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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.

Table 1
Mass spectrometric settings: Precursor, quantification and confirmation ions, declustering potentials (DP), entrance potentials (EP), collision entrance potentials (CEP), collision energies (CE) and collision cell exit potentials (CXP) for measured analytes and deuterated internal standards and retention times (RT) of detected bisphenols, parabens and estrogens in plasma. The listed values correspond to the *m/z* (Q1/Q3). The molecular formulas are shown in Fig. S1.

Analyte	Precursor ion (Q1)	Quantif. Ion (Q3)	Confirm. Ion (Q3)	DP (V)	EP (V)	CEP (V)	CE (V)*	CXP (V)	Plasma RT (min)
BPA	695	171	170	76	8	28	63(61)	4	8.92
d16BPA	709	171	170	86	7.5	28	65(65)	4	8.87
BPS	717	171	156	86	7	26	59(99)	4	7.96
d4BPS	721	171	156	81	9.5	32	61(93)	4	7.95
BPF	667	171	170	71	10.5	28	59(59)	4	8.45
BPAF	803	171	170	91	10.5	34	69(69)	4	9.32
MP	386	171	156	46	8	26	33(53)	4	6.98
d4MP	390	171	156	51	7.5	22	35(53)	4	6.96
EP	400	171	156	51	5.5	20	35(53)	4	7.30
d4EP	404	171	156	61	5.5	16	37(59)	4	7.29
PP	414	171	156	51	7	20	35(55)	4	7.63
d4PP	418	171	156	51	6	22	39(57)	4	7.62
BP	428	171	156	51	6.5	22	39(61)	4	7.97
d4BP	432	171	156	46	7	22	41(59)	4	7.96
BenzylP	462	115	156	41	6	18	67(99)	4	7.90
E1	504	171	156	71	5	22	47(75)	4	7.88
d4E1	508	171	156	71	5	24	49(77)	4	7.87
E2	506	171	156	76	5	24	49(75)	4	7.98
d4E2	509	171	430	76	5	22	49(49)	4	7.97
E3	522	171	156	76	5	22	49(79)	4	7.16
d4E3	524	171	156	71	8	17	47(77)	4	7.15

* Values for the confirmation ion are given in the parentheses.

Stock solutions of standards and deuterated standards were prepared gravimetrically in methanol at a concentration of 1 mg/mL. Working standard solutions were prepared at 10 ng/mL and 1 ng/mL by diluting the stock solutions with methanol. The calibration mixture was prepared from appropriate volumes of individual standard working solutions and methanol to obtain a solution containing 32 ng/mL MP, 12 ng/mL EP, PP, BP and BenzylP, 4 ng/mL bisphenols and 1 ng/mL estrogens. Eight-point calibration curves were constructed in the range of 0.032–4 ng/mL for bisphenols; 0.25–32 ng/mL for MP; 0.094–12 ng/mL for EP, PP, BP and BP; and 0.008–1 ng/mL for estrogens. Solutions and mixtures were stored at −20 °C and allowed to equilibrate to laboratory temperature for at least 15 min before use. All standard and internal standard (IS) solutions were first pipetted into an empty glass tube and evaporated in a vacuum evaporator to dryness. The calibration curve solutions, zero solutions (only IS added) and blank solutions (no standards added) were prepared in duplicate, substituting plasma with physiological solution.

Quality control samples were prepared in-house, using a plasma pool with appropriate volumes of steroid stock solutions. Charcoal-treated plasma used for slope and matrix tests was prepared by an in-house method employing the multistep adsorption of steroids on charcoal followed by repeated centrifugation at 10,000×*g*. The absence of steroids was checked by spiking the plasma with [^{3}H]cortisol (10,000,dpm/mL) and confirming that the remaining radioactivity was close to zero.

2.4. Application of the method

Plasma samples were obtained from patients attending the Centre of Assisted Reproduction Pronatal (Prague, CZ). Each patient had undergone a standardized ejaculate examination (spermiogram) according to the World Health Organization (WHO) criteria. A group of 58 patients was chosen according to the criteria of being of reproductive age (36.2 ± 5 years) with normospermia and normal plasma testosterone levels so that the cause of infertility if present was on the female partner. The protocol was approved by the Ethical Committee of the Institute of Endocrinology. Informed and written consent with the use of biological materials for research reasons was obtained from all subjects participating in the project. Blood was collected in collection tubes with K2EDTA, immediately centrifuged, and plasma was transferred to glass tubes and stored at −20 °C.

