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Communications to the Editor

Microscale HPLC Predicts Preparative Performance at Millionfold Scale

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Abstract:

The use of microscale HPLC for piloting the large-scale preparative chromatographic resolution of the enantiomers of a chiral pharmaceutical intermediate is reported with an example of a millionfold scale-up from a 300 μm i.d. column to a 30 cm i.d column. Performance and productivity at scale are accurately predicted by the microscale approach, which consumes only a small fraction of the material typically used for conventional loading studies. These results suggest a great potential for use of microscale HPLC loading studies during early synthetic route investigations, when only a small amount of sample is typically available.

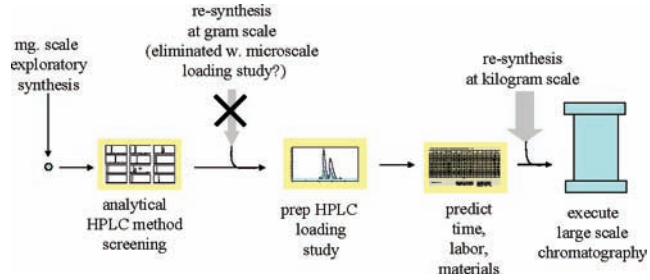

Figure 1. **Typical workflow for developing and carrying out a kilogram-scale preparative chromatographic separation. Microscale loading studies may eliminate the need for resynthesis at gram scale.**

Introduction

Preparative HPLC plays an increasingly important strategic role in the initial synthesis and subsequent scale-up of pharmaceutical candidates.[1] Chromatography provides a general, fast, and relatively inexpensive approach to a variety of challenging purification problems including the resolution of enantiomers.[2] Automated column and mobile phase screening enables rapid evaluation of chromatographic methods that can potentially be incorporated into chemical syntheses. For separations on the tens of grams and larger scale, method development is routinely followed by loading studies in which progressively larger amounts of sample are injected onto a pilot column, typically a 4.6 mm i.d. analytical column. Such loading studies are necessary because analytical chromatography alone is an insufficient predictor of preparative performance, where solubility and other more difficult to predict factors can come into play. Several hundred milligrams of a representative sample of the mixture to be purified is typically required for loading studies, from which performance at larger scale can be accurately predicted. In this study we investigate the possibility of carrying out preparative chromatographic loading studies using microscale HPLC, which reduces the overall compound requirement to a few milligrams, or even less.

Initial discovery synthesis is increasingly performed on only a few milligrams of compound, while preparative chromatographic loading studies require at least 100 mg and, more often, consume a gram or more. Consequently, gauging the feasibility of a preparative chromatography step during initial route exploration often requires scale-up of the initial pathfinding synthesis (Figure 1). This problem is further exacerbated by the fact that several preparative chromatography options are often considered for a given synthesis and that seemingly minor structural variation (e.g., methyl to ethyl ester or Boc to Cbz protecting group) can sometimes have a profound influence on chromatographic productivity.

The current need to repeat a synthesis at scale in order to investigate chromatographic feasibility is driven only by accepted practices and existing equipment, and not by any fundamental scientific or technological limitations. In this study, we investigate the potential of microscale HPLC for carrying out preparative chromatography loading studies at greatly reduced scale. Micropreparative HPLC has been used for some time in the purification of proteins and peptides.[3–5] In the small-molecule arena, we have found various forms of microscale HPLC to be useful for analysis in support of pharmaceutical process research investigations and have noted impressive (>250×) decreases in solvent usage and waste generation.[6–9] We now report on the use of microscale chromatography for carrying out preparative chromatography loading studies.

