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Note

Separation of C-17 fatty acid esters of 17β-estradiol by reversed-phase high-performance liquid chromatography

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The natural C-17 long-chain fatty acid esters of 17β-estradiol are non-polar estradiol metabolites which possess a long-acting estrogenic activity [1]. These lipoidal derivatives have been isolated from incubation of estradiol with bovine uterine endometrial slices and were fully characterized by spectrometric methods [2]. Furthermore, their presence has been detected in different estrogen-responsive tissues [2,3], human mammary cancer tissues [2,4,5] and mammary cancer cell lines [6,7].

However, measurements of the esterification activities of the different fatty acids to estradiol in human breast tumours [5] or in human mammary cancer cells in culture [7] were relatively imprecise because of unsatisfactory high-performance liquid chromatographic (HPLC) separations of the different estradiol fatty acid esters.

This report describes a new reversed-phase HPLC method that achieves complete separation of the fatty acid esters of estradiol. It has been applied to the analysis of non-polar metabolites of estradiol synthesized with bovine hepatic microsomal membranes.

EXPERIMENTAL

Materials

17β-Estradiol (E_2) was obtained from Steraloids (Wilton, NH, U.S.A.). Oleoyl coenzyme A (oleoyl CoA) and magnesium chloride were purchased from

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Sigma (La Verpillière, France). Acyl chlorides were obtained from Nu Check Prep (Elysian, MN, U.S.A.). [6,7(n)-^{3}H]E_2 (2.59 TBq/mmol) was purchased from CEA (Gif-sur-Yvette, France). All solvents were HPLC grade. Water was deionized and distilled from the glass apparatus.

HPLC equipment

The HPLC system consisted of a Philips Model PU 4100 solvent-delivery system from Pye Unicam (Cambridge, U.K.) equipped with a Model 7125 universal loop injector (100-μl volume) (Rheodyne, Cotati, CA, U.S.A.). UV absorbance of E_2 esters was measured at 280 nm on a Philips Model PU 4110 UV–VIS detector. Peak areas were recorded on a Shimadzu Model C-R3A integrating recorder (Shimadzu, Kyoto, Japan). Radioactive peaks were collected with a Gilson Model 202 fraction collector (Gilson-France, Villiers Le Bel, France). A LiChrosorb Diol column (10 μm particle size, 250 mm$\times$4.6 mm I.D.) from Chrompack (Les Ulis, France) and two Spherisorb ODS2 columns (5 μm particle size, 250 mm$\times$4.6 mm I.D.) from Société Française Chromato Colonne (Neuilly, France) were used.

Synthesis of E_2 fatty acid esters

The C-17 fatty acid esters of E_2 were synthesized as previously described [2]. They were purified by thin-layer chromatography on 0.25-mm silica gel plates (Kieselgel 60F_{254}, Merck, Darmstadt, F.R.G.) with toluene–ethyl acetate (90:10, v/v) as the developing solvent. The R_F values for E_2, E_2-3 esters, E_2-17 esters and E_2-3,17 diesters were 0.10, 0.28, 0.50 and 0.96, respectively. E_2-17 fatty acid esters were further purified by HPLC on a LiChrosorb Diol column with dichloromethane–hexane (35:65, v/v) as mobile phase at a flow-rate of 1.6 ml/min (system H1).

Reversed-phase HPLC of E_2 fatty acid esters

The conditions of separation by reversed-phase HPLC of different E_2 esters were studied with a Spherisorb ODS2 column using the two following mobile phases: (i) methanol–acetonitrile (50:50, v/v) at a flow-rate of 0.8 ml/min (system H2); (ii) methanol–acetonitrile–water (64:30:6, v/v) at a flow-rate of 1.4 ml/min (system H3). Finally, reference compounds and E_2 esters resulting from acylation of E_2 with hepatic microsomal membranes were routinely chromatographed with system H4, which consisted of two Spherisorb ODS2 columns connected in series with a stepwise elution system, viz. methanol–acetonitrile–water (64:30:6, v/v) from 0 to 28 min, methanol–acetonitrile (50:50, v/v) from 28 to 38 min and methanol–acetonitrile (80:20, v/v) from 38 to 50 min. The flow-rate of 1.8 ml/min was kept unchanged throughout the three steps. All chromatographic separations were performed at ambient temperature.

