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Separation of indenestrol A and B isomers and enantiomers by high-performance liquid chromatography

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Abstract

High-performance liquid chromatography (HPLC) methods have been developed for the separation of substituted indenestrol A and B isomers on different columns. The isomers were separated by normal-phase liquid chromatography with a silica gel column. Enantiomers of these compounds were separated by chiral HPLC and the most successful separations were achieved with a Chiralcel OJ column. Published by Elsevier Science B.V.

Keywords: Enantiomer separation; Indenestrols; Sterols

1. Introduction

Substituted indenestrols, including indenestrol A (IA) and indenestrol B (IB), which differ only in the position of the indene double bond, have been identified as high-affinity ligands for the mouse estrogen receptor (mER) [1]. These compounds are metabolites of diethylstilbestrol (DES, **1**, Fig. 1), a potent synthetic estrogen [2]. The purification [3] and activities of the enantiomers of both indenestrols A and B have been reported and it was shown that the *S* enantiomer of IA (**2a**) had a higher binding affinity for the mER than the *R* enantiomer (**2b**) [4]. The *S* enantiomer also exhibited greater estrogenic activity [5]. For the B isomer, both *S* and *R* enantiomers (**3a** and **3b**) had similar binding affinities for the mER [6]. Because of the interesting biological profiles of these compounds, analogs **4** and **5** of indenestrol A were synthesized to further explore the interactions of these derivatives with the estrogen receptor. Enantiomer separations using optically active polymers as chiral stationary phases have advanced considerably in the past decade, and chiral HPLC has become a practically useful method not only for determining their optical purity, but also for obtaining optical isomers [7–9]. We report here HPLC methods to identify and purify the A and B isomers of these indenestrol derivatives, and a comparison of several chiral HPLC methods used to purify individual enantiomers of these analogs. These procedures are important for the isolation of pure compounds and enantiomers to examine their

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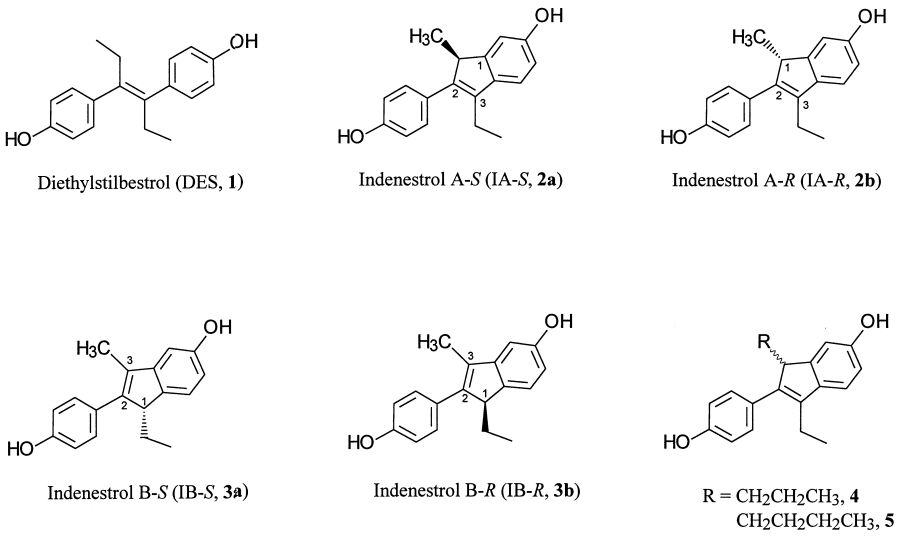

Fig. 1. Structures of diethylstilbestrol and indenestrol derivatives.

binding activities and further define interactions with the ER ligand binding region.

