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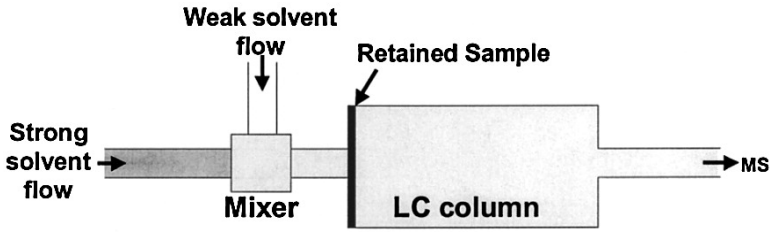

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Two-Pump at-Column-Dilution Configuration for Preparative Liquid Chromatography−Mass Spectrometry

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Preparative liquid chromatography−mass spectrometry (LC−MS) is widely used in parallel synthesis schemes to expedite purification. Recently, an alternative sample loading scheme, at column dilution, has been shown to dramatically increase the mass loading capacity of LC−MS purification methods. The prototype system utilized separate sample loading and binary gradient pumps. We report here a configuration for effecting at-column dilution using only the two pumps that provide the binary gradient flow. The advantages of a two-pump configuration are reduced cost, reduced space requirements, simplified control, and reduced service and maintenance issues. The two-pump at-column-dilution configuration is demonstrated for large- and small-scale LC−MS purifications. Purification on scales appropriate for high-throughput parallel synthesis can be achieved with small-scale chromatography using at-column dilution; purification of 20 mg of material is demonstrated using a 4.6 mm × 150 mm column and a flow rate of 3 mL/m. Reducing the scale of chromatography required for LC−MS purification has significant benefits, including the following. It requires less expensive columns, consumes less solvent, generates smaller-volume fractions (shorter dry-down time and the ability to collect into small-volume collector formats, such as 96-well plates), and has the potential for faster separations.

Introduction

High-throughput parallel and robotic synthesis techniques are used by virtually every drug discovery program in the pharmaceutical industry as a means of expediting the exploration of chemical diversity and accelerating the drug discovery process. While there are numerous strategies for utilizing these techniques, many discovery programs are moving toward more directed approaches in which smaller numbers of structurally distinct compounds are synthesized as discrete entities. This scenario generally requires relatively "pure" compounds, and some form of postsynthesis purification is needed. This purification requirement is often a significant factor limiting productivity. Mass-directed purification or preparative liquid chromatography−mass spectrometry (LC−MS) is being widely used as a means of expediting the purification process and reducing this bottleneck.

There are several instrument vendors offering preparative LC−MS systems. All of these systems appear to be reliable and effective mass-directed purification systems. Yet most of these systems have a common deficiency. The LC configurations currently provided by most vendors afford only limited mass loading capacity and are susceptible to many types of failure, including clogged injectors and transfer lines from compounds precipitating from solution, column breakthrough, and peak distortions due to the disturbance of column equilibrium by the sample diluent.

Wheat has recently introduced a gradient LC system that utilizes at-column dilution (ACD) of the sample to achieve greater mass loading.[1] The work of Wheat and others indicates that the chromatographic distortions and failures usually associated with high column loading are caused by the strong solvent(s) and/or large injection volumes required to load large amounts of sample rather than by overloading of the column packing.[2−4] The conventional loading process and proposed mechanisms of chromatographic failure are depicted schematically in Figure 1. The sample and diluent

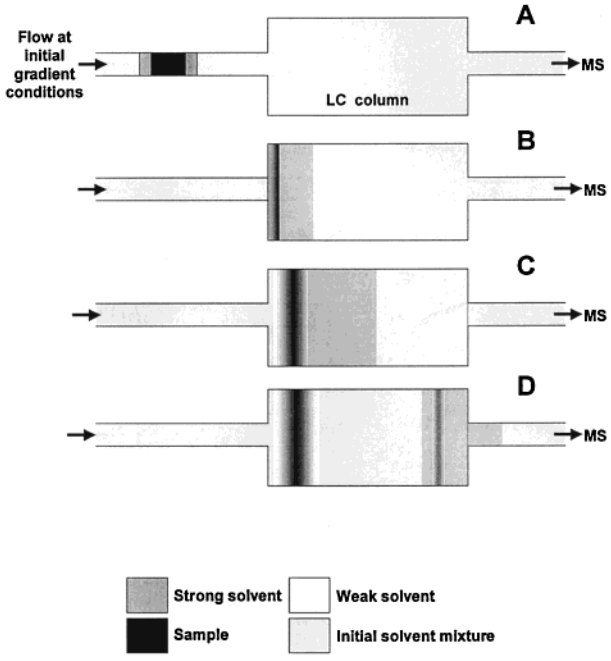

Figure 1. Schematic representation of conventional column loading process.

