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Assessment of injection volume limits when using on-column focusing with microbore liquid chromatography

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Abstract

It had been ascertained that in the use of microbore liquid chromatography (LC) and on-column focusing (peak compression), there were advantages to be had in using the strongest eluting injection solvent that would still bring about on-column focusing. In such cases, it was found that the scope for the use of on-column focusing with microbore LC was greater than previously imagined. It has been possible to explain these results by considering on-column focusing to take place in two distinct phases, i.e. focusing of the sample band due to the injection solvent, followed by further focusing of the sample band by the sample solvent/mobile phase step gradient. In this context, an equation has been developed which provides a much more accurate estimate of the maximum allowable injection volume for a given injection solvent and mobile phase than has been possible until now.

Keywords: On-column focusing; Injection volume; Indomethacin

1. Introduction

For more than a decade [1], there has been interest in the use of liquid chromatography (LC) with columns of reduced internal diameter (I.D.) compared to conventional chromatography using 4.6 mm I.D. columns. This interest arises because of several advantages including greater mass sensitivity, reduced solvent consumption and better compatibility with mass spectrometry. Despite these advantages, microbore LC has not been widely used in routine assays. One contributing factor to this is perhaps the perceived disadvantage [2] of having to use small sample injection volumes when using columns of reduced I.D. [3]. However, this problem can often be circumvented by using "on-column focusing". "On-column focusing" occurs when solutes are concentrated or "focused" onto the top of the analytical column by injecting the sample in a solvent of lower eluting strength than that of the mobile phase [4–6]. The solute band is adsorbed onto the packing material on the top of the column in a narrow band before being eluted as such by the mobile phase travelling behind the sample plug. By utilising on-column focusing, much larger injection volumes than would otherwise be possible may be used without inducing volume overload of the column [1].

The ultimate aim of this work was to develop assays using on-column focusing effected by direct injection of the eluant obtained from solid-phase extraction (SPE) cartridges. Therefore, in order to achieve maximum scope for SPE method develop-

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ment, on-column focusing would be most useful if it could be attained by minimal reduction of the solvent strength of the sample solution. Also, the use of aqueous sample solutions could potentially give rise to sample solubility problems and analyte absorption in the injection system [7,8].

In the majority of reversed-phase applications that have employed on-column focusing, the injection solvent has been an aqueous solution containing little or no organic modifier [2,5,9–13]. There has been only limited use or study of on-column focusing with reversed-phase LC using more strongly eluting organic–aqueous solutions as injection solvents [6,14–16]. For such cases, there is a need to know the limiting conditions required to produce on-column focusing. In addition, further information is required on how the effect of the retention factor (k) of the solute in the mobile phase affects the degree of on-column focusing and therefore the injection volume that can be achieved [6,15]. Therefore, as part of an evaluation of this technique with microbore LC of drugs in biological extracts [17], it was necessary to gain further insight into the limiting conditions for on-column focusing over a range of analyte k values and to determine whether or not these conditions could be predicted.

It has been proposed [1,18] that the extent of the injection volume increase arising from the use of on-column focusing may be approximated by:

$$V_{pc(max)} = V_{i(max)} \frac{(k_o + 1)}{k + 1} \quad (1)$$

where $V_{pc(max)}$ is the increased injection volume due to on-column focusing, $V_{i(max)}$ is the maximum injection volume tolerable without on-column focusing, k_o is the retention factor of the solute when eluted by the sample solvent and k is the retention factor of the solute when eluted by mobile phase.

Consequently, if the maximum injection volume using mobile phase as the solvent is 1 μl, then if the sample is dissolved in a solvent that has a $(k_o + 1):(k + 1)$ ratio of 100, then a 100-μl injection can be tolerated.

However, since most reports of on-column focusing have so far only included data concerning on-column focusing using non-eluting solvents, this relationship has not been rigorously tested in practice. Indeed, Brunmark et al. [16] have reported results that seem to be inconsistent with existing theory. In a study of the injection of derivatised toluene diisocyanate (TDI) isomers, it was found that 200 μl injection volumes could be applied to a 19 cm×0.35 mm I.D. C_{18} column in solutions containing 40% (v/v) acetonitrile while using a mobile phase that contained 70% (v/v) acetonitrile. $V_{i(max)}$ was estimated to be 0.1 μl [16]. Although values of k and k_o were not reported, it could be estimated from the data presented that k was approximately five for the 2,4-TDI isomer derivative. Therefore, from Eq. (1), k_o would have to be in the order of 13 000 to facilitate a 200-μl injection volume. Such a large k_o value arising from a solution containing only a 30% reduction in the modifier concentration compared to the mobile phase, (which gave a k of five) seems unlikely.

