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Simultaneous determination of eight estrogens and their metabolites in serum using liquid chromatography with electrochemical detection

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ABSTRACT

The liquid chromatography (LC) with electrochemical detection allows to determine, evaluate and validate the level of estrogens and their metabolites in serum. The method is fast and sensitive, and the hormones can be determined simultaneously, from one serum sample. The proposed method was successfully applied to the determination of following estrogens: estrone (E_1), 17β-estradiol (E_2), estriol (E_3) and following catecholestrogens: 2-hydroxyestradiol (2-OHE_2), 4-hydroxyestradiol (4-OHE_2), 4-hydroxyestrone (4-OHE_1), 2-methoxyestradiol (2-MOE_2) and 2-methoxyestrone (2-MOE_1).

The method of LC with electrochemical detection (LC/EC) was applied for the determination of catecholestrogens in serum sample taken from pregnant women. Estrogens and catecholestrogens were extracted from 1 mL of serum by applying diethyl ether under the specified conditions. The parameters of the procedure included using a specific mobile phase, applying the column Symmetry C18 (5 μm, 3.0 mm × 150 mm), equipping the applied electrochemical detector with the working glassy-carbon electrode, as well as applying the reference electrode Ag/AgCl.

The calibration studies on this study were performed, and a good analytical performance for E_1, E_2, E_3, 2-OHE_2, 4-OHE_2, 4-OHE_1, 2-MOE_2 and 2-MOE_1 was attained, along with low limits of detection (LOD of 0.18–0.30 ng/mL), satisfactory limit of quantification (LOQ of 0.23–0.92 ng/mL) and excellent linear dynamic range (0.6–8.0 ng/mL).

In conclusion, the presented methodology is the sensitive method of the simultaneous measurement of eight estrogens and their metabolites from one sample of blood and it might be clinically applied for testing serum of pregnant women, as well as for further studies on estrogens and their metabolites.

1. Introduction

Determining the role of estrogens and their metabolites in the regulation of reproduction is the subject of constant scientific studies.

Catecholestrogens, as natural active metabolites of estrogens, are produced by hydroxylation of estradiol and estrone at the C-2 and C-4 positions. Hydroxyestrogens can be metabolized to methylation derivatives. In our work we have studied the following estrogens and their metabolites: 4-hydroxyestrone (4-OHE_1), 2-methoxyestrone (2-MOE_1), 2-hydroxyestradiol (2-OHE_2), 4-hydroxyestradiol (4-OHE_2), 2-methoxyestradiol (2-MOE_2), estrone (E_1), 17β-estradiol (E_2) and estriol (E_3).

The effects of catecholestrogens on regulatory functions in pregnancy may be similar to or may differ from the effect of classical estrogens. Catecholestrogens belong to a group of steroids that have a characteristic, very low concentration in blood. The standard concentration amounts to around 100 pg/mL. The concentration of estrogens and their metabolites in serum blood of women may increase about 100 times during pregnancy. The measurement of low concentrations of endogenous catecholestrogens is a major analytical challenge, in spite of recent achievements in this domain [1].

In order to analyse estrogens and catecholestrogens different techniques can be applied: immunological methods, high performance liquid chromatography (LC) [2] and gas chromatography.

There are two versions of immunological method available: capillary electrophoresis immunoassay (CEIA) [3] and radioimmunoassay (RIA) [4]. LC can be performed by implementing LC technique with fluorimetric (LC/FL) [5,6], electrochemical (LC/EC) [7–10] or diode array detection (LC/DAD) [11]. While applying gas chromatography following version of this technique can be considered: gas chromatography version of mass spectrometry (GC/MS) [12] and the technique with electron-capture detection (GC-ECD) [13].

When comparing immunological methods, LC and gas chromatography, the most sensitive and precise is LC method. From the above-mentioned versions of LC (LC/FL, LC/EC, LC/DAD) the

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LC/EC is the least expensive, as it is based on applying a relatively inexpensive detector, therefore it was applied in this study.

