Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Pharmaceutical and Biomedical Analysis 38 (2005) 337–343
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Short communication

Peak capacity in gradient ultra performance liquid chromatography (UPLC)

Stephen A.C. Wren

Analytical development, PAR&D, AstraZeneca, Silk Business Park, Macclesfield, Cheshire, SK10 2NA, UK

Received 3 December 2004; received in revised form 17 December 2004; accepted 20 December 2004
Available online 9 February 2005

Abstract

A prototype commercial instrument and 2.1 mm i.d. columns packed with1.7 μm porous particles have been used to measure peak capacity in ultra performance liquid chromatography (UPLC). Peak capacity was measured for a small molecule pharmaceutical as a function of gradient time, mobile phase flow rate, and column length. For very fast analysis, the highest peak capacity is obtained from a short column operating at high linear velocities. If an even higher peak capacity is required, a longer analysis time must be employed, and a point is reached where switching to a longer column becomes the best approach.

Keywords: Ultra performance liquid chromatography; Gradient time; Peak capacity

1. Introduction

1.1. Performance of gradient HPLC

Gradient reversed phase HPLC is widely used in the analysis of pharmaceuticals in order to control quality and consistency. One assay of major importance is that of purity assessment, where the drug substance is separated from low-level impurities so that they can be quantified. Gradient methods are popular, especially in early development, as they can separate analytes with a wide range of polarities. This gives the ability to separate and quantify any residual raw materials and intermediates in addition to more closely related species such as isomers.

One important consideration in the development of control methodology is the number of components present in the sample. The number of components present, and hence the difficulty of the separation problem, can vary greatly. For example, whereas a simple small molecule may contain less than 10 synthetic impurities, a peptide map produced from a recombinant protein may well contain a considerably greater number. Because of the potential for different samples to contain different numbers of components, it is important to have an understanding of the separating power of the LC method to be used. In this work, a crude sample of a small molecule pharmaceutical (material from the synthetic route but prior to purification) was used to estimate peak capacity. The identity of the pharmaceutical is not given for commercial reasons, but it has a molecular weight and chemical and structural features which are considered to be representative.

There are a number of objective measures of chromatographic quality, for example, peak efficiency, resolution, and peak capacity. Of these, resolution and peak capacity are relevant to gradient systems. Peak efficiency, which is derived from peak width and retention time, is a concept more suited to isocratic LC. Resolution is normally used where the main interest is in a pair of analytes, for example, a critical pair. Resolution is a function of column efficiency, separation selectivity, flow rate, and gradient time [1,2]. For samples, which contain many components, peak capacity is a useful measure of the comparative separating power of different analytical systems. In gradient LC, peak capacity is a function of column efficiency, gradient time, flow rate, and analyte characteristics. A number of studies have been carried out which

E-mail address: Stephen.wren@astrazeneca.com.

0731-7085/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2004.12.028

examine the peak capacity from both experimental [3–5] and theoretical viewpoints [3,6].

1.2. Performance of gradient UPLC

Recently, here has been considerable interest in employing pressures which are much greater than those used in HPLC in order to obtain high separating efficiencies, at high linear velocities, from small diameter particles. Work in academic research laboratories has used pressures which are one order of magnitude greater than those found in HPLC [7–11], and employed non-porous packing materials with diameters in the range of 1–1.5 μm. Non-porous particles are used because of their high mechanical strength and relative ease of manufacture. In addition, fused silica capillaries with diameters in the range 30–50 μm are used to minimise the impact of frictional heating. Because of the high pressure involved special equipment is required to pump the mobile phase and to pack the columns. The term ultra high-pressure liquid chromatography (UHPLC) has been used to distinguish this procedure from conventional HPLC.

Peak capacity has also been explored in gradient UHPLC using 1.5 μm non-porous particles. This approach gave a peak capacity of over 300 for fluorescently labelled peptides produced by a tryptic digest of ovalbumin [8].

