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# The Influence of Sample Mass and Flow Velocity on Performance in Preparative Column Liquid Chromatography

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## Abstract

The *H/u* curves of two HPLC columns of 10 mm i.d., filled with silica of a particle diameter of 5 and 25–40 μm, respectively, have been determined at various amounts of injected sample. Indications of mass overload, i.e., peak broadening, occur at a sample load of 10 μg/g for the column with 5 μm silica but not until 100 μg/g for the column with coarse stationary phase. Although *H* values increase markedly with higher sample mass, the slopes of the *H/u* curves remain almost constant on both columns up to samples of 1 mg/g. This means the relative increase of the plate height with increasing flow velocity becomes nearly negligible under conditions of moderate mass overload. However, by increasing the sample load up to 5 or 10 mg/g, the *H/u* curves show a dramatic increase of slope: at high mass overload the mobile phase flow rate cannot be increased without a remarkable decrease in column performance.

## INTRODUCTION

It is a well-known fact in gas and liquid chromatography that the separation performance of a column is dependent on the linear flow velocity of the mobile phase. The separation performance is defined as the number of theoretical plates $N$ of the column which can easily be determined from a test chromatogram. The amount of sample injected for this test needs to be kept low, in liquid chromatography in the range of 1 μg of solute per gram of stationary phase. Since $N$ depends on the column length $L$, it is more useful to calculate the theoretical plate height

$H = L/N$ (also called height equivalent to a theoretical plate, HETP). The lower $H$, the better the column performance. A plot of $H$ versus the linear flow velocity $u$ of the mobile phase ($H/u$ plot or van Deemter plot) shows a minimum which lies between a steep increase of $H$ at low flow rates and a low or moderate increase at higher flow rates. An excellent column has a low minimum value of $H$ and a negligible slope of the curve at increasing flow velocities; the latter fact means that the column can be used at high flow rates without a remarkable loss of performance.

Knox (*1*) has shown that the $H/u$ curve of a liquid chromatography column follows the relationship

$$H = Au^{0.333} + B/u + Cu$$

where $A$ represents the contribution of inhomogeneous packing of the stationary phase to band broadening, $B$ accounts for longitudinal diffusion, and $C$ is the contribution of mass transfer between mobile and stationary phases. (Generally this equation is expressed with the reduced parameters $h = H/d_p$ and $\nu = ud_p/D_m$. $h$ and $\nu$ are dimensionless and represent $H$ and $u$ values corrected for particle diameter $d_p$ and diffusion coefficient of the solute in the mobile phase $D_m$. The reduced form of the equation allows to it to be discussed in a more general manner, but the relationship between plate height and flow velocity remains the same with nonreduced and reduced parameters; of course, $A$, $B$, and $C$ change their values.)

These considerations are valid if no overloading effects occur. The term "overload" means that the amount of sample injected also accounts for peak broadening. This effect is observed if a critical sample volume or sample mass or both are exceeded. According to the reason of overload phenomena, "volume overload" and "mass overload" (also called "concentration overload") are distinguished. In preparative liquid chromatography, however, both effects often occur at the same time if sample volume and mass are increased simultaneously to yield a high throughput per unit time.

Very few papers deal with the influence of mass overload on $H/u$ curves. Using some data of an older paper (*2*), DeStefano and Kirkland show a plot representing the influence of sample mass and flow velocity on the resolution of a peak pair (*3*). This is not a $H/u$ plot, but the resolution, of course, depends on peak width. The phase system was silica and chloroform with some water, particle size was 35–75 μm, and the total amount of sample was varied between 25 μg and 2.5 mg/g of stationary phase. With higher sample mass the resolution decreases, but at 2.5 mg/g the flow rate has no influence on resolution. The authors state: "when the column is overloaded... the effect of velocity on $N$ (and resolution) becomes relatively minor." In a paper of Wall (*4*) a $h/v$ plot is shown with sample loads of 0.64 and 128 μg/g, respectively. The system was 5 μm silica and petroleum ether/2-propanol 95:5. The author comments: "deviations from optimal linear velocities lead to more rapid loss of separating power at preparative load levels than at analytical levels." $h$ values of the 128 μg/g curve are much larger than those of the 0.64 μg/g curve but, in contrast to the statement cited here, the slopes seem to be very similar. Recently, Dewaele et al. presented a $H/u$ plot of 10 and 20 μm octadecyl silica (*5*, *6*). Mobile phase was acetonitrile/water 3:1 and the sample load was approximately 0.5 and 50 μg/g, respectively. Although the column with the 20 μm phase has a lower performance, both columns behave identically; at higher sample load the value of $H_{\min}$ increases but the slope of the $H/u$ curve remains nearly constant. In a study, Eisenbeiss found that in various phase systems the slope of the $H/u$ curve is not influenced by sample mass up to loads of ~1 mg/g (*7*).

