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Effects of extra-column band spreading, liquid chromatography system operating pressure, and column temperature on the performance of sub-2-μm porous particles

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ABSTRACT

The effects of extra-column band spreading, LC system operating pressure, and separation temperature were investigated for sub-2-μm particle columns using both a conventional HPLC system as well as a UPLC® system. The contributions of both volume- and time-based extra-column effects were analyzed in detail. In addition, the performance difference between columns containing 2.5 and 1.7-μm particles (same stationary phase) was studied. The performance of these columns was compared using a conventional HPLC system and a low dead volume UPLC system capable of routine operation up to 1000 bar. The system contribution to band spreading and the pressure limitations of the conventional HPLC system were found to be the main difficulties that prevented acceptable performance of the sub-2-μm particle columns. Finally, an increase in operating temperature needs to be accompanied by an increase in flow rate to prevent a loss of separation performance. Thus, at a fixed column length, an increase in temperature is not a substitute for the need for very high operating pressures.

1. Introduction

Since its first use in the mid-1970s, high performance liquid chromatography (HPLC) has become the premier separation technique in almost all analytical chemistry laboratories. It has utility in many diverse application areas including pharmaceutical R&D, pharmaceutical product quality testing, environmental analysis, clinical diagnostic testing, and food safety analysis. This is mainly due to its versatility, ease of use, compatibility with the sample, and the selectivity and resolving power of the chromatography process. The chromatographic separating power of HPLC is dependent upon the selectivity of the mobile phase/stationary phase system and the efficiency of the HPLC column. The column efficiency is dependent upon multiple factors: the diffusion coefficient of the sample in the mobile phase, which in turn depends on the mobile phase viscosity and the molecular weight of the analyte, but more importantly on the column length and the packing particle size (d_p) together with the mobile phase velocity. At a fixed velocity, the column efficiency increases in direct proportion to the column length. At the minimum of the plate height versus velocity curve, the column efficiency increases in inverse proportion with the particle size. However, practical chromatography is performed above the Van Deemter minimum, and under these circumstances, the plate count increases with a higher power of the inverse of the particle size [1]. In extreme cases (i.e., at very high velocities that can be reached only with very short columns), the plate count increases with the inverse of the particle size squared.

Over the last 40 years, the evolution of stationary phase packing materials for HPLC has seen extraordinary improvement in multiple areas: particle synthesis and characterization [2], different bonding chemistries for alternate selectivity [3], chemical and mechanical stability, and reduction in particle size [1]. The change in particle size from irregular-shaped, 30-μm particles (1970s) to sub-2-μm spherical particles today has allowed analytical scientists to use shorter columns for faster separations, or to use the smaller particles in longer columns for improved efficiency/peak capacity in a reasonable analysis time. One side effect of using smaller particles is the substantial increase in backpressure observed. As is known from the Kozeny–Carman equation, at a fixed velocity, column backpressure is inversely proportional to the square of the particle size [4,5]. Therefore, decreasing the particle size by a factor of 2 increases the backpressure by a factor of 4. In addition, at the minimum of the plate height versus velocity curve, a smaller particle requires a higher linear velocity. Under these circumstances, the pressure increases by a factor of 8 for a 2-fold reduction in particle size [6]. With pressure limitations of ~400 bar (6000 psi) or less, conven-

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tional LC instrumentation is not capable of running columns packed with particles less than 2–3-μm in size without significant sacrifice in performance. This led to the development of chromatography instruments that use significantly higher inlet pressures [7–10].

Speed also improves with elevated temperature [7,11–21]. However, one needs to keep several issues in mind. The pressure required to reach the minimum plate height does not decrease with elevated temperature; rather, it increases, albeit only mildly, at a fixed retention factor [7,19]. Furthermore, one needs to differentiate between the pure change in retention from increased temperature and the improvements in column performance due to faster diffusion and mass transfer at elevated temperature. The former leads to a deterioration of the separation space at constant mobile phase composition. The latter, however, is the key point of operating at elevated temperature, and this issue has been taken into consideration in the literature [13]. A recent review has covered the subject of using elevated temperature in great detail [11]. Of course, elevated temperature can be used in conjunction with sub-2-μm particles to combine these benefits for high efficiency and high-speed separations.

Since their commercial introduction in the mid-1990s, sub-2-μm particle columns have grown in popularity due to the implications for higher chromatographic separating power and throughput. However, using sub-2-μm particle columns at their optimal linear velocities results in pressures that exceed the pressure limit of current instrumentation. Within the last 5 years, several instruments have been commercialized that are capable of routinely operating at pressures up to 1000 bar (14,500 psi) [7–10]. These systems allow sub-2-μm particles to be used in longer column lengths and at higher linear velocities for improved speed, resolution, and sensitivity over conventional HPLC. Introduction of these systems into the marketplace was also accompanied by the development and commercialization of sub-2-μm packing materials that have excellent chemical and mechanical stability [22]. This combination of high pressure, low band spread instrumentation and columns capable of routinely withstanding high pressure, elevated temperature, and a wide pH range has been successfully used in the analytical laboratory for improved throughput, separation efficiency, and analyte detection limits.

