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SCALING-UP PROCEDURE FROM THE RANGE OF MILLIGRAMS TO GRAMS FOR THE PURIFICATION OF AMINO ACID DERIVATIVES IN DISPLACEMENT CHROMATOGRAPHY[a]

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SUMMARY

A scaling up method in displacement chromatography was applied to the purification of the amino acid derivative N^α-9-fluorenoxycarbonyl-S-tritylcysteine [Fmoc-Cys(Trt)-OH]. Once the adsorption isotherm of the compound and some related impurities had been determined, the displacement chromatographic runs were carried out in the normal phase. The influence of parameters such as particle diameter of the stationary phase, carrier composition, nature of the displacer, concentration of the loaded sample and flow-rate is reported. The eluates were analysed directly at the outlet of the preparative columns with dedicated on-line detection equipment.

The results demonstrate that, once the proper conditions have been established, it is possible to purify in one run 100 mg and 38 g of feed mixture by proportionally increasing the amount of the stationary phase, using a larger column and maintaining the linear velocity and displacer concentration constant.

INTRODUCTION

High-performance displacement chromatography has recently been proposed as a potentially useful technique for the the preparative purification of natural and synthetic compounds[1-5]. Relative to the elution mode, displacement chromatography offers the advantages of a higher loading capacity, concentrated eluates and a low consumption of solvents as eluents.

In displacement chromatography, the column is first equilibrated with an eluent, the carrier, which has to be able to dissolve the feed components and the displacer at high concentrations and has to retain the bulk of the desired products on the stationary phase. After sample loading, an eluent containing a displacer, namely a compound that is strongly adsorbed on the stationary phase, is pumped so as to push the adsorbed feed components out of the column. The main feature of a compound to be considered

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as a displacer is that its adsorption isotherm overlies those of the feed components to be displaced.

The aim of this work was to show the feasibility of a scaling-up procedure from the range of milligrams to grams in displacement chromatography for preparative purifications. The choice of N^{α}-9-fluorenoxycarbonyl-S-tritylcysteine [Fmoc-Cys-(Trt)-OH] as a model for this study came from considerations that the retention time (t_R) of the desired product falls between the t_Rs of the impurities, that the presence of a polar carboxyl group makes its purification by normal-phase elution chromatography difficult because of tailing and that the compound cannot be purified by reversed-phase chromatography because it is not soluble in aqueous solvents at high concentrations.

As the concentrations of the eluted components in displacement chromatography are so high as to saturate completely common detectors, dedicated on-line detection equipment was set up to provide the information needed for the appropriate pooling of the fractions within the time of a displacement run. The proposed equipment carries out the following functions: picking up and appropriately diluting a small amount of the eluent, injecting it into a high-speed chromatographic system and analysing the corresponding chromatographic data. The entire sequence of the events is performed in less than 2 min and can be repeated any number of times.

EXPERIMENTAL

Materials

Fmoc-Cys(Trt)-OH was synthesized following the general procedure described by Kruse and Holden[6].

Benzyltributylammonium chloride (BTBA), benzylhexadecylammonium chloride (BHDA), triphenylmethanol (Trt-OH), 9-fluorenylmethanol (Fmoc-OH) and trifluoroacetic acid (TFA) were supplied by Fluka (Buchs, Switzerland); TFA was distilled prior to use. Methanol, chloroform and acetonitrile [high-performance liquid chromatographic (HPLC) grade] were purchased from Merck (Darmstadt, F.R.G.).

Water was purified with a Milli-Q system (Millipore, Bedford, MA, U.S.A.). The aqueous eluents were filtered through a 0.45-μm cellulose acetate filter and the organic solvents through a 0.5-μm PTFE filter; all the eluents were degassed by helium purging prior to use.