2.5. Sample preparations

A plasma sample (500 µL) was spiked with 10 µL of IS mixture and diluted with 500 µL of physiological solution. Samples were extracted with 2 mL of MTBE for 1 min (liquid-liquid extraction); the water phase was frozen in solid carbon dioxide and the organic phase was transferred into a glass tube and the solvent was evaporated until dryness using a vacuum concentrator (55 °C). Control samples of known concentration of individual analytes were processed in the same way.

The derivatization reaction was performed as described in Vitku el al. [24] and Anari et al. [27]. Briefly, 50 µL of 100 mM sodium bicarbonate buffer and 50 µL of dansyl chloride in acetone (1 mg/mL) were added to dry residues, vortexed, incubated (60 °C; 5 min), cooled to laboratory temperature and evaporated using a vacuum concentrator (55 °C). The dry residues were reconstituted in 300 µL of methanol. The sample was finally diluted 1:1 with a 10 mM aqueous solution of ammonium formate and 50 µL was injected in the LC-MS/MS for analysis.

The dansyl chloride (molecular weight=269.7) monoderivatives were prepared from estrogens and parabens, while bisphenols were derivatized on both phenyl groups.

2.6. LC-MS/MS conditions

LC-MS/MS was performed using an API 3200 (Sciex, Concord, Canada) triple stage quadrupole mass spectrometer with electrospray ionization (ESI) connected to a ultra-high performance liquid chromatograph (UPLC) Eksigent ultraLC 110 system (Redwood City, CA, USA). Chromatographic separation was carried out on a Kinetex C18 1.7 µm (150×3.0 mm) column (Phenomenex, Torrance, CA, USA) with a corresponding security guard at a flow rate 0.4 mL/min at 50 °C.

Mobile phases consisted of water (solvent A) and methanol (solvent B). The following gradient was employed (all steps linear): 0 min, 50:50 (A:B); 2 min, 50:50; 6 min, 10:90; 7 min, 5:95; 8 min 80 s, 5:95; 9 min, 50:50; 11 min, 50:50 and at 11 min stop. Retention times of individual analytes are given in Table 1.

The mass spectrometer was operated in the positive ESI mode using

multiple-reaction monitoring transitions (MRMs). The conditions were as follows: curtain gas: 25 psi (172.38 kPa), ion spray voltage: 5.5 kV, vaporizer temperature: 600 °C, ion source gas 1: 40 psi (275.79 kPa), ion source gas 2: 60 psi (413.69 kPa), interface heater: on. Nitrogen was produced by a high purity nitrogen generator (Peak Scientific instruments Ltd., model NM20Z, Renfrewshire, Scotland) and employed as curtain, nebulizer and collision gas. Ion source and MS/MS conditions were optimized by the infusion of 0.2 μg/mL of the derivatized individual analytes to the mass spectrometer at 20 μL/min and are listed in Table 1. The molecular structures of all analytes are shown in Fig. S2.

2.7. Quantification of the samples

The analytes were quantified by means of calibration curves obtained by linear regression analysis using internal standardization. d16BPA was used as IS for BPA, BPF and BPAF, while d4BPS was employed for BPS. All estrogens and parabens had their own IS except BenzylP, for which d4BP was used. The data were fitted to a linear least square regression curve with a weighting index of 1/x. All analytes and IS were identified by their chromatographic retention times and by two mass transitions. All solvent blanks were free of contamination. The assay acceptance criterion for each back-calculated standard concentration was 15% deviation from the nominal value.

2.8. Quality assurance

In the analysis of endocrine disruptors, background contamination may be a problem. To avoid paraben contamination, all reusable laboratory glassware was washed in ultrapure water, acetonitrile, methanol p.a. and heated for 8 h at 400 °C. After heating, glassware was sequentially rinsed with acetonitrile, methanol and water of HPLC grade. Furthermore, gloves (Powder-Free Nitrile Examination Gloves, Genesee Scientific, USA) were worn during sample preparation, all laboratory surfaces were washed with methanol p.a. before sample processing, the laboratory floor was washed with methanol p.a. once a week, and only paraben free soap was used in the laboratory.

