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High-performance liquid chromatographic methods for monitoring of isomers of 17-hydroxy-16-hydroxymethyl-3-methoxyestra-1,3,5(10)-triene

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Abstract

Claisen condensation and consecutive reduction of 3-methoxyestra-1,3,5(10)-trien-17-one theoretically leads to four diastereomers of 17-hydroxy-16-hydroxymethyl-3-methoxyestra-1,3,5(10)-triene and their further transformations give different compounds with different biological activities. High-performance liquid chromatographic methods were developed for separation of the four isomers of 17-hydroxy-16-hydroxymethyl-3-methoxyestra-1,3,5(10)-triene: reversed-phase separation on a Nucleosil ODS C_{18} column with water–methanol as mobile phase; and normal-phase separation on an APEX Silica column with hexane–dichloromethane–2-propanol as mobile phase. The effects of eluent composition and flow-rate on the separation were investigated. This is the first chromatographic evidence for the formation of the 16α,17α isomer in the reduction of 16-hydroxymethylene-3-methoxyestra-1,3,5(10)-trien-17-one.

Keywords: 17-Hydroxy-16-hydroxymethyl-3-methoxyestra-1,3,5(10)-triene; Steroids

1. Introduction

Most of the synthetic unnatural, but biologically active steroids have a great disadvantage: limited solubility in water, which impedes the absorbance of these substances in living organisms. The introduction of hydroxy groups onto the sterane skeleton increases the hydrophilic character and hence the solubility of the steroids in water. Earlier we described a method for the synthesis of 1,3-diol derivatives of 3-methoxyestra-1,3,5(10)-trien-17-one (Fig. 1) [1]. In this reaction sequence, 3-methoxyestra-1,3,5(10)-trien-17-one (**1**) was first converted to 16-hydroxymethylene-3-methoxyestra-1,3,5(10)-trien-17-one (**2**) in a Claisen condensation reaction. In the reduction of **2,** two new stereogenic centres are formed, and thus four diastereomers of 17-hydroxy-16-hydroxymethyl-3-methoxyestra-1,3,5(10)-triene (**3–6**) can theoretically be obtained. In the early stage of investigations of the reduction of the 16-acetoxymethylene derivative of **2,** only isomers **3** and **4** were isolated; the other two diastereomers (**5** and **6**) were synthesized from **3** and **4**, respectively [1,2]. In contrast with the above method, the reduction of **2** with $NaBH_4$ in a strongly alkaline medium yielded **3, 4** and **5** in different amounts. The 16α,17α isomer (**6**) was not even detected in the reaction mixture by thin layer and column chromatographic

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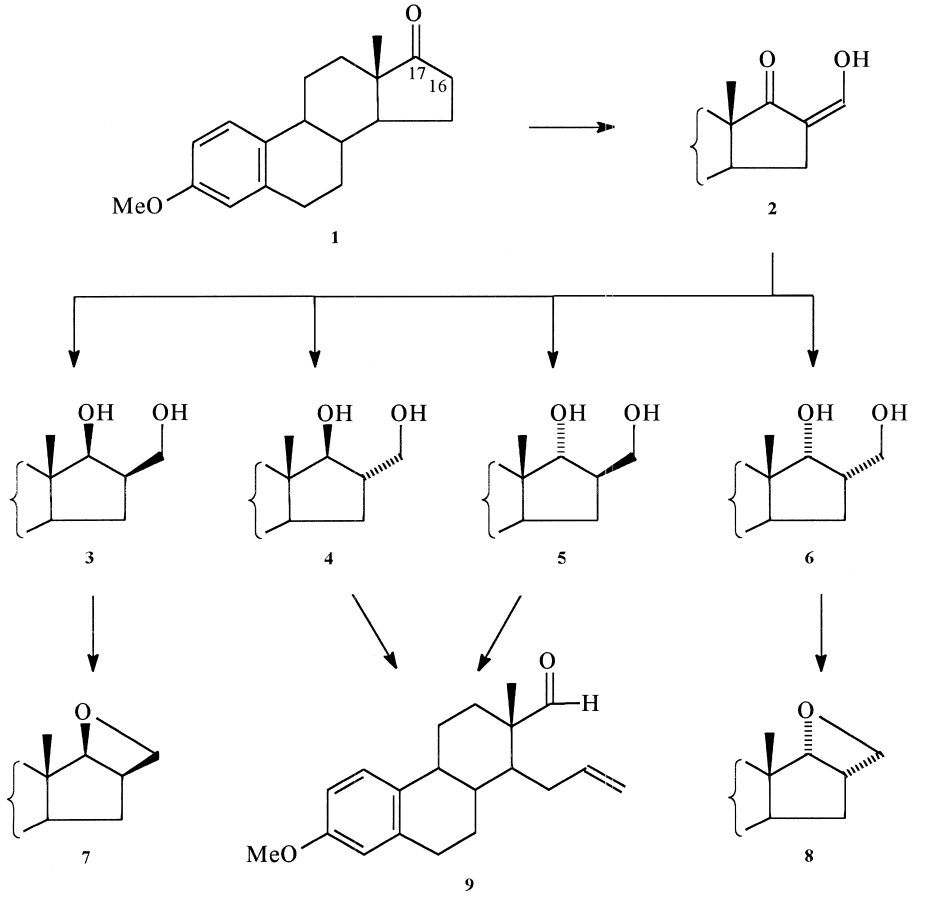

