Talanta 140 (2015) 62–67

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Development and validation of LC–MS/MS method for quantification of bisphenol A and estrogens in human plasma and seminal fluid

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ARTICLE INFO

Article history:
Received 3 February 2015
Received in revised form
3 March 2015
Accepted 6 March 2015
Available online 12 March 2015

Keywords:
Bisphenol A
Estrone
Estradiol
Estriol
Semen
LC–MS

ABSTRACT

Bisphenol A (BPA) is a widely known endocrine disruptor with estrogenic, antiestrogenic or anti-androgenic properties. BPA could interfere with estrogen metabolism as well with receptor-mediated estrogen actions. Both environmental BPA and estrogens may be traced in body fluids, of which, besides the blood plasma, the seminal fluid is of particular interest regarding their possible interactions in the testis. The method for simultaneously determining BPA and estrogens is then needed, taking into account that their concentrations in these body fluid may differ. Here the method was developed and validated for measurements of BPA, estrone (E1), estradiol (E2) and estriol (E3) in blood plasma and seminal plasma using liquid chromatography–tandem mass spectrometry. Due to the phenolic moiety of all compounds, dansyl chloride derivatization could be used. The analytical criteria of the method with respect to expected concentration of the analytes were satisfactory. The lower limits of quantifications (LLOQ) amounted to 43.5, 4.0, 12.7, 6.7 pg/mL for plasma BPA, E1, E2 and E3, and 28.9, 4.9, 4.5, 3.4 pg/mL for seminal BPA, E1, E2 and E3, respectively. The concentrations of individual steroids differed between body fluids. To the best of our knowledge, this is the first method that enabled the measurement of estrogens and BPA together in one run. The concentrations of E1, E2 and for the first time also of E3 in seminal plasma in normospermic men are reported.

1. Introduction

In recent decades, a large amount of chemicals has been introduced to the environment by anthropogenic activities. Many of them interfere with the endocrine system as so called endocrine disruptors (EDs). Bisphenol A (BPA) is one of the known EDs, which is widely used in polycarbonate and other plastics, epoxy resins, dental sealants, consumer electronics or thermal receipts [1,2]. BPA exposure has been associated with a variety of health complications including obesity, type 2 diabetes, cardiovascular disease and reproductive disorders [2,3]. Specifically in men, the relationship between BPA levels and decreased semen quality, sperm DNA damage and changes in reproductive hormones have been reported [4–7].

Multiple studies reported the determination of unconjugated BPA in serum/plasma (reviewed in [1,2]). However, a discussion has been raised about potential external contamination with BPA and method sensitivities. Therefore additional studies are needed to accurately determine BPA exposure in the general population [3]. A detailed evaluation of formerly published methods for measuring BPA in serum/plasma was reviewed by Vom Saal and Welshons [1]. On the other hand, the knowledge about BPA in seminal plasma is limited.

Research addressing the impact of BPA on steroidogenesis in men used BPA measurements in urine [7–10]. The results indicate that BPA can alter the steroid hormone pathway in men, although most of the studies dealt with infertile men. Therefore, more data are necessary to appraise the exact effects of BPA on steroidogenesis, especially in fertile men [7].

BPA possess a weak estrogenic activity similarly to endogenous estrogens, but it may also act as antiestrogen or antiandrogen [3,11,12]. Taken together, BPA effects are very complex and wide-ranging (for review see [3]). From a chemical point of view, estrogens and BPA possess phenolic moiety, therefore, it offers the

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http://dx.doi.org/10.1016/j.talanta.2015.03.013
0039-9140/

possibility for dansyl chloride derivatization. In addition, no study has reported the simultaneous determination of estrogens and BPA together, which would be useful in discovering the effects of BPA on estrogen metabolism.

The aim of the study was to develop with sufficient accuracy and sensitivity the LC–MS/MS assay for the simultaneous determination of unconjugated bisphenol A and estrogens (estrone, estradiol, estriol) in human plasma and seminal plasma. Application of this method allowed for the measurement of analytes in both matrices in 79 normospermic men. The LC–MS/MS assay was compared with the GC–MS method to determine E2 as well as with radioimmunoassay (RIA).