Also, results obtained from a study of on-column focusing following injection of solutions of indomethacin in a range of sample solvents had shown that 500 μl injection volumes could be made onto a reversed-phase microbore column with as little as a 20 to 30% reduction in the concentration of methanol in the sample solvent with respect to the mobile phase [19]. There was therefore reason to re-examine on-column focusing on a theoretical basis and to evaluate the data from the latter study [19] in a more quantitative manner, in order to establish if it might be possible to more accurately predict the maximum allowable injection volume for a given injection solvent and mobile phase.

2. Experimental

2.1. Conditions

The chromatographic system consisted of a Shimadzu LC-10AD pump, a SPD-6AV UV–Vis detector operated at 254 nm and fitted with a 2.5-μl flow cell and a C-R5A integrator (all from Dyson Instruments, Hetton-le-Hole, UK). A Rheodyne 7125 injection valve was fitted with a Tefzel rotor seal and a custom-made 0.25 mm I.D., 1-ml stainless steel external loop. The dimensions of the stainless steel connecting tubing were 9 cm×0.175 mm pre-column and 9 cm×0.175 mm post-column (Anachem, Luton, UK). A Spherisorb ODS1 (5 μm), 12 cm×

1.0 mm I.D. column (Capital HPLC, Broxburn, UK) was used with a Spherisorb Si (10 μm), 10 cm×4.6 mm I.D. pre-column in-line before the injector. Injection syringes were SGE (gas tight), fitted with LC needles (Hichrom, Reading, UK). The column temperature was maintained at 30°C using a water jacket and Tempette pump/heater, supplied by BDH (Poole, UK).

Water was glass-distilled and de-ionised (Milli-Q purification system, Millipore, Watford, UK). Other materials used were methanol (HPLC-grade), di-sodium hydrogenorthophosphate (analytical-reagent grade), orthophosphoric acid (85%), GPR, (BDH). Indomethacin was supplied by Sigma (Poole, UK).

Indomethacin solutions of varying concentration giving 5 ng on-column on injection were prepared in 0.02 *M* aqueous phosphate buffer, pH 7.0, mobile phase and a range of other methanol–0.02 *M* aqueous phosphate buffer solutions indicated later in the text. Injection volumes of 1, 5, 20, 50, 100, 250 and 500 μl were made with each of the injection solvents investigated and with mobile phases of methanol–0.02 *M* aqueous phosphate buffer, pH 7.0, (58:42, 50:50, 46:54 and 42:58, v/v) to give a range of k values. All injections were performed at least twice except those performed with $k=18.6$, because of the long analysis time. The detector flowcell throughout this experiment was 2.5 μl.

3. Theory

It is usually assumed that the displaced volume (D_v) at the front of a column covered by an unretained solute (i.e. $k=0$) is equal to the injection volume [20]. However, if a retained solute is injected in mobile phase then D_v is given by:

$$D_v = \frac{V_i}{(k+1)} \tag{2}$$

Where k is the solute retention factor for an injection in mobile phase and V_i is the injection volume.

Therefore, as the k of the solute in the injection solvent is increased, D_v, is reduced and focusing of the sample band occurs. Similarly, if the sample is injected in a solution of lower eluting strength than the mobile phase, i.e. a solution that gives larger retention (k_o), then:

$$D_v = \frac{V_i}{(k_o+1)} \tag{3}$$

Since the displaced volume is inversely proportional to the k_o value of the solute in the injection solvent it could be progressively reduced by decreasing the injection solvent elution strength. On the other hand, the injection volume could be increased while maintaining the same sample bandwidth at the top of the column, assuming that the top of the column is not overloaded by the analyte or other species in the sample mixture and that the adsorption isotherm is linear [20,21].

If the width of the displaced band at the head of the column was allowed to increase then the effective column length would be reduced, resulting in a smaller number of theoretical plates [20]. Hence, the maximum volume that may be injected ($V_{i(max)}$) is determined by the maximum pre-determined fraction (θ) of the column plate number which is lost on injection. Accordingly, the maximum sample injection volume may be defined as [1]:

$$V_{i(max)} = \frac{K\theta\pi\epsilon d_c^2 L(k+1)}{4\sqrt{N}} \tag{4}$$

where $V_{i(max)}$ is the maximum injection volume that can be made (in mobile phase) which gives a defined percentage contribution (θ) to the overall observed band dispersion. K is a constant depending on the injection technique, ϵ is the column porosity, L and d_c are the column length and diameter respectively, N is the column efficiency and k is the retention factor of the analyte when eluted by the mobile phase. The values of the constants are [1], $\theta=0.22$ ($\theta^2=0.05$); $K=2$; $\epsilon=0.7$ and therefore when k is 0, Eq. (4) may be simplified to:

$$V_{io(max)} = \frac{0.25 d_c^2 L}{\sqrt{N}} \tag{5}$$

where $V_{io(max)}$ is the maximum injection volume tolerable for an unretained solute.

