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Comparison study of porous, fused-core, and monolithic silica-based C_{18} HPLC columns for Celestoderm-V Ointment® analysis

Jinjian Zheng[a], Dolly Patel[b], Qinglin Tang[a,*], Robert J. Markovich[a], Abu M. Rustum[a]

[a] Global Quality Services-Analytical Sciences, Schering-Plough Corporation, Union, NJ 07083, USA
[b] Philadelphia College of Pharmacy, University of the Sciences in Philadelphia, 600 South 43rd St., Philadelphia, PA 19104, USA

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ABSTRACT

In this paper, three C_{18} columns with different substrates (i.e., porous ACE-3 C_{18}, 3 μm, fused-core Halo C_{18}, 2.7 μm, and monolithic Chromolith C_{18}) were compared for the analysis of a pharmaceutical product, Celestoderm-V Ointment®, that contains one active pharmaceutical ingredient, betamethasone-17-valerate and one critical pair of low level impurities, betamethasone-E-enolaldehyde and betamethasone-Z-enolaldehyde. Key column performance for the analysis of pharmaceutical products including selectivity, efficiency, separation impedance, resolution factor, sample loading capacity, linearity and lifetime from the three columns were determined. The potential applications of these three C_{18} columns for different methods for Celestoderm-V Ointment® analysis are also recommended.

1. Introduction

Analysis of pharmaceutical products using HPLC methods includes assay, estimation of impurities/degradation products, content uniformity, dissolution, etc. Each analytical method for pharmaceutical analysis has different objectives. For assay methods, only the amount of a specific compound such as active pharmaceutical ingredient (API) or preservative needs to be determined in the sample. Therefore, the assay method usually requires high selectivity for the analyte of interest and rapid analysis time. For the estimation of impurities/degradation products methods, all potential impurities, degradation products, compounds from excipients and potential contaminates (e.g., leachables) are individually quantitated at levels above quantitation limit (typically 0.05% of the label strength). Thus, the method for impurities/degradation products needs high specificity and high sensitivity for all analytes of interest. Methods for content uniformity and dissolution usually monitor one single component, such as the API, and requires testing of multiple independent samples and thus, quick analysis time is of the most importance.

The HPLC column is the heart of an HPLC method. The selection of a most appropriate HPLC column often dictates the success or failure of an HPLC method development for a particular pharmaceutical product. The performance of an HPLC column mainly depends on the substrate, the bonded stationary phase, and the column packing process. The substrate has a significant contribution to the column performance with respect to the stationary bonding density, column mechanical and chemical stability, selectivity, efficiency, pressure drop, etc. Different HPLC column substrates such as silica, polymer, zirconia, and alumina has been reported in literature [1]. Silica is the most popular substrate for HPLC columns due to the unique properties such as inertness to a wide variety of analytes, mechanical strength to withstand very high pressure, and high efficiency. Porous, non-porous, superficially porous, and monolithic silica substrates have been commercialized by various column vendors for HPLC analysis of pharmaceutical products. Various diameters and pore sizes are commercially available for HPLC column substrates. Porous silica with 3–5 μm diameters and 60–300 Å pores are the most widely used silica substrate in HPLC for the analysis of pharmaceutical products. Porous silica has high surface area and thus high carbon loading. Column packed with porous silica based stationary phase has good sample loading capacity, and excellent analyte retention characteristics. In the last few years, newer silica substrates such as monolithic silica, superficial porous or fused-core silica based HPLC columns have been introduced by commercial vendors. These newer silica substrates have different characteristics compared to conventional porous silica. The monolithic silica columns [2–5] such as Chromolith are composed of porous silica rods with ~2 μm macropores, ~130 Å mesopores and 1–2 μm skeleton (silica network) size, which provide fast mass transfer and typically generate approximately 30–40% less pressure drop compared to conventional commercial columns packed with

* Corresponding author. Tel.: +1 908 820 6747; fax: +1 908 820 6925.
E-mail address: Qinglin.Tang@spcorp.com (Q. Tang).

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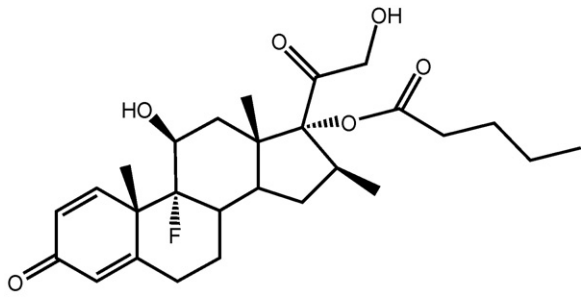
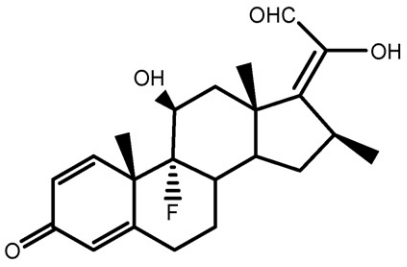
5 μm porous silica. Therefore, the monolithic HPLC column can be used at higher flow rate for high speed separation without the need for a special high pressure LC system. In addition, the mesopores have a large surface area and thus provide good sample loading capacity. The superficially porous particle was reported about half a century ago [6,7]. The rationale behind this particle design was to reduce the diffusion distance of analytes to minimize mass transfer. Kirkland [8] in 1992 introduced a new process to make small superficially porous particles and commercialized it as "Halo" silica process to make small superficially porous particles. The Halo silica contains a 1.7 μm fused-core and a 0.5 μm layer of porous silica coating (total particle diameter is 2.7 μm). Good column efficiency and fast separation using this Halo column has been reported [9,10].

Gritti et al. [11] compared the efficiency of columns packed with traditional porous silica particles and fused-core porous silica particles. Nováková et al. [12] compared the performance of monolithic C_{18} column and conventional porous silica C_{18} column for the estimation of active pharmaceutical ingredient (API), impurities and preservatives in topical pharmaceutical formulations including Estrogel gel and Ketoprofen gel. Majors [2] in a review described the approaches for fast separation using porous silica column, monolithic silica column, and fused-core Halo column under traditional HPLC equipment. To the best of our knowledge, a direct comparison of the HPLC columns based on the three different silica substrates, porous, monolithic, and fused-core silica, for the analysis of complex pharmaceutical product has not been reported.