2. Experimental

2.1. Reagents and materials

The steroids estrone (E1), 17β-estradiol (E2) and estriol (E3) and deuterated standards of estrone (d4E1) and estriol (d2E3) were purchased from Steraloids (Newport, RI, USA). Bisphenol A (BPA), deuterated BPA (d16BPA) and deuterated E2 (d3E2) were obtained from Sigma-Aldrich (St. Louis, MO, USA) as well as 99.9% tert-butyl methyl ether (MTBE), acetone, sodium bicarbonate, sodium hydroxide and dansyl chloride. Methanol and water for chromatography were purchased from Merck (Darmstadt, Germany). All solvents and reagents were of HPLC grade.

2.2. Preparation of reagents

The sodium bicarbonate buffer (100 mM, pH 10.5) was prepared by dissolving 0.42 g sodium bicarbonate in 50 mL of ultrapure water. The pH was adjusted to 10.5 with aqueous 1 M sodium hydroxide.

Physiological solution was prepared by adding 4 g of sodium chloride to 0.5 L of ultrapure water to give 0.9% solution.

2.3. Preparation of stock solutions, working standard solutions and calibration mixtures

Stock (1 mg/mL) and working solutions (1 μg/mL) in methanol were prepared for each compound and stored at −20 °C. The calibration mixture was prepared from the individual working solutions in a concentration of 4 ng/mL for BPA and 1 ng/mL for estrogens. The mixture of internal standards (IS) in methanol was prepared similarly to give final concentrations of 100 ng/mL for d16BPA and 50 ng/mL for estrogens. Mixtures were stored at −20 °C. Eight point calibration curves were constructed in the range of 0.008–1 ng/mL for estrogens and 0.032–4 ng/mL for BPA for both matrices.

2.4. Samples

Samples of plasma and seminal plasma were obtained from patients attending the Center of Assisted Reproduction Pronatal (Prague, CZ). Each patient underwent standardized ejaculate examination (spermiogram) according to the World Health Organization (WHO) criteria. The group of 79 patients was specified by reproductive age (35.8 ± 4.7 years) with normospermic spermiogram. 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 to the project. All samples were stored at −20 °C.

2.5. Extraction, derivatization and optimization

A sample of plasma (500 μL) or seminal plasma (1000 μL) was spiked with 10 μL of IS mixture and diluted with 500 μL of physiological solution. Samples were shaken and liquid–liquid extraction (LLE) using MTBE (2 mL, 1 min) was performed. The organic phase was transferred to clean glass tube and evaporated until dryness using a vacuum concentrator (55 °C).

The derivatization step was performed according to Anari et al. [13] with certain modifications. A volume of 50 μL of bicarbonate buffer (100 mM, pH 10.5) and 50 μL of dansyl chloride in acetone (1 mg/mL) was added to the dry residues and shortly vortexed. The mixture was incubated at 60 °C for 5 min and then let cool down to room temperature. Thereafter, the samples were evaporated to dryness using vacuum concentrator (55 °C). The dry residues were reconstituted with 300 μL of methanol and 50 μL of the solution was transferred to the vial with a glass insert where 50 μL of the ammonium formate in ultrapure water (10 mM) was pre-pipetted. The volume of 50 μL of the sample was injected into LC–MS/MS for analysis.

Different reagents for extraction were tested (MTBE vs diethyl ether) and also various volumes of extraction agents were tried out (1 mL, 2 mL and 3 mL for both reagents). The best recovery was obtained when 2 mL of MTBE was used.

2.6. Liquid chromatography

The ultra-high performance liquid chromatography (UHPLC) Eksigent ultraLC 110 system (Redwood City, CA, USA) equipped with a Kinetex C18 column (100 mm × 3.0 mm, 1.7 μm; Phenomenex, Torrance, CA, USA) and Security Guard ULTRA cartridge system (UHPLC C18 for 3 mm ID column; Phenomenex, Torrance, CA, USA) was used for the analysis. Column temperature was maintained at 50 °C and separation was carried out at a flow rate of 0.4 mL/min.