Apparatus

Displacement chromatographic runs in the milligram range were carried out on a LiChrocart RP-18 (10 μm) column (250 × 4.0 mm I.D. (Merck). The HPLC equipment consisted of a Model M-45 pump (Waters Assoc., Milford, MA, U.S.A.), a Model 7010 injector (Rheodyne, Cotati, CA, U.S.A.) with a 3 ml loop, a PU 4025 UV detector (Pye Unicam, Cambridge, U.K.) and a Model 2210 recorder (LKB, Bromma, Sweden). The column effluent was collected with a Model 2070 Ultrarack II (LKB).

The displacement runs in the gram range were carried out on axial compression columns of I.D. 20, 40 and 80 mm (Jobin-Yvon, Longjumeau, France) and packed with LiChroprep Si 60 silica (25–40 μm) (Merck). The pump was a Model 590 programmable HPLC pump (Waters Assoc.) and the effluents were collected with a Model 2211 Superrac fraction collector (LKB).
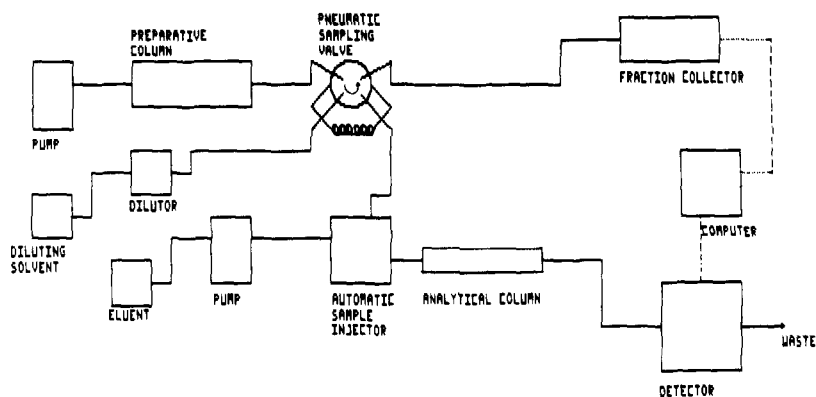
Fig. 1. Flow scheme of the on-line detection equipment used for displacement chromatographic monitoring.

The columns of I.D. 4, 20, 40, and 80 mm were equilibrated with the carrier for 20 min at a flow-rate of 0.4, 8, 32 and 128 ml/min, respectively. The same procedure, except the solvent that was methanol, was applied to regenerate the stationary phase after the displacement runs. Taking in account the steps of equilibration, elution and regeneration, the total chromatographic time for the displacement runs was about 230 min. The presence of the displacer agent in the fractions was monitored by thin-layer chromatography or spectrophotometry[7].

The on-line detection equipment (Fig. 1) consisted of a Model 401 dilutor (Gilson, Villiers-le-Bel, France), a Model 7010 injector controlled by a Model 7163 solenoid valve (Rheodyne), an isocratic HPLC system composed of a Model M-45 pump, a Model 231 sample injector (Gilson), a 100 × 2.1 mm I.D. HS C-18 (3 μm) column (Perkin-Elmer, Norwalk, CT, U.S.A.), a PU 4025 UV detector and a Model 7700 Professional Computer with Chrom 3 software (Perkin-Elmer) for data handling. This apparatus differs slightly from that described previously[8] because it permits controlled dilution of the eluate so as not to overload the analytical column.

An appropriate programme on the Model 231 sample injector directly controls the run time, the dilution volume, the injection onto the analytical column and, indirectly, through some relays, the turning of the pneumatic valve, the change of the fractions of the collector and the start of the data acquisition on the computer.

The adsorption isotherms, calculated by frontal chromatography[9], were obtained on equipment consisting of a Model 303 micro-pump (Gilson), a Model 7001 pneumatic actuator connected to two Model 7010 injectors with 1-ml loops and a solenoid valve (Rheodyne), a 51 × 2.1 mm I.D. stainless-steel column packed with LiChroprep Si 60 silica (25–40 μm), a Model 875 UV detector with a micro-cell (Jasco, Tokyo, Japan) and an Omniscribe Model D-2000 recorder (Houston Instruments, Austin, TX, U.S.A.). The column and the injection system were thermostated to the appropriate temperature in a Model 12B bath (Julabo, Seelbach, F.R.G.).

