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Supercritical Fluid Chromatography

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Supercritical fluid chromatography (SFC) has been recognized as a powerful separation technique complementing gas chromatography (GC) and high performance liquid chromatography (HPLC). Klesper *et al*. published results of the use of supercritical dichlorodifluoromethane and monochlorodifluoromethane to separate involatile nickel porphyrin in 1961. The development of the technique was limited by instrumental and experimental difficulties due to the high temperatures and pressures required to maintain the mobile phase in a supercritical state. Novotny and Lee, however, developed SFC with a capillary column (cSFC) in 1981, which led to significant advances in the technique. In 1982 Gere *et al*. developed an instrument for packed-column SFC (pSFC) based on modification of an HPLC. They demonstrated, using polycyclic aromatic hydrocarbons as probe molecules, that the resolution per unit time in pSFC was 5–10 times better than in HPLC with the same columns, due to more favourable diffusivity in supercritical fluids.

The advantages of SFC have been described elsewhere in this *Encyclopedia*. Pure carbon dioxide fluid is a solvent of inadequate polarity. For the analysis of polar compounds by SFC, alcohol is generally added to a mobile-phase fluid as a modifier. Small amounts of polar modifiers significantly increase the solvent strength of the mobile phase and make it possible to elute polar compounds. In particular, pSFC has been applied to various kinds of polar compounds such as drugs, and shown to be superior to HPLC with respect to analysis time, efficiency and selectivity.

The nonpolar steroids cholesterol and ketosteroids are easily eluted by either cSFC or pSFC with pure carbon dioxide. Synthetic corticosteroids, which are widely used therapeutically for the suppression of adrenocortical functions, inflammatory and allergic diseases, have multiple hydroxyl functional groups in the structures. In order to modify the efficacy and suppress adverse reactions, many corticosteroids have been synthesized. Thin-layer, normal and reversed-phase chromatography have been used for the analysis of these compounds. For a number of the synthetic corticosteroids used in therapy, very little work has been carried out by pSFC. These polar steroids are probably difficult to elute with pure carbon dioxide due to its poor solvent strength.

In this article, the pSFC retention behaviour of synthetic corticosteroids, possessing one to four hydroxyl groups, are focused on. The effect of several parameters (stationary phase, modifiers, pressure and temperature) on retention and efficiency are considered. The chemical structures of corticosteroids are shown in **Figure 1**. They were chromatographed using a pSFC instrument modified from a commercial HPLC system. The addition of methanol to carbon dioxide and adoption of an aminopropyl stationary phase produced both good resolution and symmetric peak shapes. Both plate number and resolution indicated that the maxima were around the critical temperature (40–50°C) of the binary fluid. The selectivity and separation of the analytes in pSFC are superior to those in existing normal and reversed-phase HPLC. Seven polar corticosteroids, possessing one to four hydroxyl groups, showed baseline separation within 6.5 min with a modifier gradient method.

Instrumentation of pSFC

Most studies have been done using commercial pSFC instruments. However, a pSFC with the same performance as a commercial instrument can easily be constructed. The HPLC for pSFC operation requires some simple adaptations to allow use of supercritical carbon dioxide as a mobile phase. The pSFC system constructed by the authors by modifying a Shimadzu HPLC is shown in **Figure 2**.