Fig. 1. Synthesis of the four diastereomers of 17-hydroxy-16-hydroxymethyl-3-methoxyestra-1,3,5(10)-triene (**3–6**) and some of their transformations.

methods. The 16-tosylates of the *cis* (**3**,**6**) and *trans* (**4**,**5**) isomers undergo different reactions on treatment with sodium methylate in methanol, and yield oxetanes **7** and **8**, and D-seco derivative **9,** respectively (Fig. 1) [3]. These compounds are key intermediates in several further transformations [4–11]. In in vitro tests, a D-homosteroid derivative of **9** with a spiroacetal moiety exhibited a strong anticancer activity against human cancer cells [10]. Since the transformations of the different diastereomers lead to different products and biological activities, it is clearly important to develop a more precise method for analysis of the reduction mixture.

Investigation of steroidal compounds by high-performance liquid chromatography (HPLC) began in the 1960s [12,13]. The HPLC methods have subsequently been widely used in several fields of steroid analysis. For a review of chromatographic methods, including HPLC, see Ref. [14] and the references cited therein. The main goal of the analyses is to determine the concentrations of clinically important steroids, such as natural hormones or synthetic derivatives [15–19]. The investigation of samples of biological origin requires a careful preliminary purification. Bowers recently reviewed the analytical advances in the detection of performance-enhancing compounds [20]. The new detection methods, e.g., MS, allow the detection limits to be lowered [20,21]. For the investigation of steroids containing an aromatic ring, such as estrone derivatives, UV detection offers an excellent possibility [15].

Systematic investigations regarding the retention behaviour of diastereomers of steroids were started by Heftmann and coworkers [22,23]. Other noteworthy publications relating to the HPLC of diastereomeric steroids include Refs. [24–27].

This paper describes the separation, identification and quantification of the diastereomers of 17-hydroxy-16-hydroxymethyl-3-methoxyestra-1,3,5(10)-triene by applying two HPLC methods: normal-phase (NP) chromatography was carried out on an APEX Silica column with hexane–dichloromethane (DCM)–2-propanol (IPA) as mobile phase, while reversed-phase (RP) chromatography was applied with a water–methanol eluent system on a Nucleosil ODS C_{18} stationary phase. The effects of the mobile phase composition and flow-rate on the separation were investigated.

2. Experimental

2.1. Chemicals and reagents

The pure diastereomers **3–6** were synthesized according to Refs. [1] and [2]. To obtain **3–6** in one step, 1 mmol 16-hydroxymethylene-3-methoxyestra-1,3,5(10)-trien-17-one (**2**) was reduced with 1 mmol $NaBH_4$ in 10 ml methanol solution at room temperature. After completion of the reaction the mixture was poured into water and neutralized with dilute hydrochloric acid, and the precipitate was filtered off, washed with water and dried. The dried sample was dissolved in eluent and before injection was filtered through on a 0.45 μm filter type HV (Millipore, Molsheim, France).

Acetonitrile, methanol, DCM, hexane, IPA and tetrahydrofuran (THF), all of HPLC grade, were purchased from Merck (Darmstadt, Germany). Eluents containing water were prepared with Milli-Q water and were further purified by filtration on a 0.45 μm filter Type HV (Millipore).