2. Experimental

2.1. Apparatus and conditions

HPLC was performed on a Beckman (Fullerton, CA, USA) HPLC system with System Gold software equipped with a Beckman 126 pump and a 168 diode array detector monitoring at 254 and 280 nm, and a Brownlee (Santa Clara, CA, USA) New Guard silica precolumn. All separations were carried out at room temperature. The HPLC columns used were: a silica gel column (250×4.6 mm, 10 μm) from IBM Instruments (Danbury, CT, USA); a Daicel (Exton, PA, USA) Chiralpak OP(+) column (250×4.6 mm, J.T. Baker) which was packed with an optically active helical polymer of diphenyl-2-pyridylmethyl polymethacrylate-coated silica gel; a Daicel Chiralcel OD column (250×4.6 mm, J.T. Baker) which was packed with an optically active cellulose *N*-dimethyl-phenylcarbamate-coated silica gel; a Daicel Chiralcel OJ column (250×4.6 mm, J.T. Baker) which was packed with an optically active cellulose toluoyl ester derivative. Preparatory columns used were: an Alltech (Deerfield, IL, USA) Econosphere silica gel column (250×10 mm, 10 μm) with a solvent system of hexane–ethyl acetate (85:15, v/v) at a flow-rate of 2.5 ml/min, and a Daicel Chiralcel OJ column (250×10 mm, J.T. Baker) with a solvent system of hexane–isopropanol (80:20, v/v) at a flow-rate of 3.0 ml/min. All figures illustrate elution profiles from 250×4.6 mm columns. HPLC analyses were performed at room temperature.

2.2. Reagents

Indenestrol A [1-methyl-2-(4-hydroxyphenyl)-3-ethyl-6-hydroxyindene], indenestrol B [1-ethyl-2-(4-hydroxyphenyl)-3-methyl-5-hydroxyindene] and the ethyl indenestrol A analog [1-ethyl-2-(4-hydroxy-phenyl)-3-ethyl-6-hydroxyindene] were obtained from ChemSyn Labs. (Lawrence, KS, USA). All other compounds were synthesized in our laboratory [10]. Compounds were dissolved in HPLC-grade ethanol and stored protected from light at −20°C. HPLC grade solvents (acetonitrile, water, hexane, isopropanol and methanol) were obtained from Fisher (Fairlawn, NJ, USA).

3. Results and discussion

HPLC elution profiles of propyl indenestrol A analog **4** using two different chiral columns were compared (Fig. 2A,B). The absolute configuration of each enantiomer was assigned to specific peaks, based on the similarities of their HPLC behavior, to the elution profiles of the IA enantiomers. The IA enantiomers were previously separated using a Chiralpak OP(+) column, and the X-ray crystal structure of the material from each fraction was carried out in order to assign absolute configuration [4]. The IA-*R* enantiomer eluted first from the chiral OP(+) column using a solvent system of methanol–water (85:15, v/v) [3]. Therefore, the first eluting peak on the chiral OP(+) column is presumably the *R* enantiomer of **4**, with the second peak assigned as the *S* enantiomer (Fig. 2A). Using the Chiralcel OD (Fig. 2B) column with a solvent system of hexane–isopropanol (90:10, v/v), the *S* enantiomer of **4** eluted first reversing the elution profile seen with the OP(+) column. Peak assignment of configuration