Therefore, if a retained solute was injected in mobile phase, the maximum injection volume would be increased accordingly and could be defined:

$$V_{i(max)} = V_{io(max)}(k + 1) \quad (6)$$

If, on the other hand, the retained solute was injected in a solution which gave a greater k than mobile phase (k_o) in order to produce increased focusing of the sample band then:

$$V_{pc} = V_{io(max)}(k_o + 1) \quad (7)$$

where V_{pc} = the injection volume possible with on-column focusing.

V_{pc} is not the maximum injection volume that may be tolerated with on-column focusing and is rather the volume that may be injected which maintains the sample band width at the pre-determined value of θ. Eq. (7) therefore does not take into account the possibility of further focusing due to the step gradient that is formed when the mobile phase comes through and displaces the sample solvent at the head of the column [5,15].

If the sample was injected in a non-eluting solvent (i.e. $k_o = \infty$) then the effect of a step gradient would be minimal since the gradient would have little effect on further reducing an already very narrow analyte band. This is analogous to the situation in preparative gradient elution LC where, if the sample is loaded under strong binding conditions, only the mass load is important and volume overloading does not occur [22]. Therefore, so long as the mass load does not cause significant band broadening and k_o is sufficiently large to minimise band migration on sample loading, then the concentration of the modifier in the mobile phase will have little effect on the degree of on-column focusing that can be achieved.

However, in a real analytical situation, the value of k_o will be considerably lower than ∞. Therefore, if larger injection volumes are made than can be compensated for by the magniitude of k_o, then increased band spreading, greater than the pre-defined value of θ, will occur. However, it may still be possible to inject these large volumes if the step gradient effect is large enough to re-compress the sample band. This again is analogous to the situation in gradient elution LC, where partial band migration can occur under high sample load. As a result, the front of a sample component traverses the column under isocratic elution, leaving behind a long tail. The compressive effect of the gradient later acts to concentrate the trailing portion of the peak [22]. The magnitude of this gradient effect is given by the ratio of the retention factors of the sample solvent and that of the mobile phase [5], i.e. $(k_o + 1)/(k + 1)$:

Therefore, to take account of the effect of the step gradient compressing the sample band, Eq. (7) must be multiplied by this factor, thus:

$$V_{pc(max)} \cong V_{io(max)}(k_o + 1)\frac{(k_o + 1)}{(k + 1)} \text{ or}$$

$$V_{pc(max)} \cong V_{io(max)}\frac{(k_o + 1)^2}{(k + 1)} \quad (8)$$

where $V_{pc(max)}$ is the maximum volume that may be injected with on-column focusing and $V_{io(max)}$ is calculated from Eq. (5).

Thus, if $k_o > k$, then focusing will occur due to the effect of the injection solvent and the step gradient formed. However, if $k = k_o > 0$, then $V_{pc(max)} = V_{i(max)}$ as calculated from Eq. (4) and although the injection volume is therefore directly proportional to k, there will be no additional injection solvent focusing. If $k = k_o = 0$, then $V_{pc(max)}$ is the same as the value for $V_{io(max)}$ calculated from Eq. (5) and is not determined by the mobile phase or injection solvent composition.

The approximation in Eq. (8) arises because this is an idealised model and in reality there would be some mixing of the sample solution with the mobile phase in the injection loop and on the top of the column forming mixing zones of lower k_o. Therefore, there would be a contribution to band dispersion as a result of this mixing effect and the degree of on-column focusing would be reduced [5,18]. The mixing effect can be minimised however by minimising the I.D. of the loop [5,19].

Also, the value of $V_{io(max)}$ depends upon the values assigned to the constants and, in particular, on the value assigned to θ, which is somewhat arbitrary [23]. The model also assumes that there is no mass overload and that there is no interference from other components in the sample. The presence of other solutes could produce non-linear adsorption isotherms and also analyte–matrix interactions and band displacement effects [24,25].

