Journal of Chromatography, 358 (1986) 268–273
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 18 514

Note

Separation of A-ring iodinated oestrogens by isocratic reversed-phase high-performance liquid chromatography

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(Received January 28th, 1986)

A-ring iodinated oestrogens are of potential interest for a number of applications, for instance as synthetic intermediates or as labelled ligands in radioimmunoassays and receptor assays. Several procedures for introducing iodine into the aromatic A-ring have been developed, which generally result in a mixture of at least three iodinated products, namely the 2- and 4-monoiodo and the 2,4-diiodo derivatives[1–3]. Homologous preparations of each of these derivatives of oestrone and oestradiol-17β (E_2) could be obtained by fractional crystallization or thin-layer or liquid chromatography. Apart from one report on regioselective iodination at the C-2 position[4], little is known, however, of the iodination of oestriol (E_3) and there is, to our knowledge, only one report on the effective separation of 2- and 4-iodooestriol[5].

Continuing our study on steroids as immunochemical probes and indicator ligands in radioimmunoassay, we were interested in the affinities of 2-iodo- and 4-iodo-E_2 and -E_3 to anti-oestradiol- and anti-oestriol-C_6-conjugate antibodies[6]. Oestradiol was iodinated and the 2- and 4-iodo isomers were obtained according to ref. 1. Oestriol was iodinated by the same simple direct procedure. The crude product mixture was resolved into its constituents by a reversed-phase high-performance liquid chromatographic (RP-HPLC) procedure[5]. Our chromatographic results, however, did not agree with those given[5] with respect to the sequence of elution of the monoiodinated oestriols. To confirm our interpretation of the chromatographic pattern obtained by RP-HPLC of iodinated oestriols, we compared this pattern with those resulting from the chromatography of analogously synthesized iodine derivatives of oestradiol on the reversed-phase and on a silica column; the latter HPLC system has already been shown to resolve iodinated oestradiols[1]. Final proof of the identity of the separated fractions of iodinated oestriols was obtained by mass spectrometry and nuclear magnetic resonance spectroscopy.

EXPERIMENTAL

Oestradiol-17β, oestriol, iodine, chloroform (LiChrosolv, stabilized with 2-methyl-2-butene) and all other chemicals (analytical reagent grade) were purchased from Merck (Darmstadt, F.R.G.). $Na^{125}I$ was obtained from Behringwerke (Marburg, F.R.G.).

0021-9673/86/$03.50

Oeastradiol and oestriol were iodinated by the procedure described in ref. 1: 0.4 mmol of iodine in 0.5 ml of tetrahydrofuran was added with stirring to a solution of 0.4 mmol of oestrogen in 2.5 ml of methanol and 0.5 ml of concentrated ammonia solution (in some experiments the iodine solution additionally contained about $5 \cdot 10^5$ cpm of $Na^{125}I$). After 30 min, the pH of the solution was adjusted to about 5 by addition of acetic acid, 5 ml of water were added and the resulting mixture was extracted with ethyl acetate; the organic phase was washed with water, dried over sodium sulphate and evaporated to dryness. The residue was dissolved in the HPLC mobile phase.

HPLC was performed using a Model 6000 A solvent delivery system, a U6K injector, a radial compression separation system equipped with either a C_{18} RP or an SI Radial-Pak cartridge (both 10 μm, 100 × 5 mm I.D.) and a Model 441 UV absorbance detector (280 nm), all from Millipore Waters Chromatographie (Eschborn, F.R.G.). Methanol–water–acetic acid [50:50:2 (E_3) or 70:30:2 (E_2)] was used as the eluent in RP-HPLC and chloroform–methanol [96:4 (E_3) or 99:1 (E_2)] in normal-phase HPLC. Chromatography was carried out at room temperature. The radioactivity of the isolated chromatographic fractions was measured with a Hydrogamma 16 γ-counter (Zinsser Analytic, Frankfurt, F.R.G.).