RESULTS AND DISCUSSION

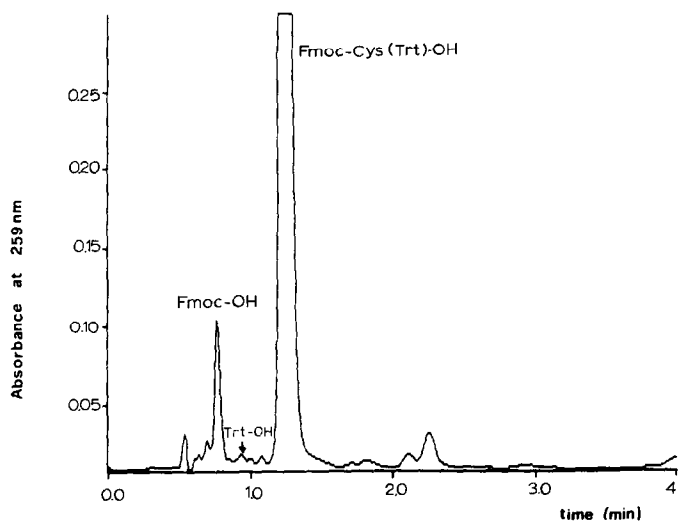Fig. 2 shows the reversed-phase analytical chromatogram of crude material to be

Fig. 2. Analytical chromatogram of crude Fmoc-Cys(Trt)-OH with isocratic elution. Column, Perkin-Elmer HS-3 C_{18} (100 mm I.D.); eluent, 83% acetonitrile with 0.1% TFA; flow-rate, 1.7 ml/min; sample, 8 μg of Fmoc-Cys(Trt)-OH mixture.

purified; 75% of the mixture was calculated to be Fmoc-Cys(Trt)-OH (retention time, t_R = 1.2 min). The impurities at t_R 0.75 and 0.87 min were Fmoc-OH and Trt-OH, respectively.
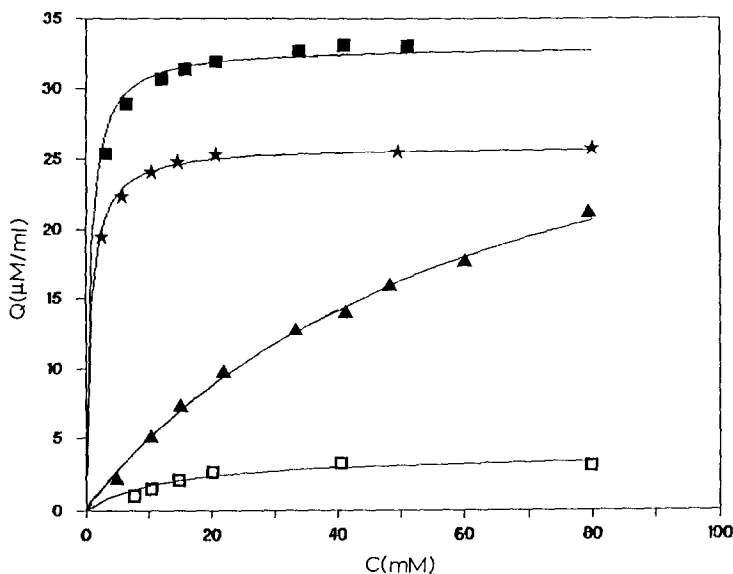

