.0 μg BPA/L, median of 1.8 μg BPA/L, BPA above LOD in 94% of samples analyzed**	(Wolff et al., 2007)
Spot urine samples form 2517 individuals, a subgroup of the population sampled in NHANES during 2003 and 2004	**SPE-extraction coupled in line to HPLC/MS-MS, glucuronidase cleavage**	**BPA above LOD of 0.36 μg/L in 92.6% of all samples, geometric mean of 2.6 μg BPA/L, ranging from 1.9 μg/L (in age group 60+) to 3.7 μg/L (in age group 6–11), 95th percentile at 15.9 μg/L for all age groups**	(Calafat et al., 2008)
Several spot urine samples from 35 children donated over 6 months	**LC/MS-MS with column switching, separate analysis for free BPA without enzymatic hydrolysis, after glucuronidase and after sulfatase treatment**	**BPA above LOD in 95% of all samples, geometric mean of 3.4 μg/L, max of 40 μg/L, 95th percentile at 7.51 μg/L**	(Teitelbaum et al., 2007)

Many studies did not report correction for creatinine excretion. Critical studies for exposure assessment are in bold.

released into aqueous solutions with low protein content such as urine from humans without renal disease (Sajiki, 2001). When specific procedures to distinguish between parent bisphenol A and bisphenol A conjugates are applied, the reported concentrations of parent bisphenol A are very low, often below the LOD (Table 5).

In all studies on urinary concentrations of bisphenol A in reference populations including studies with a large number of

individuals (up to 2517), independent of localization of the selected population, the range of urinary concentrations of bisphenol A reported reached peak levels below 16 μg/L [95th percentiles of 5.18 μg/L (Calafat et al., 2005), 11.5 μg/L (Ye et al., 2005b) and 15.9 μg/L (Calafat et al., 2008) Table 5)]; means or medians for bisphenol A concentrations in most studies are below 3 μg/L. Only one study from Korea reported a higher mean for bisphenol A concentrations of 9 μg/L, but the reason for the considerable increase in urinary bisphenol A excretion in the selected population is unknown. More recently, the same researchers have reported additional sets of data with lower mean values and have commented that the apparent decrease in bisphenol A concentrations may be due to ongoing development in analytical techniques rather than representing a true decrease (Yang et al., 2006a,b), thus suggesting the higher value reported earlier may have been an artifact. Another study from China reported levels of bisphenol A in urine (in the range of 1 mg/L) which are three orders of magnitude above those observed in other studies (Mao et al., 2004). The study also assessed the urinary excretion of several endogenous hormones with urine and also reports concentrations approximately 3 orders of magnitude above those normally reported in females, suggesting a systematic laboratory or reporting error for all analytes.

The studies available in the database also include a reasonably high number of samples from different participants (>3000 adults) thus giving robust estimates of the encountered range of exposures. For example, in a large study determining bisphenol A excretion with urine in 394 human subjects of a reference population in the US, bisphenol A was detected in 95% of the urine samples analyzed and concentrations up to 5.18 μg/L (95th percentile) were reported (Calafat et al., 2005).

The Centers for Disease Control (CDC) has also summarized the data on urinary excretion of bisphenol A using LC/MS-MS after glucuronidase cleavage with a LOD of 0.36 μg/L collected in the National Health and Nutrition Examination Survey (NHANES) 2003–2004. The bisphenol A data are from a subsample of the total population sampled at NHANES and included urine concentrations of bisphenol A for 2,517 individuals (Calafat et al., 2008). Bisphenol A was present in concentrations above the LOD in >90% of the samples. In the whole population, the geometric mean for bisphenol A concentration was 2.6 μg/L. When separating the data into age groups, concentrations of bisphenol A in urine ranged from 3.6 μg/L (geometric mean, 6–11 years) to 1.9 μg/L (60+ years). Interestingly, the concentrations reported in 2003–2004 were higher than those reported from NHANES 1999–2000 (geometric mean of 1.33 μg/L in 394 individuals) (Calafat et al., 2005). However, different analytical methods were used in the analysis of the sample sets and other study design parameters, in particular the exclusion of children and adolescents in the earlier study, which was not nationally representative, are not fully comparable and do not allow a meaningful comparison of the data from the two studies.

For comparison of the concentrations in the reference populations (1 to 3 μg/L), peak urinary concentrations of bisphenol A-glucuronide reached 4500 to 6800 μg/L (equivalents of bisphenol A) within 6 h after oral administration of 60–80 μg/kg bw (Table 1). In another controlled exposure with bisphenol A in human subjects, after administration of 25 μg bisphenol A/person, peak urinary concentrations of total bisphenol A reached approximately 9 μg/L. This observation indicates that individuals with urinary concentrations of approximately 5 μg/L (as in urine samples from most unintentionally exposed humans) ingested much less than 25 μg bisphenol A/person (Völkel et al., 2002, 2005) within a few hours before urine sampling.

Some studies also determined the excretion of bisphenol A with urine over a time interval of 24 h, which is considered most suitable for daily intake assessment. Total excretion of bisphenol A in urine samples collected over 24 h were in the range from 1.3 to 5 μg/day. Following the urinary bisphenol A excretion in five male individuals over five days, a median daily uptake of 0.02 μg/kg bw was assessed (Arakawa et al., 2004). However, the study also showed significant interday and interindividual variations in the daily excretion rates of bisphenol A in the individuals.