There are other modern modifications to LC method available as well, such as the LC method version of mass spectrometry (MS) [14–16] and liquid chromatography–electrospray ionization-mass spectrometry (LC–ESI-MS/MS) [17], however these are emerging techniques, that are considerably more expensive than the LC/EC that was applied in this study. By applying LC/EC we obtain high enough level of sensitivity and precision, while maintaining relatively low cost.

We adapted the LC method using electrochemical detection for testing blood obtained from pregnant women for presence and concentration of estrogens and their metabolites. It was possible to determine the presence and to measure concentrations of eight estrogens and catecholestrogens simultaneously, testing 1 mL of blood sample from pregnant women. For isolation of samples from serum a liquid–liquid extraction method was used. Such an approach is similar to what other groups of researchers have adapted [8].

The method reviewed in this study showed high sensitivity, sufficient to define the concentrations of estrogens and catecholestrogens in serum of women during pregnancy.

2. Experimental

2.1. Materials

Estrogens and catecholestrogens those are determined in this study: E_1, E_2, E_3, 2-OHE_2, 4-OHE_2, 4-OHE_1, 2-MOE_2 and 2-MOE_1. Chemicals used for the preparation of the mobile phase, standard solutions and application for determination of E_1 were obtained from SERVA (Heidelberg, Germany), and for other estrogens and their metabolites tested – from Sigma–Aldrich (St. Louis, MO, USA). 17β-Estradiol (E_2) had purity of no less than 98%, while catecholestrogens (2-OHE_2, 4-OHE_2, 4-OHE_1, 2-MOE_2 and 2-MOE_1), estrone (E_1) and estriol (E_3) had purities of no less than 95%. The chemical structures of these hormones are depicted in Fig. 1.

Methanol was used to prepare standard solutions for 2-OHE_2, 4-OHE_2 and 4-OHE_1. These standard solutions contained 0.05% of ascorbic acid. Diethyl ether, methanol, *n*-hexane, acetonitrile, potassium dihydrogen phosphate and ascorbic acid were obtained from J.T. Baker (B.V. Deventer, Holland).

2.2. LC method

The LC system contained the electrochemical detector INTRO (Antec Leyden, Netherlands) with VT-03 electrochemical flow-cell, the working glassy-carbon electrode and the reference electrode Ag/AgCl with oven (flow-cell, injection, pulse dampener and column: Symmetry C18 (5 μm, 3.0 mm × 150 mm) from pre-column 20 mm from Waters company, USA). Other elements included in the LC system were the pump LC-10AT with mixer SUS (Shimadzu, Japan) and the integrator (Chromax-2001, Poland). The mobile phase contained 100 mM KH_2PO_4, methanol and acetonitrile (11:4:6, v/v/v). Flow-rate of 0.5 mL/min was used. The potential applied to the electrode of the analytical cell was equal to +0.9 V. The injection volume for the LC analysis amounted to 100 μL.

2.3. Preparation of standard serum

Standard serum for validation method was received from Cormay Control Serum NH (Cormay, Poland). Serum was obtained from healthy pregnant women being in their second trimester ($N = 12$); they gave an informed consent to the Medical University, Warsaw. These blood samples were immediately transferred to test

