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Retention behavior of synthetic corticosteroids in packed-column supercritical fluid chromatography

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Abstract

The retention behavior of the synthetic corticosteroids used in a therapy was investigated using packed-column supercritical fluid chromatography (SFC) modified from a commercial liquid chromatographic system. The influence of the stationary phase, modifiers, column pressure and temperature was studied systematically. An aminopropyl-bound silica column and carbon dioxide modified with methanol were selected. The selectivity and separation efficiency in the packed-column SFC were superior to those in the existing normal- and reversed-phase liquid chromatographic conditions. Seven polar corticosteroids, possessing 1 to 4 hydroxyl groups, showed baseline separation within 6.5 min on a modifier gradient.

Keywords: Optimization; Retention behavior; Corticosteroids; Steroids

1. Introduction

Supercritical fluid chromatography (SFC) has been used as a powerful separation technique complementing or being superior to gas chromatography (GC) and liquid chromatography (HPLC) due to the use of a mobile phase which shows a low viscosity, a high diffusion coefficient and a solvating power. Especially packed-column SFC using binary or ternary mobile phases has been applied to various kinds of polar substances such as a drugs [1–10] and it was shown to be superior to HPLC with respect to the analysis time and selectivity [8–10]. To our knowledge, most studies have been done using commercial instruments. However, packed-column SFC, with the same performance as the commercial ones, is easily available in every laboratory by carrying out simple modifications to the existing HPLC method.

Synthetic corticosteroids are widely used therapeutically for the suppression of adrenocortical functions, inflammatory and allergic diseases and are important drugs in medical treatments. In order to modify the efficacy and suppress adverse reactions, a lot of corticosteroids have been synthesized. Thin-layer, normal- or reversed-phase chromatography have been used for the analysis of these corticosteroids [11]. For a number of the synthetic corticosteroids used in therapy, very little work has been carried out in packed-column SFC.

In this paper, synthetic corticosteroids possessing 1 to 4 hydroxyl groups were selected as test sub-

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stances and the effects of several parameters (i.e. modifiers, columns, pressure and temperature) on the retention were investigated systematically, and the retention in packed-column SFC was compared with that in normal- and reversed-phase HPLC. In addition, the separation was investigated using the modifier gradient. The purpose of this work is to study the application of packed-column SFC to the analysis of a wide range of polar synthetic corticosteroids.

2. Experimental

2.1. Instrumentation

A high-performance liquid chromatograph was modified for SFC operation. To facilitate introduction of the mobile phase as liquid, the carbon dioxide from the cylinder with dip tube was cooled by passing through a 1.6-mm diameter stainless steel tube in the coolant, kept at −10°C. A single plunger reciprocating pump LC-6A (Shimadzu, Kyoto, Japan) was used for delivering CO_2, and the pump head was also cooled to −10°C by circulating the coolant. A double plunger reciprocating pump LC-9A (Shimadzu) was used for delivering a modifier. Carbon dioxide and modifier were mixed by dynamic mixer MX-8010 (max. pressure: 400 kg/cm^2, volume of chamber: 1.9 ml, Tosoh, Osaka, Japan). Samples were introduced onto the column via a Rheodyne 8125 injector fitted with a 5-μl sample loop (Rheodyne, Cotati, CA, USA). The column was kept in an oven CTO-6A (Shimadzu) at a constant temperature. An SPD-6A photometric detector (Shimadzu) was used, which was equipped with a high-pressure flow cell (max. pressure: 400 kg/cm^2, cell inner volume: 3 μl). The wavelength monitored was 254 nm. To maintain supercritical conditions in the column, a Tescom backpressure regulator Model 26-1722-24-043 (Tescom Instruments, Elk River, MI, USA) was connected to the outlet of the flow cell. To prevent clogging with solid carbon dioxide, the regulator was kept at ca. 40°C on the dry-thermo-unit TAL-1G (Taitec, Osaka, Japan). The pressures at inlet and outlet of the column were monitored using pressure monitors LC-6AD (Shimadzu) equipped with a strain gauge between the dynamic mixer and the injector, and between the detector and the regulator, respectively. The chromatographic signal was recorded and processed by an Chromatopak C-R5A integrator (Shimadzu).

