Preparative Purification of Corticosteroids by HPLC; Scalability and Loadability Using Agilent Prep C18 HPLC Columns

Application

Pharmaceuticals

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Abstract

Agilent Prep reversed-phase columns with different diameters displayed the nearly linear scaleup for the gradient separation of corticosteroids. The loadability of the columns was quite good with as much as 8 mg of total corticosteroid injected onto a 4.6-mm column and 320 mg onto a 30-mm column.

Introduction

Corticosteroids are a family of drugs that include cortisol (hydrocortisone) and corticosterone, adrenal hormones found naturally in the body, as well as synthetic drugs such as triamcinolone and prednisone. Though natural and synthetic corticosteroids are both potent anti-inflammatory compounds, the synthetics exert a stronger effect. The oral forms of corticosteroids are used to treat numerous autoimmune and inflammatory conditions including bursitis, tendonitis, and Crohn's disease. Corticosteroids are available for inhalation by mouth to treat asthma conditions of restricted breathing as well as symptoms of nasal allergies. Topical forms are available to treat skin conditions such as eczema, psoriasis, and insect bites. Since corticosteroids are used in so many ways, their analysis in a variety of matrices is important. HPLC represents a convenient, rapid and sensitive technique for the analysis and quantitation of these important drugs. When investigating new synthetic or natural corticosteroids or attempting to identify trace impurities by large injections, preparative quantities are needed. So scaling up to larger columns is required. The purpose of this application note is to demonstrate the ability to perform a direct and linear scaleup from analytical to larger scale preparative HPLC using a matched set of columns.

Corticosteroids are large bulky organic molecules with very similar structures (Figure 1). Their solubilities in water vary from insoluble to very sparingly soluble. Their solubility in organic solvents such as methyl alcohol and acetonitrile is much better. Thus, these compounds are good candidates for and should be easily separated by reversed-phase liquid chromatography (LC). Even though their structures are similar, as depicted in Figure 1, chromatographic retention amongst the corticosteroids studied is quite different and a gradient is required for their separation in a reasonable time. Gradient elution is often a preferred approach to speed up analytical and preparative separations and at the same time to provide an increase in sample capacity. Since our goal was to eventually isolate tens of milligram amounts of a single corticosteroid, we initially explored analytical HPLC conditions where a minimum of solvent and sample would be required during method development.