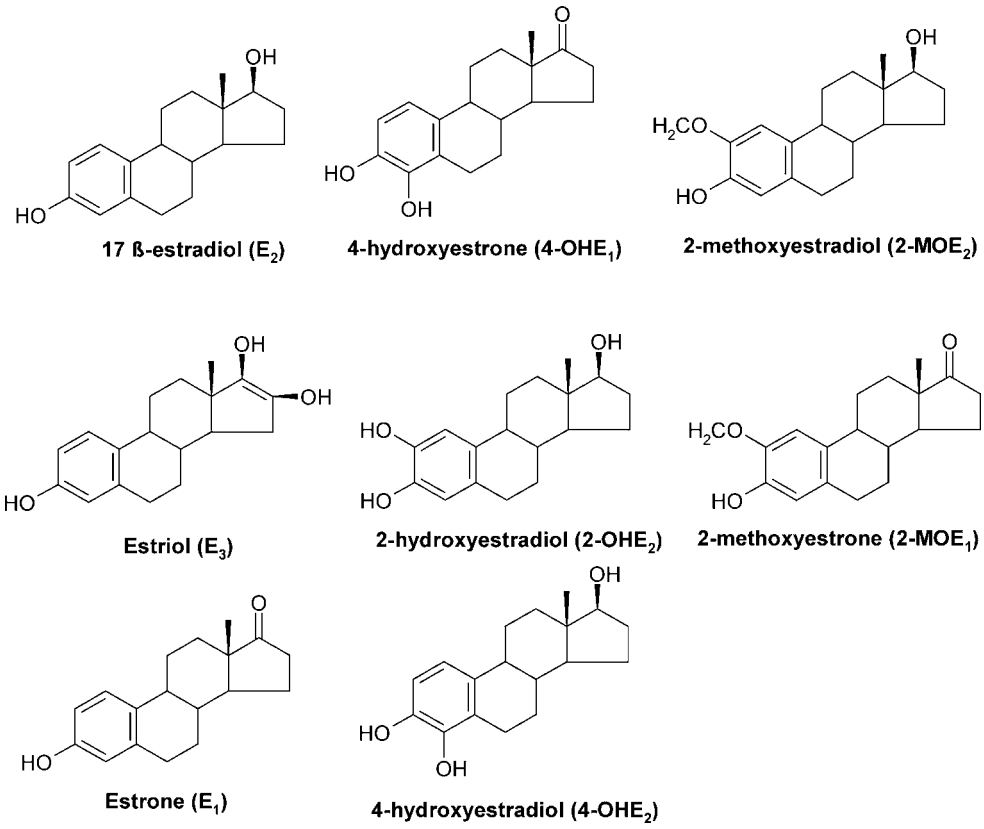

Fig. 1. Chemical structure of eight estrogens and catecholestrogens used in the present study.

tubes containing ascorbic acid. The final concentration of ascorbic acid amounted to 0.05%. Test tubes were centrifuged (3000 rpm, 10 min) and they were stored at −20 °C. Standard serum for E_1, E_2, E_2, 2-OHE_2, 4-OHE_2, 4-OHE_1, 2-MOE_2 and 2-MOE_1 was prepared as follows: 1 mL of serum (Control Serum) was spiked with methanol solution fulfilling adequate standards (1 mg/1 mL).

2.4. Method of validation

The precision and accuracy of the method were confirmed by the repetition of analyses ($n = 10$) and by analysing serum samples containing different concentrations (low, medium and high) of E_1, E_2, E_3, 2-OHE_2, 4-OHE_2, 4-OHE_1, 2-MOE_2 and 2-MOE_1. The reliability of results was ensured by performing analyses of samples under constant conditions: analyses were conducted during 1 day by one operator and applying the same testing parameters.

The intermediate precision was determined by analysing ($n = 21$) low, medium and high concentrations of E_1, E_2, E_3, 2-OHE_2, 4-OHE_2, 4-OHE_1, 2-MOE_2 and 2-MOE_1 in serum on different days by two operators (each of them analysed one sample of each concentration on 21 different days). The recovery was calculated by compiling analyses of serum samples as well as analyses of water solutions of these samples ($n = 3$ for each sample).

2.5. Extraction

One millilitre of serum and 5 ml of diethyl ether were shaken gently for 5 min. The layer of ether was decanted and evaporated under a stream of nitrogen. The residue was dissolved in 1 mL of solution containing methanol (50%) and ascorbic acid (0.05%), dissolved in water. Afterwards 1 mL of *n*-hexane was added and the sample was shaken vigorously for 2 min. The mixture was then centrifuged at 1000 rpm for 5 min, followed by drying of methanol layer at 30 °C. The next step was to dissolve the residue in 0.5 mL of distilled water in order to remove ascorbic acid. Then steroids were extracted by applying 3 mL of diethyl ether. The ether phase evaporated under a nitrogen stream in the room temperature. The residue was dissolved in 250 μL of methanol and it evaporated under a stream of nitrogen. The residue was dissolved in 250 μL of mobile phase and 100 μL of the solution was injected into the LC system.

