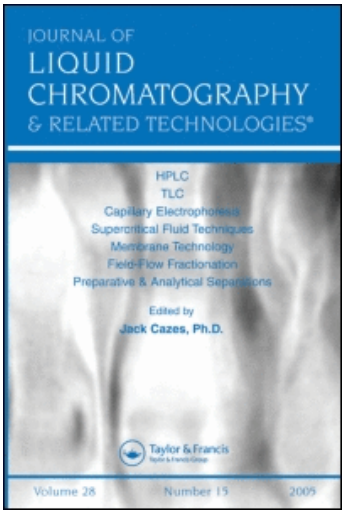

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF ESTRADIOL-17α AND -17β IN BIOLOGICAL FLUIDS; APPLICATION TO PLASMA, MILK AND URINE OF COWS

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ABSTRACT

A rapid HPLC technique was developed to separate estradiol epimers. In order to improve the sensitivity of the detection, a radioimmunoassay was used.

Estrone, estradiol-17α and estradiol-17β were separated within 20 min using 10 ml of chloroform: acetone (90:10), as the mobile phase. The efficiency of the technique was assessed with ^{3}H steroids and the assay of collected fractions with antisera specific to each estrogen. Using a non-specific radioimmunoassay, profiles of endogenous estrogens in different biological fluids (blood plasma, milk, urine) were obtained.

The efficiency of HPLC as a separation method and the high sensitivity of radioimmunoassay as a detector allows us to obtain profiles of estrogens from biological samples where steroid concentration is below 100pg/ml.

INTRODUCTION

Estrone (E_1), estradiol-17α ($E_{2\alpha}$) and estradiol-17β ($E_{2\beta}$) are known to be present in various biological fluids as free or conjugated steroids. $E_{2\alpha}$ is the major metabolite of biologically active estrogen $E_{2\beta}$ in farm animals (1); it has been measured in high concentration in blood plasma, milk and urine (2, 3). Despite the fact that it has no biological activity, $E_{2\alpha}$ can be of

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clinical interest as changes in its concentration are related to folliculogenesis (4) and development of pregnancy (3).

The major problem with the assay of E_2 is the separation of two epimers of estradiol. Previous published methods are inconvenient, and particularly in HPLC, have utilised large elution volume which increases the time (5, 6). These methods are not applicable for large number of samples as required for physiological studies. Some new methods are quicker but were evaluate only with pure steroids (7, 8). When used for biological samples, other difficulties come from the low concentration of estrogens (few pg/ml) which require efficient purification prior to quantification and high sensitivity of detection which can be reach only by radioimmunoassay.

The present paper describes an high performance liquid chromatographic separation of picogramms of estradiol epimers with subsequent quantification of purified steroid fraction by radioimmunoassay.

MATERIALS AND METHODS

Radioactive steroids 2,4,6,7-^{3}H estrone (98 curies/mmole) and 2,4, 6,7,16,17,^{3}H estradiol-17β (145 curies/mmole) were obtained from Radiochemical Centre, Amersham, Bucks, United Kingdom. 2,4,6,7-^{3}H estradiol-17α was prepared by incubation of labelled estrone with cow's erythrocytes (9). After incubation, the reaction mixture was diluted with equal volume of distilled water. 54% radioactivity was extracted with diethyl ether and the residue was delipidated with methanol: water (70:30) (10). The methanol extract was evaporated under nitrogen and the residue was chromatographed on HPLC. A ratio of ^{3}H-estrone to ^{3}H-estradiol-17α 1:12 was recovered and the synthesized radioactive estradiol-17α was further checked for its binding with specific antiserum.

All the reagents were of analytical grade. The solvents were dehydrated using a molecular sieve and were filtered through membrana filters (Membra fils 47 mm) obtained from Nucleopore Corporation, 7035 Commence Circle, Pleasanton, CA 94566 USA.