2.2. Apparatus

The HPLC system consisted of a PU-980 low-pressure gradient pump, equipped with an LG-980-02 Ternary Gradient Unit, a DG-980-50 3-Line Degasser and a UV-975 UV/VIS detector (Jasco, Tokyo, Japan). Data processing was performed on a Chromatography Station for Windows version 1.5 (Data Apex, Prague, Czech Republic). The injector was a Model 7725i (Rheodyne, Cotati, CA, USA) with a 20 μl loop.

The columns used were APEX Silica 250×4.6 mm I.D., 5 μm particle size, 100 Å pore size, and Nucleosil ODS 250×4.6 mm I.D., 5 μm particle size, 100 Å pore size (Jones Chromatography, Hengoed, Mid Glamorgan, UK).

3. Results and discussion

3.1. RP chromatography

The separations were carried out on a Nucleosil ODS C_{18} column with different water–organic modifier mixtures as mobile phases. The organic components of the mobile phases were methanol, acetonitrile or THF. Since compounds **3–6** have absorption maxima at 227 and 280 nm, while the mobile phase additives absorbs at a shorter wavelength, 280 nm was chosen as the detection wavelength. The results are summarized in Table 1 and Fig. 2.

It may be seen in Fig. 2 that the four isomers appeared in two blocks in the chromatogram: compounds **5** and **4** in the first block and compounds **6** and **3** in the second block. The two *trans* isomers eluted first, followed by the two *cis* isomers. The elution sequence was determined by cochromatography with authentic samples of the respective isomers. This elution sequence was typical on the Nucleosil ODS C_{18} column. The resolution between the two blocks was high ($R_{S;4,6}>5$) and with decreasing methanol content a further increase in R_S was observed. Results obtained in water–methanol mobile phases revealed that, with decreasing methanol content, both the retention factor and the resolution were increased (Table 1). Baseline resolution ($R_S>1.5$) could be achieved for isomers **6** and **3** in the mobile phases investigated, while for isomers **5** and **4** the resolution increased from 0.67 to 0.94 on decrease of the methanol content from 80% to 65%. A further decrease in methanol content resulted in a disadvantageously high retention time. On decrease of the flow-rate, an additional improvement in the resolution of the isomers **5** and **4** was observed. The

Table 1
Effects of experimental conditions on retention factor (k), separation factor (α) and resolution (R_S) in RP-HPLC[a]

Composition of eluent	k				$\alpha_{5,4}$	$\alpha_{6,3}$	$R_{S;5,4}$	$R_{S;6,3}$	Flow rate (ml/min)
	5	**4**	**6**	**3**					
H_2O–CH_3OH									
20:80	2.18	2.33	3.11	3.52	1.07	1.13	0.67	1.56	1.00
30:70	5.64	6.09	8.85	10.00	1.08	1.13	0.95	1.77	1.00
30:70	6.78	7.22	9.96	11.11	1.06	1.12	1.01	1.84	0.80
30:70	7.09	7.55	10.43	11.63	1.06	1.11	1.09	1.92	0.60
35:65	12.20	13.03	18.69	20.74	1.06	1.11	0.94	1.64	1.00
35:65	11.91	12.63	17.86	19.72	1.06	1.10	0.94	1.62	0.80
35:65	11.32	12.01	16.93	18.75	1.06	1.11	1.04	1.73	0.60
H_2O–CH_3OH–CH_3CN									
40:0:60	1.55	1.55	3.63	3.63	1.00	1.00	0.00	0.00	1.00
30:50:20	2.56	2.74	3.51	3.73	1.07	1.06	1.00	1.10	1.00
35:60:5	10.51	11.00	15.19	16.41	1.05	1.08	0.62	1.16	0.80
H_2O–CH_3OH–THF									
50:0:50	1.82	1.82	4.15	4.15	1.00	1.00	0.00	0.00	1.00
39:59:2	10.47	11.05	15.67	17.32	1.06	1.11	0.74	1.49	1.00
39:49:12	10.46	10.89	16.49	18.09	1.04	1.10	0.58	1.37	1.00

[a] Column: Nucleosil ODS; detection: 280 nm; mobile phase: water–methanol, water–acetonitrile, water–methanol–acetonitrile, water–tetrahydrofuran and water–methanol–tetrahydrofuran; $\alpha_{5,4}$ and $R_{S;5,4}$ represent the separation of isomers **5** and **4**; $\alpha_{6,3}$ and $R_{S;6,3}$ represent the separation of isomers **6** and **3**.