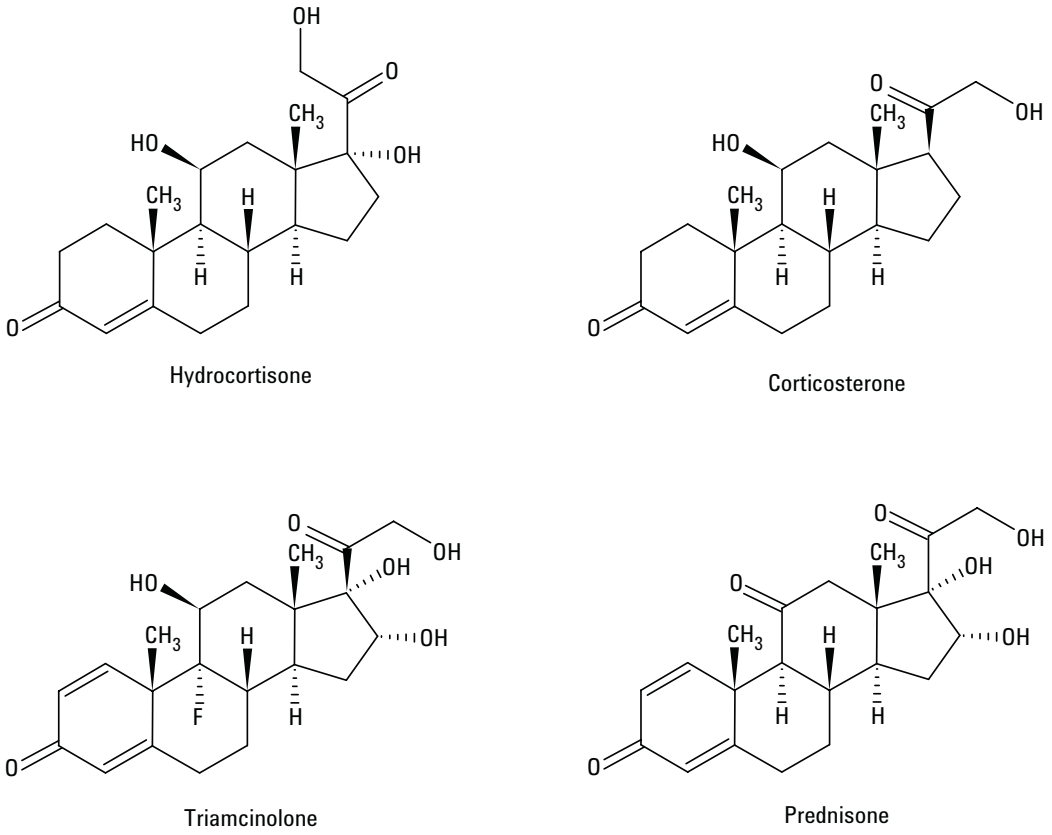

Figure 1. Structures of corticosteroids.

Chromatographic Separation of Corticosteroids on an Analytical Scale

Goals in preparative chromatography include maximizing throughput, purity and yield. The scaleup process involves injecting increasingly larger amounts of material on column until the resolution becomes so poor that purity is compromised. There are two approaches to the scaleup process. The first is to scaleup on an analytical column by injecting progressively larger sample masses until resolution is barely acceptable (arbitrarily set at R_s <1.2); then, based on the preparative mass requirement, move to a suitably-sized prep column; and adjust the chromatographic conditions dictated by the analytical separation but matched to the preparative column. The second approach is to choose the preparative column needed, then perform method development directly on this column until resolution and the resultant analyte purity is achieved. The first approach is generally preferred since the analytical method development requires less solvent, sample, and time. If the preparative column has the same chemistry as the analytical column, then the scaleup should be a linear process.

Usually the same particle size is used in both the analytical separation and the preparative separation steps. In preparative chromatography, one may work under slightly overloaded conditions where resolution may be sacrificed for yield and the efficiency advantage of small particle sizes (~5 µm) may not be required. In addition, larger particle sizes (~10 µm) will provide sufficient resolution but generate less backpressure allowing flow rates to be increased to improve separation speed. On the other hand, sometimes chromatographic resolution for critical pairs is insufficient with a 10-µm particle and having the ability to use 5-µm prep columns is advantageous. In such cases, a compromise among resolution, required purity, and speed will be required for optimum preparative performance.

Figure 2 shows the initial binary mobile phase gradient separation of the four corticosteroids on a column with reversed phase C18 packing. The column used was an Agilent Scalar C18, 4.6 mm × 150 mm, a typical size for analytical work. During method development, initial experiments involving various water (solvent “A”) and neat water-miscible organic solvent [that is, acetonitrile (ACN),

methanol (MeOH), or isopropanol (IPA)] (solvent "B") combinations were performed. However, when using pure organic solvents in a binary pumping system, selectivity was not adequate so mixtures of organic solvents were investigated. The mobile phase found to yield the best overall resolution of the corticosteroids consisted of water as the weak component (solvent "A") and 50% ACN:17% MeOH:33% IPA as the strong component (solvent "B"). The chromatographic conditions used in all the experiments described in this application note are shown in Table 1. Since the corticosteroids are neutral compounds, no buffer was required in the mobile phase system.

In preparative work, since the goal is to collect pure fractions, it is preferable to avoid the use of buffers, especially nonvolatile ones, so that further work of buffer removal from the collected fraction is not required. If the LC conditions do require a buffer to provide better chromatography, then the use of a volatile buffer, such as ammonium acetate or formic acid, is recommended. Volatile buffers may be removed by rotary evaporation or by nitrogen purging.