Due to the specialised equipment required, and the low sample capacity of non-porous particles, UHPLC has not been used routinely in pharmaceutical analysis. Sample capacity is an important issue in this field as regulatory requirements mean that impurities must be quantified down to the 0.05% level. This low quantification level means that the analyte concentration range of interest is three orders of magnitude, and so sample overload of the main drug substance can easily be a problem. Because of the high potential for sample overload, porous packing materials with high surface areas are preferred in HPLC. Sample overload typically manifests itself with a much higher degree of peak tailing for the drug substance than the low level impurities.

More recently, however, the use of 1.5 μm porous particles in UHPLC has been reported [11]. These 1.5 μm porous particles showed a much higher sample capacity than the 1.0 μm non-porous material examined, with peak widths being much less sensitive to sample concentration.

Commercial instruments capable of operating at pressures up to about 1000 bar are now available, and one manufacturer uses the term ultra performance liquid chromatography (UPLC). This pressure limit is more modest than that attained in academic laboratories but is still likely to offer significant benefits. A β-test UPLC system has been used to generate the data given in this work.

1.3. Practical measurement of peak capacity

The peak capacity of a gradient LC system can be estimated from measurement of the average peak width, and the gradient time. In this work, the analysis is considered to be finished with the completion of the gradient and so the analysis time is equivalent to the gradient time. The column re-equilibration time is not considered explicitly as it is proportionately short for all but the fastest methods.

Eq. (1) is often used for the estimation of peak capacity.

$$n = 1 + \frac{t_g}{w_b} \quad (1)$$

where n is the number of peaks, t_g the gradient time and w_b is the peak width at the baseline value.

In this work, an alternative equation is used which also takes into account the column dead time, t_0.

$$n = \frac{t_g - t_0}{w_b} \quad (2)$$

Eq. (2) is preferred in this work as it takes into account the fact that none of the components in a sample can elute before the time equivalent to the column void volume (t_0). For very fast analysis, the column void time becomes a significant proportion of the analysis time, and ignoring it leads to a serious over-estimate of how many component peaks can be fitted into the chromatogram. The factor of 1 in Eq. (1) is not significant given the peak capacity obtained from typical UPLC (and HPLC) methods, and the uncertainty associated with the choice of a value for the peak width (see discussion below).

2. Experimental

UPLC was performed using a β-test version of the Waters Acquity instrument and 50 and 100 mm columns with an i.d. of 2.1 mm packed with 1.7 μm acquity C18 BEH particles (Milford, USA). The 200 mm column was composed of two 100 mm columns joined in series. Acetonitrile was from Rathburns (Walkerburn, UK), TFA from Fluorochem (Glossop, UK). The water was de-ionised using a Millipore system (Waters, Elstree, UK). The A solvent was 0.1% Tri Fluoroacetic Acid (TFA) in water (v/v) and the B solvent 0.1% TFA in acetonitrile (v/v). For each experiment the starting conditions were 10% B, and the finishing conditions 40% B. Solvent strength was varied linearly with times ranging from 4 to 90 min being employed. The columns were thermo-stated at 40 °C. The column oven is only long enough to accommodate a single 100 mm column, and so for the experiments employing two 100 mm columns in series the second column was housed within the detector module with makeshift thermal insulation. This is not an ideal arrangement but is useable because of the moderate operating temperature. UV absorbance data were collected at 320 nm using a bandwidth of 10 nm. A data collection rate of 5 Hz was used, except for gradient times of 5 min and below when a data collection rate of 10 Hz was employed. A range of flow rates and gradient times was employed for the different column lengths with the highest flow rates being constrained by the maximum

operating pressure of the instrument. With the 100 mm column and a flow rate of 0.5 ml/min the starting pressure (10% B) was 807 bar (11,750 p.s.i.). The sample was a crude drug substance used as a System Suitability Test (SST) mixture, and was a synthetic sample extracted prior to purification. The SST sample was prepared at AstraZeneca (Macclesfield, UK) and supplied by Dr Simon de Sousa.

