Residual concentration of estriol during mouse sperm capacitation *in vitro* determined by HPLC method

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A b s t r a c t . Natural and synthetic compounds called endocrine-disrupting chemicals can interfere with normal hormone binding to convey inaccurate signals or send mixed messages that may result in altered health outcomes of both wildlife and humans. Estriol is one of natural origin endocrine disruptors and it is metabolite of 17β-estradiol. The aim of this work was to develop a method for determining free estriol available to capacitating sperm. In order to determine a status of estriol during mouse sperm capacitation *in vitro* a high performance liquid chromatography HPLC method with UV detection was used. A free estriol, and the estriol bound to the bovine serum albumin in capacitation medium can be quantified by the proposed method. A reversed-phase separation mode using a SunFire C18 column with a simple mobile phase composed of acetonitrile and water, methanol and water at the ratio 40/60 (v/v) was applied. Our results show that the level of free estriol available for mouse spermatozoa during capacitation *in vitro* can be quantified by HPLC method with UV detection. Therefore, this method represents an important tool to determine the amount of environmental estrogens, such as estriol, bound to sperm cells at the specific time point of capacitation *in vitro*.

Key words: endocrine disruptors, estriol, sperm, capacitation, HPLC

Introduction

Endocrine-disrupting chemicals (EDCs) are known as industrial and environmental contaminants, and can be found in the water of many rivers, lakes, seas, etc. EDCs interfere with the function of the endocrine system (C o l b o r n et al. 1993). It was reported that these compounds affect ecosystems, e.g. feminization of wild fish living downstream from wastewater effluent (S u m p t e r 1995). EDCs detected in environmental waters derive from factory effluent, from wastewater of plant treatment, and from residential wastewater. Some environmental estrogens have a natural origin. 17β-Estradiol (E2) and its main metabolites - estriol (E3) and estrone (E1) - along with their conjugates are naturally present at higher levels in females than in males (M i t a n i et al. 2005). Under ideal circumstances, estriol is only produced in significant amounts during pregnancy as it is made by the fetus, however, it is one of the environmental estrogens, which could have an impact on health of exposed organisms.

Environmental estrogens can also simulate the behavior of natural hormones due to binding to their receptors (D e l l a S e t a et a l . 2008). Estrogens can block the bond of hormones to receptors or simply block synthesis of certain hormones. They can also prevent

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the transport of hormones in the blood stream or alter their excretion from the body. Among others, environmental hormones are known to be responsible for decreasing the number of sperm in men (Golden et al. 1998, Fisch et al. 2000). Pesticides are one source of these emissions, which stay in the food chain for decades. Hence, estrogenous compounds, present in food, plastic wrapping and water bottles represent potentially harmful substances to the health of man (Wagner & Oehlmann 2009). Environmental estrogens bind to estrogen receptors and mimic the actions of estradiol metabolites estron and estriol (Safe et al. 2001), including inhibition hypothalamic gonadotropin-releasing (GnRH) hormone secretion, which decreases follicle-stimulating hormone (FSH) and luteinizing hormone (LH) secretion, thereby leading to a lower testicular function (Safe et al. 2001, Kumar et al. 2008). Estradiol and several estrogenic xenobiotics also act towards an increase of germ cell apoptosis and a sperm count decrease (Adeoya-Osiguwa et al. 2003). Capacitation is the key event in the study of sperm behavior prior to fertilization. Only capacitated sperm are sufficiently active and are able to fertilize. *In vivo* location of capacitation occurs in the uterus and oviducts and it is facilitated by substances of the female genital tract. For obvious reasons it is difficult to study these events *in vivo*. Therefore, *in vitro* experiments, simulating precisely an *in vivo* environment, are crucial for closer understanding of the process, when sperm gain the ability to fertilize an ovum. However, in order to study the effects of estrogen hormones such as estriol on sperm during *in vitro* capacitation, it is crucial to find out how much of the free estriol is available for cells in the sample. Therefore, how much of the estriol is bound to bovine serum albumin (BSA) present in capacitation medium due to a strong binding of estrogens to BSA (Tan & Pang 2001).