The solvent chosen for injection was dimethylsulfoxide (DMSO), particularly useful for preparative chromatography [1]. The DMSO is an excellent solvent for dissolution of many organic compounds, especially at higher sample concentrations often needed for preparative work. In addition, DMSO elutes rapidly from a typical reversed-phase column (see Figure 2) and does not overlap or interfere with the collected fractions. Near the column void volume, a small DMSO peak may appear depending on the wavelength used for effluent monitoring and on the flow cell path length.

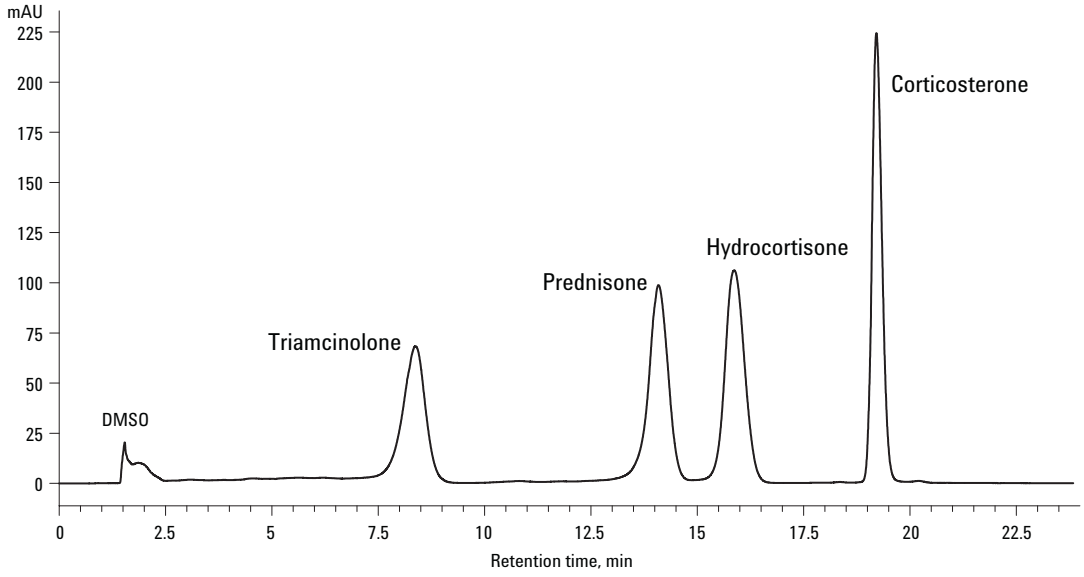

Figure 2. Analytical separation of corticosteroids on Agilent Scalar-C18 Column.

Table 1. Chromatographic Conditions for Scalability and Loadability Experiments

For 4.6-mm id	
Mobile phase A:	Water
Mobile phase B:	50% Acetonitrile: 17% Methanol: 33% Isopropanol (v/v)
Gradient:	Time 0, 25% B; 7 min, 25% B; 15 min, 34% B;15.1 min, 45% B; 20 min, 45% B; 20.1 min, 25% B; Stop
Time:	24 min
Flow rate:	1 mL/min, 4.6-mm id
Injection solvent:	DMSO
Injection volume:	4.6-mm id: 80 µL
Injection loop:	No external loop
DAD settings:	Detector wavelength: 244, 8; Reference 360, 100
Flowcell:	0.06-mm pathlength
For 21.2-mm id	
Mobile phase A:	Water
Mobile phase B:	50% Acetonitrile: 17% Methanol:33% Isopropanol (v/v)
Gradient:	Time 0, 25% B; 7 min, 25% B; 15 min, 34% B;15.1 min, 45% B; 20 min, 45% B; 20.1 min, 25% B; Stop
Time:	24 min
Flow rate:	21.2 mL/min, 21.2-mm id
Injection solvent:	DMSO
Injection volume:	21.2-mm id, 1600 µL (700-µL loop and 900-µL needle fill
Injection loop:	1500 µL
DAD settings:	Detector wavelength: 244, 8; Reference 360, 100
Flowcell:	0.06-mm pathlength
For 30.0-mm id	
Mobile phase A:	Water
Mobile phase B:	50% Acetonitrile: 17% Methanol:33% Isopropanol (v/v)
Gradient:	Time 0, 25% B; 7 min, 25% B; 15 min, 34% B;15.1 min, 45% B; 20 min, 45% B; 20.1 min, 25% B; Stop
Time:	24 min
Flow rate:	42.4 mL/min, 30.0-mm id
Injection solvent:	DMSO
Injection volume:	30.0-mm id, 3200 µL
Injection loop:	5000 µL
DAD settings:	Detector wavelength: 244, 8; Reference 360, 100
Flowcell:	0.06-mm pathlength