3. Results

3.1. Detection limit and calibration curves for E_1, E_2, E_3, 2-OHE_2, 4-OHE_2, 4-OHE_1, 2-MOE_1 and 2-MOE_2

The calibration curve was prepared on the basis of serum control containing estrogens: E_1, E_2 and E_3, catecholestrogens: 2-OHE_2, 4-OHE_2, 4-OHE_1, 2-MOE_2 and 2-MOE_1 each in concentrations of: 125, 250, 500, 750, 1000, 1500, 2000 pg (corresponding to 0.6, 1, 2, 3, 4, 6, 8 ng/mL). Standard solutions of estrogens (1 mg/1 mL) were prepared in methanol. Catecholestrogens contained 0.05% of ascorbic acid (v/v). Analytes were extracted according to the method described in Section 2.5. The calibration curves were linear in range of 0.6–8 ng/mL for E_1, E_2, E_3, 2-OHE_2, 4-OHE_2, 4-OHE_1, 2-MOE_2 and 2-MOE_1. The calibration curve was established by applying the linear regression method.

The linear regression parameters were calculated from peak areas. Parameters of curves, as well as the limit of quantification (LOQ) and the limit of detection (LOD) are presented in Table 1. LOQ and LOD were calculated according to "HPLC Methods for Pharmaceutical Analysis" by Lunn and Schmuff [18].

3.2. Chromatography

The separation of the mixture of E_1, E_2, E_3, 2-OHE_2, 4-OHE_2, 4-OHE_1, 2-MOE_2 and 2-MOE_1 was performed. Mean of retention time (min) ±SD and CV (%) were determined for each estrogen and metabolite and respectively amounted to: 4.3 ± 0.1, 3.0% for E_3, 9.3 ± 0.2, 1.9% for 4-OHE_2, 10.2 ± 0.1, 0.9% for 2-OHE_2, 13.5 ± 0.3, 2.4% for 4-OHE_1, 17.8 ± 0.3, 1.4% for E_2, 23 ± 0.5, 2.2% for 2-MOE_1, 24.5 ± 0.4, 1.7% for E_1 and 27.5 ± 0.4, 1.6% for 2-MOE_2.

Fig. 2 presents the chromatograms obtained from serum control after extraction of: (A) blank control serum and (B) serum control spiked with 0.72–4.04 ng/mL of estrogens and catecholestrogens.

3.3. The precision and accuracy

The precision and accuracy of the method are presented in Table 2. The intra-assay coefficients of variation (CV) for studied estrogens and catecholestrogens were in range of 2.3–9.6%. The inter-assay CV amounted to 1.1–18.5%. Table 3 presents the recovery of parameters. Recoveries for estrogens, catecholestrogens and 2-methoxyestrogens were between 82.2% and 98.5% at 0.6 ng/mL, between 88.6% and 97.5% at 4 ng/mL, and between 85.9% and 97.3% at 8 ng/mL.

3.4. Determination of estrogens and catecholestrogens in serum of pregnant women

Serum of pregnant women was pre-treated as described in Sections 2.3 and 2.5. The concentrations of estrogens and catecholestrogens in serum of pregnant women ($N = 12$) are presented in Table 4. Fig. 3 presents an example of chromatogram obtained from the serum of the pregnant woman (patient no. 4).

The levels of estrogens E_1, E_2 and E_3 obtained by applying the method presented above were compared to those presented in other reports [17,19] – the results were corresponding despite applying different determination methods. It confirms accuracy of the study performed.