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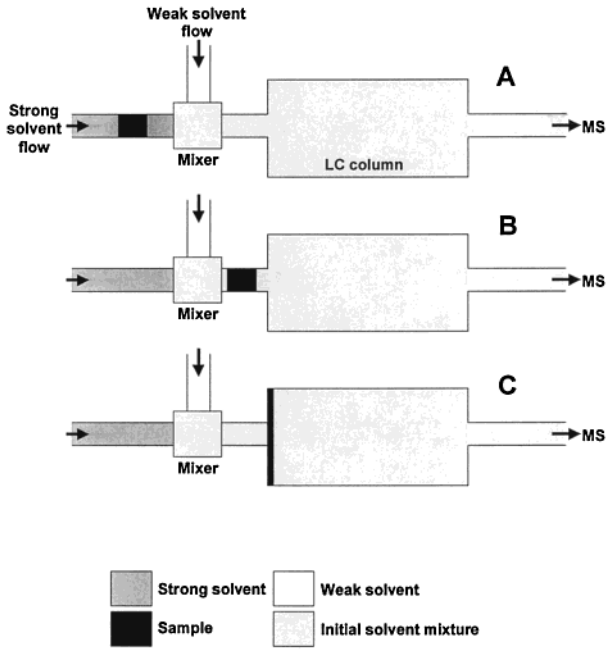

Figure 2. Schematic representation of at-column-dilution process.

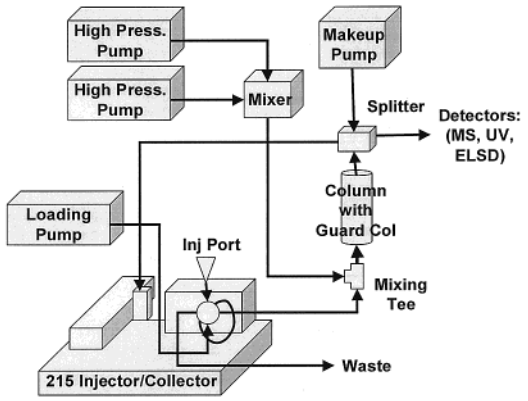

Figure 3. Three-pump at-column-dilution configuration.

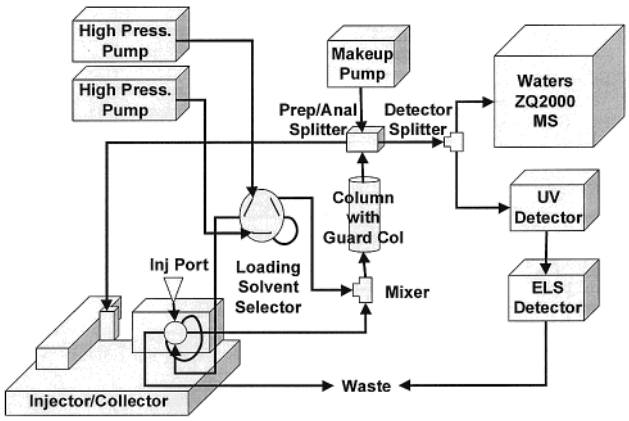

Figure 4. Two-pump at-column-dilution configuration.

are injected into a mixture of the LC mobile phases at the initial gradient conditions (Figure 1A). As the plug of sample and diluent enter the LC column, it is diluted via mixing with the mobile phase. When the concentration of the diluent has been sufficiently reduced via the mixing process, the sample is retained on the column packing (Figure 1B). However, if the diluent is too strong and/or the injection volume is too large, a greater mixing volume will be required to reduce the diluent concentration to the point at which the sample is retained by the column packing. In this situation the sample may penetrate into the column significantly before retention occurs and/or the retention band may be broad (Figure 1C). If the volume required to achieve adequate dilution is large relative to the column volume, the sample may not be fully retained on the column and a portion or all of the sample may elute with the solvent front as column breakthrough (Figure 1D). If dilution of the sample diluent is incomplete, the plug of strong solvent passing through the column may also disturb the equilibrium of the stationary phase, causing further distortion of the chromatography.