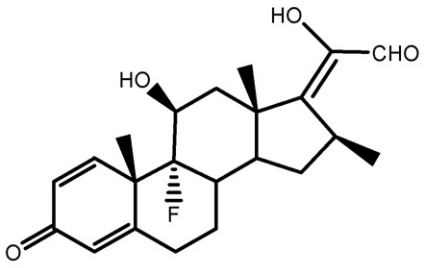
In this report, a direct comparison study of the porous (ACE C_{18}, 3 μm), fused-core (Halo C_{18}), and monolithic (Chromolith C_{18}) silica-based C_{18} columns for Celestoderm-V Ointment® analysis was presented. The critical column properties for pharmaceutical analysis such as selectivity, efficiency, separation impedance, resolution factor, sample loading capacity, linearity and column lifetime of these three columns are compared. The objective of our work was to study and to determine which silica substrate-based HPLC column is most suitable for different type of methods for analysis of finished pharmaceutical products. This information would benefit analytical scientists in pharmaceutical industry to select appropriate type of HPLC columns during method development activities of pharmaceutical products. Selection of an appropriate pharmaceutical product to obtain meaningful data for the objective of this study was an important element of this study. Celestoderm-V Ointment® was selected for this study because it contains the active pharmaceutical ingredient (betamethasone-17-valerate, BMV, Fig. 1) and one critical pair of API's degradation products (betamethasone-E-enolaldehyde, BMEE and betamethasone-Z-enolaldehyde, BMEZ, Fig. 1). It is quite challenging to obtain a baseline separation for these two isomers and therefore is a good marker to obtain selectivity information of a given stationary phase. The critical pair of degradation products also typically present at very low concentrations, which requires high sensitivity for accurate quantitation. Therefore, the data obtained from the analysis of Celestoderm-V Ointment® using the three different silica substrate columns provided all the necessary information to achieve the objectives of this study.



Betamethasone-17-Valerate (BMV)



Betamethasone-(*E*)-enolaldehyde (BMEE)



Betamethasone-(*Z*)-enolaldehyde (BMEZ)

Fig. 1. The structures of betamethasone-17-valerate, betamethasone-E-enolaldehyde and betamethasone-Z-enolaldehyde.

2. Experimental

2.1. Chemicals, reagents, and columns

Betamethasone-17-valerate (BMV), betamethasone-E-enolaldehyde (BMEE) and betamethasone-Z-enolaldehyde (BMEZ) were obtained from Global Reference Standard group of Schering-Plough (Kenilworth, NJ). The Celestoderm-V Ointment® placebo and Celestoderm-V Ointment® were obtained from Schering-Plough.

The HPLC grade acetonitrile was purchased from Thermo-Fisher (Waltham, MA). Milli-Q water (18.2 MΩ cm, TOC ≤5 ppb) was obtained from an in-house Millipore Gradient A10 Water Purification System (Bedford, MA).

The ACE C_{18} (100 mm × 4.6 mm, 3 μm) and Halo C_{18} (100 mm × 4.6 mm, 2.7 μm) columns were both purchased from Mac-Mod (Chadds Ford, PA). The Chromolith C_{18} column (100 mm × .6 mm) was purchased from Phenomenex (Torrance, CA) under trade name "Onyx". The characteristics of these three columns were listed in Table 1.

2.2. Chromatographic conditions

An Agilent 1100 Series HPLC system (Santa Clara, CA) equipped with thermostated column compartment and a UV absorbance detector was used for all the experiments. The column temperature was controlled at 40 °C. The detection wavelength was set at 240 nm, which is the λ_{max} of all three analytes. Injection volume is 20 μL. Data acquisition and data processing were performed using Agilent Chemstation software.

Table 1
Summary of characteristics of the porous ACE C_{18} column, the fused-core Halo C_{18} column, and the monolithic Chromolith C_{18} column.

Column name	Column characteristics
ACE C_{18}	Particle size: 3 μm. End capped. Pore size: 100 Å; surface area: 300 m^2/g; bonding density: 2.6 μmol/m^2; carbon loading: 15.5%
Halo C_{18}	Silica based. Particle size: 2.7 μm with 1.7 μm fused silica core. End capped. Pore size: 90 Å; surface area: 150 m^2/g; bonding density: 3.5 μmol/m^2
Chromolith C_{18}	Monolithic silica rod. Macropore: 2 μm; mesopore: 130 Å; skeleton size: 1–2 μm; end capped. Carbon loading: 17%; surface area: 300 m^2/g; bonding density: 3.6 μmol/m^2; carbon loading: 9%

Two isocratic methods were developed for BMV with retention factors (κ) ~2.5 and 8.5 by using automated HPLC method development software (Chromsword®). These two retention factors were selected to cover the retention factor ranges that are typically used for HPLC analysis of pharmaceutical products. The retention factor is obtained by adjusting the ratio of mobile phases (mobile phase A: water and mobile phase B: acetonitrile) as summarized in Table 2.

2.3. Preparation of sample solution to study column lifetime

The column lifetime evaluation solution was prepared by spiking BMEE and BMEZ into Celestoderm-V Ointment® sample and diluting it to a concentration of 0.1 mg/mL for BMV and of 0.01 mg/mL for BMEE and BMEZ in a 50-mL centrifuge tube. The centrifuge tube was capped, placed in a 75 °C water bath for 10 min, and vortexed for 1 min. The centrifuge tube is then chilled in an ice bath for 10 min, and centrifuged at 3000 rpm (approximately 1700 × g) for 10 min. An aliquot of supernatant was transferred into an HPLC vial for analysis.