A Varian model 5010 was employed for the separation of estrogens. The model contains within a single unit the component of an isocratic high performance liquid chromatography. Elution was performed using commercial pre-packed S_1-10 silice column (30 cm x4.0 mm I.D.). In order to prevent the damage of column, a small pre-column (5.0 cm x 4.0 mm) of the same material was connected before the column. Fractions were collected on fraction collector Model 568 (ISCO, Lincoln, Nebrasca USA). Radioactivity was counted in Packard Tri-Carb Model N°3385 Liquid Scintillation Spectrometer. Evaluation of RIA data was performed on Hewlett Packard Computer.

High Performance Liquid Chromatographv

Radioactive standard estrogens or samples were dissolved in the eluent. Sample (0.2 ml) was applied on Valco automatic injector and was injected directly on the column. Fractions were collected in glass tubes, dried and the radioactivity was counted in 2 ml of scintillation fluid.

Biological samples preparation

All the samples were collected around estrus from Friesian cows. Some animals were superovulated with gonadotrophin-prostaglandin $F_{2\alpha}$ treatment (11).

Blood plasma: Free estrogens were extracted thrice from blood plasma with dietyl ether. The conjugated estrogens present in the remaining plasma were enzymatically hydrolysed (12) by Helix Pomatia juice obtained from Pharmindustry, Departement Reactifs (35, avenue Jean Jaurès, 92390 VILLENEUVE LA GARENNE, France). The liberated estrogens were extracted with dietyl ether. The etherial extract was evaporated under nitrogen and the residue was delipidated using 2 ml of methanol: water

(70:30). The mixture was incubated at 40°C for 1 h and at -20°C for overnight. The lipids were compacted by centrifugation at 1500g for 30 min at -20°C and the supernatant containing estrogens was decanted and evaporated under nitrogen. The residue was dissolved in the eluent and subsequently chromatographed on HPLC.

Milk: Whole milk was centrifuged twice and the upper cream layer was removed. The defatted milk was extracted with diethyl ether for free estrogens. The conjugated estrogens present in the remaining milk were hydrolysed in the same manner as for blood plasma.

Urine: Mostly estrogens are present as conjugated steroids in the urine (13). It is, therefore, conjugated estrogens were enzymatically hydrolysed in the same manner as for blood plasma. Then, total estrogens were extracted with diethyl ether and the extract was evaporated under nitrogen. The residue was dissolved in IN NaOH and was extracted with toluene. The organic phase was discarded and the aqueous phase was mixed with equal volume of 1N HCl. The estrogens from the mixture were extracted with dichloromethane and the organic phase was dried under nitrogen. The residue was dissolved in the eluent and subsequently chromatographed.

Radioimmunoassay: Estrogens were measured using non specific antiserum by minor modifications in earlier published procedure from this laboratory (14). Estradiol-17β and ^{3}H-estradiol-17β were used to assay the various fractions of HPLC from the extract of plasma, milk and urine. The antiserum was raised against estrone 17-hydrazobenzoyl coupled with bovine serum albumin in rabbits. Using ^{3}H-estradiol-17β as labelled antigen, the antiserum showed almost equal cross reaction with estrone, estradiol-17α and estradiol-17β. The radioimmunoassay procedure in brief is as follows: HPLC fractions were dissolved in 0.6 ml phosphate buffered saline (0.1 M phosphate pH 7.0

containing 0.15 M sodium chloride, 0.015 M sodium azide and 0.1% gelatin in glass distilled water) by keeping at 40°C for 30 min and vortexing for 30 sec. For the assay, 0.2 ml was pipetted in duplicate in polypropylene tubes and was mixed with 0.1 ml ^{3}H-estradiol-17 and antiserum. The tubes were mixed and incubated at 37°C for 30 min. The bound and unbound hormones were separated by double antibody immunoprecipitation. The precipitate was washed twice with 1.5 ml of polyethylene glycol solution (6%) and finally 0.05 ml 5N HCl was added. The radioactivity was counted directly after addition of 2 ml of scintillation fluid.

