Solid-Phase Extraction of Estrogens from Bovine Blood Plasma

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Levels of Estrone (E_1) and Estradiol-17 β (E_2) in bovine blood plasma indicate precise female reproductive conditions, since these estrogens are produced in the follicles, reflect the follicular state and induce estrus [5, 8]. They are also secreted from the placenta and are essential for the maintenance of pregnancy [12]. The limited levels of estrogens in bovine peripheral blood during the estrous cycle, however, make the quantification of estrogens difficult. Therefore, many preparatory steps, such as extraction, enrichment, and defatting or purification are needed to determine the concentration of estrogens in the plasma. Several methods, mostly liquid-liquid extraction, have been used for the isolation of estrogens from biological fluids. However, they have several disadvantages.

In the present experiment, another extraction technique was investigated. The method is based on the use of reversed-phase column chromatography, which is rapid and efficient for the extraction of steroids [7, 10].

Animals and samples: Holstein cows and Japanese Black heifers, normally managed herds in our institute, were used in this study. Blood was collected by jugular venipuncture into a heparinized polypropylene tube and was immediately centrifuged (1,500 g at 4°C for 1 hr). The separated plasma was stored at -20°C until use.

Reagents: All reagents were purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan. Methanol, diethyl ether, and normal(n)-hexane were of special grade for liquid chromatography.

Labeled estrogens: [2, 4, 6, 7-³H(N)]- E_1 (4.03 TBq/mmol) and [2, 4, 6, 7, 16, 17-³H(N)]- E_2 (6.25 TBq/mmol) were obtained from New England Nuclear, Boston, Massachusetts. The labeled estrogens were stocked in methanol (approx. 0.1 MBq/ml) at -20°C. The plasma was added to the labeled estrogens after the methanol was evaporated at 45°C under N₂ gas, and was shaken for 30 minutes at room temperature. Thereafter, the mixture was equilibrated at 4°C for 12 hours. The supernatant was used for further processing after centrifugation (1500 g at 4°C for 30 min). The radioactivities of the added E_1 and E_2 ranged from 130 to 150 Bq/ml and 260 to 310 Bq/ml, respectively.

Apparatus: Octadecylsilane bonded-phase packings (SEP-PAK C₁₈ cartridges, 9-10 mm, i.d.) were obtained from Waters Associates, Inc., Milford, Massachusetts. The packings were washed with 10 ml of methanol, followed by 20 ml of distilled water before use.

Procedures: A disposable 5 ml syringe was connected to

the cartridge as a reservoir. The plasma applied into the syringe was allowed to flow down through the column spontaneously without pressure. The flow rate was approximately 0.2 to 0.5 ml/min. The column was washed with 10 ml of distilled water. The estrogens that adsorbed to the packings were eluted with 3 ml of methanol. The material with a strong affinity for the packings was collected with 3 ml of diethyl ether. Liquid scintillation cocktail was added to 1 ml from each 5 ml of the plasma effluent and the washing fluid. The cocktail was also added to the residue of the elution solvent and the diethyl ether after evaporation at 60°C under N₂ gas. The recovery rate was calculated from disintegrations per minute in each fraction, which was measured with a liquid scintillation counter for 10 minutes for each sample.

In a preliminary study, Estrogens, dissolved in phosphate buffered saline, were extracted by the cartridge. The extracts were analyzed by high performance liquid chromatography (Waters 625 LC system and 991J photodiode array detector) with a octadecylsilane column (Waters Delta-Pak C₁₈, 300 A, 3.9 × 150 mm) and proved to be not damaged by the extraction procedure.

Exp. 1. Capacity of the column

Labeled E_1 and E_2 were added to plasma samples with very high estrogen contents, which were obtained from five Holstein cows in late-pregnancy, 45 to 1 days before parturition. Plasma in volumes of 5, 10, and 15 ml was applied to the column.

Full particulars of the results are shown in Table 1 and Table 2. The recoveries of labeled E_1 and E_2 in the methanol fraction were more than 98% and 97%, respectively. No reduction in recovery was observed when the volume of plasma applied was increased from 5 ml to 15 ml. The leakage of estrogen in the plasma effluent and in the washing fluid was less than 2% and 0.5%, respectively. The radioactivity adsorbing to the column after the elution with methanol was less than 0.5%.