The mobile fluid was always fed in a constant-flow delivery mode all through this study. Since the pressure was controlled by the backpressure regulator, the inlet flow-rate could be selected completely independently from the pressure. The modifier could be mixed volumetrically with the carbon dioxide by control of the pumping rate. The system could be operated in a gradient elution mode by programming the flow-rate of the modifier.

The LC-6A pump, CTO-6A oven, SPD-6A detector and Rheodyne Model 7125 injector fitted with 20-μl sample loop were used in both normal- and reversed-phase HPLC modes.

2.2. Columns

The following columns were commercially available: Cosmosil 5NH_2 (150×4.6-mm I.D., Nacalai Tesque, Kyoto, Japan) modified with aminopropyl, Ultoron VX-SIL (150×4.6-mm I.D., Shimadzu GLC center, Osaka, Japan), Inertsil ODS-2 (150×4.6-mm I.D., GL science, Osaka, Japan) and Zorbax phenyl (250×4.6 mm-I.D., Shimadzu GLC center, Osaka, Japan). The particle size was 5 μm.

2.3. Chemicals and reagents

The liquid carbon dioxide (99.9%) was purchased from Kyoritu Shoji (Osaka, Japan). Dexamethasone acetate, triamcinolone acetonide, hydrocortisone, betamethasone and triamcinolone were obtained from Sigma, and fluocinonide and fluocinolone acetonide were obtained from the National Institute of Hygienic Science (Tokyo, Japan), and were used as the test substances. The structures of the corticosteroids used are shown in Fig. 1. All other solvents, of analytical-grade or liquid chromatography-grade, were obtained from Katayama Kagaku (Osaka, Japan).

2.4. Methods

Corticosteroids were dissolved in methanol at ca. 400 μg/ml before chromatographic operation. The hold-up time of the columns was measured from the

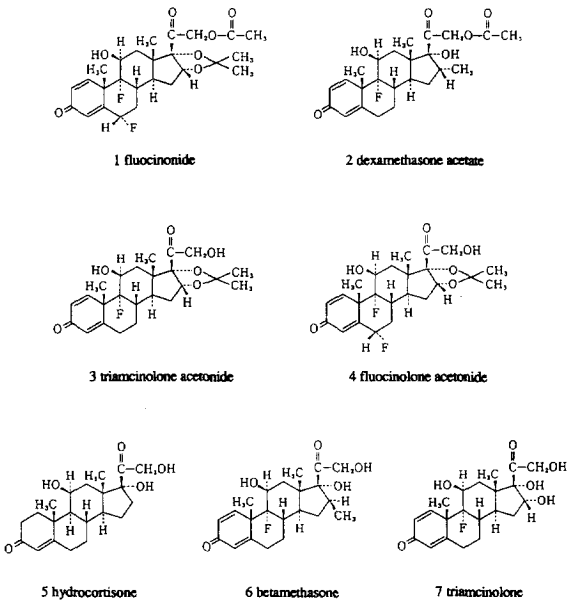

Fig. 1. Structures and symbols of synthetic corticosteroids.

point of injection to the top of the negative peak caused by methanol.

In normal-phase liquid chromatography (NP–HPLC), the mobile phase composed of water-saturated chloroform–methanol–glacial acetic acid (200:3:2), was prepared according to the monograph of fluocinolone acetonide in the Pharmacopoeia of Japan 13th edition. The separation was performed on Ultron VX-SIL packed column and at 30°C. Each corticosteroid was dissolved in the mobile phase at ca. 100 μg/ml.

In reversed-phase liquid chromatography (RP–HPLC), an aqueous mobile phase containing 55% methanol or 40% acetonitrile was selected, which showed the appropriate retention of each solute. The separation was performed on Inertsil ODS-2 and at 40°C. Each corticosteroid was dissolved in at ca. 100 μg/ml in 50% methanol.

All data were obtained in duplicate.