Specific alternative methods using antibodies such as enzyme-linked immunosorbent assay (ELISA) with a simple solid-phase extraction (SPE) were developed for the analysis of estrogens in an aquatic environment (Huang & Sedlag 2001). The limits of detection dropped down to a tenth, and in some cases, to a thousandth of a microgram per liter. Among the analytical techniques, gas chromatography-mass spectrometry (GC-MS) (Nakamura et al. 2001, Liu et al. 2004, Kawaguchi et al. 2004) and high-performance liquid chromatography-mass spectrometry (Lopez de Alda & Barcelo 2000, Rodriguez-Mozaz et al. 2004) were commonly used for determining estrogens in wastewater and soil. High-performance liquid chromatography (HPLC) with ultraviolet (UV) or fluorescence detection were also developed for determining estrones in real water samples (limit of detection ~ $\mu g \cdot L^{-1}$) (Penalver et al. 2002).

The aim of this work is to develop the HPLC method with UV detection for determining free estriol available to sperm in an *in vitro* fertilizing medium. The freshly collected mouse spermatozoa were capacitated *in vitro* within the presence of estriol. The residual concentration of estriol before, during and after capacitation was monitored on an experimental time scale.

Material and Methods

The HPLC equipment (Pye Unicam, Cambridge, UK) comprised of a PU 4015 pump, a PU 4020 UV detector and a Rheodyne injection valve Model 7725i (Cotati, CA, USA) with a 20 μl sample loop. The samples were injected with a Hamilton syringe (Reno, Nevada, USA). Signal was processed and data were handled with the CSW 32 PC software (DataApex, Prague, Czech Republic). Two commercially available steel columns - Symmetry C18 and SunFire C18 (both 150 x 4.6 mm I.D., octadecyl bonded to silica gel, particle size 5 μm) - were purchased from Waters (Milford, Massachusetts, USA).

The concentration of spermatozoa in 1 ml was assessed in a haemocytometer chamber under a 100x magnification. The motility of spermatozoa was checked under a light microscope (Zeiss, Czech Republic), and viability was evaluated under a fluorescence microscope (Olympus, Czech Republic).

Reagents and experimental conditions

Methanol (MeOH) and acetonitrile (ACN), both of them a HPLC grade purity, were purchased from Sigma-Aldrich (Chromasolv, Stienheim, Germany). Ethanol and paraffin oil were obtained from P-LAB (Czech Republic), M2 laboratory mouse *in vitro* fertilizing medium was delivered by Sigma-Aldrich (Stienheim, Germany). Deionized water (Milli-Q water purification system Millipore, Milford, MA, USA) was used in all experiments. For viability a check-up, sperm were dyed using a life-dead sperm staining kit (Molecular Probes, Invitrogen, Czech Republic).

The stock solution of estriol ($1 \mu g \cdot mL^{-1}$), (Sigma Aldrich, Czech Republic) was prepared by the dilution of an appropriate amount of standard E3 in ethanol and was stored at 5 °C. The individual concentrations of estriol for calibration measurements were obtained by the adding of an appropriate amount of stock solution into the M2 medium.

The experiments were carried out at laboratory temperature (22 ± 2 ºC). The UV detection was performed at the wavelength of 200 nm. The mobile phases containing methanol, acetonitrile, and water in various ratios were sonicated for 20 min prior to use. The mobile phase flow rate was $1 mL \cdot min^{-1}$.

Sperm capacitation *in vitro*

Laboratory BALB/c mice, purchased from Velaz (Prague, Czech Republic), were maintained and housed at the animal facilities of the Faculty of Science, Charles University, Prague. All animal procedures were carried out in strict accordance with the Animal Scientific Procedure, Art 1991, and subjected to review by the local ethics committee.