HPLC grade water (A) and methanol (B) were used as mobile phases. A gradient elution started at 50% B (0–2 min); linearly increased to 90% B (2–6 min), then to 95% B (6–7 min), maintained

Table 1
Retention times, precursor ions, fragment ions and MS optimized conditions (declustering potential, DP; entrance potential, EP; collision entrance potential, CEP; collision energy, CE; collision cell exit potential, CXP) for all analytes.

Analyte	Retention time (min)	Precursor ion	Quantification ion	Confirmation ion	DP (V)	EP (V)	CEP (V)	CE (V)[a]	CXP (V)
BPA	9.31	695.24	171.04	170.05	86	6.5	28	65 (65)	4
d16BPA	9.25	709.28	171.11	170.15	86	7.5	28	65 (65)	4
E1	8.07	504.18	171.00	156.01	71	5	22	47 (75)	4
d4E1	8.06	508.23	171.09	156.07	71	5	24	49 (77)	4
E2	8.26	506.17	170.98	155.98	76	5	24	49 (75)	4
d3E2	8.24	509.24	171.09	156.09	76	5	22	49 (73)	4
E3	7.29	522.24	171.03	156.02	76	5	22	49 (79)	4
d2E3	7.28	524.27	171.10	156.10	71	8	24	47 (77)	4

[a] Values for the confirmation ion are given in the brackets.

at 95% B (7–8.8 min), dropped to 50% B (8.8–9 min) and stayed at 50% B from 9 min to 11 min. Retention times of the analytes are given in Table 1.

2.7. Mass spectrometry

Detection of the analytes was performed on an API 3200 mass spectrometer (AB Sciex, Concord, Canada) with electrospray ionization (ESI) probe operating in a positive mode. Ion source and MS/MS conditions were optimized by infusion of 0.2 μg/mL of the individual derivatized analytes to MS at 20 μL/min. The optimal conditions were as follows: ion spray voltage of 5500 V, temperature of 600 °C, curtain gas of 25.0 psi (172.38 kPa), collision gas of 4 psi (25.58 kPa), ion source gas 1 of 40.0 psi (275.79 kPa) and ion source gas 2 of 60.0 psi (413.69 kPa). Ions were examined in multiple reaction monitoring mode (MRM). Transitions with optimized conditions for MS are listed in Table 1. Analyst 1.6 software was used for system control and data evaluation.

2.8. Validation

The analytical method was validated according to FDA Guidance for Industry [14]. Validation parameters include (1) selectivity, (2) precision, (3) recovery (analytical accuracy), (4) calibration curve, and (5) stability of the analytes in spiked samples.

Acceptable selectivity was defined as the absence of any detectable SRM LC–MS/MS ion currents at the retention time regions of each analyte and its deuterated standards in blank plasma samples (double blanks).

Accuracy, precision and recovery were determined by using 6 samples per concentration; four different concentrations were assessed for plasma analytes and three different concentrations were examined for seminal fluid analytes. Pooled plasma samples or pooled seminal fluid containing IS were used as the first concentration. The spiked concentrations were as follows: 0.02, 0.1, 0.24 ng/mL for plasma estrogens (E1, E2, E3); 0.08, 0.40, 0.96 ng/mL for plasma BPA; 0.04 and 0.2 for seminal fluid estrogens and 0.16 and 0.8 ng/mL for seminal fluid BPA. Samples were pretreated in the same way as in Section 2.5.

Intra-assay precision (repeatability) and inter-assay precision (intermediate precision) were assessed and the values are expressed as relative standard deviation (RSD).

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

Lower limit of quantification (LLOQ) was defined as 10 × standard deviation/slope of the calibration curve.

Freeze and thaw stability test, short-term temperature stability test, long-term stability test, stock solution stability test and post-preparative stability test were examined.

3. Results

3.1. Selectivity, precision and recovery

There were no detectable SRM LC–MS/MS currents at the retention time regions of all analytes. A satisfactory assay accuracy (analytical recovery) ranging from 92.3% to 104.0% for plasma and 95.0% to 103.8% for seminal fluid was obtained. Precision did not exceed 15% of the RSD at all concentrations in both matrices. The results are given in Tables 2 and 3 for plasma and seminal fluid, respectively.

Table 2
Validation parameters for analytes in plasma.