3. Results and discussions

Depending on the objective of the analysis, HPLC methods for the analysis of pharmaceutical products have different requirements on column properties such as retention factor, selectivity, efficiency, resolution factor, loading capacity, linearity, and stability. These important properties of the porous ACE C_{18}, fused-core Halo C_{18}, and monolithic Chromolith C_{18} columns were studied using the Celestoderm-V Ointment® pharmaceutical product as well as standard mixtures of the betamethasone-17-valerate (BMV) API with the related compounds betamethasone-E-enolaldehyde (BMEE) and betamethasone-Z-enolaldehyde (BMEZ).

3.1. Retention factor, selectivity, and resolution factor

The retention factor (κ) is defined in Eq. (1) as:

$$\kappa = \frac{t - t_0}{t_0} \quad (1)$$

Table 2
Summary of t_0 and V_0 of porous the ACE C_{18}, fused-core Halo C_{18}, and monolithic Chromolith C_{18} columns with 4.6 mm I.D. × 100 mm in length.

Column type	Retention factor of BMV	Mobile phase composition	
		% Water	% Acetonitrile
Halo C_{18}	8.5	58	42
	2.5	47	53
ACE C_{18}	8.5	57	43
	2.5	46	54
Chromolith C_{18}	8.5	63	37
	2.5	53	47

Table 3
Mobile phases compositions of isocratic methods for BMV at κ ~ 8.5 and κ ~ 2.5 from the ACE C_{18}, Halo C_{18} and Chromolith C_{18} columns.

Column	Flow rate 1.0 mL/min		Flow rate 0.2 mL/min	
	t_0 (min)	V_0 (mL)	t_0 (min)	V_0 (mL)
Halo C_{18}	0.960	0.960	4.785	0.957
ACE C_{18}	1.170	1.170	5.825	1.165
Chromolith C_{18}	1.520	1.520	7.653	1.531

in which t is the retention time of an analyte, and t_0 is the column zero time. To obtain the retention factor, column t_0 has to be accurately determined. The column t_0 represents the retention time of a non-retained analyte. Accurate determination of t_0 is a very challenging task, and many different approaches have bee reported [13–15]. In this study, acetone, an analyte with essentially no retention on the column, was chosen to estimate the t_0. Acetone (0.1% in acetonitrile) was injected and eluted using 100% acetonitrile at flow rates 1.0 and 0.2 mL/min at 265 nm detection wavelength, respectively. The reason for using two different flow rates is to verify the accuracy of void volume measurement. The results of the retention factor (κ) of betamethasone-17-valerate (BMV) API at two different mobile phase conditions are summarized in Table 2. The t_0 retention time of the column was measured using non-retained analyte acetone and the void volumes V_0 of the column was calculated by multiplying flow rate with t_0 retention time. The results are summarized in Table 3.

The data in Table 3 clearly demonstrate that the Chromolith column has the largest void volume (1.52 mL) which corresponds to a column porosity of 92% which was calculated by dividing the experimentally determined void volume with the column volume (column volume = 1.66 mL for the 4.6 mm × 100 mm column). The relatively large void volume for Chromolith column is mainly due to its large macropore size. The Halo column has a void volume of 0.960 mL with a calculated column porosity of 58% while the porous Ace C_{18} column has a void volume of 1.17 mL yielding a calculated column porosity of 70%. The smaller column porosity of Halo column as compared to the porous ACE column is accounted for the non-porous inner core and slightly smaller particle size of Halo column. Since the column void volume is independent of the flow rate, the t_0 at any flow rates can be calculated by simply dividing the column void volume by the specific flow rate.

Different approaches to characterize the selectivity of a HPLC column have been reported [16–19]. A recent review by Nemeth et al. [16] discussed different methods used to characterize HPLC columns. Since the columns compared in this study have significantly different designs of the substrates, tests using the above approaches most likely would yield different results for these columns. From a practical viewpoint, the relative retention (α) was used for this study, which is defined in Eq. (2) as:

$$\alpha = \frac{\kappa_2}{\kappa_1} \quad (2)$$

in which κ_2 and κ_1 are the retention factors of a pair of critical analytes, BMEE and BMEZ, respectively.

Resolution factor is an overall measurement of separation, which is depending on the selectivity (α), efficiency (N), and retention factor (κ) as expressed in Eq. (3):

$$R_s = \frac{N^{1/2}}{4} \frac{\alpha - 1}{\alpha} \frac{\kappa}{\kappa + 1} \quad (3)$$

In practice, the resolution is calculated using the retention time and peak width obtained from the chromatograms. In this report, the resolution was calculated by Chemstation using Eq. (4):

$$R_s = 1.18 \times \frac{t_2 - t_1}{w_1 + w_2} \quad (4)$$

Table 4
Summary of retention time of BMV, retention factor of BMV, selectivity of BMEE vs. BMEZ and resolution factor of BMEE vs. BMEZ from the Halo C_{18}, ACE C_{18} and Chromolith C_{18} columns.

Column property	ACE C_{18}	Halo C_{18}	Chromolith C_{18}
Retention time (BMV, min)	10.8	8.1	7.7
Retention factor (BMV)	9.2	8.5	5.0
Selectivity (BMEE vs. BMEZ)	1.07	1.06	1.04
Resolution factor (BMEE vs. BMEZ)	1.67	1.60	1.05

where t_2 and t_1 are the retention time of the critical pair of BMEE and BMEZ, respectively; w_2 and w_1 are the peak width at half peak height of BMEE and BMEZ, respectively.

The retention factor of BMV and selectivity and resolution factor for BMEZ and BMEE were evaluated by analyzing a standard mixture of these three compounds on each of the three columns under identical isocratic HPLC conditions (42:58 acetonitrile:water, v/v). Fig. 2 shows overlaid chromatograms of the standard mixture of BMV, BMEE, and BMEZ for the Chromolith C_{18} column, Halo C_{18} column, and the ACE C_{18} column. Table 4 shows the retention time and retention factor of BMV, selectivity and resolution between BMEE and BMEZ that were obtained from Fig. 2.