Discussion and conclusions

Due to the consistent numbers reported and the conclusions based on the known human toxicokinetics of bisphenol A, measurement of urinary concentrations of bisphenol A-glucuronide or total bisphenol A in urine is the most appropriate and feasible way to assess daily exposure to bisphenol A in humans from all sources. The cumulative daily human exposure can be derived from urinary excretion of bisphenol A in the form of bisphenol A metabolites since orally administered bisphenol A is completely recovered in urine within 24 h after an oral exposure (Völkel et al., 2002).

One of the major advantages of using urine in biomonitoring is ease of collection of spot (untimed) urine samples, which are widely used for biomonitoring purposes because collection of all urine samples over a 24-h period is cumbersone.

Regarding bisphenol A biomonitoring, single spot urine samples may only be appropriate to conclude on very recent exposures of an individual to bisphenol A. Due to the short half-life and rapid urinary excretion, and variations in bisphenol A concentrations in terms of frequency of food intake, time of sampling after food consumption and the last urination, and urine production rate, single spot urine samples can provide no information on long-term daily intake over extended periods of time. Therefore, high bisphenol A concentrations in some spot urine samples should also not be used to conclude on average exposure since they only indicate peak exposure occurring shortly before urine collection. An uncertainty of approximately one order of magnitude has to be accepted when using single spot urine samples to estimate total daily intake by an individual.

However, the same factors that limit the accuracy of daily intake estimates for individuals from single spot urine samples may allow estimation of reasonably accurate average daily intake values for a group of individuals. In the larger cohorts, the high number of samples analyzed will average out the variations in urinary concentrations of bisphenol A-glucuronide

arising from temporal factors within a day (e.g., time of sampling after food consumption and last urination) and across days (e.g., variable diets from day to day). Accordingly, mean values from studies reporting bisphenol A concentrations in spot urine samples with a larger number of participants correlate well with those using cumulative excretion over 24 h.

The biomonitoring data demonstrate that the average concentrations of bisphenol A in urine samples from the general population are low (at best a few µg/L) and confirm that bisphenol A is mainly present as glucuronide in human urine. The available data from Japan and the US, which contain samples from a significant number of individuals and report concentrations of bisphenol A in pooled 24 h urine samples of 1–3 µg/L thus serve as a basis for assessing daily exposures to bisphenol A in adults. Based on a total urine volume of 1.2–1.6 liters (Siegenthaler 1987; ICRP 2003) excreted over 24 h, a median daily intake of bisphenol A of 3.75–5 µg/day for adults can be concluded with maximum values up to 6–8 µg/day not including spot urine samples with high concentrations. This translates to average daily doses for 60 kg adults from 0.06 µg/kg bw to a reasonable worst case of 0.1–0.13 µg/kg bw in adults. Measured urinary concentrations of bisphenol A were recently used in Japan to define bisphenol A exposure of the population giving estimates for the daily intakes (95% confidence intervals) as 0.037–0.064 µg/kg bw/day for males and 0.043–0.075 µg/kg bw/day for females in the 95th percentile high-exposure populations (Miyamoto and Kotake, 2006). In the average exposure concentration, daily doses of bisphenol A for males were 0.028 to 0.049 µg/kg bw/day and for females 0.034 to 0.059 µg/kg bw/day.

Unfortunately, no data on the urinary excretion of bisphenol A in infants are available to determine if the higher exposures calculated from food consumption and measured bisphenol A concentrations in migration studies are consistent with actual exposures measured by biomonitoring (EFSA, 2006).

The daily exposure of humans to bisphenol A established by biomonitoring is thus well below the daily exposure as delineated from estimates of exposure based on food consumption and migration in adults, but in the same range as recent exposure assessments using food concentrations of bisphenol A and consumption patterns, e.g. 4.7 µg bisphenol A/day or 0.078 µg/kg bw/day for a 60 kg adult (Thomson and Grounds, 2005) or 0.001 µg/kg bw/day (Miyakawa et al., 2004). A low intake of bisphenol A for young children is also supported by a detailed exposure assessment of bisphenol A using measured concentrations in air, dust, and food. Delineated daily doses were between 0.052 and 0.074 µg/kg bw/day in preschool children (Wilson et al., 2007). The average daily doses of bisphenol A in adults delineated by biomonitoring and supported by the exposure assessment based on concentrations of bisphenol A in the diet are more than 500-fold below the TDI set by EFSA and the US EPA reference dose (both 50 µg/kg bw/day) suggesting that the exposure to bisphenol A does not result in a health risk to the general population. In addition, the bisphenol A exposures of the general population are also well below the daily doses of bisphenol A that sometimes have been reported to cause responses of unknown toxicological relevance in highly sensitive animal systems (20 µg/kg bw/day) (Timms et al., 2005) giving Margins-of-Exposure of 200 or more. For a comparison with human intake of other weakly estrogenic compounds, bisphenol A intake is at least 30 fold lower than that of phytoestrogens, which are more potent estrogens as compared to bisphenol A (Moors et al., 2007, Safe, 2004, Valentin-Blasini et al., 2005).

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