Exp. 2. Optimal methanol concentration for the elution of E_1 and E_2 .

Ten milliliters of the same plasma as in Exp. 1 was applied in this study. It was attempted to elute the estrogens with 3 ml of 10, 20, 30, 40, 45, 50, 55, 60, 65, 70, 75, 80, 90, and 99.5 (v/v)% methanol.

The results are summarized in Fig. 1. Most (94%) of the E_1 and E_2 were recovered in the 50% to 70% methanol fractions. In particular, 86% of E_1 and 83% of E_2 were recovered in the 60% and 65% methanol fractions.

Exp. 3. Re-usability of the column

The re-usability of the cartridge was examined by repeating the extraction five times on the same column

Table 1. Extraction of ^3H -labeled Estrone added to bovine blood plasma in late-pregnancy

Fraction		Recovery (%)		
		5 ml ^{a)}	10 ml	15 ml
Plasma effluent (5 ml)	-1	0.78±0.16 ^{b)}	0.38±0.07	0.28±0.06
	-2		0.50±0.07	0.35±0.10
	-3			0.36±0.06
Water wash (5 ml)	-1	0.17±0.07	0.10±0.02	0.07±0.02
	-2	0.04±0.03	0.03±0.04	0.04±0.03
Methanol		98.94±0.23	98.92±0.13	98.74±0.21
Diethyl ether		0.07±0.02	0.07±0.02	0.16±0.20

a) Applied plasma volume, b) Mean±S.D. (N=5).

Table 2. Extraction of ^3H -labeled Estradiol-17 β added to bovine blood plasma in late-pregnancy

Fraction		Recovery (%)		
		5 ml ^{a)}	10 ml	15 ml
Plasma effluent (5 ml)	-1	1.06±0.22 ^{b)}	0.53±0.10	0.36±0.05
	-2		0.75±0.18	0.47±0.06
	-3			0.50±0.07
Water wash (5 ml)	-1	0.22±0.08	0.13±0.05	0.10±0.02
	-2	0.02±0.03	0.04±0.01	0.05±0.01
Methanol		98.62±0.23	98.46±0.32	98.42±0.17
Diethyl ether		0.08±0.02	0.08±0.02	0.10±0.02

a) Applied plasma volume, b) Mean±S.D. (N=5).

Fig. 1. Elution patterns of E_1 and E_2 under a stepwise methanol gradient. E_1 : Estrone, E_2 : Estradiol-17 β , PI: Plasma effluent, DW: Distilled water wash, DE: Diethyl ether wash.

Table 3. Cartridge re-usability

No. of repetitions of use	Recovery (%)	
	Estrone	Estradiol-17 β
1	98.13 $\pm$ 0.13 ^{a)}	96.29 $\pm$ 1.20
2	97.70 $\pm$ 0.31	96.01 $\pm$ 1.51
3	97.94 $\pm$ 0.17	95.23 $\pm$ 1.67
4	97.55 $\pm$ 0.26	94.60 $\pm$ 2.19
5	97.41 $\pm$ 0.22	94.14 $\pm$ 2.31

a) Mean $\pm$ S.D. (N=5).

from 5 ml of the plasma samples with high (at the peak of E₂ during estrus) and low (mid-luteal phase) estrogen contents. After elution with methanol, the column was washed with 3 ml of diethyl ether, and then with 3 ml of n-hexane prior to the next generation.

The recovery of E₁ decreased only slightly through five successive extractions on the same column (Table 3). Although the recovery of E₂ decreased gradually in each extraction, 94% of added E₂ was recovered at the fifth extraction (Table 3).

Small reversed-phase cartridges (SEP-PAK C₁₈) containing octadecylsilane bonded-phase packings have been successfully used for preliminary purification, separation, and enrichment prior to quantitative determination of various substances [10, 11, 13]. They are superior for analysis of steroid profiles to Amberlite XAD-2 [1, 6], which has been used for the solid-phase extraction of steroid conjugates [2, 3].

Heikkinen *et al.* [7], using the cartridges for the extraction of estrogens and their metabolites in human urine and plasma, reported that E₁ in the urine was recovered quantitatively in the methanol fraction without pH adjustment of the urine or the washing fluid. However, the plasma, following the dilution with acetate buffer (pH 5), was treated with triethylammonium sulfate [2, 3] to recover not only E₁ but also some polar steroid conjugates.

