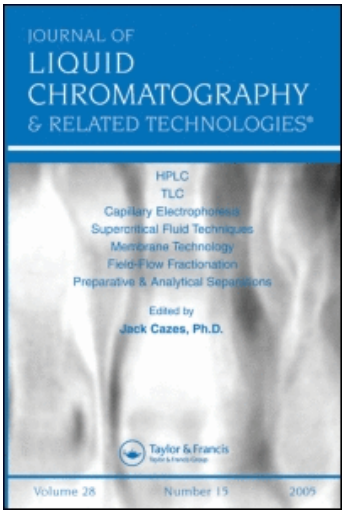

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High Speed Preparative HPLC Separation of Regioisomers of a Pharmaceutical Intermediate Using Gradients of Methanol in Ethoxynonafluorobutane

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Abstract: Highly efficient preparative HPLC separation of a mixture of regioisomers of a pharmaceutical intermediate using gradients of methanol in hexane-like ethoxynonafluorobutane on a cyano column is described. Such gradients were unique for the mixture to produce significant amounts of individual isomers in a short period of time. The quality of the separation was not affected by a two-fold increase in the flow rate used. The influence of the injection solvent, sample load, and injection volume on the separation performance was studied. Viscosity of the injection solvent and its volume appeared to play a major role in the successful outcome of the regioisomers' separation, and the polarity of the injection solvent did not seem to affect the separation.

Keywords: High speed, Preparative HPLC, Gradient, Methanol, Ethoxynonafluorobutane, Regioisomers, Cyano, Column

INTRODUCTION

Separation of various regioisomers obtained during the synthesis of potential drug candidates is a continuing challenge to medicinal chemists. Initial separation attempts typically utilize flash chromatography on silica gel because of

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the technique's speed and ease of use. However, chromatographic separation of regioisomers becomes increasingly difficult as differences in their hydrophobicities and ability to adsorb onto silica gel diminish. To utilize the full separation power of modern chromatographic purification methods, one may employ a range of separation tools taking advantage of the selectivity provided by less traditional organic solvents and modified silica gel stationary phases.

While reversed-phase (RP) HPLC remains the most commonly used technique for separation of regioisomers, it is not always the optimal method. There are several significant limitations to the technique. Organic intermediates have low solubility in aqueous solvent mixtures used in reverse-phase HPLC, making sample loading problematic. In addition, the hydrophobic properties of regioisomers are generally very similar, which may lead to insufficient separation coefficients. Reverse phase chromatography is limited to two aqueous mobile phases (aqueous acetonitrile and aqueous methanol) and to the most popular (and chemically similar) hydrophobic stationary C_8 and C_{18} phases. These issues can make reversed-phase chromatography an impractical choice for isomer separation and purification.

Given the limitations of reversed-phase chromatography, normal-phase chromatographic conditions can be better suited for the separation of structurally similar molecules, as more tools become available. These tools include a wider variety of solvents, such as non-polar hydrocarbons and chlorinated solvents mixed with polar ethers, esters, and alcohols, as well as a greater choice of column packing materials, for example, cyano-derivatized, diol-derivatized, and "bare" silica gel columns. Another advantage of normal-phase over reversed-phase HPLC is that the organic intermediates usually have higher solubility in normal-phase HPLC solvents, which also favors these solvents for preparative separations, where higher loadings are desirable. In addition, the mobile phases employed in normal-phase separations are less viscous than RP solvents, allowing for significantly higher flow rates. Increased flow rates, with minimal resolution loss for the sample components, can significantly improve the overall productivity of chromatographic purification procedures.

Normal-phase chromatography, using bare silica gel in either low or high performance formats (cartridges and HPLC columns, respectively), can have its own problems. However, these potential issues, including poor reproducibility, peak tailing, and long equilibration times, can often be avoided by utilizing bonded polar stationary phases (e.g., cyano and diol) and high speed linear gradients of polar solvent mixtures in hexane.[1] Recently, we found equal or better separation with cyano- or diol-derivatized silica can be achieved when hexane was replaced by ethoxynonafluorobutane (ENFB). Using methanol gradients in ENFB, we were able to separate such diverse synthetic and natural organic compounds as steroids, benzodiazepines, non-steroidal anti-inflamatory agents (NSAIDs), tricyclic antidepressants, β-adrenergic blocking agents, and purines and pyrimidines.[2]

As part of a project targeting G-protein-coupled receptor ligands, we were interested in aromatic systems substituted with a basic side-chain and a sulfonamide. We had targeted pyrimidines as the core aromatic system, in part because sequential displacement of the two activated chlorines in 2,4-dichloropyrimidine (**1**), first introducing a basic side chain to give regioisomers **3** and **4**, and then adding an aryl sulfonamide would lead to targets **5** and **6** (Figure 1). Heating 1-(t-butylcarboxy)-3-hydroxy-pyrrolidine **2** with **1** in the presence of sodium hydride in refluxing tetrahydrofuran (THF) provided the regioisomers, both of which were of potential interest. However, these were inseparable by flash chromatography. Heating the mixture of **3** and **4** with phenylsulfonamide, with potassium carbonate as base, provided the desired sulfonamides **5** and **6**, but again as an inseparable mixture. Because conventional flash chromatographic methods using silica gel columns failed to provide adequate separation for the regioisomers, we decided to explore other chromatographic options to separate **5** and **6**.