Eq. (8) differs from Eq. (1) in that the $(k_o + 1)$ term is squared. This shows that the value of k_o is more important than was first realised in determining the extent of on-column focusing and that by far the

most effective variable to change to facilitate a large injection volume ($V_{pc(max)}$) is the k_o value of the sample in the injection solvent. This is most easily accomplished in reversed-phase LC by reducing the concentration of the organic modifier. However, it is prudent to use the minimum reduction of the organic modifier that facilitates a given injection volume [7].

Also, in the use of Eq. (8), the value for the maximum injection volume without on-column focusing is calculated for the condition that $k=0$ (i.e. $V_{io(max)}$ is used instead of $V_{i(max)}$). This condition is necessary so that the effect of the step gradient between the sample solvent and the mobile phase is taken into account. Eq. (1) does not consider this, since the value of $V_{i(max)}$ is calculated by an expression (Eq. (4)) that contains the term $(k+1)$ and this term cancels the $(k+1)$ term in Eq. (1). Thus, Eq. (1) predicts the same value for the injection volume with on-column focusing as does Eq. (7) and therefore takes no account of the elution strength of the mobile phase. However, it is worth noting that $V_{pc(max)}$ can be obtained using $V_{i(max)}$ from:

$$V_{pc(max)} \cong V_{i(max)} \frac{(k_o + 1)^2}{(k + 1)^2} \quad (9)$$

Also, Eq. (1) would have predicted the same value for $V_{pc(max)}$ as Eq. (8), had $V_{i(max)}$ been calculated by substituting k_o for k in Eq. (4). This observation again shows that it is the elution strength of the injection solvent that is the most important determining factor for the maximum injection volume with on-column focusing. Reducing the k of the solute in the mobile phase relative to the injection solvent will also be effective up to a point, but there may be constraints to this option, such as selectivity requirements.

4. Results and discussion

The practical application of Eq. (8) was tested using volume peak standard deviation (σ_v) data derived from a previous study of the effect of the injection solvent organic modifier concentration on the degree of on-column focusing produced [19]. The value of σ_v was determined in each experiment to assess the extent of peak dispersion due to increasing injection volume. Since the peaks were symmetrical (assymmetry factor (B/A) was less than 1.4) over the range of predicted injection volumes studied, σ_v was calculated by: σ_v = peak width at half height in volume units/2.354). For example, in the experiment in which an injection solvent of methanol–0.02 *M* aqueous phosphate buffer (30:70, v/v) was used with a mobile phase of methanol–0.02 *M* aqueous phosphate buffer (58:42, v/v), (B/A) was 1.14 in the case of the 1-μl injection and 1.28 in the case of the 500 μl injection. σ_v was used instead of column efficiency (N) for this purpose, since it would be necessary to correct N values for the solute retention time lag due to the injection volume.

In order to establish values for k and k_o, it was also necessary to determine the k of indomethacin over a wide range of mobile phase concentrations of methanol. It was noted (Fig. 1) that, as expected, k became increasingly large with decreasing methanol concentration. For example, when using a 32% (v/v) methanol concentration in the mobile phase, the k for indomethacin was determined to be 40 and when using a 20% (v/v) methanol concentration in the mobile phase, the k for indomethacin was 181.

The experimental data showing the effect of k, the injection volume and the methanol concentration of the injection solvent, on indomethacin σ_v, are shown in Figs. 2–5.

It was found that when the sample injection solvent contained 20% (v/v) methanol (i.e. k_o = 181), large injection volumes (up to 500 μl) could be made onto the 1.0 mm I.D. column, over the range of k values tested, with minimal increase in peak dispersion. Further reduction of this dispersion brought about by injection solvents containing less than 20% (v/v) methanol was minimal and therefore it would be of little benefit to reduce the methanol concentration of the injection solvent below this value.

There was also minimal increase in peak dispersion with injection volumes of up to 500 μl when an injection solvent was used that contained 32% (v/v) methanol (k_o = 40) and when the mobile phase contained 58% (v/v) methanol (k = 1.6). However, when using this injection solvent with the mobile phases that contained less than 58% (v/v) methanol (i.e. increasing analyte k), significant peak dispersion