To sum up, all authors found that the influence of flow velocity on the slope of the plate height increase is negligible or low under conditions of mass overload. Indeed, there is only one study with a load of more than 1 mg/g (*2*); all others are in the μg/g range.

In this paper the influence of flow velocity on plate height is studied in the range from analytical load up to 10 mg/g on a normal phase system.

## EXPERIMENTAL

All experiments were performed with stainless steel columns of 10 mm i.d. and 25 cm length. These columns comprise rather exactly 10 g of silica. Two particle sizes of the same type of silica were used: LiChrosorb SI 60 5 μm and LiChroprep SI 60 25–40 μm, both with a mean pore diameter of 6 nm and from Merck (Darmstadt, FRG).

The fine silica was slurry-packed at 350 bar with a balanced-density slurry. At analytical conditions and with nitrobenzene in hexane, a theoretical plate number of 15,200 was determined at a flow rate of 7 mL/min; therefore $H = 16.4$ μm and $h = 3.3$. With the assumption that *p*-xylene in hexane/*t*-butyl methyl ether, 8:2, would not be retained, a hold-up volume of the column of 19.6 mL could be measured. From this a total porosity of the packing of 0.74 can be calculated and the relationship

$$u\ (\mathrm{mm/s}) = 0.287\dot{V}\ (\mathrm{mL/min})$$

results; $\dot{V}$ is the volume flow rate of the mobile phase and was determined in all experiments with stopwatch and volumetric flask.

For dry packing, Verzele and Dewaele recommend filling the pores of silica with ether (*8*). Therefore the coarse silica was wetted with *t*-butyl methyl ether and then packed dry. Measured under the conditions mentioned above, a theoretical plate number of 1070 could be determined; this means that $H = 230$ µm and $h = 7.3$ (calculated with a mean particle diameter of 32 µm). However, a glance at the *H*/*u* curve of this system, i.e., the lowest line in Fig. 2, shows that a volume flow rate of 7 mL/min, corresponding to a linear flow rate of 2 mm/s, is too high to obtain the maximum performance of this column. Indeed, the *H*/*u* minimum of this column lies at a lower value of the linear flow velocity than the lowest value investigated in this study.

The equipment used was an Altex 100 solvent metering pump (Altex, Berkeley, California), an Uvikon LCD 725 UV detector with a cell path length of 10 mm (for low sample loads), an Uvikon 720 LC UV detector with a cell path length of 1 mm (for high sample loads) (Kontron, Zürich, Switzerland), and a Rheodyne 7120 syringe loading sample injector with a 200-µL loop (Rheodyne, Berkeley, California). Detection wavelengths of 254, 285, 320, 340, and 350 nm were used depending on the sample mass. As shown in an earlier study (*9*), no volume overload occurs on 10 mm × 25 cm columns if the sample volume does not exceed 200 µL.

The mobile phase was hexane/*t*-butyl methyl ether, 8:2.

For the determination of *H*/*u* curves, the volume flow rate was varied between 3 and 20 mL/min; this corresponds to linear flow rates between 0.86 and 5.7 mm/s. In this range for each of the 7 different sample masses, 36 data points were determined. Several solutions of nitrobenzene in the mobile phase with concentrations between 85 µg/mL and 500 mg/mL were prepared and appropriate sample volumes between 20 and 200 µL were injected to obtain sample masses of 1.75 µg (this amount is called "analytical" sample), 10 µg, 100 µg, 1 mg, 10 mg, 50 mg, and 100 mg. (The "100 mg" sample was injected as 200 µL of a 0.5 g/mL solution into the 200 µL loop. It is known that by filling a loop completely, a certain amount of the sample (e.g., 10%) is lost due to the profile of linear flow. Therefore, in the "100 mg" experiments the column was loaded with less than 100 mg.)

The nitrobenzene peak was used to calculate the plate number *N* according to the equation

$$N = 5.54\left(\frac{t_R}{w_{1/2}}\right)^2$$

with $t_R$ = retention time and $w_{1/2}$ = peak width at half height. This equation is only valid for peaks with Gaussian shape. In spite of this, $N$ was used in this study as a pragmatic criterion of peak broadening and therefore this equation was also used for peaks with severe tailing or a more or less triangular shape as they occur at high sample loads.