Table 1
Calibration curve parameters, retention time (RT), the limit of detection (LOD), the limit of quantification (LOQ), linear range and correlation coefficient (R^2) for estrogens and catecholestrogens under study, by LC/EC.

Estrogens	RT[a] (min)	Linear range (ng/mL)	The regression lines[b] (slope; intercept)	R^2	LOD[c] (ng/mL)	LOQ[d] (ng/mL)
E_1	24.5	0.6–8.0	$y = 199356x - 3562$	0.9982	0.20	0.61
E_2	17.8	0.6–8.0	$y = 269956x - 36045$	0.9976	0.19	0.58
E_3	4.3	0.6–8.0	$y = 254162x - 29525$	0.9965	0.18	0.65
4-OHE_1	13.5	0.6–8.0	$y = 185742x + 33029$	0.9911	0.08	0.63
2-OHE_2	10.2	0.6–8.0	$y = 231919x + 4022$	0.9992	0.29	0.87
4-OHE_2	9.3	0.6–8.0	$y = 262713x - 10753$	0.9997	0.18	0.60
2-MOE_1	23.0	0.6–8.0	$y = 55363x + 2062$	0.9988	0.28	0.86
2-MOE_2	27.5	0.6–8.0	$y = 84290x - 11088$	0.9921	0.30	0.92

[a] Mean of retention time of six separate assay.
[b] Mean of six separate assay, $n = 48$.
[c] $S/N = 3$.
[d] $S/N = 10$.

Table 2
Precision and accuracy of determination of E_1, E_2, E_3, 4-OHE_1, 2-OHE_2, 4-OHE_2, 2-MOE_1 and 2-MOE_2.

Compound	Reference values (ng/mL)	Intra-assay		Inter-assay	
		Concentration[a] (mean ± SD)	CV (%)	Concentration[a] (mean ± SD)	CV (%)
E_1	2	2.1 ± 0.2	9.6	1.8 ± 0.3	13.4
	4	4.4 ± 0.3	7.6	4.6 ± 0.3	6.8
	8	9.4 ± 0.6	6.1	9.7 ± 0.5	5.3
E_2	2	2.0 ± 0.1	3.6	2.0 ± 0.1	3.2
	4	4.1 ± 0.4	9.3	3.1 ± 0.1	3.3
	8	9.6 ± 0.9	9.4	9.7 ± 0.3	13.1
E3	2	2.1 ± 0.0	7.1	2.1 ± 0.2	8.1
	4	3.6 ± 0.2	4.4	3.7 ± 0.0	1.1
	8	7.1 ± 0.6	8.6	8.4 ± 1.6	13.5
4-OHE_1	2	2.6 ± 0.2	9.3	2.5 ± 0.0	0.9
	4	3.8 ± 0.1	3.6	3.8 ± 0.2	4.6
	8	8.8 ± 0.8	9.4	8.7 ± 0.1	4.9
2-OHE_2	2	2.0 ± 0.1	3.2	2.0 ± 0.1	2.4
	4	3.9 ± 0.3	7.0	4.0 ± 0.2	5.7
	8	8.7 ± 0.7	7.5	8.8 ± 0.7	7.6
4-OHE_2	2	1.9 ± 0.2	8.7	2.0 ± 0.1	6.0
	4	3.8 ± 0.3	7.3	3.7 ± 0.1	1.2
	8	7.2 ± 0.6	8.0	7.2 ± 0.6	8.8
2-MOE_1	2	2.0 ± 0.2	8.3	2.0 ± 0.1	6.8
	4	3.8 ± 0.1	2.7	4.1 ± 0.2	5.5
	8	8.3 ± 0.7	8.2	8.6 ± 0.8	9.3
2-MOE_2	2	2.0 ± 0.1	6.6	2.0 ± 0.1	3.1
	4	3.7 ± 0.1	2.3	4.0 ± 0.3	7.1
	8	8.1 ± 0.8	9.4	8.9 ± 1.4	15.0

[a] Mean ± SD of six replicate analytes.