To avoid bisphenol contamination, glass equipment, e.g. Pasteur pipettes and glass syringes, were used for the dosage of all reagents as well as during sample dispensing. All samples and reagents were stored in the dark (to avoid photodegradation) in glass vials and bottles and covered with rubber or a polytetrafluor ethylene cover.

2.9. Paraben and bisphenol content in plastic laboratory caps

The majority of samples were stored in plastic caps because of better accessibility and to save space in freezers. We tested the release of bisphenols and content of parabens in three types of plastic caps used in our laboratory: (A) 1.5 mL Microcentrifuge Tubes 80–1500, polypropylene, Biologix, China (B) 1.5 mL Eppendorf Microcentrifuge tubes, polypropylene, Böttger, Germany (C) 1.0 mL Nunc CryoTube™ Vials, polypropylene, Thermo Scientific, Denmark.

For the purpose of testing the caps, three volunteers from our laboratory underwent blood collection (20 mL). Blood was collected in collection tubes with K2EDTA, immediately centrifuged, and the plasma was transferred to a glass beaker and mixed to form a plasma pool. K2EDTA tubes did not display bisphenol or paraben contamination [24]. The plasma pool as well as physiological solution were aliquoted to A, B, C caps and glass tubes. All caps and glass tubes were stored at −20 °C for 14 days, one month and 7 months. Quantitation of bisphenols and parabens was performed immediately and after each of the storage periods. No amounts of BPA, BPS, BPF, BPAF, PP or BenzylP were detected in any types of caps or glass tubes immediately or after the storage periods in plasma or physiological solution. MP, EP and PP were detected immediately in the plasma pool (MP: 0.477 ± 0.096 ng/mL, EP: 0.044 ± 0.003 ng/mL, PP: 0.188 ± 0.018 ng/mL), but there were no statistically significant changes observed after the storage periods in any types of caps and glass tube. In physiological solution, no content of MP, EP and PP was detected during the whole experiment period.

2.10. Validation

The LC-MS/MS method was validated according to the FDA Guidance for Industry [28]. Validation parameters include (1) selectivity, (2) precision, (3) recovery, (4) calibration curves, and (5) stability of the analytes in spiked samples. Acceptable selectivity was defined as the absence of any detectable MRM LC-MS/MS ion currents at the retention time regions of each analyte and its IS in blank plasma samples (double blank).

Accuracy, precision and recovery were determined by using 6 samples per concentration, and three different concentrations were examined. Pooled plasma samples containing IS were used as the first concentration. The spiked concentrations were determined to obtain low, medium and high levels expected in human plasma according to our experiences and available literature [24,29–36]. The spiked concentrations were as follows: 0.64, 3.2, 7.68 ng/mL for MP; 0.24, 1.2, 2.88 for EP, PP, BP and Benzyl P; 0.08, 0.4 and 0.96 ng/mL for bisphenols; and 0.02, 0.1, 0.24 ng/mL for estrogens. Spiked samples were processed in the same way as in Section 2.4.

Within-day precision (repeatability) and between-day precision (intermediate precision) were determined and expressed as relative standard deviation (RSD).

The recoveries for individual analytes were calculated as [(concentration of the analyte in the spiked sample – concentration in the non-spiked sample)/amounts of added analytes]×100 (%).

The lower limit of quantification (LLOQ) was defined as 10×the standard deviation/slope of the calibration curve [37].

Carry-over was determined by running a blank solvent after the highest calibrator and by injecting a control sample of known concentrations of analytes.

A freeze and thaw stability test, short-term temperature stability test, long-term stability test, stock solution stability test and a post-preparative stability test were also performed.

2.11. Data statistical analysis

Analyst software version 1.6 (Sciex, Concord, Canada) was employed for data acquisition, peak-area integration and quantitation of unknown plasma and CSF samples. Calibration curves were derived in each analytical run. Validation data were calculated using Microsoft Excel® 2013.

3. Results

3.1. Selectivity, precision, recovery, lower limit of quantification (LLOQ) and carry-over

There were no detectable MRM LC-MS/MS currents at the retention time regions of all analytes of interest. Precision (within-day and between-day) did not exceed 15% of the RSD. A satisfactory assay accuracy (recovery) ranging from 92.0% to 114.4% was obtained. The data from the precision and recovery analysis are shown in Table S2.

