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Reduction of the extra-column band dispersion by a slow transport of a sample band from the injector to the column in isocratic reversed-phase liquid chromatography

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ABSTRACT

Extra-column band dispersion during the transport of a sample band from the injector to the column can be reduced by a flow rate program starting with a low flow rate until the sample band has approached to, or just entered into the column, followed by an increased flow rate suitable for the solute separation in the column. Such a sample introduction method increased the plate counts of a 50 mm long column, 1.0 or 2.1 mmID, especially for early-eluting solutes by up to several times compared to a conventional elution method, when a 0.254 mmID, 15.2 cm connection tubing was used. Increase in plate counts of up to 50–70% was possible for solutes with retention factors smaller than 1.0 for the columns connected with a 0.13 mmID, 15 cm tube. The method also seems to reduce the contribution of the void space at the column inlet to the band dispersion. The elution method including a slow transport of the sample band in the pre-column space of 10 μL or less may require a little longer separation time than normal elution, but it was shown to be effective for increasing the observed efficiency of a small column for solutes with small retention factors.

1. Introduction

It is known that the band dispersion outside the column makes a significant contribution to the practically obtainable column efficiency in HPLC and UHPLC [1–7]. The effects of various parts of a LC system, namely the injector, the detector, and the data-processing units as well as those of connecting tubes have been reported [8–13].

The variance of a peak (the dispersion of a solute band passing through the entire apparatus, σ_{total}^2) using a certain HPLC or UHPLC instrument and a column is expressed as a sum of the dispersion of the solute band produced by the column, σ_{col}^2, and the dispersion of the solute band in the flow path outside the column, σ_{extra}^2, in Eq. (1).

$$\sigma_{total}^2 = \sigma_{col}^2 + \sigma_{extra}^2 \quad (1)$$

The extra-column dispersion of the solute band (σ_{extra}^2) consists of several factors as described in Eq. (2): (i) dispersion of the solute band in the sample injection device (inj) and in the connecting tube from the sample injector to the column inlet (tube 1), $\sigma_{inj}^2+\sigma_{tube1}^2$, (ii) dispersion of the solute bands in the tube from the column outlet to the detector (tube 2) and in the detector cell (det), $\sigma_{tube2}^2+\sigma_{det}^2$, and (iii) the dispersion of the solute peaks acquired during the data acquisition and the processing steps, σ_{data}^2.

$$\sigma_{extra}^2 = \sigma_{inj}^2 + \sigma_{tube1}^2 + \sigma_{tube2}^2 + \sigma_{det}^2 + \sigma_{data}^2 \quad (2)$$

The number of theoretical plates (N_{obs}) provided by a column can be calculated using the solute retention time (t_R) (or retention volume, V_R) and the solute band dispersion of a Gaussian peak (σ_{total}^2), as shown in Eq. (3), where $\sigma_{total,t}^2$ is the band variance calculated in the time unit, and $\sigma_{total,v}^2$ is the variance calculated in the volume unit (of the mobile phase).

$$N_{obs} = (t_R^2/\sigma_{total,t}^2) = (V_R^2/\sigma_{total,v}^2) \quad (3)$$

Compared to the number of theoretical plates that the column should show in the absence of σ_{extra}^2 (N_{col}, Eq. (4)), or under ideal conditions, to be calculated based on the band dispersion inside the column (σ_{col}^2), the plate counts (N_{obs}, Eq. (3)) obtainable by actually operating the column is smaller. This is based on the contribution of the solute band dispersion outside the column (σ_{extra}^2) shown

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in Eqs. (1) and (2). In Eq. (4), L is the column length, and $\sigma_{col,L}^2$ is the band variance value described in the length unit of the column.

$$N_{col} = (t_{R\text{-}col}^2/\sigma_{col,t}^2) = (V_{R\text{-}col}^2/\sigma_{col,v}^2) = (L^2/\sigma_{col,L}^2) \quad (4)$$

The suppression of the extra-column band spreading (extra-column effect), has been an important issue of separation science in recent years. From the late 1970's to the 1980's, the extra-column effect also attracted the attention of chromatographers, when columns packed with 5 μm particles were developed. The major subjects of research at the time was mainly the improvement of the injector and the detector in the same way as executed in recent 10 years, downsizing of various components including the injector passage and the detector cell, and increasing the detector response and the data acquisition rate to reduce the band dispersion. The development of UHPLC was made possible by an extensive reduction in the system dispersion outside the column compared to conventional HPLC instruments, along with the development of short, narrow-bore columns packed with particles around 2 μm or smaller [14]. In HPLC, the extra-column system variance, σ_{extra}^2, is often in a range, 40 μL^2 or larger, whereas in UHPLC it is typically in a range, 1–6 μL^2 [5–7]. In fact, it has been reported that downsizing of individual parts and the acceleration of data processing for a conventional HPLC equipment resulted in the increase in N_{obs} for small columns by reducing σ_{extra}^2 to 2–8 μL^2 [15,16]. Similar optimization of instruments was carried out to obtain smaller extra-column dispersions to increase N_{obs} values for small columns [17–19].