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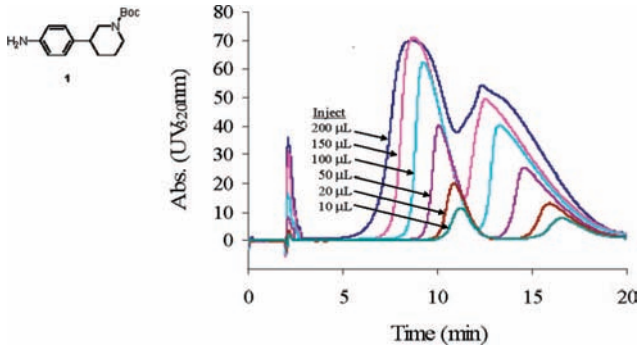

Figure 2. **Loading study on conventional analytical HPLC column (Chiralpak AD, 4.6 mm i.d. × 25 cm); particle size = 20 μm; flow rate = 1.4 mL/min; eluent 5% ethanol in heptane; injection volume = 10, 20, 50, 100, 150 and 200 μL; feed concentration = 90 mg/mL in ethanol; UV_{320} nm; ambient temperature.**

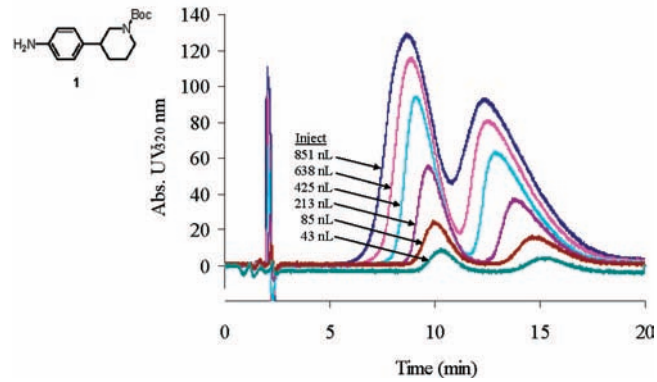

Figure 3. **Loading study on microfluidic HPLC system. Column Chiralpak AD, 300 μ i.d. × 25 cm, particle size 20 mm; flow rate 6.0 μL/min; eluent 5% ethanol in heptane; injection volume 43, 85, 213, 425, 638, 851 nL; feed concentration = 90 mg/mL in ethanol; UV_{320} nm; ambient temperature.**

Experimental Section

An Eksigent Express RT microflow HPLC system[12] was used in the study (Eksigent Technologies, LLC, Dublin, CA, U.S.A.). The standard injection loop of the system (250 nL) was replaced by a 15 cm long fused silica capillary with an i.d. of 100 μm having a volume of approximately 1.2 μL. The capillaries that lead from the injection port to the valve were also 15 cm in length with an i.d. of 100 μm. The fused silica connection capillaries, sample loop, and the micro filter had a total volume of ~2.5 μL. The injection loop was filled manually for each run to minimize the amount of sample that was needed for a complete loading study. The injection needle was flushed once with 50 μL of ethanol, and then a sample solution of 5 μL was pushed through the injection port. For each successive injection 2 μL of volume was used. The injection needle was not removed during each successive injection.

Microcolumns (300 μm, i.d. × 25 cm) were packed using a Haskel air-driven fluid pump with air-drive pressure of 100 psi. Chiralpak AD (20 μm) suspended in 1 mL of 5% IPA/heptane was added to the slurry reservoir, and the column pressure was increased to 5500 psi at a rate of 500 psi/min. The column was held at this pressure for ~10 min, and then the pressure was gradually released at a rate of ~500 psi per minute. Following addition of the final end fitting, the column was eluted with 10% IPA/heptane at a flow rate of 6 μL/min before use.

Large-scale preparative HPLC was conducted using a Biotage KP3000 system (Biotage, Charlottesville, VA, U.S.A.) and a 30 cm i.d. ProChrom LC3000 column (Novasep, Boothwyn, PA, U.S.A.) containing 10.6 kg (25 cm bed depth) Chiralpak AD 20 μm. A flow rate of 6 L/min was used with an injection cycle time of 13 min. Fraction collection was based on total volume and UV detection at 290 nm. Fractions were collected in metal drums, assayed for purity, combined, and concentrated by distillation at reduced pressure.