The at-column-dilution approach avoids these difficulties by effecting complete dilution of the sample and diluent before the column. The essential process is depicted in Figure 2. The sample and diluent are injected into a flow of strong solvent (i.e., a solvent miscible in both the sample and the diluent) (Figure 2A). The sample, diluent, and loading solvent are diluted within the mixer with weak solvent to the initial gradient conditions (Figure 2B). The initial solvent concentrations are chosen such that the sample is retained at the head of the LC column (Figure 2C). If the solvent mixing is complete within the mixer, the sample will be retained in a narrow band and there will be no front of strong solvent passing through the column to cause breakthrough or to disturb column equilibrium.

The ACD system reported by Wheat is a "three-pump" configuration depicted schematically in Figure 3. This configuration utilizes a binary gradient pump (the two high-pressure pumps) and a separate loading pump. The loading pump is operated at a flow rate 5% that of the total at-column flow rate. The loading solvent is combined with the gradient flow at the mixing tee immediately adjacent to the LC column. With this configuration, Wheat has reported mass loading capacities more than 10 times greater than could be achieved with the same LC method using a conventional loading configuration.

We recently reported an alternative "two-pump" configuration for achieving the at-column-dilution effect.[4] This configuration is shown schematically in Figure 4. In this configuration the stronger solvent (the binary gradient solvent more compatible with the sample and diluent) is directed to the injector and the sample is inserted into this flow. The switching valve selects which of the binary gradient solvents is used as the loading solvent. This loading solvent flow is then combined with the weaker solvent in the mixer immediately adjacent to the LC column.

The dedicated loading pump in the three-pump configuration permits flexibility in the choice of loading solvent, while the two-pump configuration requires that the sample and diluent are injected into one of the two LC mobile phases. While many pharmaceutically interesting compounds are not readily soluble in either of the LC mobile phases (acetonitrile or water), it appears that even slight solubility of the sample in the loading solvent and miscibility of diluent and loading solvent are sufficient for the two-pump ACD method to work well. Restricting the loading solvent to one of the LC mobile phases appears to be a minor limitation. The benefit of the two-pump configuration is that it utilizes

the same number of pumps as a conventional configuration. This is advantageous for a number of reasons, including lower cost, reduced space requirements, simpler pump programming and control, easier loading solvent switching, and reduced maintenance and service.

In this article, we will describe the two-pump configuration in detail, characterize its operation, and describe methods for three different scales of LC−MS purification.

Experimental Section

High-pressure pumping is provided by two Gilson 306 pumps fitted with 25.SC pump heads (maximum flow rate of 25 mL/m) and an 805 manometric module connected to the output of the aqueous pump. The injector/fraction collector is the Gilson 215 liquid handler. The preparative/analytical splitter is an LCPackings ACM-10-50 (1:1000 split), the makeup pump is a Waters reagent manager operating at 2 mL/m, and the detector splitter is a 0.020 in. i.d. PEEK tee with pressures adjusted to provide an approximately 1:20 split (5% flow to MS). The mixer is a static mixing tee (Upchurch, part number U-466). The switching valve is a Gilson Valvemate operating in the manual mode. All of the tubing between the high-pressure pumps and switching valve is 0.030 in. of stainless steel. (The volume of the system prior to the injector valve and mixing tee is irrelevant; thus, large-bore tubing is recommended to minimize back-pressure.) The switching valve to mixing tee and switching valve to injector valve transfer lines are 0.030 in. stainless steel tubing. The injector to mixing tee transfer line is 0.010 in. stainless steel tubing. The mixing tee to guard column connector is PEEK tubing of minimum length; the i.d. of this tubing is method-dependent and is given in the method descriptions. The column to preparative/analytical splitter transfer line is 0.020 in. PEEK tubing. All tubing after the splitter is standard Waters specification. The mass spectrometer is the Waters ZQ2000, the UV detector is the Gilson 155, and the evaporative light-scattering detector (ELSD) is the Sedex 75C. The mass spectrometer, LC, and mass-directed fraction collection are controlled via Micromass Masslynx, version 3.5, with Fractionlynx.