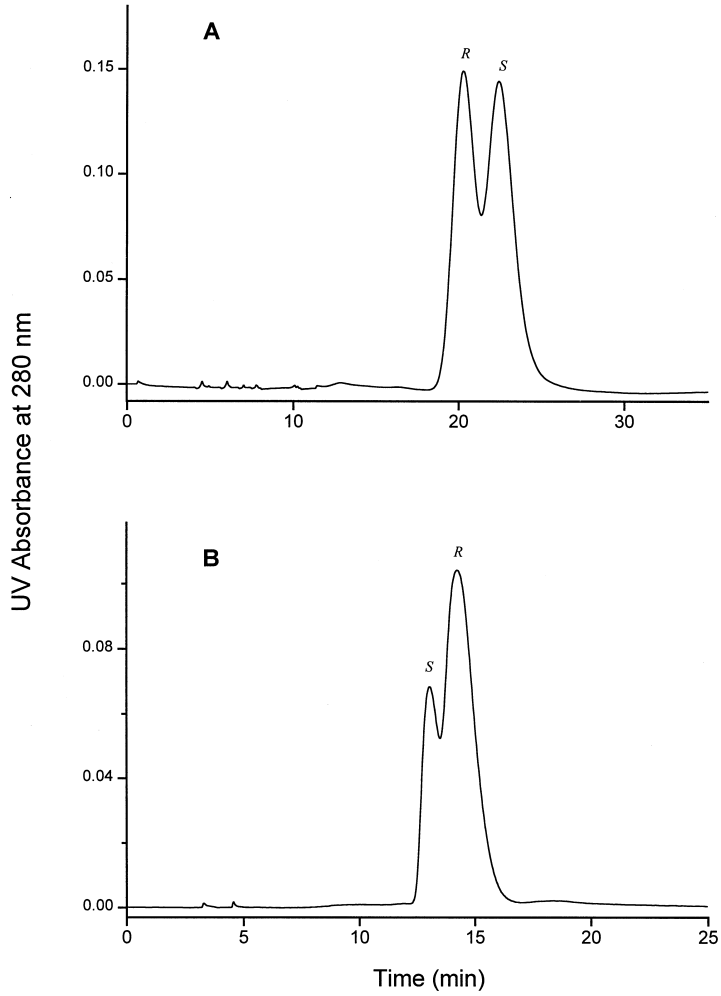

Fig. 2. Chiral HPLC elution profile of **4**. (A) Chiralcel OP(+) column with a solvent system of methanol–water (85:15, v/v) at a flow-rate of 0.5 ml/min. (B) Chiralcel OD column with a solvent system of hexane–isopropanol (90:10, v/v) at a flow-rate of 1.0 ml/min.

was determined by a comparison of the retention times of the purified enantiomers, whose configuration was determined by X-ray crystallography, with the retention times of the enantiomers as eluted from the OD column. The reversal of retention time was not unexpected since the OP(+) column is used under reversed-phase conditions and the OD column under normal-phase conditions. Both OP(+) and OD columns yielded poor resolution of the enantiomers of **4**.

When propyl analog **4** was analyzed by a silica gel column with hexane–isopropanol (90:10, v/v), it gave a single peak suggesting a homogeneous substance. However, further HPLC analysis on a silica gel column with a different solvent system, hexane–ethyl acetate (80:20, v/v), revealed two components with different UV spectra. The spectra of these components were compared with those of the known IA and IB, which are shown in Fig. 3. The spectrum of the later eluting peak corresponded with the spectrum of IA (λ_{max}=302 nm), while the spectrum of the early eluting peak corresponded with that of IB (λ_{max}=291 and 309). These observations suggested that isomerization of the indene double bond had occurred. A chiral column (Chiralcel OJ) was able to separate the two indene isomers of **4** (Fig. 4). The OJ column separated the **4** mixture into three overlapping peaks; the first eluting peak was a mixture of the *S* enantiomers of the A and B forms of **4** (as determined by ultraviolet spectra), and the slower eluting two peaks were a mixture of the *R* enantiomers of the A and B forms of **4**. The OP(+) and OD columns were unable to resolve the A and B isomers of **4** under the conditions utilized. The butyl analog **5** also existed as mixtures of A and B isomers. Isomerization of the double bonds of both **4** and **5** occurred during synthesis[1]. The stability of

[1]Isomerization occurred during the deprotection of the methoxy derivatives of the compounds to form the phenols using BBr_3 as the cleavage reagent.