Preparation of bovine hepatic microsomal membranes

Slices of liver were removed from male calves just after slaughter and kept on ice until homogenization, which was carried out within 1 h after tissue collection. Portions of liver were minced and homogenized at 4°C in a Potter–Elvehjem glass homogenizer with a PTFE pestle in 0.25 *M* sucrose buffer containing 20 m*M* Tris–HCl (pH 7.4). Homogenates were centrifuged sequentially at 10 000 *g* (15 min) and 106 000 *g* (60 min) at 4°C on a Centrikon T-1045 ultracentrifuge (Kontron, Zurich, Switzerland) to prepare the microsomal pellet. Microsomes were washed once with 0.2 *M* sodium phosphate buffer (pH 7.4) and then resuspended in a minimal volume of 0.1 *M* potassium phosphate buffer (pH 7.4) containing 0.1 m*M* ethylenediaminetetraacetic acid (EDTA) and 20% glycerol. Microsomal preparations were stored at −80°C until use. Protein determination was performed as previously described [8].

Incubation of microsomal preparations, extraction and purification of the non-polar E_2 metabolites

Microsomal membranes (2 mg) were incubated at 37°C in 1 ml of 0.1 *M* sodium phosphate buffer (pH 7.4) containing 5 m*M* magnesium chloride. Microsomes, and oleoyl CoA (0.1 m*M*) when used as cofactor, were preincubated for 3 min at 37°C, prior to the reaction, which was initiated by addition of [^{3}H]E_2 (3.7 μM, 1–1.5·10^6 dpm) in 25 μl of ethanol and stopped 1 h later by extraction with two volumes of dichloromethane. Estrogens were quantitatively extracted from the aqueous phase using two further extractions with two volumes of dichloromethane. Combined organic phases were evaporated under nitrogen, and the residue was resuspended in 250 μl of dichloromethane–hexane (35:65, v/v).

The lipoidal derivatives of E_2 have been subsequently purified by HPLC with system H1, and for each collected fraction the radioactivity of an aliquot part was counted on a Packard Tricarb Model 4430 spectrophotometer (Packard Instrument, Downers Grove, IL, U.S.A.). Data were expressed in dpm after correction for quenching. Fractions corresponding to non-polar radioactive peaks with retention times similar to those of authentic standards were pooled and separated by HPLC with system H4.

RESULTS AND DISCUSSION

Conditions of separation of E_2 fatty acid esters

With the simplest chromatographic systems, H2 and H3, the elution patterns of the polyunsaturated, monounsaturated and saturated fatty acid esters of E_2 were not different from those previously described [5,7] (Table I). However, with system H2 only E_2 oleate, E_2 palmitate and E_2 stearate were well separated. When a low proportion of water was used in the eluting mixture (system H3), the polyunsaturated esters and E_2 palmitoleate were partially

TABLE I

CAPACITY FACTORS OF E_2 ESTERS

Isocratic elution on a Spherisorb ODS2 column with a solvent mixture containing no water (system H2) or 6% water (system H3); or gradient elution on two Sperisorb ODS2 columns in series with system H4. The capacity factor (k') is determined from the retention time of the ester (t_R), using the relationship $k' = (t_R - t_0)/t_0$, where t_0 is the retention time of E_2 used as non-retained compound. The reversed-phase HPLC systems are described in Experimental.

E_2 fatty acid ester	Capacity factor		
	H2	H3	H4
$C_{22\ 6}$	1.02	3.53	5.27
$C_{18\ 3}$	1.24	3.81	5.55
$C_{20\ 4}$	1.24	4.30	6.34
$C_{16\ 1}$	1.77	5.14	7.41
$C_{18\ 2}$	1.77	5.44	7.97
$C_{18\ 1}$	2.69	8.43	10.39
$C_{16\ 0}$	3.02	9.05	10.71
$C_{18\ 0}$	4.63	15.16	13.20

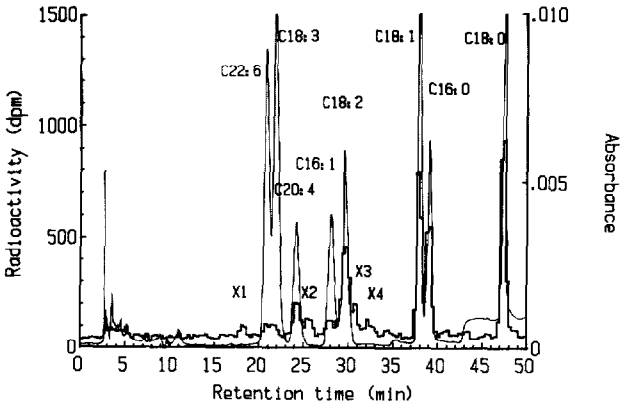

Fig. 1. Reversed-phase HPLC profile of radioactive E_2 fatty acid esters synthesized from [^{3}H]E_2 (3.67 μM, $1.2 \cdot 10^6$ dpm) by hepatic microsomes without cofactor. Chromatography was performed on two Spherisorb ODS2 columns connected in series, with system H4. The sample containing tritiated non-polar E_2 derivatives was spiked with authentic standards before injection in order to identify the radioactive metabolites by comparing their retention times with those of the reference compounds detected in parallel by UV detection at 280 nm. Stepped line, radiometric recording; smoothed line, UV recording. X1 to X4 represent four unidentified radioactive peaks.

