

High Performance Liquid Chromatographic Optimization Study of Environmental Estrogens

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ABSTRACT: Nine kinds of environmental estrogens including β -estradiol, estrone, mestranol, ethisterone, ethinyloestradiol, diethylstilbestrol, cortisone, estriol and hydrocortisone were determined using high performance liquid chromatography (HPLC). The determination conditions were optimized that ProntSIL 120-5-C18-ace-EPS column as stationary phase, methanol/water (82.5/17.5, v/v) as mobile phase and flow rate as 1.0 mL/min. These compounds could be base-line separated in 15 minutes. The commercially available milk spiked with the estrogens was performed as the real sample. The recovery rate was in the range of 92.2%-103.7% while the relative standard deviation (RSD) was less than 10%.

KEYWORDS: HPLC, estrogen, milk

1 INTRODUCTION

Activation or inhibition of the function of the endocrine system occurred when environmental estrogen entered human's body. Synthesis, release, transport, binding and metabolism of normal endocrine substances in vivo can be interference at the same time. Recently years, the research of environment estrogens has become hot topic, and reports of the harm effect of the estrogens to human body are of common occurrence. For example, estrone can lead to the damage of liver function and Myocardial Infarction; ethisterone is thought to increase the risk of an ectopic pregnancy; hydrocortisone can result in myocardial injury and cause many complications, including increasing pressure in the brain. Therefore, environment estrogens were banned strictly and can not be detected in food by China's agricultural sector in 2002. So, to establish a method for the rapid, simple, accurate determination of estrogens has great social significance.

Though there has been a lot of estrogen detection methods recently, High performance liquid chromatography (HPLC) begins to play a more and more important role in estrogens analysis. But in recent years, the number of stationary phases for HPLC rised rapidly. Up to now, more than 750 different bulk materials could be purchased on the market, and the number would be increased because of the further development.

In this study, nine kinds of environmental estrogens including β -estradiol, estrone, mestranol, ethisterone, ethinyloestradiol, diethylstilbestrol, cortisone, estriol and hydrocortisone were determined using high performance liquid chromatography (HPLC). We compared the separation effect of four kinds of chromatographic columns, and the determination conditions were optimized that ProntSIL 120-5-C18-ace-EPS column as stationary phase, methanol/water (82.5/17.5, v/v) as mobile phase and flow rate as 1.0 mL/min.

2 EXPERIMENT

2.1 Materials

Methanol and acetonitrile was purchased from J.T.Baker (America), β -estradiol, estrone, mestranol, ethisterone, ethynylestradiol, diethylstilbetrol, cortisone, estriol and hydrocortisone were purchased by J&K chemical (China), the deionized water was obtained from a water purification machine (Aquapro RM-220, China).

HPLC analysis was carried out on SHIMAZDU LC-20AT high performance liquid chromatograph (Japan) using a SIL-10AF autosampler and SPD-M20A diode array detector (DAD). PromoSIL C18 (250mm*4.6mm 5.0 μ m, Agela Technology); ProntSIL 120-5-C18-ace-EPS (250mm*4.6mm, 5.0 μ m, BISCHOFF Germany), ProntSIL 120-5-C18-AQ PLUS (250mm*4.6mm, 5.0 μ m, Agela Technology) and Thermo Hypersil Gold aQ

(250mm*4.6mm, 5.0 μ m, Thermo) were used in the study.

2.2 Practical samples collection and treatment

The pure milk samples were purchased in Walmart supermarket in Beijing. In the laboratory, the milk samples were kept in refrigerator retaining temperature 4 $^{\circ}$ C until the analysis. Acetonitrile/water (1/1, v/v) were added into 2 mL of milk samples. Vigorous shaking followed by sonication from 15 mins. The mixed sample was centrifuged (8000 rpm, 15mins) and the supernatant was collected and placed at a 15 mL centrifuge tube with 2 mL hexane. Standing and stratificated, then the upper solution was discarded. The lower solution was evaporated to dryness under a weak nitrogen stream at 40 $^{\circ}$ C. Then the residue was dissolved in 100 μ L of mobile phase. Before the analysis, samples were filtered with a 0.22 μ m nylon filter membrane.

2.3 Standard solution preparation

Nine estrogens were weighed accurately for 10 mg respectively and placed into 25 mL volumetric flask. Subsequently, acetonitrile/water (82.5/17.5, v/v) was added with the concentration was 0.4 mg/mL. The mixture was filtered by 0.45 μ m filter membrane and stored in refrigerator retaining 4 $^{\circ}$ C.

3 RESULTS AND DISCUSSION

3.1 The choice of chromatographic conditions

3.1.1 Selection of detector and the detection wavelength.

By scanning the ultraviolet absorption spectrum of 9 kinds of estrogen, acetylene progesterone, ethinylestradiol and diethylstilbestrol, cortisone, hydrocortisone had stronger absorption in the 220-260 nm, while beta estradiol, estrone, estriol, estrone had stronger absorption in the 270-290 nm, in order to achieve liquid chromatographic analysis of the nine compounds and ensure clear chromatograms, we choose our DAD detector, detection wavelength at 254 nm and 280 nm, the two wavelengths of chromatogram has carried on the stack.