Following UHPLC, micro-UHPLC (for example, Acquity class-I), i.e. UHPLC with even smaller extra-column effects, (σ_{extra}^2 = 0.5–2.5 μL^2) has become commercially available [20]. In capillary LC also, an increase in the observed column efficiency has been reported based on the reduction of the extra-column effect by downsizing the components [21,22]. Instruments that exhibit smaller extra-column effects have been shown to provide higher N_{obs} for small-size columns [18,19,23,24], although it has been discussed that the reduction of the extra-column volume may not lead to the expected reduction of the extra-column effect, and such an expectation may need an extensive improvement in the hardware fabrication [25]. In addition to the improvement in the hardware, the reduction of the extra-column effect has been attempted by concentrating the sample at the top of the column by using a mobile phase having low elution strength or utilizing the difference in the retention of solutes depending on temperature [26–28].

In spite of these improvement in the instruments and the sample introduction techniques, a considerable reduction in plate counts of a small column was observed for solutes with retention factors, k, below unity, even with micro-UHPLC [20]. The subject how to cope with the band broadening outside the column has been studied for nearly 40 years since the beginning of HPLC, and it has been proposed to downsize the components (reducing the extra-column volume) or to concentrate the sample band at the top of the column. Continuing attention has been paid to the reduction of extra-column effects [29–31].

Instruments that have smaller extra-column band broadening than current instruments are expected to provide higher observed efficiency for small columns. Smaller columns should lead to smaller retention volumes, which in turn enables the reduction in solvent consumption. Compared with 2.1 mmID, the volume of a 1.0 mmID column is 1/4.4, and the band dispersion resulting from a 1.0 mmID column showing the same plate counts as a 2.1 mmID column is only 1/19.4 (=$(1/4.4)^2$). At present, considerably lower observed column efficiency of 1 mmID columns has been reported than that of a 2.1 mmID column [18,19,24]. Instruments with a small extra-column dispersion are necessary not only for such microbore columns currently available, but also for a short microbore columns packed with ultra-high performance packing materials to be developed in the future. It has been a concern that hardware development may not be able to catch up with the column performance, unless unrealistically small extra-column volume is realized [25].

The system variance values were dependent on the individual instruments and the particular conditions including the size of a tube and the injector, the sample volume, and the detector cell volume in addition to the flow rate, the viscosity of a mobile phase, and the molecular weight of solutes [5,6,20,22–24,32,33]. The extra-column band spreading is reported to be smaller at a very low flow rate than at flow rates normally applied for current UHPLC instruments [5,6,20,22–24,32,33]. A much lower flow rate than optimum, however, has neither been practiced for UHPLC separations, nor employed for the sample introduction, because a near optimum linear velocity is commonly employed for 1–3 mmID columns packed with sub-2 μm fully porous particles or sub-3 μm core-shell particles.

We report here a sample introduction method which can reduce the extra-column effect for UHPLC leading to the increase in the obtainable plate counts for small columns. The suppression of the dispersion of a sample band in the injector and the pre-column connection tubing, σ_{inj}^2 and σ_{tube1}^2, was attempted by a slow transport of a sample band (to be abbreviated as SToSB in this report) in the pre-column space of ca. 10 μL or less. The flow rate program was studied to effect both the small dispersion of the sample band outside the column and the high-efficiency separations in the column.

2. Experimental

2.1. Instrument and columns

UHPLC instrument, LC800 (GL Sciences, Tokyo, Japan), was used. The instrument was equipped with a MU701 UV detector (GL Sciences) and an automated valve-loop injector with a 5 μL sample loop. The detector, operated at 240 nm, employs a single piece, 0.05 mmID, 0.375 mmOD, 20 cm long, fused silica capillary tube (the detection point at 10 cm from the inlet, tube volume to the detection cell = 0.2 μL), a part (3 mm portion) of which is used as a flow cell (cell volume = 6 nL). The column was connected to the injector with a 0.254 mmID, 15.2 cm PEEK tube (volume = 7.7 μL, to be abbreviated as tube A), or with a 0.13 mmID, 15 cm Viper tube (Thermo Fisher Scientific, Germering, Germany, volume = 2.0 μL, tube B). The experiment with a 0.0635 mmID, 15.0 cm PEEK pre-column tube (volume = 0.48 μL, tube C) was undertaken for the revision of the manuscript. Another Viper tube, 0.13 mmID, 6.5 cm (0.86 μL), connected the column outlet to the detector inlet with a zero dead-volume union (ZDU) in all cases. Following columns were employed: InertSustain C18, (2 μm particles, 1.0 mmID, 5 cm long, GL Sciences) and Kinetex C18, (2.6 μm particles, 2.1 mmID, 5 cm long, Phenomenex, Torrance, CA. USA).

2.2. Chromatographic measurement

A mixture of acetonitrile/water, 65/35 (v/v), was used as a mobile phase. A mixture of the same composition was used as a weak-wash solvent for the sample injector. The extra-column band variance values associated with the LC800 system were measured at various flow rates (5–800 μL/min) in the presence of ZDU in place of a column by injecting 0.2 μL of uracil solution (0.2 mg/mL in an acetonitrile-water mixture (65/35=v/v), sample I). For the column performance measurement, a reversed-phase column performance test mixture (Agilent, Little Fall, DE, USA), containing acetanilide, acetophenone, propiophenone, butyrophenone, benzophenone, valerophenone, hexanophenone, heptanophenone,

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