Fig. 3. Adsorption isotherms of (■) BTBA, (★) Fmoc-Cys(Trt)-OH, (▲) Fmoc-OH and (□) Trt-OH on a 51 × 2.1 mm I.D. LiChroprep Si 60 (25–40 μm) column with chloroform at 25°C. Q = μmol/ml of adsorbent in the column.
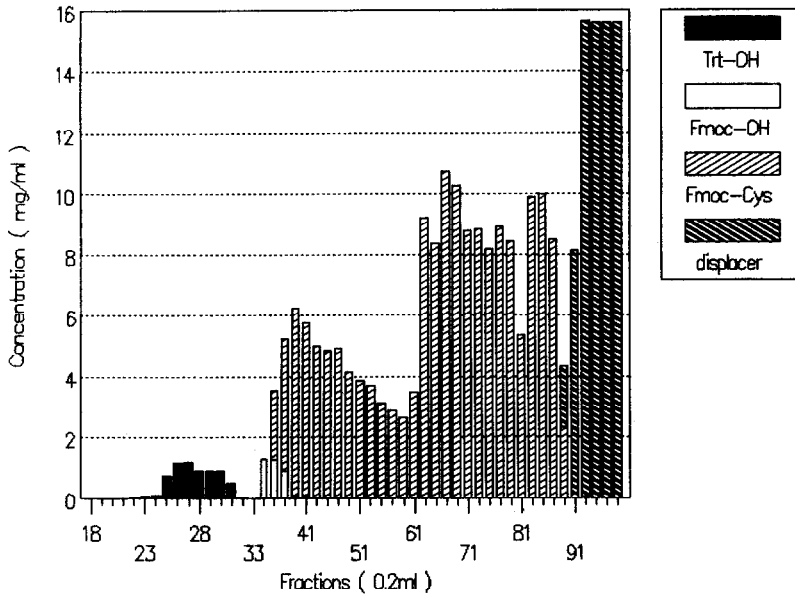
Fig. 4. Displacement chromatography of crude Fmoc-Cys(Trt)-OH. Column, 10 μm LiChrocart Si 60 (250 × 4.0 mm I.D.); carrier, chloroform; displacer, 50 m*M* BTBA in chloroform; flow-rate, 0.1 ml/min; temperature, 23°C; feed, 100 mg of Fmoc-Cys(Trt)-OH mixture in 2 ml of chloroform.
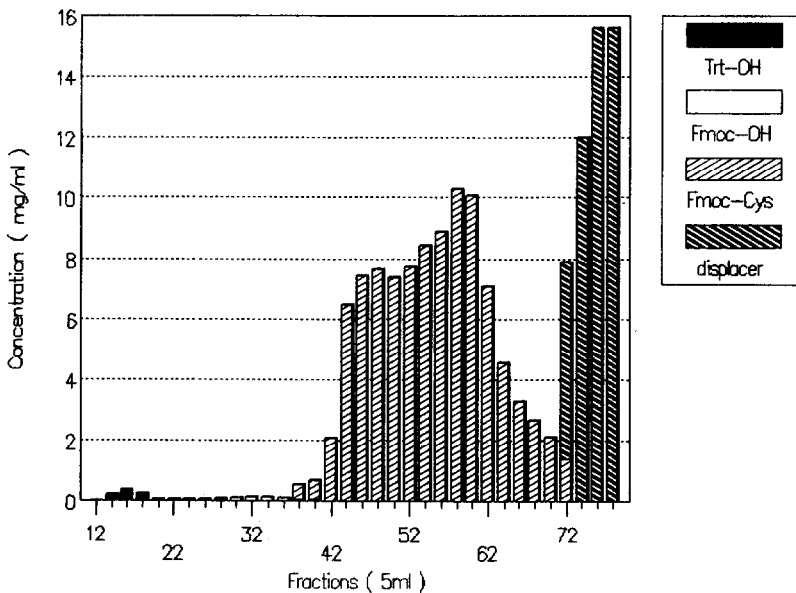

Fig. 5. Gram-scale displacement chromatography. Column, 25–40 μm LiChroprep Si 60 (230 × 20 mm I.D.; carrier, displacer and temperature as in Fig. 4; flow-rate, 2 ml/min; feed, 1.5 g of Fmoc-Cys(Trt)-OH mixture in 30 ml of chloroform.

