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Development and application of novel human biomonitoring methods for chemicals of emerging health relevance

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Erklärung

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DANKE

Meinen Eltern Margit & Thomas Stöckelhuber

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1) Introduction

1.1 History of human biomonitoring

Human biomonitoring (HBM) of occupational exposures started in the 1890s by determining lead in urine and blood samples of factory workers to evaluate critical exposure to lead and consequently to prevent an acute lead poisoning [1, 2]. First more systematic measurements of chemical exposure in workplaces took place in the 1930s by determining lead and benzene metabolites in different biological fluids [3, 4]. Since that time, more powerful analytical techniques became available allowing the determination of lower concentrations of chemical substances in several matrices [5] and with higher throughput. With those improvements it was possible to measure lead levels in blood samples of the general population of industrialized countries [5, 6] resulting in an alarming outcome of lead levels and the first European "Directive on biological screening of the general population for lead" in 1977 [6].

In the U.S., a first systematic biomonitoring survey was conducted with the "National Health and Examination Survey I (NHANES I)" from 1971 to 1974 [7], followed by two further surveys (NHANES II and III) until 1999 [8]. In 1999 the survey became a continuous program with changing focus on a variety of health and nutrition measurements with approximately 5,000 participants per year from the whole U.S. [9].

The first "German Environmental Survey (GerES)" was carried out in 1985 and 1986 in Western Germany (GerES I) [10] and also in 1985, the "German Environmental Specimen Bank (ESB)" was launched with the aim to collect and archive human and environmental samples in order to document trends in exposure to environmental pollutants [11]. Since GerES I, four further surveys took place (GerES II-V) and currently, data are collected for GerES VI, which should be conducted between 2018 and 2022 [12]. Many other countries like Belgium, France, Russia, Spain, Denmark, and Norway also conducted human biomonitoring (HBM) surveys [13].

Since 2017, the “HBM4EU” project is ongoing with the aim to protect human health in Europe. The project is supported by the European Environment Agency, the European Commission and 28 countries and is coordinated by the German Environment Agency [14]. This increase in human biomonitoring projects emphasizes the importance and necessity of human biomonitoring in modern industrialized countries.

1.2 Definition and requirements for human biomonitoring

Human biomonitoring can be defined as “a systematic continuous or repetitive activity for collection of biological samples for analysis of concentrations of pollutants, metabolites or specific non-adverse biological effect parameters for immediate application, with the objective to assess exposure and health risk to exposed subjects, comparing the data observed with the reference level and — if necessary — leading to corrective actions’’ [15] or as “one method for assessing human exposure to chemicals by measuring the chemicals or their metabolites in human tissues or specimens, such as blood and urine“ [2].

Approaches in HBM can be categorized into dose monitoring, biochemical effect monitoring and biological effect monitoring. Dose monitoring means the quantification of hazardous compounds or their metabolites in biological matrices. Biochemical effect monitoring is used to determine reaction products of substances with biological molecules such as DNA or proteins. Biological effect monitoring is the investigation of early biological effects caused by chemicals, for instance sister chromatid exchange rates, micronuclei aberration, enzyme activities [5]. All HBM applications presented in this thesis can be categorized as dose monitoring applications (internal exposure, cf. Figure 1).

Human biomonitoring needs to be delimited from ambient monitoring (AM) which means “determination of chemical substances in environmental matrices like air, water soil, food, etc.” [5]. In contrast to HBM, AM does not consider all possible

routes of absorption, and concentrations measured do not represent the internal dose taken up [16]. Nevertheless, both approaches are needed to protect humans from the exposure of chemicals which are hazardous to health. While AM identifies the source of exposure, HBM determines the amount of chemical taken up by an individual. AM and HBM complement each other, but HBM only can assess the dose really taken up (internal dose) [5], which is directly related to effects on human health.

However, some requirements have to be fulfilled in order to conduct HBM studies. First, a suitable biological matrix, preferably easily accessible and available, needs to be found. The analyte concentration in the matrix selected should be able to reflect internal exposure and biochemical or biological effects. Furthermore, validated and reliable analytical methods, which are kept under control by quality assurance need to be available for analysis, and reference and limit values need to be established in order to interpret the results [5, 17].

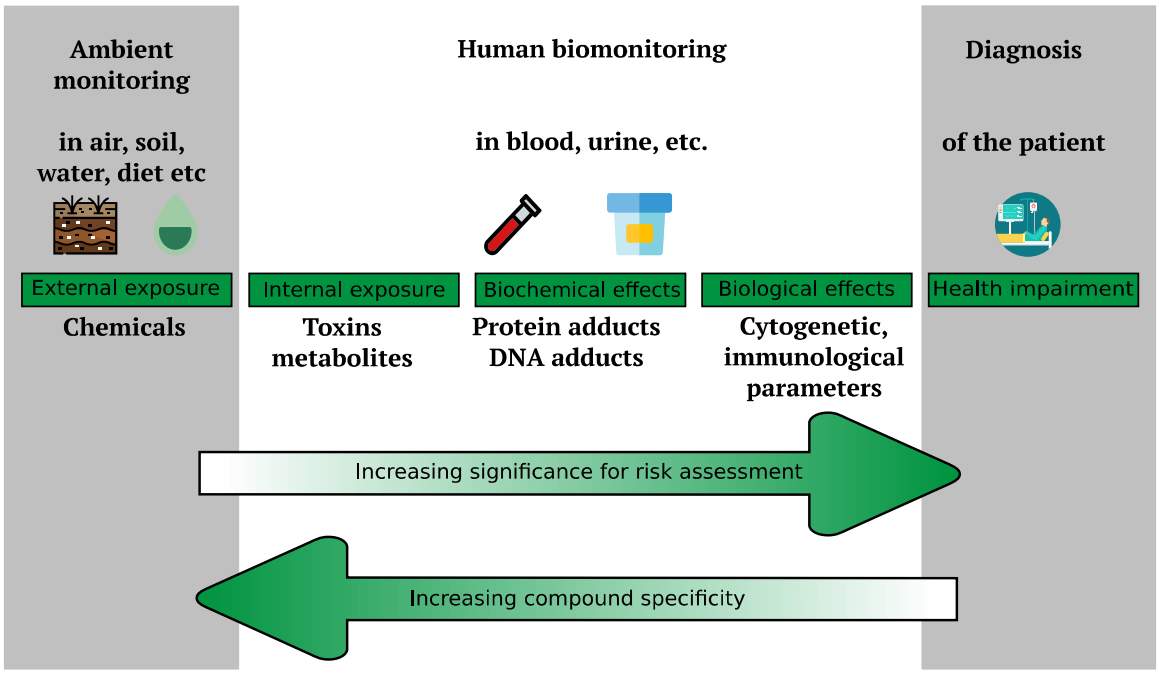

Figure 1: Classification of ambient and biological monitoring (adapted from Angerer, 2007 [5] and Budnik, 2009 [18])

1.3 Selection of the biological matrix

Environmental chemicals are primarily entering the human body by ingestion, inhalation or dermal absorption [19, 20]. After uptake, a substance can be excreted without transformation, excreted after metabolization or stored in tissues or bones [13, 19], or undergo a combination of these processes [21]. The physicochemical properties of a chemical highly affect its metabolism and excretion routes which influence the selection of a biological matrix for HBM [13]. For instance, substances, being not absorbed from the body are excreted directly in the feces and can be determined in blood or feces for a short time after intake only [21]. In contrast, substances with a long half-live ranging from several weeks up to years can be measured in blood, adipose tissue, teeth or breast milk [20-22]. Certainly, the selection of biological matrices is limited by several additional factors: The xenobiotic analyzed should be determinable in a level and in a biological matrix which are meaningful in terms of systemic exposure and health risks [17], sufficient amounts of the sample material should easily be accessible and sampling should not cause unacceptable discomfort or health risks for the subjects [5]. For these reasons, the most common matrices used in occupational medicine and human biomonitoring are blood and urine, whereby urine is easier accessible [5, 19, 21].

As the central compartment of the human body, blood is in equilibrium with all organs [5, 19, 21]. This enables the determination of persistent chemicals which are stored in deposition sites such as adipose tissue, bones or in blood or its components [20, 21]. On the contrary to the storage compartments, the recovery of blood samples is by far less invasive resulting in higher participation rates in epidemiological studies [21]. To ensure specificity for a certain chemical it can be necessary to analyze the original chemical and/or one or more of its metabolite(s) in blood instead of a transformation product in another matrices, especially if a given metabolite may originate from more than one parent chemical [20]. Determining the parent com-

pound is also favorable if the chemical itself possesses toxicological relevant properties [23]. A classical group of synthetic chemicals which are measured in human blood samples in order to assess systemic exposure are organochlorine pesticides [2, 13, 17, 24].

Urine is also a very common matrix for HBM, especially for water-soluble chemicals and metabolites [19, 25]. The big advantage of urine as biological sample is that sample collection is noninvasive while it is continuously accessible in large volumes [5]. Large sample volumes can help to determine very low concentrations of chemicals, because analytes can be concentrated by evaporating the solvent (after extraction) and reconstitution of the analytes in smaller solvent volumes [5]. On the other hand urine samples can strongly differ in their compositions and volumes [26]. To overcome misinterpretation of analytical data and to take urine dilution into account, different approaches to standardize urinary concentrations determined have been established[11]. The most common standardization method is to relate analyte concentrations on creatinine assuming that creatinine is constantly excreted into urine [27]. In some cases, relating concentrations to the amount of creatinine excreted is not purposeful, for example, for human exposure measurements of metals like cadmium and thallium which significantly affect kidney function and thereby creatinine excretion [28, 29]. Alternative methods for standardization of urinary concentrations are specific gravity [30], conductivity [31], osmolality [32] and total urine volume [33]. Due to the easy sampling of urine, this matrix is particularly well suited for studies which require repeated sampling [26].

Another important aspect which must be considered while determining urinary metabolites is that xenobiotics are often excreted as conjugates in order to increase their water solubility and hence facilitate excretion [34]. Possible conjugates include sulfate, glycine and glutathione conjugation, but the major elimination pathway in mammals is the conjugation of glucuronic acid to an oxygen, nitrogen, or less often, sulfur or carbon atom of the xenobiotic [35]. The glucuronidation reaction is catalyzed by glucuronosyltransferases which are located in the endoplasmic reticulum of cells

mainly of the liver, but also in other organs, like kidney, intestine, skin, lung, spleen, prostate and brain [34, 35]. Drug (metabolite) glucuronidation in humans can be affected by multiple factors, like age, diet, cigarette smoking, diseases, drug therapies, ethnicity, genetic and hormonal factors [36] and therefore, the ratio between unconjugated and glucuronidated xenobiotics varies from individual to individual making it necessary to determine the whole amount (free and conjugated) of a compound. To capture the whole amount of a compound, glucuronides can be hydrolyzed by β-glucuronidases, esterases and serum albumin to give the respective aglycone [35, 37].

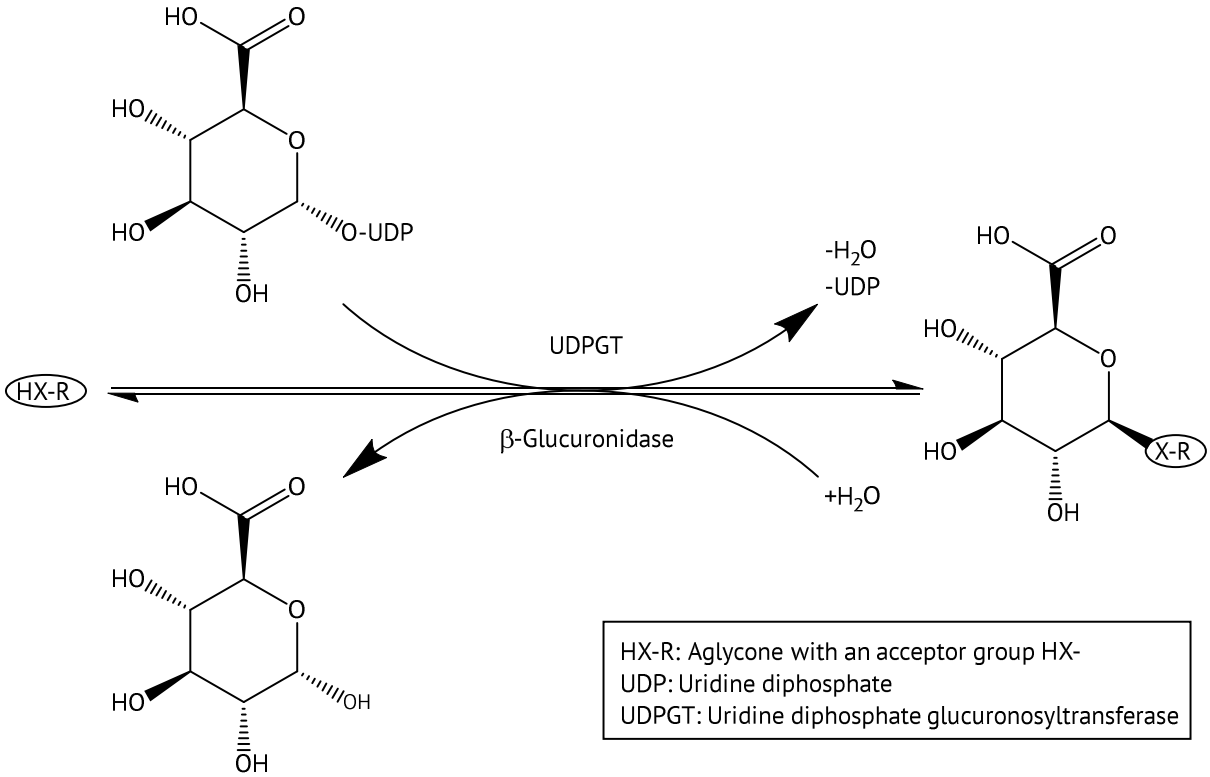

Figure 2: Scheme for the conjugation of an aglycone (R-XH) with glucuronic acid. The acceptor group HX- may contain oxygen, nitrogen, sulfur or carbon atoms. The configuration of glucuronic acid is inverted from alpha- to beta form during the conjugation reaction (adapted from Kroemer, 1991 [34] and Miners, 1991 [36]).

Urine as matrix is usually used for non-persistent chemicals such as phenols, parabens, phthalates, volatile organic compounds and polycyclic aromatic hydrocar-

bons [13], especially if a metabolite of the chemical monitored is responsible for possible detrimental effects [21]. Due to the big advantages of urine as biological matrix, all HBM methods presented in this thesis are based on urine as sample matrix.

1.4 The German Human Biomonitoring Initiative

To improve the knowledge about chemicals taken up from the human organism, the German Human Biomonitoring Initiative was started in 2010 [38, 39]. This project is a cooperation between the German Federal Ministry for the Environment, Nature Conservation and Nuclear Safety (Bundesministerium für Umwelt, Naturschutz und nukleare Sicherheit, BMU) and the German Chemical Industry Association (Verband der chemischen Industrie, VCI) with the aim to develop novel HBM methods for up to 50 chemicals within ten years until 2020 [40]. In order to achieve sufficient specificity and sensitivity for all analytes, the physical and chemical properties of the respective chemical and its metabolites need to be considered. Therefore, every method development requires an unique approach, which is demanding and needs time and that is why the cooperation was extended until 2025 [41].

For the selection of chemicals of relevance, a HBM Expert Panel consisting of representatives from the Federal Institute for Risk Assessment, the Federal Institute for Occupational Safety and Health and the BMU, as well as of experts from chemical industry enterprises and from universities and other scientific institutions was built [42]. Chemicals of relevance were selected based on their toxicology, their (expectedly) good bioavailability (health relevance), a high likelihood of consumer exposure (consumer relevance) and non-existence or unsuitability of an existing HBM method [40, 42]. While the VCI bears responsibility for method development, the BMU, supported by the German Environment Agency (Umweltbundesamt, UBA) is responsible for the application of the methods in suitable investigations like GerES [40, 43].

In order to obtain suitable methods, some requirements were imposed on the laboratories developing the monitoring methods [42]:

1. To facilitate sample collection, especially in studies addressing children, the method should be developed for urine (non-invasive matrix).

2. Since the method will be applied to a large number of samples, the method should run in fast routine using state of the art techniques.

3. Sensitivity of the method should allow to evaluate the environmental exposure of the general population even at very low exposure. Therefore, LOQ must be well below the LOQ for occupational exposure. In general, a LOQ of 0.1 µg/L in urine or plasma should be reached.

4. Application of the method to samples of 30-40 not occupationally exposed participants is part of the method development.

Since March 2020, all 50 substances were selected by the HBM expert panel for the development of an HBM method. Method development was successfully completed for 22 chemicals, four method developments had to be aborted, and 17 method developments are still in progress (state: October 2019) [44]. Substances selected possess numerous application fields and are a highly heterogeneous group as displayed in Figure 3.

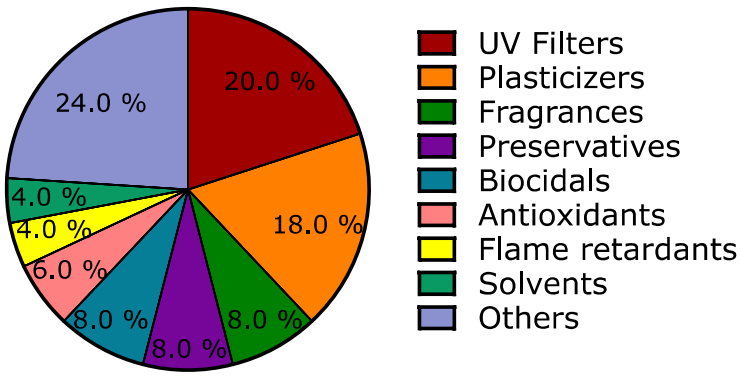

Figure 3: Categorization of the 50 substances selected for the German HBM Initiative

The large progress in German HBM in the last decade ensured that the UBA was commissioned with the coordination of the first Europe-wide HBM program "HBM4EU" which shall help to harmonize and refine HBM within Europe [40].

2) Aims and scope

The amount of chemicals the populations of industrial and developing countries are exposed with in their everyday lives is steadily increasing. From 2002 to 2019, the number of chemicals registered in the Chemical Abstract Service Registry was growing from 20 million to 156 million chemicals [45]. The development and usage of new chemicals for specific application fields supports the improvement of our living standard, but also leads to an increased exposure of the environment and human beings to anthropogenic chemicals. Besides the direct exposition of humans to chemicals, this inevitably causes human exposure to pesticides, industrial chemicals, pharmaceuticals and other synthetic chemicals via consumer products or the food chain [42, 45]. Numerous of these substances taken up by the human body by inhalation, dermal contact or oral absorption through the food chain or drug ingestion can induce detrimental effects [18], therefore the producers and governments have to protect the population and the environment against unwanted effects [42].

In the present work, human biomonitoring methods were developed for three chemicals selected within the German Human Biomonitoring Initiative. The substances processed in this work possess manifold chemical and physical properties, because of their different application fields which had to be taken into account during method development.

The first chemical processed in this work was the fragrance 7-hydroxycitronellal (CAS number: 107-75-5) which was selected as chemical of interest in 2015 by the HBM expert panel. This synthetically manufactured diterpene is frequently used in cosmetics, washing and cleaning agents and was shown to cause skin irritation and skin sensitization [46].

In 2016, the UV filter Uvinul A plus® (CAS number: 302776-68-7) was chosen for the development of an HBM method and selected as second chemical within this work.

Uvinul A plus® belongs to a widespread class of UV filters possessing a benzophenone basic structure being present in many sunscreens and anti-aging products [47]. Although it could not be confirmed for Uvinul A plus®, a considerable number of benzophenone based UV filters has been shown to act as endocrine disruptors and affect the hormone systems of animals and humans [48].

The third chemical investigated in this work is ethoxyquin (CAS number: 91-53-3), which was selected by the HBM expert panel in 2017. Ethoxyquin is often added to animal feed, because of its antioxidative properties and is preferred over other antioxidants commercially available because of its low costs of production [49]. This quinoline-based antioxidant was shown to induce detrimental effects after acute or chronic exposure [49] and some transformation products of ethoxyquin are suspected to be carcinogenic [50].

The chemical structures of the three substances investigated in this work are displayed in Figure 4.

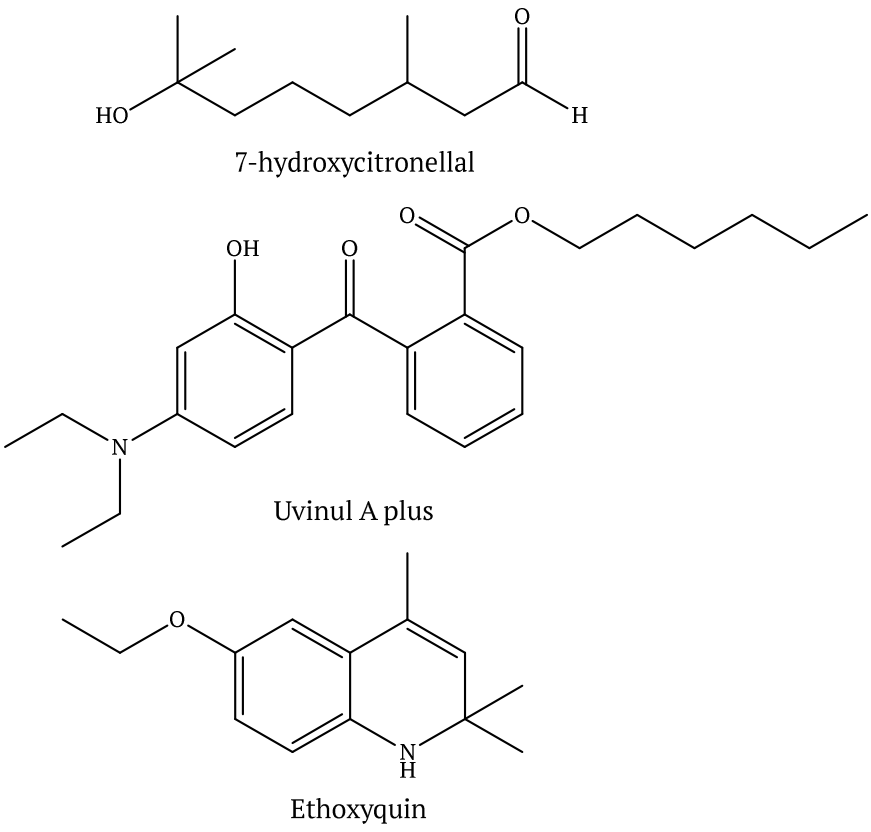

Figure 4: Chemical structures of the three substances investigated in this work

In order to build up powerful HBM methods, metabolites which could serve as suitable biomarkers of exposure had to be identified for every chemical investigated and subsequently, HBM methods were optimized to the selected biomarkers and validated. As most appropriate sample matrix, urine was selected for all three HBM methods (cf. 1.3 Selection of the biological matrix). Since no information on human metabolism of the chemicals of interest was available, biomarkers were postulated based on results of *in vitro* and/or *in vivo* experiments and human metabolism was investigated within the frame of method development by orally (and dermally) applying one of the three test compounds to five healthy volunteers. Potential biomarkers not commercially available were self-synthesized. Sample preparation included enzymatic hydrolysis of conjugated metabolites (cf. 1.3 Selection of the biological matrix), extraction of the analytes into an organic solvent and concentration of the analytes before measurement. Subsequently, analytes were separated from the sample matrix by means of ultra high-performance liquid chromatography (UHPLC). UHPLC guaranteed a satisfactory separation efficiency for all analytes and was preferred over gas chromatography (GC), because GC analysis is limited to volatile compounds and some biomarkers selected were not volatile enough for GC analysis, which is especially hold true for polar metabolites even though they were analyzed in their unconjugated forms. Detection of the analytes was achieved by coupling the UHPLC system with a quadrupole mass spectrometer leading to a selective and sensitive determination of the analytes.

The HBM methods developed, including selection of the biomarkers, sample preparation, chromatographic separation and detection were fully validated according to FDA guidelines [51, 52]. As a proof of concept, a number of 40 to 58 samples of the general population were analyzed with the new HBM methods.

3) Development and application of novel human biomonitoring methods for chemicals of emerging health relevance

3.1 Human biomonitoring for 7-hydroxycitronellal

***Stoeckelhuber M**, Krnac D, Pluym N, Scherer M, Leibold E, Scherer G. A validated UPLC–MS/MS method for biomonitoring the exposure to the fragrance 7-hydroxycitronellal. Journal of Chromatography B. 2017;1068-1069 (Supplement C):261-7.*

***Stoeckelhuber M**, Krnac D, Pluym N, Scherer M, Peschel O, Leibold E, Scherer G. Human metabolism and excretion kinetics of the fragrance 7-hydroxycitronellal after a single oral or dermal dosage. International Journal of Hygiene and Environmental Health. 2018;221(2):239-45*

7-Hydroxycitronellal (CAS number: 107-75-5) is a synthetic fragrance which can be found in a wide variety of consumer products such as cosmetics, washing and cleaning agents [53-56]. It is also utilized as flavoring agent for food [55], because it has a sweet, floral and lily-type odor [56]. 7-Hydroxycitronellal is manufactured and/or imported in 1,000 – 10,000 tons per year within the EU and is supplied under various trade names such as citronellal hydrate, cylclosia, laurinal, laurine or lilyl aldehyde [46]. In 2002, the usage of 7-hydroxycitronellal in Europe was estimated 88 tons per year with an average percentage of 35 % used in households [56]. In deodorants an increase in the generally high occurrence of 7-hydroxycitronellal from 50 % in 1998 [57] to 70 % in 2007 [54] could be observed. Overall, it could be detected in 10.6 % of the consumer products investigated in 2007 [54]. In the same year Buckley et al. determined an occurrence of 17 % of 7-hydroxycitronellal in consumer products in the UK, which meant that it was the 8th most frequently labeled fragrance in consumer products in the UK at that time [53]. A large survey addressing the occurrence of fragrance contact allergens in >5,000 cosmetic products was conducted within the EU from 2015 to 2016: The fragrance allergen 7-hydroxycitronellal was labelled at 7.1 % of the cosmetic products. The product category most often labelled with 7-hydroxycitronellal were perfumes with 41.2 % [58].

Although the substance is widely used in consumer products, 7-hydroxycitronellal possesses a topical toxicity leading to skin irritation and skin sensitization [46] and is listed by the European Commission in regulation EC No 1223/2009 as one of 26 contact allergens used as fragrance ingredients, which have to be declared on cosmetic products [59, 60]. No other harmful effects causing acute toxicity could be found after dermal or oral exposure of rabbits or rats. Genotoxicity was also tested in various *in vitro* and *in vivo* models without any hints on mutagenic activity [46]. The LD_{50} value in rats was found to be higher than 6,400 mg/kg body weight [46] and a "No Observed Effect Level" (NOEL) of 250 mg/kg/d was established by The Joint FAO/WHO Expert Committee on Food Additives (JECFA) [56]. Following the toxicological findings, the International Fragrance Association (IFRA) updated the restriction limits in the finished product in January 2020 to 0.11-15 % depending on the product category [61]. The lowest restriction limit with 0.11 % in the finished product affects deodorants, the highest restriction limits were set for hard surface cleaners (15 %) [61, 62]. Levels observed in consumer products vary from 0.015-0.478 % in perfumes [54] and 0.0001-0.1 % in deodorants [57].

Even though 7-hydroxycitronellal is a widely used chemical which the major part of the general population is exposed with, no data concerning human exposure was available until recently and human metabolism of 7-hydroxycitronellal was not elucidated as well. That's why a human biomonitoring was developed, and human metabolism was investigated within the frame of a small metabolism study with five participants.

The metabolism study revealed two urinary metabolites of 7-hydroxycitronellal: the reduced form 7-hydroxycitronellol and the corresponding carboxylic acid 7-hydroxycitronellylic acid (see Figure 5). The non-metabolized aldehyde could not be determined in any sample.

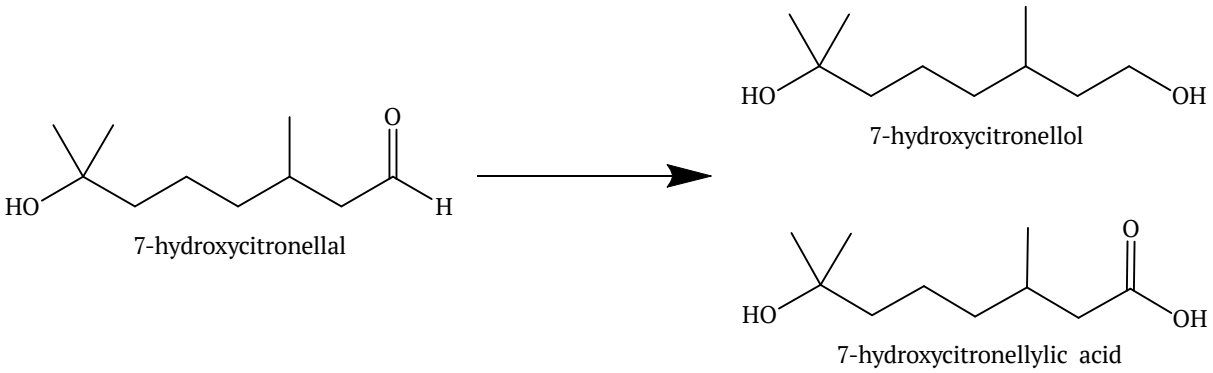

Figure 5: Pathway for the 7-hydroxycitronellal metabolism in humans

Out of these two metabolites, 7-hydroxycitronelllylic acid only was found to be a suitable biomarker of exposure for 7-hydroxycitronellal since 7-hydroxycitronellol levels were about 1,000 times lower compared to 7-hydroxycitronellylic acid levels. Hence, the biomonitoring method was optimized for 7-hydroxycitronellylic acid and validated according to guidelines issued by the German Research Foundation (Deutsche Forschungsgemeinschaft, DFG) and the US Food and Drug Administration (FDA)[51]. The final analytical method is carried out by using stable isotope-labeled 7-hydroxycitronellylic acid as internal standard (IS) and includes a hydrolysis step of possible metabolite conjugates with an enzyme mix of β-glucuronidase and arylsulfatase. Sample cleanup was achieved by liquid-liquid extraction (LLE) of the urine samples with dichloromethane.

The validated method was applied to all samples of the metabolism study and 40 urine samples collected from adult volunteers from the general population. Maximum excretion rates were reached after three to five hours after oral administration and after ten hours after dermal administration. Skin absorption for 7-hydroxycitronellal reduced the amount of urinary excreted 7-hydroxycitronellylic acid within the first 24 hours after gavage to 9 % after dermal application in comparison to 50 % after oral application. From the data of the human metabolism study, a conversion factor was calculated in order to estimate the exposure of the general population to 7-hy-

droxycitronellal from the urinary concentration of 7-hydroxycitronellylic acid determined. From the samples of 40 volunteers from the general population a daily average exposure dose of approximately 93 µg per day could be determined. The metabolite 7-hydroxycitronellylic could be quantified in all 40 samples proving the suitability of the method for future human biomonitoring projects.

3.2 Author contributions

The results achieved were published in two journals. The method development and validation of the method was published in the Journal of Chromatography B, and the human metabolism study including the calculation of toxicokinetic parameters were published in the International Journal of Hygiene and Environmental Health.

3.2.1 Contributions to "A validated UPLC–MS/MS method for biomonitoring the exposure to the fragrance 7-hydroxycitronellal"

Dusan Krnac (chemist) and I developed the analytical method. The validation of the human biomonitoring method was carried out by me with the support of Dusan Krnac. I collected 40 samples of the general population within the greater Munich area and analyzed them according to the method described for the determination of 7-hydroxycitronellylic acid. The manuscript was prepared by Prof. Dr. Gerhard Scherer, Dr. Max Scherer, Dr. Nikola Plum, Dr. Edgar Leibold (BASF), and me.

Own contribution:

Development of the human biomonitoring method	40 %	
Validation of the human biomonitoring method	90 %	
Sample collection from the general population	100 %	
Analysis of study samples	100 %	
Submission and revision of publication 1	80 %	

3.2.2 Original article: "A validated UPLC–MS/MS method for biomonitoring the exposure to the fragrance 7-hydroxycitronellal"

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A validated UPLC–MS/MS method for biomonitoring the exposure to the fragrance 7-hydroxycitronellal

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ABSTRACT

7-Hydroxycitronellal is a synthetic fragrance (CAS No. 107-75-5) which is used commonly in cosmetics, washing- and cleaning agents and as flavoring in foods. Due to its broad application in various fields, 7-hydroxycitronellal was selected for the development of a biomonitoring method for the quantitative exposure assessment within the frame of the cooperation project of the German Federal Ministry for the Environment, Nature Conservation, Building and Nuclear Safety (BMUB) and the German Chemical Industry Association (VCI). For this purpose, an ultra performance liquid chromatography combined with tandem mass spectrometry (UPLC–MS/MS) based method was developed for the determination of potential biomarkers of 7-hydroxycitronellal (7-HC) in human urine samples. 7-Hydroxycitronellylic acid (7-HCA) turned out to be the quantitatively most important metabolite of 7-HC in human urine, occurring in 1000 times higher amounts than 7-hydroxycitronellol (7-HCO) or other potential metabolites. Therefore, an analytical method for 7-HCA was developed using stable isotope-labeled 7-HCA as internal standard (IS). The method includes a cleavage step of possible metabolite conjugates with an enzyme mix of ß-glucuronidase and arylsulfatase. Subsequent sample cleanup was performed by liquid–liquid extraction (LLE) with dichloromethane. The method was calibrated by calculating the linear regression between the analyte/IS ratio and the nominal 7-HCA concentrations in water. The method was validated according to approved standard guidelines and proved to be robust, reliable and sensitive for the human biomonitoring of 7-HC. The method was applied to urine samples of 40 adult volunteers from the general population. 7-HCA was quantifiable in urine of all subjects. Thus the developed method proved to be suitable for assessing the background exposure to 7-HC in the general population.

1. Introduction

7-Hydroxycitronellal (7-HC, chemical structure **1** in Fig. 1), also known as citronellal hydrate, laurinal or laurine (IUPAC: 7-Hydroxy-3,7-dimethyl-octanal, CAS No. 107-75-5, chemical structure shown in Fig. 1) is a synthetic odorant which is frequently used in cosmetics, washing- and cleaning agents and also approved as flavoring for foods in Germany [1]. It has a scent of lilac, lily and lily of the valley. 7-HC (**1**) is listed as one of 26 contact allergens used as fragrance ingredients, which have to be declared on cosmetic products due to its skin sensitizing effects according to the Scientific Committee on Consumer Safety (SCCS) [2] and, therefore, the International Fragrance Association (IFRA) restricted the levels in finished products to 0.1–3.6% [3]. In rats a LD_{50} over 6400 mg/kg body weight was determined [4] and The Joint FAO/WHO Expert Committee on Food Additives (JECFA) established a No Observed Effect Level (NOEL) of 250 mg/kg/d [5].

According to the SCCS, 7-HC (**1**) is typically applied in its racemic form in concentrations between 0.015–0.478% in perfumes [2]. Several market surveys on the presence of fragrance substances reported between 10 and 50% occurrence of 7-HC (**1**) in cosmetics and detergents [2,6,7].

The wide scope and common use of cosmetics imply that the general population is probably exposed to this chemical. From studies in rabbits it can be deduced that 7-HC (**1**) is converted to two primary metabolites: Its alcohol 7-hydroxycitronellol (7-HCO, **2**) and its carboxylic acid 7-hydroxycitronellylic acid (7-HCA, **4**) (Fig. 1). No statements to kinetics and percental distribution of the metabolites or the excretion of unchanged 7-HC (1) was made [8]. These metabolites containing free hydroxy groups or carboxylic moieties are likely to be conjugated by sulfation or glucuronidation before excretion into urine.

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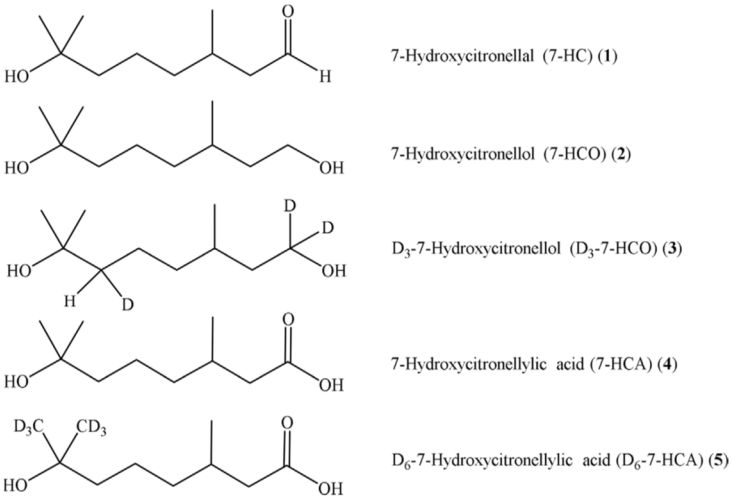

Fig. 1. Chemical Structures of 7-hydroxycitronellal, its major metabolites and the internal standards.