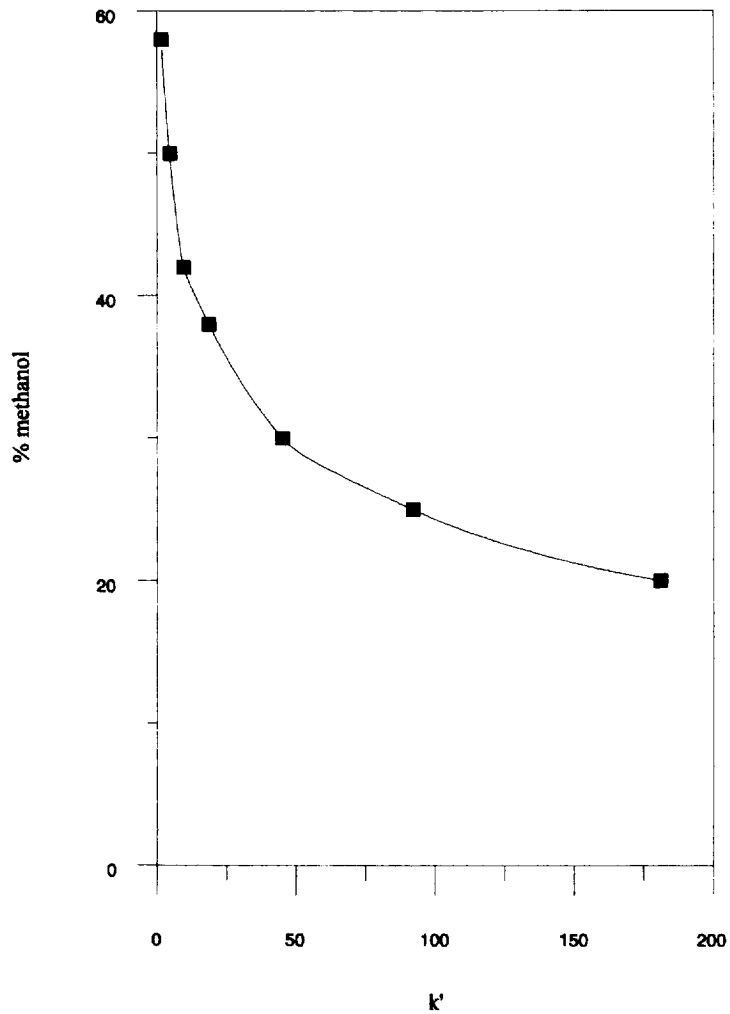

Fig. 1. Effect of methanol concentration in the mobile phase on the k of indomethacin. Column: 12 cm × 1.0 mm I.D., Spherisorb ODS1 (5 μm). Mobile phase: methanol–0.02 M aqueous phosphate buffer, pH 7.0; flow-rate, 0.071 ml min^{-1}.

was noted in some cases, with much smaller injection volumes.

For example, it was found (Figs. 3–5), that when the mobile phase contained 50% (v/v) methanol, then up to 250 μl could be injected before there was a significant increase in σ_v. When the mobile phase contained 42% (v/v) methanol, an injection volume of up to 100 μl could be tolerated. Peak dispersion increased markedly with injection volumes of over 50 μl when the mobile phase contained 38% (v/v) methanol.

These results confirmed that there was a minimum difference between the methanol concentration of the mobile phase and the methanol concentration of the injection solvent which could provide adequate on-column focusing for a large injection volume. In order to determine if these limiting conditions could be predicted, the values for $V_{pc(max)}$, when using an injection solvent containing 32% (v/v) methanol ($k_o = 40$) with the above mobile phases, were calculated using Eqs. (8,1). These results are shown in Table 1. $V_{io(max)}$ was calculated from Eq. (5) to be 0.78 μl, which is in line with values calculated by others [1,26–28] for a 1.0 mm I.D. column. In order to use Eq. (1), $V_{i(max)}$ was calculated at each k from Eq. (4).