3.2. Calibration curves and matrix effect

Charcoal-treated plasma was first used as a blank matrix for the calibration curves. However, it contained small amounts of BPA, MP and PP, apparently from the manufacturing processes, and could therefore not be used for the construction of calibration curves for BPA, MP and PP. Hence, we examined five sets of calibration curves containing IS in charcoal-treated plasma and five sets of calibration

Table 2
Concentration ranges of the analytes and medians of measured samples (*n=58*) in plasma.

Analyte	Concentration range (ng/mL)	Median (ng/mL)
BPA	< LLOQ – 8.17	0.09
BPS	< LLOQ	not detected
BPF	< LLOQ – 6.23	0.28
BPAF	< LLOQ	not detected
MP	< LLOQ – 16.00	0.52
EP	< LLOQ – 0.36	0.36
PP	< LLOQ – 0.75	0.56
BP	< LLOQ	not detected
BenzylP	< LLOQ	not detected
E1	< LLOQ – 0.17	0.06
E2	< LLOQ – 0.22	0.02
E3	< LLOQ – 0.14	0.02

curves in physiological solution according to Higashi et al. [38]. The regression lines were constructed with 1/x weighting and reached good linearity ($r > 0.9992$) and acceptable reproducibility of slopes: [0.6515 ± 0.0196] (slope ± standard deviation) and 3.0% (RSD) for BPA; [0.2088 ± 0.0058] and 2.8% for MP; [0.458 ± 0.011] and 2.4% for PP. Slopes of the lines obtained from physiological solution and charcoal-treated plasma did not differ significantly. Therefore, we concluded that the plasma matrix had no significant impact on the determination of BPA, MP and PP.

Calibration curves for all other analytes constructed in physiological solution exhibited good linearity and satisfactory correlation coefficients ($r > 0.9992$) and reproducibility of slopes. [1.045 ± 0.0536] (slope ± standard deviation) and 5.1% (RSD) for E1; [1.1875 ± 0.0363] and 3.0% for E2; [1.1047 ± 0.0854] and 7.7% for E3; [0.3705 ± 0.0224] and 6.1% for BPS; [0.8565 ± 0.0312] and 3.7% for BPF; [0.2718 ± 0.0185] and 6.8% for BPAF; [0.527 ± 0.0222] and 4.2% for EP; [0.4725 ± 0.0059] and 1.2% for BP; [0.0747 ± 0.0068] and 9.1% for BenzylP. Slopes of the calibration curves were compared by the same manner as described in the previous paragraph. There was no significant difference observed for any bisphenols, parabens or estrogens, and therefore the plasma matrix did not display any matrix effect for the determination of target analytes.

The correlation coefficients as well as LLOQs and calibration ranges of individual analytes are summarized in Table S3. No carry-over was detected.

3.3. Stability tests

3.3.1. Freeze and thaw stability test

The stabilities of individual analytes were evaluated after three freeze and thaw cycles. Three aliquots in charcoal-treated plasma of each low and high concentrations in plasma were processed; 1.6 and 19.2 ng/mL for MP, 0.6 and 7.2 ng/mL for EP, PP, BP and BenzylP, 0.2 and 2.4 ng/mL for bisphenols, 0.05 and 0.6 ng/mL for estrogens. No significant differences among the analyte concentrations in corresponding samples before and after the test were observed.

3.3.2. Short-term temperature stability test

This test was performed with the same set of sample concentrations as described in Freeze and thaw stability test. The samples were analyzed after 20 h at laboratory temperature and compared with samples that were processed immediately after thawing. No significant differences among the analyte concentrations in corresponding samples before and after performing the test were observed.

3.3.3. Long-term stability test

The test was performed with similar concentrations as described in Freeze and thaw stability test. Samples were used in duplicates through all experiments, and their concentrations were monitored continuously. Concentrations of the samples were compared with those prepared at the first day of testing. The analyte concentrations of corresponding samples did not differ significantly during the test performance.

3.3.4. Stock solution stability test

The stability of stock solutions of the analytes as well as IS was determined after letting the solutions sit for 24 h at laboratory temperature. Two calibration curves were constructed using these solutions and compared with two calibration curves constructed from freshly prepared solutions. The instrument responses remained unchanged.

3.3.5. Post-preparative stability

The stability was assessed after letting samples sit for 24 h in the instrument autosampler (7 °C). The instrument response remained unchanged.