Due to its ubiquitous occurrence in consumer products, 7-HC (**1**) was selected as a chemical of interest for the ten year human biomonitoring (HBM) project, a joint cooperation between the German Federal Ministry for the Environment, Nature Conservation, Building and Nuclear Safety (BMUB) and the German Chemical Industry Association (VCI) with the scope to develop and apply HBM methods for 50 chemicals with widespread occurrence in the environment and probable exposure of the general population [9]. In the frame of this project, we developed and validated a suitable HBM method for the quantitative evaluation of the exposure to 7-HC (**1**) in the general population. Furthermore, the validated method was applied to 40 urine samples from adult volunteers. Results on the analytical method, its performance and application are described in this paper. As human metabolism and toxicokinetics of 7-HC (**1**) have not yet been investigated in humans, we also conducted and evaluated a human metabolism study using defined oral and dermal administration of the flavor ingredient in order to identify the major metabolites and to determine the toxicokinetics of this chemical. The results of the clinical study will be reported in a separate publication (currently in preparation).

2. Materials and methods

2.1. Chemicals

7-Hydroxycitronellal (CAS No. 107-75-5, **1**) was purchased from Sigma-Aldrich (Taufkirchen, Germany). 7-Hydroxycitronellol (107-74-4, **2**) was obtained from Pfaltz & Bauer (Waterbury, CT, USA). 7-Hydroxycitronellylic acid (**4**), deuterated 7-hydroxycitronellol (**3**) and deuterated 7-hydroxycitronellylic acid (**5**) were custom-synthesized by Vladimir Belov (Max-Planck-Institute Göttingen, Germany). Principle of syntheses as well as characterizations of these compounds are described in the Supplementary material.

3-Nitrophthalic acid anhydride (3-NPA), used for derivatization of 7-HCO (**2**), and phosphoric acid 85% in water was supplied by Sigma-Aldrich (Taufkirchen, Germany). Water, hexane, heptane, toluene and dichloromethane were obtained from Promochem (Wesel, Germany). Acetic acid was purchased from Fluka (Deisenhofen, Germany). HPLC-grade water with 0.1% formic acid and methanol with 0.1% formic acid and all other analytical grade organic solvents were from LGC standards (Wesel, Germany). Other chemicals and reagents used were at least of analytical grade. ß-Glucuronidase/arylsulfatase (30 units/mL ß-glucuronidase and 60 units/mL arylsulfatase) enzyme mix from *Helix pomatia* was purchased from Merck-Millipore (Darmstadt, Germany) or from Roche (Mannheim, Germany) (4.5 or 14 units/mL, respectively). ß-Glucuronidase (500,000 Units/mL) from *E. coli* was purchased from Sigma-Aldrich.

2.2. Urine sample preparation

One mL of urine (either fresh or thawed at room temperature from samples frozen at < −20 °C) was spiked with 10 µL of the internal standards D_3-7-HCO (**3**) (1 µg/mL in water) and D_6-7-HCA (**5**) (1 µg/mL in water). The mixture was enzymatically hydrolyzed by adding 0.5 mL of 1 M acetate buffer pH 5.1 and 10 µL of a β-glucuronidase/arylsulfatase mixture (4.5 or 14 units/mL). The higher concentrated enzyme mixture from Merck-Millipore (30 and 60 units/mL) was diluted 7-fold with water to obtain similar enzyme concentrations. The samples were incubated at 37 °C for 3 h. After the addition of 50 µL of 4 M phosphoric acid, the solution was extracted with 1.5 mL dichloromethane for 30 min and centrifuged (15 min at 1900 × *g*). The organic phase was evaporated to dryness using a SpeedVac centrifuge (Thermo Fisher, Dreieich, Germany) without heating. The residue was derivatized by adding 1 mL 3-NPA solution in ethyl acetate (100 µg/mL) and incubated for 30 min at 80 °C. The mixture was evaporated to dryness (SpeedVac, no heating) and re-dissolved in methanol/water (1:1, v/v). The extract was transferred into an HPLC microvial. In the final analytical method, which comprises only the quantification of 7-HCA (**4**), the addition of the IS D_3-7-HCO (**3**) and the derivatization step with 3-NPA were omitted.

2.3. UPLC–MS/MS analysis

Five µL of the extract were injected into an ultra performance liquid chromatography combined with tandem mass spectrometry (UPLC–MS/MS) system consisting of an ACQUITY UPLC I-Class with a binary pump (Waters, Eschborn, Germany) and a triple quadrupole mass spectrometer Xevo TQ-S (Waters, Eschborn, Germany). Chromatography was performed on a UPLC column ACQUITY UPLC BEH C8, 1.7 µm; 2.1 × 150 mm (Waters, Eschborn, Germany) at 40 °C column temperature with a flow rate of 0.5 mL/min and a gradient consisting of A (water + 0.1% formic acid) and B (acetonitrile + 0.1% formic acid) solutions. During method development chromatographic separation was also tested with a UPLC C18 column with the same dimensions from Waters (ACQUITY UPLC BEH C18, 1.7 µm, 2.1 mm × 150 mm). Gradient elution was performed starting with 80%

Table 1
Retention times, mass transitions, and MS/MS parameters for 7-hydroxycitronellylic acid (7-HCA, **4**) and its IS (D_6-7-HCA, **5**).

Analyte or IS	Retention time (min)	Mass transition (*m/z*)	Role	Measuring time (ms)	Cone (V)	Collision Energy (V)
7-HCA (**4**)	2.73	187.0 → 127.1	Quantifier	0.025	24	16
		187.0 → 57.0	Qualifier	0.025	24	20
D_6-7-HCA (**5**)	2.73	193.0 → 63.0	IS	0.025	24	20

A for one minute and then decreasing A linearly to 20% over four minutes. Conditions were hold for half a minute and then A was increased again linearly to 80% in one and a half minutes.

Negative electrospray ionization (ESI^-) was applied with a source temperature of 150 °C and a desolvation temperature of 600 °C. The MS/MS detector was run in the multiple reaction monitoring (MRM) mode. Applied mass transitions and MS/MS parameters as well as retention times for all analytes and the internal standards (ISs) integrated in the original method are shown in Table 1.

2.4. Calibration

Calibration was tested in water and in different urine samples. There was no significant difference in the slopes between the calibration standards prepared in water and those prepared in urine matrix. We observed background levels of 7-HCA (**4**) in all investigated urine samples. For this reason, calibration was performed by spiking water with 0.2, 0.5, 1.0, 2.0, 5.0, 10, 40, 100, 500, 1000 and 2000 µg/L of 7-HCA (**4**) and 7-HCO (**2**). Ten µL of aqueous solutions of the ISs D_6-7-hydroxycitronellylic acid (**5**) (1 mg/L) and D_3-7-HCO (**3**) (1 mg/L) were added. The spiked samples were worked-up as described above. Calibration functions were calculated by linear regression of the area ratios analyte to IS and the nominal concentrations. The regression lines were forced through the origin.

2.5. Method validation

Method validation was performed according to the guidelines issued by the German Research Foundation (DFG) [10] and the US Food and Drug Administration (FDA) [11].

Selected human urine samples with low amounts of the analyte were spiked in three different concentration levels (low, medium and high) covering the calibration range for method validation.

Selectivity was proven for the applied mass transitions. *Intra*-day precision was determined by analyzing three analyte concentrations (low, medium, and high) in authentic human urine samples five times within one day. For the determination of the *inter*-day precision, the same samples were analyzed once on six different days within two weeks. Acceptance criteria for precision were relative coefficients of variation (CVs) of ± 15% (± 20% at levels up to three times the limit of quantification (LOQ)).

Accuracies were determined with human urine samples spiked at low, medium, and high levels. Each level was analyzed five times. Acceptance criteria were met with accuracy rates of 85–115% (80–120% at levels up to three times the LOQ). Recovery rates (indicating analyte losses during sample work-up) were determined by comparing the analyte concentrations at three levels, measured when a urine extract was spiked after sample work-up (reference, corresponding to 100%), and when the same urine sample was spiked before the sample work-up procedure. IS was added before sample work-up.

The LOQ was determined as lowest concentration which does not deviate more than 20 % from the nominal value. The LOD was calculated by dividing the LOQ by 3. The accuracy for samples after dilution was tested by spiking a urine sample with a concentration of 7-HCA (**4**) exceeding the highest calibration level and diluting it with water. The calculated and the determined concentration should not differ by more than 20%. Matrix effects were investigated by comparing spiked and processed urine extracts (according to the sample work-up preparation procedure) at low and high concentrations of analyte with water samples spiked and processed in the same way. Additionally, a blank (unspiked) sample of the same was analyzed and the result was subtracted from the result of the spiked urine in order to correct for the urinary background level. The difference to 100% (=signal in water) was defined as matrix effect. Carryover effects were evaluated by repeated (N = 5) injections of urine extracts with high analyte concentrations, followed by two blank (methanol) injections. Stability of analytes was verified upon storage at room temperature, 10 °C and at −20 °C over different time periods.

2.6. Application of the method to human urine samples

All human studies within this study were approved by the responsible ethical commission. In a pilot study for the identification of suitable biomarkers of 7-HC (**1**) with a volunteer (male nonsmoker, 67 years old), 4 g sun cream spiked with 40 mg 7-HC (**1**) were applied to the skin (arms and breast). One spot urine immediately before and all urine voids within 48 h after the dermal application were collected. The urine samples were prepared with a preliminary version of the analytical method and checked for the presumable metabolites 7-hydroxycitronellol (**2**) and 7-hydroxycitronellylic acid (**4**). Data from this study were used for identifying suitable biomarkers of exposure for the final analytical method and also for estimating the urinary concentration of 7-HC (**1**) metabolites excreted under real-life conditions.

For enzymatic hydrolysis experiments, additional four subjects (both sexes, nonsmokers, 33–70 years old) were exposed orally with 1.9–2.6 g 7-HCA (**1**), depending on their body weight. One spot urine immediately before and all urine voids within 48 h after the oral application were collected. All samples were prepared according to the final analytical method. The two samples from every subject with the highest 7-HCA (**4**) levels were reanalyzed twice, with and without enzymatic hydrolysis.

The validated analytical method was applied to spot urine samples collected by 40 healthy human volunteers, 23 males and 17 females between 18 and 83 years old. The time of the urine sample collection was also annotated. Subjects gave their informed consent for measuring chemicals in their provided urine samples. The 40 spot urine samples were analyzed for the 7-HC metabolite 7-HCA (**4**) with the validated analytical method described above. Urinary levels were standardized for creatinine, which was determined according to a published method [12].

2.7. Statistical analyses

All statistical analyses including tests and correlations described below were carried out using Prism (GraphPad, Version 7.03, La Jolla, CA, USA) software package. The non-parametric Mann–Whitney *U* test was used to determine the statistical significance of differences between subgroups. Non-parametric tests were applied in order to avoid disturbing effects of extreme values and of non-normal distributions. P-values of < 0.05 were regarded as statistically significant.

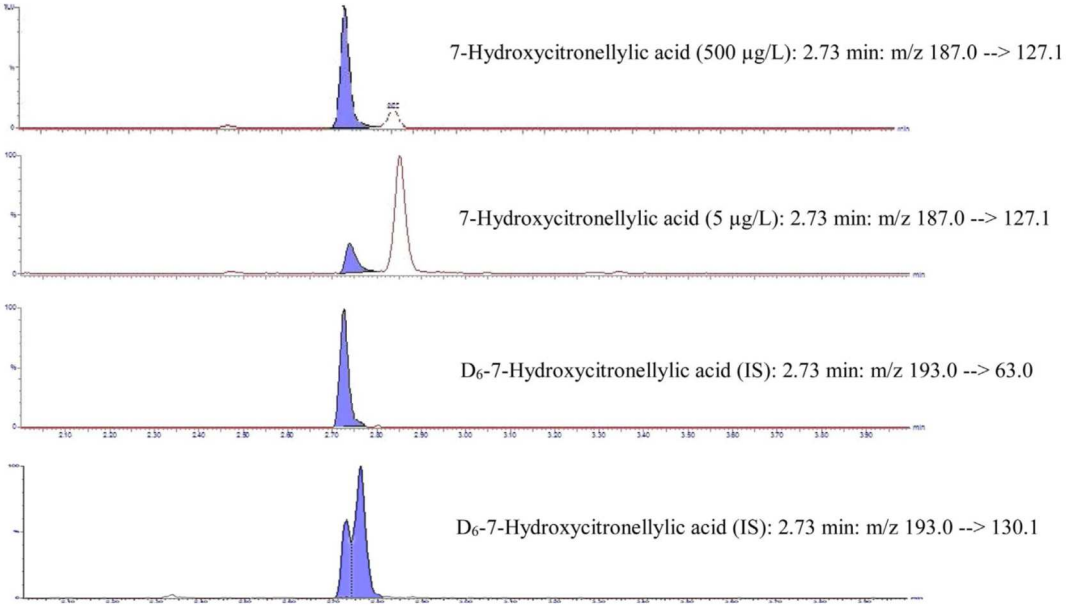

Fig. 2. MRM chromatograms of the major 7-hydroxycitronellal metabolite 7-HCA (**4**) and the corresponding internal standard (IS, D_6-7-HCA, **5**) in spiked urine samples (5 and 500 µg/L) and corresponding IS MRM transitions. IS was spiked to a level of 10 µg/L. The bottom chromatogram shows an interference of the mass transition 193.0 → 130.1 of the 7-HCA-IS (**5**) with the urinary matrix.

3. Results

3.1. Performance of the analytical method

7-Hydroxycitronellylic acid, both in unlabeled (**4**) and labeled form (**5**), can be ionized efficiently by electrospray ionization in negative mode (ESI^-). Applied mass transitions and MS/MS parameters are shown in Table 3. As the transition from *m/z* 187.0 to 127.1 was the most intense one and selective for 7-HCA (**4**), we chose this transition as quantifier. MRM chromatograms for 7-HCA (**4**) and the corresponding IS D_6-7-HCA (**5**) spiked to urine are shown in Fig. 2. The quantifier mass transition of the analyte (*m/z* 187.0–127.1) does not show any interferences with other urinary constituents. However, for the internal standard (IS, D_6-7-HCA, **5**), the corresponding transition from *m/z* 193.0–130.1 showed an interference with an unknown compound which could not be separated from the IS peak with the current method (Fig. 2, bottom). Consequently, we chose the transition *m/z* 193.0–63.0 (which is the analogue to the qualifier mass transition of the analyte) for the quantification of the IS. This mass transition was found to be highly selective and sensitive enough for the quantification of 10 µg/L IS.

In the pilot study, 7-HC (**1**) was applied to the skin of a single volunteer and all urine fractions of the following 48 h were collected in order to identify suitable biomarkers of exposure. The samples were prepared following a preliminary version of the analytical method including an additional derivatization step with 3-NPA after the LLE. As we also expected to find 7-hydroxycitronellol (7-HCO, **2**), a derivatization of the primary hydroxyl group was necessary in order to improve sensitivity, because 7-HCO (**2**) was found to be barely ionized in negative ESI mode. 3-NPA was chosen due to its selective reaction with primary and secondary alcohols to form a derivative which is permanently negatively charged at physiological pH [13]. The selectivity of the reaction in the presence of a tertiary alcohol was proven by means of MS experiments both for 7-HCO (**2**) and 7-HCA (**4**) (data not shown). The negatively charged carboxylic acid obtained from the derivatization step was ionized efficiently in ESI^-, allowing the simultaneous determination of 7-HCA (**4**) and 7-HCO (**2**).

Urine samples from five subjects, when analyzed with and without prior enzymatic hydrolysis revealed varying amounts of 7-HCA (**4**) in the unconjugated form (range: 11–69%). This implies that a substantial part of the total amount (unconjugated + conjugated) of 7-HCA (**4**) is excreted in conjugated form, for example as glucuronide or sulfate. Enzymatic hydrolysis of the conjugates prior to LLE is therefore regarded as a necessary step of the sample work-up procedure for quantification of urinary 7-HCA (**4**) excretion.

The performance of both enzyme mixtures from *Helix pomatia* (see Experimental section) was tested by incubating a urine sample over 0, 1, 2, 3, 4, 5, 6, 18 and 24 h. The amount of measurable (unconjugated) 7-HCA (**4**) was observed to not further increase after 2 h, suggesting complete deconjugation after this time period. An incubation time of 3 h was, therefore, considered to be sufficient to ensure complete hydrolysis. Enzymatic hydrolysis with an acetate buffer pH 6.4 and ß-glucuronidase (250,000 units/mL) from *E. coli* lead to slightly less 'noise' in the chromatograms, probably due to less cleavage of molecules with sulfate residues. However, the enzyme mixture from *Helix pomatia* resulted in satisfying results and was therefore, used in the final sample preparation.

Necessity and yield of the LLE were tested using dichloromethane, hexane, heptane and toluene as solvents, with dichloromethane leading to the best signal to noise ratios for 7-HCA (**4**). Chromatographic separation was tested with two different column materials (C18 and C8) comprising the same dimensions regarding particle size, length and diameter. The C18 column was chosen for final method development because of better peak symmetry and resolution as compared to the C8 material.

Relevant fragments generated from 7-HCA (**4**) are shown in Fig. 3. We hypothesize that, as a first step, the carboxylic group is eliminated as carbon dioxide. The resulting fragment (m/z = 143) was observed in the mass spectrum but intensity was too low for quantification or qualification purposes. The most frequent fragment used for quantification with m/z = 127 occurs due to additional elimination of methane. The mass transition resulting from elimination of the hydroxyl

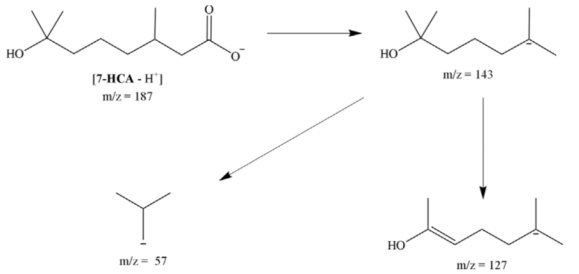

Fig. 3. Mass fragmentation pattern for 7-HCA (**4**) in ESI⁻ mode (for explanation, see text).

group and yielding a C_4 residue (*m/z* 57) was applied as qualifier. Mass transitions for the deuterated IS (*m/z* 193 → 130 and 193 → 63) are in accordance with the mass transitions for quantification (*m/z* 187 → 127) and qualification (*m/z* 187 → 57) of the analyte described above.

In the pilot experiment with dermal application, concentrations of 7-HCO (**2**) were found to be about 1000 times lower compared to 7-HCA (**4**) and, therefore, 7-HCA (**4**) was identified as the only suitable biomarker of exposure to 7-HC (**1**). The alcohol (7-HCO, **2**) was not considered in the final analytical method, which allowed us to omit the derivatization step from the final method.

The method performance data are shown in Table 2. *Intra*-day precision was evaluated by relative standard deviations (CVs), which were below 5% for all three concentrations tested. The CVs for the *inter*-day precision were also below 5% for high and medium and below 10% for low concentrations. The acceptance level was set to 15% CV according to FDA guidelines on bioanalytical method validation [11] and was adhered for every level tested. *Intra*-day accuracy rates were found to be between 85 and 115%. Recovery values indicating losses during sample cleanup were in an acceptable range of 75–100%. The LOQ for 7-HCA (**4**) amounted to 0.5 µg/L and can be rated as sufficiently low for HBM purposes, since all urine samples analyzed up to now (in total, 50 from the general population) contained 7-HCA (**4**) levels above the LOQ. The LOD calculated from the LOQ amounted to 0.17 µg/L. The calibration was found to be linear in the range of 0.5–2000 µg/L. Higher concentrated samples can be diluted up to six-fold with water prior to sample preparation yielding accurate results. We observed a rather strong signal suppression by urine matrix yielding a decrease by 79 and 76% at high and low concentrations, respectively, compared to water. Still, method application to urine samples from the general population proved sufficient sensitivity of the method. The washing procedure of the UPLC–MS/MS device was optimized in a way that no significant carryover effects were observed even after five injections of high levels of the analyte. 7-HCA (**4**) and its internal standard (D_6-7-HCA, **5**) were found to be stable in urine at room temperature (22 °C) for 24 h and at −20 °C for at least five weeks. Sample extracts ready for injection can be stored at 10 °C for at least two weeks. Long-term stability studies over longer storage periods are currently in progress.

Table 2
Method performance data for the quantification of 7-hydroxycitronellylic acid (7-HCA, **4**) in urine.

LOD (µg/L)	~0.17
LOQ (µg/L)	0.5
Calibration range (µg/L)	0.5–2000
Precision (*intra*-day, N = 5), µg/L (CV, %)	6.3 (4.5)
	73.8 (3.5)
	515.1 (2.8)
Precision (*inter*-day, N = 6), µg/L (CV, %)	5.9 (9.6)
	71.8 (3.8)
	503.7 (3.7)
Accuracy (intra-day, N = 5), µg/L (%)	1.0 (97.8)
	5.0 (106.0)
	500.0 (102.9)
Recovery (indicates losses during sample work-up), µg/L (%)	1.0 (95.5)
	5.0 (88.2)
	500.0 (76.7)
Matrix effects (deviation from 100%), µg/L (%)	2.0 (78.8)
	40.0 (76.2)
	IS, 10.0 (77.0)
Carryover effects	none

Table 3
7-Hydroxycitronellylic acid (7-HCA, **4**) levels in the spot urine samples collected from 40 volunteers from the general population.

	µg/L	µg/g creatinine
Mean ± SD	33.6 ± 96.2	34.4 ± 85.2
Median	14.2	15.1
min–max	3.1–627.0	4.9–557.0
< LOQ, N (%)	0 (0)	0 (0)

3.2. Urinary excretion of 7-hydroxycitronellylic acid in a small group of the general population

Forty spot urine samples collected from volunteers from the general population in Germany (see the experimental section) were analyzed with the validated analytical method. The subjects were not intentionally exposed to 7-HC (**1**) containing consumer products but were advised to behave as usual. Results are summarized in Table 3 and Fig. 4.

Urinary levels of 7-HCA (**4**) are shown both as concentrations (µg/L) and as normalized values to creatinine (µg/g creatinine) in order to consider the variability in the dilution of the urine samples. 7-HCA (**4**) levels were above LOQ in all samples. Levels of the metabolite ranged from 5–557 µg/g creatinine with a median of 15 µg/g creatinine. All study samples showed levels up to approx. 75 µg/g creatinine except for one sample with an extremely high value of 557 µg/g creatinine. Minimum level of 7-HCA (**4**) was 4.85 µg/g creatinine corresponding to a concentration of 3.05 µg/L, which is six times above the LOQ. 7-HCA (**4**) levels in different subgroups were compared using the Mann–Whitney *U* test. Significantly higher levels of 7-HCA (**4**) were obtained in females compared to males. No difference in 7-HCA (**4**) levels were found between younger (age 18–30) and older (age 31–83) subjects. The times of collection were grouped into morning to noon (6 am–12 am) and noon to about midnight (12 am–1 am). No statistical significant differences due to sample collection time were observed.

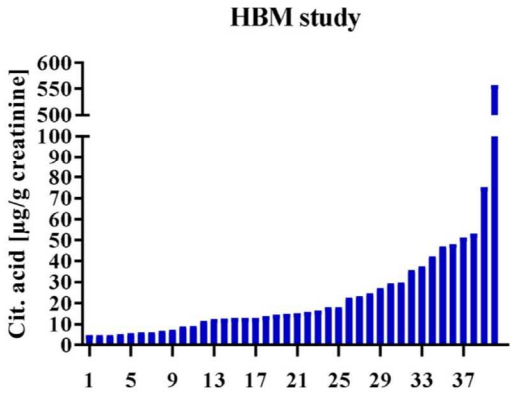

Fig. 4. Urinary excretion of the 7-hydroxycitronellal major metabolite 7-HCA (**4**) in 40 volunteers from the general population arranged from low to high 7-HCA (**4**) levels.

4. Discussion

7-Hydroxycitronellal (**1**) is a synthetic fragrance added to many cosmetics and hygiene products. It is also used as flavoring ingredient in food. Several studies have reported 7-HC (**1**) as contact allergen [2,14,15]. As a consequence, lower permissible concentration limits of 7-HC (**1**) were set by the International Fragrance Association (IFRA) in 2013 [3]. However, more profound data regarding the metabolism and degree of exposure to 7-HC (**1**) in humans is needed to allow a solid risk assessment. Considering the widespread use of this chemical, an HBM study with a well suited analytical method would be the most appropriate approach to assess the exposure in the general population.

7-HC (**1**) is part of the cooperation project between the German BMUB and the German VCI. This joint project between the German government and the German chemical industry aims to develop suitable HBM methods and investigate the toxicokinetics in humans, which is a prerequisite for establishing valid HBM methods. The methods should preferably be applicable to human urine samples, as urine is a non-invasive biological matrix with usually no limitations in terms of sample volume. Here, we report on the method development and validation of an HBM method for the fragrance 7-HC (**1**) together with some preliminary applications to human urine samples. The results of a metabolism study in humans, which we have also conducted, will be published in a separate publication (currently in preparation). To date, no analytical method for the determination of 7-HC metabolites has been published. For the determination of unchanged fragrances, including 7-HC (**1**) in consumer products, GC–MS methods [16–18] and HPLC methods with diode array detection (DAD) [19] were applied. As the excreted 7-HC metabolites are expected to be more polar than the unchanged fragrance, we decided to develop an UPLC–MS/MS method for the analysis of the major 7-HC metabolites in urine. The metabolism of 7-HC (**1**) has not been investigated in humans, yet. Reduction as well as oxidation of the aldehyde moiety to yield the corresponding alcohol 7-hydroxycitronellol (7-HCO, **2**) and the carboxylic acid 7-hydroxycitronellylic acid (7-HCA, **4**) (cf. Fig. 1) were postulated as these two metabolites were observed in *in vivo* experiments with rabbits after oral application of 7-HC (**1**) [8]. Both metabolites are likely to be conjugated by sulfation or glucuronidation at their carboxylic or alcoholic groups. Our aim was to develop a sensitive method for both analytes in their free form. As first experiments showed partial excretion of conjugated 7-HCA (**4**), we included an enzymatic hydrolysis prior to the sample work-up procedure in order to quantify the total urinary 7-HCA (**4**).

Both 7-HCO (**2**) and 7-HCA (**4**) were successfully detected in sub µg/L levels. The acid (**4**) was identified to be the major metabolite, yielding approximately 1000-fold higher concentrations in urine than the alcohol (**2**) after a controlled dermal exposure to 7-HC (**1**) in a volunteer. The potential occurrence of two cyclic metabolites of 7-HC (**1**) was also investigated (more details will be reported in the publication currently in preparation). As we were not able to detect further possible metabolites, we decided to optimize the method with respect to the quantification of 7-HCA (**4**). Thus, the derivatization step with 3-NPA, which was initially introduced for the efficient ionization of 7-HCO (**2**) by means of ESI^-, was omitted, which accelerated the sample preparation. 7-HCA (**4**) eluted already after 2.7 min with a total analytical run time of seven minutes allowing a high analytical throughput by means of UPLC–MS/MS. The relatively long post-run time after elution of the analyte was necessary for complete re-equilibration of the chromatographic column prior to the next injection.

Method performance for the sensitive quantification of 7-HCA (**4**) for HBM was proven during the method validation in terms of sensitivity (LOD and LOQ), precision (*inter*-day and *intra*-day), accuracy, and linear range. In addition, no background signals or carryover effects were observed due to the extensive washing of the column during gradient elution. A limitation is the fact that there is an interference from urinary matrix of the analyte-analogue mass transition of the IS (*m/z* 193.0–130.1). As a substitute, the analyte qualifier mass transition (*m/z* 193.0–63.0) was applied.

The validated analytical method was applied to 40 spot urine samples collected by volunteers from the general population (23 males, 17 females, age between 18 and 83 years). The means, standard deviations, medians, and ranges of both urinary concentrations and creatinine-normalized levels are summarized in Table 3. Urinary levels of 7-HCA (**4**) were significantly higher in females compared to males. A reasonable explanation might be that women probably tend to use more hygiene and cosmetic products compared to men. No association with subjects' age and time of sample collection was found, indicating that 7-HC (**1**)-containing consumer products are widely used in all age groups with about equal distribution over the day. The latter conclusion (equal distribution of use over the day) is deduced form the excretion half-life of about 3–5 h after dermal application (publication currently in preparation). The latter finding was in some contrast to our expectation, according to which there should be more frequent use of 7-HC (**1**)-containing consumer products in the morning. However, the number of urine samples analyzed up to now is certainly too low for any solid conclusions in this respect. All samples showed 7-HCA (**4**) levels above the LOQ with a mean concentration of 34.4 µg/g creatinine (33.6 µg/L), indicating that exposure to 7-HC (**1**) is common in the population in Germany. Yet, this has to be confirmed in a larger HBM study.

In conclusion, we have developed and validated a UPLC–MS/MS method for the quantification of 7-HCA (**4**) in human urine as a biomarker of exposure to the widely used fragrance 7-HC (**1**). The method is characterized by its high precision, robustness, and accuracy. Furthermore, the method shows sufficient sensitivity and specificity for assessing the exposure to 7-HC (**1**) by HBM in the general population.

Compliance with ethical standards

Studies with human subjects were approved by the Ethic Committee of the Bayerische Landesärztekammer, Munich, Germany.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgments

The development of this method of analyzing 7-hydroxycitronellal metabolites in human urine (including the synthesis of reference compounds) was funded by the Chemie Wirtschaftsförderungsgesellschaft, Frankfurt/Main, Germany. The method was developed during an ongoing 10-year project on human biomonitoring. The project is a cooperation agreed in 2010 between the Federal Ministry for the Environment, Nature Conservation, Building and Nuclear Safety (BMUB) and the Verband der chemischen Industrie e.V. (German Chemical Industry Association (VCI)); it is administered by the Federal Environment Agency (UBA). Experts from government authorities, industry, and science accompany the project in selecting substances and developing methods.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jchromb.2017.10.040.

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3.2.3 Contributions to “Human metabolism and excretion kinetics of the fragrance 7-hydroxycitronellal after a single oral or dermal dosage”

The ethical approval for the metabolism study was obtained by Prof. Dr. Gerhard Scherer. The metabolism study was implemented by Prof. Dr. Gerhard Scherer, Dusan Krnac and me, Dr. Oliver Peschel led the medical surveillance. I analyzed all samples according to the method described for the determination of 7-hydroxycitronellylic acid and evaluated all toxicokinetic parameters with the support of Prof. Dr. Gerhard Scherer. The manuscript was prepared by Prof. Dr. Gerhard Scherer, Dr. Max Scherer, Dr. Nikola Plum, Dr. Edgar Leibold (BASF), and me.

Own contribution:

Writing application for the ethical approval	0 %	
Implementation of the metabolism study	20 %	
Analysis of study samples	100 %	
Evaluation of toxicokinetic parameters	90 %	
Submission and revision of publication 2	80 %	

3.2.4 Original article: "Human metabolism and excretion kinetics of the fragrance 7-hydroxycitronellal after a single oral or dermal dosage"

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Human metabolism and excretion kinetics of the fragrance 7-hydroxycitronellal after a single oral or dermal dosage

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ABSTRACT

7-Hydroxy-3,7-dimethyl-1-octanal, also known as 7-hydroxycitronellal (7-HC, CAS No. 107-75-5) is a synthetic fragrance widely used in cosmetic and hygiene products. Due to its large scope, 7-HC was selected for the development of a biomonitoring method suitable for the general population within the frame of the cooperation project between the German Federal Ministry for the Environment (BMUB) and the German Chemical Industry Association (VCI). In a human study with 5 healthy subjects who received single dermal and oral doses 7-HC, suitable metabolites and their urinary excretion kinetics was investigated. Two metabolites of 7-hydroxycitronellal were identified in urinary fractions after dermal and oral dosing: The alcohol 7-hydroxycitronellol (7-HCO) and the corresponding acid 7-hydroxycitronellylic acid (7-HCA). Only 7-HCA proved to be a suitable biomarker of exposure to 7-HC, since 7-HCO was quantifiable in only a minority of urine samples collected from the general population. Quantification of 7-HCA was conducted by means of a newly developed UPLC–MS/MS (ultra-high pressure liquid chromatography combined with tandem mass spectrometry) method. Peak excretion of 7-HCA occurred between 3 and 5 h after oral application and about 10 h after dermal administration. Due to the limited skin absorption of 7-HC, 7-HCA concentrations after dermal application were much lower than levels after oral application. After 24 h, about 9% and 50% of the dermally and orally applied dose, respectively, were excreted as 7- HCA. With the conversion factors derived from the controlled human study, we estimated median exposure doses in a group of 40 human volunteers from the general population of approximately 93 µg 7-HC per day. In conclusion, the 7-HC metabolite 7-HCA in urine is a suitable biomarker of exposure and can be applied for biomonitoring of the general population.

1. Introduction

7-Hydroxy-3,7-dimethyl-1-octanal, also known as 7-hydroxycitronellal (7-HC, CAS No. 107-75-5, chemical structure **1**, see Fig. 1) is a synthetic fragrance with a smell of lilac, lily and lily of the valley. It is widely used in cosmetic and hygiene products like deodorants, perfumes, shampoos and creams. It is also approved as flavoring in foods (Bundesministerium der Justiz und für Verbraucherschutz, 1981). 7-HC (**1**) is a chiral molecule but it is generally deployed as the racemate (BASF SE, personal communication, 2017). Several market surveys on the presence of fragrance substances reported between 10 and 50% occurrence of 7-HC (**1**) in cosmetics and detergents (Buckley, 2007; Rastogi et al., 1998; Scientific Commitee on Consumer Safety, 2012). According to the SCCS, typical application levels are between 0.015-0.478% in perfumes (Scientific Commitee on Consumer Safety (SCCS), 2012). Topical toxicity leading to skin irritation and skin sensitization was found (European Chemicals Agency, 2017) and, therefore, the International Fragrance Association (IFRA) restricted the levels in finished products to 0.1-3.6% (International Fragrance Association, 2017). 7-HC (**1**) is listed as one of 26 contact allergens used as fragrance ingredients, which have to be declared on cosmetic products (Heisterberg et al., 2011). No acute toxicological effects after dermal or oral administration in rabbits or rats were observed (European Chemicals Agency, 2017). 7-HC was not found to be genotoxic in a variety of *in vitro* and *in vivo* tests (European Chemicals Agency, 2017).

Metabolism and toxicokinetics of 7-HC (**1**) in humans have not yet been explored. Studies in rabbits revealed that 7-HC is converted to two primary metabolites: reduction to the alcohol 7-hydroxycitronellol (7-HCO, **2**) and oxidation to the carboxylic acid 7-hydroxycitronellylic acid (7-HCA, **3**) (Ishida et al., 1989) (Fig. 1). Those metabolites which

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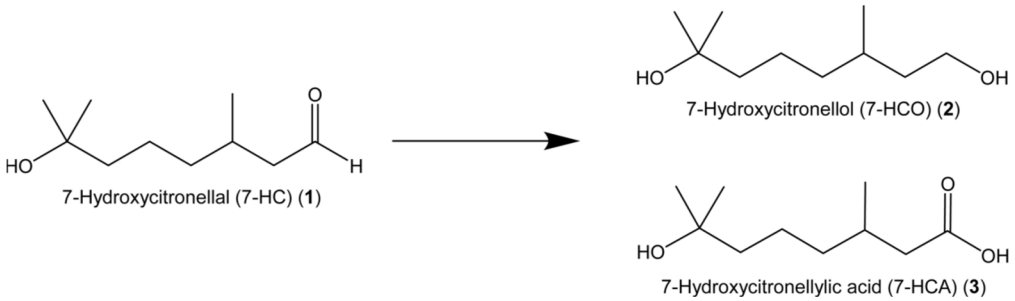


Fig. 1. Hypothetical pathway for the 7-hydroxycitronellal (7-HC, **1**) metabolism in humans. 7-Hydroxycitronellol (7-HCO, **2**) and 7-hydroxycitronellylic acid (7-HCA, **3**) were reported earlier in rabbits (Ishida et al., 1989) and could now be confirmed in humans. Conjugated metabolites are not shown in this figure.

contain hydroxy groups or carboxylic groups are likely to be conjugated predominately to glucuronic acid before excretion into urine. In this study, conjugated metabolites were hydrolyzed prior to their analysis.

The widespread and common use of cosmetics and hygiene products imply that the general population is exposed to this chemical. The exposure can most suitably be assessed by human biomonitoring (HBM) which is an integral measure of all exposure sources and routes of uptake. 7-HC (**1**) was selected as a chemical of interest for the 10-year HBM project, which is a cooperation between the German Federal Ministry for the Environment, Nature Conservation, Building and Nuclear Safety (BMUB) and the German Chemical Industry Association (VCI). Within the scope of this cooperation, we developed and validated a suitable HBM method for assessing quantitatively the exposure to 7-HC (**1**) in the general population by determining specific 7-HC (**1**) metabolites in urine (Stoeckelhuber et al., 2017). The validated method was then applied to 40 urine samples from adult volunteers. In this paper, we report on a human metabolism study using defined dermal and oral administrations of 7-HC (**1**) to 5 subjects, in order to identify the major human metabolites, determine their urinary elimination kinetics and select the metabolites which are most suitable as biomarkers of exposure to 7-HC (**1**). In addition, urinary conversion factors (CF) are deduced from the urinary excretion of potential biomarkers after oral application of 7-HC (**1**), which allows the back-calculation of the virtual exposure doses of 7-HC of 40 volunteers from the general population hitherto investigated.

