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Determination of selected bisphenols, parabens and estrogens in human plasma using LC-MS/MS

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ABSTRACT

In this study, a novel liquid chromatography - tandem mass spectrometry method for the simultaneous determination of bisphenols (BPA, BPS, BPF, BPAF), parabens (methyl-, ethyl-, propyl-, butyl-, benzyl-paraben) and estrogens (estrone, estradiol, estriol) in human plasma is presented. Since all analytes possess the phenolic group, dansyl chloride derivatization was applied in order to gain high sensitivity. The method was validated according to FDA guidelines, and all validation requirements were satisfactory. The lower limits of quantifications were 41.6, 54.9, 43.5 and 150.8 pg/mL for BPA, BPS, BPF and BPAF; 172, 149, 171, 134 and 202 pg/mL for methyl-, ethyl-, propyl-, butyl- and benzyl-paraben; 10.5, 6.7 and 9.4 pg/mL for estrone, estradiol and estriol, respectively. This is the first method allowing the determination of plasma bisphenols, parabens and estrogens in one run, and also the first determination of BPF levels in human plasma. The method was used to examine the plasma levels of healthy normospermic men, where three times higher plasma levels of BPF than BPA were found.

1. Introduction

In recent decades, an increased incidence of various hormonal disorders has been reported, associated with widespread pollution and the presence of chemicals in the environment as well as the food chain. Many chemicals have the ability to interfere with the endocrine system, and these substances have thus been termed endocrine disruptors (EDs) [1]. The primary routes that EDs enter an organism are the intake of contaminated food and fluids, breathing contaminated air, and transdermal absorption [2]. EDs may affect hormone biosynthesis, altering their genomic and non-genomic effects, control and regulatory mechanisms, as well as epigenetic manifestations.

One of the most widely discussed EDs is the estrogen mimic bisphenol A (BPA). This chemical is released from epoxy resins, polycarbonate and other plastics used for food and cosmetic packaging, toys, various paper products such as thermal receipts, and from composites used in dentistry. BPA is known to affect hormonal homeostasis, binding to estrogen receptors and leading to a combination of agonistic and/or antagonistic actions depending on the target tissue. In addition, BPA interacts with the androgen receptor (with anti-androgenic activity), the pregnane X receptor, and the thyroid and glucocorticoid receptors [3–5]. Thousands of studies have demonstrated the harmful effects of BPA on living organisms, and therefore its usage is now limited or even prohibited, especially in products intended for children [6]. During the past few decades, many “BPA free” products have been introduced to the market. These plastic products are presented as being safe; however, in the majority of “BPA free” products other BPA analogues can be found, in particular bisphenol S (BPS) and bisphenol F (BPF) [4,7,8].

BPS is used in polycarbonate plastic and thermal paper products (“BPA free” papers) because of its higher thermal stability [9]. BPS is chemically more stable than BPA, but is worse in terms of biodegradability and has higher dermal penetration. BPF has been used to make protective coatings for food and beverage cans, and BPF epoxy resins are also used for several consumer products such as liners, lacquers, adhesives and dental sealants [10]. Both bisphenols have been detected in many products of daily use such as personal care products (e.g. toothpaste, body wash, shampoo), paper products (e.g. money, tickets,

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receipts) and food (e.g. meats, vegetables, canned foods). BPS and BPF have also been detected in indoor dust, surface water, sediment, and sewage effluent [10].

As alternatives to the harmful compound BPA, these substitutes should be ideally inert or at least less toxic than the original compound. Unfortunately, many of them were not tested before their introduction to the market, and they can be similar or even more potent EDs than the original. Such a replacement is called "regrettable substitution" [11]. Results of many various studies have indicated that the potencies, metabolism and mechanism of actions of BPS and BPF are similar to BPA including hormonal activities (with antiandrogenic, estrogenic, and aryl hydrocarbon activity, as well as inhibitory hormonal signaling in adipocytes), and thus they may pose similar potential health risks as BPA [8]. Surprisingly, BPF was found to be possibly even more potent than BPA [10]. Despite these concerns, monitoring and evaluation of the effects of these alternative bisphenols is still limited, and their use has not yet been regulated.

Another BPA analogue, bisphenol AF (BPAF), is a fluorinated BPA analogue used in the production of polycarbonates, polyamides, polyamines and polyesters [6]. It is also present in electronic materials and gas permeable membranes [12]. BPAF is known to possess higher estrogenic activity than BPA and comparable anti-androgen activity. BPAF is considered potentially more harmful to living organisms because of its higher electronegativity and reactivity of the CF_3 moiety comparing to the CH_3 moiety present in the BPA [13].

Complicating matters, the human organism is usually exposed to a mixture of bisphenols along with other EDs, which may have additive or even synergic effects. Some of the most important of these are parabens, effective antimicrobial agents used as preservatives mainly in cosmetics and pharmaceuticals, but also in food commodities and industrial products. They are esters of p-hydroxybenzoic acid with alkyl substituents ranging from methyl to butyl or benzyl groups. The most commonly used are methylparaben (MP), and propylparaben (PP), but ethylparaben (EP), butylparaben (BP) and benzylparaben (BenzylP) are of interest as well. After the discovery of their estrogenic potential, parabens were listed as EDs, and the European Union authorized their use in only limited amounts [14]. In addition to their estrogenic properties, some parabens have been reported to display anti-androgenic activity by binding to androgen receptors and causing the inhibition of testosterone-induced transcription. Their usage in now limited in the EU, the USA and Canada to 0.4% content for a single paraben and 0.8% for mixtures of all parabens [15], but in accordance with current legislation, parabens are still extensively used and generally characterized as "safe". Taking into account the possibility of exposure to a combination of EDs and their possible synergic effects, however, guaranteed "safe doses" may not be as safe as they appear. Reflecting this concern, there are an increasing number of "paraben-free" products available, especially in personal care products intended for children.