RESULTS

The crude product mixture resulting from iodination of E_3 with an equivalent amount of iodine was resolved into four fractions by isocratic RP-HPLC with methanol–water–acetic acid (50:50:2) as the eluent (Fig. 1A). As could be shown by co-chromatography with E_3, the fraction eluting first (peak 1) contained unreacted educt. When the iodination of E_3 was performed in the presence of a small amount of $Na^{125}I$, radioactivity was recovered almost quantitatively in the fractions corresponding to peaks 2–4; the ratio between radioactivity of the fraction and peak area was nearly the same for peaks 2 and 3 but was about twice as high for peak 4 (Table I). Peak 4 was the prevalent peak when E_3 was reacted with two equivalents of iodine instead of one. These findings suggest that peaks 2 and 3 may be assigned to two isomers of monoiodo-E_3 and peak 4 to a diiodo-E_3.

For comparative purposes the product mixture obtained by the analogous iodination of E_2 was also analysed by RP-HPLC. As E_2 and its iodinated derivatives were hardly soluble in the solvent used with E_3, chromatography was carried out using methanol–water–acetic acid (70:30:2) as the eluent. Under these conditions, the iodination products of E_2 were also resolved into four fractions, which from the same experimental evidence as outlined above could be assigned tentatively to unreacted E_2 (peak I), two isomers of monoiodinated E_2 (peaks II and III) and a diiodinated E_2 (peak IV) (Fig. 1B, Table I).

In order to confirm this assignment and to specify the two monoiodo derivatives of E_2, the product mixture was analysed additionally by HPLC on a silica column using chloroform–methanol (99:1) as the eluent. This HPLC system has been shown to separate (in the sequence of elution) 2,4-diiodo-E_2, 2-iodo-E_2, 4-iodo-E_2 and E_2[1]. Our chromatographic results (Fig. 2B, Table I) were in agreement with those in ref. 1 with respect to both the number of peaks and their retention times. When fractions I, II, III and IV obtained by RP-HPLC of the E_2 iodination mixture

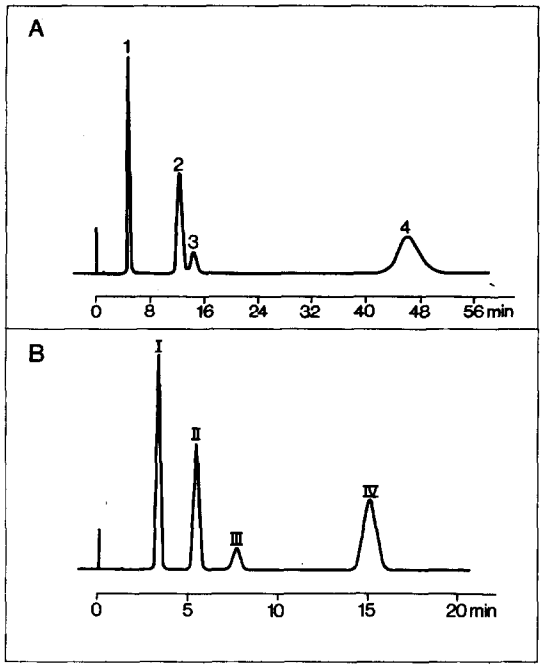

Fig. 1. Reversed-phase HPLC of the iodination products of (A) oestriol and (B) oestradiol. Column, 10 μm C_{18} Radial-Pak cartridge (100 × 5 mm I.D.); mobile phase, methanol–water–acetic acid, (A) (50:50:2) and (B) (70:30:2); flow-rate, 1 ml/min; detection, absorbance at 280 nm, 0.1 a.u.f.s.

TABLE I

HPLC ANALYSIS OF IODINATION PRODUCTS OF OESTRADIOL AND OESTRIOL

Chromatography as shown in Figs. 1 and 2. Capacity factors (k') and relative peak areas are mean values and mean values ± standard deviations, respectively, of at least two chromatographic analyses of the products of three independent iodinations of each oestrogen. Radioactivity values are those of a typical experiment; the amount of radioactivity applied to the column was 5000 cpm in the case of iodinated E_2 and 6000 cpm in the case of iodinated E_3.