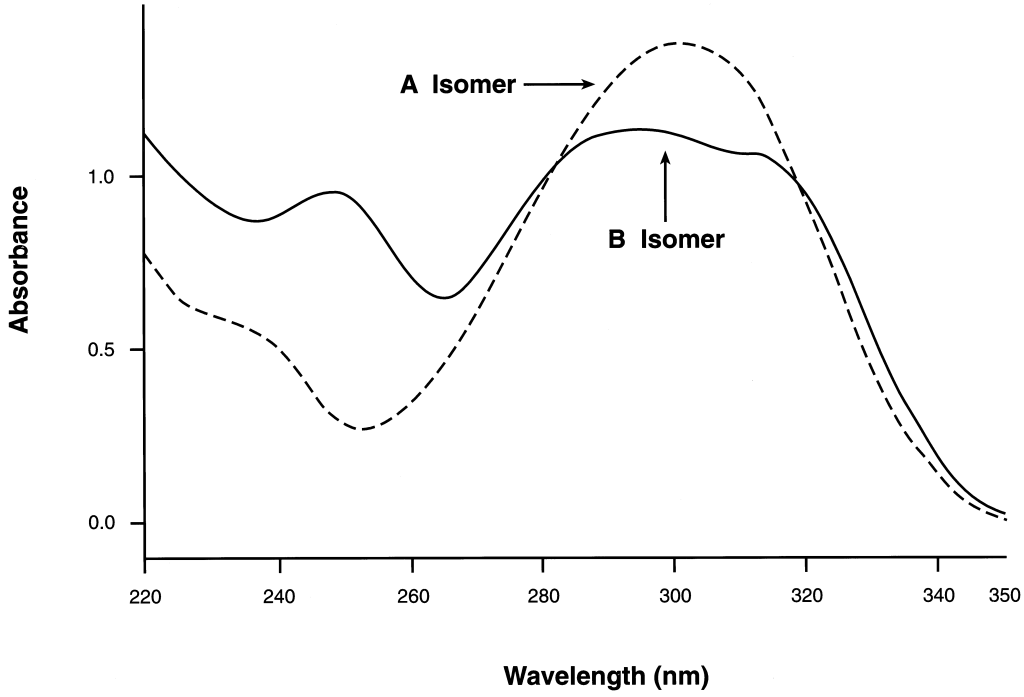

Fig. 3. Ultraviolet scans of indenestrol A (- - -) and B (———) were recorded from the diode array detector after each isomer was separated using a silica gel column with a solvent system of hexane–ethyl acetate (80:20, v/v) at a flow-rate of 1.5 ml/min.