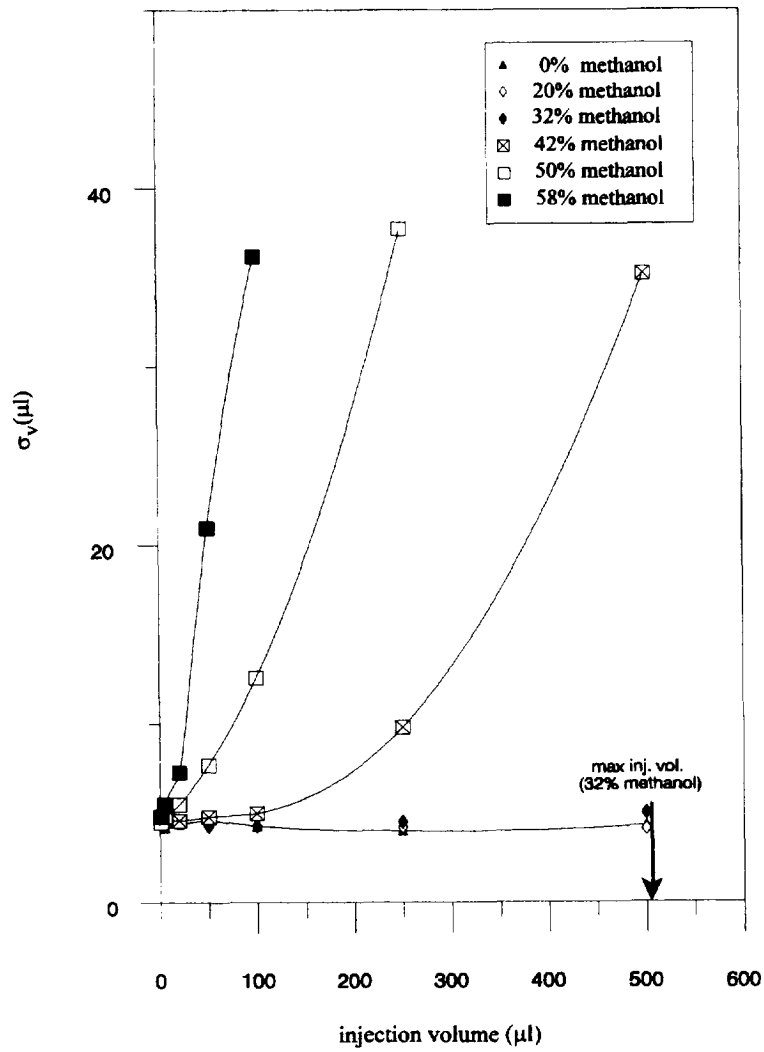

Fig. 2. Effect on σ_v of indomethacin ($k = 1.6$) of increasing injection volume and varying the percentage of methanol in the sample injection solvent. Arrow denotes the calculated maximum injection volume (Eq. (8)) with an injection solvent containing 32% (v/v) methanol. Column: 12 cm×1.0 mm I.D., Spherisorb ODS1 (5 µm). Mobile phase: methanol–0.02 *M* aqueous phosphate buffer, pH 7.0 (58:42, v/v); flow-rate, 0.071 ml min^{-1}.

It was apparent (Table 1) that the predicted values of $V_{pc(max)}$ from Eq. (8) were considerably larger than those predicted from Eq. (1). Also, the predicted value from Eq. (1) remains the same over the range of k values studied. The experimental data (Figs. 2–5) are therefore in fairly good agreement with the predicted values from Eq. (8), but are grossly out of line with those predicted from Eq. (1). Also, the experimental data and the predicted results using Eq. (8) show that the maximum injection volume with on-column focusing is reduced with increasing k of the mobile phase. This observation is in line with the reported findings of others [6,15], but it has been shown here that on-column focusing is effective with large sample volumes (up to 500 µl) with k values of up to ~twenty (Fig. 5) providing that k_o is large enough.