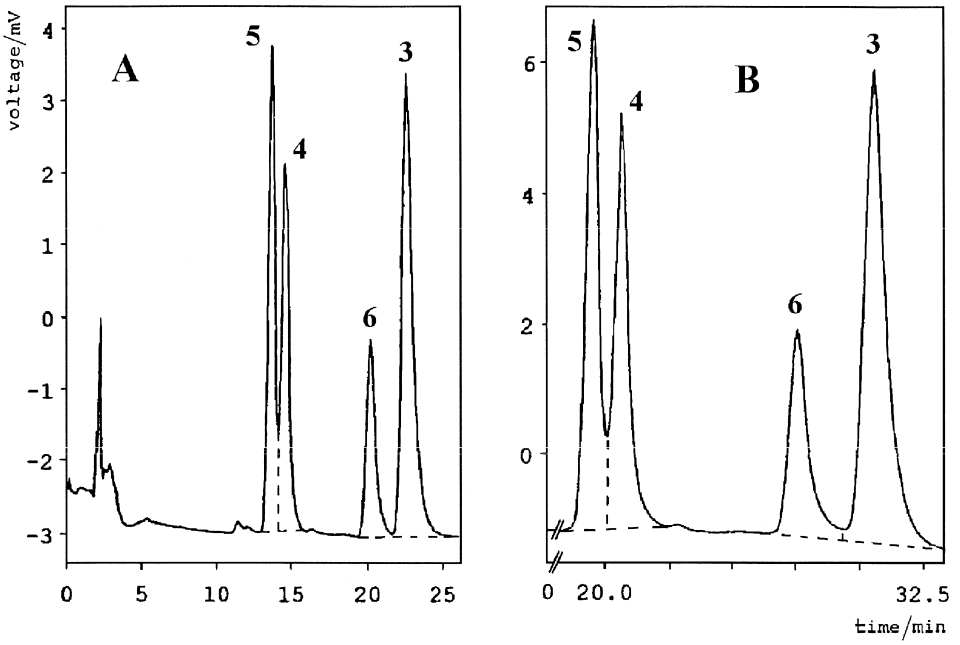

Fig. 2. Reversed-phase chromatograms of isomers **3–6**. Column, Nucleosil ODS; mobile phase, water–methanol, **A**,**B**, 30:70 (v/v); flow-rate, **A**, 1 ml/min, **B**, 0.8 ml/min; detection, 280 nm.

chromatogram of a reaction mixture is depicted in Fig. 4A (below). In the reaction mixture, diastereomer **6** has the lowest concentration and the resolution of isomers **6** and **3** in the RP case is best in the water–methanol mobile phase system. The detection of **6** was therefore excellent in the reaction mixture: less than 0.05% of **6** could be determined in the presence of a large excess of **3**.

Change of the organic modifier in the mobile phase from methanol to acetonitrile impaired the separation (Table 1). Any variation in acetonitrile concentration resulted in two peaks in the chromatograms instead of four. At low acetonitrile concentration (<30% v/v), the unresolved peaks started to separate, but the resolution remained far from that desired. In ternary systems containing water–methanol–acetonitrile, resolutions were poorer than that obtained in simple water–methanol (Table 1). Decrease of the flow-rate in the ternary systems resulted in band broadening and poorer R_S values.

The application of THF in the mobile phase gave results similar to those with acetonitrile (Table 1). In ternary systems containing 2–12% (v/v) THF, isomers **6** and **3** were separated well, while the resolution of **5** and **4** remained below $R_S<1$.

The precision of the procedure was examined by analysing ten replicate injections of isomer mixtures and comparing the peak areas. The relative standard deviations for the peak areas of the four isomers at the 10 μ*M* level were 1.9% (**3**), 2.1% (**4**), 1.3% (**5**) and 1.5% (**6**). The detection limit at a signal-to-noise ratio of 3 was 50 nM. The correlation coefficients for concentration versus response in the concentration range 0.25–50 μ*M* were $r^2>0.997$.