Mouse spermatozoa were recovered from the *cauda epididymidis* by placing its very distal region into the M2 *in vitro* fertilizing medium containing 4.0 g/l of bovine serum albumin (BSA) for 10 minutes at 37 °C in 5% CO_2 in air. Sperm stock was diluted for the required concentration (5×10^6 sperm$\cdot mL^{-1}$) into the 100 µL drop of M2 medium under paraffin oil in 35 mm Petri dishes. Each drop contained estriol of the concentration $200 \mu g \cdot L^{-1}$. The motility of the sperm population was checked throughout the whole experiment by a light inverted microscope with a thermostatically controlled stage at 37 °C. Spermatozoa were capacitated for up to 90 minutes. At time 0 and then after 30, 60 and 90 min of capacitation a drop with spermatozoa was collected, and centrifuged for 5 min at 10 000 rpm. Individual supernatants were further investigated for the remaining amounts of free estriol. Parallel controls without presence of estriol were run throughout the whole experiment to check whether sperm viability and motility was not altered by an addition of estriol.

Determining of the residual concentration of estriol in M2 medium

Supernatants collected at time 0, 30, 60 and 90 min of sperm capacitation *in vitro* (1989) were investigated for the residual concentration of estriol. Under optimized separation

conditions, the residual estriol concentrations were monitored at each experimental time. In order to eliminate inaccuracies caused by manipulation with samples, appropriate controls (estriol + M2) without sperm were prepared in parallel. The analysis of estriol in the samples (supernatants) was performed in triplicates.

Results and Discussion

Optimization of separation conditions

Based on the results reported for the separation of estron and estradiol (contained in drug preparations) in the literature (Pacáková et al. 2009, Nováková et al. 2004), the reverse-phase separation system consisted of a C_{18} stationary phases and methanol, acetonitrile and water as mobile phase constituents were selected for determining estriol in a specific biological sample – M2 medium (Sigma-Aldrich, Czech republic) supernatant after centrifugation of capacitated sperm. The M2 medium is a complex mixture, containing inorganic and organic components, which especially BSA (4.0 g/l) can cause difficulties during the separation process. To attain the sufficient resolution between estriol and the other constituents of M2 media, two types of C18 stationary phases were used and various volume ratios of MeOH, ACN and water were tested as mobile phase compositions. As the compromise among analysis time, resolution and peak symmetry, column SunFire C18 (150 x 4.6 mm, particle size 5 μm, Waters) and the mobile phase composed of ACN/water 40/60 (*v*/*v*) were selected for the determining and subsequent quantification of estriol (t_R = 4.36 min) in the M2 medium (see Fig. 1).

Calibration of estriol

At optimized separation conditions, the calibration of estriol in M2 medium was carried out. With respect to the biological purpose, the highest measured concentration of estriol in M2 medium was 200 $\mu g \cdot L^{-1}$. The calibration curve was measured over a concentration range from 50 to 200 $\mu g \cdot L^{-1}$. Measurements at all concentration levels were repeated four times and the mean values of the peak areas were subjected to linear regression (see Fig. 2). A satisfactory fit between the experimental points and linear calibration curve was observed.

Residual concentration of estriol in M2 medium after sperm capacitation *in vitro*

Mean peak areas (A) of estriol in experimental and parallel control samples are listed in Table 1. The mean peak areas of the experimental samples were implemented into a calibration equation for the calculation of residual concentrations of estriol after the time-dependent capacitation of sperm *in vitro*. Dependency of the residual concentration of estriol on the time elapsed from capacitation of sperm is depicted in Fig. 3. The attained results clearly demonstrate the almost parabolic decrease of estriol concentration in experimental samples within this time.

Conclusion

The importance of ability to detect and measure an exact amount of estrogens present in the environment especially in water growing with a knowledge of an involvement of endocrine

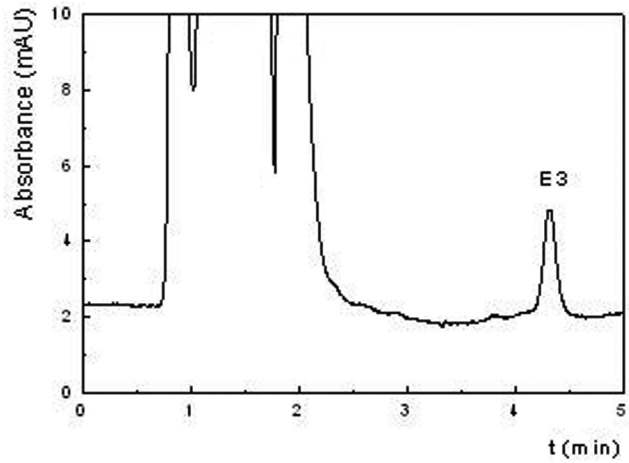

Fig. 1. Chromatogram of separation of 200 μg L^{-1} estriol in M2 medium, experimental conditions: SunFire C18 column, mobile phase composition – ACN /water 40/60 (v/v); flow rate 1 mL ·min^{-1}, λ = 200.