The specific antiserum of estrone was raised in rabbits against estrone-6-(O-carboxy) methyloxine conjugated to bovine serum albumin. The antibodies cross-reacted at a level of 0.2% with estradiol-17α and 0.95% with estradiol-17β. The antiserum to estradiol-17α was raised against estradiol-17α-6-O-Carboxy methoxine coupled with BSA, in rabbits. The antiserum cross reacted 1% with estrone and negligible with estradiol-17β (15). The antiserum against estradiol-17β was raised in rabbits against estradiol-17β -6 (O-Carboxy) methyloxine bovine serum albumin and cross reacted 0.7% with estrone and 0.88% with estradiol-17α (16).

RESULTS

As there were variations in the retention time of estrogens due to the moisture present in the solvents (17), therefore, it was felt necessary to dehydrate the solvents with a molecular sieve just before using into HPLC system. In order to obtain a suitable mobile phase for separation of $E_{2\alpha}$ and $E_{2\beta}$, a number of solvent systems known to be efficient in TLC (18) were tested (Table 1). Two mobile phases containing chloroform resolved the estradiol epimers with appreciable difference in the retention time.

TABLE 1
Chromatographic Elution of Various Radiolabelled Estrogens Using Various Mobile Phases, Flow Rate 0.5 ml/min; Temperature 30°C; Fraction Volumne 0.5ml.

Mobile phase	Retention time (min)		
	E_1	$E_{2\alpha}$	$E_{2\beta}$
Ethyl Acetate: Cyclohexane (50:50)	5	6	6.5
Chloroform: Ether (75:25)	5	7.5	8.5
Dichloromethane: Acetone (75:25)	4	5	5.5
Chloroform: Acetone (80:20)	4	6.0	7.0

As the system chloroform: acetone (80:20) was rapid as compared to chloroform: ether (75:25), therefore, it was further tested using various percentages of acetone in chloroform (Table. 2). There was increase in the difference of retention time between various estrogens with the decreasing percentage of acetone in chloroform. When the flow rate was further decreased, there was increase in the retention time of estrogens with improved resolution (Table. 3).

The HPLC profiling of radioactive estrogens using chloroform: acetone (90:10) has been shown in Fig. 1-4A. The estrogens were separated in 20 min using 10 ml of mobile phase. Recoveries of ^{3}H-estrone, ^{3}H-estradiol-17α and ^{3}H-estradiol-17β from HPLC were 81.6%, 85.2% and 74.8% respectively. The pattern of radiolabelled estrogens on HPLC was not affected using plasma (Fig 2A), milk (Fig. 3A) and urine (Fig. 4A) extracts. The

TABLE 2
Effect of Decreasing Percentage of Acetone in Solvent System Chloroform: Acetone on Separation of Estrogens. Parameters as in Table 1.

Percentage of Acetone	Retention time (min)		
	E_1	$E_{2\alpha}$	$E_{2\beta}$
50	4	4	4.5
30	4	5	5.5
20	4	6	7
10	5	7.5	9
5	6	10	11

TABLE 3
Effect of Flow Rate on the Separation of Estrogens Using Chloroform: Acetone (90:10). Parameters as in Table 1.

Flow rate ml/min	Retention time (min)		
	E_1	$E_{2\alpha}$	$E_{2\beta}$
1.5	3.5	5.0	6.0
1.0	5.0	7.5	9.0
0.5	10.0	14.5	16.5