2.5. Calculation of dipole moments

The dipole moments of 4 corticosteroids, i.e. triamcinolone acetonide, fluocinolone acetonide, hydrocortisone and betamethasone, were calculated as described below. At first, the three-dimensional molecular structures of these molecules were determined by modifying the absolute structure of fluocinonide which could be obtained from the Cambridge Structural Database System (Cambridge Crystallographic Centre, UK). Then the conformation energies of the obtained structures were minimized by molecular mechanics calculations (MM2). The dipole moments of each molecule were calculated by molecular orbital calculation (MOPAC, PM3) based on minimized structures of MM2 calculation.

3. Results and discussion

3.1. Selection of stationary phase

Corticosteroids were separated on some stationary phases of different polarity. As shown in Fig. 2, the aminopropyl column exhibited the best selectivity and peak shape with a reasonable retention time in comparison with the others. ODS and phenyl columns showed poor separation, the former did not retain any solutes (data not shown) and the latter did not separate under the operating conditions used. On the silica support, the solutes showed appropriate retention but poor separation and peak shape. Although the retention times of test substances 3, 4, 5 and 6 were almost the same as those on the aminopropyl column, the separation factor, α, of the two pairs – steroids possessing two hydroxyl groups, and steroids possessing three hydroxyl groups – decreased remarkably on silica. A reversed elution order, however, was observed on the silica, which showed that the changing of the selectivity of retention is possible by selection of the stationary phase.

3.2. Effect and optimization of modifier

No corticosteroids were eluted from the column packed with Cosmosil $5NH_2$ with pure carbon dioxide as the mobile phase because of the low polarity of the supercritical carbon dioxide. Therefore a modifier had to be added to the mobile phase. The effect of modifiers with different polarities on the retention of corticosteroids was examined as shown in Fig. 3. The addition of 11.8% (v/v)

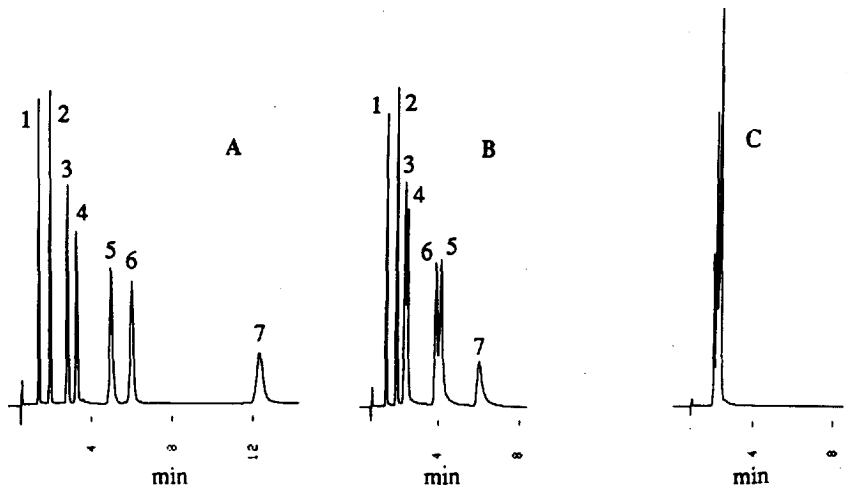

Fig. 2. Effect of column on retention of corticosteroids. A: Cosmosil 5NH_2, B: Ultron VX-SIL, C: Zorbax phenyl. Operating conditions: mean pressure 213 kg/cm^2, flow-rate of CO_2 3 ml/min, flow-rate of methanol 0.4 ml/min, temperature 40°C. Peaks: as in Fig. 1.

methanol to carbon dioxide showed the best resolution and symmetric peak shapes within 14 min. In comparison with the addition of 99.5% ethanol, that of 95% ethanol reduced the resolutions but improved the peak shape of the most polar triamcinolone in the test substances remarkably. This should be attributed to deactivation of the active sites on the silica support by the water [12].