3.2. NP chromatography

Since the resolution of isomers **5** and **4** in the case of RP chromatography was critical, the NP method was applied to find conditions for an improved of separation of these isomers. The NP separation was carried out on an APEX Silica column. The apolar part of the mobile phase was hexane, while the polar part was chosen after preliminary experiments in which the four isomers were run in eluents with different polar additives. Polar organic compounds such as chloroform, carbon tetrachloride, DCM, ethanol, *n*-propanol and IPA were tested as mobile phase additives. The best results were obtained with mobile phases containing DCM and IPA as polar components in hexane. The ratio of DCM and IPA was optimized and results are presented in Table 2 and Fig. 3. The detection wavelength was the same as in the case of RP chromatography, i.e., 280 nm.

It may be seen in Fig. 3 that here too the four isomers appeared in two blocks in the chromatogram: compounds **6** and **3** in the first block and compounds **5** and **4** in the second block. The elution sequence was also determined by cochromatography with authentic isomers. This elution sequence was typical on the APEX Silica column and was different from that observed in the case of RP chromatography. Here the two *cis* isomers eluted first, followed by the two *trans* isomers. The resolution between the two blocks was higher than in the case of RP chromatography ($R_{S;3,5}>10$) and was practically unaffected by variation of the mobile phase composition. For NP chromatography, it is characteristic that, to achieve a resolution similar to that in the RP

Table 2
Effects of experimental conditions on retention factor (k), separation factor (α) and resolution (R_S) in NP-HPLC[a]

Composition of eluent Hexane–DCM–IPA	k				$\alpha_{6,3}$	$\alpha_{5,4}$	$R_{S;6,3}$	$R_{S;5,4}$	Flow rate (ml/min)
	6	**3**	**5**	**4**					
87:5:8	2.13	2.27	4.29	4.57	1.06	1.06	0.98	1.14	1.00
82:10:8	2.19	2.33	4.43	4.73	1.06	1.07	1.13	1.38	1.00
77:15:8	2.22	2.35	4.54	4.85	1.06	1.07	1.18	1.42	1.00
84:10:6	2.84	3.05	6.41	6.72	1.08	1.05	1.29	1.00	1.00
87:10:3	5.50	5.98	17.94	18.48	1.09	1.03	1.49	0.53	1.00
82:10:8	2.37	2.51	4.58	4.88	1.06	1.07	1.16	1.34	0.80
82:10:8	2.57	2.70	4.74	5.01	1.05	1.06	1.15	1.45	0.60

[a] Column: APEX Silica; detection: 280 nm; mobile phase: hexane–dichloromethane–2-propanol; $\alpha_{6,3}$ and $R_{S;6,3}$ represent the separation of isomers **6** and **3**; $\alpha_{5,4}$ and $R_{S;5,4}$ represent the separation of isomers **5** and **4**.