Three LC methods of differing scales are reported here. For all three methods solvent A is water with 0.1% TFA added and solvent B is acetonitrile with 0.1% TFA added. The "small-scale" LC method uses a 4.6 mm × 150 mm Zorbax C-18, 5 μm particle size column with a 2.1 mm × 10 mm C8 guard column. The mixer to guard column and guard column to LC column connectors are both of 0.005 in. PEEK tubing of minimum length. The flow rate is 3 mL/min, and the initial mobile-phase composition is 10% B. The gradient method is given in Table 1. Note that a short wait time is used prior to beginning the gradient to ensure that the sample is fully loaded onto the column well before the elution composition is reached. The maximum injection volume used with this method was 1 mL; approximately 2.1 min is required to load this volume onto the column (see below) with this method. This was adequate for the compounds used in this study, which were all reasonably well retained on the C-18 column. For compounds that are not well retained, it would be necessary to lengthen the wait time so that the sample is fully loaded under initial conditions. At the initial conditions (10% acetonitrile, 3 mL/m), the loading time for a 1 mL injection is about 3.3 min. The loading time is generally negligible for the methods utilizing higher flow rates.

Table 1

time (min)	% B	flow (mL/m)
	Small-Scale Method	
0	10	0.2
0.2	10	3.0
1.0	10	3.0
6.0	100	3.0
7.5	100	3.0
7.6	10	3.0
9.8	10	3.0
10	10	0.2
	Midscale Method	
0	10	0.2
0.2	10	8.0
5.0	100	8.0
6.5	100	8.0
6.6	10	8.0
9.8	10	8.0
10	10	0.2
	Large-Scale Method	
0	10	0.2
0.3	10	25
7.0	100	25
8.5	100	25
8.6	10	25
9.7	10	25
10	10	0.2
	Flow Gradient (Small-Scale) Method	
0	10	0.2
3.0	55	3.0
6.0	100	3.0
7.5	100	3.0
7.6	10	3.0
9.8	10	3.0
10	10	0.2

The volume of loading solvent that has moved through the injector at any given time into the gradient run is

$$V_{\text{loading solvent}} = C_i F t_w + C_i F t_g + G F t_g^2$$

where C_i is the initial fraction of the flow composed of the loading solvent, F is the flow rate, t_w is the wait time, t_g is the gradient time, and G is the gradient (rate of change in composition). The loading time is approximately the time required for the volume of loading solvent passing through the injector to equal the injection volume. The loading time can thus be estimated by setting $V_{\text{loading solvent}}$ equal to the injection volume and solving the quadratic equation for t_g; the loading time is then equal to $t_w + t_g$.

The "midscale" LC method uses a 10 mm × 100 mm YMC ODS, 5 μm particle size column and a 10 mm × 10 mm guard column. The mixer to guard column and guard column to LC column connectors are 0.010 in. i.d. PEEK tubing of minimum length. The maximum injection volume is 2 mL. The flow rate is 8 mL/m, and the gradient method is shown in Table 1.

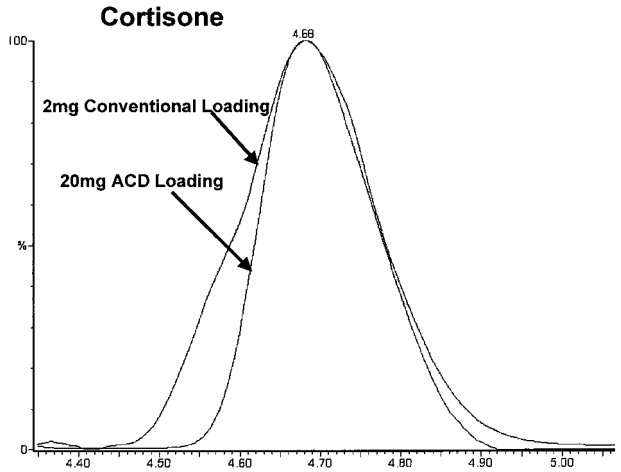

Figure 5. Peak shape for cortisone using small-scale method (4.6 mm × 150 mm column; 3 mL/m flow rate): 2 mg with conventional loading and 20 mg loading with at-column dilution.