The levels of catecholestrogens 4-OHE_1, 2-OHE_2, 4-OHE_2, 2-MOE_1 and 2-MOE_2 vary considerably among different patients (as presented in Table 4). As the detailed data concerning the history of pregnancy and delivery of women who were patients for this study was not available, the more detailed and in-depth analysis of the regulatory function of different levels of catecholestrogens was not conducted. However, the diversity of these levels was confirmed, which could be the basis for further studies on the dependence between these variations and abnormalities in the course of pregnancy and delivery.

While analysing the serum of pregnant women we have detected the presence of other, not mentioned in this study, metabolites of estrogens that appeared after 32 min of retention. Nevertheless, these metabolites were not the subject of this study, and as their standards were not at our disposal, we have not determined them. These were probably such metabolites as 4-MOE_1, 4-MOE_2 and others.

Table 3
Recovery of estrogens and catecholestrogens determination.

Compound	Expected concentration (ng/mL)	Recovery (% ± SD)[a]
E_1	2	92.6 ± 14.9
	4	97.5 ± 11.9
	8	87.2 ± 11.3
E_2	2	98.5 ± 1.0
	4	92.1 ± 5.3
	8	89.5 ± 12.4
E_3	2	97.6 ± 4.0
	4	88.6 ± 4.4
	8	89.2 ± 8.6
4-OHE_1	2	91.0 ± 8.1
	4	91.5 ± 11.6
	8	85.2 ± 2.4
2-OHE_2	2	97.0 ± 2.9
	4	93.8 ± 5.8
	8	93.5 ± 12.1
4-OHE_2	2	96.1 ± 8.7
	4	91.3 ± 3.6
	8	89.5 ± 8.0
2-MOE_1	2	82.2 ± 5.4
	4	93.8 ± 2.6
	8	85.9 ± 1.8
2-MOE_2	2	90.3 ± 11.4
	4	91.1 ± 2.3
	8	97.3 ± 4.6

[a] Mean recovery ± SD of six replicate analytes.

4. Discussion

The method of LC with the electrochemical detection was used for the determination of 17β-estradiol, estrone, estriol, 2-hydroxyestradiol, 4-hydroxyestradiol, 4-hydroxyestrone, 2-methoxyestrone and 2-methoxyestradiol. The validation parameters were satisfactory; therefore the method can be put into practice.

Numerous publications describe the measurement of separate estrogens, e.g. the measurement of estriol [8,20], or the measurement of all three "classical" estrogens simultaneously E_1, E_2 and E_3 [16,17,21,22]. Additionally the measurements presented in the literature are performed by both electrochemical detection and diode array detection [11], or by the liquid chromatography–electrospray ionization-mass spectrometry [17].

Catecholestrogens are mentioned in the literature less often. Such studies are quite rare and they refer mainly to individual derivatives of estrogens, such as 2-MOE_1 [23], 2-OHE_1 [4], 2-OHE_2 and 4-OHE_2 [13], 2-OHE_1, 4-OHE_2, 2-OHE_2 and 4-OHE_1 [24]. The above-mentioned catecholestrogens were detected by applying techniques different from LC/EC. The major advantage of LC/EC when compared to other techniques is detecting five catecholestrogens simultaneously – from one serum sample taken from pregnant