Loadability of Corticosteroids on Agilent Preparative Reversed-Phase Columns

Loadability for a preparative separation is defined as the maximum amount of analyte that can be injected onto a column that no longer permits the isolation of product at the desired level of purity or recovery levels; in other words, it is the maximum sample capacity of a particular column for the analyte(s) of interest. In the reversed-phase mode for a column of fixed dimensions, loadability is affected mostly by bonded phase coverage, chromatographic resolution, and chromatographic conditions such as mobile phase, injection solvent, temperature, etc. In preparative chromatography, the size of the column is dictated by the amount of purified material needed for further investigation. Generally, the larger the column, the greater

amount of sample can be injected onto the column. Having a wide range of column dimensions with the same chemistry allows the chromatographer greater flexibility in obtaining the desired amount of purified material.

For a fixed chromatographic method with the same stationary phase and column length, the loadability (sample capacity) is directly related to the mass of packing in a column as depicted in Equation 1.

$$x_2 = \frac{x_1 \bullet r_2^2 \bullet C_L}{r_1^2} \qquad (1)$$

where:
x2 is the maximum amount (mass) of sample injected on column 2 (the larger id column)
x1 is the maximum amount (mass) of sample injected onto column 1 (the smaller id column)
r_1 and r_2 are the radii of columns 1 and 2, respectively
C_L is the ratio of column lengths of column 2: column 1 (in this case both lengths are the same)

It can easily be deduced that the sample mass that can be injected is directly proportional to the radius ratios squared. For a linear scaleup, the chromatographic flow conditions should be adjusted to reflect this ratio and maintain an equivalent linear velocity. For example, for the scaleup of a separation done in a 4.6-mm id analytical column with a flow rate of 1.0 mL/min to an equivalent preparative column with a 21.2-mm id, the flow rate should be adjusted to $(21.2/4.6)^2 \times$ 1.0 mL/min or to 21.2 mL/min in order to have the same separation time.

To investigate the loadability of Agilent Prep C18 columns of various dimensions for these corticosteroids, we obtained three 150-mm long columns of 4.6, 21.2, and 30-mm internal diameters (ids) (Agilent Technologies, Inc., Wilmington, DE, USA) and injected increasing volumes of a stock solution of the four test corticosteroids (50-mg/mL concentration of each). For one of the dimensions, the 30-mm id column, Figure 3 shows a series of total corticosteroid injected sample masses from 2.5 mg up to 320 mg. At 320 mg of total injected corticosteroid (80 mg each), column overloading began to appear and peaks became distorted, typical of overload conditions. Here overloading is defined, for a given column dimension, as the mass of sample injected where the resolution of critical compound pairs appears to deteriorate thus precluding collection of pure fractions.