The data points were fitted on a personal computer with the simplex procedure to find the parameters of the Knox equation:

$$H = Au^{0.333} + B/u + Cu$$

With the same program, the data points and the fitted curve could also be plotted. The Knox equation was developed for HPLC columns at analytical loadings. It seems to be unlikely that it is still valid for overloaded columns, and it can be assumed that $A$, $B$, and $C$ lose their meanings as was explained in the Introduction. Nevertheless, for lack of a better one, the Knox equation was also used for curves at high load.

## RESULTS AND DISCUSSION

The results are shown in Fig. 1 for the column with fine silica and in Fig. 2 for the column with coarse silica.

At all loads the column with a 5-μm stationary phase performs better than the one with a 25–40-μm phase, i.e., its $H$ values are always smaller. Although this effect can be expected from theory, the superiority of the microparticulate phase decreases with increasing sample load: $H$ values are 10-fold smaller for analytical sample load but only 3 to 4 times lower at loaded conditions.

On Fig. 1 the curve resulting from a sample load of 10 μg is not shown because it is almost identical with the analytical curve. ($H_{min}$ of the analytical curve is 18 μm, for the 10 μg curve it is 19 μm, whereas this value is 25 μm for the 100-μg curve.) On Fig. 2 the curves for the 10 and 100 μg loads are missing for the same reason. ($H$ at 0.86 mm/s is 190 μm at analytical conditions, 190 μm at 10 μg load, 200 μm at 100 μg, and 260 μm at 1 mg load.) This means that the column with 5 μm silica is overloaded at lower sample masses than the column with coarse silica.

Under conditions of beginning mass overload, the $H_{min}$ values of Fig. 1 always increase at a factor of 3 if the sample mass is raised 10-fold: $H_{min}$ is 25 μm for 100 μg, 78 μm for 1 mg, and 240 μm for 10 mg load.

The slopes of the curves remain constant for low and medium sample loads. This effect is very clearly seen in Fig. 1 up to the 10-mg samples. It

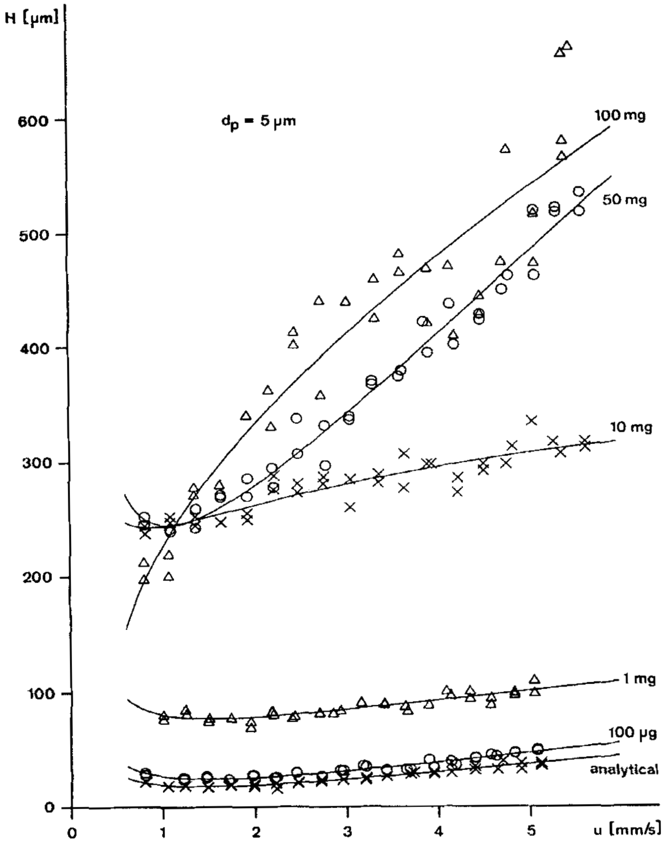

FIG. 1. Influence of the sample mass on the *H*/*u* curve of a column with microparticulate silica. Column: 10 mm × 25 cm. Stationary phase: LiChrosorb SI 60 5 µm. Mobile phase: hexane/*t*-butyl methyl ether 8:2. Sample: nitrobenzene. The column comprises 10 g of silica.

is also true for the coarse silica although the 10-mg curve of Fig. 2 is difficult to interpret. This means that the relative decrease of the column efficiency with increasing flow rate is remarkable for conditions where no overload occurs (e.g., from a plate height of 18 µm up to 40 µm for the analytical curve of Fig. 1) but is negligible at medium loads (from 240 µm up to 330 µm for the 10-mg curve of Fig. 1). This is the effect also reported by other studies (*2–7*). From these results one could deduce that overloaded columns can be used at any high flow rate without a remarkable decrease in efficiency.