In packed-column SFC, the addition of a modifier to a mobile phase should be considered from the viewpoint of its effect either on the stationary phase or on the mobile phase. Berger et al. [13] studied the effect of column and mobile phase polarity using steroids. They concluded that polar modifiers tended to decrease the intensity of the solute–silanol interaction and the more polar stationary phases produced greater retention, requiring the use of modifiers to obtain a reasonable retention time. Blilie and Greibrokk [14] described that the modifiers functioned as deactivation agents by direct interactions with residual silanol groups and also as modifiers of the eluting power of the mobile phase. Janssen et al. [15] confirmed that the effect of a few percent of modifier in packed-column SFC was largely deactivation of residual silanol groups on the silica support and the accessibility to the active sites was found to depend strongly on the size and structure of the modifier molecules. According to Janssen et al. [15] almost the same volume percentage of tetrahydrofuran (THF) and methanol was needed to cover 95% of the surface. No corticosteroid was eluted under these conditions when methanol was replaced with THF (data not shown); this suggests that the effect of the modifier on retention of corticosteroids consists in the enhancement of the solvent strength of the mobile phase rather than the deactivation of the active sites on the silica support.

The effect of the methanol concentration in the range of 9.1 to 16.7% (v/v) was investigated. The capacity factor of any corticosteroid decreased 2- to 4-fold with an 1.8-fold increase in methanol concentrations. All solutes were eluted within 5 min using carbon dioxide modified with 16.7% (v/v) and the resolutions among them were more than 1.6.

The repeatabilities of the retention time, capacity factor and the area of each solute were determined by repeated injections. Calculated relative standard deviations (R.S.D.) of 0.35–0.70% for t_R, 0.82–1.47% for k' and 0.50 to 1.34% for peak area were obtained (Table 1).

3.3. Effect of pressure

The effect of pressure on the retention of 7 corticosteroids was studied. The capacity factor of each solute decreased by a factor of two with an increase in the range of 107 to 223 kg/cm^2. A few researchers measured the densities of modified super-

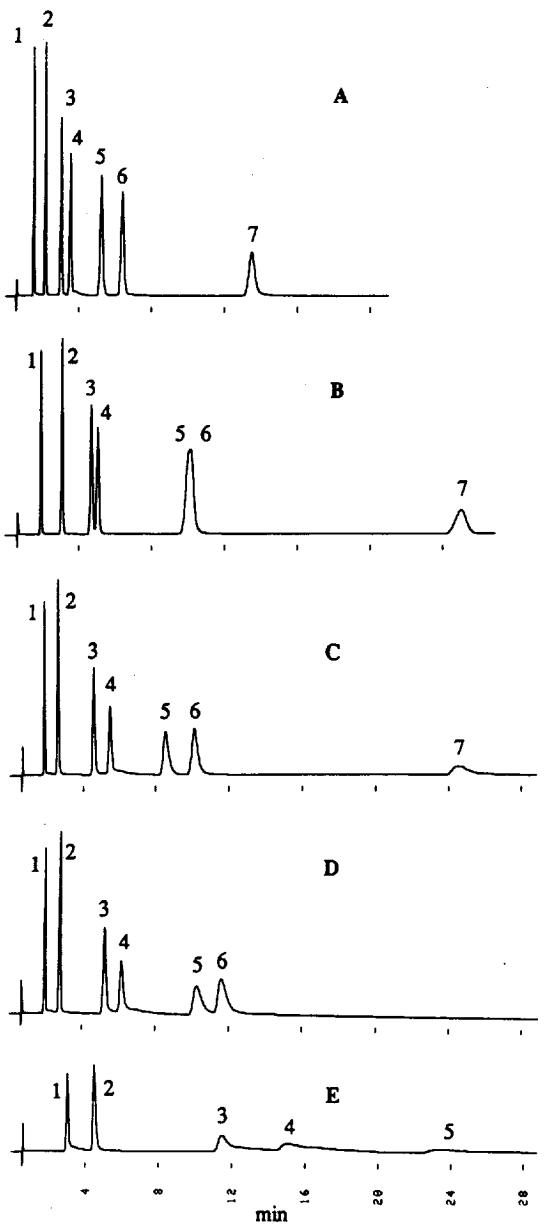

Fig. 3. Effect of modifiers on retention of corticosteroids. A: methanol, B: ethanol (95%), C: ethanol (99.5%), D: 1-propanol, E: 2-propanol. Operating conditions: column Cosmosil 5NH_2, inlet pressure 224 kg/cm^2, outlet pressure 191 kg/cm^2, flow-rate of CO_2 3 ml/min, flow-rate of modifier 0.4 ml/min, temperature 40°C, Peaks: as in Fig. 1.