Plasma		Precision (%)		Recovery (%)
Compound	Added (ng/ml)	Intra-day	Inter-day	
BPA	0	11.55	8.8	
	0.08	10.57	7.6	93.7
	0.4	7.86	3.5	92.3
	0.96	8.69	8.3	103.0
E2	0	8.23	2.2	
	0.02	8.7	0.5	97.7
	0.1	5.92	5.1	103.2
	0.24	6.05	5	98.1
E1	0	10.99	8.4	
	0.02	7.12	6	103.4
	0.1	8.92	2.9	102.0
	0.24	5.77	7	103.3
E3	0	10.98	14.3	
	0.02	8.86	9.4	99.6
	0.1	7.35	3.4	104.0
	0.24	5.57	3.1	102.0

Table 3
Validation parameters for analytes in seminal fluid.

Seminal fluid		Precision (%)		Recovery (%)
Compound	Added (ng/ml)	Intra-day	Inter-day	
BPA	0	9.25	6.6	
	0.16	6.89	6.5	99.7
	0.8	5.36	1.7	103.8
E2	0	13.96	3.2	
	0.04	8.82	3.3	96.1
	0.2	5.61	6.6	98.6
E1	0	12.21	11.1	
	0.04	8.02	5.6	95
	0.2	4.27	2.9	96.7
E3	0	12.46	6.9	
	0.04	5.27	6.2	100.7
	0.2	4.14	2.4	99.8

3.2. Calibration curve and matrix effect

Charcoal-treated plasma was used as blank matrix for plasma estrogens calibration curve. The plasma contained a small amount of BPA, apparently during the manufacturing process, therefore it could not be used for preparation of the calibration curves for BPA in plasma. Hence, we examined five sets of calibration curves containing IS in charcoal-treated plasma and five sets of calibrations in physiological solution similarly as Higashi et al. [15]. The regression lines were constructed with $1/x$ weighting and reached good linearity ($r > 0.9991$) and acceptable reproducibility [0.567 ± 0.0645 (mean ± SD) and 11.39% (RSD)]. Slopes of the lines obtained from physiological solution did not significantly differ from slopes from standard-added plasma.

Similarly, we compared the slopes of calibration curves prepared in physiological solution and pooled seminal plasma due to the impossibility to produce or buy the seminal plasma stripped of steroids and BPA. BPA calibration prepared in a physiological solution showed good linearity ($r > 0.9990$] and reproducibility

[1.2040 ± 0.0585 (mean ± SD) and 4.9% (RSD)]. Slopes showed no significant difference and we therefore concluded that plasma and seminal plasma matrix have no significant impact on BPA determination.

Calibration curves for estrogens constructed in a physiological solution exhibited good linearity with satisfactory correlation coefficients ($r > 0.9991$ for each estrogen) and reproducibility of slopes [2.19 ± 0.1511 (mean ± SD) and 6.9% (RSD) for E2; 2.15 ± 0.1196 and 5.6% for E1; 2.01 ± 0.18 and 8.8% for E3]. Slopes of calibration curves were compared in the same manner as BPA with slopes of calibration curves constructed in pooled seminal plasma. There was no significant difference observed for any estrogen. Therefore, seminal plasma did not display any matrix effect for estrogen determination.

Calibration ranges and LLOQs for each steroid are provided in Table 4.

3.3. Stability tests

3.3.1. Freeze and thaw stability test

We evaluated the stability of the samples after three freeze and thaw cycles. Three aliquots of each of the low (0.05 ng/mL and 0.2 ng for estrogens and BPA, respectively) and high (0.6 ng/mL and 2.4 ng/mL for estrogens and BPA, respectively) concentrations of plasma were used. Due to the limited amount of seminal fluid, we used two aliquots of low concentration (0.125 ng/mL for BPA, 0.005 ng/mL for E1, 0.007 ng/mL for E2 and 0.03 for E3) and two aliquots of high concentration (1.26 ng/mL for BPA, 0.285 ng/mL for E1, 0.287 ng/mL for E2 and 0.31 ng/mL for E3). After performing the tests, there were no statistical differences between analyte concentrations in these groups.