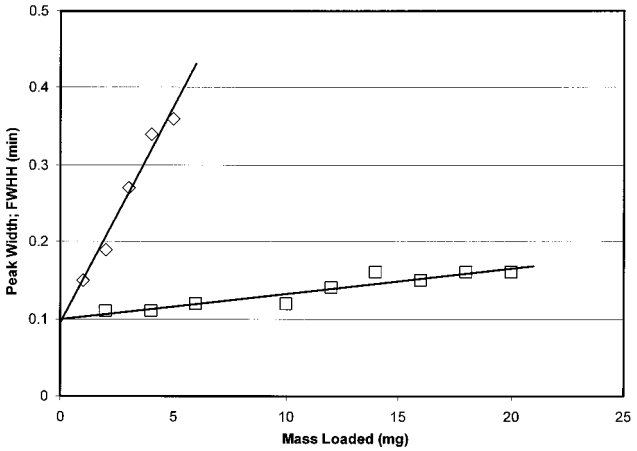

Figure 6. Dependence of peak width (full width at half-height) on mass loading for cortisone using small-scale method: (◇) conventional loading; (□) at-column dilution.

The "large-scale" method uses a 20 mm × 100 mm YMC ODS, 5 μm particle size column and 10 mm × 10 mm guard column. The connectors are 0.020 in. i.d. PEEK tubing of minimum length. The flow rate is 25 mL/m, and the gradient is shown in Table 1. The maximum injection volume is 2 mL.

Results

Figure 5 compares chromatographic peak shapes observed with the small-scale method (4.6 mm × 150 mm column) for 2 mg of cortisone loaded via the conventional method and 20 mg of cortisone loaded with the two-pump ACD method. At 2 mg, the peak shape obtained using conventional loading is already showing indications of significant broadening and asymmetry while the peak observed for the 20 mg loading using ACD is sharp and symmetric. The results of systematic loading studies for several compounds are depicted in Figures 6 and 7. In Figure 6 the peak width observed for cortisone, expressed as the full width at half-height, is shown as a function of the mass loading using the conventional configuration and the two-pump ACD configuration. In both cases the peak width increases in an approximately linear manner with increasing mass loading.

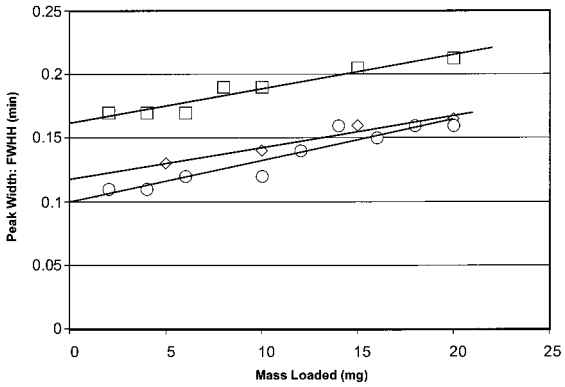

Figure 7. Peak width vs mass loading for three compounds using small-scale method with at-column dilution: (□) reserpine; (◇) furosemide; (○) cortisone.

Extrapolating the peak widths to zero mass loading (intercepts for least-squares linear fits of the data) gives approximately the same result for both loading methods (0.100 min for ACD data and 0.095 min for conventional loading data). The zero mass peak width is independent of the loading method, as might be expected. However, the slopes of the linear plots are significantly different for the conventional and two-pump ACD loading methods. The peak width increases at a rate of 0.045 min/mg with the conventional loading method and at 0.0033 min/mg with the two-pump ACD loading; the rate of peak broadening with increasing mass loading is approximately 14 times greater with the conventional loading configuration as it is with the ACD configuration.

Figure 7 shows mass loading studies for three compounds using the small-scale method with ACD. The peak widths of these and all compounds investigated exhibit approximately linear dependencies on mass loading. The intercepts or zero mass peak widths are strongly compound-dependent, varying over a range from about 0.08 to 0.18 min (for all compounds investigated using this column and the LC method). The slopes or rates of peak broadening may also be compound-dependent, but the range of variation appears to be much smaller; approximately 0.0025−0.0035 min/mg for all compounds investigated. The observed variation in slopes may be less than the uncertainties in the estimated slopes.

The largest mass loading used with the small-scale method and ACD to date is 20 mg. At this mass loading, column breakthrough and peak distortion (i.e., excessive peak broadening or asymmetry) have not been observed for any compound studied. The loading limit of the 4.6 mm × 150 mm Zorbax column is clearly greater than 20 mg; however, the actual mass loading limit for this column and method has not been systematically established.