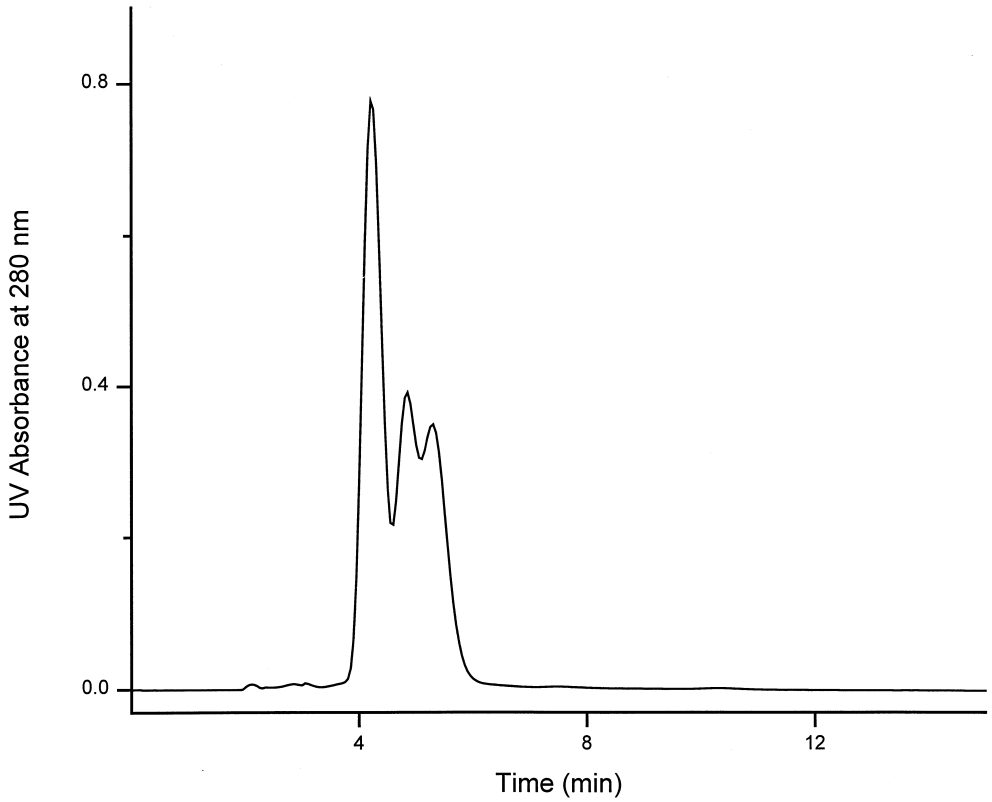

Fig. 4. HPLC elution profile of a mixture of A and B isomers of propyl analog **4** on a Chiralcel OJ column with a solvent system of hexane–isopropanol (80:20, v/v) at a flow-rate of 2.0 ml/min.

these compounds in ethanol solution was monitored by HPLC, which showed that no isomerization occurred during storage at −20°C. The A and B isomers of propyl analog **4** were separated by preparative HPLC with a silica gel column and a hexane–ethyl acetate solvent system, as shown in Fig. 5, and the isomers of butyl analog **5** were also separated in a manner similar to **4**.

Fig. 6 shows the chiral HPLC elution profiles and enantiomeric separations of the methyl, ethyl, propyl and butyl indenestrol A analogs using the OJ column. With this column, we observe that, as the length of the side chain increases from methyl to butyl, the retention time and enantiomeric resolution decrease. The absolute configuration of the indene A enantiomers was preliminarily assigned on the basis of the elution profile of IA enantiomers whose configurations have been determined by X-ray crystal analysis [4]. Using the OJ column, the *S* enantiomer eluted before the *R* enantiomer. Also identified in the chromatogram of the methyl IA analog is the presence of a small amount of the IB isomer, an artifact of the synthetic procedure. The structural assignment of the IB isomer is based upon retention time and UV spectrum. When enantiomeric resolution by the three chiral columns, OP(+), OD and OJ (Fig. 2A, Fig. 2B and Fig. 6, respectively), is compared, the best resolution is seen with the OJ column. Accordingly, the separation and purification of the propyl and butyl enantiomers were accomplished from multiple runs using the Chiralcel OJ column and a hexane–isopropanol solvent system.