3.4. Determination of analytes in plasma samples

In normospermic men, BPA was detected in 45% and BPF in 40% of plasma samples, while BPS and BPAF were not detected. Plasma parabens were detected in 40%, 5% and 28% of samples for MP, EP and PP, respectively. BP and BenzylP were not detected. Plasma E1, E2 and E3 were detected in 84%, 79% and 59% of samples. Concentration ranges of measured analytes and corresponding medians in plasma are summarized in Table 2. Chromatograms of estrogens and measurable EDs in plasma sample are shown in Fig. 1, chromatograms of non-detected EDs are shown in spiked samples in Fig. 2.

4. Discussion

The governmental bodies of the European Union, United States (FDA), Canada (Health Canada) and other national authorities determine the thresholds for allowable contents of individual EDs. However, living organisms are exposed to combinations of these compounds, and such exposure to multiple agents present at or even below allowed limits may result in unanticipated effects on the human organism [14,39]. Parabens and bisphenols may occur together in various personal care products, food and beverages, either as preservatives or released from the plastics used in the packaging.

Studies monitoring levels of EDs often encounter problems during sampling, handling and storage of biological samples because of their possible contamination with EDs, as described previously for BPA [24]. Samples can be contaminated by parabens as well, mainly from various laboratory equipment washing and cleaning procedures. Therefore, in this study all reusable laboratory glassware was washed with organic solvents and water and heated 8 h at 400 °C in order to decompose all parabens. Samples need to be stored in glass vessels or vessels that do not leak bisphenols. We therefore tested the release of bisphenols and the content of parabens in three types of plastic caps used in our laboratory. All tested caps were confirmed to not release bisphenols or contain parabens. We had already tested the K2EDTA tubes used for plasma collection, with no contamination by bisphenol or parabens found.

Derivatization of analytes is effective to improve the sensitivity on LC-MS/MS and enabling us the measurement of EDs and estrogens in the pg/mL order of magnitude. Without derivatization, more sophisticated LC-MS/MS instruments would be necessary which are not easily accessible for many research and healthcare laboratories. 500 μL of plasma is routinely used amount for the majority of medical devices. The method performance characteristics for newly determined alternative bisphenols and parabens fulfilled the FPA guideline criteria and enable the simultaneous measurement of those analytes with high sensitivity and reliability together with previously published BPA and estrogens [24]. Compared to the previous method, the new method