As seen from Fig. 2 and Table 4, the elution order is the same for these three compounds on all columns. BMV has similar retention time on the Halo C_{18} and Chromolith C_{18} columns while the ACE C_{18} column retained BMV approximately 30% longer. The longer retention of BMV on the ACE C_{18} column is due to the higher carbon loading of the ACE C_{18} column as shown in Table 1. The retention factors of BMV are similar on the ACE C_{18} and Halo C_{18} columns while the Chromolith C_{18} column gives only slightly more than half of the retention factor for BMV. The retention factor of BMV is a ratio of the retention time of BMV and t_0. The retention time of BMV on the ACE C_{18} column is about 30% longer than that on the Halo C_{18} column as seen from the data in Table 4 and the t_0 of the ACE C_{18} column is also approximately 20% more than that of the Halo C_{18} column as seen from the data in Table 3. Therefore, the retention factors of BMV are similar on both the ACE C_{18} and Halo C_{18} columns. The retention time of BMV on the ACE C_{18} column is about 30% longer than that on the Chromolith C_{18} column as seen from the data in Table 4 and the t_0 of the ACE C_{18} column is approximately 20% less than that of the Chromolith C_{18} column as seen from the data in Table 3, which leads to approximately 45% less retention factors of BMV on Chromolith C_{18} than on ACE C_{18}. The selectivity of BMEE vs. BMEZ are 1.07, 1.06, and 1.04 for ACE C_{18}, Halo C_{18}, and Chromolith C_{18} column, respectively. The difference of selectivity for BMEE and BMEZ obtained from the three columns could be attributed to the different characteristics of the silica substrate since all three columns have the C_{18} bonded phase, and the HPLC conditions were kept identical. Under the same mobile phase conditions, the resolution factor of BMEE vs. BMEZ are 1.67, 1.60, and 1.05 for ACE C_{18}, Halo C_{18}, and Chromolith C_{18} column, respectively. While BMEE and BMEZ are baseline separated on ACE C_{18} and Halo C_{18} column, they are only partially separated on Chromolith C_{18} column. The higher resolution obtained from ACE C_{18} and Halo C_{18}, than that from Chromolith C_{18} column are due to not only the higher retention factor and slightly better selectivity but also the column efficiency that is discussed in detail in Section 3.2. The higher retention factors, slightly better selectivity, and better resolution factors exhibited by both the ACE C_{18} and Halo C_{18} column make these two columns better for complex sample separations as compared to the Chromolith C_{18} column.

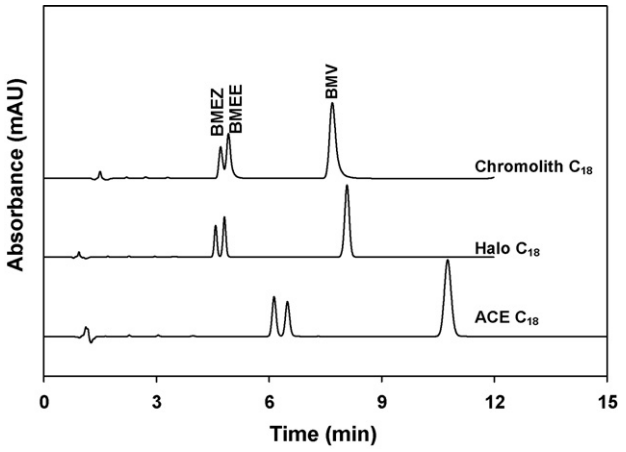


Fig. 2. Overlay of the chromatograms of a standard mixture of BMV, BMEE, and BMEZ using ACE C_{18} (bottom), Halo C_{18} (middle), and Chromolith C_{18} (up) columns. HPLC conditions: acetonitrile:water 42:58 (v/v) at 1.0 mL/min. Other HPLC conditions are the same as those in Section 2.

3.2. Efficiency

The column efficiency is another key factor for column selection. The column efficiency, expressed as the plate number, is calculated by dividing the column length to the "height equivalent to a theoretical plate". According to the van Deemter equation [20] as listed below:

$$H = A + \frac{B}{\upsilon} + C\upsilon \tag{5}$$

"height equivalent to a theoretical plate" (H) is determined by term A, Eddy diffusion or nonuniformity of the packed bed; term B, longitudinal diffusion of the analyte, and term C, mass transfer of the analyte in mobile phase and stationary phase. Silica substrate can contribute to all the three A, B, and C terms and, thus, affect the column efficiency. At high velocity, C term (mass transfer term) has the biggest contribution to the H.

The plate height from mass transfer can be expressed using Eq. (6) as listed below [21–23]:

$$H = H_M + H_S = \left(\frac{1}{96} \frac{d_c^2}{D_M} \frac{11k^2 + 6k + 1}{(k+1)^2} + \frac{2}{3} \frac{d_S^2}{D_S} \frac{k}{(k+1)^2} \right) \nu \tag{6}$$

in which H_M is the resistance to mass transfer in mobile phase ($H_m = C_m \cdot \upsilon$), H_S is resistance to mass transfer in stationary phase ($H_S = C_s \cdot \upsilon$), and H is the sum of total resistance to mass transfer.

In literature, the column efficiency is often reported using non-retained compounds such as thiourea, sodium nitrate and, etc. as a probing analyte running under isocratic condition. As indicated by Eq. (6), those reported efficiency will be higher than that obtained with retained compounds. In practical HPLC analysis of pharmaceutical products, compounds are always well retained. Therefore, the efficiency for retained compounds is more relevant and meaningful. As shown in Eq. (6), the resistance to mass transfer H is a function of retention factor κ. Therefore, it is important to use the same compound with the same retention factors (κ) from the columns to be compared in column efficiency. In this study, the column efficiency of BMV at two retention factors, $\kappa \sim 8.5$ and $\kappa \sim 2.5$, from each column was obtained and compared. The two retention factors, 2.5 and 8.5 covered the typical retention factor range in pharmaceutical analysis. The isocratic conditions to obtain the two retention factors for BMV at ~0.2 mg/mL in diluent (30/70 water acetonitrile, v/v) in the three columns are listed in Table 2. The flow rates were varied from 0.2 to 0.4, 0.6, 0.8, 1.0, 1.2, 1.5, 1.8, 2.0, 2.3, 2.6, and 3.0 mL/min for all three columns. In addition, flow rates at 3.3, 3.6, and 4.0 mL/min were used for ACE C_{18} column, and flow rates at 3.3, 3.6, 4.0, 4.3, 4.6, and 5.0 mL/min were used for Chromolith C_{18} column. Higher flow rates were not explored due to the pressure