In this study, it was found that E₁ is easily extracted from bovine plasma without pH adjustment or any other treatment. Although the method was not tried for human plasma, these results suggest that E₁ could be also extracted from human plasma without pH adjustment.

E₂ in blood plasma was thought to be hardly retained by reversed-phase packings, as it is specifically bound to the sex hormone binding globulin (SHBG) [4]. Heikkinen *et al.* [7] reported that in man, as much as one half of E₂ is lost in the plasma effluent during the extraction with the cartridge, when the plasma is directly applied to the column. To ensure a quantitative recovery of E₂ by releasing from SHBG, d-norgestrel, which competes with E₂ for the binding sites of SHBG [14], was added to the plasma.

In our preliminary examination with human plasma of two men and two women by the same method for bovine blood plasma, the loss of E₂ in the plasma effluent was

only 8% and 10% in men and 12% and 20% in women. The recovery of E₂ in the methanol fraction was not quantitative, 90% and 88% in men and 85% and 76% in women, but was higher than the results of Heikkinen *et al.* [7]. Considering that the flow rate is important to obtain high recovery in a solid-phase extraction system [15], this difference in recovery may be due to the flow rate. It was speculated that E₂ may gradually separate from the SHBG and then be adsorbed by the packings while the plasma passes through the column, because the affinity of E₂ to the binding protein is weaker than to octadecylsilane-bonded packings. Therefore, the slower the plasma flows, the higher the recovery [15]. The flow rate (approx. 2 ml/min) by Heikkinen *et al.* [7] might be too fast for most estrogens bound to the proteins in the plasma to be retained in the column.

In this study, E₂ was recovered quantitatively from bovine plasma without any treatment before application. There was little interference of the binding proteins or other obstacles with the recovery of E₂. In addition, individual variation of the interference in cattle was less than in man. These results indicate that the form of E₂ in the blood is different between man and cattle. Most E₂ in bovine blood seems not to be bound to SHBG.

Salter and Shrimanker [9] reported that the relative binding affinity of E₂ for bovine SHBG was one eighth that of testosterone and only 9% that of 5 α -dihydrotestosterone. Although they employed a standard quantity of bovine serum as the SHBG, the serum also involved low affinity non-specific proteins. Taken together, it was considered that the absolute binding affinity of E₂ for SHBG may be very low, and SHBG may not be the major carrier of E₂ in bovine blood.

The optimal methanol concentration for elution of each estrogen was determined to prevent the extracts from contamination by unnecessary materials. It is particularly desirable that E₁ and E₂ is separated from each other for quantitative determination with radio-immunoassay (RIA), because the cross-reactions of the antibodies can not be disregarded. However, the optimal methanol concentration of both estrogens was approximately the same (65(V/V)%), so that they could not be separated from each other. The results, nevertheless, suggest that the cartridge could be re-washed with 40% to 45% methanol after a water wash, and then 70% to 75% methanol could be used for the elution of the estrogens to make the purity of extracts higher.

The cartridge can be used for the extraction of E₁ and E₂ from bovine plasma at least five times after proper regeneration. These results were similar to those for human urine previously reported by Heikkinen *et al.* [7] and Shackleton and Whitney [10]. The capacity of the cartridge may be sufficient for further extraction, since almost all of the labeled estrogen added to the plasma was recovered in the fifth extraction. However, repeated application of the cartridge would increase the contaminants in the recovered solvent, such as octadecyldimethylsilane detached from the packing, which would interfere

with the RIA. In the present study, the purity of the extracts in each experiment was not examined, and the influence of repeated use of the same cartridge on the value of RIA is not known. Therefore, for practical use, it would be better to examine whether further purification of the extracts is required for RIA analysis.

In summary, the solid-phase extraction with the small reversed-phase C₁₈ cartridge is useful for the extraction of E₁ and E₂ in bovine blood plasma and has considerable advantages over previous methods. The extraction time is significantly shortened over liquid-liquid extraction methods, since the plasma can be applied to the column without any preliminary management except for centrifugation. Moreover, the cartridge can be used for extraction at least five times.

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