Figure 8 shows the mass loading dependence of the peak width for cortisone using the midscale LC method (10 mm × 100 mm column) with ACD loading. This method was designed for use with plate-to-plate purification; thus, the flow-rate-to-column cross-section ratio is less than that required for optimal peak width. As a result, the zero mass peak width is slightly greater than that observed with the small-scale method (0.12 vs 0.10 min). However, the rate

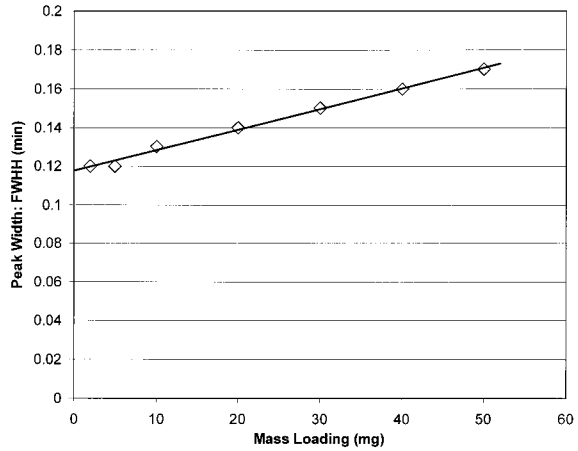

Figure 8. Peak width vs mass loading for cortisone using midscale method (10 mm × 100 mm column, 8 mL/m flow rate) with at-column dilution.

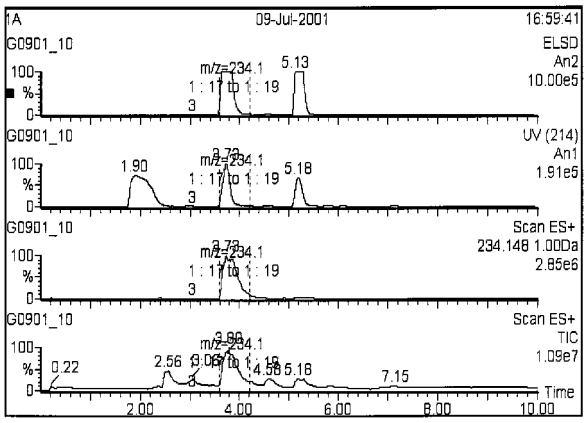

Figure 9. Purification of approximately 250 mg of material using the large-scale method (20 mm × 100 mm column, 25 mL/m flow rate) with at-column dilution.

of increase in peak width with increasing mass loading is about what would be expected from the ratio of the column volumes; the ratio of column volumes [(10 mm × 100 mm column)/(4.6 mm × 150 mm column)] is 3.15, while the ratio of slopes for linear fits of the peak width data is 3.0. The largest loading used in this study was 50 mg. At this loading, there is no evidence of column breakthrough or significant peak distortion. The mass loading limit for this column and method has not been systematically established.

No systematic mass loading studies have been performed using the large-scale LC method with ACD loading. However, in the purification of synthesis mixtures, mass loadings greater than 250 mg have been successfully accomplished using this method with ACD. An example of a purification of approximately 250 mg of material is shown in Figure 9. At this loading, the chromatographic peak shapes observed with UV and ELSD are narrow and symmetric. However, significant tailing is observed on the mass spectrometric detector, apparently due to overloading of the electrospray source. Extrapolation of the small-scale loading studies suggests it may be possible to achieve mass loadings in excess of 250 mg with this LC method; however, purification of this much material may require the use of a larger preparative/analytical split ratio in order to reduce peak broadening on the mass spectrometer.

Application of the two-pump at-column-dilution method is not limited to compounds that are readily soluble in the LC mobile phases (i.e., acetonitrile or water). Compounds with limited solubility in the loading solvent can work well by this method if the sample diluent is miscible with the loading solvent. For example, reserpine is slightly soluble in acetonitrile (less than 5 mg/mL) while cortisone is readily soluble in acetonitrile (ca. 40 mg/mL). The loading and chromatographic behaviors for reserpine loaded from a 50 mg/mL solution in DMSO and using the acetonitrile phase as the loading solvent are comparably to those for cortisone. No difficulties were encountered loading up to 20 mg of reserpine with the small-scale method (Figure 7) and up to 50 mg with the midscale method. Systematic loading studies with other standards and purifications of diverse synthesis mixtures indicate that this result is general.