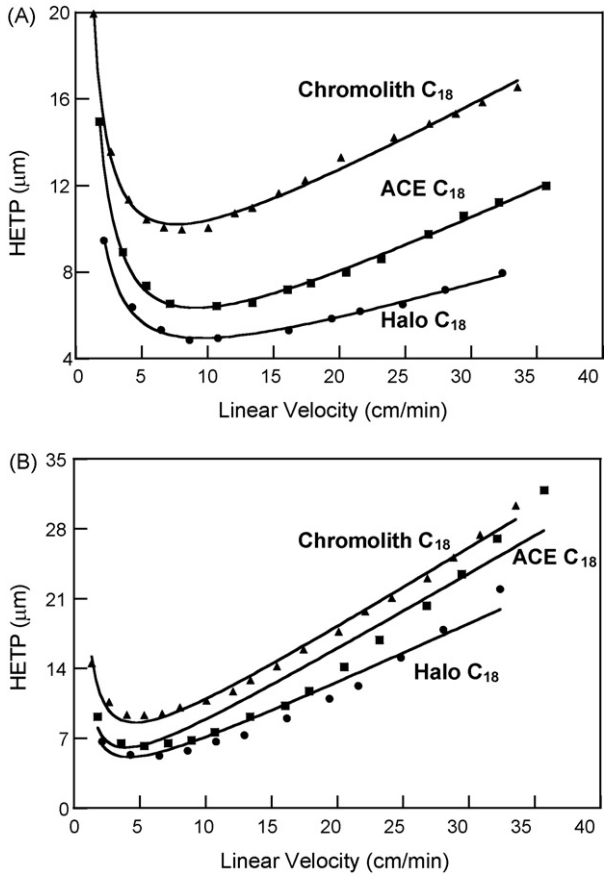


Fig. 3. The van Deemter plots for betamethasone-17-valerate (BMV) at $\kappa \sim 8.5$ (A) and $\kappa \sim 2.5$ (B) from the ACE C_{18}, Halo C_{18} and Chromolith C_{18} columns. HPLC conditions: the same as that in Section 2 except the flow rate that varied from 0.2 to 3.0 mL/min for the Halo C_{18} column, 0.2 to 4.0 mL/min for the ACE C_{18} column, and 0.2 to 5.0 mL/min for the Chromolith C_{18} column.

and flow rate limit of Agilent HPLC instrument, which has a maximum pressure rating of 6000 psi and flow rate of 5.0 mL/min. The Van Deemter plot of calculated plate height vs. flow rate for BMV at retention factors $\kappa \sim 8.5$ and $\kappa \sim 2.5$ from each of the three columns are shown in Fig. 3A and B, respectively. The data obtained by fitting the Van Deemter plot using Van Deemter equation were presented in Table 5.

As shown in Fig. 3A and B, and Table 5, the Van Deemter plots from the three columns fitted well for BMV at $\kappa \sim 8.5$. However, the Van Deemter plots for BMV at $\kappa \sim 2.5$ for the Halo C_{18} and ACE C_{18} columns did not fit as well as those at $\kappa \sim 8.5$. The most probable cause for the poor Van Deemter curve fitting at $\kappa \sim 2.5$ could be due to the effect of extra column volume for the less retained compounds. As predicated by Eq. (6), the Van Deemter plots of BMV at different retention factors are different. Also, for all three columns, the plate height increases with the increase of linear velocity at high flow rate at $\kappa \sim 2.5$ is much faster that that at $\kappa \sim 8.5$, which indicated that the mass transfer in stationary phase is dominant over the mass transfer in mobile phase by Eq. (6).

As seen from Table 5, the Halo C_{18} column shows the smallest plate height and, thus, the highest efficiency ($\sim 2.0 \times 10^5$ plates/m) at both retention factors among three columns. The ACE C_{18} column gave an optimum column efficiency of 1.6×10^5 plates/m for BMV, which is ~20% lower than that obtained for the Halo C_{18} column. The Chromolith C_{18} column generated the highest optimum plate height and, thus, lowest column efficiency (1.0×10^5 to 1.2×10^5 plates/m) among three columns. For a well packed column, the optimum plate height should be equal to approximately 2 times of particle size. For example, the plate height should be 6 μm (i.e., 1.7×10^5 plates/m) for a well packed column with 3 μm particles and 10 μm (i.e., 1.0×10^5 plates/m) for a column packed with 5 μm particles. Although monolithic column is not a true packed column with particles, the optimum column efficiency of BMV obtained from the Chromolith C_{18} column is comparable with columns packed with 5 μm particles. The particle size of the Halo C_{18} column is 2.7 μm, which is about 90% of the particle size of the ACE C_{18} column (3 μm). Therefore, it is expected that the Ace C_{18} column generates approximately 10% lower optimum column efficiency than the Halo C_{18} column. The extra 10% higher optimum column efficiency of Halo C_{18} than Ace C_{18} could be due to Halo column's unique silica substrate. The silica substrate in Halo C_{18} column has fused-core with superficially coated 0.5 μm porous layer that has less stagnant mobile phase in the pores of fused-core silica than the same size porous silica, resulting in a smaller B term and much smaller C term as shown in Table 5. The C term for BMV on the Halo C_{18} column at $\kappa \sim 2.5$ is approximately 2 times higher than that at $\kappa \sim 8.5$. This indicates that at high flow rate of the mobile phase, Halo C_{18} column is more suitable for fast separation for highly retained compounds but it may not be the best choice for less retained compounds.