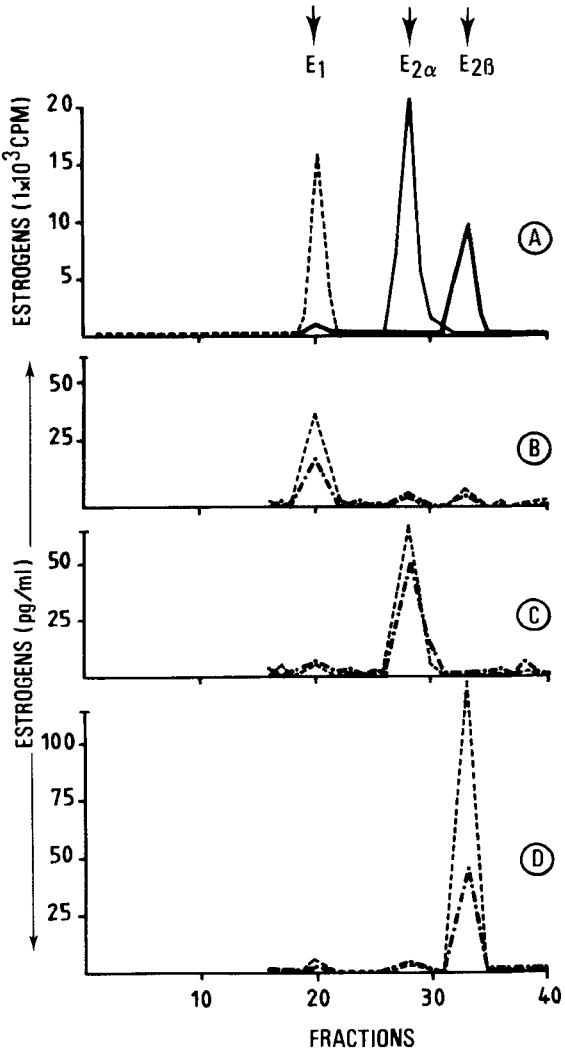

Figure 1: HPLC prolifing of estrogens. Mobile phase, chloroform: acetone (90:10); flow rate, 0.5 ml/min; temperature 30°C, pressure, 23 atmosphere; fraction volume, 0.25 ml. ^{3}H-radiolabelled (·----) E_1, (——) $E_{2\alpha}$ and (——) $E_{2\beta}$ (A). Immunoreactive profile of various estrogens in plasma of two superovulated animals (– – –) and (–·–·–) using specific antiserum against estrone (B), estradiol-17 α (C) and estradiol-17β (D) across the tritiated hormones using the entire elute.