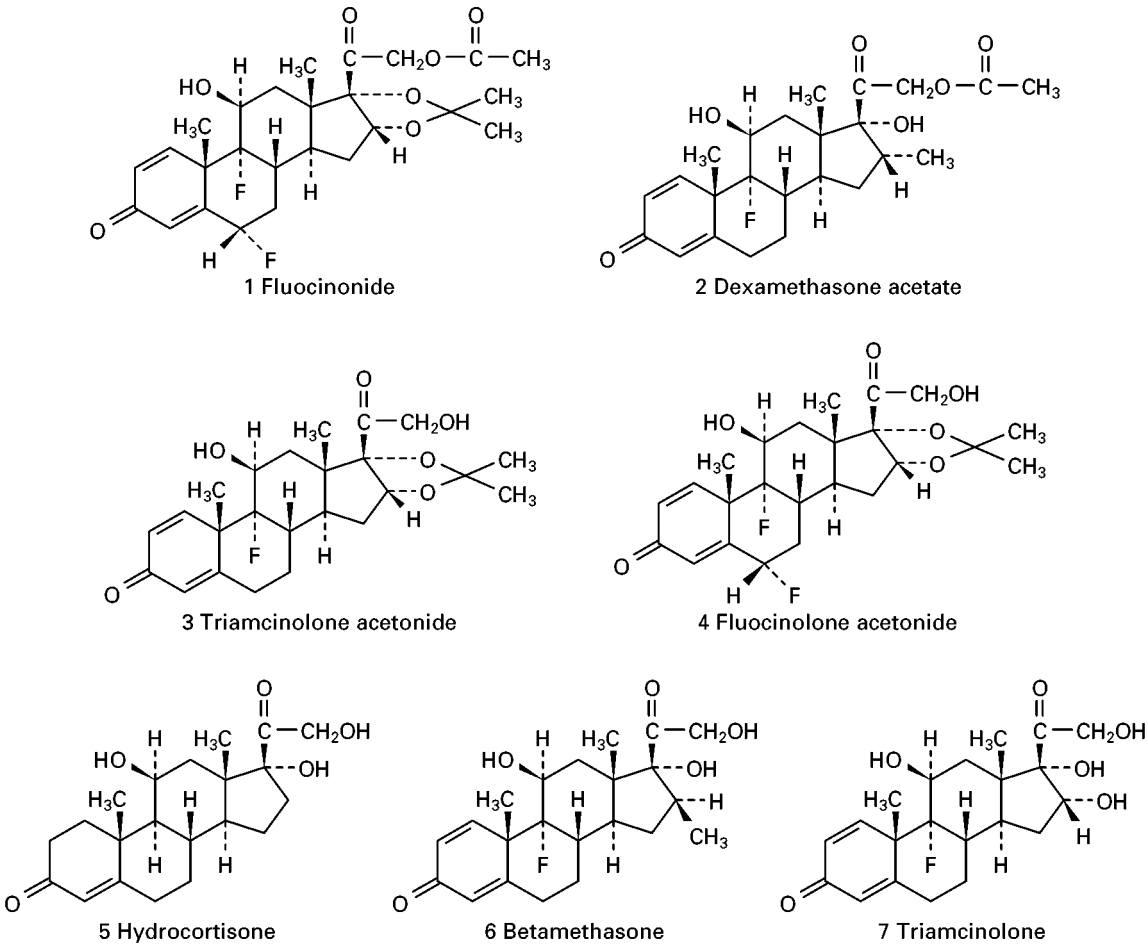

Figure 1 Structures and symbols of synthetic corticosteroids.

Effect of Analytical Parameters

Stationary Phase

Peak shapes of polar solutes on a packed column are often poor when pure carbon dioxide is used as the mobile phase. The separation of steroids has been performed using columns with phenyl, nitrophenyl, diol, aminopropyl, octadecyl and cyanopropyl-modified silica and pure silica as packing materials. It is likely that only polar modifiers used with polar stationary phases produce both good resolution and symmetrical peaks. As shown in **Figure 3**, an aminopropyl column exhibited the best selectivity and peak shape with a reasonable retention time in comparison with the others. Octadecyl and phenyl columns showed poor separation: the former did not retain any solutes 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 triamcinolone acetonide, fluocinolone acetonide, hydrocortisone and betamethasone 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 it is possible to change selectivity by selecting the stationary phase.