Based on the adsorption isotherms on silica of Fmoc-Cys(Trt)-OH, Fmoc-OH, Trt-OH and BTBA, measured in chloroform and shown in Fig. 3, BTBA was selected as the displacer compound for the Fmoc-Cys(Trt)-OH mixture. Chloroform was chosen as the carrier eluent because it dissolved large amounts of crude material and it did not elute Fmoc-Cys(Trt)-OH in normal-phase chromatography.

The histogram of displacement chromatography for the purification of 100 mg of Fmoc-Cys(Trt)-OH mixture on a 250 × 4.0 mm I.D. LiChrocart Si 60 column, constructed on the basis of the reversed-phase analysis of the fractions, is shown in Fig. 4. The displacement conditions are given in the caption. About 65 mg of pure Fmoc-Cys(Trt)-OH were recovered from the pool of fractions 39–87 with a collected volume of 9.8 ml. The chromatographic yield was 95%, calculated as the ratio of pure collected compound to the total amount present in the crude sample.

In the first step of the scaling-up procedure, a 20 mm I.D. column packed with 30 g of LiChroprep Si-60 silica (25–40 μm) was employed. With respect to the chromatographic conditions in Fig. 4, 1.5 g of Fmoc-Cys(Trt)-OH mixture, dissolved in 30 ml of chloroform, was loaded to maintain a ratio of 50 mg of loaded sample per gram of stationary phase and a concentration of 50 mg/ml of injected sample. The flow-rate was increased to 2 ml/min to obtain the same breakthrough time of the displacer front.

Fig. 5 shows the relative histogram; 1.08 g of pure Fmoc-Cys(Trt)-OH was collected in 145 ml of eluate from fractions 42–70 with a recovery of 95%.

As under the last chromatographic conditions the separation efficiency and the yield were as good as those obtained on the analytical column, the diameter of the
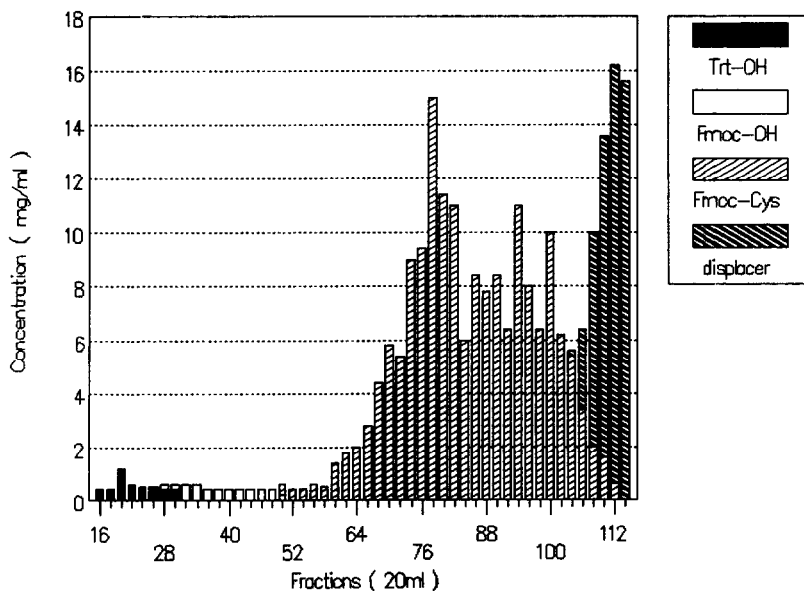

Fig. 6. Gram-scale displacement chromatography. Column, 25–40 μm LiChroprep Si 60 (270 × 40 mm I.D.); carrier, displacer and temperature as in Fig. 4; flow-rate, 8 ml/min; feed, 9.5 g of Fmoc-Cys(Trt)-OH mixture in 190 ml of chloroform.

preparative column was increased further to 40 and 80 mm I.D. The two columns were packed with 200 and 800 g of LiChroprep Si-60 silica (25–40 μm), respectively, and, to maintain the same ratio of sample per gram of stationary phase, 9.5 and 38 g of Fmoc-Cys(Trt)-OH mixture, respectively, were injected. The flow-rate was increased 4- and 8-fold, respectively, all the other chromatographic parameters being maintained constant.