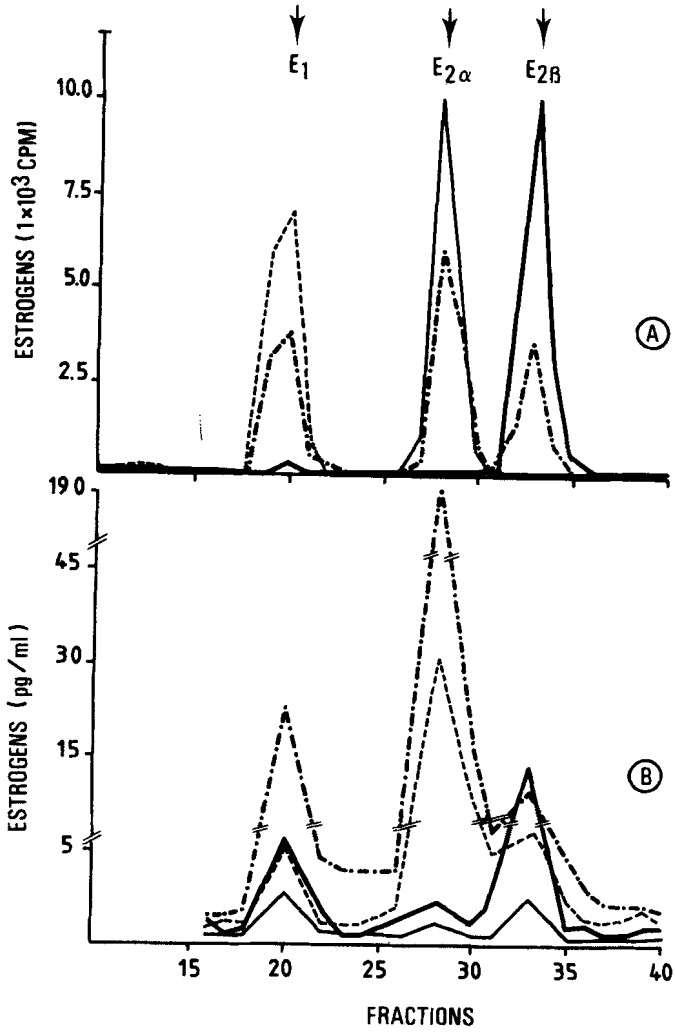

Figure 2: Chromatogram of estrogens. Parameters as in Fig. 1.
A: Radioactive profile (-----) E_1, (——) $E_{2\alpha}$, (——) $E_{2\beta}$ and (—·—) mixture of estrogens extracted from plasma.
B: Plasma immunoreactive estrogens in normal animal (——) free and (- - -) conjugated; superovulated animal (——) free and (—·—) conjugated.

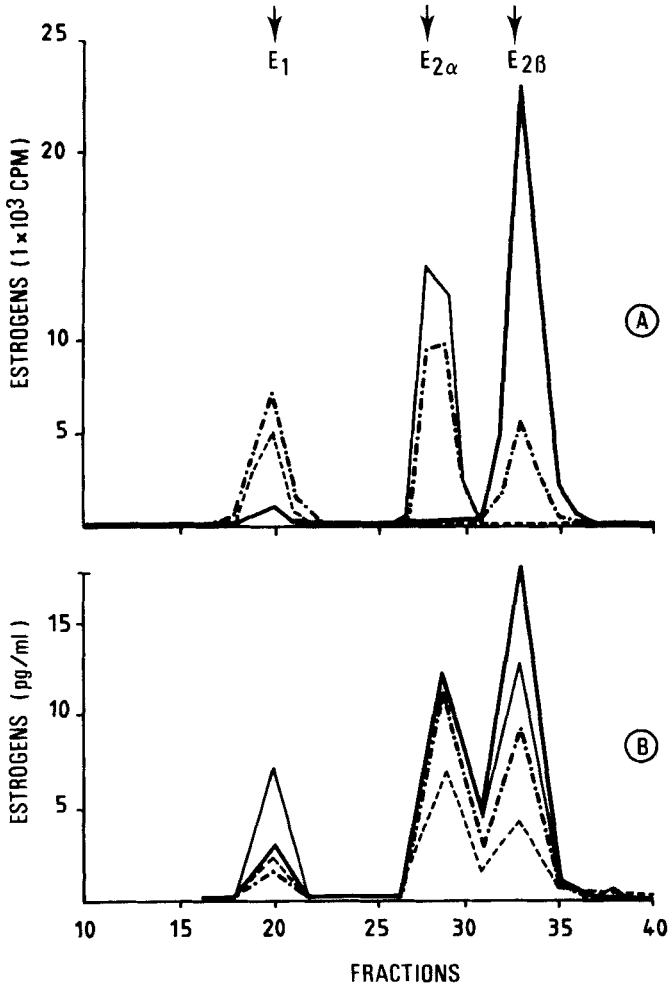

Figure 3: Chromatogram of estrogens. Parameters as in Fig. 1.
A: Profile of radioactive estrogens as in Fig. 2 and (—·—·) mixture of estrogens extracted from milk.
B: Milk immunoreactive conjugated estrogens in (— — —) normal animal; (——), (——) and (—·—·) superovulated animals.