Modifier

In pSFC, the addition of a modifier to a mobile phase should be considered from the viewpoint of its effect on either the stationary phase or on the mobile phase. Berger *et al.* have 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 more polar stationary phases produced greater retention, requiring the use of modifiers to obtain reasonable retention times. Blilie and Greibrokk indicated 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.

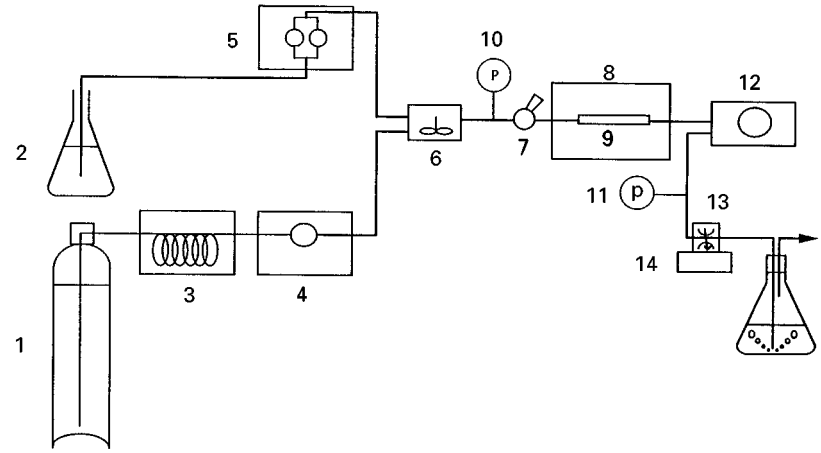

Figure 2 Schematic diagram of packed-column supercritical fluid chromatography. 1, Carbon dioxide cylinder; 2, modifier; 3, cooling bath; 4, LC-6A pump; 5, LC-9A pump; 6, dynamic mixer; 7, injector; 8, oven; 9, packed column; 10 and 11, pressure monitor; 12, detector; 13, back-pressure regulator; 14, dry thermo unit.

No corticosteroids were eluted from the column packed with Cosmosil 5NH_2 with pure carbon dioxide as the mobile phase and a modifier had to be added. The effect of modifiers with different polarities on the retention of corticosteroids is shown in **Figure 4**. The addition of 11.8% (v/v) methanol to carbon dioxide gives the best resolution and symmetrical peak shapes within 14 min. In comparison, the addition of the same amount of 99.5% ethanol, and of 95% ethanol reduced resolution but remarkably improved the peak shape of the most polar compound, triamcinolone, in the corticosteroids. This should be attributed to deactivation of the active sites on the silica support by the water in the 95% ethanol.

Janssen *et al.* confirmed that the effect of a few per cent of modifier in pSFC is largely due to deactivation of residual silanol groups on the silica support, and the accessibility to the active sites depends strongly on the size and structure of the modifier molecules. According to Janssen *et al.*, the same volume percentage of tetrahydrofuran (THF) and methanol was needed to cover 95% of the surface, but since no corticosteroid was eluted under these conditions when methanol was replaced with THF, the effect of the modifier on retention of corticosteroids consists in the enhancement of the solvent strength of the mobile phase rather than deactivation of the active sites on the silica support.

The capacity factor of every corticosteroid decreased two- to fourfold with a 1.8-fold increase in methanol concentrations over the range 9.1–16.7% v/v. All solutes were eluted within 5 min using carbon