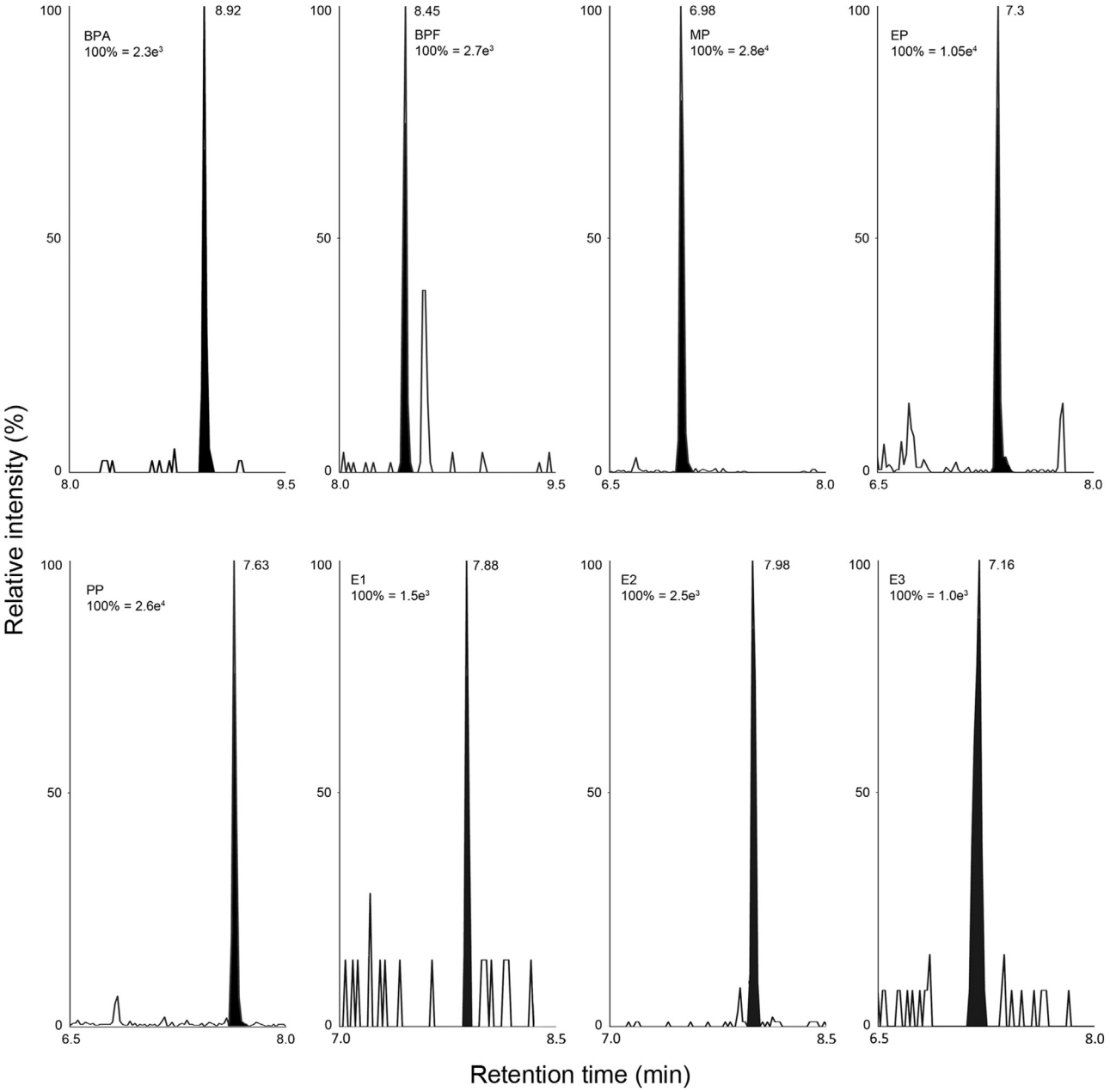

Fig. 1. Chromatograms of bisphenols, parabens and estrogens measured in plasma. The measured concentrations were BPA – 0.355 ng/mL; BPF – 0.225 ng/mL; MP – 0.523 ng/mL; EP – 0.193 ng/mL; PP – 0.669 ng/mL; E1 – 0.126 ng/mL; E2 – 0.061 ng/mL; E3 – 0.077 ng/mL.

provides an extension of eight EDs, which are actually of interest. Reflecting the previously found association between BPA and E2 plasma levels [33], simultaneous determination of bisphenol alternatives will let us possibility to search for associations between estrogen metabolism and mentioned EDs in the future.

The plasma levels of BPA and estrogens measured here are in accordance with our previous observations [24,33,34]. Of the alternative bisphenols only BPF was detected; interestingly, its plasma levels were three times higher than BPA. This indicates an increased usage of BPF as a consequence of restrictions in the use of BPA. We speculate that BPF is likely present in various “BPA free” products, from which it may leach into food or beverages. The diglycidyl ether of BPF was recently detected in human plasma and adipose fat samples [7], and BPF has been found in surface water, wastewater and sewage sludge [40–42], reflecting its widespread occurrence in the environment. Taking into account the higher possible potency of BPF [10] and the three-times higher BPF plasma levels compared to BPA found here, further studies are needed to examine the sources, transport, effects, environmental and biological consequences of BPF usage. Concerning determination of alternative bisphenols in plasma/serum, we have recently found only one study dealing with estimation of BPS in cashiers. Serum BPS was detected by LC-MS/MS in 40.6% of cashiers exposed to BPS receipts in order of hundredths of ng/mL [35]. The LC-MS/MS method enabled determination of BPS from 50 μL of serum, but is it designed for determination of this one ED only.

Of the parabens tested, MP was found in the highest percentage (40%) of analyzed samples, followed by PP (28%) and EP (5%). In one of the samples, MP was found in the concentration of 16 mg/mL. These extremely high levels may be caused by ingestion of dietary supplements containing MP as a preservative [43]. Frederiksen et al. [29] measured MP, EP, PP and BP in plasma samples of young Danish men by LC-MS; EP and BP were not detected, while MP and PP levels were in accordance with our observations. Ye et al. [30] determined the levels of MP, EP, PP, BP and BenzylP in 15 commercial human serum