2. Materials and methods

2.1. Human study

The dermal and the oral dosage were conducted with 5 adult, healthy volunteers (3 males, 2 females, age between 33 and 70) at the Analytisch-Biologisches Forschungslabor (ABF) Munich, Germany. The study was performed in accordance with the ethical standards of the Declaration of Helsinki (World Medical Association, 2013) and was approved by the Ethics Commission of the Bavarian State chamber of Physicians (Reg. No.: 16002). The subjects gave their informed, written consent to their participation. Subjects' characteristics are shown in Table 1. For the dermal application, a commercially available sunscreen was spiked to a final concentration of 1% (m/m) 7-HC (**1**) and a defined amount depending on the body weight was applied to a chest surface area of about 200 cm^2. Subject 1 received a 10 times higher dermal dose than subjects 2–5 to capture also subordinate metabolites. The oral application was performed by administering a dose of 7-HC (**1**) standardized for the body weight (0.03 mg/kg body weight), dissolved in ~ 100 mL water. Immediately before both administrations the subjects collected a urine sample. Subjects provided between 13 and 22 urine voids during 48 h after dosing. Voids were completely collected in separate fractions with the time of voiding being free, however the subjects were asked to empty their bladder about every 2 h during the first 12 h after dosing. Exact times of 7-HC administration, urine voiding and urine volumes for all subjects were recorded. Urine samples were frozen (< −20 °C) immediately after collection until analysis. There was at least 1 week between the dermal and the oral administration to ensure that all 7-HC from the last administration was eliminated. None of the subjects showed any physiological reaction after either the oral or dermal application of 7-HC. Furthermore, no conditions such as diarrhea or vomiting occurred during the human study which could have influenced the resorption, metabolism or excretion of the test compound.

The derived conversion factors (CFs) were applied to spot urine samples of 40 health subjects (23 males, 17 females and ages 18–83). More details on these subjects are provided in our previous report on 7-HC (Stoeckelhuber et al., 2017).

2.2. Chemicals and reference compounds

7-HC (CAS No.107-75-5, **1**), (+)-*cis*-*p*-Menthane-3,8-diol (42822-86-6) and (−)-Isopulegol (104870-56-6, **5**) were purchased from Sigma-Aldrich (Taufkirchen, Germany). 7-HCO (107-74-4, **2**) was obtained from Pfaltz & Bauer (Waterbury, CT, USA). 7-HCA (**3**), deuterated 7-hydroxycitronellol (D_3-7-HCO) and deuterated 7-hydroxycitronellylic acid (D_6-7-HCA) were custom-synthesized by Vladimir Belov (Max-Planck-Institute Göttingen, Germany). (+)-*trans*-*p*-Menthane-3,8-diol (3564-98-5, **4**) was purchased from Chemos GmbH & Co. KG (Regenstauf, Germany).

3-Nitrophthalic acid anhydride (3-NPA), used for derivatization of 7-HCO and phosphoric acid 85% in water was supplied by Sigma-Aldrich (Taufkirchen, Germany). Water and dichloromethane were obtained from Promochem (Wesel, Germany). Acetic acid was

Table 1
Characteristics of the subjects and dosages of 7-hydroxycitronellal (7-HC, **1**) applied in the human study.

Subject	Age [Years]	Gender	Body weight [kg]	Oral 7-HC Dose [mg]	Urine fractions collected (oral)	Dermal 7-HC Dose [mg]	Urine fractions collected (dermal)
1	67	Male	87.5	2.63	15	40.40	13
2	70	Female	74.5	2.24	23	4.47	20
3	33	Male	77.0	2.31	19	4.62	16
4	33	Female	86.5	2.60	14	5.19	14
5	33	Male	62.0	1.86	22	3.72	18

purchased from Fluka (Deisenhofen, Germany). HPLC-grade water with 0.1% formic acid and acetonitrile with 0.1% formic acid and all other analytical grade organic solvents were from LGC Standards (Wesel, Germany). Other chemicals and reagents used were at least of analytical grade. ß-Glucuronidase/arylsulfatase (30 units/mL ß-glucuronidase and 60 units/mL arylsulfatase) enzyme mix from *Helix pomatia* were purchased from Merck-Millipore (Darmstadt, Germany) and from Roche (Mannheim, Germany) (4.5 and 14 Units/mL), respectively.

2.3. Analytical methods

The determination of urinary 7-HC metabolites by a newly developed UPLC–MS/MS method has been published elsewhere (Stoeckelhuber et al., 2017). Briefly, 1 mL of urine was spiked with 10 µL of the internal standard (IS) D_6-7-HCA (1 µg/mL). For enzymatic hydrolysis, 0.5 mL of acetate buffer (1 M, pH 5.1) and 10 µL of a β-glucuronidase/arylsulfatase mixture (4.5 and 14 units/mL) were added. The sample was incubated at 37 °C for 3 h. The mixture was subjected to liquid-liquid-extraction (LLE) with 1.5 mL dichloromethane and 50 µL phosphoric acid (4 M). The mixture was extracted on a roller mixer for 30 min, centrifuged at 1900 x g for 15 min. The organic phase was evaporated to dryness (SpeedVac, no heating). The residue was derivatized by adding 1 mL 3-NPA solution in ethyl acetate (100 µg/mL) and incubated for 30 min at 80 °C. The mixture was evaporated to dryness (SpeedVac, no heating) and re-dissolved in 100 µL methanol/water (1:1, v/v). The extract was transferred into an HPLC microvial. In the final analytical method, which comprises only the quantification of 7-HCA (**3**), the addition of the IS D_3-7-HCO and the derivatization step with 3-NPA were omitted.

For analysis, 5 µL of the extract was injected into a UPLC–MS/MS system (Acquity UPLC I-Class with a binary pump connected to a Xevo TQ-S triple quadrupole mass spectrometer, Waters, Eschborn, Germany). Chromatography was performed on a UPLC column (Acquity UPLC BEH C8 1.7 µm; 2.1 × 150 mm, Waters, Eschborn, Germany) at 40 °C column temperature with a flow rate of 0.5 mL/min and a gradient consisting of A (water + 0.1% formic acid) and B (acetonitrile + 0.1% formic acid) solutions. Negative electrospray ionization (ESI^-) was applied with a source temperature of 150 °C and a desolvation temperature of 600 °C. The MS/MS detector was run in the multiple reaction monitoring (MRM) mode. In the 174 urine samples collected in the human study described above, the major metabolite of 7-HC, 7-HCA (**3**), was quantified.

The analytical method containing the derivatization step to determine 7-HCO (**2**) was applied to all 13 samples of Subject 1 after dermal application and additionally to all 15 samples of Subject 1 after oral application.

To obtain the ratio between the unconjugated form of 7-HCA (**3**) and the total amount of 7-HCA (unconjugated + conjugated form), two high concentrated samples from each subject were prepared twice. The first preparation was done according to the regular sample preparation to detect the total amount of 7-HCA. In a second experiment sample preparation was conducted without adding the enzyme mixture and incubating to assess unconjugated 7-HCA (**3**) exclusively.

For the analysis of the two potential 7-HC metabolites (+)-*trans*-*p*-Menthane-3,8-diol (**4**) and (−)-isopulegol (**5**) the same sample work-up procedure was used, but chromatographic parameters have been changed. Ten µL of the extract was injected into an HPLC–MS/MS system consisting of an Agilent 1200 HPLC system (Agilent Technologies, Waldbronn, Germany) with a binary pump (G1312B), a column oven (G1316 B) and a degasser (G1379B) coupled with a mass spectrometer API 5000 from AB Sciex (Darmstadt, Deutschland). The instrument was equipped with a HTC PAL autosampler (CTC Analytics, Zwingen, Switzerland). As analytical column a Kinetex PFP (2.6 µm; 3.0 × 100 mm, Phenomenex, Aschaffenburg, Germany) was used at a column temperature of 30 °C and a flow rate of 0.5 mL/min. The solvent gradient consisted of A (water + 0.01% ammonium acetate) and B (acetonitrile) starting with 95% A and terminating with 5% A after a total run time of 16 min. Positive electrospray ionization (ESI^+) was applied with a source temperature of 250 °C. In a preliminary test, the two samples from every Subject after oral application with the highest levels of 7-HCA (**3**) were analyzed for (+)-*trans*-*p*-menthane-3,8-diol (**4**) and (−)-isopulegol (**5**). Subsequently all urine samples collected by Subjects 3 and 5 after oral application (in total, 44 samples) were analyzed with this method, because they showed highest 7-HCA (**3**) levels from all subjects in the preliminary test.

2.4. Data evaluation

The toxicokinetic variables for the urinary excretion of the 7-HC metabolite was evaluated individually for each subject. Where appropriate, means, standard deviations (SD) and medians were calculated. The amount of metabolite excreted after 3, 6, 12 and 24 h were obtained by linear interpolation between the amounts excreted at the time points directly before and after the time point of interest. Urinary excretion of the metabolite was assumed to be complete after 48 h. The time of oral 7-HC (**1**) administration (which is virtually identical with the time of voiding the first urine sample before administration of 7-HC) was set to zero and all following urine voids were related to this time point. The urinary elimination constant (k_{el}) and half-life ($t_{½}$) were determined with the 'sigma-minus' method (Klotz, 1984), with the total amount excreted ($A_e(\infty)$) being the amount excreted within 48 h. The conversion factor (CF) was defined as the ratio between the oral dose of 7-HC (in nmol) divided by the amount of the metabolite 7-HCA (**3**) excreted after 24 h ($A_{7\text{-HCA-24h}}$ in nmol): CF = oral dose/$A_{7\text{-HCA-24h}}$.

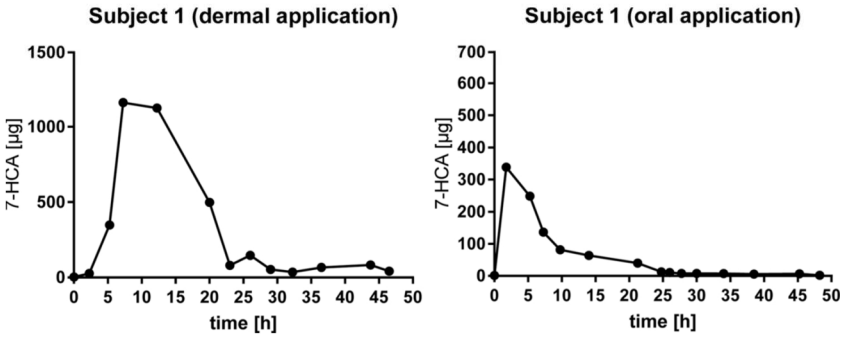


Fig. 2. Time courses of the urinary excretion of 7-hydroxycitronellylic acid (7-HCA, **3**) after dermal (40.4 mg) and oral (2.63 mg) administration of the fragrance 7-hydroxycitronellal (7-HC, **1**) to Subject 1.

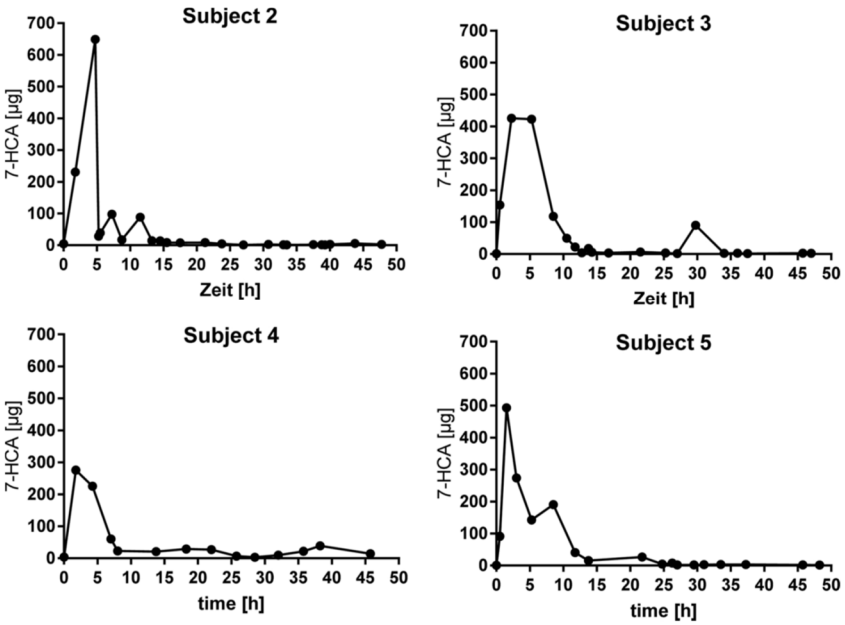


Fig. 3. Time courses of the urinary excretion of 7-hydroxycitronellylic acid (7-HCA, **3**) after oral administration of 7-hydroxycitronellal (7-HC, **1**) for Subjects 2–5.

3. Results

3.1. Toxicokinetics of 7-hydroxycitronellal after dermal and oral administration

A comparison between the time courses of urinary excretion of the major 7-HC metabolite 7-HCA (**3**) after dermal and oral application for Subject 1 is shown in Fig. 2. The maximum (peak) amounts of 7-HCA were excreted about 10 and 3 h after the dermal and oral administration, respectively. After 24 h, more than 90% (dermal) and 94% (oral) of 7-HCA were excreted in the urine. Excretion of 7- HCA can be regarded as being virtually complete by 48 h after the oral uptake of the chemical. Subject 1 received a ten times higher dermal dose as compared to Subjects 2–5. The dermal study with Subject 1 was performed prior to the other human studies with 7-HC in order to identify also minor metabolites. For Subjects 2–5 the applied dermal dose proved to be too low for a solid evaluation of the urinary elimination kinetics. Time courses of urinary excretion of 7-HCA (**3**) after oral administration for Subjects 2–5 are shown in Fig. 3. Time profiles similar to Subject 1 were observed. Table 2 summarizes means, SDs, medians and min to max ranges for some toxicokinetic parameters of the five subjects after oral administration of the fragrance. On average, about 50% of the 7-HC dose is excreted as 7-HCA (**3**), which, therefore, can be regarded as the major metabolite. The average time for peak excretion (t_{max}) was found to be 2.7 h. On average, 94% of 7-HCA (**3**) were excreted within 24 h after the single oral 7-HC dose. The mean elimination half-life is calculated to be 3.3 h. A mean conversion factor (CF) of 2.24 was calculated as described in the section 'Data evaluation'.

7-HCA was excreted in unconjugated (free) form and as a conjugate, probably with glucuronic acid. Ratios of free to total (free + conjugated form) amounts of 7-HCA for each subject are shown in Table 3. The data in Table 3 were obtained from the urine samples with the highest concentration after oral administration of 7-HC (**1**), which have been analyzed for 7-HCA (**3**) with and without prior enzymatic hydrolysis. Occurrence of the unconjugated form seems to be highly variable in the subjects with ranges amounting to approximately 10–70%.

The extent of dermal resorption is shown in Table 4. Dermal resorption was calculated on the basis of urinary excretion of 7-HCA (**3**) after oral application assuming 100% oral resorption rate. Dermal resorption was found to range from 6 to 10% for Subjects 2–5. Subject 1, who received a 10 fold higher dermal dose, showed a resorption of 25%.

Table 2
Aggregated toxicokinetic results for 7-HCA (**3**) obtained from 5 subjects orally dosed with 7-hydroxycitronellal (7-HC, **1**).

	Mean ± SD Median, Min-Max
Amount excreted after 48 h (µmol)(= Ae(∞))	**6.62 ± 1.10** 7.16, 5.00–7.57
Percent of oral dose $P_{Mx}D$ (%)	**49.95 ± 11.20** 50.22, 36.98–65.86
t_{max} (h)	**2.70 ± 1.36** 1.75, 1.75–5.25
Excreted after 3 h (% of dose)	**51.75 ± 9.04** 51.34, 40.64–65.86
Excreted after 6 h (% of dose)	**75.31 ± 5.18** 77.16, 65.69–80.18
Excreted after 12 h (% of dose)	**89.07 ± 4.88** 89.38, 81.36–94.69
Excreted after 24 h (% of dose)	**94.36 ± 3.43** 94.59, 89.06–98.03
Elimination constant k_{el} (h^{-1})	**0.22 ± 0.05** 0.22, 0.16–0.28
Elimination half-life $t_{½}$ (h)	**3.28 ± 0.73** 3.10, 2.47–4.38
$A_{7\text{-HCA-24h}}$ (µmol)	**6.27 ± 1.17** 7.01, 4.45–7.42
Conversion factor, CF	**2.24 ± 0.55** 2.03, 1.54–2.90

7-HCO (**2**) was detectable in samples containing high amounts of 7-HCA (**3**), but 7-HCO (**2**) levels were about 1000 times lower compared to 7-HCA (**3**) levels. 7-HCO (**2**) was detectable in seven samples from Subject 1 after dermal application and in one sample from Subject 1

Table 3
Percentage of free 7-HCA (**3**) related to total (free + conjugated) amount of 7-HCA excreted in urine by 5 subjects. The data originate from the urine samples with the highest 7-HCA levels after oral dosage.

Subject	Free/total amount [%]
1	10.6
2	37.3
3	69.2
4	42.1
5	51.1
Average ± SD	42.1 ± 21.4
Min-Max	10.6–69.2
Median	42.1

Table 4
Estimation of the dermal resorption for 7-HC (**1**).

Applied dose [μmol]	Excreted amount of 7-HCA (**3**) [μmol]	Excretion rate of 7-HCA (**3**) after oral application [%]	Dermal resorption
234.5	21.292	37.1	24.5%
21.8	1.090	66.1	7.6%
26.8	1.031	57.6	6.7%
25.9	0.591	38.4	5.9%
30.1	1.565	50.2	10.4%

after oral application with concentrations in the range between 0.8–6.3 μg/L.

(+)-*trans*-p-Menthane-3,8-diol (**4**) was found in all samples from Subject 3 and 5 after oral application. Time courses of 7-HCA (**3**) and (+)-*trans-p*-menthane-3,8-diol (**4**) after oral administration of 7-HC (**1**) for Subject 5 are shown in Fig. 4. Levels of (+)-*trans-p*-menthane-3,8-diol (**4**) did not show an excretion time pattern similar to 7-HCA (**3**). The same samples from Subjects 3 and 5 were also analyzed for (−)-isopulegol (**5**), but (−)-isopulegol was below LOD in all samples.

3.2. Virtual exposure dose of 7-HC in subjects of the general population

The conversion factor (CF) calculated from the oral dosage study with 7-HC (**1**) (Table 2) was applied to 40 urine samples collected by subjects of the general population (Stoeckelhuber et al., 2017). Creatinine standardized urinary 7-HCA (**3**) levels were used for back-calculation of the uptake dose. For this purpose, daily creatinine excretion rates were assumed to amount to 1.2 for females and 1.5 g for males (Barr et al., 2005).

Table 5 summarizes the estimated daily uptake doses ('equivalent oral dose') of 7-HC (**1**) for the 40 subjects. On average, 236 nmol HCA/d were excreted by the 40 volunteers resulting in an equivalent oral dose of 529 nmol (= 93 μg) 7-HC (**1**) per day. The average urinary concentration in the 40 subjects was found to be about 30 μg/L, eliciting a signal/noise ration of approximately 60.

4. Discussion

We conducted a metabolism study of 7-HC (**1**), a widely used synthetic fragrance, deploying five subjects, who received one oral and one dermal dose of the chemical. It was ensured that there was a minimum interval of one week between the two administrations, in order to avoid carry-over effects from one application type to the other. The collected urine samples (in total, N = 174) were analyzed by applying a recently developed UPLC–MS/MS method (Stoeckelhuber et al., 2017). To the best of our knowledge, this is the first time that the 7-HC metabolism has been studied in humans after a defined administration of the chemical. The aim of this study was to identify the major metabolites of 7-HC as well as their amounts excreted and their urinary elimination kinetics. From the results of the oral study, a conversion factor (CF) was deduced, allowing the estimation of the daily uptake (exposure dose) of the chemical (Koch et al., 2007). Our results confirm the metabolism of 7-HC (Fig. 1) previously observed in rabbits (Ishida et al., 1989), showing that the aldehyde group of 7-HC (**1**) is either reduced to the alcohol 7-HCO (**2**) or oxidized to the carboxylic acid 7-HCA (**3**). In humans, we found 7-HCO (**2**) in concentrations, which were about 1000 times lower than those of 7-HCA (**3**). Unfortunately, no quantitative data for 7-HCO (**2**) and 7-HCA (**3**) were reported in the rabbit study (Ishida et al., 1989) so that the metabolic ratio (7-HCA/7-HCO) cannot be compared to that observed in humans. Thus 7-HCA (**3**) can be regarded as being by far the most important metabolite of 7-HC (**1**) in quantitative terms. We also screened for urinary excretion of the parent compound (7-HC) with a preliminary version of the analytical method and optimized MRM transitions for 7-HC, but no 7-HC (**1**) was detectable neither in 13 samples from Subject 1 after dermal application nor in eight samples from Subjects 2–5 after oral application of 7-HC.

Due to its very low excretion rates in urine, 7-HCO (**2**) is not a suitable biomarker of exposure to 7-HC (**1**) and it is expected that 7-HCO is below the limit of detection (LOD) in most samples from subjects of the general population. For this reason 7-HCO (**2**), was not considered in our recent method development and validation (Stoeckelhuber et al., 2017).

It is highly likely that the carboxylic group of 7-HCA (**3**) is, at least partly, conjugated with glucuronic or sulfonic acid prior to the excretion into urine. We found that 7-HCA is partly (10–70%) excreted in urine in its conjugated forms in all 5 subjects. The ratio between the conjugated and the free forms of the metabolite varied significantly between the subjects. This *inter*-individual variation has no impact on our data evaluation, since the preceding enzymatic hydrolysis in our analytical method ensures that only total (free + conjugated) 7-HCA is considered in our analysis.

We could recover approximately 50% of the oral dose excreted in urine via the major metabolite 7- HCA (**3**), but only around 9% of the dermal dose excreted from Subject 1. These results suggest that dermal resorption in humans is relatively low (about 6–25 %), which explains why dermal doses for subjects 2–5 were too low to observe any effects. However with the data available we can only guess that the resorption

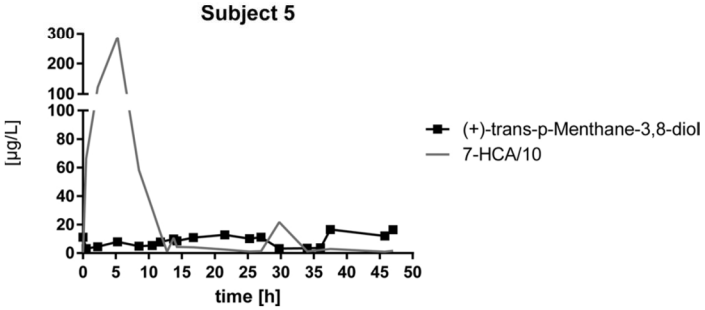
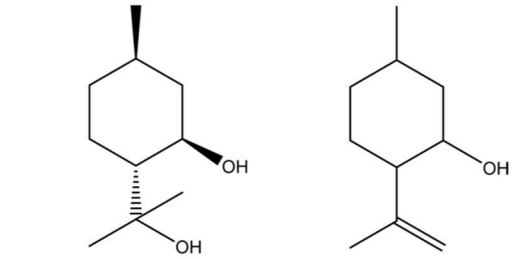


Fig. 4. Time courses of the urinary excretion of 7-hydroxycitronellylic acid (7-HCA, **3**) and (+)-*trans-p*-Menthane-3,8-diol (**4**) after oral administration of 7-hydroxycitronellal (7-HC, **1**) to Subject 5. For a clearer illustration 7-HCA concentrations are divided by 10.

Table 5
Amount of excreted 7-hydroxycitronellylic acid (7-HCA, **3**) and estimates of the absolute exposure dose to 7-hydroxycitronellal (7-HC, **1**) in 40 volunteers (23 males, 17 females) of the general population by applying the average conversion factor (CF) shown in Table 2. Daily urinary amounts excreted where calculated by assuming creatinine excretion rates of 1.5 and 1.2 g/d for males and females, respectively.

			7-Hydroxycitronellylic acid (7-HCA, **3**)	7-Hydroxycitronellal (7-HC, **1**)
Urinary excretion	nmol/g creatinine	Mean ± SD	184.1 ± 455.7	–
		Median (min-max)	80.7 (25.9–2978)	–
	nmol/d	Mean ± SD	236.1 ± 545.8	–
		Median (min-max)	103.7 (39.3–3574)	–
Equivalent oral dose	nmol/d	Mean ± SD	–	528.8 ± 1223
		Median (min-max)	–	232.2 (88.0–8005)
	µg/d	Mean ± SD	–	93.4 ± 215.9
		Median (min-max)	–	41.0 (15.5–1414)



(+)-trans-p-Menthane-3,8-diol (**4**) Isopulegol (**5**)

Fig. 5. Chemical structures and molecular weights (MW) of two potential cyclic metabolites of 7-hydroxycitronellal (7-HC, **1**).

might be dose dependent and that higher doses might be absorbed better from the skin.

We also examined whether 7-HC (**1**) is metabolized to two cyclic species shown in Fig. 5. For this purpose, a different LC–MS/MS was applied to a selection of urine samples collected in the human metabolism study. (+)-*trans-p*-Menthane-3,8-diol (**4**) is known as a metabolite of citronellal (Ishida et al., 1989) and menthol (Yamaguchi et al., 1994) and could be detected in the samples due to the widespread use of menthol in everyday life. No correlation between 7-HCA (**3**) excretion and (+)-*trans-p*-menthane-3,8-diol (**4**) levels was found. No excretion time pattern similar to that of 7-HCA (**3**) was observed for this metabolite. Therefore, (+)-*trans-p*-menthane-3,8-diol (**4**) cannot be seen as a metabolite of 7-HC (**1**). (−)-Isopulegol (**5**) is a metabolite of citronellal (Ishida et al., 1989) and also formed through the cyclisation of citronellal in chemical synthesis (Chuah et al., 2001). Its structure is similar to that of (+)-*trans-p*-menthane-3,8-diol (**4**) and other monoterpenes. (−)-Isopulegol (**5**) was below the LOD in all samples analyzed, thus providing no evidence that (−)-isopulegol (**5**) is a metabolite of 7-HC (**1**) in humans.

It can be assumed that 7-HC (**1**) could be formed due to the hydration of citronellal in its metabolism. No experimental evidence was found in the metabolism of bacteria (Joglekar and Dhavlikar, 1969) or rabbits (Ishida et al., 1989). Therefore, a formation of 7-HC (**1**) from citronellal appears to be unlikely, but cannot be completely excluded.

As a consequence, solely 7-HCA (**3**) qualifies as a suitable biomarker of exposure to 7-HC (**1**). It can be regarded as specific, since currently there is no evidence for any other source or precursor for 7-HCA (**3**).

Urinary excretion of 7-HCA (**3**) is relatively fast. 24 h after both administration forms around 90% of 7- HCA is excreted and urinary excretion is virtually complete after 48 h.

The CF calculated represents the ratio between the total dose of 7-HC (**1**) orally administered (D) and the amount of 7- HCA (**3**) excreted within 24 h ($CF = D/A_{7\text{-}HCA\_24h}$). The CF was applied to 40 spot urine samples collected from volunteers of the general population (23 males, 17 females, age between 18 and 83). Creatinine normalized 7-HCA levels for these 40 samples have been previously reported (Stoeckelhuber et al., 2017). Significant higher concentrations of 7-HCA were observed in females than in males. Age and time of the sample collection do not seem to have an influence on 7-HCA levels. The levels of 7-HCA (**3**) in 40 subjects of the general population and the calculated virtual exposure doses for 7-HC (**1**) show a high *inter*-individual variation (Table 5).

The back-calculated exposure doses (termed 'equivalent oral dose' in Table 5) are virtual oral uptake doses, while in everyday life, 7-HC (**1**) uptake predominately occurs via dermal absorption and possibly, to a smaller extent, also by inhalation. Results from the dermal administration study suggest that the dermal resorption in the range of 6–25% (Table 4).

5. Conclusion

7-HCO (**2**) and 7-HCA (**3**) were found to be urinary metabolites of the widely used fragrance 7-HC (**1**) in humans. Due to its approximately 1000 time higher levels in urine compared to 7-HCO (**2**) and other potential 7-HC metabolites, 7-HCA (**3**) qualified as a (quantitative) biomarker for assessing the everyday exposure to 7-HC in HBM studies. 7-HCA (**3**) is representing about 9 % of the dermal dose and 50% of the oral dose and can be regarded as a specific metabolite for 7-HC (**1**). Urinary excretion kinetics showed that the elimination is fast (elimination half-life: 3–4 h after oral administration). After dermal and oral administration, the peak excretion occurred around 10 and 3–5 h later, respectively. Back-calculation of the exposure dose in 40 subjects from the general population applying the CF resulted in a median daily dose of 93 µg 7-HC (**1**). With the recently developed analytical method for 7-HC metabolites in urine (Stoeckelhuber et al., 2017) and the toxicokinetic parameters determined in this study, the prerequisites for assessing the exposure to the widely used fragrance 7-HC (**1**) in a larger population study by HBM are available.

Conflict of interest

The authors in general declare that they have no conflict of interest.

One of the authors (E.L.) is employed by a manufacturer of 7-hydroxycitronellal, but the opinions expressed in the paper are those of the author and the chemical manufacturer had no role in the study design, data collection, analysis or interpretation of the study findings. All other authors declare no conflict of interest.

Acknowledgments

This study is part of an ongoing 10-year project on the advancement of human biomonitoring in Germany. The project is a cooperation agreed in 2010 between the Federal Ministry for the Environment, Nature Conservation, Building and Nuclear Safety (BMUB) and the Verband der chemischen Industrie e.V. (German Chemical Industry

Association – VCI); it is administered by the German Environment Agency (UBA). Within this cooperation project the analytical method development and the human metabolism study are financed by the Chemie Wirtschaftsförderungsgesellschaft, Frankfurt/Main, Germany. Experts from government authorities, industry, and science accompany and advise the project in selecting substances and developing methods.

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3.3 Human biomonitoring for Uvinul A plus®

Stoeckelhuber M, *Pluym N, Bracher F, Leibold E, Scherer G, Scherer. A validated UPLC-MS/MS method for the determination of urinary metabolites of Uvinul® A plus. Analytical and Bioanalytical Chemistry. 2019;411:8143-8152.*

Stoeckelhuber M, *Scherer M, Peschel O, Leibold E, Bracher F, Scherer G, Pluym N. Human metabolism and urinary excretion kinetics of the UV filter Uvinul A plus® after a single oral or dermal dosage. International Journal of Hygiene and Environmental Health. 2020;227*

Hexyl 2-[4-(diethylamino)-2-hydroxybenzoyl]benzoate (CAS number: 302776-68-7; DHHB) is a synthetic, organic UV filter available on the market under its trading name Uvinul A plus®. It is one of 27 UV filters approved by the European Commission allowing cosmetic products to contain a maximum concentration of 10 % (w/w) [63]. Furthermore, it is approved for use in Australia, New Zealand, Brazil, Japan and South Africa (concentrations up to 10 %) [64, 65]. Since DHHB possesses its maximum of absorption at 354 nm [64, 66], it is used for protection of the skin against UV-A radiation mainly in sunscreens, but also in other cosmetics like anti-aging products [47, 67], and over 1000 tons per year are produced within the EU [68].

From 2006 to 2009, a product survey for cosmetics marketed in Germany was conducted. Overall, 4447 cosmetic products were screened for the International Nomenclature of Cosmetic Ingredients (INCI) names of all UV filters approved within the EU. The products investigated can be categorized into seven groups: Sunscreens, creams, hair styling products, make-ups, nail polishes, cosmetics for the lips, and perfumes. DHHB was declared in 1.8 % (80/4447) of all products and in 2.6 % (12/462) of the sunscreens investigated. The highest percentage occurrence of DHHB could be observed in perfumes with 4.8 % (2/42), however, with a small number of samples. DHHB was not declared on any sample in the categories creams, make-ups and nail polishes [47]. In a shop survey conducted by the Danish Environmental Protection Agency, DHHB was identified in 18.2 % (53/291) by analyzing the list of ingredients of the cosmetic products, including 46 sunscreens [67]. Once applied, UV filters can

reach waters as a consequence of washing off from the skin [69]: DHHB has already been detected in coastal waters from beaches in Gran Canaria in concentrations up to 229 ng/L [70]. Until now, no DHHB could be detected in seafood commercialized in the European Union [71].

The median lethal dose after oral dosing of rats with Uvinul A plus® was found to be higher than 2000 mg/kg [72]. Although, cases of contact dermatitis could be observed [73], several studies in animals and a human repeated insult patch test with 10 % DHHB showed no skin-irritating or sensitizing effects [68]. While most of the UV filters possessing a benzophenone basic structure, like benzophenones-1-4, are known to act as endocrine disruptors primarily influencing the estrogenic and androgenic hormone system [48, 74-77], recent studies did not indicate that DHHB influences the hormone system [67].

Varying results concerning the dermal absorption of DHHB were obtained depending on the experimental design. In *in vitro* experiments with porcine skin [72, 78, 79] and human skin [80] conducted with Franz diffusion cells, no DHHB could be detected in the receptor fluid indicating that DHHB was not able to permeate through the skin. Another *in vitro* study using Franz diffusion cells and human skin calculated an absorption rate of 0.5 % [68]. No *in vivo* data are available, but in general, *in vivo* absorption rates can be assumed as lower as in *in vitro* experiments [81, 82].

As mentioned above, DHHB is used in many cosmetic products in comparably high concentrations and residuals have even been detected in coastal waters, but no reliable data concerning human exposure to this chemical are available. In order to assess the human exposure to DHHB, the compound was selected for the development of a HBM method within the German HBM Initiative including the elucidation of its human metabolism as well as the identification of suitable biomarkers of exposure.

Since the human metabolism of DHHB was unknown, a metabolism study with five participants was conducted and three major and four minor urinary metabolites of Uvinul A plus® have been identified (see Figure 6). The only metabolite of DHHB

previously known from *in vivo* experiments in rats was 2-[4-(diethylamino)-2-hydroxybenzoyl]benzoic acid (DHB), which is formed by simple hydrolysis of the *n*-hexyl ester moiety [68]. 2-[4-(Amino)-2-hydroxybenzoyl]benzoic acid (AHB) and 2-[4-(ethylamino)-2-hydroxybenzoyl]benzoic acid (EHB) could be identified as urinary metabolites of DHHB for the first time. Furthermore, four minor, additionally hydroxylated species could be determined by means of QTrap® experiments. Indeed, no chemical reference standards for the additionally hydroxylated metabolites were available and urinary levels were assumed to be approximately 100-fold lower in comparison to urinary levels of the major metabolites. The precise location of the additional hydroxyl moieties could not be clarified, but the fragmentation patterns observed in QTrap® experiments suggest that these moieties are exclusively located in the aniline ring. Unchanged DHHB could not be determined in any sample. Thus, the four minor metabolites were rated as unsuitable for a HBM of DHHB, because of the low amounts detected in the samples of occupational exposed participants and the final HBM method was optimized for the three major metabolites AHB, EHB and DHB.

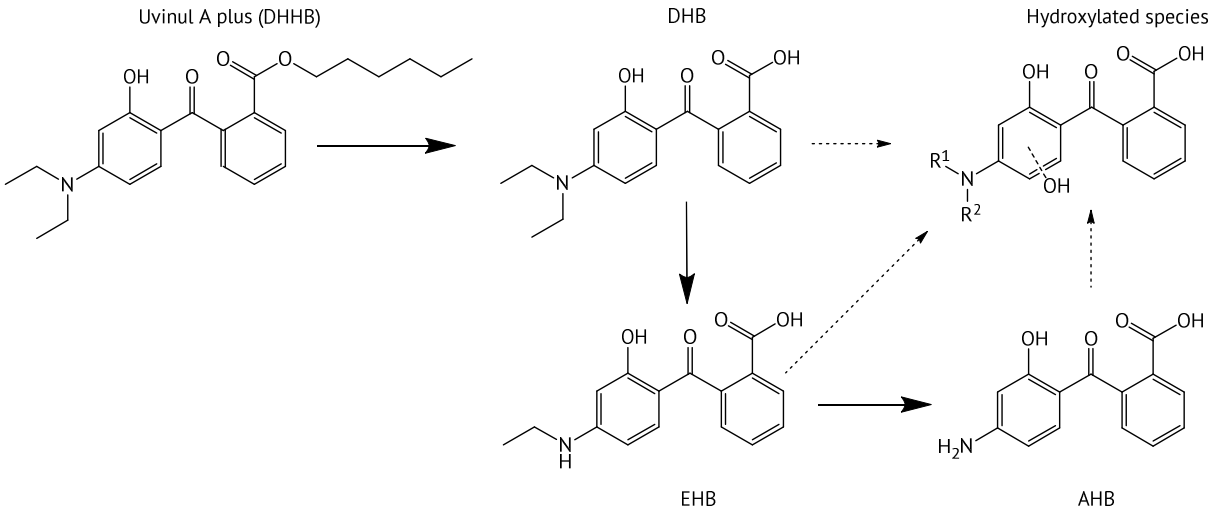

Figure 6: Proposed pathways for the Uvinul A plus® metabolism in humans (R^1 = H or ethyl, R^2 = H or ethyl)

All three major metabolites were simultaneously determined by using authentic stable isotope-labelled standards (D_4-AHB, D_5-EHB and D_{10}-DHB). After conjugates of the metabolites were enzymatically hydrolysed with β-glucuronidase from *E. coli*, the

analytes were extracted *via* LLE with ethyl acetate, the organic layer was evaporated to dryness and reconstituted in methanol. The extracts were injected into a UPLC-MS-MS system and AHB, EHB and DHB were determined. The final HBM method was validated according to guidelines issued by the DFG and the FDA [52].