3. Results and discussion

3.1. Choice of a value for peak width

A key consideration in estimating peak capacity from experimental data is the choice of the peak width value to be used in Eq. (2). The SST sample used in this work contains a number of impurities although most are present at very low levels. Fig. 1 shows one of the chromatograms generated using a 50 mm column, a gradient time of 4.0 min, and a flow rate of 0.5 ml/min. The chromatogram shown in Fig. 1 contains a number of components with the peaks marked a, b, and c being present at levels of approximately 0.4, 0.3, and 1.1%, respectively, relative to the drug substance. Direct determination of baseline peak width as a function of gradient time, flow rate, and column length posses some problems. One difficulty is that peaks can shift, split, overlap, and merge as changes in analysis conditions result in minor changes in selectivity. Because of these difficulties the approach adopted here is to determine the peak width at half height and to extrapolate to a "baseline value". Impurities such as a, b, and c, are suitable for this measurement as their much greater levels mean that their peak widths at half height will be little affected by any changes in the overlap with the minor components that surround them. Extrapolation to peak width at "baseline" is achieved by assuming that the peaks can be described by normal probability distributions [12]. Peak width at half height is then defined as 2.35σ (where σ is the standard deviation), and that at 13.4% height as 4.0σ (the baseline value dictated by convention). On this basis, the measured peak width at half height is multiplied by a factor of 4/2.35 to give "baseline" width. Other values for baseline width could also be justified, for example 5.0σ (corresponding to a height of 4.4%). The value chosen for the baseline width will depend upon the range of concentrations of the different analytes, and the degree of uncertainty that would be acceptable in quantification.

In Fig. 1 the different components in the sample give different peak widths at half height, with peaks a, b, and c, giving values of 1.73, 1.25, and 1.53 s, respectively. As different components give different peak widths, a representative component must be chosen to provide an estimate of overall peak capacity. The width of peak b is chosen for this work. The use of either peak a or c to provide an estimate of peak width would lead to different values for peak capacity, but give the same observations about trends in the variation in peak capacity with gradient time.

In this work, the peak capacity is estimated from measuring the peak width of a single component. This is a practical approach to estimating how peak capacity will vary as a function normal operating parameters. A more rigorous approach is to use mathematical de-convolution to measure the peak widths of all the components and so the actual peak capacity [8]. This later approach reflects the fact that peak widths vary across the chromatogram, but will require much greater time.

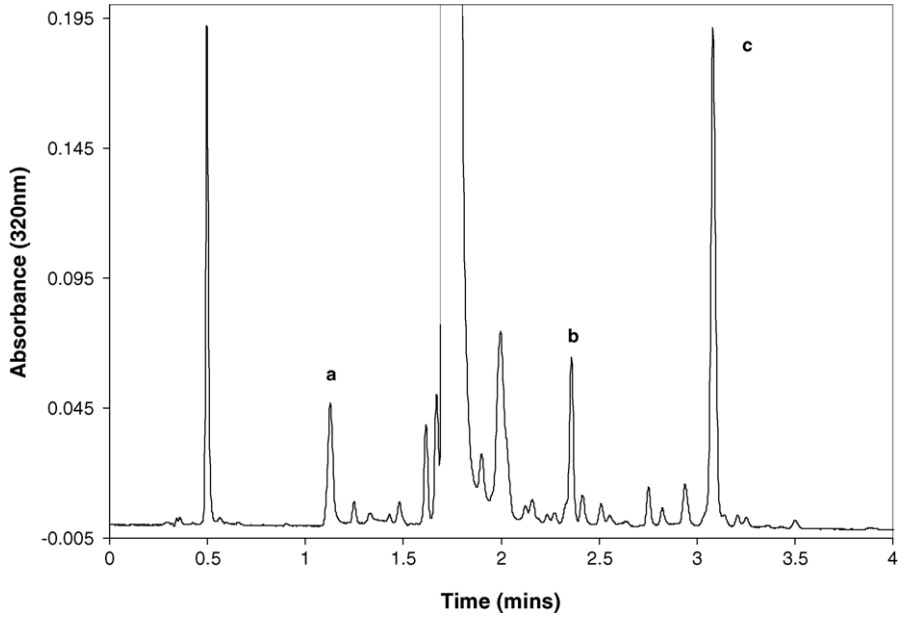

Fig. 1. Chromatogram produced by using a 50 mm long column with a flow rate of 0.5 ml/min and a gradient time of 4 min.