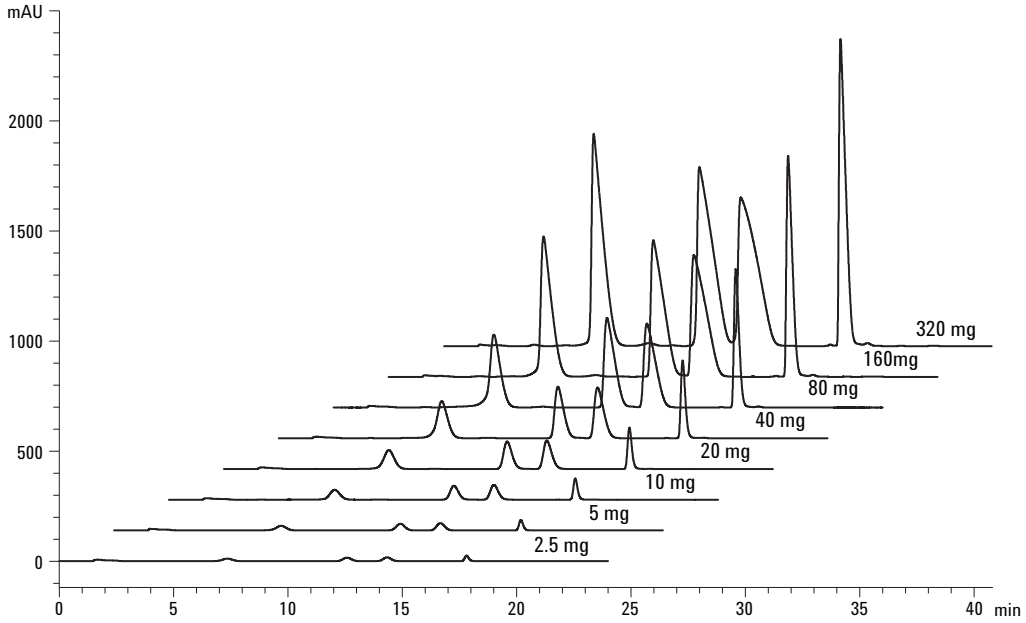

Figure 3. Corticosteroids on Agilent Prep-C18, 30.0 × 150 mm Loadability: 2.5 → 320 mg total.

To further illustrate this behavior, Figure 4 shows the resolution for peaks 2 and 3 (critical pair) as a function of loading on the three different sized columns. For each column, the injected mass was equivalent to the radius ratios squared so that an "apples to apples" comparison could be made. As can be seen in Figure 4, as the sample size increases, at low loading, resolution remains relatively constant. However, as the injection mass increases, resolution decreases. Eventually around R_s = 1.2, peaks become severely overlapped so that individual peak purity is compromised. Although there are some variations in the absolute resolution due to slight selectivity differences, it is apparent that the columns behave quite similarly and that column id is really a determinant of how much can be injected onto a preparative column prior to overload. When resolution becomes less than 1.2, overlap between the adjacent peaks occurs, precluding collection of a pure fraction. Obviously, even with poor resolution, use of heart-cutting techniques enables pure analyte to be obtained, but at the cost of yield.

Scalability for a Matched Set of Columns

The scalability of a chromatographic process is the reproducibility of results on columns of different ids when using the same particle size and bonded phase. In order to assess scalability for the Agilent Prep columns, we determined the maximum loadability of total analyte for each column dimension; and then compared all three column dimensions to see if the performance was similar.

The effect of overloading each size column can clearly be seen in Figure 5 that shows the maximum amount of corticosteroids 2 and 3 that can be injected under the chromatographic conditions employed without significant peak overlap. At the point of overload, peak shapes also begin to deteriorate with visible tailing occurring. Nevertheless, at least 80 mg of each steroid (320 mg total) could be successfully injected onto the largest (30 mm) id prep column. This scalability experiment demonstrated the excellent sample capacity of the Agilent Prep phase as well as reproducibility for the corticosteroids at all column dimensions.