Compound	*Reversed-phase column*				*Silica column*			
	Peak	*k'*	*Relative area (%)*	*Radioactivity (cpm)*	*Peak*	*k'*	*Relative area (%)*	*Radioactivity (cpm)*
	Methanol–water–acetic acid (70:30:2)				*Chloroform–methanol (99:1)*			
E_2	I	2.3	29.0 ± 3.4	–	D	10.8	30.0 ± 2.9	–
2-I-E_2	II	4.5	27.5 ± 2.3	1360	B	5.4	25.3 ± 3.0	1310
4-I-E_2	III	6.7	6.5 ± 1.3	290	C	6.2	6.3 ± 1.3	320
2,4-di-I-E_2	IV	14.0	37.5 ± 4.5	3300	A	4.4	38.5 ± 2.3	3230
	Methanol–water–acetic acid (50:50:2)				*Chloroform–methanol (96:4)*			
E_3	1	3.8	28.5 ± 3.5	–	d	7.8	30.2 ± 3.4	–
2-I-E_3	2	11.3	29.8 ± 4.5	1570	b/c	6.5	34.2 ± 1.8	1960
4-I-E_3	3	13.2	6.2 ± 2.8	330				
2,4-di-I-E_3	4	45.0	35.3 ± 5.0	3900	a	5.3	35.6 ± 3.6	3780

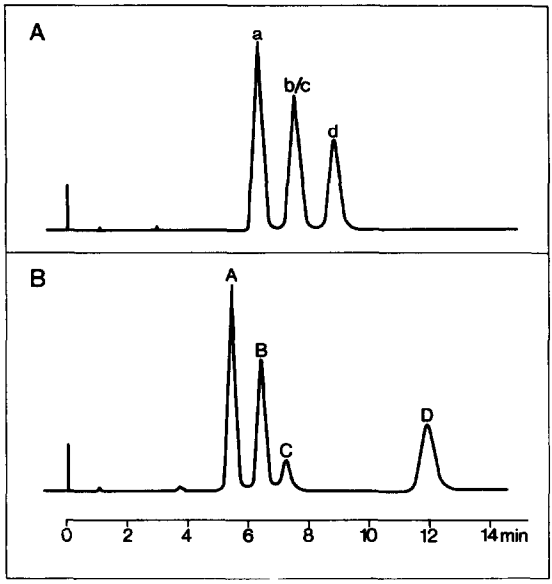

Fig. 2. Normal-phase HPLC of the iodination products of (A) oestriol and (B) oestradiol. Column, 10 μm SI Radial-Pak cartridge (100 × 5 mm I.D.); mobile phase, chloroform–methanol, (A) (96:4) and (B) (99:1). Other details as in Fig. 1.

were isolated and rechromatographed on the silica column, they co-eluted with peaks D, B, C and A, respectively. Hence, the sequence of elution of the products of E_2 iodination from the reversed-phase column could be established as E_2 iodination from the reversed-phase column could be established as E_2, 2-iodo-E_2, 4-iodo-E_2 and 2,4-diiodo-E_2.

The analogous chromatography on silica of the products of E_3 iodination resulted in the separation of only three fractions; the compounds, however, dissolved incompletely in the chloroform–methanol (99:1) eluent. By increasing the proportion of methanol in the eluent to 4%, complete dissolution of all analytes could be achieved but again only three fractions, a, b/c and d, were obtained by HPLC (Fig. 2A). When the fractions isolated by RP-HPLC of the products of E_3 iodination were rechromatographed on the silica column, fraction 1 co-eluted with peak d, fractions 2 and 3 with the peak b/c and fraction 4 with peak a. The silica HPLC system, although resolving the isomers of monoiodinated E_2 efficiently, apparently does not separate the isomers of monoiodinated E_3; it therefore could not be used to provide further support for the identification of the monoiodinated oestriol peaks obtained by RP-HPLC.

In order to identify definitively the fractions obtained by RP-HPLC of the products of E_3 iodination, they were analysed by spectroscopic methods[7]. Mass spectrometry revealed molecular ion peaks (relative intensities 100%) at the following *m/e* values: fraction 1, 288 (E_3); fractions 2 and 3, 414 (monoiodo-E_3); and fraction 4, 540 (diiodo-E_3). Proton nuclear magnetic resonance spectroscopy showed a 2-substituted oestriol in fraction 2 [two singlets in the aromatic region at about 6.7 and

7.6 ppm (acetone-d_6) or 7.0 and 7.9 ppm (pyridine) corresponding to 4-H and 1-H, respectively], a 4-substituted oestriol in fraction 3 [a pair of doublets at about 6.8 and 7.2 ppm (acetone-d_6) corresponding to 2-H and 1-H, respectively] and a 2,4-disubstituted oestriol in fraction 4 [a singlet at about 7.9 ppm (pyridine) corresponding to 1-H]. According to these results, the products of E_3 iodination, like those of E_2 iodination, elute from the reversed-phase column in the sequence unreacted oestriol, 2-iodo, 4-iodo and 2,4-diiodo derivative.