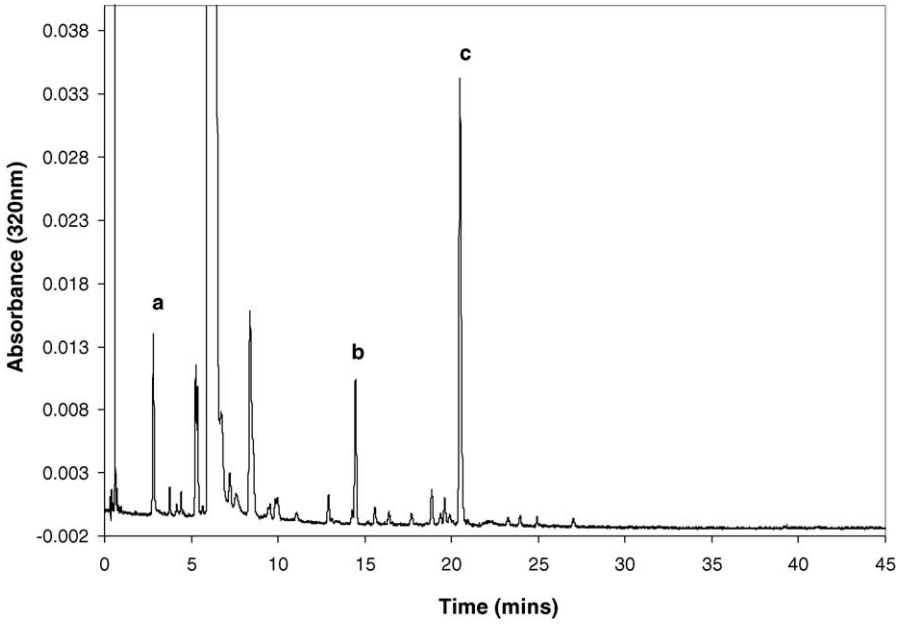

Fig. 2. Chromatogram produced by using a 50 mm long column with a flow rate of 0.5 ml/min and a gradient time of 45 min.

3.2. Variation in peak capacity

3.2.1. Column length of 50 mm

Fig. 2 shows the chromatogram of the SST sample obtained using a 50 mm column, a gradient time of 45 min, and a flow rate of 0.5 ml/min. As in Fig. 1, and indeed in all experiments, the mobile phase composition changed linearly between 10% B and 40% B. A comparison of Figs. 1 and 2 shows that increasing the gradient time has lead to a significant increase in peak capacity. The increase in peak capacity is due to the peaks being proportionately sharper, and the proportion of the chromatogram which occurs before the void volume being smaller. Thus, the number of components separated in Fig. 2 is greater, and there is more available free space in the chromatogram for additional peaks to be seen. There are also some changes in selectivity on increasing the gradient time but that is outside the scope of this paper.

The change in peak capacity for the 50 mm column at a flow rate of 0.5 ml/min and as a function of gradient time is shown in Fig. 3. From Fig. 3, it is seen that the peak capacity increases with increasing gradient time, but also that the increase is non-linear. The initial rapid increase in peak ca-