Figs. 6 and 7 show the relative displacement histograms. From the 40 mm I.D. column 6.42 g of pure material were obtained from fractions 50–102 (1040 ml of collected eluate, yield 92%), whereas from the 80 mm I.D. column 25.4 g were recovered in fractions 59–79 (2520 ml of collected eluate, yield 89%). After each displacement run, the stationary phase was washed with 3.7 ml of methanol per gram of stationary phase and no reduction in the column efficiency was detected after the regeneration cycles.

The influence of various operational parameters was investigated. Fig. 8 shows a displacement chromatogram obtained under the same conditions as in Fig. 5 except that the flow-rate was increased 3-fold. A 960-mg amount of pure material was recovered from fractions 40–60 with a yield of 86%.

The replacement of LiChroprep Si-60 (25–40 μm) silica with LiChroprep Si-60 (40–60 μm) silica or with Baker 30–60 μm silica significantly increased the area of the overlapping zones, reducing the yield to 30%. When BTBA was replaced with BHDA, the breakthrough volume of the displacement train increased, but no appreciable differences in the recovery of the products were detected. An elution rather than a displacement mechanism was observed when chloroform, used as the carrier and the
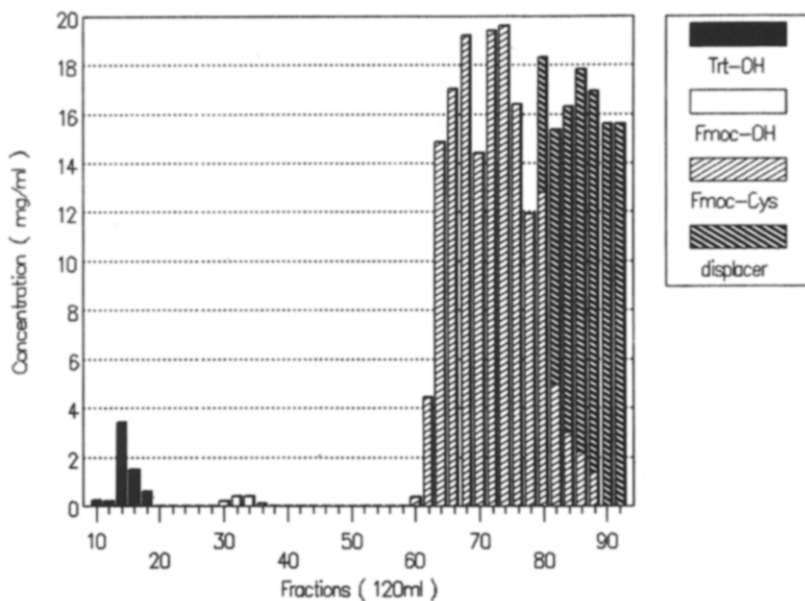