3.3. Separation impedance

It is well known that, for packed column, the higher efficiency obtained with smaller particles comes with a price of higher column back pressure. With the pressure limit of a typical HPLC system at ~6000 psi, a better measurement for overall column performance is to use separation impedance E, a dimensionless factor proposed by Bristow and Knox [24], which is defined by Eq. (7) as shown below:

$$E = \frac{t_0 \Delta P}{N^2 \eta} \quad (7)$$

where t_0 is the zero time, ΔP is the column back pressure, N is the plate count, and η is the viscosity.

The separation impedance, E, is typically around 5000 for a typical HPLC column, but can be as low as 3000 for a well manufactured column. The separation impedance vs. linear velocity plots on the ACE C_{18}, Halo C_{18}, and Chromolith C_{18} columns for BMV at $\kappa \sim 8.5$ and $\kappa \sim 2.5$ are shown in Fig. 4A and B, respectively.

Table 5
Efficiency parameters from van Deemter plot from the Halo C_{18}, ACE C_{18} and Chromolith C_{18} columns.

κ	Column (4.6 mm × 100 mm)	R	A (cm × 10^{-4})	B (cm^2/min × 10^{-4})	C (min/cm^2 × 10^{-4})	υ_{opt} (cm/min)	H_{opt} (μm)	N_{opt} (plates/m)
8.5	ACE C_{18}	0.998	1.07	24.10	0.29	9.1	6.4	1.6×10^5
	Halo C_{18}	0.996	1.49	16.57	0.18	9.6	5.0	2.0×10^5
	Chromolith C_{18}	0.996	5.13	19.34	0.33	7.6	10.2	1.0×10^5
2.5	ACE C_{18}	0.982	0.0	11.94	0.77	3.9	6.1	1.6×10^5
	Halo C_{18}	0.979	0.0	10.79	0.60	4.2	5.1	2.0×10^5
	Chromolith C_{18}	0.996	1.00	17.56	0.82	4.6	8.6	1.2×10^5

κ = retention factor; R = correlation coefficient of van Deemter fitting; A = eddy diffusion constant; B = longitudinal diffusion constant; C = mass transfer constant; υ_{opt} = optimum linear velocity; H_{opt} = optimum plate height; N_{opt} = optimum column efficiency.

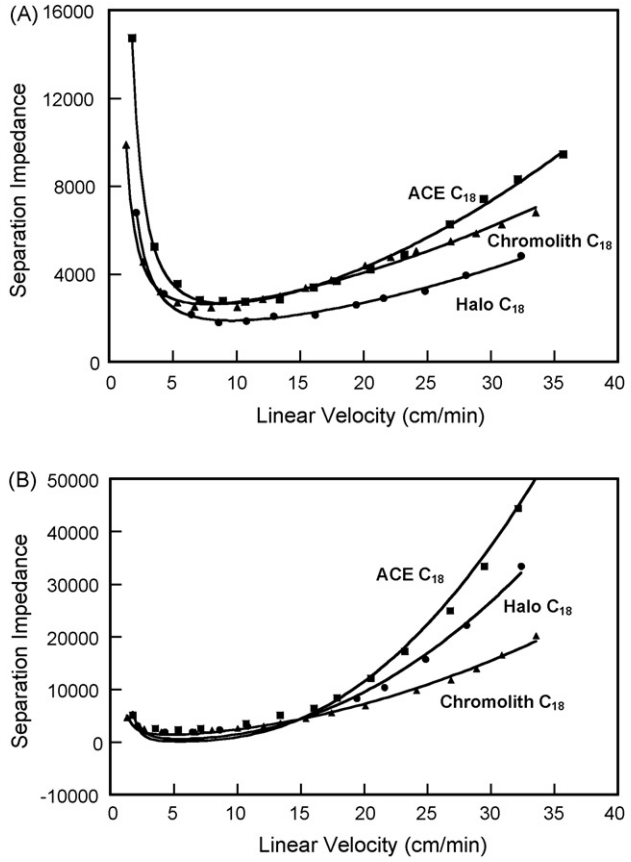


Fig. 4. Plots of separation impedance vs. linear velocity for betamethasone-17-valerate (BMV) at $\kappa \sim 8.5$ (A) and $\kappa \sim 2.5$ (B) from the ACE C_{18}, Halo C_{18} and Chromolith C_{18} columns. HPLC conditions: the same as that in Section 2 except the flow rate that varied from 0.2 to 3.0 mL/min for Halo C_{18} column, 0.2 to 4.0 mL/min for ACE C_{18} column, and 0.2 to 5.0 mL/min for Chromolith C_{18} column.

As shown in Fig. 4A, at $\kappa \sim 8.5$, the separation impedance of Halo C_{18} reached as low as 1800 when the linear velocity is 9 cm/min and increased to 4800 at a linear velocity of 32 cm/min. The separation impedances of the ACE C_{18} column range from 2700 at 9 cm/min to 4200 at 20 cm/min, and the separation impedances of the Chromolith C_{18} column range from 2500 at 10 cm/min to 4400 at 20 cm/min, which suggests that the total column performance for highly retained compounds on these two columns at similar flow rates are comparable. The Halo C_{18} column generated the lowest optimum separation impedance for BMV at $\kappa \sim 8.5$, which further confirmed the conclusion from efficiency study that the Halo C_{18} column is most suitable for fast separation of highly retained compounds.

Fig. 4B shows that all three columns had similar optimum separation impedance at low flow rate for BMV at $\kappa \sim 2.5$. However, the separation impedance of the Chromolith C_{18} column increased slowest with the increase of mobile phase flow rates. Therefore, the Chromolith C_{18} column is more suitable for fast separation of less retained compounds under high flow rate, which would be ideal for simple methods such as dissolution test, content uniformity, high throughput screening, etc.

3.4. Sample loading capacity

Sensitivity of an HPLC method for the analysis of pharmaceutical product to conduct stability studies of the products is very critical. The quantitation limit of a typical method has to be less than or equal to the drug impurity/degradant reporting threshold according to International Conference on Harmonization (ICH) guidelines.