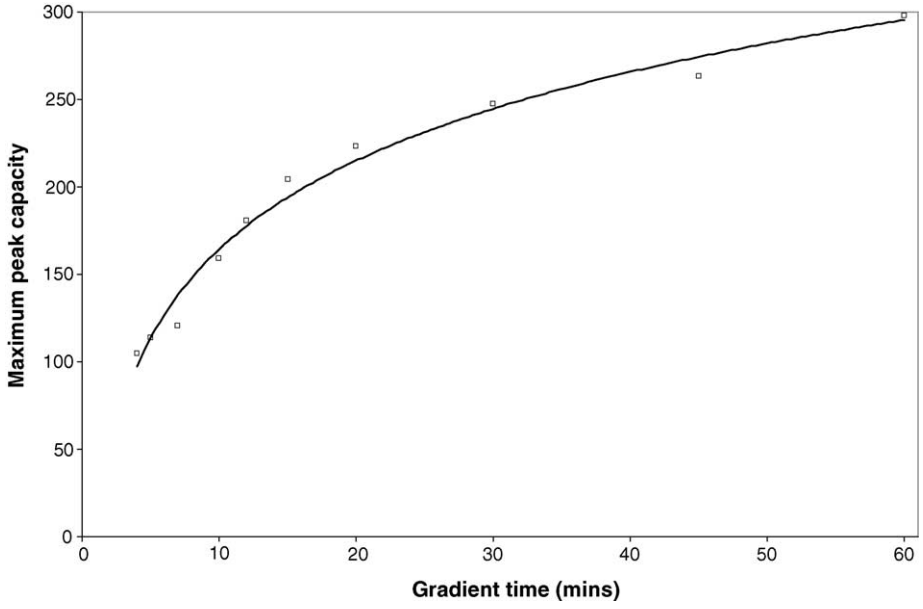

Fig. 3. Maximum peak capacity as a function of gradient time for a 50 mm long column with a flow rate of 0.5 ml/min.

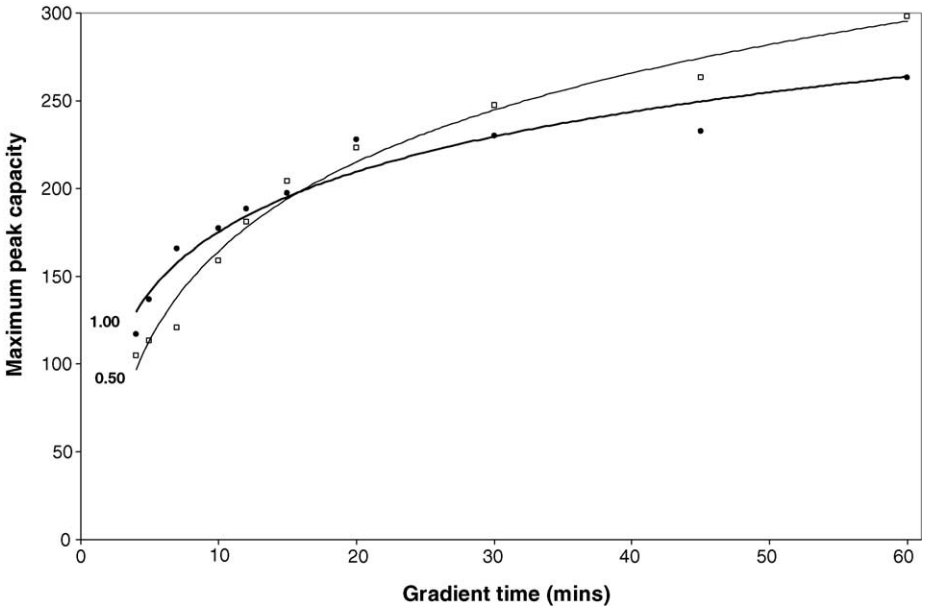

Fig. 4. Maximum peak capacity as a function of gradient time for a 50 mm long column with flow rates of 0.5, and 1.0 ml/min.

pacity with time soon flattens out and there is little increase in peak capacity on doubling the gradient time from 30–60 min. The curve shown in Fig. 3 is of the same general form as those shown in theoretical studies on peak capacity [6,3], and in experimental studies on resolution as a function of gradient time [2]. The data shown in Fig. 3 can be fitted to a logarithmic relationship (Peak capacity = 73.2ln (time) − 4.2) with an R^2 value of 0.98.

The use of different flow rates gives rise to curves of a similar form but with different slopes to that shown in Fig. 3. For example, Fig. 4 compares the peak capacity curves for the 50 mm column that arise from flow rates of 0.50 and 1.00 ml/min, respectively. It is interesting to see that whilst the higher flow rate gives a higher peak capacity with very short gradient times, the reverse holds true at longer gradient times. The switch to higher peak capacity being generated at the lower flow rate occurs with a gradient time of about 16 min. The reason for this change in behaviour is not certain but it may be associated with artifacts caused by operation at close to maximum pressure. It is noticeable that baseline noise was significantly greater at the very highest operating pressures. Additional noise will lead to a larger and more uncertain peak width measurement. A higher noise level would be expected to be proportionately more sig-