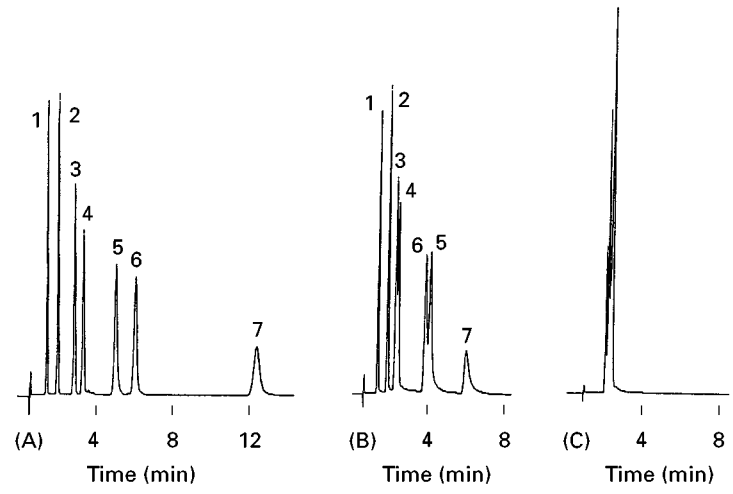

Figure 3 Effect of column on retention of corticosteroids. (A) Cosmosil NH_2; (B) Ultron VX-SIL; (C) Zorbax phenyl. Operating conditions: mean pressure 213 kg cm^{-2}, flow rate of CO_2 3 mL min^{-1}, flow rate of methanol 0.4 mL min^{-1}, temperature 40°C. Peaks: as in Figure 1.

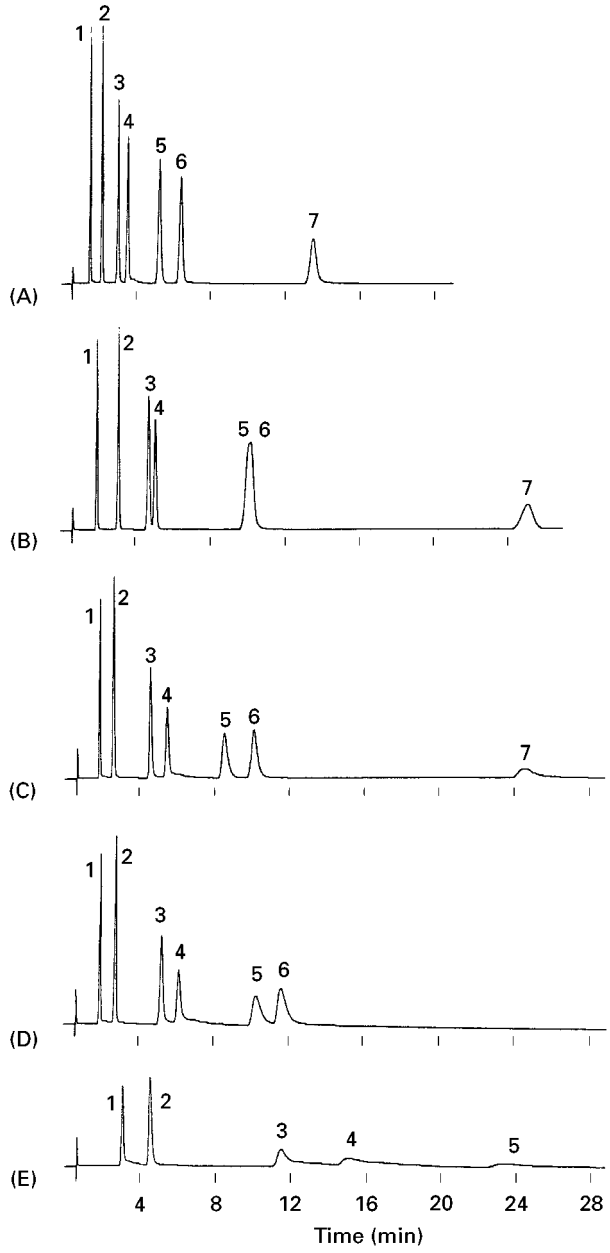

Figure 4 Effect of modifiers on retention of corticosteroids. (A) Methanol; (B) ethanol (95%); (C) ethanol (99.5%); (D) 1-propanol; (E) 2-propanol. Operating conditions: column Cosmosil $5NH_2$, inlet pressure 224 kg cm^{-2}, outlet pressure 191 kg cm^{-2}, flow rate of CO_2 3 mL min^{-1}, flow rate of modifier 0.4 mL min^{-1}, temperature 40°C. Peaks: as in Figure 1.

dioxide modified with 16.7% (v/v), and the resolutions among them were more than 1.6.