separated. So, to improve the separation of the polyunsaturated fatty acid esters of E_2, the number of theoretical plates was enhanced by using two Spherisorb ODS2 columns in series. Thus, to obtain a convenient chromatographic procedure for the separation of E_2 esters, stepwise elution (system H4) was performed as follows: the first eluting mixture (from 0 to 28 min) was the same

as for the system H3 and ensured the complete separation of the polyunsaturated E_2 esters; the second eluting mixture (from 28 to 38 min) was system H2 to separate E_2 oleate from E_2 palmitate; finally E_2 stearate was rapidly eluted in the third step (from 38 to 50 min) by increasing the elution strength of the solvent mixture (methanol–acetonitrile, 80:20, v/v) (Fig. 1, Table I).

Analysis of E_2 fatty acid esters synthesized with bovine hepatic microsomes

In the course of purification of non-polar radioactive estrogens on a Li-Chrosorb Diol column with the eluting system H1, a group of estrogens was eluted with the same retention times as those of E_2-17 fatty acyl esters (13–15 min), but no radioactivity was eluted at the retention times of E_2-3,17 diesters (3 min) or E_2-3 esters (4.5 min). The amounts of non-polar E_2 metabolites synthesized after incubation of E_2 (3.7 μM) for 1 h with hepatic microsomes without cofactor and with oleoyl CoA (0.1 mM) were, respectively, 26.7 ± 1.9 and 71.9 ± 3.5 pmol/h/mg of protein (mean $\pm$ S.D.). These radioactive E_2 fatty acid esters were further chromatographed with system H4 (Fig. 1, Table II). Thus, twelve metabolites have been separated. Among these, in absence of any cofactor, five major metabolites, which together account for more than 80% of

TABLE II

HPLC QUANTIFICATION OF RADIOACTIVE NON-POLAR METABOLITES OF E_2 SYNTHESIZED WITH HEPATIC MICROSOMES WITHOUT COFACTOR OR WITH OLEOYL CoA

E_2 fatty acid ester	E_2 ester composition[a] (%)	
	No cofactor ($n=2$)	Oleoyl CoA ($n=2$)
$C_{22\ 6}$	1.5	1.2
$C_{18\ 3}$	1.9	1.3
$C_{20\ 4}$	6.6	6.2
$C_{16\ 1}$	2.8	1.0
$C_{18\ 2}$	18.4	9.6
$C_{18\ 1}$	19.2	62.3
$C_{16\ 0}$	14.0	4.1
$C_{18\ 0}$	24.1	7.5
Total identified fraction	88.5	93.2
Total unidentified fraction	11.5	6.8
HPLC recovery[b] (%)	95.3	94.2

[a]The E_2 ester composition is expressed as a percentage of the total radioactivity measured after chromatography.

[b]The recovery is expressed as a percentage of the injected radioactivity.

the total injected radioactivity, could be isolated; the C-17 fatty acid moieties were arachidonate, linoleate, oleate, palmitate and stearate.

This composition is quite different from that obtained previously by incubation of bovine uterine endometrial tissue, where the poly- and monounsaturated fatty acid esters of E_2 were predominantly biosynthesized [2], but are in a good agreement with the relatively high contents of monounsaturated and saturated fatty acids measured in triglycerides [9] and cholesteryl esters [10] in bovine liver.

When oleoyl CoA is added to the incubation buffer as an acyl donor, E_2 oleate is recovered as the predominant metabolite, with 62.3% of the total biosynthesized E_2 esters. Concomitantly, the polyunsaturated esters (E_2 docosahexaenoate, E_2 linolenate and E_2 arachidonate) are decreased by only $19.3 \pm 13.0\%$ (mean $\pm$ S.D.), whereas all other less polar esters are lowered by $63.0 \pm 10.4\%$ (mean $\pm$ S.D.) of the values obtained without cofactor (Table II). Thus, for the first time in bovine hepatic microsomes, the existence of a basal esterification activity of E_2 has been shown which is strikingly increased in the presence of oleoyl CoA as cofactor.

In conclusion, a simple, direct and unambiguous analysis of the C-17 long-chain fatty acid esters of E_2 can be performed by reversed-phase HPLC. This method can be easily applied to reliable quantitative analysis of the biosynthesized E_2 esters. The variation of such activity, depending on the presence of cofactors in various biological membrane preparations, is under investigation in our laboratory.

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