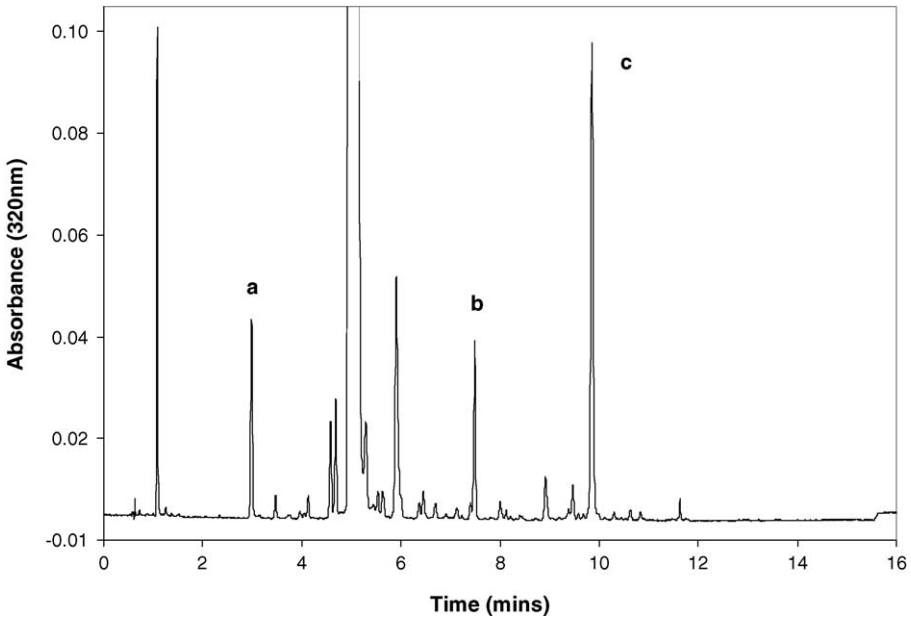

Fig. 5. Chromatogram produced by using a 100 mm long column with a flow rate of 0.5 ml/min and a gradient time of 15 min.

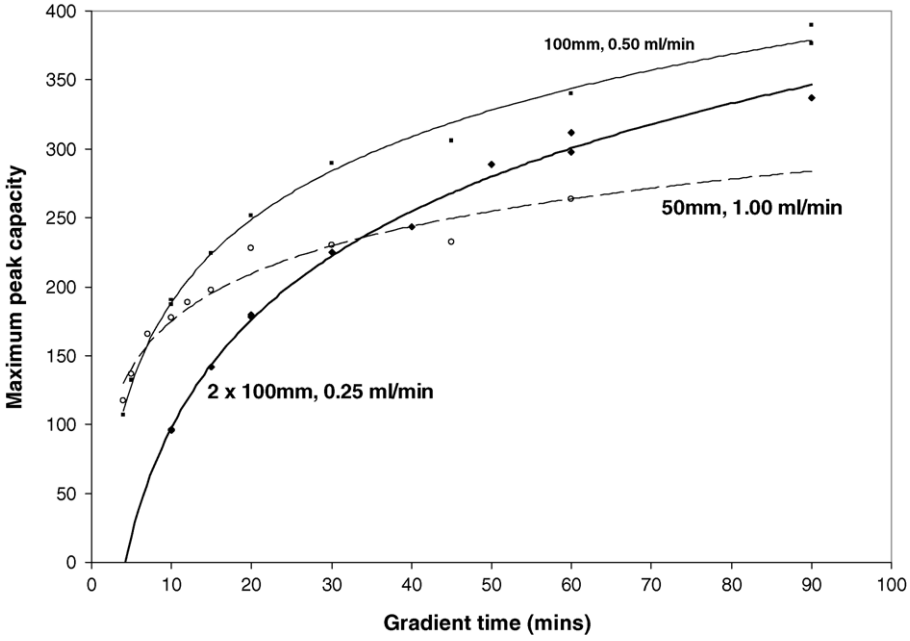

Fig. 6. Maximum peak capacity as a function of gradient time for 50, 100, and 2 × 100 mm long columns with a flow rates of 1.00, 0.50, and 0.25 ml/min, respectively.

nificant with the more dilute sample bands associated with longer run times. This is consistent with the observation of more scatter in the data generated at the higher flow rate (for 0.5 ml/min $R^2 = 0.98$ with $n = 10$, and for 1.0 ml/min $R^2 = 0.92$ with $n = 16$).