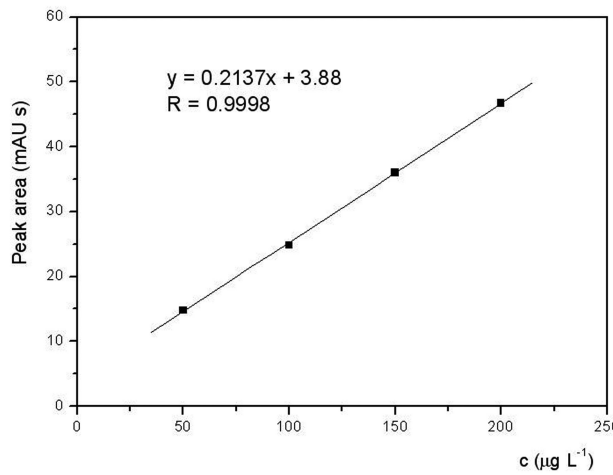

Fig. 2. Calibration curve of estriol, experimental conditions: SunFire C_{18} column, mobile phase composition - 40/60 (v/v) ACN /water; flow rate 1 mL ·min^{-1}, λ = 200 nm.

Table 1. Average values of the peak area (*A*) of estriol in supernatants. Experimental conditions: SunFire C_{18} column, mobile phase composition - 40/60 (v/v) ACN /water; flow rate 1 mL ·min^{-1}, λ = 200 nm.

Time of capacitation [min]	***A* [mAUs]**	
	Experimental sample	**Control sample**
0	47.07	46.72
30	46.41	46.89
60	45.12	47.10
90	42.92	47.11

Experimental sample – supernatant 200 μg·L^{-1} of estriol + M2 + sperm.
Control sample – supernatant 200 μg·L^{-1} of estriol + M2 without sperm added.

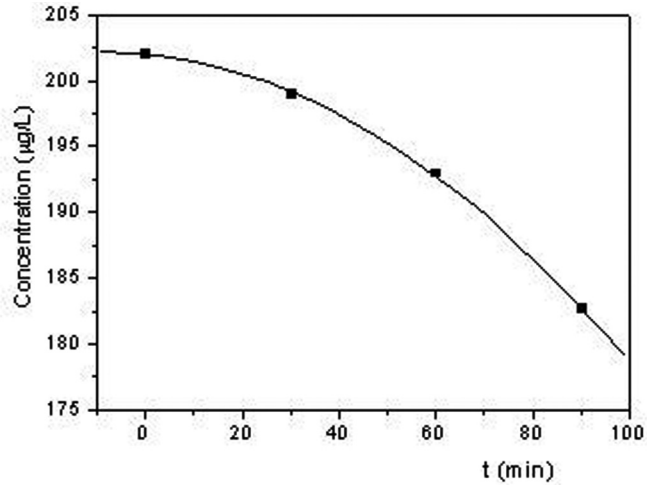

Fig. 3. Dependency of a residual concentration of estriol on the time of sperm capacitation *in vitro*.

disruptors in rising fertility problems in human. However, there is still call for methods, which could helps to monitor a concentration of these EDCs in *in vitro* experiments, as well as in daily life sources such as drinking water. The proposed analytical method proved to be suitable for the detection and quantification of the residual concentration of estriol in sperm capacitation medium containing BSA. Our results show that the level of free estriol available for cells in the medium can be determined throughout the ongoing capacitation of mouse spermatozoa *in vitro*. Comparing experimental samples with controls, a quantity of the estriol bound to the BSA can also be ascertained. Therefore, in conclusion, HPLC method with UV detection represents an important tool to determine the amount of environmental estrogens, in our case estriol, bound to sperm cells at the specific time point of capacitation *in vitro*. This study, therefore, can help to monitor the environmental effects of EDCs on human health.

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