3.3.2. Short-term temperature stability

We evaluated short-term temperature test with the same set of sample concentrations as in freeze and thaw stability test. After 19 h in laboratory temperature, the samples were analyzed and compared with samples, which were immediately processed after thawing. We did not observe any statistical differences between analyte concentrations in these groups.

Table 4
Calibration ranges and lower limits of quantifications of all analytes in plasma and seminal fluid.

Analyte	Calibration range (ng/ml)	Seminal fluid LLOQ (pg/ml)	Plasma LLOQ (pg/ml)
BPA	0.032–4	28.9	43.5
E2	0.008–1	4.9	4.0
E1	0.008–1	4.5	12.7
E3	0.008–1	3.4	6.7

3.3.3. Long-term stability testing

Charcoal treated plasma with two different concentrations added (0.2 and 2.4 ng/mL for BPA and 0.05 and 0.8 ng/mL for estrogens, respectively) was prepared at the beginning of the validation tests and aliquoted. These samples were used in duplicates through all validation experiments and also for measuring plasma samples from normospermic men. Peak intensities of the samples were monitored continuously. Concentrations of the samples were compared with the appropriate concentrations from the first day of testing. The values in each concentration did not differ significantly.

Similar results were observed for seminal fluid samples. Pooled seminal fluid was used as the low concentration (0.125 ng/mL for BPA, 0.005 ng/mL for E1, 0.007 ng/mL for E2 and 0.03 ng/mL for E3) and the pooled seminal fluid with the addition of appropriate standards was used as the high concentration (2.4 ng/mL for BPA, 0.57 ng/mL for E1 and E2, 0.60 ng/mL for E3).

3.3.4. Stock solution stability

The stability of stock solutions of analytes and deuterated standards was evaluated after 15 h at room temperature. Two calibration curves were prepared using the stock solution left in a room temperature for the relevant period and subsequently compared by two calibration curves constructed from freshly prepared solutions. The instrument responses remained unchanged.

3.3.5. Post-preparative stability

The stability of samples was assessed after 24 h of remaining in the autosampler. The test showed that instrument responses of the samples remain unchanged at least for 1 day.

3.4. Comparison of the method with gas chromatography–mass spectrometry (GC–MS) method and radioimmunoassay (RIA)

We compared E2 in 20 plasma samples measured by the present method and the published GC–MS method [16]. In addition, plasma E2 was compared with the commercial RIA kit from Cisbio Bioassays. Both reference methods showed strong correlations with the present method ($r=0.93$ and $r=0.90$ for GC–MS and RIA respectively). Regression showed the equations $y=1.04x - 0.004$ and $y=0.2x+0.004$ for comparison with GC–MS and RIA, respectively. Plots of fitted models are shown in Fig. 1. The slope of the second regression analyzes indicated considerable overestimation by RIA.

3.5. Determination of estrogens and BPA in plasma and seminal fluid in normospermic men

Plasma BPA, E2, E1 and E3 were detected in 73%, 90%, 100% and 82% samples, respectively. Seminal BPA, E2, E1 and E3 were detected

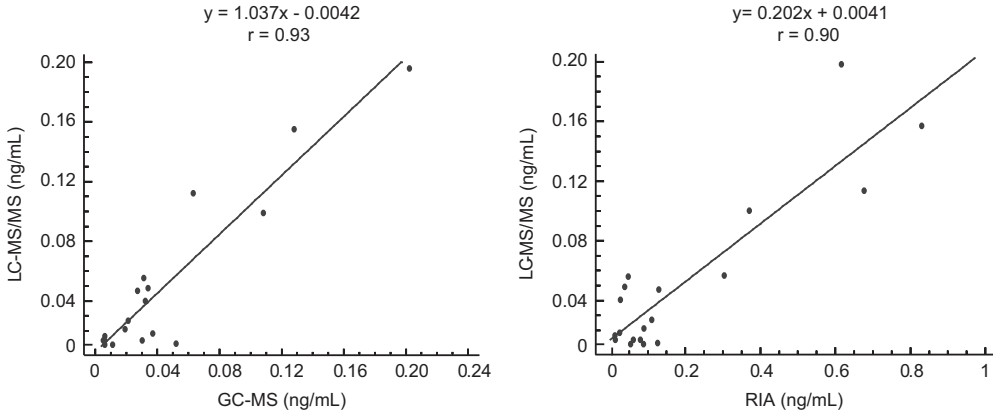

Fig. 1. Simple regression for E2. GC–MS and RIA, respectively, were selected as reference methods (x) and LC–MS/MS as a test method (y).

in 87%, 72%, 85% and 99% samples, respectively. Concentration ranges of each analytes and medians of measured samples in both fluids are given in Table 5. Examples of chromatograms obtained from the samples are shown in Fig.2.