Calculated relative standard deviations (RSD) of 0.35–0.70% for t_R, 0.82–1.47% for k and 0.50–1.34% for peak area are shown in **Table 1**, indicate that the pSFC modified from a commercial HPLC system has a good performance and is useful for a routine analysis.

Pressure

In a study of seven corticosteroids, the capacity factor of each solute decreased by a factor of two with an increase in the range of 107–223 kg cm^{-2}. A few researchers have measured the densities of modified supercritical fluids experimentally. Berger measured the density of binary fluids using a U tube densitometer and drew the constant density lines in plots of the pressure against the composition for methanol–carbon dioxide system at three temperatures. The densities of CO_2–methanol (12%, v/v) at different pressures can be calculated by extrapolating the lines in the pressure range 105–180 bar. The plots of $\ln k$ against the binary fluid density revealed that there is a linear relationship between them in SFC, as expected.

Except for fluocinonide, theoretical plate numbers (N) reached maximum values at 126 and 144 kg cm^{-2}, as shown in **Figure 5**. The maximum N values were *c.* 4700–9800. Corresponding to the behaviour of the N values, the resolutions between the adjacent solutes also showed a maximum at 126–162 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 pressure is one of the significant parameters for optimizing the operating conditions.

Temperature

The retention of corticosteroids increases with an increase in the temperature (decrease in the density). The N values of each solute also increase with temperature as shown in **Figure 6**, and reach maximum values at 39 or 49°C, with the exception of hydrocortisone. The maximum N value for triamcinolone is *c.* 8400 at 39°C but only *c.* 3200 at 58°C, corresponding to about a 60% decrease. Although little variation in the separation factor (α) of any pair of neighbouring solutes was observed over the wide range of temperature measured, resolution reached maximum values at 39–49°C, corresponding to the behaviour of the N values. The critical temperature and pressure

Table 1 Repeatability (RSD%, $n = 6$)

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 Figure 3.

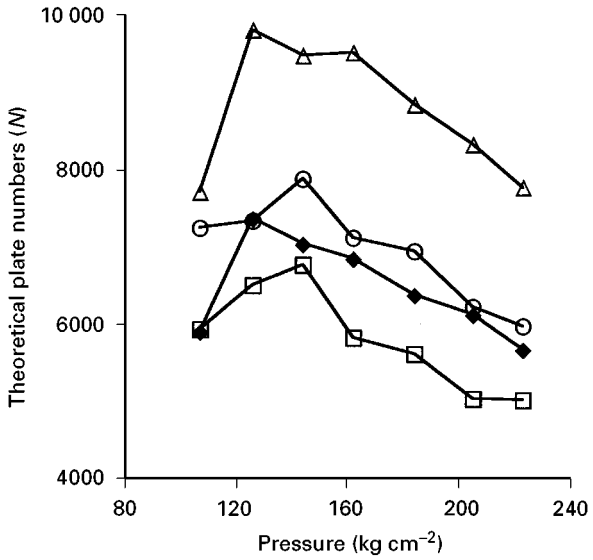

Figure 5 Relationship between theoretical plate numbers and pressure. Operating conditions: mean pressure 107, 126, 144, 162, 184, 205 and 223 kg cm^{-2}; other conditions as in Figure 4. Symbols: squares, hydrocortisone; diamonds, fluocinolone acetonide; circles, betamethasone; triangles, triamcinolone.