Results and Discussion

The predictable scale-up of preparative chromatography is one of the most valuable features of this technology. The fact that chromatography loading studies using conventional 4.6 mm i.d. columns can be used to accurately predict performance at larger scale is widely appreciated; thus, the use of even smaller 300 μ i.d. microcolumns for carrying out loading studies is a concept that should work, in principle. However, we were aware of several possible barriers to success, including clogging, wall effects, possible differences in column porosity, and differences in instrument injection styles. Accordingly, a study was set up to evaluate the approach in a real world preparative chromatography study.

Compound **1**, an early intermediate in the synthesis of a recent pharmaceutical development candidate from these laboratories, was chosen for study (Figure 2). While a number of synthetic routes to enantiopure **1** are possible in theory, none were readily suitable for producing this compound at kilogram scale with high enantiopurity in the short time available for this development project. Furthermore, initial chromatographic screening showed acceptable enantioseparation of **1** on several commercial chiral stationary phases, suggesting this would be a good candidate for preparative resolution.

A conventional loading study for the preparative separation of the enantiomers of **1** on the Chiralpak AD CSP (20 μm particle size) using a standard 4.6 mm i.d. × 25 cm analytical column is illustrated in Figure 2. The peaks corresponding to the two enantiomers are completely resolved for the smaller injection volumes of 10, 20 and 50 μL, which at a feed concentration of 90 mg/mL correspond to injection amounts of 0.9, 1.8, and 4.5 mg. At a higher feed volume of 100 μL (9 mg) the bands are incompletely resolved, while at 200 μL (18 mg) substantial peak overlap is observed. Preparative chromatography at the "touching band" level—corresponding to the 100 μL injection in Figure 2—is often chosen for early development, as high recovery and acceptable purity can be obtained under these conditions without the need to deal with mixed fractions. In contrast, even greater loads are often used for large-scale separations that employ steady-state recycling,[10] multicolumn chromatography (MCC),[11] or other techniques that enable recycle of mixed fractions. The choice of which loading

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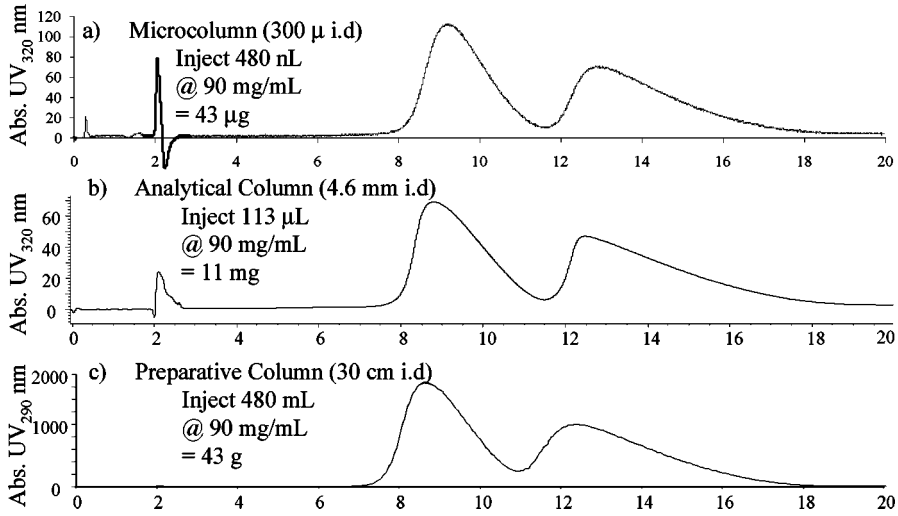

Figure 4. **Preparative HPLC injection of 1 at three different scales a) microscale (300 μ i.d.) conventional scale (4.6 mm i.d.) and c) Preparative scale (30 cm. i.d.) Comparable performance from smallest to largest scale, spanning a range of 10^6 suggests that microscale loading studies can accurately predict performance at preparative scale.**

study chromatogram to use as the basis for scale-up also depends on factors such as the required level of purity and recovery and considerations of whether the first or second peak, or both, are to be collected. In the present case, the second eluted enantiomer was required with a target enantiopurity of 98% ee and a target recovery of 85% of the theoretical 50% obtainable from the racemate.