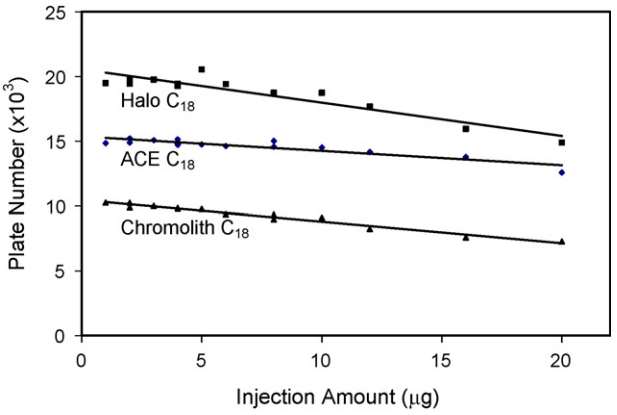


Fig. 5. Plot of column plate number vs. the injection amounts of betamethasone-17-valerate (BMV) on the ACE C_{18}, Halo C_{18} and Chromolith C_{18} columns. HPLC conditions: the same as that in Section 2 for BMV at $\kappa \sim 8.5$ except the injection volume varied from 10 to 100 μL.

The ICH reporting threshold of drug impurities/ degradants is typically at least 0.1% for drug substance and 0.2% for drug product. One way to increase the method sensitivity is to increase sample load on the column by injecting large volume of sample solution or by increasing the concentration of sample solution or a combination of both. However, the amount of active pharmaceutical ingredient (API) is typically 100–2000 times higher than the drug impurites/degradants; this situation could overload the column, which will result in loss of resolution of the critical pairs, loss of column efficiency, deteriorated peak shape and/or nonlinear response for the active pharmaceutical ingredient. Therefore, for pharmaceutical analysis such as for a stability indicating test method it is often required for HPLC column to have large sample loading capacity.

Typically, the sample loading capacity is defined as the maximum amount of sample load that will result in less than 10% loss of column efficiency [22]. It was found that the injection volumes from 10 to 100 μL had no impact on column efficiency. In this study, the sample loading capacities for these three columns were evaluated by a combination of BMV standard concentration (0.1, 0.2, 0.3, 0.4 and 0.5 mg/mL) and injection volume (10 and 40 μL) because BMV has limited solubility in diluent. The sample loading capacity of the three columns was evaluated by injecting BMV ranging from 1 to 20 μg. The plate numbers of BMV were plotted vs. absolute injection amounts (μg) from all injections as shown in Fig. 5.

As seen from Fig. 5, all three columns showed efficiency loss with the increase of sample load. However, the efficiency loss on the fused-core Halo C_{18} and monolithic Chromolith C_{18} columns are higher than that on the porous ACE C_{18} column. The calculated sample loading capacities for the ACE C_{18}, Halo C_{18}, and Chromolith C_{18} columns with a 4.6 mm × 100 mm column dimension are 17.9, 11.7, and 7.4 μg, respectively. The difference of sample loading capacity from the three columns can be attributed to total surface area

Table 6
Summary of linearity results for BMV and BMEE from the Halo C_{18}, ACE C_{18} and Chromolith C_{18} columns.

	BMEE	BMV
Halo C_{18}	$y = 57{,}187x - 1.2$ $R^2 = 1.0000$	$y = 37{,}568x + 0.2$ $R^2 = 1.0000$
ACE C_{18}	$y = 47{,}725x - 0.7$ $R^2 = 1.0000$	$y = 31{,}370x - 9.1$ $R^2 = 1.0000$
Chromolith C_{18}	$y = 37{,}078x - 1.3$ $R^2 = 0.9998$	$y = 25{,}259x - 22.1$ $R^2 = 1.0000$

Table 7
Summary of retention time, plate numbers, and tailing factor of BMV, and resolution factor of BMEE vs. BMEZ from injections #1 and #320 using the Halo C_{18}, ACE C_{18} and Chromolith C_{18} columns.

Column type	RT (min)			Tailing factor			Plate number			Resolution factor of BMEE vs. BMEZ		
	#1	#320	Δ%	#1	#320	Δ%	#1	#320	Δ%	#1	#320	Δ%
ACE C_{18}	8.37	8.41	0.5	1.05	1.04	−1.0	15,294	14,159	−7.7	1.60	1.56	−2.6
Halo C_{18}	8.70	8.58	−1.4	0.97	0.96	−1.0	19,620	19,348	−1.4	1.74	1.75	0.5
Chromolith C_{18}	9.38	9.36	−0.2	1.42	1.42	0.0	10,292	10,426	+1.3	1.54	1.53	−0.6

of packing materials in each column. The total surface of a column is proportional to the surface area and the bulk density of packing material. As shown in Table 1, the surface area of the ACE C_{18} column is 300 m^2/g, compared to 150 m^2/g for the Halo C_{18} column. Although the bulk density of the Halo C_{18} column stationary phase is about 20–30% heavier than the ACE C_{18} stationary phase (according to manufacturer) because the Halo silica has a fused-core and the ACE column has a totally porous silica, the total surface area per column for the halo column is still about 30% less than that for the ACE column. On the other hand, the Chromolith C_{18} column has comparable surface area to the ACE C_{18} column (300 m^2/g); however, the bulk density of the monolith of Chromolith C_{18} is much smaller than the silica of the ACE C_{18} column because of the large macropore structure of the monolith. This leads to lower sample loading capacity for the Chromolith C_{18} column than that of the ACE C_{18} column. The large sample loading capacity of the porous ACE C_{18} column suggests that the ACE C_{18} column is more suitable for analyses that would require high sample loading to obtain adequate sensitivity for the intended purpose of the method while the Chromolith column is least preferred for such application.