Because the loading solvent is compatible with the sample and/or diluent, the usual problems associated with sample precipitation (i.e., clogged injector valves and transfer lines) are avoided with ACD. However, some compounds will precipitate when they encounter the weaker solvent in the mixer. Since the volume of the mixer-to-guard column connector is minimal (see below), the sample transfer time is generally small compared to the time required for precipitation and precipitates will form and collect on the guard column. This process does generally result in increased back-pressure. But since the cross-sectional area of the guard column is relatively large, the increase in the back-pressure is usually modest, temporary, and not problematic. In extreme cases, the increase in back-pressure can exceed the limits of the pumps and/or LC plumbing. In these cases the increase in pressure can usually be maintained within system tolerances by using a flow gradient superimposed on the compositional gradient. A flow gradient method was required for loading more than 5 mg of furosemide onto the 4.6 mm × 150 mm column (Figure 7). The flow gradient method used for furosemide is given in Table 1. To ensure good chromatographic peak shape and recovery in fraction collection, the full flow rate must be reached well in advance of the elution time of the compound. (More specific criteria for selecting appropriate flow gradients have not yet been established.)

At the large mass loading possible with at-column dilution, the chromatographic retention time can exhibit a significant dependence on the mass loading. Figure 10 shows the variation in retention time with increasing mass loading for three compounds using the small-scale LC method. The observed effect is strongly compound-dependent and is likely to be column- and gradient-method dependent as well.

Achieving complete mixing of the loading and diluting solvents is critical to obtaining good peak shape with the two-pump at-column-dilution configuration. (Mixing effects have not been reported for the three-pump ACD configuration.) The specific type of mixer required to achieve adequate mixing depends on the flow rate being used. At higher flow rates adequate mixing can usually be achieved using a simple tee. The large-scale method (flow rate of 25 mL/m) data in Figure 9 were obtained using a standard PEEK tee with 0.020 in. i.d. For the midscale and small-scale methods (flow rates

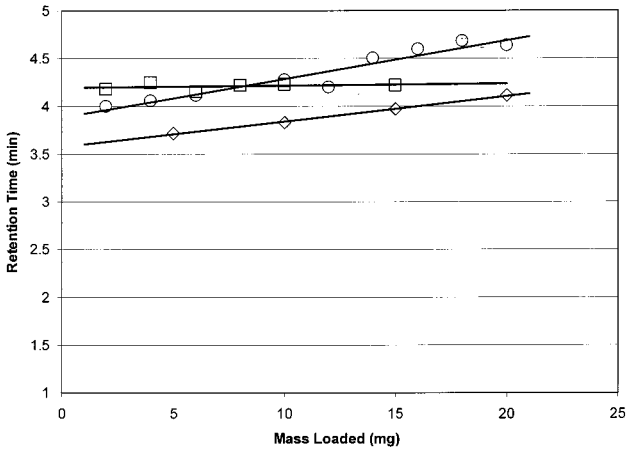

Figure 10. Retention time dependence on mass loading using small-scale method with at-column dilution: (□) reserpine; (◇) furosemide; (○) cortisone.

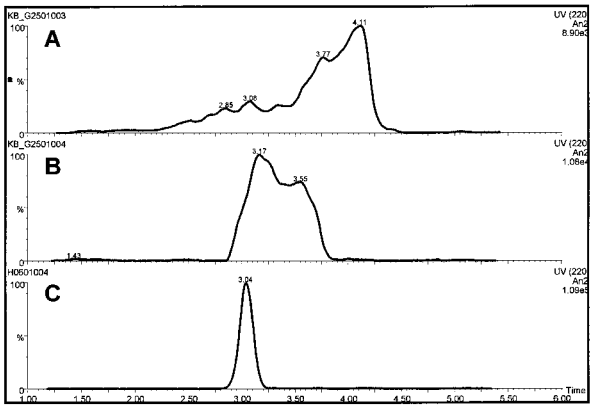

Figure 11. Peak shape observed for reserpine using midscale method with at-column dilution using different mixers: (A) 10 mg loaded using 0.02 in. i.d. PEEK tee mixer; (B) 5 mg loaded using 0.02 in. i.d. PEEK tee mixer; (C) 10 mg loaded using static tee mixer.

of 8 and 3 mL/m, respectively), the standard tee does not provide adequate mixing. Parts A and B of Figure 11 are representative of the peak shapes obtained for reserpine using a standard PEEK tee for the ACD mixer at 8 mL/m (midscale method). The peak shapes are very poor, and the exact shape obtained depends on the volume injected, sample concentration, initial composition of mobile phase, etc. Chromatogram C shows the peak shape for reserpine using the same method but using a static mixing tee (see Experimental Section). The static mixing tee can provide good peak shape with ACD at flow rates as low as 0.5 mL/m.