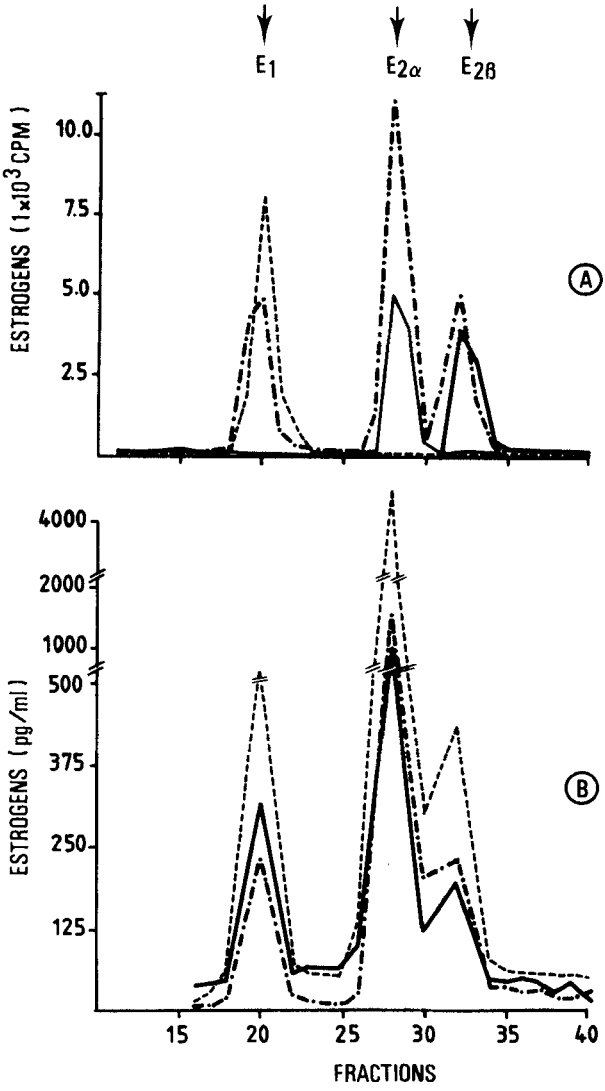

Figure 4: Chromatogram of estrogens. Parameters as in Fig. 1.
A: Profile of radioactive estrogens as in Fig. 2 and (—·—·—) mixture of estrogens extracted from urine.
B: Total immunoreactive estrogens of urine in (—·—·—) normal animal, (- - - -) superovulated animal and (——) non-responsed superovulated animal.

elution profile of endogenous estrogens across radioactive estrogens were confirmed by RIA using specific antiserum against estrone (Fig. 1B), estradiol-17α (Fig. 1C) and estradiol-17β (Fig. 1D).

The effectiveness of the developed HPLC technique was assessed by its application to various biological samples. The separated estrogens on HPLC were measured by RIA using non specific antiserum in blood plasma (Fig. 2B), milk (Fig. 3B) and urine (Fig. 4B).

DISCUSSION

The present high performance liquid chromatographic system that we have described for the separation of estradiol epimers is rapid and more convenient and sensitive than earlier available methods (5, 6, 7, 8). The separation is completed within 20 min using small volume (approximately 10 ml) of mobile phase over the earlier methods which requires long column and/or large elution volume. The method is more convenient than indirect method (12) where $E_{2\alpha}$ was separated from $E_{2\beta}$ by enzymatic oxidation of estradiol-17β to estrone.

The sequence of elution of E_1, $E_{2\alpha}$ and $E_{2\beta}$ is in accordance with their polarities. By creating a change in the polarity in the mobile phase, the resolution of the two epimers were improved. The changing in the polarity in chloroform: acetone, though increased the retention time, but the separation was better. The decrease in flow rate also increased the retention time, but the elution volume was decreased with improved resolution.

The estrogens concentrations in blood plasma of normal animal are in agreement with earlier report from this laboratory (11) and other workers (3, 19, 20, 21). Estradiol-17β concentrations in milk of cyclic cows are in the range of those

reported previously (22, 23); moreover, the conjugated estrogenic activity partly in the ketonic and mainly in non-ketonic fraction of defatted colostrum was also reported by previous worker (24). Similarly, all the 3 estrogens were present in urine of normal animal with the predominance of $E_{2\alpha}$ which agrees with other workers (2, 25).

It is, therefore, concluded that the present HPLC method can be utilised for the simultaneous quantification of free and conjugated E_1, $E_{2\alpha}$ and $E_{2\beta}$. The rapid and precise separation of estradiol epimers makes it an invaluable technique in the clinical diagnosis.

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