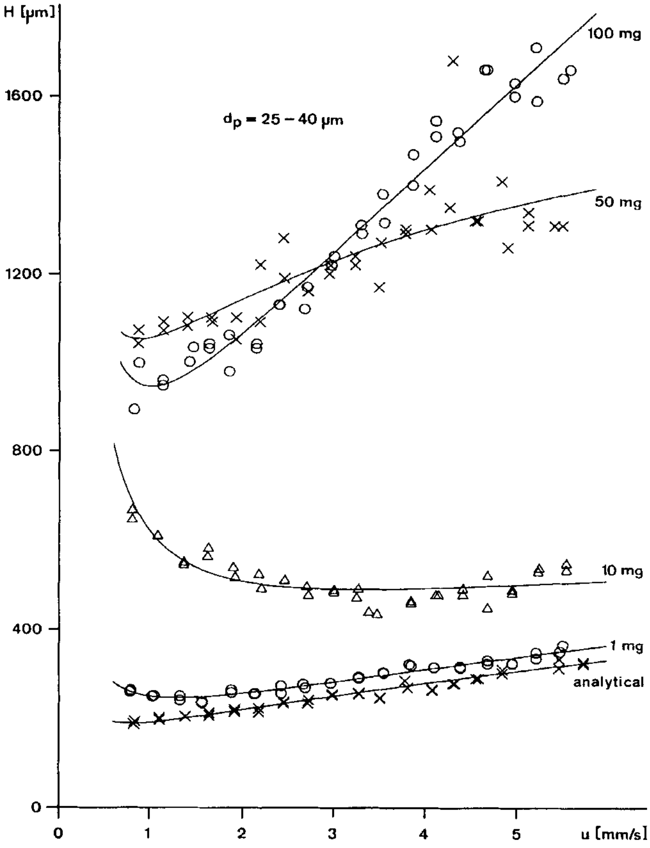

FIG. 2. Influence of the sample mass on the *H*/*u* curve of a column with coarse silica. Conditions as in Fig. 1 except for the stationary phase: LiChroprep SI 60 25–40 µm.

This situation grossly changes at still higher sample loads. With samples of 50 and 100 mg which correspond to loads of 5 and 10 mg/g, a considerable increase of the theoretical plate height with the flow rate is observed. The column with the coarse stationary phase behaves slightly better (*H* increases from 900 to 1700 µm at 100 mg load) than the column with the fine silica (*H* increases from 200 to 600 µm).

(Note: The *H*/*u* minima indicated in some curves at higher load are a consequence of the fit according to the Knox equation. They do not have an experimental validation.)

## CONCLUSIONS

The system investigated in this study consists of silica, hexane/*t*-butyl methyl ether, 8:2, and nitrobenzene as a sample. Column dimensions are 10 mm × 25 cm. It can be concluded that:

1. A stationary phase of 5 μm shows indications of mass overload at a sample load of 10 μg/g. With the 25–40 μm stationary phase, this effect occurs at a load of 100 μg/g.
2. With an analytical sample load up to 1 mg/g, the slope of the *H*/*u* curve remains constant.
3. For a load of 10 mg/g or less, the separation performance is injured markedly at increasing flow velocity.

## REMARKS

It is difficult or impossible to generalize the results obtained by a single test system if the study deals with a mass overloaded chromatographic system. The reason is that the adsorption isotherm of the test compound in this particular system should be known; any generalization would only be valid for compounds with a similar isotherm. Therefore, the above-mentioned results need not be valid for systems with different chromatographic properties; e.g., reversed-phase systems, ion exchangers, or samples like proteins.

Moreover, the results obtained with a single test compound are of limited value for the prediction of the behavior of a peak pair or of "real-life" separation problems which may contain dozens or hundreds of compounds.

Finally, the role of sample application and column dimension is not dealt with in this paper. It is obvious that geometrical prerequisites need to be paid attention in overloaded chromatography. Dewaele et al. found that the column inner diameter affects the shape of *H*/*u* curves if the columns are thick enough (*5*, *6*). Whereas columns of 4.6 and 22 mm i.d. behaved similarly, the slope of the *H*/*u* curve was increased considerably on a 44-mm i.d. column due to temperature effects.

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