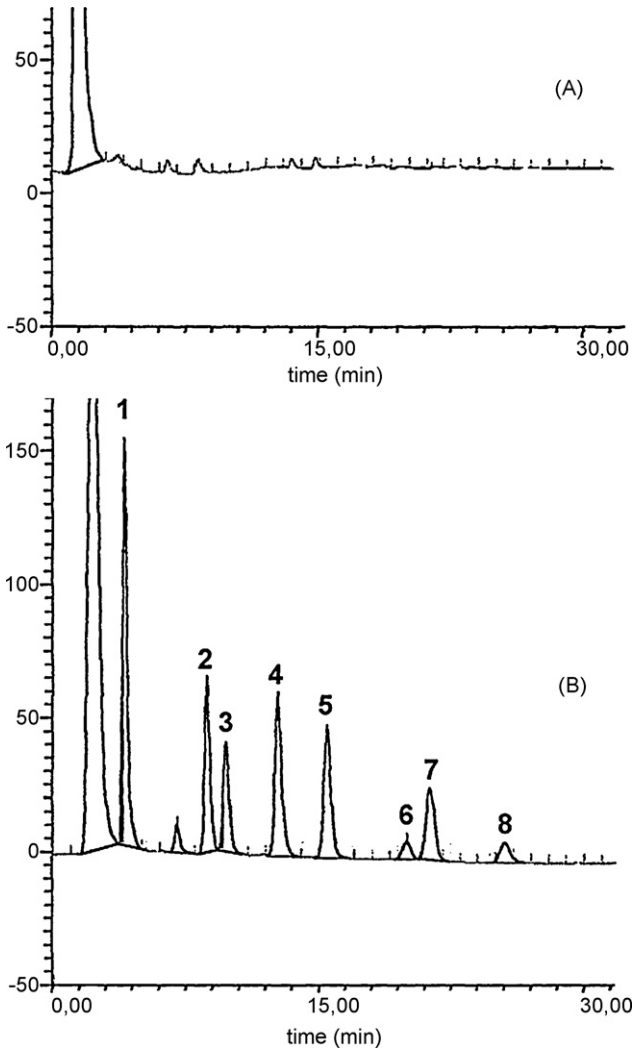

Fig. 2. Chromatograms obtained from serum control after extraction: (A) blank serum control and (B) serum control spiked with 4.04 ng/mL E_3 (1), 0.94 ng/mL 4-OHE_2 (2), 0.72 ng/mL 2-OHE_2 (3), 1.65 ng/mL 4-OHE_1 (4), 0.95 ng/mL E_2 (5), 0.74 ng/mL 2-MOE_1 (6), 0.83 ng/mL E_1 (7) and 0.72 ng/mL 2-MOE_2 (8).

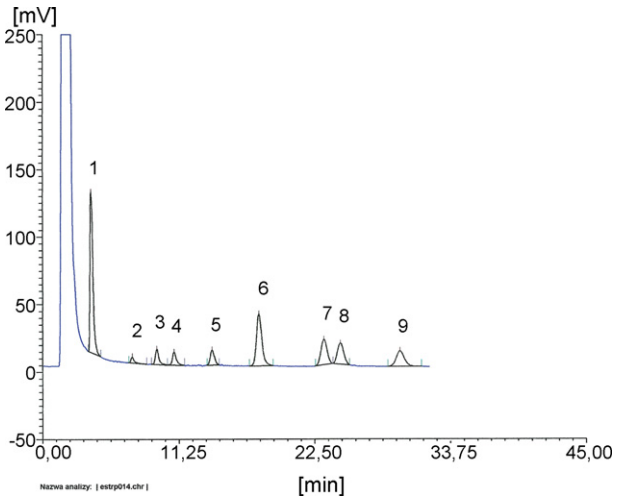

Fig. 3. Chromatogram of serum from the pregnant woman (serum no. 4, Table 4). The concentrations of the serum were: 3.7 ng/mL for E_3 (1), 0.37 ng/mL for 4-OHE_2 (3), 0.39 ng/mL for 2-OHE_2 (4), 0.59 ng/mL for 4-OHE_1 (5), 1.92 ng/mL for E_2 (6), 1.21 ng/mL for 2-MOE_1 (7), 4.95 ng/mL for E_1 (8) and 2.77 ng/mL for 2-MOE_2 (9).

woman. Additionally LC/EC is the technique of relatively inexpensive detector.

LC/EC was applied in the study of Nakagomi and Suzuki [9] for determination of E_2 and 4-OHE_2. However, authors claimed that chromatograms showed a considerable background response and the accuracy of the method was not established. Devanesan et al. [10] determined 4-OHE_2 in hamsters' tissues and applied LC/EC as well as LC/SM, however detection limits by neither technique was evaluated. In our study the results of the analysis were validated and the outcome of validation was satisfactory.