All steps in protocol were performed using glass equipment e.g. Pasteur pipettes, glass syringes and glass tubes and controlled for contamination. Collection tubes were the only plastic that the blood came into contact with. The dwell time at collection tubes was reduced to the minimum. Nevertheless, we tested whether it could lead to contamination. Plasma collection tubes with K2EDTA did not display BPA contamination. Surprisingly, collection tubes for serum appeared to be a significant source of BPA. We could hypothesize that this is a consequence of BPA leakage from the separator gel. We therefore used collection tubes with K2EDTA. Total BPA contamination was below the limit of detection.

4. Discussion

Sex steroids (androgens and estrogens) are not only synthesized in the testis, but also act there through their receptors in both testicular cell types [17]. In the seminal fluid, in concert with its other constituents, they participate in maintainance of a unique milieu for protection and maturation of germ cells. The composition of testicular fluid may differ considerably from that of blood plasma and it is valid as well for steroids [18,19].

In this paper we attempted to determine and compare the concentrations major estrogens together with BPA in both body fluids. Our results of E2 in blood plasma are in accordance with those of other authors in normospermic men [20–22]. In this case, the results from immunoassays are comparable with instrumental methods. Few studies have determined E2 in seminal fluid in normospermic men [20,21,23–25] (for review see [18,19]). Seminal E2 concentrations were substantially higher than concentrations in blood plasma when using competitive immunoassays [20,21,23,26]. It was concluded that immunoassays are suitable for blood plasma determinations of E2, but are not applicable for seminal plasma E2 determination without more selective chromatographic separation [24].

When comparing our results to chromatographic determinations, the results are more similar [24,25]. None of these authors, however, measured simultaneously plasma levels of E2, therefore it was not possible to correlate concentrations of E2 in both biological fluids.

E1 is a biologically less active estrogen with the concentration range in plasma/serum of 9–60 pg/mL [22,27–29]. The role of E1 in men is unclear. Of interest may be the recent finding of Jasuja et al. with a cohort of 1458 men that plasma E1 was associated with diabetes risk [29]. Here we reported a median of 25 pg/mL in blood plasma. To our knowledge, only three studies measured E1 in seminal fluid. The reported mean concentration in normospermic men amounted as much as 150–178 pg/mL [24,26] in comparison to only 5 pg/mL found by us. Our data are almost 30 times less than results published in the 80s probably due to the instrumentation at the time.

E3 is the third, weakest bioactive estrogen. Mean concentrations in blood plasma in healthy men were reported between 19.1 [30] and 36.8 pg/mL [31], which is in accordance with our findings. To our knowledge, until now, nobody has measured E3 in the seminal fluid. The nearly twice higher concentration of E3 in seminal fluid than in blood plasma (74 pg/mL in seminal fluid vs 38.4 pg/mL in plasma) could be a result either of more intensive metabolic degradation of E1 in the testes or in seminal fluid, or of easier transport of E3 to seminal fluid.

Many studies reported serum/plasma levels of BPA in men ([32–37]; for review see [2,38]). The determination of BPA in body fluids is more problematic in comparison with measurements of steroids due to the possible BPA contamination of samples during collection, handling and storage. It is necessary to check if BPA is leaking from the laboratory equipment. In our study, we evaluated potential contamination of the sample during the entire process and concluded that the total BPA contamination is below the limit of detection. Therefore, our method provides sensitive and accurate measurements.

Previously, three methods determined BPA in semen with inconsistent results [33,39,40]. Inoue et al. developed the instrumental method to quantify BPA in human semen without a derivatization step, that is why the LOD was relatively high – 100 pg/mL and LOQ 500 pg/mL [39]. The authors compared their method with the ELISA method and concluded that the ELISA results may give inaccurate

Table 5
Concentration ranges of each analyte and medians of measured samples ($n=79$) in both fluids.