All samples derived from the metabolism study and 58 urine samples collected from adult volunteers from the general population were analysed according to the validated method. Similar excretion patterns for AHB, EHB and DHB were obtained with a maximum excretion after about four to five hours after oral dosing. After 72 h, 54 % of the oral dose given was excreted as EHB (33 %), AHB (16 %) and DHB (5 %). Urinary excretion for all three major metabolites after dermal dosing began after approximately three hours and wave-like excretion patterns with maximum excretion rates at 24, 43 and 56 hours could be observed. Overall, less than 0.02 % of the dermal dose administered could be determined, resulting in a calculated skin absorption between 0.003 and 0.02 % for the five subjects. At least one of the three major metabolites of DHHB could be determined in 35 % of the 58 samples from non-occupationally exposed participants and an average exposure dose for DHHB between 8.1 and 9.3 µg/d was calculated depending on the conversion factor used for calculation.

3.4 Author contributions

The research data obtained within this study were summarized in two publications. The first article comprises the development and validation of the HBM method and was published in Analytical and Bioanalytical Chemistry. The second article was published in the International Journal of Hygiene and Environmental Health and deals with the application of the HBM method to the samples of the metabolism study and the calculation of toxicokinetic parameters.

3.4.1 Contributions to “A validated UPLC-MS/MS method for the determination of urinary metabolites of Uvinul® A plus”

The development as well as the validation of the human biomonitoring method was conducted by me. I carried out the synthesis of AHB under the supervision of Prof. Dr. Franz Bracher. David Schmidl (BSc), who worked as a trainee under my supervision and I collected the samples from the general population, and I analyzed all study samples. The manuscript was prepared by Prof. Dr. Gerhard Scherer, Prof. Dr. Franz Bracher, Dr. Max Scherer, Dr. Nikola Plum, Dr. Edgar Leibold (BASF) and me.

Own contribution:

Development of the human biomonitoring method	100 %	
Validation of the human biomonitoring method	100 %	
Sample collection from the general population	100 %	
Analysis of study samples	100 %	
Submission and revision of publication 1	70 %	

3.4.2 Original Article: "A validated UPLC-MS/MS method for the determination of urinary metabolites of Uvinul® A plus"

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https://doi.org/10.1007/s00216-019-02201-6

RESEARCH PAPER

A validated UPLC-MS/MS method for the determination of urinary metabolites of Uvinul® A plus

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Abstract
Uvinul® A plus (DHHB) is a synthetic benzophenone derivative mainly used in sunscreens, and also in other skin care products. The compound is authorized by the EU as UV filter and a maximum concentration of 10% in consumer products is permitted. Despite its high production volume and usage in consumer products,to date, no information about the systemic exposure to Uvinul® A plus in humans is available. Therefore, we developed a human biomonitoring method which allows the simultaneous determination of three major metabolites of Uvinul® A plus in human urine samples. Furthermore, three minor metabolites of Uvinul® A plus were identified by ion trap experiments. Urine samples were enzymatically hydrolyzed, extracted via liquid-liquid extraction with ethyl acetate, and analyzed by means of UPLC-MS/MS. The final method was validated according to FDA guidelines and applied to 58 urine samples retrieved from the general German population. The three major and specific metabolites of Uvinul® A plus were found in about 36% of the samples, proving the suitability of the method for future human biomonitoring studies.

Keywords Uvinul® A plus · Urine · UPLC-MS/MS · Human biomonitoring (HBM)

Introduction

Hexyl 2-[4-(diethylamino)-2-hydroxybenzoyl]benzoate (DHHB; CAS No. 302776-68-7), better known under the trading name Uvinul® A plus, is a synthetic, organic UV filter possessing its maximum of absorption at 354 nm [1]. It is mainly used in sunscreen formulations as protection against UV-A radiation, but it is also present in other skin care products [2, 3]. Since 2005 DHHB is approved as UV filter by the European Commission with a maximum concentration in consumer products of 10% [4]. The compound is registered under the EC number 443-860-6 at the European Chemicals Agency and is described to be manufactured in 100–1000 tons per year within the European Economic Area [5].

The high production volume of the substance as well as the long skin contact time after application of cosmetic products along with the relatively high concentrations of DHHB in the skin care products led to the assumption that the general population might be exposed significantly to this compound. Therefore, Uvinul® A plus was selected as a chemical of interest as part of a 10-year human biomonitoring (HBM) project conducted in cooperation with the German Federal Ministry of the Environment, Nature Conservation and Nuclear Safety (BMU) and the German Chemical Industry Association (VCI). This joint cooperation aims to establish new HBM methods for 50 chemicals with an assumed wide range of exposure to the general population [6].

A method for the simultaneous determination of three major and specific metabolites of DHHB (Fig. 1) was developed and validated: 2-(4-amino-2-hydroxybenzoyl)benzoic acid (AHB; CAS No.: 67414-64-6), 2-(4-ethylamino-2-hydroxybenzoyl)benzoic acid (EHB), and 2-(4-diethylamino-2-hydroxybenzoyl)benzoic acid (DHB; CAS No.: 5809-23-4). The targeted metabolites were postulated based on metabolism pathways of compounds with structures

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00216-019-02201-6) contains supplementary material, which is available to authorized users.

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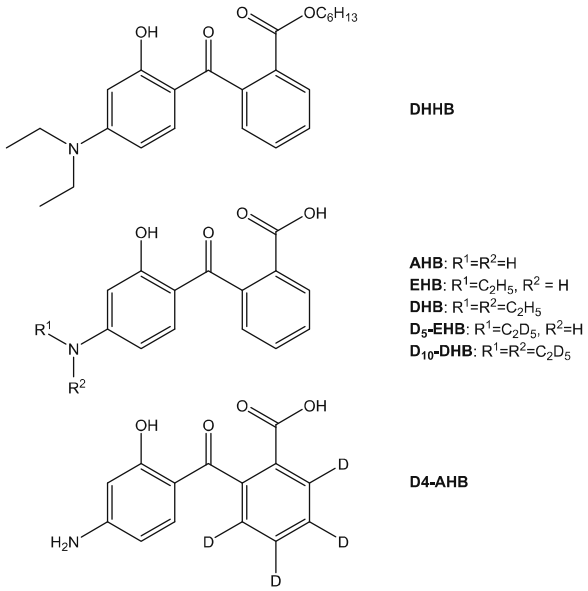

Fig. 1 Chemical structures of DHHB, its major metabolites (DHB, EHB, AHB), and the internal standards

similar to DHHB [7, 8] and identified as urinary metabolites of DHHB as part of a human metabolism study after oral and dermal application (publication in preparation).

The validated method was applied to 58 urine samples from healthy volunteers in order to estimate DHHB exposure within the population.

Experimental

Chemicals

2-(4-(Diethylamino)-2-hydroxybenzoyl)benzoic acid (DHB) was purchased from Sigma-Aldrich (Taufkirchen, Germany). 2-(4-Amino-2-hydroxybenzoyl)benzoic acid (AHB) was synthesized in our lab (for details, see Electronic Supplementary Material (ESM)); 2-(4-(ethylamino)-2-hydroxybenzoyl)benzoic acid (EHB) and the deuterated internal standards D_4-AHB, D_5-EHB, and D_{10}-DHB were custom synthesized (for additional information, see ESM). 4-Methylumbelliferyl-β-D-glucuronide dihydrate (MUG) was obtained from Santa Cruz Biotechnology (Dallas, TX, USA).

Ethyl acetate and methanol were purchased from Th. Geyer (Renningen, Germany). Ethanol, hexane, heptane, dichloromethane, and chloroform were from LGC Standards (Wesel, Germany), LC-MS grade acetonitrile with 0.1% formic acid and formic acid were obtained from Biosolve Chimie (Dieuze, France). Aqueous phosphoric acid (85%), potassium dihydrogen phosphate, and disodium hydrogen phosphate were supplied by VWR International (Leuven, Belgium). The water used was filtered through a Sartorius arium (Göttingen, Germany) water system. For enzymatic hydrolysis β-glucuronidase from *E. coli* was obtained from Megazyme (Bray, Ireland). During method development SPE was tested with the following cartridges: Oasis MAX, 3 mL, 60 mg sorbent per cartridge (Waters, Eschborn, Germany); Oasis MCX, 3 mL, 60 mg sorbent per cartridge (Waters); Oasis HLB 3 mL, 60 mg sorbent per cartridge (Waters); and Chromabond C18, 6 mL, 1000 mg sorbent per cartridge (Macherey-Nagel, Düren, Germany).

Urine sample preparation

Urine samples were stored at − 20 °C, thawed at room temperature, and shaken for homogenization prior to analysis. To 1.0 mL of urine, 10 µL of an internal standard solution, containing 1 µg/mL D_4-AHB, D_5-EHB, and D_{10}-DHB in water and 10 µL of an aqueous solution of 4-methylumbelliferyl-β-D-glucuronide dihydrate (MUG; 50 µg/mL), was added. For enzymatic hydrolysis the sample was diluted with 0.5 mL phosphate buffer (1/9 M, pH = 6.4) and 10 µL of β-glucuronidase was added. Subsequently, the samples were kept at 37 °C for 1.5 h. After incubation, pH adjustment of the samples was achieved by adding 25 µL of phosphoric acid (8.5% v/v). The sample was mixed with 2 mL of ethyl acetate and centrifuged for 10 min at 1900×*g*. The organic layer was separated and evaporated to dryness using a SpeedVac centrifuge (Christ, Osterode am Harz, Germany) and the residue was reconstituted in 100 µL of methanol and transferred into a 1.5-mL HPLC microvial with a 0.3-mL insert. Reagent blank samples were prepared following the sample preparation described under the "Urine sample preparation" section, by replacing 1 mL of urine with 1 mL of deionized water.

Urine samples for method validation were obtained from volunteers at ABF. Additionally, 58 samples from the general population were collected in the greater Munich area. For our metabolism study five subjects were given DHHB once oral and once dermal and all urine fractions were collected for the following 2 days.

UPLC-MS/MS analysis

Five microliters of the extract was injected into a liquid chromatography Nexera X2 UHPLC system (Shimadzu, Neufahrn, Germany). Chromatography was performed on an Acquity UPLC BEH Phenyl column (1.7 µm, 2.1 × 100 mm; Waters, Eschborn, Germany) equipped with an Acquity UPLC BEH Phenyl VanGuard (1.7 µm, 2.1 × 5 mm) at 40 °C and at a flow rate of 0.5 mL/min. Water (eluent A) and acetonitrile (eluent B) each containing 0.1% formic acid were used for gradient elution. Gradient elution was achieved starting at 90% of A for 2 min and then decreasing A linearly to 0% A over two more minutes. 0% of A was maintained for

1.5 min followed by an increase immediately to 90% A and held for 1 min until the end of the run at 6.5 min. For the detection of DHHB metabolites, a 6500$^+$ QTrap® from Sciex (Darmstadt, Germany) was used in ESI positive mode and scheduled MRM. All modules were controlled via Analyst software (Sciex, Version 1.6.3).

Nitrogen was used for all instrument gas flows. Curtain gas was set to 50 psi; ion source gas 1 and 2 were set to 45 psi. Source was heated to 500 °C, ion spray voltage was 5000 V, and entrance potential (EP) was set to 10 V for all analytes. Detailed information for the MRM transitions is summarized in Table 1.

Calibration

Calibration standards were freshly prepared and treated similar to urine samples as described in the "UPLC-MS/MS analysis" section by using an analyte-free urine pool mixed from at least three different analyte-free urine samples and measured immediately after sample work-up. A total of eleven calibrators were prepared by spiking increasing amounts of analytes to urine from 0.1 to 200 ng/mL (0.1; 0.2; 0.5; 1; 2; 5; 10; 20; 50; 100; and 200 ng/mL) for AHB and EHB and 0.05 to 100 ng/mL (0.05; 0.1; 0.2; 0.5; 1; 2; 5; 10; 20; 50; and 100 ng/mL) for DHB. Linear calibration was achieved by applying 1/*x* weighting. Linearity of all calibrators was proven during method validation.

Method validation

Method validation was performed according to the guidelines issued by the US Food and Drug Administration (FDA) [9]. Quality control samples (QCs) were prepared by pooling native human urine samples with known concentrations of AHB, EHB, and DHB. Where appropriate, analytes were spiked to the QC material. Three different concentration levels (low, medium, and high) were prepared to cover the entire range of expected concentrations. QCs were stored at − 20 °C until analysis. Two QC samples of each level were analyzed in combination with a calibration every day to monitor the robustness of the method. The target values of the QC samples for each analyte were determined prior to the method validation by analyzing six QC samples per level.

Selectivity was proven for the applied MRM transitions for each analyte and the corresponding internal standard. Therefore six analyte-free urine samples (i.e., not containing AHB, EHB, or DHB) were cleaned up as described in the "Urine sample preparation" section and analyzed by LC-MS/MS. The respective mass transitions were screened for interfering peaks. Additionally, the same six urine samples were spiked with 10 ng/mL AHB, EHB, and DHB and accuracy was determined. Accuracy and precision were determined by spiking analyte-free native human urine samples at four different concentration levels (LOQ, low, medium, high). Inter-day accuracy and precision were determined by analyzing five spiked urine samples for each concentration level on three different days. Intra-day accuracy and precision values were derived from five spiked samples per level of 1 day. Intra- and inter-day precisions were evaluated calculating the relative coefficients of variation (CVs), which should not exceed 15% for concentrations > three times the LOQ and 20% for concentrations up to three times of the LOQ, respectively. Acceptance criteria were met with accuracy rates of 85–115% (80–120% at levels up to three times the LOQ). Sample work-up-dependent losses of analytes were expressed by the recovery. Recovery rates were determined by comparing the analyte concentrations at three levels, measured when analyte-free urine samples ($N = 3$) were spiked after sample work-up (reference, corresponding to 100%), and when the same urine samples were spiked before the sample work-up procedure. LOQs were validated within accuracy and precision experiments and were set to the lowest concentrations fulfilling the requirements of accuracy and precision. The LODs were calculated by dividing the LOQ by 3. Accuracy after dilution was investigated by spiking three different analyte-free urine samples with concentrations of AHB, EHB, and DHB above their highest calibration level (ULOQ) and diluting the samples

Table 1 Retention times, mass transitions, and MS/MS parameters for DHHB metabolites and their deuterated IS

Analyte or IS	Retention time (min)	Mass transition (*m/z*)	Role	DP (V)	CE (V)	CXP (V)
AHB	3.9	258.0 ➔ 110.1	Quantifier	21	13	14
AHB	3.9	258.0 ➔ 149.0	Qualifier	21	21	18
D_4-AHB	3.9	262.0 ➔ 110.1	IS	21	13	14
EHB	4.1	286.0 ➔ 138.0	Quantifier	21	15	16
EHB	4.1	286.0 ➔ 149.0	Qualifier	21	25	22
D_5-EHB	4.1	291.1 ➔ 143.0	IS	21	15	16
DHB	4.3	314.1 ➔ 240.1	Quantifier	36	37	24
DHB	4.3	314.1 ➔ 149.0	Qualifier	36	33	18
D_{10}-DHB	4.3	324.1 ➔ 245.1	IS	36	37	24

with water. Three different dilutions (1:100, 1:10, and 1:5) were tested and accuracy was found to be acceptable in the range between 85 and 115%.

Occurring matrix effects (MEs) were evaluated by comparing spiked and processed urine samples at low and high concentrations of the analytes with aqueous reference standards possessing the same concentrations of analytes (reference, corresponding to 100%). MEs are described as ratios from processed samples to references. Ratios > 1 indicate a signal enhancing ME; signals < 1 indicate a signal suppressing ME. Carryover effects were tested by repeated injection ($N = 5$) of the highest calibrator followed by injection of methanol as a blank sample. Stability of analytes during storage was investigated at room temperature, 10 °C, and − 20 °C over different time periods ranging from 1 day up to several months.

Application of the method to human urine samples

The validated method was applied to 58 human urine samples collected in the greater Munich area from volunteers not occupationally exposed to DHHB. In total, 27 female and 31 male healthy volunteers aged between 18 and 83 years participated in this study. The study was approved by the Ethic Committee of the national Medical Association of Bavaria.

UPLC-QTRAP experiments for the identification of minor metabolites of DHHB

Samples from a human metabolism study (manuscript currently in preparation) were processed with a preliminary method (see ESM) and at first analyzed for the major metabolites of DHHB (AHB, EHB, DHB). Samples with high concentrations of AHB, EHB, and DHB (<ULOQ = 100 μg/L) were selected for the investigation of further additionally hydroxylated metabolites.

Samples were chromatographically separated using the gradient described in the "UPLC-MS/MS analysis" section. At first an Enhanced MS Scan (EMS) was conducted with focus on *m/z* of + 16 or + 32 of the parent ions from AHB, EHB, and DHB, in order to screen for additionally mono- or dihydroxylated species. Afterwards Enhanced Product Ion Scans (EPIs) based on the molecular weights found in EMS were carried out. False positive transitions were excluded by comparison with a sample showing levels of AHB, EHB, and DHB below LOQ. MRM transitions for additionally hydroxylated metabolites were created based on the parameters established for AHB, EHB, and DHB since no reference standards were available and mass fragmentation was comparable (see Fig. 3). Plausibility of retention times was checked by comparing retention times of AHB, EHB, and DHB with the peaks attributed to the additionally hydroxylated species as these should elute right before the respective major metabolite. Finally, the generated MRMs were optimized with respect to the potentials and energies by means of flow injection analysis (FIA).

Statistical analyses

All statistical analyses were performed with Prism (GraphPad, Version 8.0.1, La Jolla, CA, USA) software package. Values below LOQ were set to LOQ/2 for calculations. The non-parametric Mann-Whitney *U* test was applied for the determination of statistical differences between subgroups. For the calculation of non-parametric coefficients of correlation between urinary levels of DHHB metabolites, Spearman's correlations were used. Non-parametric tests were chosen to avoid distorted statistics caused by extreme values. *p* values of < 0.05 were regarded as statistically significant.

Results and discussion

Performance of the analytical method

The newly developed and optimized method described above enables the simultaneous, sensitive, and precise determination of three major metabolites of DHHB. The metabolic dealkylation of DHHB to AHB and EHB was postulated based on the biotransformation of similar compounds like the UV filter EDP (2-ethylhexyl 4-(N,N-dimethylamino)benzoate) whose dimethylated aniline function is demethylated either once or twice at the aniline group resulting in a secondary or primary amine derivative [7, 8]). The same metabolic pathway was proven for the diethylated aniline function of DHHB which can be deethylated once (EHB) or twice (AHB). Additionally, the hydrolysis of the ester moiety to the respective carboxylic acid is also a pathway observed for EDP [8] or drugs like the ACE inhibitors trandolapril or quinapril [10].

DHHB itself could not be detected in any sample of the metabolism study showing that DHHB is not excreted in human urine. Therefore, DHHB was not taken into account for the final analytical method. DHB is the only metabolite of DHHB which has already been reported as a metabolite of DHHB in in vivo experiments in rats [11]. All three of the suggested metabolites of DHHB could be confirmed within a human metabolism study with oral and dermal application of DHHB to five participants (manuscript in preparation).

Furthermore, the study revealed the need of an enzymatic hydrolysis step before the sample preparation procedure, since all analytes were partly present in their conjugated form as glucuronides. As DHHB itself possesses an ester moiety, an enzyme extract without esterase activity was used in order to avoid formation of DHB from DHHB during the sample preparation which may lead to an overestimation of urinary DHB concentrations. The enzyme extract β-glucuronidase/arylsulfatase from *Helix pomatia* was previously reported to

contain lipase and esterase activities besides the glucuronidase activity [12]. Hence, this enzyme mixture was not suited for the purpose of this method. Alternatively, β-glucuronidase from *E. coli* without esterase activity was used. As a consequence the sum of unconjugated and glucuronidated metabolites is captured in this method while possibly occurring sulfated metabolites are not detectable. However, a comparison between the two enzyme extracts did not show different concentrations of the metabolites suggesting that sulfated conjugates were only present to a very low extent, if any. A phosphate buffer (1 M) was used in order to maintain a stable pH value of 6.4 for all samples, which is the optimal working pH for the β-glucuronidase from *E. coli* (5.0 and 7.5 according to the manufacturer).

In addition, chemical hydrolysis with 6 N HCl was tested as an alternative approach yielding lower analyte concentrations, presumably due to analyte degradation during acidic incubation. The kinetics of the enzymatic hydrolysis were evaluated by processing highly concentrated samples derived from a metabolism study (manuscript in preparation) with different incubation times. Figure 2 shows the dependency of the enzymatic hydrolysis on the incubation time. After 15 min, hydrolysis of the glucuronidated analytes appears to be complete. To assure reproducible results and a complete hydrolysis, incubation time was set to 1.5 h. Additional experiments revealed a decrease of analyte concentrations in samples after more than 6 h of incubation (data not shown). Deconjugation efficiency was monitored by spiking all samples with 500 ng of MUG in order to monitor the enzymatic hydrolysis [13]. In case no MUG was detectable in the UPLC-MS/MS analysis the enzymatic hydrolysis was complete.

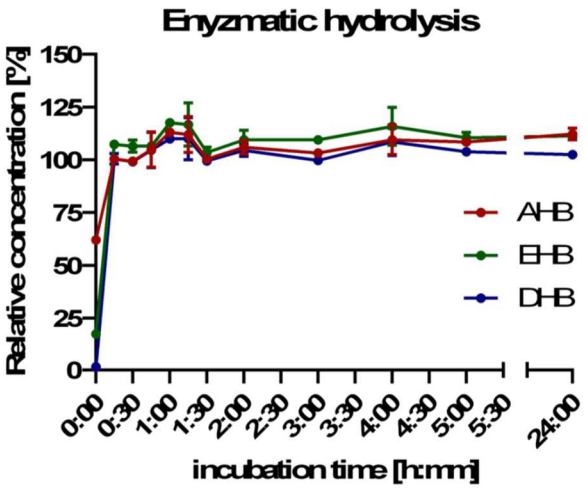

Fig. 2 Enzymatic hydrolysis of a sample with high concentrations of endogenous AHB, EHB, and DHB glucuronides, in relation to the incubation time. Values were analyzed in triplicates. Concentrations in %, 100% refer to the final incubation time used in the validated method (1.5 h)

Due to matrix suppression and interferences in the chromatography an extraction step was necessary. Liquid-liquid extraction (LLE) was tested with dichloromethane, chloroform, ethyl acetate, diethyl ether, methyl *tert*-butyl ether (MTBE), and hexane. Best recoveries were obtained by extracting with ethyl acetate and MTBE, respectively. Pipetting steps were executed more conveniently using ethyl acetate because of its lower vapor pressure. Hence, ethyl acetate was used in the final method instead of MTBE. As all analytes possess both a basic amine group and an acidic carboxyl moiety, pH adjustment in proximity of the isoelectric point (pH 4.6 ± 0.2) was crucial to achieve satisfactory yields during LLE. Moderate to satisfying recoveries between 40 and 80% were achieved after optimization of the LLE regarding pH and solvent. Apparently, the presence of zwitterions with a positive charge at the amine group and a negative charge at the carboxylic group may influence the yield during extraction. To increase recovery rates solid-phase extraction (SPE) was also tested with MAX, MCX, and HLB cartridges from Waters and with Chromabond C18 columns from Macherey-Nagel (Düren, Germany). This approach leads to higher recovery rates, but signal intensities decreased due to a stronger matrix suppression. Thus, LLE was superior in terms of overall sensitivity.

Efficient ionization of the compounds was achieved with positive electrospray ionization (ESI^+). Applied mass transitions and MS/MS parameters are shown in Table 1. Mass transitions used as quantifiers were chosen considering intensity and selectivity of the transition. Figure 3 a shows structure suggestions for the most prominent fragments from AHB, EHB, and DHB. Cleavage of the molecules at the ketone bridge seems to be very likely, because both fragments resulting from this cleavage were detectable. Furthermore, the elimination of the carboxyl group as observed in DHB is thinkable. Similar fragmentation patterns are observed for the analogous hydroxylated species (see Fig. 3b).

For internal standards (IS), the corresponding mass transitions were evaluated. MRM chromatograms for all analytes and their internal standards are shown in Fig. 4. IS concentrations were set in the middle of the calibration range to 10 µg/L and the applied transitions (corresponding to the quantifier mass transition) were found to be selective with sufficient sensitivity.

Method performance data are shown in Table 2. Parameters reported were evaluated according to FDA guidelines on bioanalytical method validation [9]. LOQs were set to 0.1 µg/L for AHB and EHB and to 0.05 µg/L for DHB being the lowest measurable concentrations with intra- and inter-day precisions below 20% CV. Intra- and inter-day accuracies at LOQ were in the acceptable range of 80–120%. The LOD was calculated from the LOQ by dividing the LOQ through 3. Calibration was found to be linear in a range from 0.1 to 200 µg/L for AHB and EHB and in a range from 0.05 to

100 µg/L for DHB, respectively (correlation coefficients $R^2 > 0.995$).

Samples above ULOQ can be diluted up to 100-fold with water still yielding in accurate results (85–115% accuracy).

Intra- and inter-day precisions for all analytes at all concentration levels tested had CVs between 0.4 and 18.8% fulfilling the acceptance criteria. Intra- and inter-day accuracies ranged between 86.2 and 107.0%. Recovery values including losses during sample extraction were in a range between 42 and 79%. AHB signals in prepared urine matrix were suppressed by the matrix yielding in 25–42% signal area compared with an aqueous standard solution. DHB signals were also suppressed by the matrix, but to a lower extent (49–87% signal area compared with an aqueous standard). In contrast, a moderate signal enhancement of 110–116% could be observed for EHB.

Reagent blank samples were injected together with tested samples, no blank values >LLOQ for any analyte could be determined. Despite optimization of the washing method for the UPLC-MS/MS system between runs, small carryover effects in the range of the LOQs were observed after five injections of the highest calibrator for all analytes. However, high values close to the ULOQ have not been measured in the urine samples of non-occupationally exposed individuals so far. Therefore, in the case of a sample with high concentrations

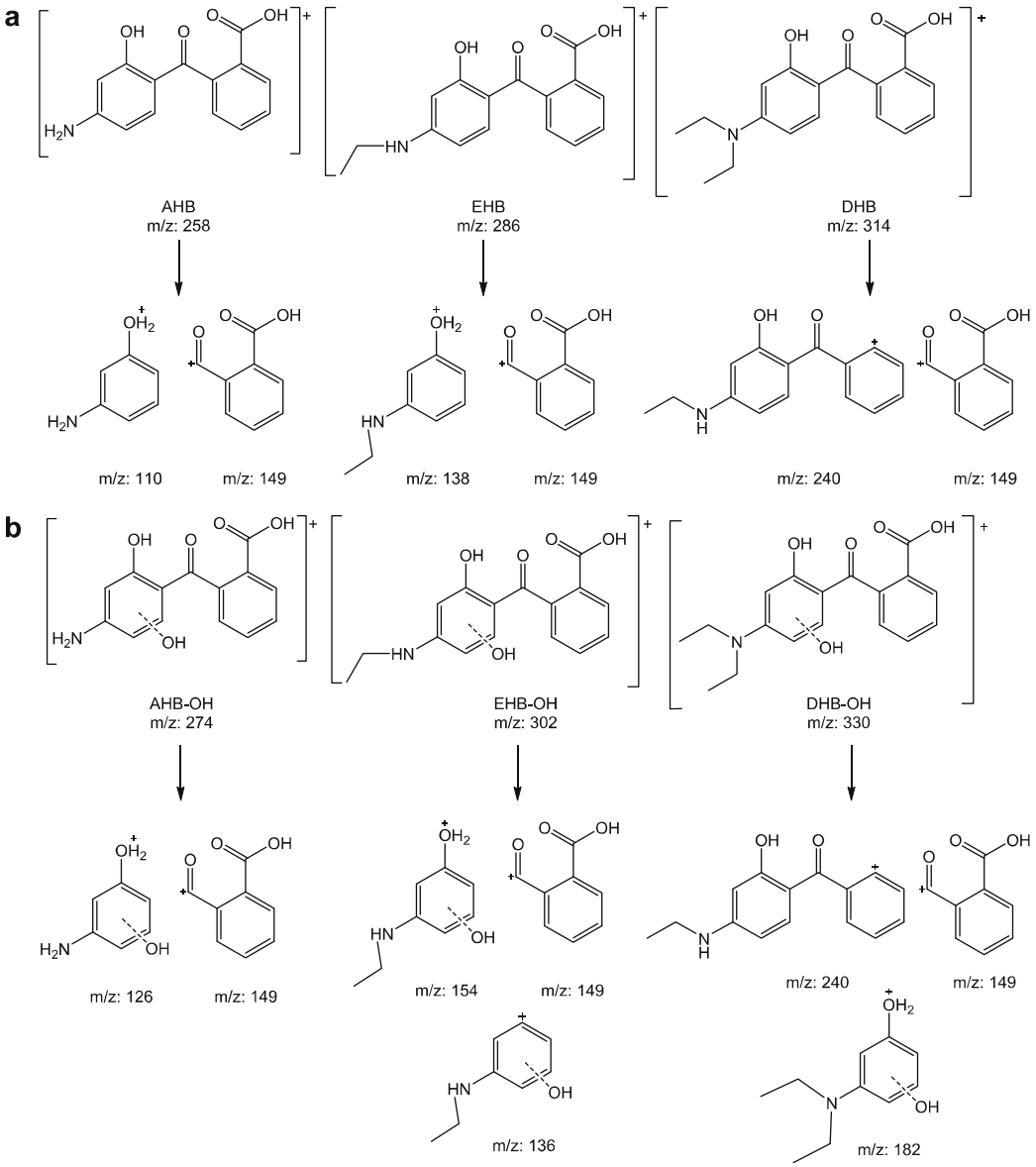

Fig. 3 Suggested mass fragmentation pattern for major (**a**) and minor (**b**) metabolites of DHHB in ESI^+ mode

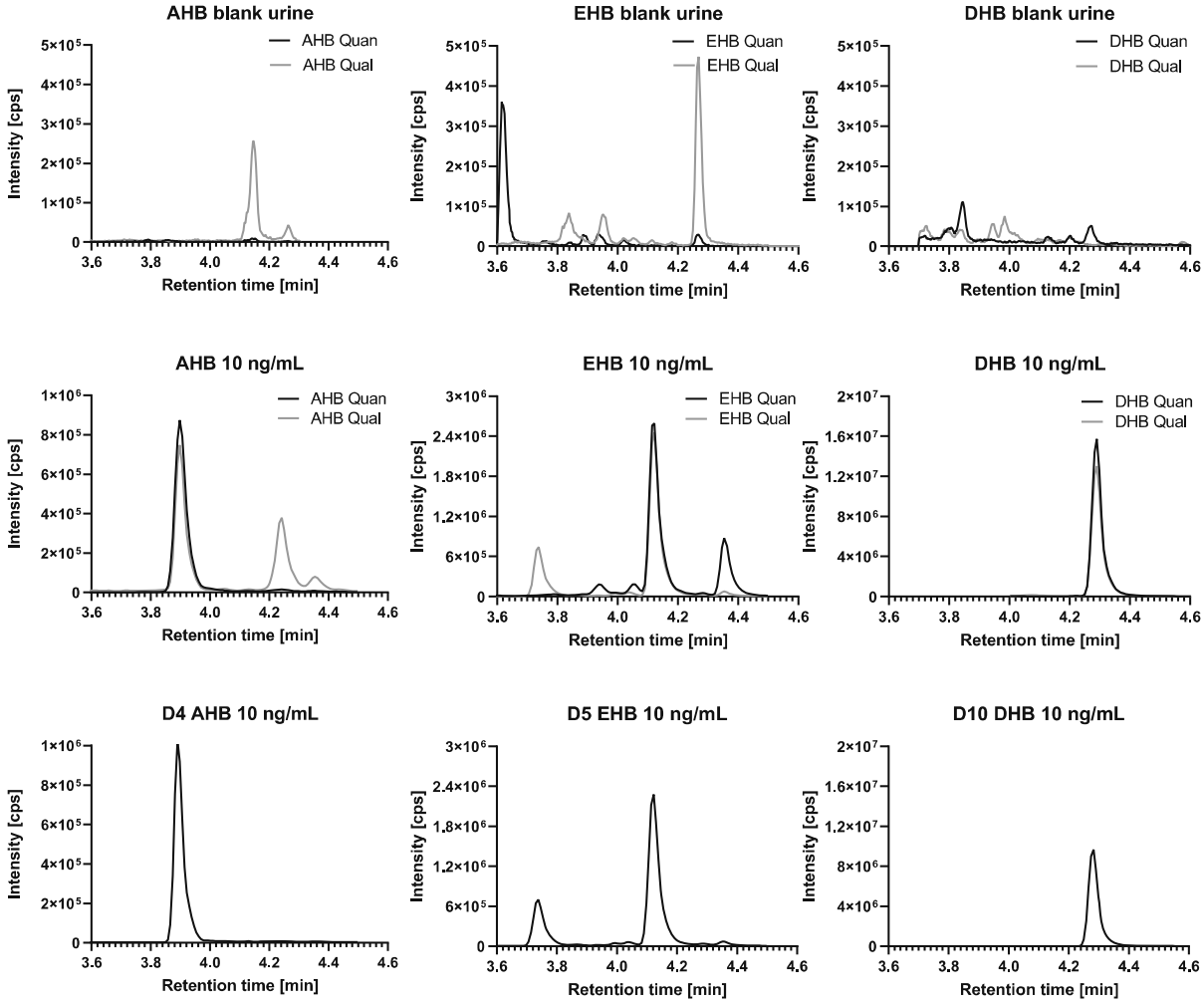

Fig. 4 Chromatograms of the analytes and their internal standards. Blank urine samples (top), same sample spiked with 10 ng/mL of each analyte (middle), and their deuterated internal standards (bottom)

close to ULOQ, the following sample shall be reinjected to overcome false positive results.

In-house and custom synthesized standards were checked for impurities with 1H-NMR and HPLC-UV. No impurities were detectable and therefore, a purity of > 95% was assumed. Experiments addressing the stability of the standards will follow in the near future, since there is only a limited amount of each standard available.

All analytes were found to be stable in urine at room temperature (22 °C) for at least 18 h. Prepared samples can be stored at − 20 °C for at least 3 weeks and for a maximum of five freeze-thaw cycles. Post-preparative stability was proven for at least 1 week stored in the autosampler at 10 °C. Long-term stability studies over longer storage periods are currently in progress.

A few methods haven been published on the determination of DHHB, e.g., in sunscreens [14, 15], chlorinated water [16], and porcine skin samples [15]; however, to the best of our knowledge, the current method describes for the first time the simultaneous analysis of the three major DHHB metabolites in one method.

Urinary excretion of DHHB metabolites in a small group of the general population

To evaluate potential exposure due to the usage of DHHB containing consumer products in everyday life, 58 spot urine samples were collected from volunteers who were not exposed occupationally with DHHB and analyzed with the validated method. The subjects were not intentionally exposed to DHHB-containing consumer products but were advised to behave as usual. There were no restrictions with regard to selection of the volunteers and the time of urine collection. In total, 27 females and 31 males aged between 18 and 83 took part in this study.