critical fluids experimentally [16,17]. Berger [16] measured the density of binary fluids using an 'U' tube densitometer and drew constant density lines in

Table 1
Repeatability (R.S.D.%, $n=6$)

Corticosteroids	t_R (min)	k'	Peak area
Fluocinonide	0.37	1.15	1.01
Dexamethasone acetate	0.35	1.10	0.75
Triamcinolone acetonide	0.49	1.39	1.08
Fluocinolone acetonide	0.60	1.47	1.34
Hydrocortisone	0.64	1.39	0.67
Betamethasone	0.70	1.39	1.09
Triamcinolone	0.45	0.82	0.50

Operating conditions as in Fig. 3.

plots of the pressure against the composition for the methanol–carbon dioxide system at three temperatures. The densities of CO_2–methanol (12%, v/v) at different pressures used were calculated by extrapolation of the lines in the pressure range from 105 to 180 bar. The plots of ln k' against the evaluated binary fluids density revealed that there is a linear relationship between them in the packed-column SFC using the modified mobile phase, as expected.

The theoretical plate numbers (N) of solutes were calculated on each pressure. Except for fluocinonide, N values reached the maximum values at 126 and 144 kg/cm^2 as shown in Fig. 4. The maximum N values were ca. 4700–9800. Corresponding to the behavior of N values, the resolutions between the adjacent solutes also showed a maximum at 126–162

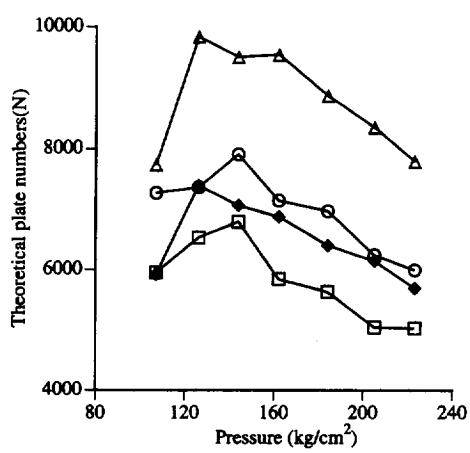

Fig. 4. Relationship between theoretical plate numbers and pressure. Operating conditions: mean pressure 107, 126, 144, 162, 184, 205 and 223 kg/cm^2, other conditions as in Fig. 3, Symbols: (□) hydrocortisone, (♦) fluocinolone acetonide, (○) betamethasone, (△) triamcinolone.

kg/cm^2. Since the mass flow-rate was kept constant, the linear velocity varied with pressure. The minimum plate height was obtained in this pressure range. These results reveal that the pressure is one of the significant parameters optimizing the operating conditions.

3.4. Effect of temperature

The effect of a temperature on the retention of corticosteroids at a constant pressure was investigated. The retention of the solutes increased with an increase in temperature (decrease in density). On the other hand, the N values of each solute, as shown in Fig. 5, increased with temperature and reached maximum values at 39 or 49°C except for hydrocortisone. Especially, the maximum N value for triamcinolone was ca. 8400 at 39°C but only ca. 3200 at 58°C, corresponding to about a 60% decrease. Although little variation of the separation factor (α), of any couple of the neighboring solutes, was observed over the wide range of the temperatures measured, the resolutions reached maximum values at 39–49°C corresponding to the behavior of the N values. The critical temperature and pressure were reported to be 36.85°C and 80 bar and 50°C and 95 bar for 2% methanol and 16% methanol in carbon dioxide, respectively [16]. So, the critical temperature for 12% methanol in carbon dioxide, which was used as mobile phase by the authors, can be assumed to be in the range of 40 to 50°C; the maximum N and resolution were obtained around the critical temperature.