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. So, the critical temperature for 12% methanol in carbon dioxide, which we used as the mobile phase, can be assumed to be in the range of 40–50°C: the maximum

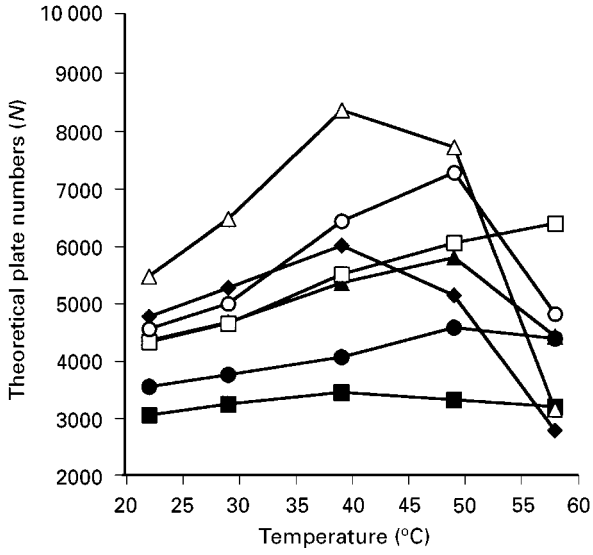

Figure 6 Relationship between theoretical plate numbers and temperature. Operating conditions: temperature 22, 29, 39, 49 and 58°C, mean pressure 213 kg cm^{-2}, other conditions as in Figure 4. Symbols: filled squares, fluocinonide; filled circles, dexamethasone acetate; filled triangles, triamcinolone acetonide; diamonds, fluocinolone acetonide; open squares, hydrocortisone; open circles, betamethasone; open triangles, triamcinolone.

N and resolution were obtained around the critical temperature.

Separation with Modifier Gradient

A wide range of polar corticosteroids has been separated in a modifier gradient elution mode. As shown in **Figure 7**, 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 baseline without drift and noise is considered to be due to the good mixing process of the binary fluid. In pSFC, a modifier gradient is one of the most effective techniques for the analysis of polar steroids.

Comparison with HPLC

The retention of a wide range of corticosteroids by pSFC using an aminopropyl silica column has been compared with that in normal and reversed-phase

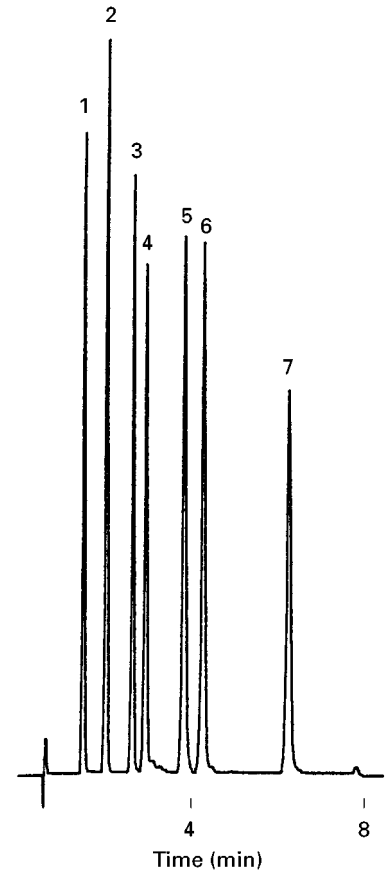

Figure 7 Gradient elution of corticosteroids. Operating conditions: column Cosmosil $5NH_2$, flow rate of CO_2 3 mL min^{-1}, methanol gradient 11.8–17.0% (v/v) at 0.52% (v/v) per min, mean pressure 206 kg cm^{-2}, temperature 40°C. Peaks: as in Figure 1.