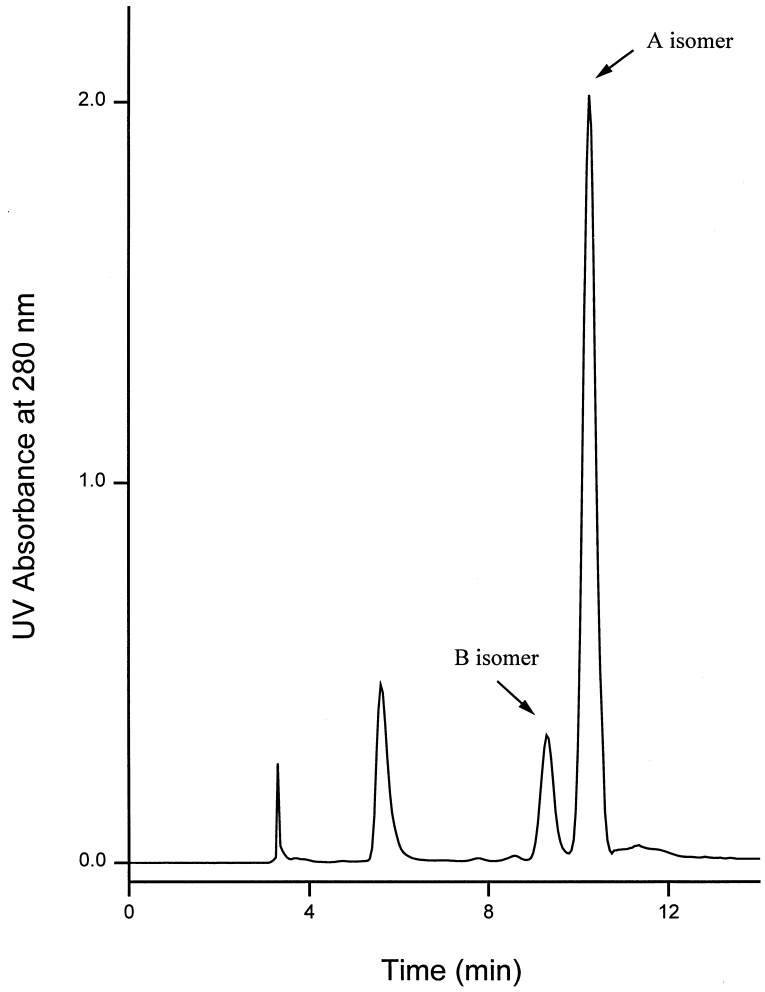

Fig. 5. HPLC profile of separation of propyl indenestrol A and B isomers using a silica gel column (250×4.6 mm) and a solvent system of hexane–ethyl acetate (80:20, v/v) at a flow-rate of 1.5 ml/min.

The absolute configuration of the enantiomers of the indene B isomers from the OJ column was assigned based on the elution profile of IB enantiomers whose configurations have been determined by X-ray crystal analysis [11].

Rechromatography of the purified individual enantiomers of both the A and B isomers of **4** from the preparatory chiral HPLC using the OJ column indicates that the propyl IA isomers are approximately >97% enantiomerically pure (Fig. 7A,B). Similar results were obtained for the butyl analog. The OJ column yielded better resolution of the enantiomers of the B isomer than the enantiomers of the A isomer of both compounds **4** and **5**.

4. Conclusions

HPLC analysis of indenestrol A analogs revealed the presence of their B isomers, as a result of the synthetic procedure. These isomers were separated