Fig. 7. Gram-scale displacement chromatography. Column, 25–40 μm LiChroprep Si 60 (270 × 80 mm I.D.); carrier, displacer and temperature as in Fig. 4; flow-rate, 32 ml/min; feed, 38 g of Fmoc-Cys(Trt)-OH mixture in 760 ml of chloroform.
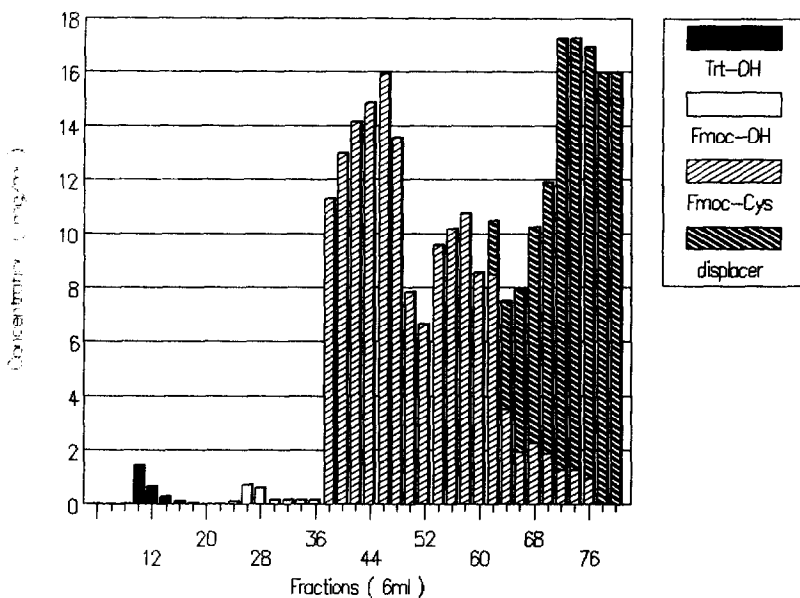
Fig. 8. Effect of flow-rate on the recovery. Conditions as in Fig. 5 except flow-rate, 6 ml/min.

solvent for the displacer solution, was replaced with ethyl acetate–chloroform–hexane (33:57:10, v/v/v), an eluent that under analytical conditions gave for a similar performance to chloroform with respect to the Fmoc-Cys(Trt)-OH mixture. A small reduction in the yield occurred when the concentration of the loaded sample was increased.

The reported separations demonstrate that a scaled-up purification of an amino acid, i.e., Fmoc-Cys(Trt)-OH, can be carried out in the displacement mode from 100 mg up to 38 g simply by proportionally increasing the amount of the stationary phase and adjusting the flow-rate to the column and the particle diameter. Indeed, the advantageous performance of displacement chromatography, namely a high recovery (90%), a high sample loadings [50 mg of crude Fmoc-Cys(Trt)-OH per gram of silica], concentrated eluates (10–20 mg/ml) and low consumption of solvents as eluents and displacer washing (0.5 ml/mg of purified material), were maintained over the whole range of purified material.

The need to minimize the cost of the preparative columns prompted us to replace the expensive 10 μm stationary phase with the less expensive 25–40 μm material. This substitution did not have any significant effect on the displacement performance and also permitted the use of a reduced velocity in the run in Fig. 5 that was faster than that employed in Fig. 4. A further increase in the flow rate (Fig. 8) caused a decrease in the recovery from 97% to 86%[10]. However, as 86% might still be a satisfactory yield, it is a matter of optimization to decide whether the lower recovery could be justified by a reduction in the run time. Silica with particle diameters larger than 40 μm did not seem to be usable because the recovery was drastically reduced.

The change in the elution mechanism observed when the chloroform was

replaced with ethyl acetate–chloroform–hexane could be explained by the fact that when the silica is equilibrated with a mixed eluent, the solvent with the highest polarity in the mixture is preferentially absorbed on the stationary phase[11]. During the displacement run this solvent is displaced out of the stationary phase by the injected sample and mainly by the displacer solution, so that close to the displacer front the eluent becomes richer in the more polar solvent, causing the elution of the products.

CONCLUSION

The major requirements in a scaling-up procedure in preparative chromatography are that, in order to save time and materials, the conditions for purification should be optimized in milligram-scale runs and that the same conditions should be applied to the gram or kilogram scales without any extra experiment to adjust the chromatographic parameters. In this work it has been demonstrated that these requirements are fulfilled by displacement chromatography; indeed, when parameters such as the composition of the carrier, nature of the displacer, stationary phase, amount of sample loaded per gram of stationary phase, concentration of the injected sample, linear velocity and regeneration procedure were maintained constant in all preparative experiments in runs in which from 100 mg to 38 g of crude mixture were loaded, similar performances were obtained.

ACKNOWLEDGEMENTS

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