3.1.2 Selection of chromatographic column.

With four kinds of chromatographic column as stationary phase, methanol/water (82.5/17.5, v/v) as mobile phase, flow rate of 1.0 mL/min, detection wavelength of 254 nm and 280 nm, column temperature stable at 30 $^{\circ}$ C, taking nine kinds of estrogen mixture 10 μ L sample into test under the method of 2.3, the result is shown in figure 1.

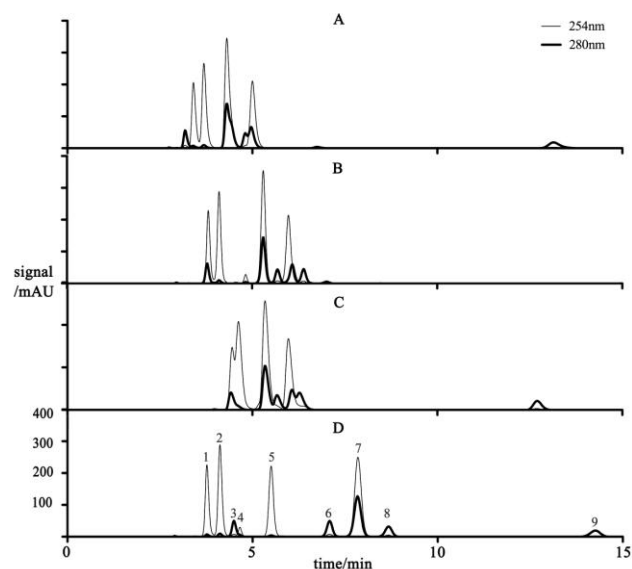


Figure 1 Result of the mixed standard estrogens solution on different chromatographic column chromatogram. (a) C18 column; (b) 120-5-C18-AQ PLUS column; (c) Hypersil Gold aQ column; (d) 120-5-C18-ace-EPS column.

The results show that with C18, C18 - AQ PLUS and Hypersil Gold aQ column can't separate the nine estrogen baseline, while using C18 EPS column can complete the nine environmental estrogen baseline separation.

3.1.3 The choice of mobile phase flow rate

Velocity affects the size of the column system pressure, the degree of separation and elution time among these components. In order to further optimize the experiment, a 120-5 - EPS column as stationary phase, using C18 - ace - with the mobile phase at 1.2 mL/min and 1.2 mL/min of nine compounds are the prototype under two kinds of flow velocity in mixed solution, in order to achieve better separation effect, as shown in figure 2.

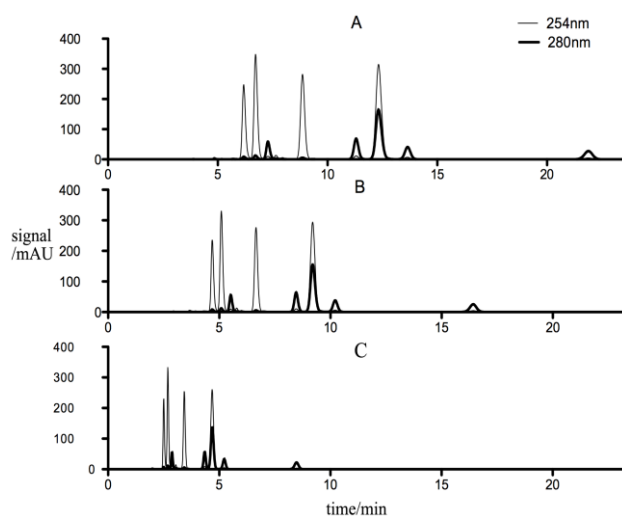


Figure 2 estrogen mixed standard solution in the chromatogram of different mobile phase A - C was the separation results under flow rate of 0.6 ml/min, 1.0 ml/min, 1.2 ml/min condition respectively.

According to the figure 1D, figure 2A, 2B and the formula 1:

$$R = \frac{2(t_{R_2} - t_{R_1})}{(W_2 + W_1)} \quad (1)$$

Compounds separation degree is calculated, of which the tR2 after one of the adjacent two peak retention time, tR1 for two adjacent peak retention time of the top piece, W1 and W2 respectively two adjacent peak peak width, the calculation results were shown as table 1.