While Eq. (8) gave a good estimate of the

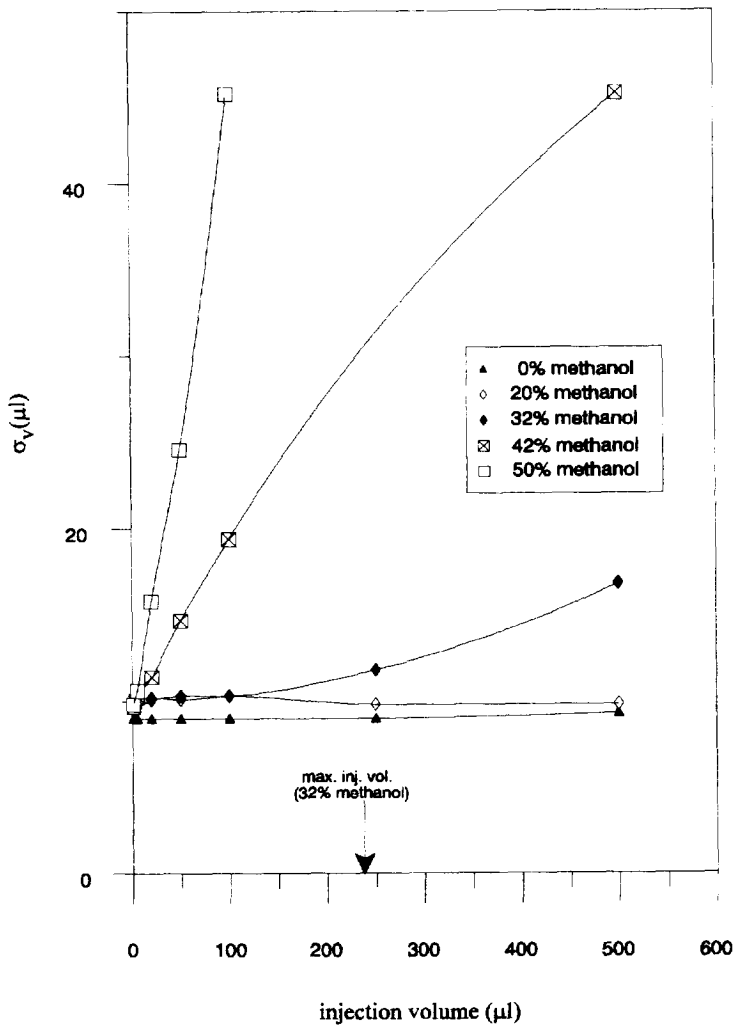

Fig. 3. Effect on σ_v of indomethacin ($k=4.5$) of increasing injection volume and varying the percentage of methanol in the sample injection solvent. Arrow denotes the calculated maximum injection volume (Eq. (8)) with an injection solvent containing 32% (v/v) methanol. Column: 12 cm×1.0 mm I.D., Spherisorb ODS1 (5 µm). Mobile phase: methanol–0.02 *M* aqueous phosphate buffer, pH 7.0, (50:50, v/v); flow-rate, 0.071 ml min^{-1}.

maximum injection volumes in this study, which used non-extracted solutions of indomethacin, these injection volumes could be reduced in other applications, depending on the nature of the sample matrix. For example, it would be expected that more complex samples could potentially produce mass overload problems.

This is more likely to be a problem with microbore LC than with conventional LC, since a microbore column can tolerate a reduced sample mass in proportion to the square of the column I.D. [26].

5. Conclusions

A better understanding has been developed of the

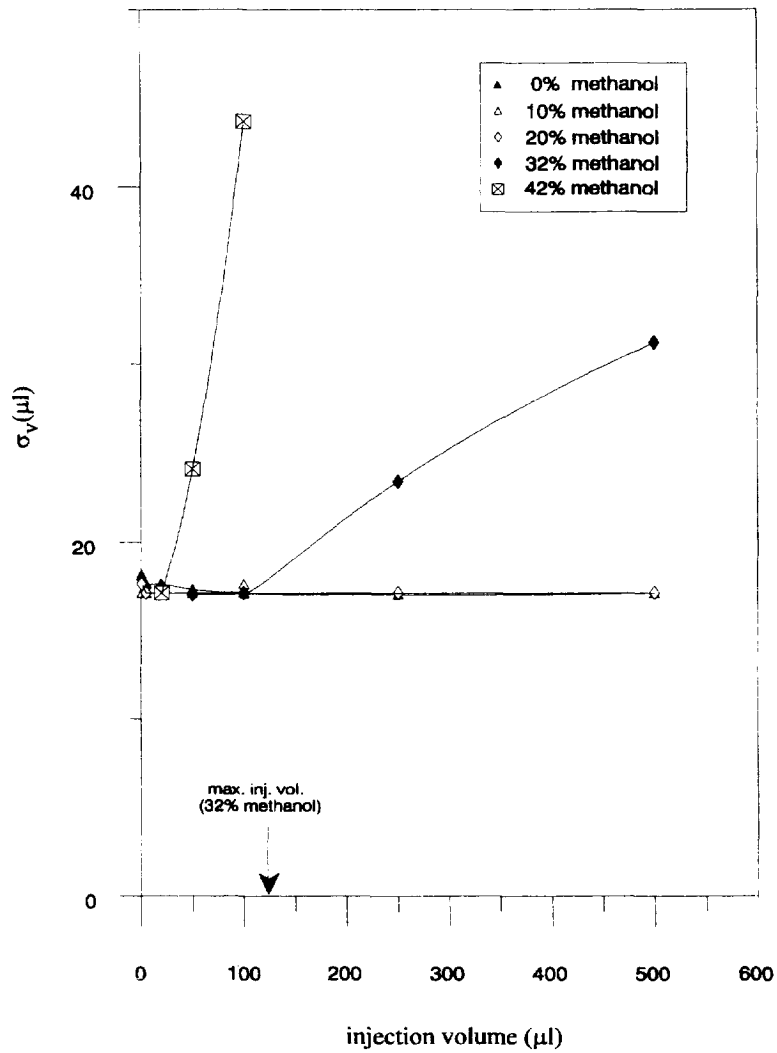

Fig. 4. Effect on σ_v of indomethacin ($k=9.6$) of increasing injection volume and varying the percentage of methanol in the sample injection solvent. Arrow denotes the calculated maximum injection volume (Eq. (8)) with an injection solvent containing 32% (v/v) methanol. Column: 12 cm×1.0 mm I.D., Spherisorb ODS1 (5 µm). Mobile phase: methanol–0.02 *M* aqueous phosphate buffer, pH 7.0, (42:58, v/v); flow-rate, 0.071 ml min^{-1}.