3.5. Linearity

Linearity is a critical criterion for quantitative pharmaceutical analysis particularly for stability indicating test methods where small amount related compounds must be quantitated with the presence of large amount of active pharmaceutical ingredient. The linear response range is mainly dependent upon the detector; however, the column itself is also a key attribute to the method linearity. Columns with peak tailing and adsorption of analytes will significantly impact the linearity of related compounds at low concentration level.

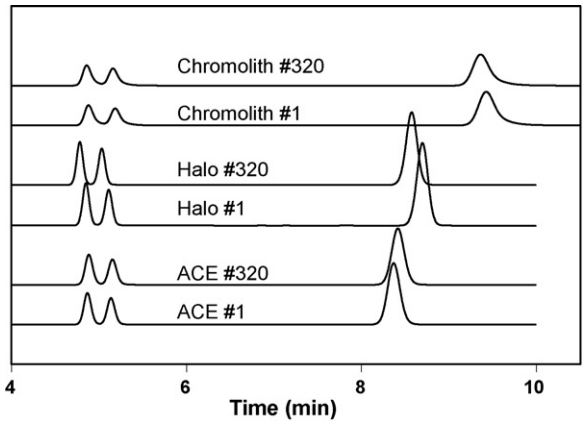


Fig. 6. Overlay of chromatograms of the injections #1 and #320 of a Celestoderm-V Ointment® placebo spiked with BMV, BMEE and BMEZ from ACE C_{18}, Halo C_{18}, and Chromolith C_{18} columns from bottom to top. HPLC conditions: the same as that in Section 2 for BMV at $\kappa \sim 8.5$.

The linearity solutions that contains 0.8, 1.6, 2.4, 3.2, and 4.0 μg/mL BMEE (corresponding to 0.2–1.0% of label claim of Celestoderm-V cream) and 0.1, 0.2, 0.3, 0.4 and 0.5 mg/mL BMV (corresponding to 25–125% Label Claim of Celestoderm-V Ointment®) were prepared by dissolving reference materials in diluent. To study linearity, the isocratic methods for BMV at $k \sim 8.5$ as given in Table 2 were used. The flow rates were 1.0, 1.2, and 1.5 mL/min for the Halo C_{18}, ACE C_{18} and Chromolith C_{18} columns, respectively. The flow rates were adjusted for different columns so that similar linear velocities were maintained to obtain similar retention times for BMV. The linearity was assessed by linear regression analysis for related compounds, BMEE, at 0.2–1.0% of label claim and for active pharmaceutical ingredient, BMV, at 25–125% of label claim. The coefficient of determination (R^2) and y-intercept for BMEE and BMV were calculated and summarized in Table 6.

As shown in Table 6, the coefficient of determination (R^2) for BMEE and BMV are all greater than 0.999 on all three columns. On all three columns, the y-intercept values indicated no significant bias for the analysis of BMEE and BMV.

3.6. Column lifetime

A column after extensive usage and aging can show retention time change, efficiency loss, and/or peak tailing, and, thus may fail the method's system suitability requirements such as the resolution of the critical pair and the precision of repeatable standard injections, the peak identification via retention time or relative retention time, method specificity, and accurate quantitation. In quality control analysis of a pharmaceutical product, diluent blank, system suitability solutions, and sample solutions are typically prepared for each sample set. A robust column should endure hundreds or even thousands of injections of solutions which would help to control the cost per analysis in quality control labs.

The column lifetime was studied by consecutively injecting the column lifetime evaluation solution. For each column, 320 consecutive injections were made using the isocratic method at $\kappa \sim 8.5$ as listed in Table 2. Total run times for 320 injections were 3200 min for Halo C_{18} and ACE C_{18} column, and 3840 min for Chromolith C_{18} column.

An overlay of chromatograms from the first injection and the 320th injection for the ACE C_{18}, Halo C_{18} and Chromolith C_{18} columns is shown in Fig. 6. The retention time, tailing factor, and plate number of BMV, and the resolution factor between BMEE and BMEZ from the 1st injection and the 320th injection were calculated, and summarized in Table 7. Visual inspection of the chromatograms in Fig. 6 suggests that there is no significant change in elution pattern and chromatogram after 320 injections, which is consistent to similar values in retention time and tailing factor as shown in Table 7. The retention time of BMV on Halo C_{18} column decreased about 0.12 min or about 1.4% after 320 injections, largest among the three columns studied. The efficiencies of the Halo C_{18} and Chromolith C_{18} columns were almost unchanged. However, the efficiency of the ACE C_{18} column decreased about 7.7% over 320 injections, which is consistent to the decrease of resolution factor between BMEE and BMEZ

from 1.60 to 1.56. From the results in Table 7, it is clear that key system suitability requirements such as resolution and tailing factor can still be met after 320 consecutive injections. Therefore, it is concluded that all three columns can be used for at least 320 injections of Celestoderm-V Ointment® sample solutions.

4. Conclusions

In this study, the performance of the fused-core silica Halo C_{18} column, the monolithic silica rod Chromolith C_{18} column, and porous silica ACE C_{18} column was compared for Celestoderm-V Ointment® analysis. The data obtained from this study for three different types of silica based C_{18} columns clearly demonstrated that the ACE C_{18} column has the longest retention time for BMV, best selectivity between BMEE and BMEZ, and also has the highest sample capacity compared to the other two columns. Therefore, ACE C_{18} column is more suitable for stability indicating analytical methods for Celestoderm-V Ointment® that would require high sensitivity to determine low level impurities and/or degradation products in pharmaceutical products. On the other hand, the fused-core Halo C_{18} column has the highest column efficiency and smallest mass transfer resistance for highly retained compounds, and therefore, is most suitable for analytical methods that would require fast analysis such as assay and stability indicating method for Celestoderm-V Ointment®. As increasing flow rates of the mobile phase has less impact on the separation impedance of the Chromolith C_{18} column for less retained compounds, this column is most suitable for fast and simple analytical methods such as assay for Celestoderm-V Ointment®. The low back pressure of the Chromolith C_{18} column also provides an option to use solvents in the mobile phase with high viscosity (e.g. isopropanol) which can provide more flexibility to obtain the optimum separation conditions of a method.

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