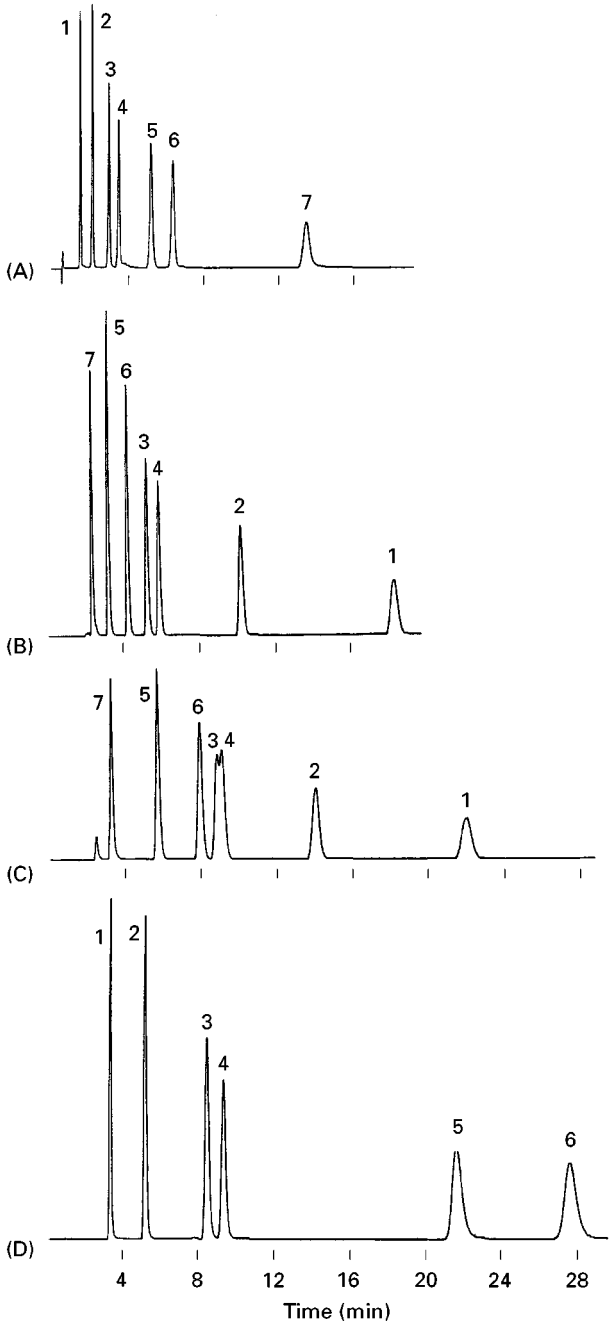

Figure 8 Chromatograms of corticosteroids. (A) Packed-column SFC; operating conditions as in Figure 3; (B) reversed-phase-HPLC (40% acetonitrile), (C) reversed-phase-HPLC (55% methanol); (D) normal-phase-HPLC. Peaks: as in Figure 1.

HPLC. The observed chromatograms are shown in **Figure 8**. Since the most polar corticosteroid among the analytes, triamcinolone, was not dissolved in the mobile phase, it could not be eluted in the normal-phase mode. The elution order of corticosteroids in pSFC is the same as that in normal-phase HPLC and is mainly determined by the number of hydroxyl groups present in the compound: firstly, fluocinonide with a single OH group; dexamethasone acetate, triamcinolone acetonide and fluocinolone acetonide with two OH groups; then hydrocortisone and betamethasone with three OH groups; and finally triamcinolone, with four OH groups. Corticosteroids are eluted almost in reversed order in reversed-phase HPLC, but it is noteworthy that the pairs of triamcinolone acetonide and fluocinolone acetonide, and hydrocortisone and betamethasone are eluted in the same order as in pSFC.

The elution order of these compounds with the same number of OH groups seems to be closely related to their dipole moment. The estimated dipole moments were 1.19 and 2.04 debye for triamcinolone acetonide and fluocinolone acetonide, and 0.52 and 2.24 debye for hydrocortisone and betamethasone.