Many analytical approaches have been reported for measuring parabens and BPA separately, and several publications report measurements of parabens and BPA in various biological fluids in one run [16–19]. However, reflecting the recent trend to substitute BPA with its alternatives, the determination of alternative bisphenols has become an important analytical challenge. Several LC-MS/MS methods have been developed to measure the levels of alternative bisphenols (BPS, BPF, BPAF), but within biological applications predominately in urine [20–22]. To the best of our knowledge, no methods have yet been published for the determination of various alternative bisphenols (BPS, BPF and BPAF) in human plasma or for estimating parabens and alternative bisphenols in one run.

All mentioned estrogen mimicking substances may interplay with the natural physiologic estrogens estrone (E1), estradiol (E2) and estriol (E3). They are mainly known as female hormones responsible for development and maintenance of female secondary sex characteristics, however they also play important role in the male organism. The estrogen biosynthesis is located in the men testicular cells as well as specific men hormone – testosterone. It is thought, that the absence of estrogen receptors in testicular cells may cause adverse effects in the spermatogenesis as well as steroidogenesis [23].

Here we present the extension of previously developed LC-MS/MS method enabling estimation of BPA, E1, E2 and E3 in human plasma [24]. The method was extended and validated for simultaneous determination of alternative bisphenols (BPS, BPF, BPAF) and parabens (MP, EP, PP, BP, BenzylP) together with previously published BPA and estrogens.

2. Experimental

2.1. Chemicals and reagents

Standards of methylparaben (MP), ethylparaben (EP), propylparaben (PP), butylparaben (BP), benzylparaben (BenzylP), bisphenol A (BPA), bisphenol S (BPS), bisphenol F (BPF), bisphenol AF (BPAF) and deuterated standards of BPA (d16BPA), 17β-estradiol (d3E2) were purchased from Sigma-Aldrich (St. Louis, MO, USA) as were 99,9% tert-butyl methyl ether (MTBE), acetone, acetonitrile, sodium bicarbonate, sodium hydroxide, potassium hydroxide, 10% palladium on charcoal and dansyl chloride. The deuterated standards of MP (d4MP) and PP (d4PP) were obtained from Chiron (Trondheim, Norway). Deuterated standards of EP (d4EP) and BP (d4BP) were from EQ Laboratories GmbH (Augsburg, Germany). The steroids estrone (E1), 17β-estradiol (E2), estriol (E3) and deuterated standards of estrone (d4E1) and estriol (d4E3) were purchased from Steraloids (Newport, USA). LC-MS grade methanol and water for chromatography were from Merck AG (Darmstadt, Germany). Physiological solution was from Ardeapharma, a.s. (Ševětín, Czech Republic). Methanol p.a., acetic acid p.a., chloroform p.a., tetrahydrofurane p.a., ethanol p.a., bromine, sodium sulfite p.a., and tert-butyl alcohol p.a. were from Lach-Ner, s.r.o. (Neratovice, Czech Republic). [1,2,6,7-3H]Cortisol, specific radioactivity 3.04 TBq/mmol, was from Amersham Biosciences, Inc. (Amersham, UK). Deuterium 2.8 was purchased from Linde Gas a.s. (Munich, Germany).

2.2. Synthesis of d4BPS

d4BPS was synthesized via catalytic dehalogenation mediated by palladium on charcoal from tetrabrominated BPS, prepared from commercially available BPS according to Vibhute et al. and Garchar et al. [25,26] with modified conditions. High-resolution mass spectrometry with electrospray ionization (HR-MS ESI) (Exactive Plus Orbitrap, Thermo Fisher Scientific, Waltham, MA USA) indicated that the relative abundance of d4BPS was higher than 90%. In the HR-MS ESI spectra there were peaks characteristic for tetradeuterated BPS, typical adduct $[M\text{-}H]^-$ with formula $C_{12}H_6{}^2H_4O_4S$ and tetrabrominated BPS, typical adduct $[M\text{-}H]^-$ with formula $C_{12}H_6O_4S^{79}Br_2{}^{81}Br_2$. The observed isotopic pattern corresponded with calculated formulas and the theoretical estimation of *m/z* observed ions. Tetrabrominated as well as deuterated BPS were also demonstrated using NMR data (Bruker Avance III, Bruker Daltonics GmbH, Bremen, Germany), in ^1H NMR a singlet signal of phenol hydrogens (H-2 a H-6) of tetrabrominated BPS at δ 8.19 ppm was observed, and in ^{13}C NMR four aromatic carbon signals were identified between 112 and 156 ppm. A detailed description of the synthetic procedure is shown in the Supplementary material.

2.3. Preparation of reagents, stock solutions, calibration mixture and quality control samples

The sodium bicarbonate buffer (10 mM, pH 10.5) was prepared by dissolving 0.42 g of sodium bicarbonate in 50 mL of ultra-pure water. pH was adjusted to 10.5 with an aqueous 1 M solution of sodium hydroxide.