The loading study in Figure 2 requires a minimum of 500 μL of sample feed, which corresponds to only about 50 mg of sample. However, in practice, significantly more material is often used, owing to the fact that repeated injections are often required and loading studies for several different column/mobile phase combinations are often carried out side by side. In addition, the preparative method illustrated here has a relatively modest productivity of only ~0.2 kkd (kilograms of isolated product per kilogram of stationary phase per 24 h day of operation), while chromatographic productivities of 1 or even 2 kkd are now becoming routine, and consume correspondingly more sample for loading studies.

We next performed the same loading study using a 300 μ i.d. × 25 cm microscale HPLC column packed with the same stationary phase. The injection volumes and flow rate were scaled down by a factor of 235, corresponding to the cross-sectional areas of the conventional and microbore columns. The results of the loading study on the 300 μ i.d. microbore column (Figure 3) are very similar to those obtained with the conventional 4.6 mm i.d. column. For the injection volumes of 43, 85, and 213 nL corresponding to 10, 20, and 50 μL on the conventional 4.6 mm i.d column, comparable baseline resolution of the two bands is observed, while the 425 nL injection (corresponding to 100 μL on the conventional 4.6 mm i.d. column) the beginning of peak overlap can be noted, with larger injection volumes leading to significant peak overlap in both cases. Overall, the peak shapes and retention times of the loading chromatograms in the two studies are well matched, suggesting that microscale HPLC loading studies may indeed be a useful predictor of larger-scale preparative separations.

The loading studies obtained with the microscale 300 μ i.d. HPLC column can be used to predict conditions for the actual preparative separation, which in this instance was carried out on a 30 cm i.d. × 25 cm column. Note that the scaling factor between the 300 μ i.d. microflow column and the 30 cm i.d. preparative column corresponds to the square of the ratio of the diameters of the two columns, i.e. $1000^2 = 1{,}000{,}000$. Consequently, a flow rate of 6 L/min was used for the preparative separation (vs 6 μL/min for the microscale loading study). On the basis of the loading studies, an injection size of 425 mL was predicted as optimal for the pilot-plant separation. The final separation conditions used in the actual preparative campaign employed a slightly larger load of 480 mL and a cycle time of 13 min to afford 3.5 kg of product with an enantiopurity of >98%ee and a recovery of 92%.

A comparison of chromatograms obtained with the three different column sizes under comparable load is shown in Figure 4. The remarkable degree of agreement between the three chromatograms spanning a range of 10^6 in scale emphasizes the potential value that the microscale HPLC approach may have for carrying out preparative chromatography loading studies. The close agreement also suggests that our initial concerns about wall effects, differences in column porosity, *etc.* may be relatively unimportant. Finally, the freedom from problems in this study and in subsequent investigations suggests that clogging may not be a general problem.

These findings have a number of interesting implications for the use of preparative chromatography to support organic synthesis. In principle, the microcolumn loading study described in this study could be carried out with less than a milligram of compound. There is a growing interest in miniaturization and "Laboratory on a Chip" approaches to reduce to an absolute minimum the compound requirements for drug discovery and development.[13] The ability to perform preparative chromatographic loading studies using only a few micrograms of sample could help to enable the interesting possibility of carrying out new synthetic route investigation at the submilligram scale, with the confidence that the separation could be scaled up for kilogram-scale production, if needed.

Conclusions

The results presented in this study clearly demonstrate the potential for the use of microscale HPLC for the predictive modelling of large scale preparative chromatographic separations. A significant benefit of the new approach is the potential for directly evaluating the feasibility of preparative chromatography during the initial pathfinding synthesis, without the need for resynthesis at larger scale.

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