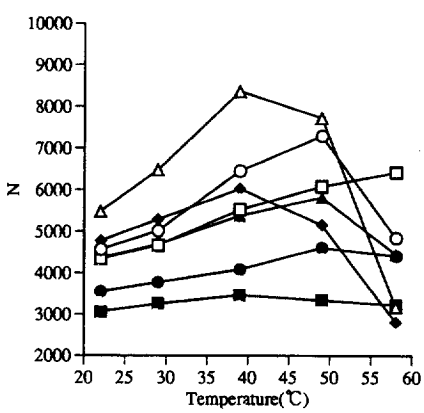

Fig. 5. Relationship between theoretical plate numbers and temperature. Operating conditions: temperature 22, 29, 39, 49 and 58°C, mean pressure 213 kg/cm^2, other conditions as in Fig. 4, Symbols: (■) fluocinonide, (●) dexamethasone acetate, (▲) triamcinolone acetonide, (♦) fluocinolone acetonide, (□) hydrocortisone, (○) betamethasone, (△) triamcinolone.

3.5. Separation with modifier gradient

A wide range of polar corticosreroids was separated in a modifier gradient elution mode. As shown in Fig. 6, all solutes were eluted within 6.5 min by increasing the methanol content from 11.8% (v/v) to 17.0% (v/v) at 0.52% (v/v) per min and keeping the CO_2 flow-rate constant. Good peak shapes, completely baseline separated, were observed. The stable

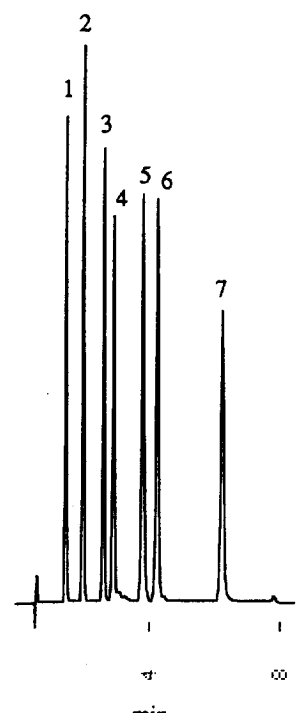

Fig. 6. Gradient elution of corticosteroids. Operating conditions: column Cosmosil 5NH$_2$, flow-rate of CO_2 3 ml/min, methanol gradient 11.8–17.0% (v/v) at 0.52% (v/v)/min, mean pressure 206 kg/cm^2, temperature 40°C, Peaks: as in Fig. 1.

baseline without drift and noise is considered to be due to the good mixing process of the binary fluid.

3.6. Comparison of NP- and RP-HPLC

The retention of the corticosteroids, possessing a wide range of polarities, in packed-column SFC using an aminopropyl silica column was compared with that in NP–HPLC and RP–HPLC. The observed chromatograms are shown in Fig. 7. Since the most polar among the substances, triamcinolone, was not dissolved in the mobile phase, it could not be eluted in the NP mode. The elution order in SFC was the same as that in NP–HPLC, as expected. It was determined mainly by the number of hydroxyl groups in the solutes, as follows: at first compound 1 with a single OH group, subsequently compounds 2,3 and 4 with two OH groups, then compounds 5 and 6 with three OH groups and at last compound 7 with four OH groups. Corticosteroids were eluted almost in reversed order in RP–HPLC but it is noteworthy that the pairs of compounds 3 and 4 and 5 and 6 were eluted in the same order as in SFC. The elution order of these compounds with the same number of OH groups seems to be closely related to their dipole moment. The dipole moments of 4 corticosteroids were estimated based on a molecular orbital method (MOPAC); 1.19 and 2.04 debye for compound 3 and 4, respectively, and 0.52 and 2.24 debye for compound 5 and 6, respectively.

The same range of N values was obtained in each chromatography, i.e. ca. 3600–8000 in packed-column SFC, ca. 4800–8700 in NP–HPLC and ca. 2300–11 000 in RP–HPLC. The separation of compounds 3 and 4, which showed the lowest resolution in the solutes and the same elution order in the normal- and reversed-phase systems, were comparable. The resolutions of these solutes were 2.73 in packed-column SFC, 2.04 in NP–HPLC, 0.53 in RP–HPLC (methanol mixture, mobile phase) and 2.20 in RP–HPLC (acetonitrile mixture, mobile phase). In addition, the elution time of all solutes in packed-column SFC was about 4 times faster than in NP–HPLC and about 1.5 times faster than in RP–HPLC.