Table 2 Method validation data for the quantification of AHB, EHB, and DHB in urine

	AHB	EHB	DHB
LOD (μg/L)	0.033	0.033	0.017
LLOQ (μg/L)	0.1	0.1	0.05
Calibration range (μg/L)	0.1–200	0.1–200	0.05–100
Precision (intra-day, $N = 3 \times 5$), μg/L (CV, %)	0.10 (5.4–18.8)	0.10 (5.0–13.7)	0.05 (6.8–14.6)
	0.15 (4.3–5.5)	0.15 (3.3–8.2)	0.15 (1.6–4.7)
	10.0 (2.6–3.8)	10.0 (1.4–3.9)	10.0 (0.4–4.2)
	200 (1.2–8.8)	200 (3.2–8.2)	100 (2.4–4.6)
Precision (inter-day, $N = 15$), μg/L (CV, %)	0.10 (13.7)	0.10 (12.8)	0.05 (13.8)
	0.15 (4.3)	0.15 (6.6)	0.15 (7.6)
	10.0 (2.6)	10.0 (10.8)	10.0 (11.7)
	200 (2.8)	200 (7.3)	100 (10.6)
Accuracy (intra-day, $N = 3 \times 5$), μg/L (%)	0.10 (96.0–100.8)	0.10 (89.4–106.0)	0.05 (88.4–106.8)
	0.15 (97.5–99.2)	0.15 (97.2–106.4)	0.15 (86.8–101.7)
	10.0 (93.2–100.9)	10.0 (93.3–94.9)	10.0 (93.1–100.7)
	200 (90.2–103.0)	200 (89.1–99.2)	100 (86.2–107.0)
Accuracy (inter-day, $N = 15$), μg/L (%)	0.10 (97.9)	0.10 (98.1)	0.05 (96.7)
	0.15 (98.2)	0.15 (102.9)	0.15 (94.1)
	10.0 (96.6)	10.0 (94.2)	10.0 (98.3)
	200 (95.5)	200 (94.0)	100 (94.6)
Recovery ($N = 3$), μg/L (%)	0.15 (60.2)	0.15 (62.4)	0.15 (61.2)
	10.0 (72.0)	10.0 (78.9)	10.0 (79.2)
	200 (41.9)	200 (46.9)	100 (58.7)
Matrix effects (aqueous standard = 100%) ($N = 3$), μg/L (%; CV, %)	0.15 (24.6; 2.6)	0.15 (109.5; 2.0)	0.15 (48.9; 1.7)
	200 (41.9; 5.2)	200 (116.2; 0.9)	100 (87.4; 2.3)
	IS 0.15 (27.4; 0.5)	IS 0.15 (86.9; 1.0)	IS 0.15 (63.6; 1.0)
	IS 200 (39.2; 3.7)	IS 200 (107.2; 2.8)	IS 200 (77.0; 1.9)
Carryover effects (μg/L)	0.08	0.09	0.07

The results are shown in Table 3. Urinary concentration levels of DHHB metabolites are shown as concentrations (μg/L). Additionally, values were normalized for the creatinine level as micrograms per gram creatinine taking into account the varying dilutions of the spot urine samples.

The metabolite determined with the highest concentrations was EHB with an average of 1.39 μg/g creatinine, followed by AHB (0.65 μg/g creatinine) and DHB (0.38 μg/g creatinine) with the lowest concentrations. AHB was found above LOQ in 19 samples (33%), EHB in 20 samples (36%), and DHB in 14 samples (26%). Thus, in approximately two-thirds of the study samples, no DHHB metabolites were detectable indicating a low exposure to DHHB in non-occupationally exposed subjects.

All three compounds were found to be strongly correlated (Spearman $r = 0.81$–0.92) showing that AHB, EHB, and DHB are specific metabolites to monitor DHHB exposure.

Levels of DHHB metabolites were compared for different subgroups and statistical significance was evaluated by means of the Mann-Whitney *U* test. Subgroups were divided as follows for comparison: gender (male/female), age (18–35/50–83), daytime of sample collection (4 am–2 pm/2:30 pm–1 am), seasons of sample collection (winter, spring/summer), age of the spot urine (> 1 year/< 1 year). No difference in DHHB metabolite levels could be observed for any subgroup comparison.

Table 3 DHHB metabolite levels in spot urine samples collected from 58 volunteers

	AHB	EHB	DHB
μg/L			
Mean ± SD	0.65 ± 3.36	1.44 ± 8.22	0.39 ± 2.24
Median	0.05	0.05	0.025
Min–max	0.05–25.9	0.05–63.1	0.025–17.2
<LOQ, *N* (%)	67.2	65.5	74.5
μg/g creatinine			
Mean ± SD	0.65 ± 2.59	1.39 ± 6.45	0.38 ± 1.72
Median	0.12	0.13	0.05
Min–max	0.02–19.2	0.02–46.9	0.01–12.8

For results <LOQ, values were set to LOQ/2 for descriptive statistics

It has to be noted that only a small number of samples from the Munich area was analyzed not being necessarily representative for the exposure of the general population. To assess the exposure to DHHB in the general population, human biomonitoring studies with a larger sample size in a representative environment (rural/urban, locations throughout the country/region of interest) will be necessary.

Determination of additionally hydroxylated metabolites using a UPLC-QTrap system

Following the procedure described in the "UPLC-QTRAP experiments for the identification of minor metabolites of DHHB" section, EMS experiments lead to four plausible molecule masses: $m/z = 273$ for AHB-OH, $m/z = 289$ for AHB-$(OH)_2$, $m/z = 301$ for EHB-OH, and $m/z = 329$ for DHB-OH. Subsequently conducted EPI experiments lead to multiple product ions which were translated into MRM transitions. Furthermore, retention times of the evaluated peaks were reviewed and regarded as valid if the additionally hydroxylated species eluted immediately before their analogue possessing just one hydroxyl group. At this point the occurrence of AHB-$(OH)_2$ was rejected, because retention times of the peaks observed in EPI experiments were found to be 0.2 min after those of AHB and no MRM transitions could be generated from the found product ions. Plausible mass fragments for additionally hydroxylated metabolites of DHHB are shown in Fig. 3b. They are fragmented in analogy to AHB, EHB, and DHB (Fig. 3a). Cleavage of the molecules again occurred at their ketone functions leading to mainly two fragments. The fragment with m/z 149 was observed for every additionally hydroxylated metabolite as well as for AHB, EHB, and DHB. The second fragments of the additionally hydroxylated metabolites differ in the degree of alkylation of the amine function, leading to $m/z = 126$ for AHB-OH, 154 for EHB-OH, and 182 for DHB-OH. Those mass fragments correspond to the mass fragments of AHB (110), EHB (138), and DHB (166) which are lacking of the additional hydroxyl group (− 16). Therefore, the fragmentation patterns observed suggest that the additional hydroxyl moieties are exclusively located in the aniline ring. The exact positions of the hydroxyl functions could not be clarified without authentic reference material.

Eight plausible MRM transitions for the three compounds AHB-OH, EHB-OH, and DHB-OH were determined without the use of a standard by applying suitable EMS, EPI, and FIA experiments as summarized in Table 4. As cell exit potentials (CXPs) could only be adjusted to a maximum of 15 V in FIA experiments, CXPs obtained from the manual tuning of AHB, EHB, and DHB, respectively, were implemented in the final MRM transitions. Concentration levels of AHB-OH, EHB-OH, and DHB-OH were estimated to be 100-fold lower compared with the major metabolites AHB, EHB, and DHB based on the comparison of the area ratios. In conclusion, it was shown that the additionally hydroxylated species were found in negligible concentrations in urine, and hence, these metabolites are not suited as biomarkers of exposure to DHHB.

Table 4 MRM parameters for additionally hydroxylated DHHB metabolites

Analyte	Q1	Q3	DP (V)	EP (V)	CE (V)	CXP (V)
AHB-OH	274.2	126.1	24	9	11	54
		149.1	24	10	28	54
EHB-OH	302.3	136.1	34	15	31	26
		149.0	34	11	29	26
		154.2	34	15	11	26
DHB-OH	330.2	149.0	42	10	34	30
		182.4	42	14	23	30
		240.1	38	14	9	30

Conclusion

We developed and validated an UPLC-MS/MS method with sufficient sensitivity for the simultaneous determination of the three major and specific DHHB metabolites AHB, EHB, and DHB in urine of non-occupationally exposed subjects. All three major metabolites were detectable in approx. one-third of the urine samples from a pilot study with 58 healthy adult volunteers. Further minor metabolites with an additional hydroxyl function in the aniline ring were detected based on EMS experiments. However, these minor metabolites were not included in the final method due to their very low abundance. In conclusion, the new method proved its suitability to determine the three major metabolites AHB, EHB, and DHB in HBM studies to assess the DHHB exposure in the general population. This method will help to evaluate the exposure to DHHB in future HBM studies in larger populations.

Funding information The method development for the determination of urinary DHHB metabolites (including the synthesis of part of the reference compounds) was funded by the Chemie Wirtschaftsförderungsgesellschaft, Frankfurt/Main, Germany. The method was developed as part of a 10-year human biomonitoring initiative between the Federal Ministry for Environment, Nature Conservation and Nuclear Safety (BMU) and the Verband der chemischen Industrie e.V. (German Chemical Industry Association (VCI)).

Compliance with ethical standards

Studies with human subjects were approved by the Ethic Committee of the Bayerische Landesärztekammer, Munich, Germany. All subjects gave their informed consent.

Conflict of interest The authors MS, NP, FB, GS, and MS declare that they have no conflict of interest. EL is employed by a company manufacturing DHHB.

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Analytical and Bioanalytical Chemistry

Electronic Supplementary Material

A validated UPLC-MS/MS method for the determination of urinary metabolites of Uvinul® A plus

Markus Stoeckelhuber, Nikola Pluym, Franz Bracher, Edgar Leibold, Gerhard Scherer, Max Scherer

Additional information to: Experimental

Synthesis of analytical standards

2-(4-Amino-2-hydroxybenzoyl)benzoic acid (AHB)

This compound was prepared by alkaline cleavage of rhodamine 110 according to WO 2017/205350 Al (PCT/US2017/033961). Hereby 272 mg (0.74 mmol) rhodamine 110 were dissolved in 12 N potassium hydroxide solution (5 mL) and stirred under reflux for 48 h. The mixture was acidified to pH 2 with concentrated HCl, methanol (10 mL) was added and the mixture was filtrated through a qualitative filter paper 413 from VWR International. The methanol was removed *in vacuo* and the mixture was extracted with dichloromethane (3 x 15 mL). The combined organic layers were dried over anhydrous sodium sulfate and the dichloromethane was evaporated *in vacuo*. After washing with hexane the total yield was 50 mg (23 %) of the title compound as a pale yellow solid.

2-(4-Amino-2-hydroxybenzoyl)-[D_4]-benzoic acid (D_4-AHB)

3-(*N*,*N*-Dibenzylamino)phenol was synthesized from 3-aminophenol (0.55 g, 5.0 mmol) and benzyl bromide (Sigma; 1.71 g; 10.0 mmol) using $NaHCO_3$ (1.68 g, 20 mmol) as a base (stirring in 40 mL acetonitrile at 40 °C for 20 h. After removal of the solvent *in vacuo*, water was added to the residue and the organic compounds extracted with diethyl ether (*Bioorg. Med. Chem.* **2016**, *24*, 6131-6138). The product (0.83 g, 57 % yield) was isolated as a colorless solid by repetitive (2 times) flash column chromatography (cartridge with 25 g spherical SiO_2; 1-10 % of ethyl acetate in the mixture of hexane – dichloromethane, 3:1).

2-[4-(*N*,*N*-Dibenzylamino)-2-hydroxybenzoyl]-[D_4]-benzoic acid was prepared according to the method described by A. Butkevich et. al. (*Chem. Eur. J.* **2017**, *23*, 12114–12119). [D_4]-Phthalic anhydride (132 mg, 0.87 mmol) was heated with 3-(*N*,*N*-dibenzylamino)phenol (293 mg, 1.01 mmol) in glacial acetic acid (3 mL) with 0.01 mL of conc. H_2SO_4 (catalyst) at 120 °C (bath temperature) for 5 h. Acetic acid was removed *in vacuo*, and the deep red residue was mixed with chloroform and aqueous citric acid buffer (pH = 3-4). The organic layer was evaporated, subsequent purification by flash column chromatography (cartridge with 25 g of spherical silica gel; gradient of 1-10 % methanol in dichloromethane) afforded 2-[4-(*N*,*N*-dibenzylamino)-2-hydroxybenzoyl]-[D_4]-benzoic acid (130 mg, impure, slightly contaminated with the corresponding rhodamine dye, resulting from reaction of phthalic anhydride with two equivalents of the aminophenol; <34 % yield). No D-H exchange was observed under these reaction conditions.

2-(4-Amino-2-hydroxybenzoyl)-[D_4]-benzoic acid. 2-[4-(*N*,*N*-dibenzylamino)-2-hydroxybenzoyl]-[D_4]-benzoic acid (130 mg, impure, contaminated with the corresponding rhodamine dye; ca. 0.29 mmol; see above) was dissolved in 2-propanol (10 mL), flushed with argon, then $PdCl_2$ (72 mg, 0.41 mmol) was added followed by 60 mg of Pd/C (10 % Pd, oxidized form; VWR International). The reaction mixture (in a Schlenk flask with a rubber septum) was flushed with argon, then with hydrogen, and vigorously stirred under hydrogen at atmospheric pressure for 24 h. The course of the reaction was monitored by TLC (dichloromethane – methanol – 25 % aq. NH_3 = 95:5:0.2). When the reaction was complete, hydrogen gas was

substituted by argon, the catalyst removed through Celite and washed with 2-propanol. The title compound was isolated by preparative HPLC from the residue obtained after removal of 2-propanol *in vacuo* (RP column 20 x 250 mm with C18-modified silica gel (Interchim), flow rate 20 mL min-1, gradient 20 – 100% acetonitrile in water (+0.1 % TFA) in 25 min). Yield: 32 mg of the title compound (as trifluoracetate salt).

2-[4-(Ethylamino)-2-hydroxybenzoyl]benzoic acid (EHB)

3-(*N*-Benzylamino)phenol was prepared in high yield by reductive amination of benzaldehyde with 3-aminophenol in methanol with addition of acetic acid using sodium cyanoborohydride as the reducing agent, following WO 2014/195507b (PCT/EP2014/061921). 3-[(*N*-Benzyl-*N*-ethyl)amino]phenol was prepared in low yield by reductive amination of acetaldehyde with 3-(*N*-benzylamino)phenol in methanol with addition of acetic acid and sodium cyanoborohydride as the reducing agent as described in the same patent (WO 2014/195507b (PCT/EP2014/061921)). 2-[4-(Ethylamino)-2-hydroxybenzoyl]benzoic acid was prepared in low yield by heating of phthalic anhydride (148 mg, 1.00 mmol) with 3-[(*N*-benzyl-*N*-ethyl)amino]phenol (227 mg, 1.00 mmol) in toluene (10 mL, 130 °C bath temperature) for 16 h followed by chromatographic isolation of 2-[[4-(*N*-benzyl-*N*-ethyl)amino]-2-hydroxybenzoyl]benzoic acid and cleaving of *N*-benzyl group (tetrahydrofuran, 10 % Pd/C (oxidized form VWR International, 30% to the mass of the substrate), hydrogen, atmospheric pressure). The final compound (20 mg, 6 %) was isolated as trifluoroacetate salt by preparative HPLC (RP, C18 column, 21 x 250 mm, 30 – 100 % acetonitrile gradient in 30 min (0.1 % TFA in water and 0.1 % TFA in acetonitrile), flow rate 20 mL/min).

2-[4-(*N*-[D_5]-ethyl)amino]-2-hydroxybenzoyl]benzoic acid (D_5-EHB)

3-[(*N*-Benzyl-*N*-[D_4]-ethyl)amino]phenol was prepared in low yield (108 mg, 13% from 730 mg of the starting material) by reductive amination of [D_4]-acetaldehyde (Sigma) with 3-(*N*-benzylamino)phenol in 1,2-dichloroethane with addition of molecular sieves (4 Angström) using sodium triacetoxyborohydride as the reducing agent, as described by Abdel-Magid et al. (*J. Org. Chem.* **1996**, *61*, 3849-3862). Due to low yield, we decided to use another method and prepared 3-[(*N*-Benzyl-*N*-[D_5]-ethyl)amino]phenol from 3-(*N*-benzylamino)phenol (2.0 g, 10 mmol) and [D_5]-ethyl iodide (Sigma; 1.85 g; 11.4 mmol) using K_2CO_3 (1.45 g, 11 mmol) as a base (stirring in 20 mL DMF at 70 °C for 24 h). DMF was removed in vacuo, the residue mixed with water and diisopropyl ether, organic layer separated, washed with brine, dried (Na_2SO_4), and the solvent evaporated *in vacuo*. The product (1.25 g, 53% yield) was isolated by flash column chromatography on silica gel (elution with hexane – ethyl acetate, 3:1).

2-[4-(*N*-Benzyl-*N*-[D_5]-ethyl)amino]-2-hydroxybenzoyl]benzoic acid was prepared according to the method described by A. Butkevich et al. (*Chem. Eur. J.* **2017**, *23*, 12114–12119). In detail, phthalic anhydride (518 mg, 3.5 mmol) was heated with 3-[(*N*-benzyl-*N*-[D_5]-ethyl)amino]phenol in glacial acetic acid (5 mL) with 2 drops of conc. H_2SO_4 (catalyst) at 100 °C (bath temperature) overnight. Acetic acid was removed *in vacuo*, and the deep red residue was mixed with dichloromethane and aqueous citric acid buffer (pH = 3-4). The title product (in the organic layer) has practically the same R_f as the rhodamine dye (obtained from 2 moles of the amine and 1 mol phthalic anhydride). *o*-Phthalic acid (formed upon hydrolysis of phthalic anhydride) has a

lower R_f(hexane – ethyl acetate with 1% v/v formic acid) and can be separated by chromatography. Flash column chromatography (cartridge with 25 g of spherical silica gel; gradient of 10-50 % ethyl acetate in hexane; +1% HCOOH) afforded pure title compound (>98 % HPLC area, 440 mg, 50% yield). Diagnostically important ^{13}C-NMR (CD_2Cl_2, 101 MHz, δ, ppm): 198.4, 169.7, 165.3, 154.9, 141.1, 137.5, 134.4, 132.8, 131.0, 129.2 (2x), 128.4, 127.5, 127.1, 126.2 (2x), 110.3, 104.2, 97.6, 53.4.

2-[4-(*N*-[D_5]-ethyl)amino]-2-hydroxybenzoyl]benzoic acid was prepared from 2-[4-(*N*-benzyl-*N*-[D_5]-ethyl)amino]-2-hydroxybenzoyl]benzoic acid by cleaving of the *N*-benzyl group (tetrahydrofuran, 10 % Pd/C (oxidized form VWR International, 30 % to the mass of the substrate), hydrogen, atmospheric pressure). The catalyst was removed by filtration of the reaction mixture through Celite™. Evaporation of the solvent *in vacuo* followed by drying in high vacuum (0.1 mbar) afforded the pure product in quantitative yield.

2-[4-(*N*,*N*-Di[D_5]-ethyl)amino]-2-hydroxybenzoyl]benzoic acid (D_{10}-DHB)

Similarly, 3-[*N*,*N*-di([D_5]-ethyl)amino]phenol was synthesized from 3-aminophenol (0.85 g, 7.8 mmol) and [D_5]-ethyl iodide (Sigma; 2.70 g; 16.8 mmol) using K_2CO_3 (2.16 g, 15 mmol) as a base (stirring in 40 mL DMF at 70 °C for 24 h). The product (0.95 g, 69 % yield) was isolated after the work-up (see above) by flash column chromatography on silica gel (elution with hexane – ethyl acetate, 3:1). Some amount of 3-[*N*-([D_5]-ethyl)amino]phenol (with lower R_f, separable by chromatography) has been detected.

2-[4-(*N*,*N*-Di[D_5]-ethyl)amino]-2-hydroxybenzoyl]benzoic acid was prepared from 3-[*N*,*N*-di([D_5]-ethyl)amino]phenol (535 mg, 3.0 mmol) and phthalic anhydride (670 mg, 4.5 mmol) according to the method described by Butkevich et al. (*Chem. Eur. J.* **2017**, *23*, 12114–12119) and described above in more detail. Extraction with dichloromethane (200 mL) from the slightly acidic buffer solution (pH 3-4) followed by chromatographic separation (cartridge with 50 g of spherical SiO_2, application in dichloromethane; elution with 10-50 % ethyl acetate in hexane; +1 % HCOOH) afforded ca. 100 mg of the pure fraction and ca. 50 mg of the mixed fraction which was recrystallized from acetonitrile. Total yield: 150 mg (15 %) of the title compound.

Preliminary method used for the determination of additionally hydroxylated metabolites

Urine samples were thawed at room temperature and shaken manually for homogenization before analysis. To 1.0 mL of urine was added 10 µL of an IS solution containing 1 µg/mL of each D_4-AHB, D_5-EHB and D_{10}-DHB in water. Samples were diluted with 0.5 mL acetate buffer (1 M, pH = 5.1) and 10 µL of ß-glucuronidase/arylsulfatase from *Helix pomatia* were added. Subsequently the samples were heated to 37 °C for 18 hours. After incubation pH adjustment of the samples was achieved by adding 25 µL of phosphoric acid (20 % v/v). The samples were mixed with 1.5 mL of ethyl acetate and centrifuged for 10 minutes at 1,900 x g. The organic layer was separated and evaporated to dryness using a SpeedVac centrifuge and the residue was reconstituted in 100 µL methanol and transferred into a 1.5 mL HPLC microvial with a 0.3 mL insert.

3.4.3 Contributions to “Human metabolism and urinary excretion kinetics of the UV filter Uvinul A plus® after a single oral or dermal dosage”

The application for ethical approval was written by me and reviewed and submitted by Prof. Dr. Gerhard Scherer. I set up and conducted the metabolism study, Dr. Oliver Peschel led the medical surveillance. All samples obtained from this study were analyzed by me and I calculated all toxicokinetic parameters with the support of Prof. Dr. Gerhard Scherer. The manuscript was prepared by Prof. Dr. Gerhard Scherer, Prof. Dr. Franz Bracher, Dr. Nikola Pluym, Dr. Max Scherer, Dr. Edgar Leibold (BASF) and me.

Own contribution:

Writing application for the ethical approval	70 %	
Implementation of the metabolism study	90 %	
Analysis of study samples	100 %	
Evaluation of toxicokinetic parameters	90 %	
Submission and revision of publication 2	70 %	

3.4.4 Original Article: "Human metabolism and urinary excretion kinetics of the UV filter Uvinul A plus® after a single oral or dermal dosage"

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Human metabolism and urinary excretion kinetics of the UV filter Uvinul A plus® after a single oral or dermal dosage

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ABSTRACT

Hexyl 2-[4-(diethylamino)-2-hydroxybenzoyl]benzoate, better known under its trading name Uvinul A plus® is a UV filter mainly used in sunscreens, but also present in other cosmetic products with a maximum concentration of 10% (w/w) according to the EU directive. In this study we investigated the human metabolism after a single oral and a single dermal dose of Uvinul A plus®, respectively. Samples collected within 72 h of administration were analyzed with a newly developed UHPLC-MS/MS method. Results of the study revealed three major urinary metabolites, namely 2-(4-amino-2-hydroxybenzoyl)benzoic acid (AHB), 2-(4-(ethylamino)-2-hydroxybenzoyl)benzoic acid (EHB) and 2-(4-(diethylamino)-2-hydroxybenzoyl)benzoic acid (DHB), representing 52% of the administered oral dose. The three major metabolites are further converted into four minor metabolites with an additional hydroxyl group in the aniline moiety. Toxicokinetic parameters (amount excreted, t_{max}, elimination constant and half-life $t_{1/2}$) and conversion factors were determined for the three major metabolites. The conversion factors were used to estimate the mean daily exposure to Uvinul A plus® in spot urine samples from 58 volunteers not intentionally exposed to Uvinul A plus® derived from a pilot study. The three major metabolites were quantifiable in 26% of the samples. In 35% of the samples, at least one major metabolite could be quantified. The daily systemic exposure to Uvinul A plus® was estimated to approximately 8.1–9.3 µg/d by applying the combined conversion factor for all three major metabolites. In conclusion, a very low systemic exposure to DHHB was observed with regard to the no observed adverse effect level (NOAEL) as an established threshold for chronic uptake.

1. Introduction

UV-blocking substances are used to protect the human skin from the UV radiation of the sun, e.g. in sunscreens. Yet, UV filters are not exclusively used in sunscreens, but also in other cosmetic products like lipsticks, body lotions or hair-care products in order to protect the skin (Chisvert et al., 2012; Mikkelsen et al., 2015). Hexyl 2-[4-(diethylamino)-2-hydroxybenzoyl]benzoate (DHHB, CAS No. 302776-68-7), also known as Uvinul A plus® is one of 27 UV filter substances authorized in the EU. The maximum concentration allowed in cosmetic products was set to 10% (w/w) (The European Commission, 2009). According to the European Chemicals Agency, Uvinul A plus® (EC number 443-860-6) is produced in 100–1000 tons per year within the European Economic Area (European Chemicals Agency, 2019). Uvinul A plus® possesses its maximum of absorption at 354 nm (Vielhaber et al., 2006) and therefore mainly provides protection against UVA radiation (315–380 nm). In contrast to many other UV filters, Uvinul A plus® is photostable (Klimová et al., 2013; Kockler et al., 2012). Studies addressing the skin tolerance of Uvinul A plus® revealed a rare occurrence of contact dermatitis (The European Multicentre Photopatch Test Study (EMCPPTS) Taskforce, 2012) and weakly pronounced skin irritations (Kerr et al., 2009). However, studies in animals and a human repeated insult patch test with 10% of Uvinul A plus® showed no skin-irritating or sensitizing effects (European Chemicals Agency, 2019). Although many benzophenones and their derivatives were shown to act as endocrine disruptors (Wang et al., 2016), recent studies did not indicate that Uvinul A plus® influences the hormone system (Mikkelsen et al., 2015).

Despite the high production volume and usage within the EU, data

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on human exposure to Uvinul A plus® as well as the human metabolism of Uvinul A plus® is scarce. *In vivo* studies in rats revealed the cleavage of the ester moiety, leading to 2-(4-(diethylamino)-2-hydroxybenzoyl) benzoic acid (DHB) (European Chemicals Agency, 2019) as the only known metabolite so far. In addition the de-ethylation reaction at the amino group to form 2-(4-amino-2-hydroxybenzoyl)benzoic acid (AHB) and 2-(4-(ethylamino)-2-hydroxybenzoyl)benzoic acid (EHB) can be regarded as a plausible metabolic transformation. Hence, we developed a quantitative Ultra High Performance Liquid Chromatography-tandem mass spectrometry (UHPLC-MS/MS) method for the simultaneous determination of AHB, DHB, and EHB in urine and conducted a pilot study with 58 participants to estimate the exposure to DHHB in the general population, where at least one DHHB metabolite was observed in 35% of the samples collected. Moreover, four additionally hydroxylated DHHB metabolites were identified as minor metabolites (Stoeckelhuber et al., 2019). However, to date, data on urinary excretion kinetics and quantitative formation of urinary DHHB metabolites do not exist. Thus, we conducted a human metabolism study with five participants. The subjects were dermally and orally administered DHHB to identify the urinary metabolites that are suitable as biomarkers of exposure to DHHB, determine these biomarkers urinary elimination kinetics, and calculate conversion factors. We applied these conversion factors to estimate the DHHB dose in a group of 58 volunteers.

2. Materials and methods

2.1. Human studies

Studies were performed in accordance with the ethical standards of the Declaration of Helsinki (World Medical Association (WMA), 2013) and were approved by the Ethics Commission of the Bavarian State Chamber of Physicians (Reg. No.: 16091).

The method development and validation as well as the human studies presented for Uvinul A plus® are part of the large HBM project coordinated by the German Federal Ministry for the Environment, Nature Conservation and Nuclear Safety (BMU) and the German Chemical Industry Association (VCI). This cooperation aims to develop up to 50 new HBM methods for chemicals of concern in order to increase the knowledge on the internal exposure of the general population to those chemicals (Kolossa-Gehring et al., 2017).

Five healthy volunteers, 3 males and 2 females, aged between 28 and 68 were administered a single oral or dermal dose of Uvinul A plus®. Healthy male and female subjects aged between 18 and 70 years were considered for this study. More detailed information about the subjects is compiled in Table 1. The dermal and the oral administration of Uvinul A plus® was conducted at the Analytisch-biologisches Forschungslabor (ABF) in Planegg, Germany. The amount of Uvinul A plus® for oral administration was standardized for the body weight of the volunteers (0.295 mg/kg body weight Uvinul A plus®). The oral dosage was based on the estimated human exposure to Uvinul A plus® from sunscreens and other cosmetic products published by the Danish Environmental Protection Agency (Mikkelsen et al., 2015). The respective amount was dissolved in 4 mL of ethanol (99%) and further diluted in 100 mL water prior to the oral dosage. The dermal application was performed by spiking a commercially available Uvinul A plus®-free sunscreen with the test compound to a total amount of 10% (w/w). A defined amount of 0.59 g/kg body weight of the spiked sunscreen was applied to the arms, neck and chest of the subjects. Subjects were asked not to wash the relevant body parts until the cream was completely absorbed into the skin (approx. 15–30 min). The dermal dosage was adapted from the oral dosage by using an estimated dermal absorption rate of 0.5% (Mikkelsen et al., 2015). A urine sample was collected before each application to determine the background exposure of the subjects. After application, all urine fractions of the following 72 h were collected and stored at −20 °C until analysis. Collection times of all urine fractions and times of administration were recorded. All subjects were advised to omit sunscreens and anti-aging products containing UV-blockers throughout the conduct of the study. A wash out period of at least one week was kept between oral and dermal treatment of the subjects to ensure that DHHB and its metabolites from the previous administration were completely eliminated. The metabolism study was performed in February and March 2018. All subjects collected all urine voids of the following 72 h after oral or dermal application. All samples were immediately frozen after sample collection and stored at −20 °C until analysis. Storage time between sample collection and first analysis did not exceed five months.

Spot urine samples from 58 volunteers who were not exposed occupationally to DHHB were collected and analyzed with the validated method to estimate the exposure to DHHB in the general population (Stoeckelhuber et al., 2019). Samples were obtained from 27 female and 31 male healthy volunteers aged between 18 and 83 living in the greater Munich Area. No additional restrictions regarding the selection of the volunteers and the time of the urine collection were set.

2.2. Chemicals and reference compounds

Uvinul A plus® (log P_{OW} 6.2, aqueous solubility < 0.01 mg/L at 20 °C (Scientific Commitee on Consumer Safety (SCCS), 2008)) was obtained from BASF SE (Ludwigshafen, Germany). 2-(4-(Diethylamino)-2-hydroxybenzoyl)benzoic acid (DHB) was supplied from Sigma-Aldrich (Taufkirchen, Germany). 2-(4-Amino-2-hydroxybenzoyl)benzoic acid (AHB) was synthesized as reported elsewhere (Stoeckelhuber et al., 2019). 2-(4-(Ethylamino)-2-hydroxybenzoyl)benzoic acid (EHB) and the deuterated internal standards D_4-AHB, D_5-EHB and D_{10}-DHB were custom synthesized according to the protocol given in Stoeckelhuber et al. (2019). 4-Methylumbelliferyl-ß-D-glucuronide-dihydrate (MUG) was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Ethyl acetate and methanol were obtained from Th. Geyer (Renningen, Germany) and ethanol was from LGC Standards (Wesel, Germany). LC-MS grade acetonitrile with 0.1% formic acid and formic acid were purchased from Biosolve Chimie (Dieuze, France). Aqueous phosphoric acid 85%, potassium dihydrogen phosphate and disodium hydrogen phosphate was supplied by VWR International (Leuven, Belgium). The water used was filtered through a Sartorius arium (Göttingen, Germany) water system. For enzymatic hydrolysis β-glucuronidase from *E. coli* was obtained from Megazyme (Bray, Ireland) and β-glucuronidase/arylsulfatase from *H. Pomatia* was purchased from Sigma-Aldrich. Other chemicals and reagents used were at least of analytical grade.

Table 1
Characteristics of the subjects taking part in the metabolism study.

Subject	Age [years]	Gender	Body weight [kg]	Oral DHHB dose [mg]	Urine fractions collected over 72 h (oral)	Dermal DHHB dose [g]	Urine fractions collected over 72 h (dermal)
1	68	male	88	26.0	18	5.19	17
2	28	female	90	26.6	22	5.31	14
3	30	male	85	25.1	15	5.02	15
4	35	male	78	23.0	31	4.60	29
5	34	female	62	18.3	27	3.66	27

2.3. Analytical methods

1 mL urine was spiked with 10 µL of an aqueous internal standard mixture (D_4-AHB, D_5-EHB and D_{10}-DHB, each 1 µg/mL) and 10 µL of an aqueous solution containing 4-methylumbelliferyl glucuronide (MUG, 50 µg/mL). 0.5 mL of a phosphate buffer (pH 6.4, 1/9 M) and 10 µL enzyme mix with β-glucuronidase from *E. coli* (125000 units/mL) were added for enzymatic hydrolysis and the sample was incubated at 37 °C for 1.5 h. Subsequently, extraction of analytes was achieved by liquid-liquid-extraction (LLE). For this purpose, the sample was acidified with 25 µL phosphoric acid (8.5% w/w) to a pH of 4.7 ± 0.2 and extracted with 2 mL ethyl acetate. The organic phase was evaporated to dryness using a Speed Vac concentrator without heating and reconstituted in 100 µL of methanol. Due to the high analyte concentrations expected in the samples of the metabolism study, reduced urine volume was used for analysis. 10 µL instead of the established 1 mL of urine were used and diluted with 1 mL water. The dilution factor of 100 was considered for the calculation of the results. Samples with concentrations below LOQ for any analyte were repeated with the original sample volume of 1 mL urine without dilution. Accuracy after dilution was proven during method validation (accuracy within 85–115% after 100-fold dilution) (data not shown).

To carry out the chromatographic separation a Nexera X2 UHPLC system (Shimadzu, Neufahrn, Germany) was used together with an Acquity UPLC BEH Phenyl column (1.7 µm, 2.1 × 100 mm; Waters, Eschborn, Germany) equipped with an Acquity UPLC BEH Phenyl VanGuard (1.7 µm, 2.1 × 5 mm). The column was operated at 40 °C and the flow rate was set to 0.5 mL/min. Water (eluent A) and acetonitrile (eluent B) each containing 0.1% formic acid were used for gradient elution. Gradient elution was achieved starting at 90% of A for two minutes and then decreasing A linearly to 0% over two more minutes. 0% of A was maintained for 1.5 min followed by an immediate increase to 90% A and held for one minute until the end of the run at 6.5 min. The UHPLC system was connected to a 6500+ QTrap® from Sciex (Darmstadt, Germany). The mass spectrometer was operated in ESI positive mode and scheduled MRM was applied to determine the analytes.

To ensure data integrity and quality, internal quality control samples (QCs) were prepared in three different concentration levels (low, medium and high) and stored at −20 °C until analysis. Two QCs per concentration level were prepared and analyzed in each analytical run. Samples were quantified by linear calibration using 1/x weighting. The analytical run was regarded as valid if accuracy of the QCs and calibrators was within 85–115% and 80–120% for concentrations below three times LOQ. All acceptance criteria were fulfilled and the analytical batches were accepted as valid.

The three highest concentrated samples after oral application and the highest concentrated sample after dermal application of each subject were analyzed in duplicates. We analyzed the samples twice: first, using the above procedure to obtain the total concentrations; and second, using the above procedure but omitting the enzymatic hydrolysis step to quantify the unconjugated forms of DHHB metabolites. The enzymatic hydrolysis was performed using β-glucuronidase from *E. coli* and β-glucuronidase/arylsulfatase from *H. Pomatia,* respectively, to estimate the amounts of sulfate and glucuronide conjugates. With this approach the ratios between the free and the conjugated (glucuronidated or sulfated) forms of the metabolites AHB, EHB and DHB could be determined to obtain the percentage of the conjugated phase II metabolites. Due to the high analyte concentrations expected, all samples were analyzed with 10 µL instead of 1 mL of urine diluted with 1 mL water.

For the analysis of additionally hydroxylated metabolites a preliminary method was used for the sample preparation. To 1 mL of urine, 0.5 mL acetate buffer (pH = 5.1, 1 M) and 10 µL of an enzyme mixture containing β-glucuronidase and arylsulfatase (4.5 and 14 units/mL) were added. The sample was incubated overnight and acidified with 25 µL of phosphoric acid (4 M). The sample was extracted with 1.5 mL ethyl acetate and the organic phase was evaporated to dryness and reconstituted in 100 µL of methanol. 5 µL of the extract were injected into the UPLC-MS/MS system and analyzed for additionally hydroxylated metabolites by conducting Q-Trap experiments according to the method described above. Q-Trap experiments included an Enhanced MS Scan (EMS) to screen for mono- or dihydroxylated species of the metabolites followed by Enhanced Product Ion Scans (EPIs) to identify suitable mass transitions for MS/MS detection. For further details refer to (Stoeckelhuber et al., 2019).