Analyte	Plasma		Seminal fluid	
	Concentration range (ng/ml)	Median (ng/ml)	Concentration range (ng/ml)	Median (ng/ml)
BPA	< LLOQ–7.23	0.093	< LLOQ–10.9	0.085
E2	< LLOQ–0.073	0.022	< LLOQ–0.258	0.005
E1	< LLOQ–0.065	0.025	< LLOQ–0.063	0.006
E3	< LLOQ–0.298	0.018	< LLOQ–0.360	0.043

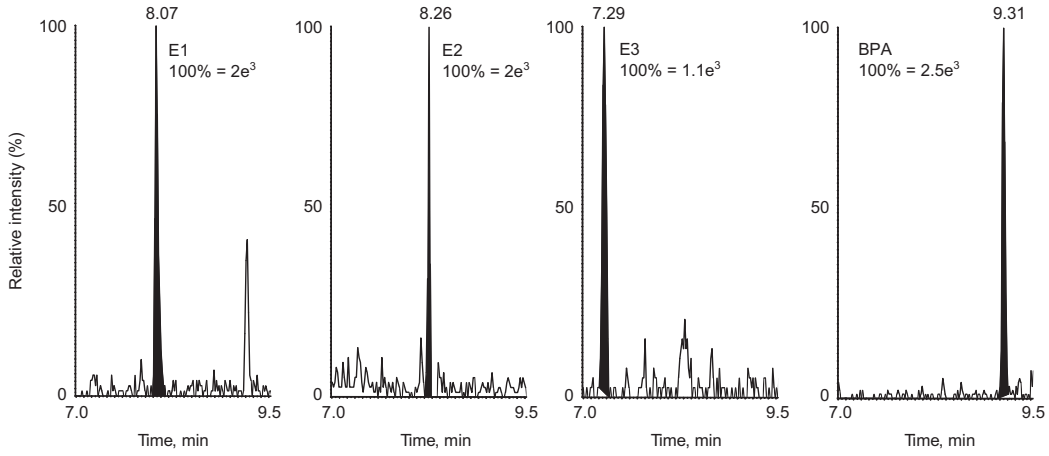

Fig. 2. Chromatograms of estrogens in plasma and BPA in seminal fluid. The measured concentrations were as follows: E1 – 0.148 ng/ml; E2 – 0.056 ng/ml; E3 – 0.149 ng/ml; BPA – 0.0723 ng/ml.

results due to the matrix effect and insufficient specificity of anti-BPA antibody [33,39,41]. Other authors also measured BPA in seminal plasma, but all of their samples were surprisingly below LOD (1 pg/mL) [40]. The third study concerned BPA levels in follicular and seminal fluids by RIA in 28 randomly selected couples attending in vitro fertilization. The concentration range of BPA in semen varied from 80 pg/mL to 1 ng/mL [33].

Our method with LLOQ 28.9 pg/mL enabled us to measure unconjugated BPA in seminal fluid in 87% of samples. Median of BPA concentration in seminal fluid is similar with the concentrations of BPA in human plasma (see Table 5), which indicates that the transmission from blood through blood–testis barrier to seminal plasma occurs. Nevertheless, plasma and seminal fluid concentrations of BPA did not correlate with each other, which emphasized that seminal plasma and testis represent quite a different milieu. It points to importance of simultaneous determination of BPA as an ED and steroids in semen. Furthermore, a trend of increasing seminal E2 and E1 levels as well as plasma E2 levels towards the highest BPA quartile was observed (data not shown).

5. Conclusion

To the best of our knowledge, this is the first method that allows estrogens and BPA to be measured together in one run. The concentrations of E1, E2 and for the first time also of E3 in seminal plasma are reported. The method will be beneficial for the monitoring of estrogen metabolism within the context of BPA exposure in men and their fertility status. The method enables highly sensitive BPA and estrogens to be determined and the examination disruption of estrogen metabolic pathways by BPA not only in the circulation but also directly in testes.

Acknowledgements

This work was supported by Internal Grant Agency Project no. NT/13369 from the Czech Ministry of Health.

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