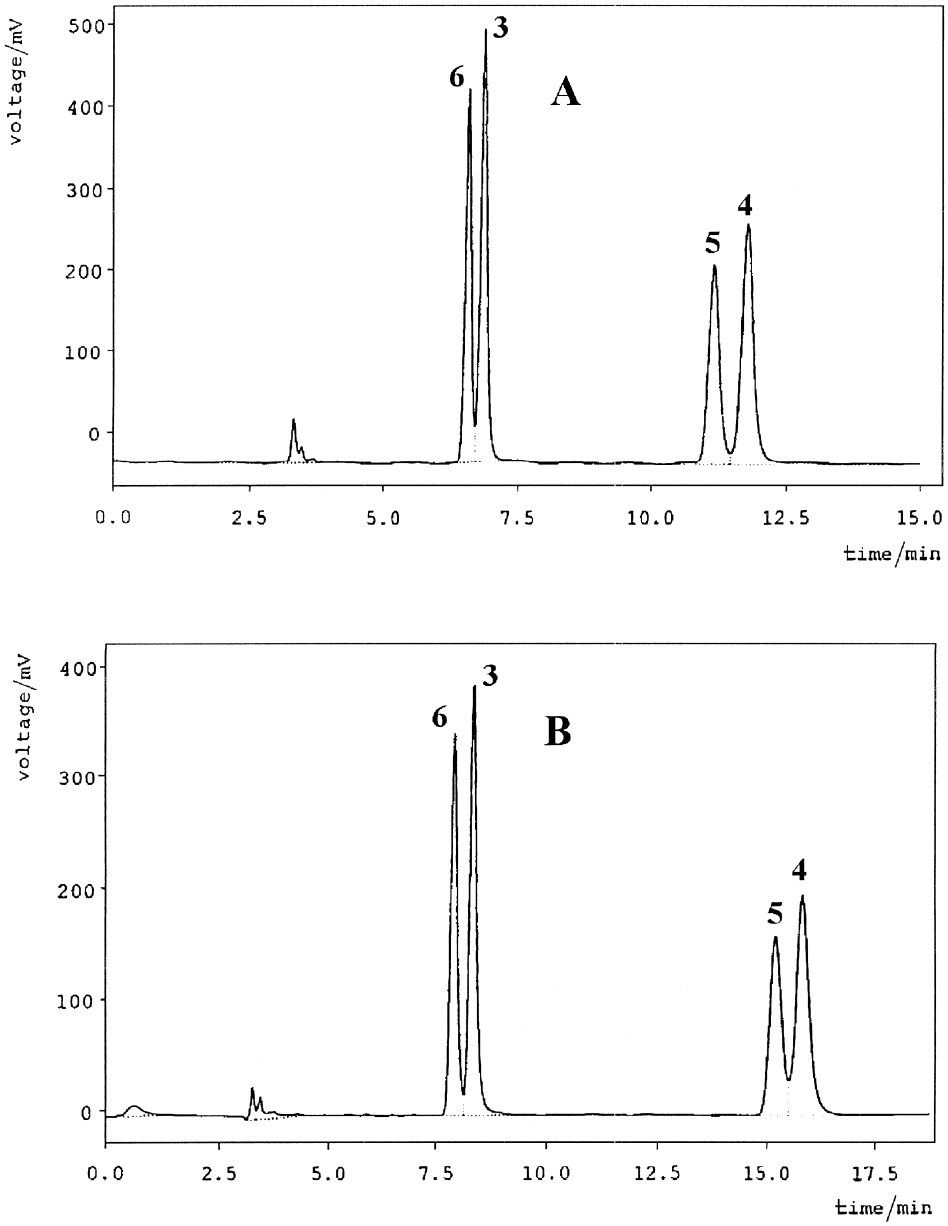

Fig. 3. Normal-phase chromatograms of isomers **3–6** Column, APEX Silica; mobile phase, hexane–dichloromethane–2-propanol, **A**, 82:10:8 (v/v/v), **B**, 84:10:6 (v/v/v); flow-rate, 1 ml/min; detection, 280 nm.

case, a lower retention time is required. Isomers **6** and **3,** and **5** and **4** sometimes underwent only partial separation, and the separation should therefore be optimized. For optimization, the ratio of the polar components in the eluent was varied. Variation of the DCM concentration had little effect on the retention, but the resolution of isomers **6** and **3**, and **5** and **4** improved with increasing DCM concentration (Table 2). The optimal amount of DCM was found to be about 10% (v/v). At a mobile phase composition hexane–DCM–IPA (82:10:8 v/v/v), the first pair of isomers displayed a partial separation ($R_{S;6,3}\approx 1.1$), while the second pair of isomers exhibited almost the baseline separation ($R_{S;5,4}\approx 1.4$). The amount of IPA