2.4. Data evaluation

Toxicokinetic parameters (the amount excreted after 72 h, the percentage of the total oral dose excreted, the time of the maximum excretion, the percentage doses excreted after defined time points (3, 6, 12, 24 and 48 h), the elimination constant and the elimination half-life) for the urinary excretion of DHHB metabolites were evaluated individually for each subject. Where appropriate, means, standard deviations (SD) and medians were calculated. The amount of metabolites excreted 3, 6, 12, 24 and 48 h after application of DHHB were calculated by linear interpolation between the closest urine voiding time points. Urinary excretion of each metabolite was assumed to be complete after 72 h. The time points of dermal and oral administration of DHHB was defined as baseline and all following urine samples collected were related to those time points. The blank samples were collected right before dermal and oral administration and calculated blank concentrations of DHHB metabolites were subtracted. Urinary elimination constants (k_{el}) and elimination half-lives ($t_{1/2}$) were calculated using the 'sigma-minus' method (Klotz, 1984), with the total amount excreted ($Ae(\infty)$) being the amount excreted within 72 h. Conversion factors (CFs) are defined as the ratio between the total dose applied (D in mol) and the molar amount of a metabolite (or the sum of several metabolites) excreted within the first 72 h after oral uptake ($CF = D/A_{MX72h}$). For statistical analysis, values below LOQ were set to LOQ/2. The LOQ for AHB and EHB was 0.1 ng/mL while DHB was measured with an LOQ of 0.05 ng/mL.

3. Results

3.1. Toxicokinetic parameters after oral application

The subjects received a body weight-standardized dose of 0.295 mg/kg bw Uvinul A plus®, resulting in a total amount of 18.3–26.6 mg Uvinul A plus® administered orally to the 5 subjects (Table 1). Those samples, collected shortly before application (U0), showed no or only small amounts, mostly below LOQ, of the analytes. The highest value at baseline was observed for subject 1 with 1.5 ng/mL of EHB. Baseline concentrations were subtracted from the concentrations for each sample after administration. Fig. 2shows the time courses of the excretion of DHHB metabolites from subject 1 after oral application as excreted analytes in µg per hour (A) and cumulated excretion (B) throughout the entire study. Similar time courses were obtained for subjects 2–5 and can be found in the Supplementary Files. As expected, the parent substance DHHB was not detected in any sample of this study. Ratios between conjugated and non-conjugated forms of the major DHHB metabolites are shown in Table 2. DHB is almost exclusively occurring as glucuronide (90%) while EHB and AHB are conjugated to glucuronic acid to only 63 and 31%, respectively. Sulfated conjugates could not be detected in a significant amount. Ratios between conjugated and non-conjugated forms were similar in samples collected after oral or dermal application within the subjects. Inter-individual variations in the ratios between conjugated and non-conjugated forms among the subjects were found to be between 11 and 18% for the three analytes. The three major metabolites exhibit similar excretion patterns with a maximum excretion after about four to five

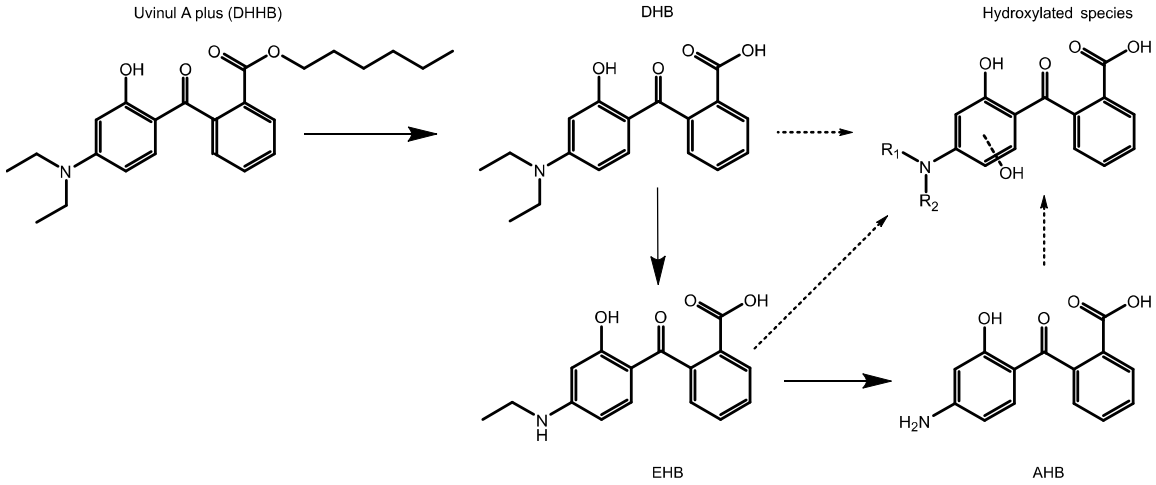

Fig. 1. Postulated metabolic pathway for DHHB in humans. Conjugated metabolites are not shown; R_1 = H or ethyl. R_2 = H or ethyl.

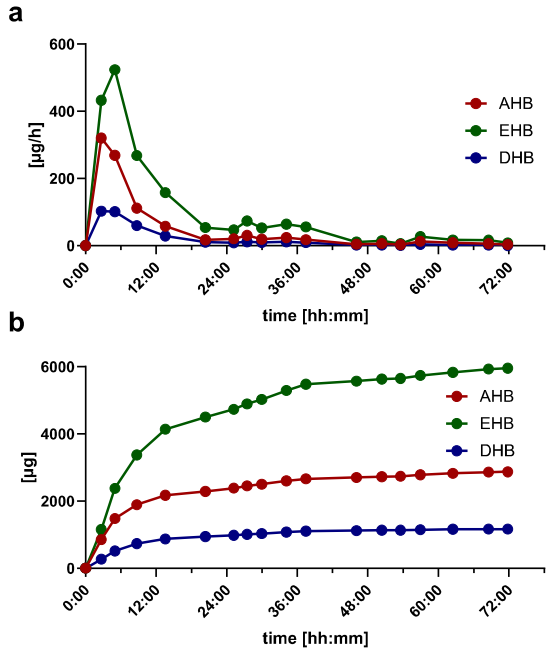

Fig. 2. Time courses of the urinary excretion of DHHB metabolites after oral administration of DHHB to subject 1 displayed as **A** excretion rate in µg/h and **B** cumulative excretion in µg.

hours. EHB was found at the highest concentrations of all three metabolites and accounts for about 33% of the total dose given, followed by AHB (16%) and DHB (5%). Elimination half-lives were found to be around 4.5 h across all analytes. The amounts excreted showed a fast decline after the first 24 h of oral application. After 48 h over 95% of the total amount was excreted, however even after 72 h minor levels of AHB and EHB were quantifiable, which make up to 12 µg and 50 µg, respectively. After 72 h, 54% of the orally applied dose was recovered through the metabolites AHB, EHB and DHB. Conversion factors were calculated for each single metabolite and all possible combinations (see Table 3).

Table 2
Ratios between conjugated and non-conjugated forms of the major DHHB metabolites. Values were obtained from the three highest concentrated samples after oral application and the highest concentrated sample after dermal application of each subject.

Subject	Amount of conjugated form [%]		
	AHB	EHB	DHB
1	43	84	97
2	31	75	98
3	10	32	61
4	38	67	95
5	32	56	100
Mean ± SD	31 ± 11	63 ± 18	90 ± 15
Min-Max	9–54	19–89	45–100
Median	32	67	97

3.2. Toxicokinetic parameters after dermal application

The subjects received a body weight-standardized dose of 0.059 g/kg bw Uvinul A plus®, resulting in a total amount of 3.66–5.31 g Uvinul A plus® administered dermally in the form of a DHHB free sunscreen spiked with 10% (w/w) of Uvinul A plus® (Table 1). Even though there was a wash out period of at least one week between the oral and dermal application, metabolite levels of up to 4 µg/mL for AHB and 9 µg/mL for EHB were observed in the urines collected at baseline of the dermal application (U0) for subjects 1–3. Consequently, baseline levels were taken into account for the calculation of the respective metabolite concentrations after application in analogy to the oral dosing. As a consequence, the wash out period was prolonged to at least three weeks for subjects 4 and 5. Subsequently, baseline urines were empty for DHB, and AHB as well as EHB showed significantly lower levels compared to subjects 1–3. Fig. 3 shows the time courses of the excretion of DHHB metabolites from subject 1 after dermal application as amounts excreted per hour (A) and cumulated excretion (B) throughout the entire collection period. Similar time courses were obtained for subjects 2–5 and can be found in the Supplementary Files. Urinary excretion began approximately three hours after dermal application for the three major metabolites with a first explicit increase in metabolite excretion after 24 h. Excretion across analytes appeared in waves with maximum excretion rates after 24, 43 and 56 h still being not complete after 72 h. As for the oral application, no DHHB was detected in any sample. After 72 h, less than 0.02% of the dermally applied dose was absorbed based

Table 3
Toxicokinetic results from the metabolism study with 5 subjects after oral application of DHHB.

		AHB	EHB	DHB
Amount excreted after 72 h [µmol] ($A_{e(\infty)}$)	**Mean ± SD**	**9.7 ± 2.4**	**19.4 ± 4.8**	**3.1 ± 0.8**
	Median; Min-Max	9.9 (7.1–13.4)	19.4 (13.8–27.7)	3.1 2.0–3.8)
Percent of oral dose PMxD [%]	**Mean ± SD**	**16.3 ± 3.9**	**32.8 ± 8.6**	**5.1 ± 1.1**
	Median; Min-Max	15.5 (11.3–23.2)	31.9 (21.9–47.8)	5.4 (3.5–6.5)
t_{max} [h]	**Mean ± SD**	**4.4 ± 1.8**	**4.9 ± 1.5**	**4.9 ± 1.5**
	Median; Min-Max	4.0 (2.6–6.8)	5.0 (2.6–6.8)	5.0 (2.6–6.8)
Excreted after 3 h [%]	**Mean ± SD**	**32.9 ± 5.2**	**23.4 ± 4.7**	**21.6 ± 3.6**
	Median; Min-Max	32.8 (27.5–42.5)	22.3 (17.3–31.3)	21.4 (17.3–26.4)
Excreted after 6 h [%]	**Mean ± SD**	**52.8 ± 10.1**	**43.5 ± 10.6**	**40.4 ± 8.1**
	Median; Min-Max	48.1 (44.1–71.4)	43.4 (33.3–62.6)	41.1 (29.3–49.5)
Excreted after 12 h [%]	**Mean ± SD**	**71.9 ± 10.5**	**67.2 ± 8.3**	**65.4 ± 3.6**
	Median; Min-Max	72.5 (57.0–84.8)	65.3 (58.6–80.7)	69.3 (47.7–72.4)
Excreted after 24 h [%]	**Mean ± SD**	**85.8 ± 7.7**	**83.4 ± 7.4**	**84.1 ± 7.0**
	Median; Min-Max	83.7 (74.4–95.6)	79.3 (75.0–92.5)	84.6 (71.7–92.5)
Excreted after 48 h [%]	**Mean ± SD**	**96.0 ± 3.1**	**95.4 ± 3.4**	**96.0 ± 3.6**
	Median; Min-Max	96.1 (90.9–99.7)	94.6 (90.3–99.6)	96.5 (89.2–99.5)
Elimination constant k_{el} [h^{-1}]	**Mean ± SD**	**0.17 ± 0.05**	**0.17 ± 0.05**	**0.17 ± 0.05**
	Median; Min-Max	0.17 (0.11–0.24)	0.16 (0.11–0.24)	0.16 (0.11–0.25)
Elimination half-life $t_{1/2}$ [h]	**Mean ± SD**	**4.58 ± 1.39**	**4.58 ± 1.39**	**4.33 ± 1.21**
	Median; Min-Max	4.16 (2.84–6.28)	4.45 (2.91–6.42)	4.37 (2.80–6.31)
Conversion factor (72 h, single)	**Mean ± SD**	**6.5 ± 1.4**	**3.3 ± 0.8**	**20.6 ± 4.4**
	Median; Min-Max	6.5 (4.3–5.9)	3.1 (2.1–4.6)	18.7 (17.5–28.9)
Conversion factor (72 h, combined)	**Mean ± SD**	**2.6 ± 0.8**		
AHB + EHB	Median; Min-Max	2.0 (1.4–3.0)		
Conversion factor (72 h, combined)	**Mean ± SD**	**2.8 ± 0.6**		
EHB + DHB	Median; Min-Max	2.7 (1.8–4.0)		
Conversion factor (72 h, combined)	**Mean ± SD**	**5.9 ± 1.7**		
AHB + DHB	Median; Min-Max	5.0 (3.4–6.8)		
Conversion factor (72 h, combined)	**Mean ± SD**	**2.0 ± 0.4**		
AHB + EHB + DHB	Median; Min-Max	1.8 (1.3–2.7)		

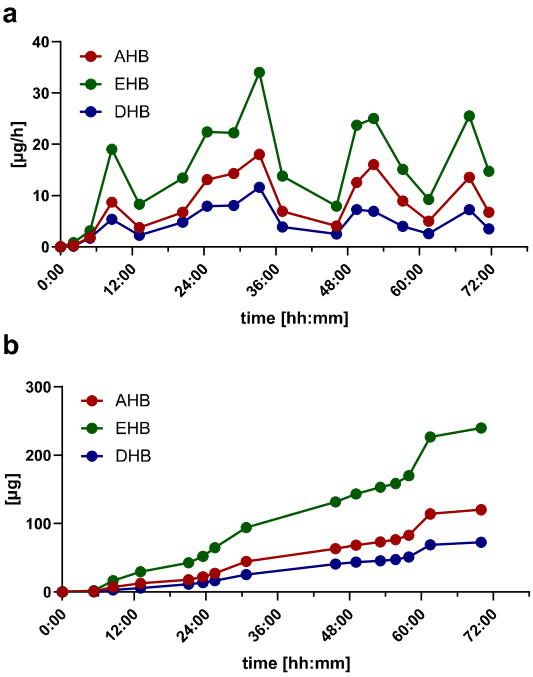

Fig. 3. Time courses of the urinary excretion of DHHB metabolites after dermal administration of DHHB to subject 1 displayed as **A** excretion rate in µg/h and **B** cumulative excretion in µg.

on the determined amounts of AHB, EHB and DHB. Skin absorption rates between 0.003 and 0.02% were calculated for the 5 subjects based on the cumulated concentrations of AHB, EHB and DHB (see Table 4).

3.3. Estimated dose of DHHB in non-occupationally exposed subjects

Spot urine samples from 58 subjects were analyzed for AHB, EHB and DHB (Stoeckelhuber et al., 2019). For the back-calculation of the virtual uptake dose, urinary metabolite concentrations were standardized to creatinine. Daily creatinine excretions were assumed to be in the range of 1.2 and 1.5 g for females and males, respectively (Barr et al., 2005). The highest excretion rates were determined for EHB with 7.1 nmol/d, followed by AHB (3.6 nmol/d) and DHB (0.6 nmol/d). Conversion factors (CF) were calculated from the results of the oral application of the metabolism study for each of the 3 metabolites and all combinations thereof. Table 5 summarizes the estimated daily uptake doses of DHHB, calculated from a combined conversion factor considering AHB, EHB and DHB. At least one of the three DHHB metabolites was observed in 35% of the urine samples. Calculations of the virtual systemic uptake doses using the different single or combined conversion factors led to similar average exposure doses for DHHB from 8.1 to 9.3 µg/d. As an exception, an average exposure dose of 4.5 µg/d DHHB was determined applying the conversion factor based on DHB only. As DHB was the least occurring of the three metabolites resulting in more samples below LOQ, the calculation of the conversion factor for DHB may be less accurate.

3.4. Determination of additionally hydroxylated metabolites

Samples from subjects 1 and 3 were analyzed for additionally hydroxylated species using a preliminary sample preparation (Stoeckelhuber et al., 2019). The identified secondary metabolites of AHB, EHB, and DHB containing an additional hydroxyl function are summarized in Fig. 1. The additionally hydroxylated species were identified by means of QTrap experiments. For this purpose Enhanced MS Scan and Enhanced Product Ion Scan experiments were conducted (Stoeckelhuber et al., 2019). However, the position of the hydroxyl moiety could not be unambiguously determined due to the lack of authentic reference standards (Stoeckelhuber et al., 2019). Fig. 4 shows

Table 4
Estimation of the dermal absorption for DHHB. The DHHB dose dermally applied was multiplied with the percentage excreted after oral application (resulting in the virtual amount with 100% dermal absorption). For the calculation of the dermal absorption, the amount actually excreted after dermal application was divided by the value corresponding to 100% absorption.

Subject	DHHB dose dermally applied [µmol]	Amount excreted after dermal application [µmol]	Percentage excreted after oral application [%]	Dermal absorption
1	65406	7.66	54.7%	0.0214%
2	66915	4.48	48.0%	0.0139%
3	63141	1.54	36.6%	0.0067%
4	57859	1.54	77.5%	0.0034%
5	46035	2.07	54.3%	0.0083%

the excretion pattern for the additionally hydroxylated species AHB-OH, EHB-OH and DHB-OH. For DHB-OH two peaks with similar retention times were quantified indicating two different hydroxylated species of DHB. All four metabolites were excreted in a very similar pattern compared to the major metabolites. Amounts of the hydroxylated analytes were estimated based on the observed peak areas and referred to the peak areas of the corresponding major metabolites AHB, EHB and DHB, assuming similar MS responses of the analytes. Based on these assumptions, the secondary, hydroxylated metabolites would be present at about 100-fold lower concentrations than the major metabolites.

4. Discussion

In this study the urinary excretion of metabolites of the UV-filter Uvinul A plus® after oral and dermal application was investigated in five subjects. All urine samples collected were analyzed by applying a recently developed UHPLC-MS/MS method (Stoeckelhuber et al., 2019). The present paper is, to our knowledge, the first study, which investigated systematically the human metabolism of Uvinul A plus® after a defined oral and dermal dose. The data obtained were used to identify the major metabolites of DHHB and their urinary elimination kinetics as well as to calculate the absolute systemic uptake of DHHB based on the CF deduced from the oral study (Koch et al., 2007).

As the human metabolism of DHHB was unknown, only assumptions concerning the chemical structures of urinary DHHB metabolites could be made. DHB was previously detected in a metabolism study in rats (European Chemicals Agency, 2019). DHB, therefore, was very likely to be formed in humans as well by ester hydrolysis of DHHB being a common metabolic route catalyzed by ubiquitous esterases (European Chemicals Agency, 2019). The prodrug strategy for instance, takes advantage of this pathway in several orally applied drugs like the ACE inhibitors trandolapril or quinapril (Hajnal et al., 2016). AHB and EHB were postulated to be formed by ester hydrolysis followed by *N*-de-

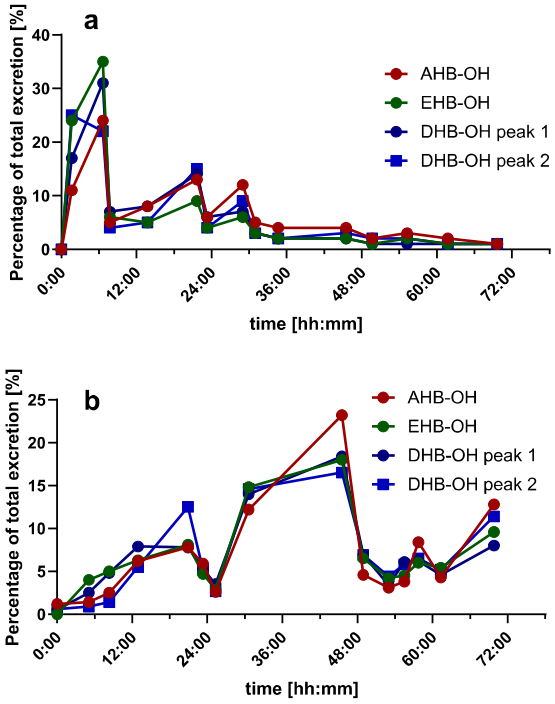

Fig. 4. Time courses of the urinary excretion of additionally hydroxylated metabolites after oral (**A**) and dermal (**B**) administration of DHHB from subject 3. The total amount of one analyte excreted after 72 h refers to 100%.

Table 5
Amounts of excreted DHHB metabolites in samples of a small population (n = 58). Values were corrected for dilution by standardizing for creatinine excretion. Single and combined CFs after 72 h were used for calculations.

CF		Urinary excretion		Equivalent oral dose	
		nmol/g crea	nmol/d	nmol/d	µg/d
AHB	**Mean ± SD**	**2.5 ± 10.1**	**3.6 ± 15.1**	**23.3 ± 97.5**	**9.3 ± 38.8**
	Median (min - max)	0.5 (0.1–74.8)	0.6 (0.1–112.1)	4.2 (0.6–724.1)	1.7 (0.2–287.9)
EHB	**Mean ± SD**	**4.9 ± 22.6**	**7.1 ± 33.9**	**23.2 ± 110.5**	**9.2 ± 43.9**
	Median (min - max)	0.5 (0.1–164.4)	0.6 (0.1–246.7)	1.9 (0.3–804.0)	0.8 (0.1–319.6)
DHB	**Mean ± SD**	**1.2 ± 5.5**	**0.6 ± 2.6**	**11.3 ± 53.2**	**4.5 ± 21.2**
	Median (min - max)	0.2 (0.03–40.8)	0.1 (0.01–19-2)	1.4 (0.2–395.2)	0.5 (0.1–157.1)
AHB + EHB	**Mean ± SD**	**7.4 ± 32.6**	**10.7 ± 49.0**	**23.2 ± 105.9**	**9.2 ± 42.1**
	Median (min - max)	0.9 (0.1–239.2)	1.3 (0.2–358.8)	2.7 (0.4–776.5)	1.1 (0.2–308.7)
AHB + DHB	**Mean ± SD**	**3.7 ± 15.6**	**4.2 ± 17.7**	**20.4 ± 86.8**	**8.1 ± 34.5**
	Median (min - max)	0.6 (0.1–115.6)	0.7 (0.1–131.3)	3.5 (0.5–644.7)	1.4 (0.2–256.3)
EHB + DHB	**Mean ± SD**	**6.1 ± 28.1**	**7.7 ± 36.5**	**21.5 ± 102.4**	**8.6 ± 40.7**
	Median (min - max)	0.5 (0.1–205.3)	0.7 (0.1–265.9)	1.9 (0.3–746.2)	0.8 (0.1–296.6)
AHB + EHB + DHB	**Mean ± SD**	**8.6 ± 38.1**	**11.3 ± 51.5**	**22.1 ± 100.8**	**8.8 ± 40.1**
	Median (min -max)	1.1 (0.1–280.0)	1.3 (0.2–378.0)	2.6 (0.4–739.2)	1.0 (0.1–293.8)

ethylation of the amino group, which is a frequent metabolic pathway observed in similar compounds like 2-ethylhexyl 4-(*N*,*N*-dimethylamino)benzoate (EDP) (León et al., 2010) and Chloroquine (Bergqvist and Domeij-nyberg, 1983). Our study revealed that EHB was excreted at the highest levels followed by AHB in similar concentrations and DHB at considerably lower quantities (EHB > AHB ≫ DHB). In conclusion, ester hydrolysis and *N*-dealkylation of one or both alkyl chains of DHHB yielded the major metabolites EHB and AHB (Fig. 1). Hence, the postulated metabolic pathways could be confirmed in our study with human subjects. Additionally, all metabolites possess a carboxylic and a hydroxy function which can be conjugated to form glucuronides or sulfates. To investigate the presence of conjugates and to capture the whole amount of DHHB metabolites (conjugated + unconjugated), an enzymatic hydrolysis step was performed before sample extraction. For enzymatic hydrolysis, β-glucuronidase from *E. coli* was used converting glucuronidated, but not sulfated metabolites into their unconjugated forms. For this reason, another enzyme mixture from *Helix pomatia* combining β-glucuronidase as well as arylsulfatase activity was used resulting in similar levels of unconjugated DHHB metabolites, indicating that DHHB metabolites do not contain sulfates in significant amounts (Stoeckelhuber et al., 2019). The enzyme extract from *Helix pomatia* was found to possess additional esterase and lipase activities (Blount et al., 2000), the products which could interfere with the determination of DHHB metabolites. Particularly, the esterase activity of the enzyme extract could lead to an ester cleavage of DHHB, resulting in overrated DHB concentrations. Therefore, β-glucuronidase from *E. coli* was used generally for enzymatic hydrolysis as precaution even though DHHB could not be detected in our experiments. Since all metabolites are at least partly conjugated to glucuronic acid (Table 2), enzymatic hydrolysis was found to be a necessary step in sample preparation in order to capture the whole amount of conjugated and unconjugated metabolites.

Apparently, the polarity of the compounds triggers the percentage of conjugation. DHB which is the most lipophilic metabolite is almost completely converted into the more polar conjugate before urinary excretion, whereas AHB as the most polar metabolite apparently is being already excreted in its free form to a high percentage. Moreover, conjugation of the metabolites strongly varies between the subjects, e.g. DHHB metabolites in samples from subject 3 are conjugated to a much smaller extent as compared to the other subjects. No difference in the conjugation pattern between samples after oral or dermal application was observed. Additionally, hydroxylated species were excreted in a similar time pattern compared to their analogues AHB, EHB and DHB. Therefore, it is very likely that the species detected are subordinate metabolites of DHHB. We tried to decipher further metabolites containing one or two additional hydroxyl groups in the major DHHB metabolites. We could only identify species possessing one additional hydroxyl group (Fig. 1). For additionally hydroxylated DHB (DHB-OH), two peaks could be detected in the MRM transitions, chromatographically eluting shortly after each other, suggesting two different positions of the hydroxylation (Stoeckelhuber et al., 2019). In fact, additionally hydroxylated metabolites could be only determined in samples with very high concentrations of the major metabolites (EHB, AHB, and/or DHB) after oral or dermal administration. As there was no reference material available during the course of our investigations, the amounts were estimated assuming a similar detector response as for the corresponding major metabolites. The additionally hydroxylated species were estimated to represent 0.5–1% of their major metabolites which would be equal to about 0.5% of the DHHB dose. Thus, the metabolites bearing an additional hydroxyl moiety are not suitable biomarkers of exposure to DHHB due to their very low abundance and can therefore be neglected for a human biomonitoring. In contrast EHB, AHB, and DHB proved their suitability as biomarkers of DHHB exposure in our study with 58 non-occupationally exposed subjects from the general population (Stoeckelhuber et al., 2019).

Since DHHB as well as its three major metabolites possess a variety of functional groups at the basic benzophenone structure all three biomarkers are likely to be specific for DHHB exposure. Especially the benzophenone structure combined with an (ethyl-)amino group is unique to DHHB in contrast to other UV-filters based on the benzophenone structure which lack the aniline group (Chisvert et al., 2012). In addition, a strong correlation between AHB, EHB and DHB was found in urine samples obtained from 58 volunteers emphasizing the specificity as well (Stoeckelhuber et al., 2019). Although half-lives calculated from the metabolism study after oral application of DHHB (see Table 3) were found to be short, DHHB metabolites were quantified in 35% of the samples from non-occupationally exposed subjects (Stoeckelhuber et al., 2019).

Maximum excretion rates after oral application were already observed in the first urine fractions voided 4–5 h after DHHB administration. Half-lives were estimated to range from 4.3 to 4.6 h, proving the fast excretion kinetics for all three metabolites. Nevertheless, even after 72 h low amounts of DHHB metabolites were measured showing that excretion still was not complete. Yet, the concentrations found in the last fractions (after 24–36 h) were very low and only contributed to a negligible extent to the calculated toxicokinetic parameters. Even one week after oral application low levels in the range of the LOQ of AHB and EHB were observed in the urine samples indicating a continuous, slow excretion of the residual metabolites even one week after the peak excretion.

By quantifying AHB, EHB and DHB in the urine samples of the metabolism study, approximately 54% (37–78%) of the oral DHHB dosage could be absorbed but only approximately 0.008% of the administered dose could be found after dermal exposure on average. Apparently, dermal absorption was very low at least for the formulation applied in the dermal study (0.003–0.02%). In *in vitro* experiments with human skin which always yield higher absorption rates as compared to in vivo, a slightly higher skin absorption rate of 0.5% was reported (Mikkelsen et al., 2015; van Ravenzwaay and Leibold, 2004a, b). DHHB is a nonpolar compound with low solubility in water below 0.01 mg at pH 6–7 and 20 °C (Scientific Commitee on Consumer Safety (SCCS), 2008) favoring a long retention on the skin rather than penetrating through the skin. It has to be emphasized that besides the chemical properties of DHHB itself, the formulation which is applied has a high influence on the skin absorption. Since DHHB is applied to protect the skin from UV irradiation sunscreens are formulated to remain on the skin and a low permeation is favorable. In addition, the low polarity of the molecule suggests a slow penetration through the skin (Souza and Maia Campos, 2017). Indeed, longer elimination kinetics of AHB, EHB and DHB were observed after dermal administration (Fig. 3). This may be caused by a poor skin absorption and slow penetration of DHHB resulting in a continuous systemic uptake after dermal exposure (Hiller et al., 2019). The wavelike elimination kinetics may be explained by a subcutaneous or cutaneous reservoir (Hiller et al., 2019), which releases and resumes DHHB and/or its metabolites decelerating the systemic uptake resulting in decreased urinary excretion rates of AHB, EHB and DHB as compared to the oral administration.

Conversion factors were calculated based on the observed metabolite concentrations for the oral administration in order to estimate the amount of DHHB uptake by subjects from the general population. The conversion factors are defined as the ratio between the total dose applied (D in mol) and the amount of a metabolite (or the sum of several metabolites; A_{MX72h}) excreted within the first 72 h after oral uptake ($CF = D/A_{MX72h}$). CFs were calculated for the most abundant DHHB metabolite as well as for various combinations of molar sums of the major metabolites (Table 3). Applying CFs to urinary metabolite concentrations result in exposure doses which correspond to a virtual equivalent oral dose (Table 5). Dermal absorption calculated from the data of the metabolism study after dermal application was found to be in the range between 0.003 and 0.02%. This low skin absorption suggests that most of the DHHB dermally applied in our study was not absorbed by the subjects from the sunscreen formulation used. The

equivalent oral doses calculated with the different single and combined CFs are listed in Table 5 and range from 8.1 to 9.3 µg/d. Only the equivalent oral dose calculated with the CF for DHB (4.5 µg/d) clearly differs from the other equivalent oral doses calculated. This may be caused by the low concentrations measured for DHB, with several results below LOQ, and, therefore, a higher uncertainty. The most suitable CF for the back calculation of DHHB exposure was the combined conversion factor including the molar sum of AHB, EHB and DHB, since all three major metabolites are rated as suitable biomarkers of exposure to DHHB. Their combination into one CF should lead to more reliable values compared to the individual CFs. An average equivalent oral dose of approximately 9 µg per day was calculated for the 58 samples from the general population by means of the applied CF. No Observed Adverse Effect Levels (NOAELs) for rats after oral gavage of Uvinul A plus ® for 90 days were reported to be between 1250 mg/kg body weight/d for male and 1450 mg/kg body weight/d for female rats (European Chemicals Agency, 2019). Studies concerning the reproductive toxicity in rats lead to NOAELs in the range between 100 and 1000 mg/kg body weight/d (European Chemicals Agency, 2019). The lowest NOAEL reported with 100 mg/kg body weight/d is about 100,000 fold higher than the calculated daily intake of Uvinul A plus ® found within our studies giving a very high margin of safety.

5. Conclusion

In our human study, we identified seven metabolites of the UV filter Uvinul A plus®. Three major metabolites (AHB, EHB and DHB) were analyzed in all samples after oral or dermal administration of DHHB. Four additionally hydroxylated metabolites were determined at presumably 100-fold lower concentration levels as compared to the major metabolites. Due to the negligible levels of the minor metabolites, only the three major metabolites were regarded as suitable biomarkers of exposure to DHHB. We evaluated the exposure to DHHB in a small group of 58 subjects from the general population. The three major DHHB metabolites were detected in 35% of the samples of this group. Applying the CFs determined in the study with a defined oral dose of DHHB resulted in a virtual systemic daily exposure dose of 9 µg/d DHHB for the 58 subjects. Based on this first pilot study, the systemic exposure to DHHB appears to be well below the suggested exposure limits. Larger human biomonitoring studies are required to confirm these observations in a representative population.

Funding

This study is part of an ongoing 10-year project on the advancement of human biomonitoring in Germany. The project is a cooperation agreed in 2010 between the Federal Ministry for the Environment, Nature Conservation, and Nuclear Safety (BMU) and the German Chemical Industry Association (Verband der chemischen Industrie e.V. – VCI) and managed by the German Environment Agency (UBA). Experts from government authorities, industry, and science accompany and advise the project in selecting substances and developing methods. Within this cooperation project the analytical method development and the human metabolism study are financed by the Chemie Wirtschaftsförderungsgesellschaft, Frankfurt/Main, Germany.

Declaration of competing interest

The authors in general declare that they have no conflict of interest. One of the authors (E.L.) is employed by a manufacturer of Uvinul A plus®, but the opinions expressed in the paper are those of the author and the chemical manufacturer had no role in the study design, data collection, analysis or interpretation of the study findings.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijheh.2020.113509.