A similar range of N values was obtained in each chromotographic mode, i.e. *c.* 3600–8000 in pSFC, *c.* 4800–8700 in normal-phase HPLC and *c.* 2300–11 000 in reversed-phase HPLC. The separation of triamcinolone acetonide and fluocinolone acetonide, which show the lowest resolution and the same elution order in the normal- and reversed-phase systems, are comparable. The resolution of these solutes is 2.73 by pSFC, 2.04 by normal-phase HPLC, 0.53 by reversed-phase HPLC with a methanol mixture, mobile phase, and 2.20 by reversed-phase HPLC with an acetonitrile mixture, mobile phase. The elution time of all solutes in pSFC is about four times faster than in normal-phase HPLC and about 1.5 times faster than in reversed-phase HPLC.

These results suggest that the pSFC conditions used give a higher selectivity and better separation efficiency than normal-phase and reversed-phase HPLC for the analysis of corticosteroids which possess one to four hydroxyl groups.

Conclusion

PSFC is useful for the analysis of polar steroids, and its application as a rapid method for quality control and routine analysis can be expected in the future.

Acknowledgements

This article was adapted from Yaku K, Aoe K, Nishimura N, Sato T and Morishita F (1997) Retention behavior of synthetic corticosteroids in packed-column supercritical fluid chromatography. *Journal of Chromatography A* 773: 277–284. Copyright 1997, with kind permission from Elsevier Science.

Further Reading

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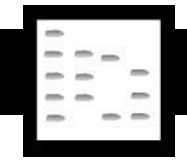
STEROLS



Supercritical Fluid Chromatography

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Introduction

In supercritical fluid chromatography (SFC) the mobile phase is a fluid subjected to pressures and temperatures near or above its critical point. This fact determines the mobile phase properties (e.g. diffusivity, density, viscosity, etc.) that are intermediate between those of gases and liquids and can be varied and controlled by small changes in the pressure or temperature. The most common fluid used in SFC is carbon dioxide, which has a critical temperature of 31°C, allowing the separation of thermally labile compounds under mild conditions. SFC can be carried out with open tubular and packed columns, with differences in selectivity, detection and need of modifier addition to the carbon dioxide. Both types have been employed in the separation of sterols in a wide variety of samples. Supercritical carbon dioxide has an adequate solvating power for sterol separation with both column types without the need of modifier addition. It is therefore possible to separate sterols in complex samples at lower temperatures than gas chromatography and in shorter times than liquid chromatography.

Importance of the Analysis of Sterols

The analysis of sterols is of great significance from the health point of view and for the quality control of numerous food products.

With respect to quality control of food and nutrition studies, the sterols of vegetable origin (phytosterols) are found in complex mixtures in numerous plants, with the mixture containing some major sterols and a great variety of minor compounds (**Figure 1**). Thus, the sterol profile can be indicative of the origin, or species or variety of food from vascular plants, as well as from fungi or marine organisms. Additionally, these compounds are fundamental in the study of several metabolic pathways.

In the animal world, the variety of sterols is not so broad, and the main constituents are cholesterol and derivative esters. For that reason, supercritical carbon dioxide has been employed for the selective extraction of cholesterol from meat products and edible animal fats, to obtain healthier food for human intake.

On the other hand, some sterol oxides (oxysterols) are known for their toxicity, mutagenicity or carcinogenic properties, a fact that makes the determination of their concentrations in natural matrices very necessary, especially in studies of food quality and physiology.

Characteristics of the Separation of Sterols Using Supercritical Fluids

The main properties of supercritical fluid chromatography which affect sterol separation are related to the high solvating power of supercritical fluids and a low viscosity, which yields a high resolving power and rapid throughput. In addition to its other advantages, the ability of SFC to resolve complex mixtures of low volatility compounds allows the direct injection of samples that contain sterols with no or little pretreatment.

Some sterols can be degraded or lost during exposure to light, heat or extreme values of pH. In the SFC of sterols, all these factors can be avoided, providing a separation under mild conditions that preserves the integrity of the sample.

Finally, the relatively good solubility of compounds with intermediate polarity and volatility such as the sterols has also frequently been utilized in