These results suggest that the packed-column SFC conditions used give a higher selectivity and better

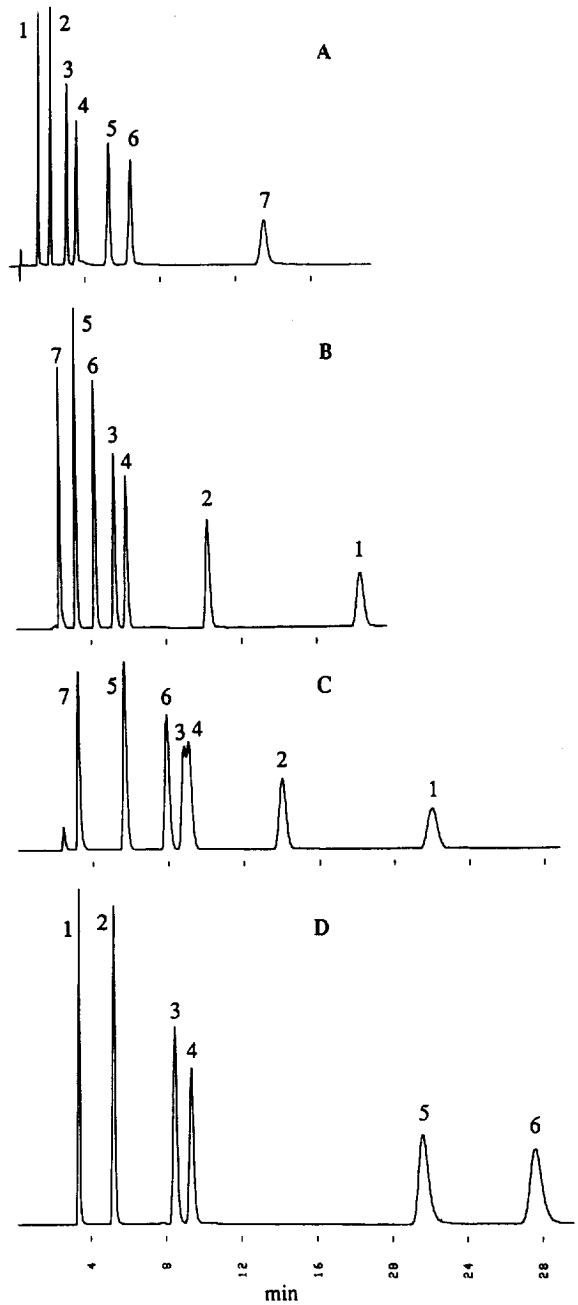

Fig. 7. Chromatograms of corticosteroids. A: packed-column SFC, operating conditions: as in Fig. 3, B: RP–HPLC (40% acetonitrile), C: RP–HPLC (55% methanol), D: NP–HPLC, Peaks: as in Fig. 1.

separation efficiency than the existent NP- and RP-HPLC ones, in the analysis of corticosteroids which possess 1 to 4 hydroxyl groups.

4. Conclusions

The retention behavior of synthetic corticosteroids used in therapy was investigated using packed-column SFC modified from a commercial HPLC system. The addition of methanol to carbon dioxide as the mobile phase and adoption of the aminopropyl stationary phase showed the best resolution and symmetric peak shape at 40°C. Both plate number and resolution indicated that the maxima were around the critical temperature (40–50°C) of the binary fluid used. The retention in packed-column SFC showed the highest separation efficiency (analysis time and resolution) compared with that in NP- and RP-HPLC. In addition, 7 synthetic corticosteroids, possessing 1 to 4 polar functional hydroxyl groups, were completely separated within 6.5 min with the modifier gradient.

The packed-column SFC modified from a commercial HPLC system is useful for the analysis of polar drugs, and its application as a rapid method for quality control and routine analysis can be expected.

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