Nevertheless, none of these studies mentioned above based the determination of estrogens and their metabolites on the blood serum, the method which is less invasive and easier to apply for pregnant women.

The advantage of our work is the possibility of the simultaneous measurement of eight estrogens and their metabolites from one sample of blood. Although Xu et al. [25] analysed seven endogenous estrogens and their metabolites using LC method and 15 estrogens [26] – using the capillary gas chromatography–mass spectrometry method, but their research was done in human urine.

It seems that, in the context of already existing publications, our method of simultaneous measurement of eight estrogens and catecholestrogens is clinically applicable for the analysis of serum of pregnant women. To confirm the possibility of using the above described method for clinical purposes we measured the following classical estrogens: E_1, E_2, E_3, catecholestrogens: 2-hydroxyestradiol (2-OHE_2), 4-hydroxyestradiol (4-OHE_2), 4-hydroxyestrone (4-OHE_1), and methoxyestrogens: 2-methoxyestradiol (2-MOE_2) and 2-methoxyestrone (2-MOE_1). The measurement was performed on the basis of serum of healthy pregnant women before labour. Our study allows to analyse the concentration of each individual estrogens and catecholestrogens in serum of pregnant woman, which is important for the clinical diagnosis of the fetoplacental function.

The monitoring of fetoplacental function during heavy pregnancy is usually aided by analysing functions of "classical" estrogens [2,17], despite the fact that Berg and Kuss [19] claim in their study that catecholestrogens and their methylethers are metabolites without any regulatory function in pregnancy. As far

Table 4
The concentration of estrogens and catecholestrogens in serum of pregnant women ($N = 12$).

	Serum's no.												Mean ± SD (ng/mL) $N = 12$
	1	2	3	4	5	6	7	8	9	10	11	12	
Estrogens (ng/mL)													
E_1	2.91	4.96	5.52	4.95	0.47	2.75	3.40	6.36	1.64	2.84	0.56	2.38	3.38 ± 1.8
E_2	3.07	2.16	2.31	1.93	5.07	3.35	2.32	4.45	5.38	2.90	3.14	3.63	3.16 ± 1.1
E_3	0.43	2.01	3.82	3.70	2.83	2.23	1.96	3.01	2.33	1.43	2.11	2.27	2.37 ± 0.9
4-OHE_1	1.15	0.28	0.28	0.59	0.70	0.60	0.43	1.17	1.16	0.42	1.31	1.52	0.79 ± 0.4
2-OHE2	0.96	4.61	2.01	0.39	0.58	0.49	0.64	0.61	2.32	1.33	0.84	3.90	1.97 ± 1.4
4-OHE2	0.39	2.17	0.26	0.37	0.67	0.28	0.38	0.56	1.93	0.59	0.93	0.45	0.80 ± 0.6
2-MOE_1	1.45	0.83	1.21	1.21	1.70	2.65	1.08	3.56	0.77	1.75	2.64	1.43	1.98 ± 1.2
2-MOE_2	1.81	2.13	2.32	2.77	3.07	4.02	3.08	1.80	2.64	3.33	2.75	2.22	2.39 ± 0.9

as the usefulness of the method of measuring catecholestrogens is concerned, Barnea and Fakih [27] communicated the role of 2-OHE_1 in placental steroidogenesis. Martuchii [28] described the study on 2-MOE_1 and 2-OHE_1 in the fetal and neonatal rat and the role of catecholestrogens in the beginning of the delivery. Moreover, Biswas et al. [29] suggest that in human pregnancy catecholestrogens, through their stimulating effects on prostaglandin synthesis, participate in the initiation of labour.

It seems that the method suggested in our work of simultaneous measurement of estrogens and catecholestrogens may be clinically applied and supplies valuable information about the pregnancy and the delivery, as well as it may be used for further study of these metabolites.

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