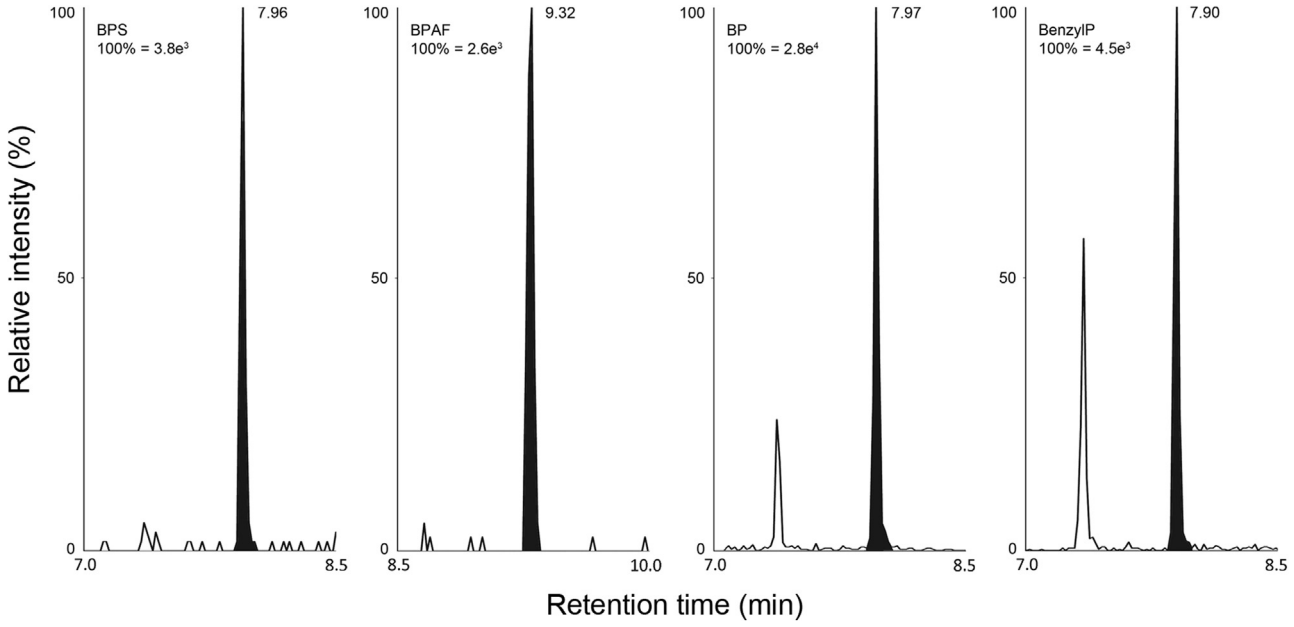

Fig. 2. Chromatograms of bisphenols and parabens in plasma sample spiked by 0.08 ng/mL of BPS and BPAF and 0.24 ng/mL of BP and BenzylP.

samples (4 men, 11 women) for method validation using the same order of magnitude as our samples, and EP, BP and BenzylP were under the limit of detection. An MP plasma concentration on the order of ng/mL units was reported by Sandanger et al. [31] in woman using cosmetic products. A more recent study [36] measured plasma paraben levels in women using a paraben-containing lipstick. Their serum paraben levels were much higher than in men. Overall, plasma and serum paraben levels were summarized in Boberg et al. [32], with levels depending on the study group as well as the use of paraben-containing products.

Meeker et al. [44] reported a relationship between urinary paraben levels and DNA damage in sperm. Urinary MP and PP concentrations were associated with increased BMI in a Korean population (*n=2541*), and there were 1.44 and 1.69 times higher levels of BP and PP in male compared to female adolescents (3–18 years) [45]. These findings suggest the need for further studies of the effects of parabens and monitoring their levels in humans. The combined effects of parabens and bisphenols on the human organism will likely be an interesting topic for future investigations.

5. Conclusion

The LC-MS/MS method proposed here is applicable for determination of bisphenols (BPA, BPS, BPF, BPAF), parabens (MP, EP, PP, BP, BenzylP) and estrogens (E1, E2, E3) in 11 min per sample run from 500 µL of human K2 EDTA plasma stored in glass tubes. The method met the FDA validation criteria. It was successfully applied for determination of abovementioned analytes in plasma from healthy men. To the extent of our literature search, this is the first report on the plasma analysis for simultaneous determination of selected bisphenols, parabens and estrogens and also the first determination of BPF in human plasma.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.talanta.2017.05.070.

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