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Supplementary information to:

Human metabolism and urinary excretion kinetics of the UV filter Uvinul A plus after a single oral or dermal dosage

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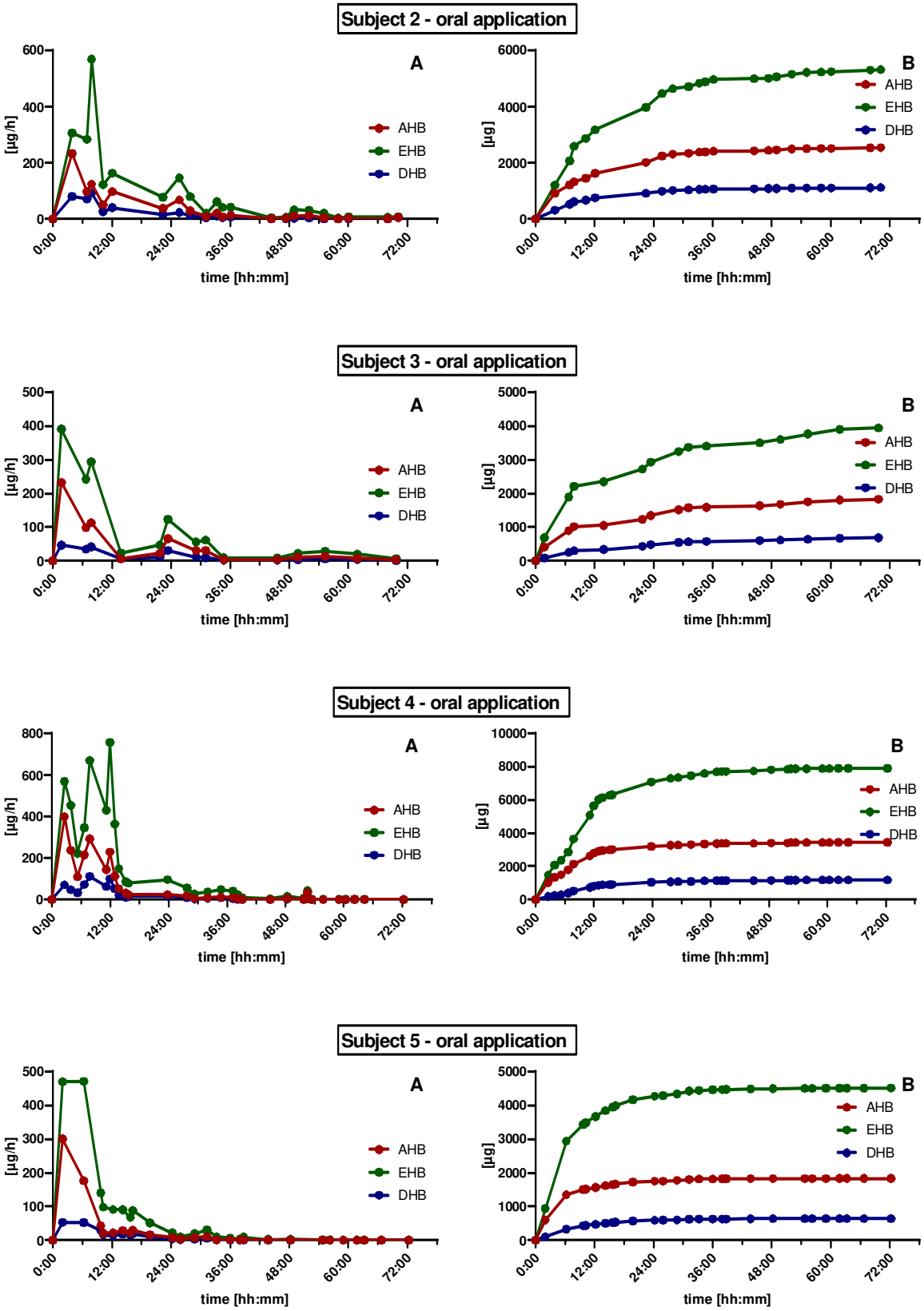

Fig. S1. Time courses of the urinary excretion of DHHB metabolites after oral administration of DHHB to subjects 2-5 displayed as **A** excretion rate in µg/h and **B** cumulative excretion in µg

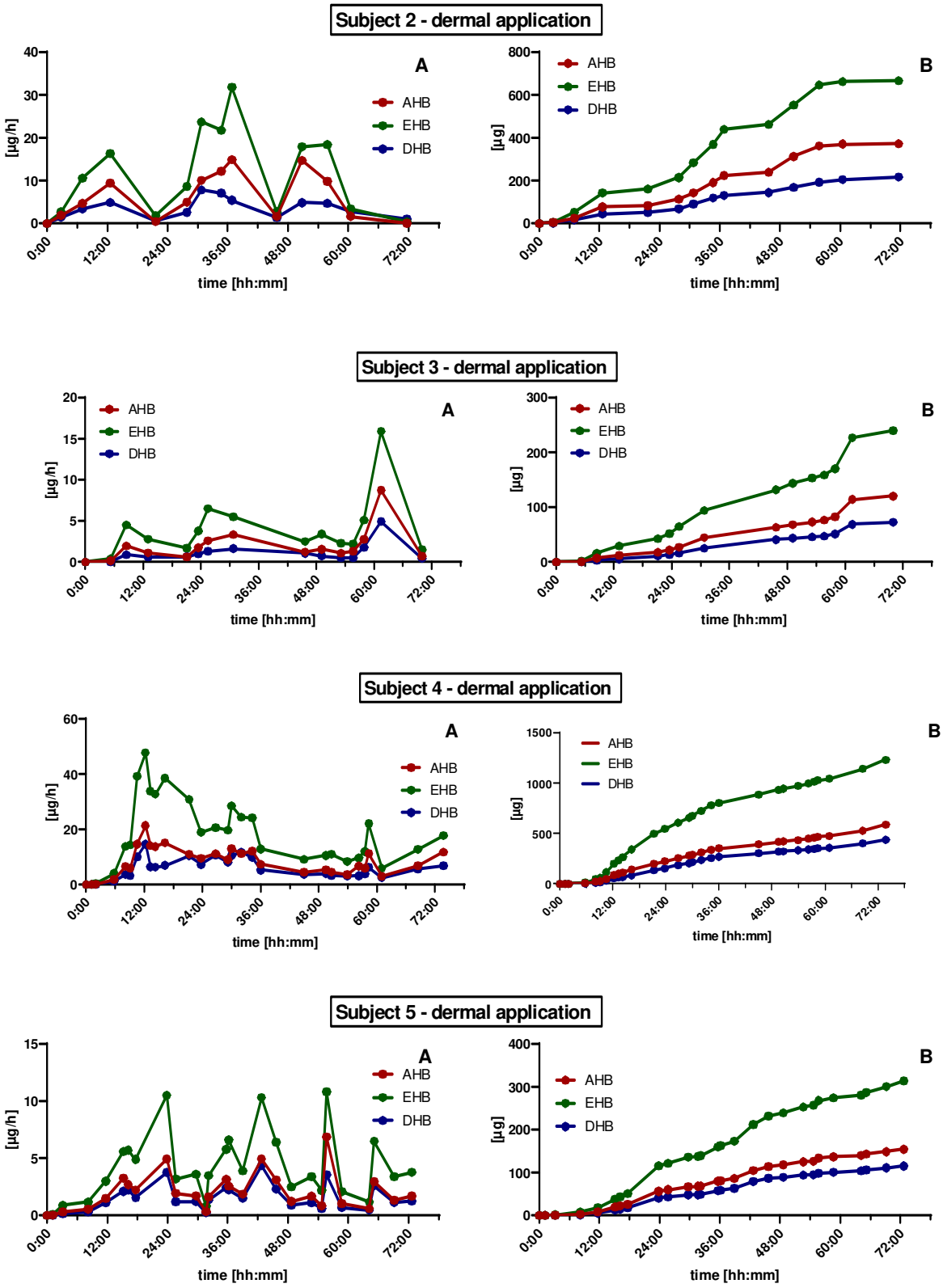

Fig. S2. Time courses of the urinary excretion of DHHB metabolites after dermal administration of DHHB to subjects 2-5 displayed as **A** excretion rate in µg/h and **B** cumulative excretion in µg

3.5 Human biomonitoring for ethoxyquin

Ethoxyquin (CAS number: 1-53-2; EQ) is a synthetic quinoline-based antioxidant which is widely used as an additive in animal feed and as preservative in dried forage crops and spices [49, 83, 84]. In the past, EQ was also used as an anti-scalding agent in pears and apples [85], but the authorization within the EU of EQ as pesticide was stopped in 2011 [86]. EQ is often preferred over other antioxidants because of its low production costs and its high antioxidant capacity [49, 87, 88]. This can be explained by its high reactivity towards radicals formed during lipid peroxidation [89-91] inhibiting further oxidation of lipids, stabilizing liposoluble vitamins [49, 92] and preventing the self-ignition of fishmeal during shipping and storage [93].

Despite the fact that the usage of EQ is prohibited nowadays in any food for human consumption within the EU, residues of EQ and its transformation products could be detected in food of animal origin coming from the usage of EQ as animal feed additive [84, 94, 95]. Consequently, EQ could be determined in meat, eggs and farmed fish [92], but as a result of its high reactivity, higher levels of the transformation products of EQ can be determined in food of animal origin. The main transformation product detected in fish is the 1,8'-ethoxyquin dimer (EQDM) which was quantified in farmed salmon with up to 1450 µg/kg, while EQ levels up to 167 µg/kg could be determined [96]. The formation of EQDM in fish feed containing EQ could also be proven [83]. Besides EQ and EQDM, two further major transformation products of EQ could be identified as 1,2-dihydro-2,2,4-trimethyl-6-quinolinol (*O*-deethylated EQ; 6-OH-EQ) and 2,2,4-trimethyl-6(2H)-quinolinone (quinone imine; EQI) in Atlantic salmon [90]. Due to the additional usage of EQ as pesticide outside the EU, EQ could also be detected in some surface waters of Vietnam in concentrations up to 0.29 µg/L [97].

Oral exposure of different animals including fish and dogs with EQ containing feed led to several detrimental effects including weight loss, changes in liver, kidney,

alimentary duct and urinary bladder and effects on the immune system [49, 84, 94, 98]. Further studies reported, that EQ itself is not genotoxic or carcinogenic [50]. No data on the toxicology of EQ metabolites are available with the exception of EQDM, whose toxicological profile is considered to reflect that of EQ [87, 99]. Although the toxicity of EQI has not been investigated yet, structural alerts for mutagenicity, carcinogenicity and DNA binding were found in a structure-activity analysis [50]. Because of the lack of data on the toxicology of EQ metabolites, especially for EQI, the safety of EQ for any target animals, for consumers and for the environment has not yet been proven. Thus, the authorization of EQ as a feed additive within the EU was suspended by 30th September 2019 with certain exemptions resulting in a step-wise removal of EQ from animal feed by 30th June 2020 [100].

Although residues of EQ have been detected in consumer products and the toxicological profile of EQ and its transformation products is incomplete causing concerns regarding animal and human health, no data on human exposure to EQ are available. Therefore, the chemical was selected for the development of an HBM method within the frame of the German Human Biomonitoring Initiative including the investigation of the human metabolism of EQ by a human metabolism study and the selection of one or more suitable biomarkers of exposure.

The human metabolism study revealed the formation of the two urinary metabolites 6-OH-EQ and its oxidized form EQI, small amounts of unmetabolized EQ could also be determined as displayed in Figure 7. EQDM could not be determined in any urine sample.

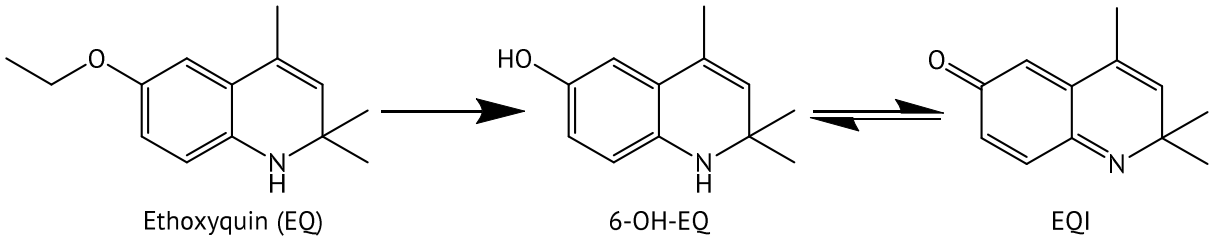

Figure 7: Pathway for the urinary ethoxyquin metabolism in humans

Thus, the HBM method was optimized for EQ and the redox pair 6-OH-EQ/EQI with special attention to the stability of the redox-active analytes during sample preparation. In order to prevent unwanted redox reactions of the analytes in the samples, ascorbic acid was used as antioxidant [95]. Nevertheless, oxidation of 6-OH-EQ to EQI could not be prevented, but further oxidation of EQI to its *N*-oxide was omitted [101, 102]. Furthermore, oxidation of 6-OH-EQ to EQI could be observed in the ionization chamber of the mass spectrometer, which was also reported by Skaare and Solheim [103]. Therefore, the whole amount of 6-OH-EQ in the samples was deliberately oxidized in the course of sample preparation and determined as EQI. After addition of ascorbic acid, glucuronidated 6-OH-EQ was enzymatically hydrolyzed with β-glucuronidase from *E. coli* and subsequently the analytes were extracted by a salt-assisted liquid-liquid extraction (SALLE) approach. Due to the removal of water-soluble ascorbic acid from the extracts by SALLE, oxidation of 6-OH-EQ to EQI could occur in the extracts obtained which were subsequently analyzed by means of UPLC-MS/MS.

Within method validation conducted following guidelines of DFG and FDA [52], stability of EQ in samples prior to sample preparation was found to be low while stability of EQI, mainly present as glucuronidated 6-OH-EQ in urine samples, was found to be sufficient. Therefore, EQI only could be evaluated as suitable biomarker of exposure for EQ.

The validated method was applied to all urine samples of the human metabolism study. EQI is rapidly excreted after oral administration of EQ with a maximum excretion at about 1.3 h and could be quantified in all samples. After 48 h, 28.5 % of the total dose of the EQ administered were urinary excreted as 6-OH-EQ and EQI (both determined as EQI). Additionally, the validated method was applied to 53 samples from volunteers in the greater Munich area. EQI could be quantified in eleven samples (20.8 %) with an average concentration of 290 ng/L proving sufficient sensitivity and the suitability of the method for the HBM of EQ.

3.5.1 Contributions to "Development of a human biomonitoring method for assessing the exposure to ethoxyquin in the general population"

The results of this project including method development and validation and the application of the newly developed HBM method to the samples of the human metabolism study and the pilot HBM study were summarized in a manuscript which was submitted to Archives of Toxicology.

The development and validation of the human biomonitoring method was conducted by me with the support of my trainees David Schmidl (BSc) and Bernhard Köppl (BSc) who both worked under my supervision. I carried out the synthesis of EQI under the supervision of Prof. Dr. Franz Bracher. The application for ethical approval was submitted by Prof. Dr. Gerhard Scherer and me. Bernhard Köppl and I collected all samples from the general population and we analyzed all samples of the human metabolism study and the human biomonitoring pilot study. Dr. Nikola Pluym and I implemented the metabolism study and Dr. Oliver Peschel led the medical surveillance. All toxicokinetic parameters were evaluated by me with the help of Prof. Dr. Gerhard Scherer. The results of this work were summarized in a manuscript which was submitted to the Journal Archives of Toxicology and was prepared by Prof. Dr. Gerhard Scherer, Prof. Dr. Franz Bracher, Dr. Max Scherer, Dr. Nikola Plum, Dr. Edgar Leibold (BASF) and me.

Own contribution:

Development of the human biomonitoring method	100 %	
Validation of the human biomonitoring method	100 %	
Writing application for the ethical approval	70 %	
Sample collection from the general population	100 %	
Implementation of the metabolism study	80 %	
Analysis of study samples	90 %	
Evaluation of toxicokinetic parameters	90 %	
Submission of the publication	70 %	

3.5.2 Submitted Article: "Development of a human biomonitoring method for assessing the exposure to ethoxyquin in the general population"

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Development of a human biomonitoring method for assessing the exposure to ethoxyquin in the general population

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Keywords: Ethoxyquin, LC-MS/MS, Urine, Human metabolism, Toxicokinetics, Human Biomonitoring

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Abstract

Ethoxyquin (EQ) is commonly used as an antioxidant in animal feeds. Although EQ is not permitted for usage in food products for humans within the EU, residues of EQ and its transformation products could be determined in food of animal origin. Despite its widespread use and concerns on its toxicological profile, no information about the systemic exposure to EQ in the general population is available. Hence, we developed and validated a human biomonitoring (HBM) method based on UHPLC-MS/MS comprising enzymatic glucuronide hydrolysis and salt-assisted liquid-liquid extraction for the determination of suitable biomarkers of exposure to EQ. Our approach included a metabolism study with five subjects, who were administered an oral dose of 0.005 mg EQ/kg body weight. 1,2-dihydro-2,2,4-trimethyl-6-quinolinol (6-OH-EQ) was identified as the major excretion product mainly present as the glucuronide (6-OGlu-EQ) in urine, while unchanged EQ was only identified in highly exposed subjects. Remarkably, 6-OGlu-EQ is rapidly oxidized to 2,2,4-trimethyl-6(2H)-quinolinone (EQI) after enzymatic hydrolysis during sample preparation. Thus, EQI was assessed in the newly developed UHPLC-MS/MS method to evaluate the toxicokinetic parameters of EQ. In addition, the analytical method was applied to 53 urine samples from the general population. EQI could be quantified in 11 (21 %) of the samples, proving the suitability of the developed method for the intended purpose.

Introduction

The quinoline-based, synthetic antioxidant ethoxyquin (6-ethoxy-1,2-dihydro-2,2,4-trimethylquinoline; CAS Number 91-53-2; EQ) possesses a broad range of applications because of its antioxidant capacity and its low production costs (Błaszczyk et al. 2013; Rodríguez-Gómez et al. 2018). It is globally used as an additive in animal feed, as preservative in dried forage crops and spices and was used as an anti-scalding agent in pears and apples in the past (Błaszczyk et al. 2013; Negreira et al. 2017). The anti-oxidative properties of EQ can be explained by its ability to trap radicals formed during lipid oxidation (Bohne et al. 2006; Rössing et al. 1985) inhibiting further oxidation of lipids, stabilizing fat soluble vitamins (Błaszczyk et al. 2013; Merel et al. 2019) and preventing the self-ignition of fishmeal during shipping and storage (International Maritime Organization 2017).

Although EQ is not authorized in any food for human consumption within the EU the use of EQ as animal feed additive has been reported to lead to residues in food of animal origin (Egloff and Pietsch 2018; EU Reference Laboratory Requiring Single Residue Methods (EURL-SRM) 2016; Pradhan et al. 2019). Due to its high reactivity, the occurrence of several transformation products of EQ in food of animal origin was demonstrated. EQ itself could be detected in meat, eggs and farmed fish (Merel et al. 2019). While up to 167 µg/kg EQ could be quantified in farmed fish, the main transformation product, 1,8'-ethoxyquin dimer (EQDM), was determined in concentrations up to 1450 µg/kg (Lundebye et al. 2010). EQDM has also been detected in fish feed (Błaszczyk et al. 2013; Negreira et al. 2017). Two further transformation products in Atlantic salmon were identified as 2,2,4-trimethyl-6(2H)-quinolinone (quinone imine; EQI) and 1,2-dihydro-2,2,4-trimethyl-6-quinolinol (deethylated EQ; 6-OH-EQ) (Bohne et al. 2006). Currently, more than 30 biotransformation products of EQ are known (Negreira et al. 2017).

Several adverse effects after acute or chronic treatment of animals with EQ were reported including weight loss and changes in liver, kidney, alimentary duct and urinary bladder (Błaszczyk et al. 2013). Furthermore, EQ was evaluated as itself not genotoxic or carcinogenic (EFSA Panel on Additives Products or Substances used in Animal Feed 2015). The toxicological effects of the main transformation product EQDM were found to be similar to the findings on EQ (Ørnsrud et al. 2011). Although, the toxicity of EQI has not been evaluated yet, structural alerts for mutagenicity, carcinogenicity and DNA bindings were found in a structure-activity analysis (EFSA Panel on Additives Products or Substances used in Animal Feed 2015). Hence, the safety of EQ for any target animals, for consumers and for the environment could not be proven and the authorization of EQ as a feed additive was suspended by 30th September 2019 with certain exemptions resulting in a step-wise removal of the additive from animal feed by 30th June 2020 (The European Commission 2017). The suspension is to be reviewed by 31st December 2020, depending on further data to be submitted by industry and their evaluation by EFSA.

Despite the wide usage of EQ and the great concerns regarding animal and human health, no data on human exposure of EQ are available. Therefore, we developed a method for the human biomonitoring (HBM) of EQ in the general population. For the identification of urinary human metabolites of EQ, five participants were orally administered with EQ. An analytical

method was developed and validated for the most promising EQ metabolites to be used as biomarkers of exposure. This method was applied to all samples from the human metabolism study in order to determine the toxicokinetic parameters. Additionally, the method was applied to 53 spot urines from healthy volunteers of the general population in order to estimate their EQ exposure.

This project is part of a 10-year ongoing HBM initiative conducted in cooperation with the German Federal Ministry of the Environment, Nature Conservation and Nuclear Safety (BMU) and the German Chemical Industry Association (VCI). Ethoxyquin was selected as one out of 50 chemicals of interest for which a HBM method should be developed. All chemicals are selected because of their widespread occurrence in the environment and the probable exposure of the general population (Verband der chemischen Industrie e.V. 2016).

Materials and methods

Reagents

Ethoxyquin (EQ), 1,2-dihydro-2,2,4-trimethyl-6-quinolinol (6-OH-EQ), 6-ethoxy-2,2,4-trimethyl-1,2,3,4-tetrahydroquinoline (DHEQ), 1,8'-ethoxyquin dimer (EQDM) and D_{10}-ethoxyquin (D_{10}-EQ, structure see Fig. S2) were purchased from HPC Standards (Cunnersdorf, Germany). 2,2,4-Trimethyl-6(2H)-quinolinone (EQI) was synthesized by ourselves (for details, see Electronic Supplementary Material), D_{10}-2,2,4-trimethyl-6(2H)-quinolinone (D_{10}-EQI, structure see Fig. S2)) was custom-synthesized by Aptochem (Montreal, Canada). Ethoxyquin containing <2.5 ppm p-phenetidine (a common contaminant in EQ preparations) was received from an approved qualified supplier in the industry (USA). 4-Methylumbelliferyl-β-D-glucuronide dihydrate (MUG) was purchased from Santa Cruz Biotechnology (Dallas, TX; USA), 4-methylumbelliferone (MU) from Sigma Aldrich (Taufkirchen, Germany). Acetonitrile and ethyl acetate for residue analysis and LC-MS grade acetonitrile with 0.1 % formic acid were obtained from Th. Geyer (Renningen, Germany), formic acid was received from Biosolve Chimie (Dieuze, France). Aqueous phosphoric acid (85 %) was purchased from VWR International, acetic acid from Alfa Aesar (Kandel, Germany) and sodium hydroxide from Merck (Darmstadt, Germany). The water used was filtered through a Sartorius arium water system (Göttingen, Germany). β-Glucuronidase from *E. coli* was obtained from Megazyme (Bray, Ireland) and β-glucuronidase/arylsulfatase from *H. pomatia* was purchased from Sigma Aldrich. Ascorbic acid, magnesium sulfate and sodium chloride were received from Sigma Aldrich. Bondesil PSA bulk sorbent was obtained from Agilent Technologies (Waldbronn, Germany).

Human studies

A human metabolism study with ethoxyquin was conducted at the Analytisch-Biologisches Forschungslabor (ABF), Planegg, Germany. Five adult, healthy volunteers (three males, two females aged between 26 and 70 years, for further details, see Table 1) were orally administered a single ethoxyquin dose of 0.005 mg/kg body weight. Ten mg ethoxyquin were dissolved in 10 mL (non-denatured) ethanol and diluted to 100 mL with water resulting in a

0.1 mg/mL solution of ethoxyquin. The subjects drank a bodyweight-standardized volume of this solution (3.1 – 4.1 mL). A urine sample was collected immediately before administration and all urine voids of the following 48 h, with the exception of Subject 1 (72 h urine collection), were collected in separate fractions with the time of voiding being free. All samples were stored below -20 °C until analysis. The exact time of administration as well as the time of voiding and volume of each fraction were recorded.

For a first assessment of the exposure to EQ in the general population 53 spot urine samples were collected and analyzed with the validated method. The study group consisted of 40 males and 13 females, between 18 and 63 years old, living in Bavaria (Germany) and not occupationally exposed to EQ.

Urine sample preparation

Frozen urine samples were thawed at room temperature and homogenized by shaking. Aliquots of 3 mL were mixed with 10 µL of an aqueous solution of ascorbic acid (100 mg/mL), 10 µL of an aqueous solution of MUG (50 µg/mL) and 10 µL of an IS solution consisting of D_{10}-EQ and D_{10}-EQI (1 µg/mL each in acetonitrile). For enzymatic hydrolysis, 0.5 mL of an acetate buffer (1 M, pH 5.1) and 10 µL of β-glucuronidase from *E. coli* were added and samples were incubated at 37 °C for one hour. Subsequently, a salt assisted liquid-liquid extraction (SALLE) was conducted by transferring the samples into a 4 mL glass vial containing 1.0 g of a mixture of $MgSO_4$ and NaCl (4/1, w/w) and adding 100 µL sodium hydroxide solution (14.3 N) and 400 µL ethyl acetate. The samples were shaken for 10 min at 2500 rpm, centrifuged for 10 min at 4000 rpm, and 100 µL of the organic layer were transferred into a micro vial with a 300 µL insert. Samples with EQI concentrations >ULOQ were reanalyzed by deploying only 100 µL urine sample diluted with 2900 µL water. Linearity after dilution was found to be accurate during method validation.

Analytical methods

Chromatographic separation was achieved by injecting five microliters of the sample extract into a liquid chromatography Nexera X2 UHPLC system (Shimadzu, Neufahrn, Germany) equipped with an Acquity BEH C18 analytical column (1.7 µm, 2.1 × 100 mm; Waters, Eschborn, Germany) connected to an upstream guard column (Waters Acquity UPLC BEH C18 VanGuard, 1.7 µm, 2.1 × 5 mm). Chromatography was conducted at 40 °C and a flow of 0.5 mL/min with a gradient consisting of eluent A (water with 0.1 % formic acid) and eluent B (acetonitrile with 0.1 % formic). The gradient started at 90 % of A for 1 min, decreasing to 10 % over 3 min, holding for 2 min, followed by an immediate increase to 90 % and keeping this level until the end of the analysis at 8.0 min. Mass spectrometric detection of the analytes was accomplished by using an MS/MS instrument (Model 6500$^+$ QTrap® from AB Sciex, Darmstadt, Germany) operated in ESI positive and multiple reaction monitoring (MRM) mode. All modules were controlled via Analyst software (AB Sciex, Version 1.6.3).

The instrument gases (nitrogen) were set as follows: Curtain gas 50, ion source gas 1 80, ion source gas 2 60 and collision gas medium. The ion source was heated to 500 °C, ion spray voltage was set to 4000 V, and entrance potential (EP) was set to 10 V for all analytes. All analyte-specific parameters for the MRM transitions are summarized in Table 2.

Quality control samples (QCs) were prepared in three different concentration levels (low, medium, high) and two QCs per concentration level were analyzed in every analytical batch (consisting of up to 100 samples) to ensure data integrity and quality. Quantification of samples and QCs was conducted by linear calibration with 1/x weighting. Acceptance criteria were fulfilled and the analytical series was regarded as valid if accuracy of the QCs and calibrators was within 85 – 115 % and 80 – 120 % for concentrations below three times LOQ.

Data evaluation and statistics

All toxicokinetic parameters evaluated were calculated with Microsoft Excel Home and Business 2013 (Version 15.0, Unterschleißheim, Germany), all statistical analysis were carried out with Prism (GraphPad, Version 8.3.1, La Jolla, CA, USA). Linear interpolation between the closest urine voiding times was conducted for the calculation of metabolites excreted 3, 6, 12, 24 and 48 h after application of EQ. After 48 h, urinary excretion of the metabolites was assumed to be virtually complete (which is supported by the excretion kinetics over 72 h for Subject 1). Urinary elimination constants (k_{el}) and elimination half-lives ($t_{1/2}$) were calculated using the 'sigma-minus' method (Klotz 1984), with the total amount excreted ($Ae(\infty)$) being the amount excreted within 48 h. For the back calculation of the human exposure to EQ, a conversion factor (CF) was calculated as the ratio between the total dose applied (D in mol) and the molar amount of the metabolite excreted within the first 24 h after oral uptake ($CF = D/A_{MX24h}$). In the pilot HBM study with 53 volunteers, non-parametric tests were conducted instead of parametric tests to limit the impact of extreme values. Differences between subgroups were determined by applying Mann-Whitney U test, coefficients of correlation between urinary levels of EQ metabolites were calculated using Spearman's correlation. P values of <0.05 were regarded as statistically significant, values below LOQ were set to LOQ/2.

Results and discussion

Identification of urinary metabolites

The metabolism of ethoxyquin (EQ) was extensively investigated in different animal species and plants. Skaare and Solheim identified 1,2-dihydro-2,2,4-trimethyl-6-quinolinol (6-OH-EQ), its oxidation product 2,2,4-trimethyl-6-quinolinone (EQI) and some minor metabolites in urine and faeces of rats dosed with EQ (Skaare and Solheim 1979). Sheep administered an EQ-containing diet were found to excrete EQ and a not further characterized hydroxylated EQ metabolite in urine. Deethylation of EQ leading to 6-OH-EQ or oxidation giving EQI was not observed in this study (Kim et al. 1992). Following metabolism studies in rats and mice confirmed the findings of Skaare and Solheim: Deethylation of EQ was found to be the major metabolic pathway followed by sulfation or glucuronidation resulting in the corresponding

conjugates of 6-OH-EQ. Additionally, 1,2-dihydro-6-ethoxy-8-hydroxy-2,2,4-trimethylquinoline glucuronide was found as major metabolite of EQ in rats and mice after oral gavage or intravenous injection (Burka et al. 1996). In several investigations on the biotransformation of EQ in fish, the formation of 6-OH-EQ, EQI and the dimer (EQDM) was observed (Bohne et al. 2006; Bohne et al. 2007; Bohne et al. 2008). In plants, three different metabolites of EQ were determined: 6-ethoxy-1,2,2,4-tetramethyl-1,2-dihydroquinoline, 6-ethoxy-2,4-dimethylquinoline and 6-ethoxy-2,2,4-trimethyl-1,2,3,4-tetrahydroquinoline (DHEQ) (Gupta and Boobis 2005). Although, metabolites of ethoxyquin are well known, no information on the human *in vivo* metabolism is available until now. Therefore, we conducted a metabolism study with five participants. The subjects were orally administered between 310 and 410 µg EQ and collected between 11 and 22 urine fractions (93 fractions in total, Table 1). To identify urinary metabolites of EQ, we worked-up all samples of Subject 1 using a preliminary method (see Supplementary Material) and screened them for possible metabolites. We chose the metabolites shown in Fig. 1 for screening, because we rated them as most likely occurring metabolites and commercially available. DHEQ and EQDM could not be determined in any sample (data not shown), whereas 6-OH-EQ, EQI and EQ were found in the first fractions after oral dosage.

Method development and performance

The final method was optimized for EQ and EQI which could be determined in the samples of the metabolism study and validated according to the guidelines issued by the US Food and Drug Administration (FDA, for further details see Supplementary Material) (U.S. Food and Drug Administration (FDA) 2018). Method validation data can be found in Table S1. Acceptance criteria regarding accuracy and precision were met for both analytes. The calibration range was set to 0.03 – 20.0 µg/L. Both analytes show a linear response in this range. Probably due to the forcedly low volume of ethyl acetate used for analyte extraction, recovery rates of EQ and EQI are poor (10 - 15 %). On the other hand, matrix effects are low (78 – 103 %, Table S1) so that acceptable LOD and LLOQ levels were achieved with the approach taken. For EQ, no carry-over effects were observed. Carry-over for EQI after five injections of a sample with 20 µg/L EQI was found to be 0.006 µg/L, which is far below the respective LOQ of 0.03 µg/L. Due to the low stability of EQ in samples prior to sample preparation, EQ was found to be less suitable as a biomarker of exposure. On the other hand, EQI turned out to be stable during the course of the sample analysis.

Special attention was given to the stability of both analytes during sample preparation. Both analytes are known for their redox activities requiring control of redox reactions for example by adding ascorbic acid as antioxidant (EU Reference Laboratory Requiring Single Residue Methods (EURL-SRM) 2016). Still, adding ascorbic acid could not prevent the oxidation of the free 6-OH-EQ to EQI, presumably because water-soluble ascorbic acid is removed from the extract by SALLE. However, further oxidation of EQI to its *N*-oxide was omitted (Pico et al. 2010; Thorisson et al. 1992). Also, oxidation of 6-OH-EQ to EQI was reported to occur in the ionization chamber of the mass spectrometer (Skaare and Solheim 1979) and this behavior was also observed in our lab (see Fig. S4). Our observations suggest, that 6-OH-EQ is converted

to EQI during sample preparation. The conversion was proven by preparing analyte-free urine samples spiked with 6-OH-EQ: The nominal concentration of the spiked 6-OH-EQ could be recovered as EQI while 6-OH-EQ could not be found.

Even though an increase of EQI levels after enzymatic hydrolysis could be observed, the analyte is present in urine as its reduced and glucuronidated form 6-OGlu-EQ which was also reported in urine samples of mouse and rat (Burka et al. 1996). After enzymatic hydrolysis, 6-OH-EQ oxidizes during sample preparation, presumably due to autoxidation (Thorisson et al. 1992)(see also Fig S5). Therefore, all EQI concentrations reported reflect the whole amount of 6-OH-EQ (glucuronidated + non-conjugated 6-OH-EQ) and EQI.

In order to enhance analyte concentrations and to obtain cleaner extracts, an extraction step was necessary. Because EQI was found to be volatile, solvents could not be removed by evaporating them limiting the choice of extraction solvents to those suitable for the subsequent chromatographic separation (acetonitrile and ethyl acetate). Three milliliters urine extracted with 0.4 mL ethyl acetate yielded the highest analyte concentrations in the extracts without noticeably suppressing the MS signals. A mixture of $MgSO_4$ and NaCl, known from the QuEChERS sample preparation (Anastassiades et al. 2003; EU Reference Laboratory Requiring Single Residue Methods (EURL-SRM) 2016; Plössl et al. 2006) was used in order to achieve a sharp separation of the two phases. Improved signal intensities for EQI were observed by increasing the pH before extraction. Therefore, an aqueous solution of sodium hydroxide was added before extraction resulting in precipitation of $Mg(OH)_2$ in the aqueous phase and a stable pH of 9.1.

β-Glucuronidase from *E. coli* and β-glucuronidase/arylsulfatase from *H. pomatia* were tested for enzymatic hydrolysis of possible conjugates. Higher signal intensities for EQI were obtained after enzymatic hydrolysis with β-glucuronidase from *E. coli* in comparison to hydrolysis with β-glucuronidase/arylsulfatase from *H. pomatia* indicating that sulfated conjugates are not built to an appreciable extent and that the usage of *H. pomatia* enzyme leads to a higher signal suppression caused by a differing composition of the matrix. To evaluate the ratio between conjugated (glucuronidated) and non-conjugated (free) 6-OH-EQ, the three samples of each participant with the highest EQI concentrations measured were analyzed twice: Firstly, by including the enzymatic hydrolysis with β-glucuronidase from *E. coli* to capture the total amount of 6-OH-EQ and secondly, by omitting the enzymatic hydrolysis, thus assessing the unconjugated form of 6-OH-EQ only. Table 1 shows the ratios between the conjugated and non-conjugated form of 6-OH-EQ. In all but one participants, 6-OH-EQ is nearly exclusively (> 97 %) present as conjugate, presumably as glucuronidated 6-OH-EQ (6-GluO-EQ), in all samples investigated before hydrolysis and subsequent oxidation to EQI (cf. Fig S5). Subject 1 showed a slightly lower glucuronidation ratio of 90 %.

To evaluate the kinetics of the enzymatic hydrolysis, the first urine fraction of Subject 2 voided after oral administration of EQ was enzymatically hydrolyzed by applying different incubation times. The dependency of free EQ and EQI concentrations on the incubation time are shown in Fig. S6. Hydrolysis for EQI appears to be already complete after the first time point (0.5 h). To assure a complete hydrolysis, the incubation time was set to 1 h in the final method. Efficiency of the enzymatic hydrolysis in sample series was monitored by spiking all samples

with 500 µg/L of MUG (Grignon et al. 2017). Enzymatic hydrolysis was regarded as complete if MU was detectable with a minimum area of 10^6 counts for the respective MRM in the samples.

Toxicokinetic parameters after a single oral dose of ethoxyquin

All samples collected during the metabolism study with 5 subjects were analyzed by applying the validated method (described in the Analytical methods section). Exemplary chromatograms from samples of Subject 4 for EQI and its internal standard (IS) before and 3 h after oral application are shown in Fig. 2A+B. EQI was found in all samples after oral dosage. In samples collected immediately before oral administration of EQ, EQI could only be determined in the sample of Subject 2 (0.12 µg/L). Despite the low stability of EQ, it could be quantified in the first fractions up to 5.5 h after oral administration (0.03-1.18 µg/L), but evaluation of toxicokinetic parameters was not meaningful. EQ was not measurable in the samples collected before administration of EQ.

The time course of the urinary excretion of EQI from Subject 1 is shown in Fig. 3, both as excretion rate (µg/h) and cumulated amount (µg) excreted over the entire study. Time courses for Subjects 2-5 are similar to the time course of Subject 1 and are shown in the Supplementary Material (Fig. S1). EQI is rapidly excreted after absorption with a maximum at about 1.3 hours. Toxicokinetic parameters are summarized in Table 3. On average, 28.5 % of the total dose were excreted 48 h after dosing as EQI and thereof, 99.4 % were excreted in the first 24 h after dosing. The mean elimination half-life amounted to 0.83 h. The conversion factor for EQI was calculated to be 3.62, which means that 1 µg EQI excreted in 24 h corresponds to a daily exposure dose of 3.62 µg EQ.

Pilot HBM study

The validated method was applied to 53 spot urine samples collected in January 2020 from the general population in the greater area of Munich. Fig. 2C shows an exemplary chromatogram of one sample collected during this study in comparison with the sample of Subject 4 before oral dosing (A) and the first fraction of Subject 4 after oral dosing (B). In order to take large variations in urine dilution into account, values were standardized to creatinine. For estimations of the daily EQ uptake, creatinine excretion rates of 1.2 g/d for females and 1.5 g/d for males were assumed (Barr et al. 2005). Table 4 summarizes the estimated daily exposure doses of EQ for the 53 subjects. EQI could be quantified in eleven samples (20.8 %) with an average concentration for theses 11 samples of 290 ng/L. The average for all 53 samples was 72.3 ng/L (for samples < LOQ, LOQ/2 = 15 ng/L was used as EQI concentration). Overall, an average creatinine-standardized concentration of 0.41 nmol/g creatinine was calculated. To calculate the systemic uptake dose of EQ the conversion factor was applied to the standardized EQI concentrations determined resulting in an average exposure dose of EQ of 0.43 µg/d. Significantly higher concentrations of EQI were observed in females than in males. However, men are over-represented in this study and the number of participants is by far too low for drawing any reliable conclusions.

The most relevant source of human uptake of EQ seems to be fish. Levels of EQ in commercially farmed fish such as salmon, trout, halibut and cod were reported to range from 9.5 to 55 µg/kg (Lundebye et al. 2010). By assuming a consumption of one portion of fish (200 g), this would result in an intake dose of 2-11 µg EQ. Assuming the consumption of one fish meal per week would yield in a daily average EQ intake of 0.3-1.6 µg/d. This estimate is in line with the average exposure dose determined and both values are far beyond the acceptable daily intake dose of 5 µg/d/kg bw derived by the World Health Organization (WHO) as a pesticide residue level in food (Gupta and Boobis 2005; World Health Organization 2005). However, only little amounts of unmetabolized EQ are present in fish. Most of the EQ taken up by fish is transformed to EQDM, resulting in much higher EQDM levels up to 2593 µg/kg compared to the EQ levels reported (Lundebye et al. 2010). The human metabolism of EQDM is not elucidated yet and EQDM as well as its transformation products are not captured with the method developed except the dimer would be cleaved to EQ in the human body. EQDM could not be determined in urine samples since it is too lipophilic for urinary excretion and presumably is stored in adipose tissues of the body or excreted via another excretion route.