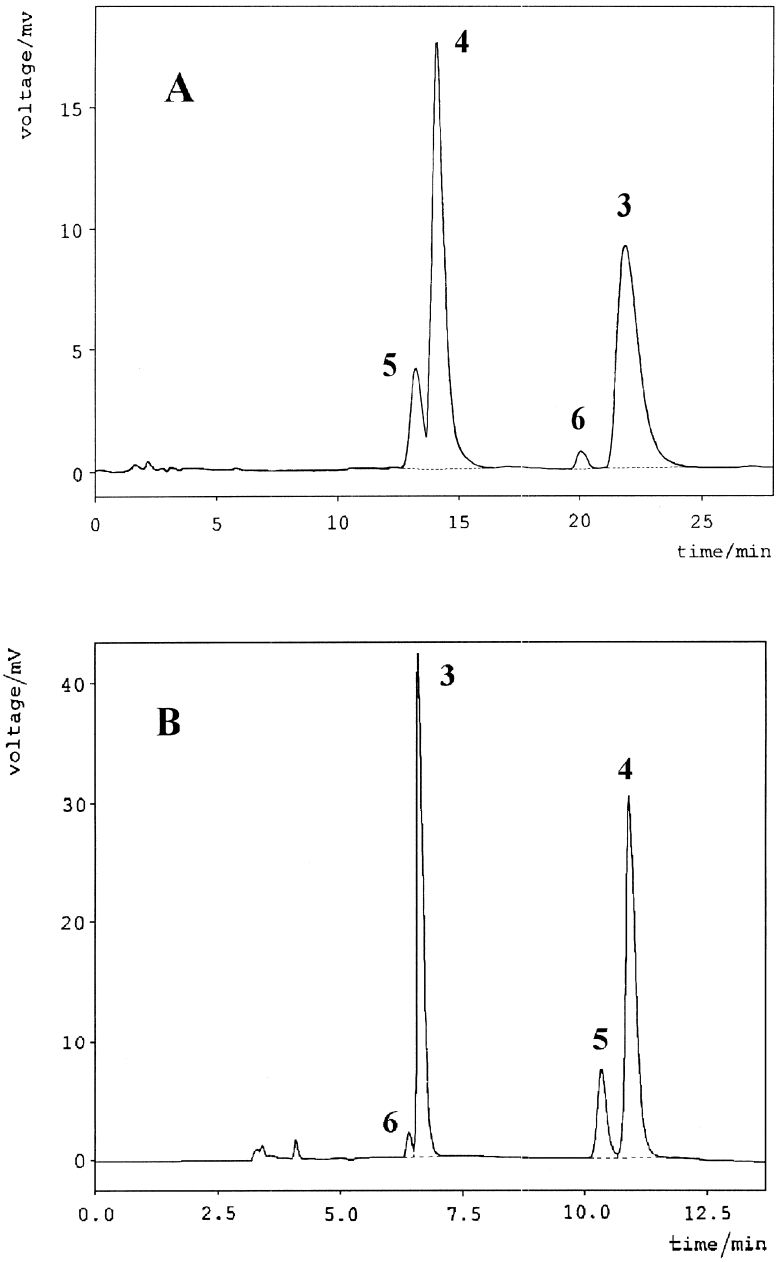

Fig. 4. Normal- and reversed-phase chromatograms of an actual reaction mixture. Column, (A), Nucleosil ODS, (B), APEX Silica; mobile phase, (A), water–methanol, 30:70 (v/v), (B), hexane–dichloromethane–2-propanol, 82:10:8 (v/v/v); flow-rate, 1 ml/min; detection, 280 nm.

in the mobile phase had a determining effect on the resolution. A higher IPA content was advantageous for the separation of isomers **5** and **4,** and simultaneously the total analysis time was below 15 min. A lower IPA content was favourable for the resolution of isomers **6** and **3,** but it was associated with a poor resolution of **5** and **4** and with a longer analysis time. Decrease of the flow-rate improved the resolution, but $R_{S;5,4}$ remained below the optimal $R_S = 1.5$ and the higher time of analysis was dis-

advantageous. The chromatogram of an actual reaction mixture is depicted in Fig. 4B. Since the elution sequence is **6** and **3** and isomer **6** is formed in the lowest concentration, application of a higher IPA content was advantageous for the separation of both pairs of isomers (**6** and **3**, and **5** and **4**). By this method, less than 0.1% of **6** can be determined in the presence of a large excess of the other isomers.

The precision of the procedure was determined in a similar way as in the case of RP chromatography. The relative standard deviations for the peak areas of the four isomers at the 10 μ*M* level were 3.9% (**3**), 6.5% (**4**), 3.6% (**5**) and 5.3% (**6**), which is somewhat higher than in the case of RP chromatography. The detection limit at a signal-to-noise ratio of 3:1 was 500 n*M*, which is also higher than in the RP case and depends somewhat on the impurities in the solvents and the sample matrix. The correlation coefficients for concentration versus response for all four isomers in the concentration range 2.5–150 μ*M* were $r^2 > 0.996$.

4. Conclusions

The described procedures can be applied for the separation and quantification of the four isomers of 17 - hydroxy - 16 - hydroxymethyl - 3 - methoxyestra-1,3,5(10)-triene. By means of NP chromatography, good separation was achieved for the four diastereomers with hexane–DCM–IPA mobile phase systems. On RP-HPLC, isomers **6** and **3** were separated well, while the resolution of **5** and **4** isomers did not exceed $R_S \approx 1.1$ in the water–methanol eluent system. The application of acetonitrile or THF as mobile phase additives did not lead to the desired resolution of the diastereomers. This is the first chromatographic evidence for the formation of the 16α,17α isomer in the reduction of 16-hydroxymethylene-3-methoxyestra-1,3,5(10)-trien-17-one.

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