Table 1 separation degree of nine kinds of estrogen

	1-2	2-3	3-4	4-5	5-6	6-7	7-8	8-9
Degree of separation	1.7	1.5	1.5	3.5	5.0	2.0	2.5	5.5

From table 1 shows, when the velocity was in the 1.0 mL/min, the compound isolation were greater than 1.5, to satisfy the demand of the "People's Republic of China pharmacopoeia" for HPLC

Table 2 Regression equation and Correlation coefficient

Analytes	Regression equation	Linear interval	Correlation coefficient
cortisone	$Y=1.6 \times 10^7 x + 1.0 \times 10^4$	0.001-0.1	R=1.000
hydrocortisone	$Y=4.1 \times 10^7 x + 2.0 \times 10^4$	0.001-0.1	R=1.000
estriol	$Y=4.1 \times 10^6 x + 3.9 \times 10^3$	0.001-0.1	R=1.000
ethynylestradiol	$Y=3.9 \times 10^6 x + 4.6 \times 10^3$	0.001-0.1	R=0.999
ethisterone	$Y=2.2 \times 10^7 x + 2.4 \times 10^5$	0.001-0.1	R=1.000
estrone	$Y=6.6 \times 10^6 x + 2.5 \times 10^4$	0.001-0.1	R=0.999
diethylstilbetrol	$Y=3.0 \times 10^7 x + 2.4 \times 10^5$	0.001-0.1	R=1.000
β -estradiol	$Y=4.4 \times 10^6 x + 1.2 \times 10^3$	0.001-0.1	R=0.999
mestranol.	$Y=4.4 \times 10^6 x + 5.5 \times 10^3$	0.001-0.1	R=1.000

3.3 Recovery rate and precision

We detected pretreatment milk sample under above HPLC detection condition, and we did not detect any estrogens. In order to determine the feasibility of this method in the actual samples test, the adding standard experiments was conducted. Three groups of blank milk samples were mixed with different concentrations of estrogen standard solution

separation degree, proving that this method to separate 9 kinds of estrogen can achieve very good effect.

3.2 Detection limit and linear range

Setting the mixture concentration as 0.001 mg/ml, 0.005 mg/ml, 0.01 mg/ml, 0.05 mg/ml, 0.1 mg/ml of standard mixed solution. Using ProntSIL 120-5 - C18 - ace - EPS column, mobile phase with 2.1.1 selected in the mobile phase, sample 10 μ L, concentration of each sample three times respectively, chromatographic peak area average, nine compounds estrogen standard curve drawing, respectively, the abscissa for compound concentration, ordinate for high performance liquid chromatogram peak area, calculation of linear equation and correlation coefficient. Results show that the use of liquid phase under the condition of 9 kinds of estrogen in 0.001 mg/mL ~ 0.1 mg/mL concentration range inside sex is good. Results were shown in table 2.

respectively. Recovery and RSD datas for the determination of nine kinds of estrogens in spiked milk were calculated using the formula 2 and the results were shown in table 3.

$$P = \frac{(C_1 - C_2)}{C_3} \cdot 100\% \quad (2)$$

Table 3 Recovery and RSD for the determination of nine kinds of estrogens in spiked milk

Analytes	Spiked amount (mg/mL)	Recovery (%)	RSD (%)	Spiked amount (mg/mL)	Recovery (%)	RSD (%)	Spiked amount (mg/mL)	Recovery (%)	RSD (%)
cortisone	0.1	93.1	2.26	0.05	96.9	1.62	0.025	93.5	1.91
hydrocortisone	0.1	93.3	1.91	0.05	101.6	2.03	0.025	100.1	1.78
estriol	0.1	96.9	4.72	0.05	101.9	4.33	0.025	98.4	1.99
ethynylestradiol	0.1	93.4	3.04	0.05	100.4	2.08	0.025	99.1	1.99
ethisterone	0.1	93.9	1.84	0.05	100.5	3.31	0.025	100.1	2.01
estrone	0.1	93.5	1.96	0.05	103.7	1.61	0.025	98.5	1.77
diethylstilbetrol	0.1	94.2	2.03	0.05	98.6	1.53	0.025	98.5	1.76
β -estradiol	0.1	92.2	2.11	0.05	95.5	1.79	0.025	97.7	1.56
mestranol	0.1	92.4	2.07	0.05	100.1	1.91	0.025	89.3	1.49

In table 3, according to the results of standard addition of nine kinds of estrogens in milk recovery range from 92.2% to 103.7%, relative standard deviation less than 10%, the results are satisfactory.

4 CONCLUSION

We used ProntoSIL 120-5 - C18 - ace - EPS chromatographic column and methanol/water (82.5/17.5, v/v) as mobile phase while setting flow rate of 1.0 mL/min, diode array detector was used at the same time. Results showed that nine kinds of estrogen including β -estradiol, estrone, mestranol, ethisterone, ethinyloestradiol, diethylstilbestrol, cortisone, estriol and hydrocortisone can be determined simultaneously within 15 min, proving that high performance liquid chromatography (HPLC) method, the optimal chromatographic conditions can be put into use in the field of a variety of environmental estrogen detection. Meanwhile, this method is simple, high degree of separation, so it can be applied to analysis of environmental

estrogen residues in milk and standards of environmental estrogen residues in dairy products.

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