Conclusion

We successfully developed and validated a human biomonitoring method for the determination of urinary EQ metabolites. EQI was quantified in all samples after oral administration of EQ. Unchanged EQ was detectable in samples of highly (experimentally) exposed persons only. These findings and the low stability of EQ lead us to the conclusion that EQ itself is not a suitable biomarker of exposure. EQI was quantifiable in eleven (21 %) of the 53 samples from the non-occupationally exposed general population, proving the sufficient sensitivity of the method and the suitability of EQI as biomarker of exposure for EQ. By applying the CF obtained from the human metabolism study, an exposure of approximately 0.43 EQ µg/d was estimated suggesting a low risk with respect to the threshold of 5 µg/d/kg bw. Larger human biomonitoring studies are required in order to obtain representative results for the exposure to EQ of the general population.

Declarations

Funding

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Compliance with ethical standards

Studies with human subjects were approved by the Ethic Committee of the Bayerische Landesärztekammer, Munich, Germany in accordance with ethical standards laid down in the Declaration of Helsinki from 1964 and its later amendments. All subjects gave their informed written consent.

Conflict of Interest

Except for EL, the authors declare that they have no conflict of interest. EL is working for a company using EQ in part of their products in the past.

Availability of data and material

Not applicable.

Code availability

Not applicable.

Tables and figure captions

Table 1: Characteristics of the subjects taking part in the metabolism study, including their glucuronidation rate for 6-OH-EQ

Subject	Age [years]	Gender	Body weight [kg]	Oral EQ dose [µg]	Urine fractions collected	Percentage of conjugated 6-OH-EQ in urine (%)
1	70	Male	82	410	22	90.5
2	26	Male	77	385	18	98.5
3	36	Male	80	400	21	97.2
4	31	Female	64	320	11	99.2
5	36	Female	62	310	21	98.4

Table 2: Retention times, mass transitions, declustering potentials (DP), collision energies (CE) and cell exit potentials (CXP) for EQI and EQ, their deuterated IS and the hydrolysis control MU

Analyte or IS	Retention time (min)	Mass transition (*m/z*)	Role	DP (V)	CE (V)	CXP (V)
EQI	3.4	188.0 → 173.1	Quantifier	13	21	10
EQI	3.4	188.0 → 145.1	Qualifier	13	35	10
D_{10}-EQI	3.3	198.0 → 152.1	IS	13	35	10
EQ	3.5	218.1 → 160.1	Quantifier	30	47	16
EQ	3.5	218.1 → 173.9	Qualifier	30	39	18
D_{10}-EQ	3.5	228.1 → 166.2	IS	30	47	16
MU	2.9	177.1 → 76.9	Control of enzymatic hydrolysis	36	49	10

Table 3: Toxicokinetic parameters for EQI derived from the metabolism study with 5 subjects after oral application of EQ

	Mean ± SD	Median	Min-Max
Amount excreted after 48 h [nmol/kg bw] ($A_{e(\infty)}$)	6.56 ± 1.01	6.13	5.25-8.36
Percent of oral dose PMxD [%]	28.50 ± 4.73	26.66	22.80–36.32
t_{max} [h]	1.32 ± 0.86	0.83	0.67–3.00
Percent of total EQI excreted after 3 h [%][1]	85.50 ± 9.41	85.92	68.59–96.15
Percent of total EQI excreted after 6 h [%][1]	95.12 ± 2.72	96.68	90.35–97.57
Percent of total EQI excreted after 12 h [%][1]	98.45 ± 0.80	98.96	97.41-99.30
Percent of total EQI excreted after 24 h [%][1]	99.43 ± 0.34	99.59	98.97–99.83
Elimination constant k_{el} [h^{-1}]	1.31 ± 0.64	1.64	0.32–1.93
Elimination half-life $t_{1/2}$ [h]	0.83 ± 0.68	0.42	0.36–2.15
Conversion factor (24 h)	3.62 ± 0.58	3.76	2.76-4.43

[1]: The percentage refers to the total amount excreted after 48 hours of 6.56 nmol/kg bw

Table 4: Amount of excreted EQI and estimates of the absolute exposure doses to EQ in 53 volunteers of the general population calculated by applying the average conversion factor (CF) shown in Table 3. Values <LOQ (79 %) were set to LOQ/2 (0.015 µg/L) for calculation.

Urinary excretion of EQI	nmol/g creatinine	Mean ± SD	0.41 ± 0.96
		Median (min-max)	0.10 (0.02-4.90)
	nmol/d	Mean ± SD	0.55 ± 1.20
		Median (min-max)	0.16 (0.03-5.88)
Estimated oral dose of EQ	nmol/d	Mean ± SD	1.98 ± 4.34
		Median (min-max)	0.57 (0.12 – 21.27)
	µg/d	Mean ± SD	0.43 ± 0.94
		Median (min-max)	0.12 (0.03 – 4.62)

Fig. 1: Ethoxyquin and some major metabolites identified in animals and plants. The left pathway (EQ → 6-OH-EQ → EQI) was confirmed for humans. Conjugated metabolites are not shown (see also Fig. S5).

Fig. 2: Chromatograms of EQI and its internal standard. A: First urine fraction (t=0 h) from Subject 4 (c = 0.00 µg/L) before oral administration of EQ; B: First fraction (t=3.0 h) from Subject 4 (c = 9.9 µg/L) after oral administration of EQ; C: Sample from a subject of the pilot study not intentionally exposed to EQ (c = 0.53 µg/L).

Fig. 3: Time courses of the urinary excretion of EQI after oral administration of EQ to Subject 1 displayed as **A** excretion rate in µg/h and **B** cumulative excretion in µg over 72 h.

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Figure 1

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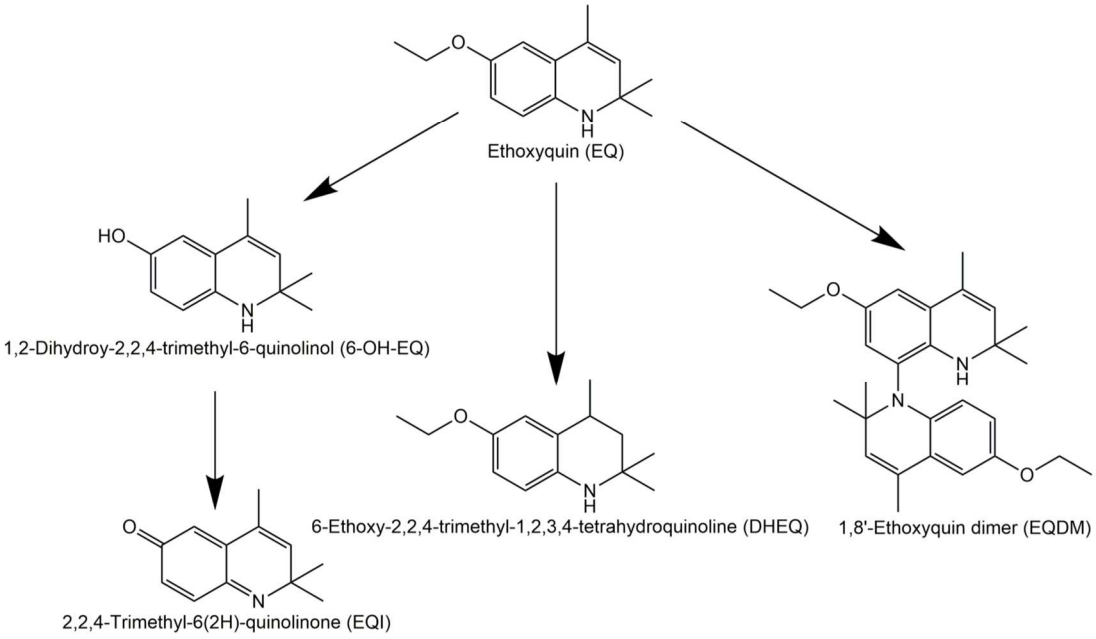

Figure 2

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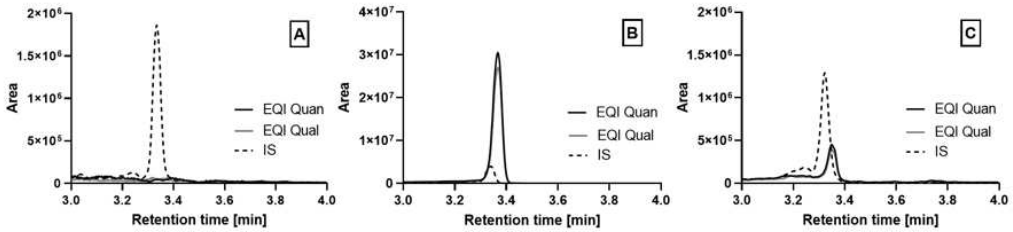

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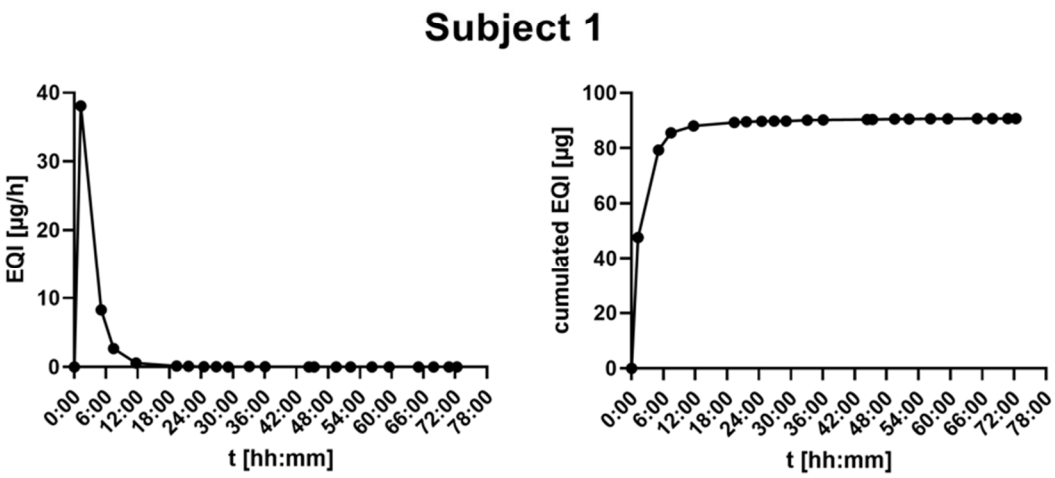

Supplementary information to:

Development of a human biomonitoring method for assessing the exposure to ethoxyquin in the general population

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Keywords: Ethoxyquin, UPLC-MS/MS, Urine, Human metabolism, Toxicokinetics, Human Biomonitoring

Reagents

Synthesis of 2,2,4-trimethyl-6(2H)-quinolinone (EQI)

2,2,4-Trimethyl-6(2H)-quinolinone (EQI) was prepared by oxidation of acetonanile with Fremy's salt according to Teuber and Glosauer (Teuber and Glosauer 1965). Hereby, a solution of 524 mg (3.00 mmol) acetonanil in 100 mL methanol and a solution of 1.7 g (6.3 mmol) potassium nitrosodisulfonate (Fremy's salt) in 200 mL phosphate buffer pH 7 (0.066 M) were mixed under stirring in an ice bath, and stirring was continued for one hour at this temperature. Subsequently, the product was extracted with chloroform (3 × 25 mL) and dried over sodium sulfate. After evaporation of the solvent the product was recrystallized from iso-hexane. The crystallized product was further purified by means of flash column chromatography on silica gel (elution with dichloromethane/ethyl acetate 90/10) yielding 220 mg (39 %) of the title compound as reddish yellow solid.

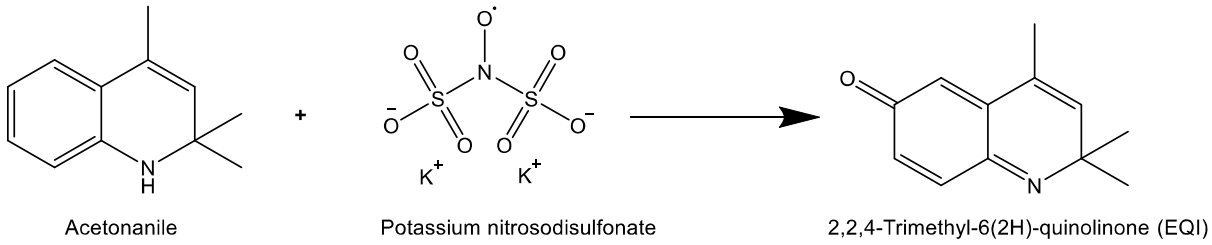


Fig. S1: Synthesis of 2,2,4-trimethyl-6(2H)-quinolinone (EQI)

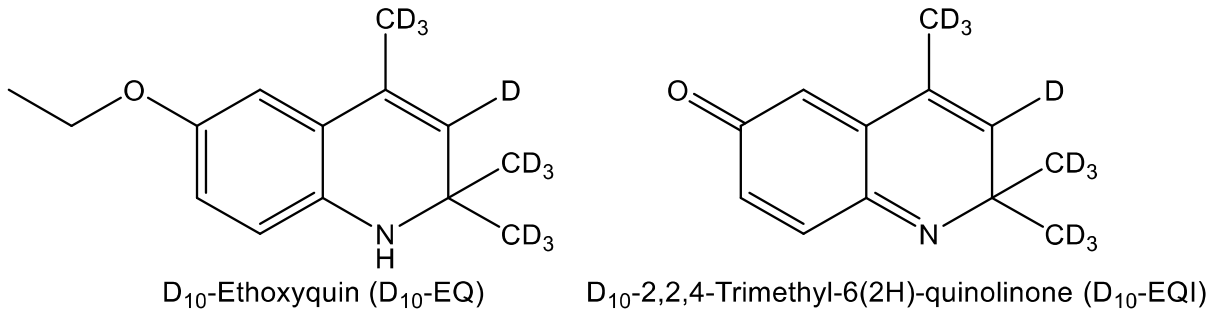


Fig. S2: Chemical structures of the deuterated internal standards

Identification of urinary metabolites and method development

Preliminary method

Frozen urine samples were thawed at room temperature and homogenized by shaking before samples were aliquoted in two milliliters urine sample fractions. To the urine samples were added 0.5 mL of an acetate buffer (pH 5.1, 1 M) and 10 µL of β-glucuronidase from *E. coli* and the samples were incubated for 18 h at 37 °C. Subsequently a modified QuEChERS method was applied (EU Reference Laboratory Requiring Single Residue Methods (EURL-SRM) 2016). Hereby, the samples were transferred into 4 mL glass vials containing 1.0 g of a mixture of $MgSO_4$ and NaCl (4/1 w/w) and 500 µL ethyl acetate were added. After shaking (10 min, 2000 rpm) and centrifugation (10 min, 4000 rpm), 300 µL of the organic layer were transferred 1000 µL piston stroke pipette into a 1.5 mL polypropylene tube containing a mixture of potassium sulfate and Bondesil PSA (4/1 w/w). After shaking (10 min, 2000 rpm) and centrifugation (10 min, 4000 rpm), 100 µL of the extract were transferred with a 100 µL piston stroke pipette into a micro vial with a 300 µL insert.

Validation

Method validation was performed according to the guidelines issued by the US Food and Drug Administration (FDA) (U.S. Food and Drug Administration (FDA) 2018). Quality control samples (QCs) were prepared by pooling native human urine samples with known concentrations of EQ and EQI. Where appropriate, analytes were spiked to the QC material. Three different concentration levels (low, medium and high) were prepared to cover the entire range of expected concentrations. QCs were stored at -20 °C until analysis. Two QC samples of each level were analyzed in combination with a calibration every day to monitor the robustness of the method. The target values of the QC samples for each analyte was determined prior to the method validation by analyzing six QC samples per level.

Selectivity was proven for the applied MRM transitions for EQ and EQI and the corresponding internal standard. Therefore six analyte-free urine samples (i.e. not containing EQ or EQI) were cleaned-up as described in the “Urine sample preparation” section and analyzed by LC-MS/MS. The respective mass transitions were screened for interfering peaks. Additionally, the same six urine samples were spiked with 1 µg/L EQ and EQI for accuracy determination. The mass transition of D_{10}-EQI m/z 188.0 → 182.1 corresponding to the quantifier MRM for EQI showed interfering peaks in some blank samples. That is why we chose the transition m/z 188.0 → 152.1 corresponding to the qualifier MRM for EQI as MRM for measurements of the internal standard. No interfering peaks could be detected for all other MRMs listed in Table 2.

Accuracy and precision were determined by spiking analyte free native human urine samples at four different concentration levels (LOQ, low, medium, high). Inter-day accuracy and precision were determined by analyzing five spiked urine samples for each concentration level on three different days. Intra-day accuracy and precision values were derived from five spiked samples per level of one day. Intra- and inter-day precision was evaluated calculating the relative coefficients

of variation (CVs), which should not exceed 15 % for concentrations > three times the LOQ and 20 % for concentrations up to three times of the LOQ, respectively. Acceptance criteria were met with accuracy rates of 85–115 % (80–120 % at levels up to three times the LOQ). Sample work-up dependent losses of analytes were expressed by the recovery. Recovery rates were determined by comparing the analyte concentrations at three levels, measured when analyte free urine samples (N=3) were spiked after sample work-up (reference, corresponding to 100 %), and when the same urine samples were spiked before the sample work-up procedure. LOQs were validated within accuracy and precision experiments and were set to the lowest concentrations fulfilling the requirements of accuracy and precision. The LODs were calculated by dividing the LOQ by three. Accuracy after dilution was investigated by spiking three different analyte free urine samples with concentrations of EQ and EQI above their highest calibration level (ULOQ) and diluting the samples with water. Three different dilutions (1:30, 1:6 and 1:3) were tested and accuracy was found to be acceptable in the range between 85 and 115 %.
Occurring matrix effects (ME) were evaluated by comparing spiked and processed urine samples at low and high concentrations of the analytes with aqueous reference standards possessing the same concentrations of analytes (reference, corresponding to 100 %). MEs are described as ratios from processed samples to references. Ratios > 1 indicate a signal enhancing ME, signals < 1 indicate a signal suppressing ME. Carry over effects were tested by repeated injection (N = 5) of the highest calibrator followed by injection of methanol as a blank sample. Stability of analytes during storage was investigated at room temperature, 10 °C and -20 °C over different time periods ranging from one day up to several months. Results of the method validation are summarized in Table S1.

Calibration

For calibration an analyte free urine pool consisting of at least three different urine samples was used and calibration standards were freshly prepared according to the validated method described. A total of ten calibrators were prepared by spiking increasing amounts of EQ and EQI to urine from 0.03-20 µg/L (0.03; 0.05; 0.1; 0.2; 0.5; 1.0; 2.0; 5.0; 10; 20 µg/L). Linear calibration was achieved by applying 1/x weighting.

Table S1: Method validation data for the quantification of EQ and EQI in urine

	EQ	EQI
LOD (µg/L)	0.01	0.01
LLOQ (µg/L)	0.03	0.03
Calibration range (µg/L)	0.03 – 20	0.03 – 20
Precision (*intra*-day, N=3x5), µg/L (CV, %)	0.03 (2.7 – 5.5)	0.03 (1.8 – 5.8)
	0.10 (4.7 – 8.8)	0.10 (0.5 – 6.4)
	1.00 (1.3 – 4.3)	1.00 (0.6 – 2.4)
	10.0 (1.2 – 3.0)	10.0 (1.3 – 1.9)
Precision (*inter*-day, N=15), µg/L (CV, %)	0.03 (6.2)	0.03 (5.3)
	0.10 (6.3)	0.10 (3.5)
	1.00 (4.3)	1.00 (3.1)
	10.0 (2.8)	10.0 (3.4)
Accuracy (*Intra*-day, N=3x5), µg/L (%)	0.03 (101.3 – 111.3)	0.03 (102.0 – 110.7)
	0.10 (100.4 – 105.2)	0.10 (106.4 – 108.4)
	1.00 (101.4 – 109.3)	1.00 (107.0 – 111.7)
	10.0 (107.8 – 112.0)	10.0 (99.5 – 107.0)
Accuracy (*Inter*-day, N=15), µg/L (%)	0.03 (104.4)	0.03 (106.0)
	0.10 (102.6)	0.10 (107.5)
	1.00 (103.9)	1.00 (109.8)
	10.0 (110.0)	10.0 (103.0)
Recovery (N=3), µg/L (%)	0.10 (13.0)	0.10 (13.3)
	1.00 (12.8)	1.00 (14.6)
	10.0 (6.6)	10.0 (17.1)
Matrix effects (aqueous standard = 100 %) (N=3), µg/L (%; CV, %)	0.10 (78.1; 1.7)	0.10 (103.1; 0.5)
	10.0 (95.3; 1.4)	10.0 (93.1; 1.6)
	IS 0.10 (84.6; 3.5)	IS 0.10 (99.1; 2.8)
	IS 10.0 (78.5; 1.9)	IS 10.0 (98.1; 2.1)
Carry-over effects (µg/L)	none	0.006
Stability at 20 °C, 20 h	No	Yes
Freeze-thaw-stability, 6 cycles -20 to 20 °C	Yes	Yes
Post preparation stability at 10 °C, 4 days	Yes	Yes
Stability of stock solutions at -20 °C, 11 months	No	Yes

Toxicokinetic parameters after a single oral dosage of Ethoxyquin

Fig S3: Time courses of the urinary excretion of EQI after oral administration of EQ to Subjects 2-5 displayed as **A** excretion rate in µg/h and **B** cumulative excretion in µg

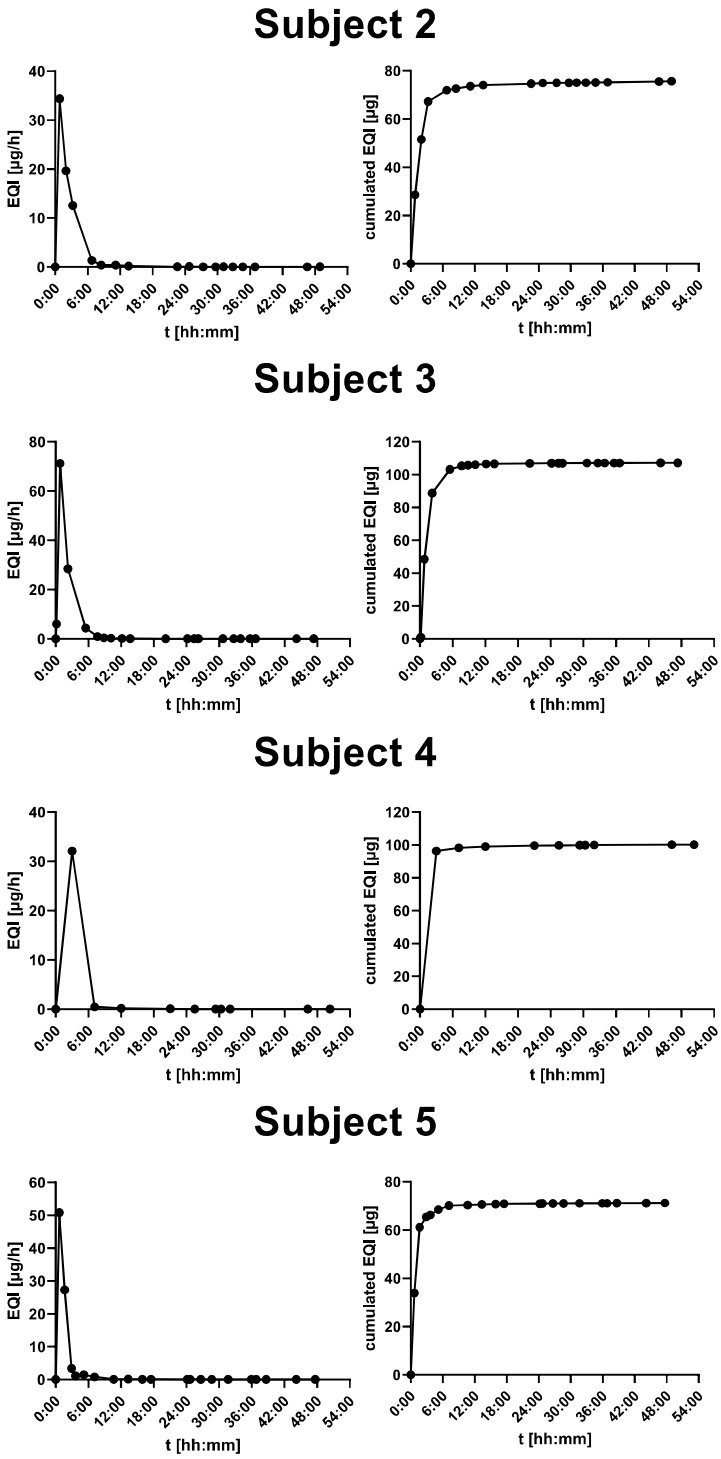


Fig. S4:Overlaid chromatograms for MRM transitions of EQI (black) and 6-OH-EQ (grey) after injection of 10 µg/L 6-OH-EQ. Although the sample originally did not contain EQI, two peaks could be identified in the MRM transition for EQI. The first peak at 2.4 minutes is retained at the same time as in the MRM transition for 6-OH-EQ indicating that 6-OH-EQ was oxidized to EQI in the ionization chamber of the mass spectrometer. In addition, a small fraction of 6-OH-EQ oxidized to EQI before the chromatographic separation took place (smaller peak at 2.8 minutes (in black) in MRM transition of EQI).

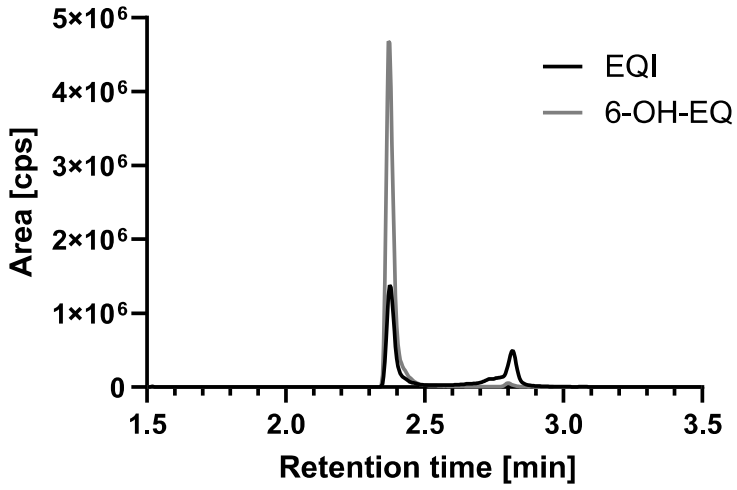


Fig. S5: Transformation pathway for the glucuronidated 6-OH-EQ (6-OGlu-EQ) during sample preparation. After enzymatic hydrolysis, the unconjugated 6-OH-EQ is converted into its oxidized form.

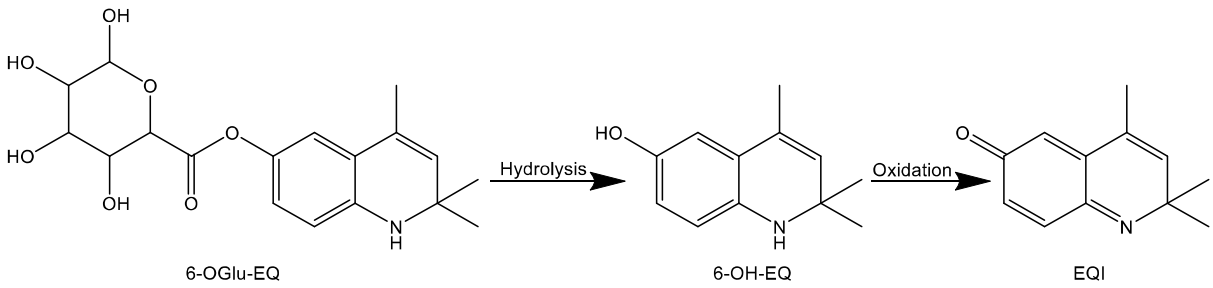
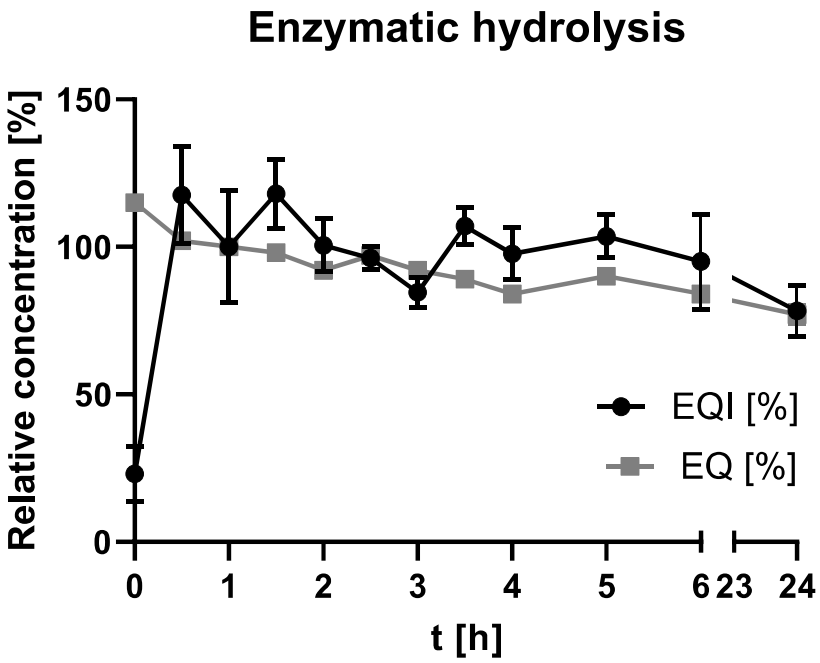


Fig. S6: Extent of enzymatic hydrolysis of a sample with high concentrations of 6-OH-EQ glucuronide determined as unconjugated EQI and of EQ, respectively (first urine fraction of Subject 2 after oral administration of EQ), in relation to the incubation time. Values were analyzed in triplicates. Concentrations in %, 100% refers to the incubation time used in the validated method (1.0 h).



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4) Summary

Due to the progressive development of our society, the amount of chemicals used to facilitate our everyday lives is steadily rising with the consequence of an increasing exposure of an average person with anthropogenic chemicals, especially in industrialized countries. Several chemicals used in our everyday lives can cause detrimental effects even though they are authorized by national or international institutions. Detrimental effects of a chemical must be evaluated during the authorization process and in some cases, concentration limits for products are established in order to ensure product safety and to protect human health. But even if concentration limits for products are set, this does not allow a conclusion on the real human exposure of an individual to this chemical. Since these data can be accessed via HBM only, HBM was gaining in importance over the last decades resulting in more and more HBM initiatives like the German Human Biomonitoring Initiative (Deutsche Human-Biomonitoring Initiative; cf. 1.4 The German Human Biomonitoring Initiative) which aims to develop HBM methods for up to 50 chemicals with emerging health relevance.

The aim of this work was to develop HBM methods for the chemicals 7-hydroxycitronellal, Uvinul A plus® and ethoxyquin, which were all selected within the German Human Biomonitoring Initiative. The first chemical, 7-hydroxycitronellal, is frequently used as fragrance in cosmetics and cleaning and washing agents. Uvinul A plus® mostly serves as UV filter in sunscreens and anti-aging products, and ethoxyquin is an antioxidant frequently found in farmed fish products like salmon.

At the beginning of this work, human metabolism was unknown for all three compounds. That is why human metabolism had to be investigated and suitable biomarkers of exposure had to be identified before optimizing the HBM method. For this purpose, five healthy volunteers each were orally administered with one of the chemicals, urine voids of the following 48 or 72 h after administration were collected and analyzed for possible metabolites. For 7-hydroxycitronellal and Uvinul A plus®, the

same five volunteers received an additional dermal dose of the test substance in form of a spiked sunscreen in order to depict a real exposure scenario, because these two chemicals are mostly used in cosmetics and the main exposure occurs *via* the skin.

After selection of suitable metabolites, authentic deuterated internal standards were obtained from a contract laboratory, the HBM methods were optimized and validated according to DFG and FDA guidelines. All three HBM methods contain an enzymatic hydrolysis step before samples extraction, because urinary metabolites are partly present as conjugates, e.g. as glucuronides (cf. 1.3 Selection of the biological matrix) and only unconjugated metabolites were determined after sample preparation. Necessity of an enzymatic hydrolysis was confirmed for all three methods and ratios between conjugated and unconjugated forms in high concentrated samples of every participant could be calculated. The deconjugated metabolites were extracted by using the solvent which was experimentally found to give the highest recovery rates. After sample preparation, the sample extracts were analyzed by means of UPLC-MS/MS.

UHPLC was used to achieve a satisfactory separation efficiency for the analytes and was preferred over GC in all three methods, because with UHPLC non-volatile analytes could be analyzed as well as volatile analytes. Additionally, shorter measuring times can be achieved by using UHPLC instead of GC, which is an essential parameter for a high throughput method. Positively charged analyte ions were generated by means of electrospray ionization, and a selective determination of the analytes was achieved by conducting MRM experiments.

The validated methods were applied to all samples of the respective human metabolism study and to the samples of a small collective not occupationally exposed to one of the original chemicals in order to prove the sufficient sensitivity of the methods and for a first rough estimation of the biomarker levels to be expected in further studies. For every original substance, at least one suitable biomarker of exposure could be

identified and toxicokinetic parameters such as the amount excreted, maximum excretion time and elimination half-life and conversion factors were calculated for every biomarker. Conversion factors allow to extrapolate from the urinary concentrations of one or more biomarkers of exposure to the systemic exposure of an individual with a chemical.

The completed methods are cross-examined regarding their reproducibility including basic validation parameters like linearity, accuracy, precision and recovery by a second laboratory from the ranks of the scientific working group "Analysis in Biological Materials" of the DFG. For this purpose, a second laboratory gets instructed and if the second laboratory can confirm the feasibility and suitability of the method, the method including the results of the cross-examination will be published online in open access by the DFG [42, 43] . This procedure was already successfully conducted for 7-hydroxycitronellal, the methods for Uvinul A plus® and ethoxyquin are currently under revision.

For the three substances, 7-hydroxycitronellal, Uvinul A plus® and ethoxyquin, HBM methods were successfully developed and first estimations for the human exposure with these chemicals could be carried out proving the suitability of the methods for the intended use. Indeed, human exposure levels obtained from the pilot studies cannot be interpreted as representative values, because of the small number of samples analyzed and the small collection area (greater Munich area) , but the measurements suggest that a not negligible part of the general population is exposed to these chemicals. Therefore, HBM studies with more representative study cohorts and a larger number of samples must be conducted.

For 7-hydroxycitronellal, a first larger HBM study with 329 study samples derived from the German Environmental Specimen Bank has already been conducted. The method developed within this work could be successfully applied to all study samples and a decrease in the human exposure to 7-hydroxycitronellal from the year 2000 to 2018 in Germany could be observed. This study was also conducted with a view to

GerES VI (cf. 1.1 History of human biomonitoring), where even a larger number of samples from German adults will be analyzed for 7-hydroxycitronellal levels [104]. Presumably, similar approaches will be carried out for Uvinul A plus® and ethoxyquin, but at this time, no information on HBM actions for both substances are available.

HBM values for the three chemicals investigated will be derived from large epidemiological studies like GerES by analyzing the samples with the HBM methods developed. These values will contribute to an improved evaluation of the substances investigated concerning their effects on human health and responsible authorities will be able to adapt concentrations limits in consumer products.

5) List of abbreviations

6-OH-EQ	1,2-Dihydro-2,2,4-trimethyl-6-quinolinol
AHB	2-[4-(Amino)-2-hydroxybenzoyl]benzoic acid
AM	Ambient monitoring
BMU	German Federal Ministry for the Environment, Nature Conservation and Nuclear Safety (Bundesministerium für Umwelt, Naturschutz und nukleare Sicherheit)
DFG	German Research Foundation (Deutsche Forschungsgemeinschaft)
DHB	2-[4-(Diethylamino)-2-hydroxybenzoyl]benzoic acid
DHHB	Hexyl 2-[4-(diethylamino)-2-hydroxybenzoyl]benzoate (Uvinul A plus®)
EHB	2-[4-(Ethylamino)-2-hydroxybenzoyl]benzoic acid
EQ	1,2-Dihydro-6-ethoxy-2,2,4-trimethylquinoline (Ethoxyquin)
EQDM	1,8'-Ethoxyquin dimer
EQI	2,2,4-Trimethyl-6(2*H*)-quinolinone
FDA	US Food and Drug Administration
GC	Gas chromatography
GerES	German Environmental Survey
HBM	Human biomonitoring

IFRA	International Fragrance Association
INCI	International Nomenclature of Cosmetic Ingredients
JECFA	The Joint FAO/WHO Expert Committee on Food Additives
LLE	Liquid-liquid extraction
LOQ	Low limit of quantification
MRM	Multiple reaction monitoring
MS/MS	Tandem mass spectrometry
NHANES	National Health and Examination Survey
NOEL	No Observed Effect Level
SALLE	Salt-assisted liquid-liquid extraction
UBA	German Environment Agency (Umweltbundesamt)
U(H)PLC	Ultra (high) performance liquid chromatography
VCI	German Chemical Industry Association (Verband der Chemischen Industrie)
WHO	World Health Organization

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