scope for using the technique of on-column focusing to reduce band broadening and increase sample injection volumes with microbore LC. In particular, the equation (Eq. (8)) developed from consideration of the principles underlying dispersion due to the volume of sample injected, provided a much more accurate estimate of the maximum allowable injection volume for a given injection solvent and mobile phase than has been possible to date. If relatively "clean" samples can be obtained so as to avoid mass overload, analyte matrix interactions and displacement effects, then the scope for the use of on-column focusing in the analysis of drugs in biological fluids is greater than previously imagined.

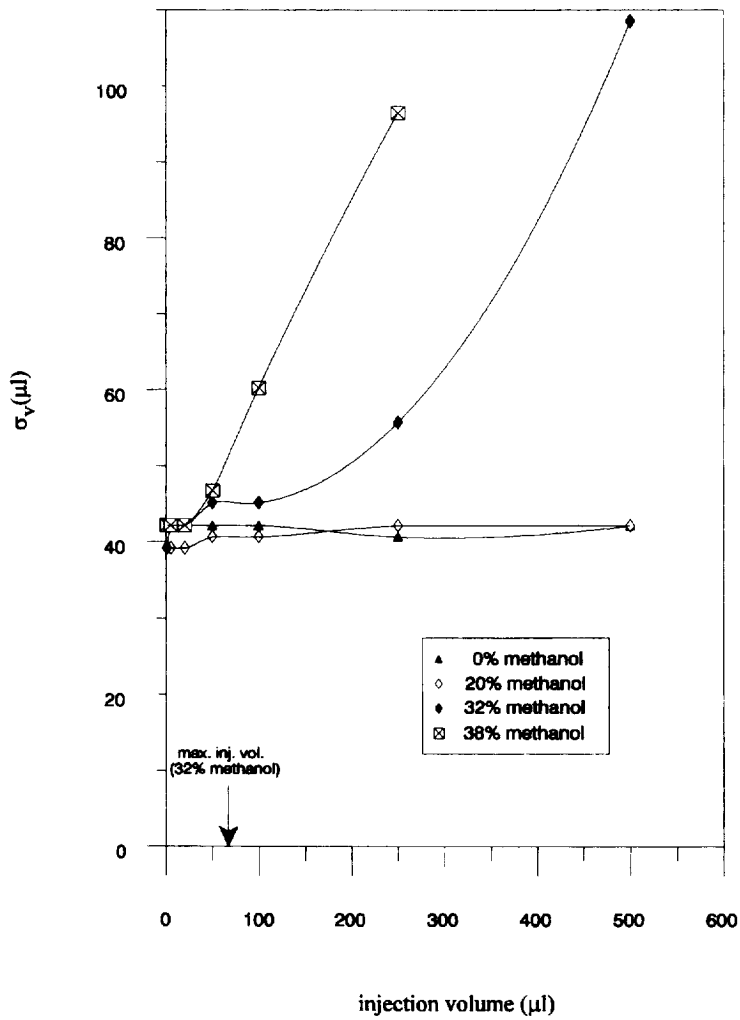

Fig. 5. Effect on σ_v of indomethacin ($k=18.6$) of increasing injection volume and varying the percentage of methanol in the sample injection solvent. Arrow denotes the calculated maximum injection volume (Eq. (8)) with an injection solvent containing 32% (v/v) methanol. Column: 12 cm×1.0 mm I.D., Spherisorb ODS1 (5 µm). Mobile phase: methanol–0.02 *M* aqueous phosphate buffer, pH 7.0, (38:62, v/v); flow-rate, 0.071 ml min^{-1}.

Table 1
Predicted injection volumes ($V_{pc(max)}$) over a range of k values with on-column focusing

Methanol in the mobile phase (%)	k of indomethacin in mobile phase	Eq. (8) $V_{pc(max)}$ (µl)	Eq. (1) $V_{pc(max)}$ (µl)
58	1.6	504	32
50	4.5	238	32
42	9.6	124	32
38	18.6	67	32

$V_{pc(max)}$ was calculated from Eqs. (8,1). Injection solvent contained 32% (v/v) methanol ($k_o=40$).

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