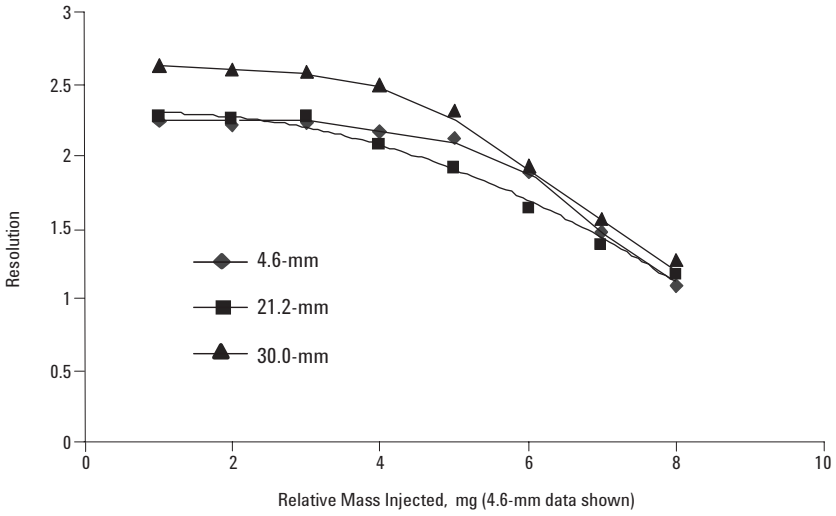

Figure 4. Resolution vs. loading for corticosteroids on different id columns.

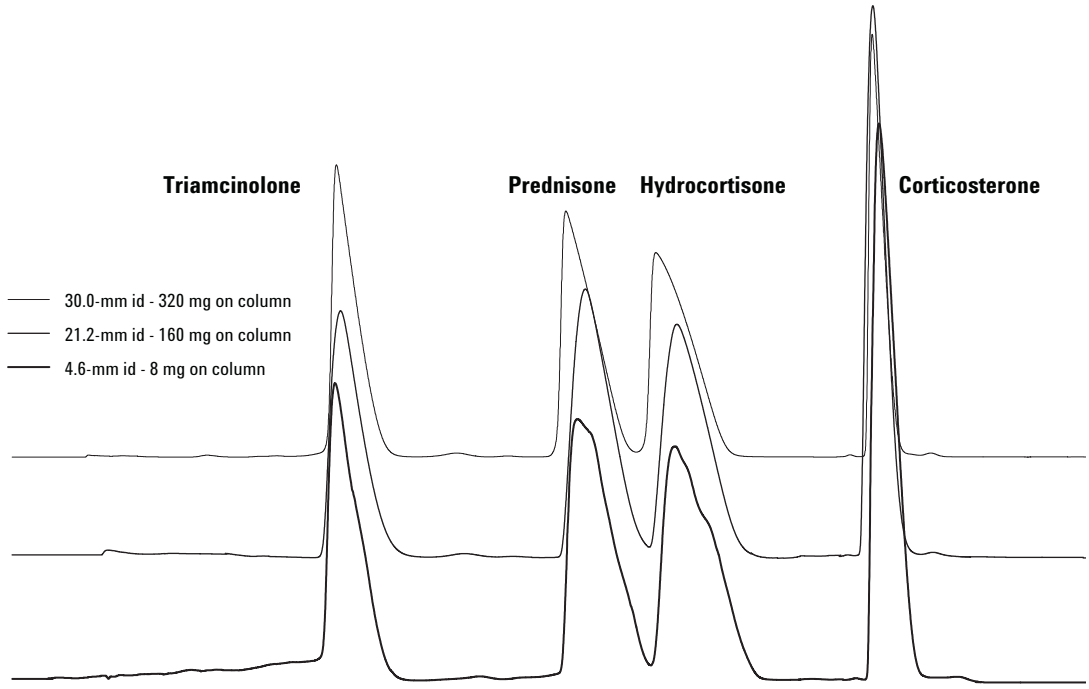

Figure 5. Scalability of Agilent Prep-C18: overlay of chromatographic results from 4.6 mm id to 30.0 mm id.

Conclusions

The use of Agilent Prep reversed-phase columns with different diameters displayed the nearly linear scaleup for the gradient separation of corticosteroids. The loadability of the columns was quite good with as much as 8 mg of total corticosteroid injected onto a 4.6-mm column and 320 mg onto a 30-mm column.

Reference

1. Angelika Kaufmann, Ulrike Jegle, Alexander Dreyer, and Ronald E. Majors, (2005) “Using DMSO as an Injection Solvent to Increase Sample Load in Preparative LC Applications”, Agilent Technologies, publication 5989-2485EN www.agilent.com/chem

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