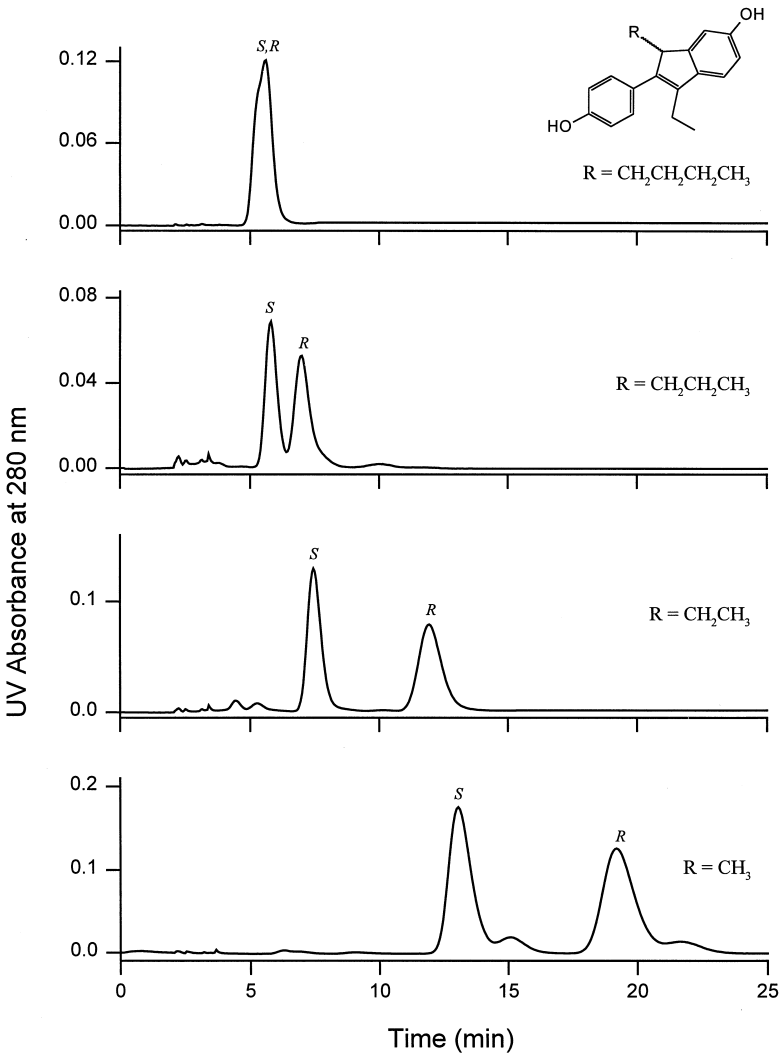

Fig. 6. HPLC of indenestrol A analogs on the Chiralcel OJ column using a solvent system of hexane–isopropanol (85:15, v/v) at a flow-rate of 2.0 ml/min.

by normal-phase HPLC using a silica gel column, while their enantiomers were separated via chiral HPLC with the Chiralcel OJ column yielding the best separation. A procedure to identify and purify the A and B isomers and their enantiomers is important because of the difference in their binding affinities for the estrogen receptor and subsequent biological activities.

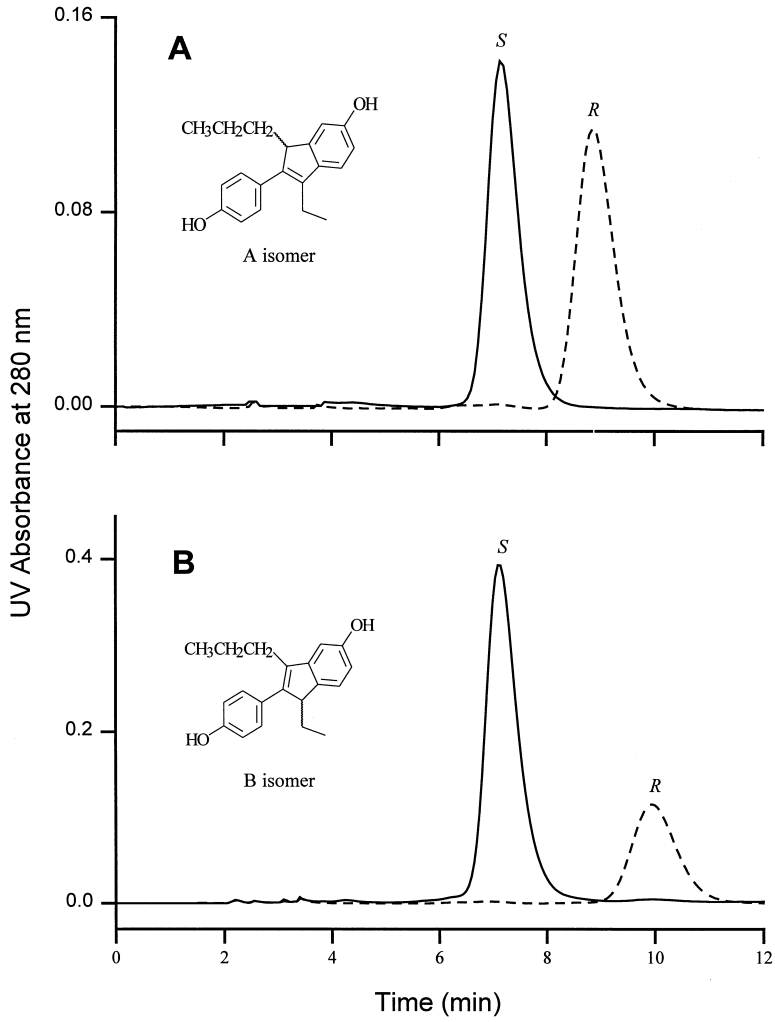

Fig. 7. HPLC elution profiles of purified *S* and *R* enantiomers of the (A) propyl indenestrol A isomers, and (B) propyl indenestrol B isomers, using the Chiralcel OJ column with hexane–isopropanol (85:15, v/v) at a flow-rate of 1.5 ml/min.

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