Extra-column band spreading affects the measured performance of columns packed with smaller particles, especially for columns with an internal diameter smaller than the classical standard of 4.6 mm. Recently, several published reports characterizing the performance of sub-2-μm particle columns have identified extra-column effects as a major factor that negatively impacts their performance [12,23–26]. These reports either used UPLC/UHPLC systems with low band spread values for sub-2-μm particle column performance evaluations, or conventional HPLC systems with corrections for extra-column effects. In other reports, sub-2-μm particle columns with a small internal diameter have been compared to columns packed with 2–3-μm particles in larger diameter columns without taking into account the detrimental effects of extra-column band spreading [27–29]. In the current paper, the influence of extra-column effects on column performance is described and clarified. In the same context, the influence of elevated pressure and temperature on column efficiency will be revisited.

2. Experimental

2.1. Chemicals

Water (18.2 MΩ-cm) was purified in-house using a Milli-Q Academic system (Millipore, Billerica, MA, USA). Ammonium acetate (98.2%), HPLC grade acetonitrile (ACN) and methanol (MeOH), and tert-amylbenzene were from ThermoFisher Scientific (Waltham, MA, USA). Glacial acetic acid was from J.T. Baker (Phillipsburg, NJ, USA). Ammonium hydroxide (28–30%), thiourea, and acenaphthene were supplied by Sigma Aldrich (St. Louis, MO, USA). The didanosine system suitability mixture was obtained from the U.S. Pharmacopeia (Rockville, MD, USA).

2.2. Chromatographic conditions

Separations were performed either on an unmodified, commercially available ACQUITY UPLC® system equipped with an ACQUITY UPLC® PDA detector, or an Alliance® 2695 Separations Module equipped with a 2998 PDA detector (all from Waters, Milford, MA, USA). The Alliance® 2695 system was used as the conventional HPLC system for all experiments. All data were collected using Empower software (Waters). The measured system volume for the ACQUITY UPLC® system was 105 μL, and band spreading was $\sigma_v = 2.8$ μL. The measured system volume for the Alliance® 2695 system was 876 μL, and band spreading was $\sigma_v = 7.2$ μL.

The system volumes for both the ACQUITY UPLC® system and the Alliance® 2695 system were measured using the following protocol: using a low dead volume union in place of the chromatographic column, the signal at 254 nm was collected for an isocratic flow of 100% ACN for 5 min, at which time a step gradient to 100% ACN containing 0.05 mg/mL uracil was performed. The UV trace at 254 nm was collected for additional 5 min. The absorbance difference between the flat portions of the UV chromatogram was measured, and the time (in minutes) at 50% of this absorbance difference was recorded. The start time of the step gradient was then subtracted from this value, and the time difference was multiplied by the flow rate to give the system volume. The flow rate for measuring the ACQUITY UPLC® system volume was 0.2 and 1.0 mL/min for the Alliance® system.

All band spreading measurements were made by injecting a 0.01 mg/mL solution of thiourea in 65/35 ACN/H_2O. Flow rates used were 0.05, 0.1, 0.15, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8, and 2.0 mL/min. Peak width was measured at 13.4% peak height (4σ). The diffusion coefficient for thiourea was estimated to be 1.8×10^{-5} cm^2 s^{-1} using the Wilke–Chang equation.

For Van Deemter measurements using acenaphthene as the test probe, two different columns were evaluated: an ACQUITY UPLC® BEH C_{18} column, 2.1 mm × 50 mm, 1.7 μm, and an XBridge™ C_{18} column, 2.1 mm × 50 mm, 2.5 μm (both from Waters). The mobile phase was 65/35 ACN/H_2O. Separations were performed isocratically in the flow rate range of 0.05–1.0 mL/min. Column temperature was maintained at 30 °C. Detection was performed at 254 nm using a sampling rate of 40 Hz and no filter time constant. The sample consisted of 0.01 mg/mL thiourea (void marker) and 0.2 mg/mL acenaphthene in 65/35 ACN/H_2O. Injection volume was 1 μL. For the ACQUITY UPLC® system, injection was performed using a 1 μL sample loop in the full loop injection mode. The Alliance® 2695 was equipped with a 100 μL sample loop. A 250 psi restrictor was placed on the outlet side of the 2998 PDA detector connected to the Alliance® system to generate suitable backpressure when the column was replaced with a zero-volume union.

Separation of the didanosine system suitability mixture was performed using XBridge™ C_{18} columns in two different dimensions: 4.6 mm × 150 mm, 5 μm and 4.6 mm × 100 mm, 3.5 μm. An ACQUITY UPLC® BEH C_{18} column (2.1 mm × 50 mm, 1.7 μm) was also used for separation of this mixture. Mobile phase A was 10 mM ammonium acetate with 0.02% acetic acid (pH 5.1). Mobile phase B was 100% ACN. The gradient for the 4.6 mm × 150 mm, 5 μm dimension was as follows: initial hold at 2% B for 0.54 min, then to 15% B in 6.7 min, reset to 2% B at 7.94 min, equilibrate until 10.76 min. Flow rate was 1.0 mL/min. Injection volume was 28.8 μL. The sample was prepared at a concentration of 0.5 mg/mL