3.2.2. Column length of 100 mm

Fig. 5 shows the chromatogram produced using the 100 mm column with a gradient time of 15 min and a flow rate of 0.5 ml/min. The chromatogram in Fig. 5 has a peak capacity that is between the values measured for Figs. 1 and 2 (see later discussion). A comparison of Figs. 5 and 2 also shows that the use of the longer column results in better resolution because of greater retention and selectivity. As discussed earlier, the optimisation of conditions for a particular sample is not solely determined by peak capacity, but the maximum available peak capacity is an important factor in the initial choice of conditions. Examination of different gradient times and different flow rates with the 100 mm column gave rise to curves of a similar form to those shown in Fig. 3, although with different slopes and limiting values.

3.2.3. Comparison of column lengths

The performance of different column lengths is explored in Fig. 6. Fig. 6 shows the peak capacity curves for the 50, 100, and 200 mm column lengths using flow rates of 1.00, 0.50, and 0.25 ml/min, respectively. These different column lengths and flow rates were chosen to compare performance at the same operating pressure. The different column lengths give rise to curves of similar shape but with different degrees of curvature and limiting values of peak capacity. For example, the 50 mm column gives the greatest initial increase in peak capacity with time but has the lowest peak capacity with long gradient times. The choice of column length to maximise peak capacity will depend upon the time available for the analysis. Below a time of about 7 min the 50 mm column gives the highest peak capacity of the three columns. If times longer than 7 min are available for analysis, then switching to the 100 mm column is the best approach. It is possible that the 200 mm column may become preferred for very long analysis times but further experiments are required to determine this with any degree of certainty.

4. Conclusions

Peak capacity has been determined in UPLC as a function of gradient time, flow rate, and column length. For any column length and flow rate, the peak capacity varies with gradient time in an asymptotic fashion. Peak capacity is low with very fast gradients but increases rapidly as the gradient times are increased. With longer gradient times, the peak capacity tends to a limiting value. The choice of column length to maximise peak capacity will depend upon the analysis time available. For very short analysis times, the highest peak capacity can be obtained from short columns operating at very high linear velocities. For longer analysis times it is beneficial to switch to longer columns and lower linear velocities.

Acknowledgment

I wish to thank Waters for the loan of the beta test instrument and columns and for technical support.

References

[1] L.R. Snyder, J.W. Dolan, J.R. Gant, J. Chromatogr. 165 (1979) 3–30.
[2] K.A. Cohen, J.W. Dolan, S.A. Grillo, J. Chromatogr. 316 (1984) 359–372.
[3] J.W. Dolan, L.R. Snyder, N.M. Djordjevic, D.W. Hill, T.J. Waeghe, J. Chromatogr. A 857 (1999) 1–20.
[4] H. Minakuchi, N. Ishizuka, K. Nakanishi, N. Soga, N. Tanaka, J. Chromatogr. A 828 (1998) 83–90.
[5] D.V. McCalley, J. Chromatogr. A 1038 (2004) 77–84.
[6] U.D. Neue, J.R. Mazzeo, J. Sep. Sci. 24 (2001) 921–929.
[7] J.E. MacNair, K.C. Lewis, J.W. Jorgenson, Anal. Chem. 69 (1997) 983–989.
[8] K. Lan, J.W. Jorgenson, Anal. Chem. 71 (1999) 709–714.
[9] J.A. Lippert, B. Xin, N. Wu, M.L. Lee, J. Microcolumn Sep. 11 (1997) 631–643.
[10] Y. Xiang, B. Yan, C.V. McNeff, P.W. Carr, M.L. Lee, J. Chromatogr. A 1002 (2003) 71–78.
[11] J.S. Mellors, J.W. Jorgenson, Anal. Chem. 76 (2004) 5441–5450.
[12] J.M. Miller, Chromatography: Concepts and Contrasts, Wiley, New York, 1988.