DISCUSSION

As was previously pointed out for oestriol[5] and shown in detail in this work for both oestriol and oestradiol, isocratic RP-HPLC with methanol–water–acetic acid as the mobile phase efficiently resolves the iodo derivatives of oestrogens. In accordance with data for oestradiol already published in the literature, 2-iodo-, 4-iodo- and 2,4-diiodooestriol and -oestradiol turned out to be the main products of iodination[1–3]; although both our chromatographic and spectroscopic results argue against the existence of iodinated oestrogens other than those mentioned above, they do not exclude, of course, the possibility that such compounds may be present additionally in the crude product mixtures in minor amounts. The relative yields of the respective iodinated oestradiols as determined by RP-HPLC agree well with those obtained by normal-phase HPLC on silica which, in turn, are comparable to the values reported in ref. 1; the product distribution of iodinated oestriols closely resembles that of iodinated oestradiols (Table I).

There are, however, some discrepancies between the results given in ref. 5 and our results with respect to the chromatographic pattern of monoiodooestriols. RP-HPLC of the products of E_3 iodination with carrier-free $Na^{125}I$ by the chloramine-T procedure was reported in ref. 5 to result in the separation of unreacted oestriol (retention time about 4.7 min) and two subsequent radioactive fractions (retention times about 7.5 and 9.7 min); based on chromatographic findings with the corresponding nitro derivatives and on immunological binding studies (not published in detail) the first of these two fractions was tentatively assigned to [4-^{125}I]E_3 and the second to [2-^{125}I]E_3. Although using an almost identical HPLC equipment except for a shorter RP column, we were not able to reproduce this pattern. On the contrary, we obtained the inverse elution sequence of monoiodooestriols, namely 2-iodo-E_3 eluting prior to 4-iodo-E_3; this was confirmed by comparison with results derived from the chromatography of analogously iodinated oestradiol, by mass spectrometry and by nuclear magnetic resonance spectroscopy. Previously, RP-HPLC with acetonitrile–water as the eluent was used to resolve the monobromooestradiols; these experiments also showed 2-bromo-E_2 eluting prior to 4-bromo-E_2[8]. Further, we never observed a spearation of the monoiodo-E_3 peaks as good as that shown in ref. 5, but rather had difficulties in approaching baseline separation. When the RP column was used for more than 20–30 runs the 4-iodo-E_3 peak appeared as a shoulder on the 2-iodo-E_3 peak only. Finally, in contrast to ref. 5, but in agreement with other reports showing the direct iodination at position 2 of oestrogens to be favoured over that at position 4, the relative yield of 4-iodo-E_3 was found to be much less than that of 2-iodo-E_3. At present we have no clear explanation to account for these discrepancies. It should be noted, however, that substantially the same chromatographic

pattern as was seen with oestriol reacted with unlabelled iodine was also obtained in preliminary experiments in which the iodination of oestriol was carried out by the procedure cited in ref. 5 using carrier-free $Na^{125}I$ (not shown).

Overall, RP-HPLC proved to be suitable for the analysis of iodinated oestriols and oestradiols. Because of its high resolving power, rapidity and ease of operation, this technique likewise is suited to the preparation of homogeneous iodo derivatives of oestrogens. RP-HPLC may also be used as a powerful tool in immunological and receptor binding studies, which demand pure iodinated oestrogens as ligands.

ACKNOWLEDGEMENTS

The authors are indebted to Dr. K. Jacob, Universität München, Klinikum Grosshadern, for the mass spectrometric analyses and to Prof. Dr. D. N. Kirk and Dr. C. J. Slade, Queen Mary College, University of London, for the nuclear magnetic resonance spectroscopic analyses. The technical assistance of R. Beck and G. Lautenbacher is gratefully acknowledged.

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