The volume of the mixer-to-column connector can also have a significant effect on the peak shape obtained with the two-pump ACD configuration. The volume of this connector should be minimized to achieve optimum peak shape. The effects are flow-rate-dependent, becoming more prevalent at lower flow rates. Figure 12 shows data using the at-column-dilution configuration in an analytical application. (The advantage of ACD in analytical applications is the elimination of column breakthrough for compounds dissolved in strong solvents.) The column is a 2.1 mm × 50 mm Zorbax C18, 5 μm particle size, and the flow rate is 0.625 mL/m. Traces A and B show the UV and extracted ion chromatograms observed for the analysis of 200 ng of

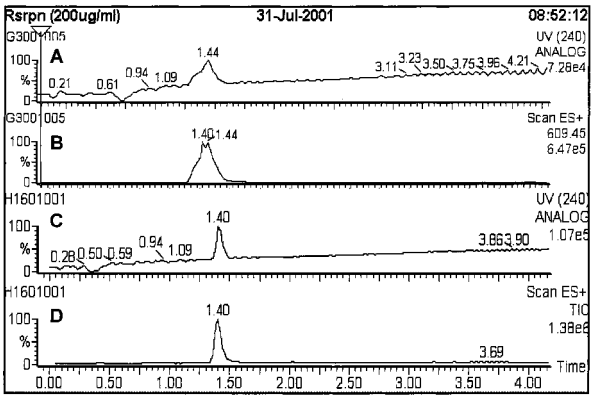

Figure 12. A 200 ng sample of reserpine using analytical scale method (2.1 mm × 50 mm column, 0.625 mL/m flow rate) with at-column dilution using different volume mixer-to-column connectors: (A, B) UV and extracted mass chromatograms observed with 20 cm × 0.007 in. i.d. PEEK connector; (C, D) UV and extracted mass chromatograms observed with 5 cm × 0.005 in. i.d. PEEK connector.

reserpine using a 20 cm length of 0.007 in. i.d. PEEK for the mixer-to-column connector. Traces C and D show the analysis performed using a 5 cm length of 0.005 in. i.d. PEEK tubing for the mixer-to-column connector. The reduction in tubing volume has improved the peak width by more than a factor of 2.

Conclusions

At-column dilution significantly increases the amount of sample that can be purified with a given column and flow rate relative to conventional loading methods. The two-pump configuration presented here is an effective means for achieving the at-column-dilution advantage. In this work, maximum loading capacities were not determined, but satisfactory chromatography was demonstrated with the two-pump ACD configuration at mass loadings more than 10 times greater than those at which the chromatography with conventional loading failed (i.e., exhibited excessive peak broadening, peak asymmetry, and/or column breakthrough). The rate of mass-loading-dependent peak broadening is sample-dependent but appears to be generally a factor of 15−20 times smaller with ACD than with conventional loading. Compounds with limited solubility in the loading solvent can work well with the two-pump ACD method, providing the sample diluent and loading solvent are miscible. Column breakthrough has not been observed for any compounds using ACD at the mass loading reported here. No distortions due to disturbance of column equilibrium by diluent have been observed using ACD. Compounds have been observed to precipitate on the guard column using ACD, but the resulting increase in back-pressure is usually tolerable or manageable via flow gradient methods.

At-column dilution makes large-scale LC−MS purification feasible. However, the more important applications of the technique will most likely involve reducing the scale of the chromatography required to perform high-throughput LC−MS purification of parallel synthesis products in the small-scale (<20 mg) to midscale (<50 mg) range. The ability to use smaller columns and lower flow rates to effect a

purification for a given mass of material has many benefits, including use of less expensive columns, consumption of less solvent, production of smaller-volume fractions (shorter dry-down times and the ability to collect into small-volume collector formats, such as 96-well plates), and perhaps, the ability to permit faster separations.

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