In this communication, we describe our investigation of high performance liquid chromatographic approaches to the separation of two regioisomers and the optimization of the best technique to larger scale separations. After preparative HPLC separation, both isomers were further elaborated to produce two sets of pharmacologically distinctive derivatives.

EXPERIMENTAL

All chemicals used in this study were purchased from Aldrich-Sigma (St. Louis, MO).

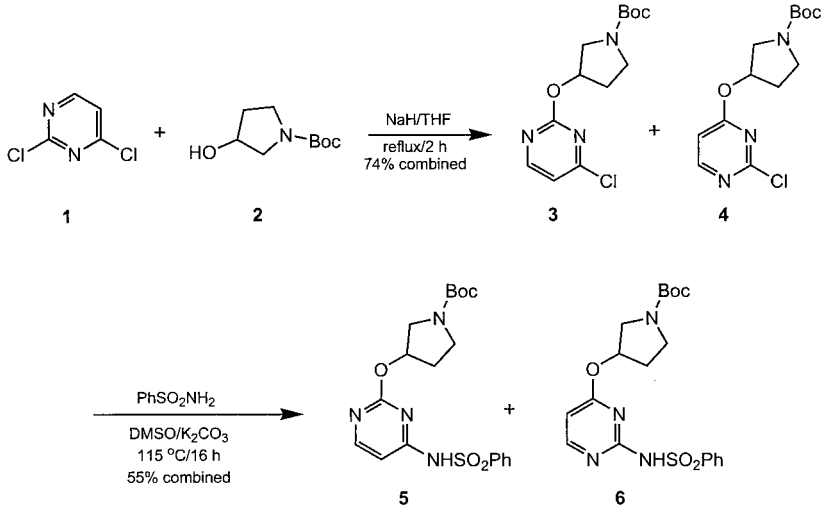

Figure 1. Synthesis of a mixture of regioisomers **5** and **6**.

All chemicals used in this study were purchased from Aldrich-Sigma (St. Louis, MO). Ethoxynonafluorobutane (ENFB) was obtained as 3M NovecTM Engineered Fluid HFE-7200 from 3M Company (St. Paul, MN). All other solvents were of HPLC grade and purchased from EM Science (Gibbstown, NJ).

The 1100 (Agilent Technologies, Palo Alto, CA) liquid chromatograph equipped with an autosampler, a thermostatted column compartment, and a diode-array detector was used for analytical HPLC.

Preparative HPLC was performed on a Varian (Walnut Creek, CA) Star preparative HPLC system consisting of two SD-1 pumps, a Rheodyne manual injector (2 mL sample loop), a UV-1 detector equipped with SuperPrep cell, and an FC-1 fraction collector.

All columns were purchased from Phenomenex (Torrance, CA). Analytical normal-phase HPLC separations were carried out on 0.2×15 cm Primesphere SIL (5 μm particles, flow rate 0.2 and 0.5 mL/min) and Luna CN column (3 μm, 0.6 mL/min), RP separations on a Primesphere C_{18} 0.2×15 cm column (3 μm, 0.2 mL/min).

Preparative HPLC was performed using a 2×15 cm Luna CN column (5 μm) at 38 mL/min flow rate. Linear gradients (10 min) of methanol (solvent A) and a mixture of methylene chloride with methanol (8 : 2, B) in non-polar ENFB (C) and hexane (D) were used for analytical and preparative HPLC work. Triethylamine (TEA) was added at 0.1% to all solvents (A–C) to reduce peak tailing. Aqueous mixtures of ACN and MeOH with 0.1% trifluoroacetic acid were employed for analytical RP HPLC.

RESULTS AND DISCUSSION

A mixture of regioisomers **5** and **6** (Figure 1) was found to be practically inseparable by RP analytical HPLC using either 30% acetonitrile in water or 40% methanol in water. In both cases, the resolution achieved was not adequate ($R_s \sim 2$) to merit preparative separation under RP mode.

Low resolution was also observed while trying to separate the mixture with 40% methyl-tert-butylether (MTBE) in hexane or with 1.5% methanol in methylene chloride ($R_s = 1.25$ and 0.3, respectively) on a Primesphere silica gel HPLC analytical column.

Excellent analytical separation of regioisomers was finally achieved on a Luna CN 2×100 mm column with 10 min linear gradients of methanol and methylene chloride in hexane (5–40% B in D at 0.8 mL/min) or methanol in EFNB (5–20% A in C at 0.6 mL/min) under conditions described previously.[2] Resolutions obtained with both solvent systems ($R_s \sim 7$ and ~6) were adequate for a preparative HPLC scale-up. Higher than conventional flow rates employed in those experiments did not seem to be detrimental to the overall quality of the separation and encouraged the use of increased flow rates for preparative HPLC work.

The first attempt to separate **5** and **6** using a hexane-based gradient (5–30% B in D in 12 min), and injecting ~100 mg of the mixture in ~1 mL of methylene chloride onto a preparative Luna CN column resulted in severe chromatographic system clogging due to the sample's low solubility in hexane.

No such problem with sample solubility in mobile phase was observed when hexane was replaced with ENFB, solvent B with MeOH (A) and

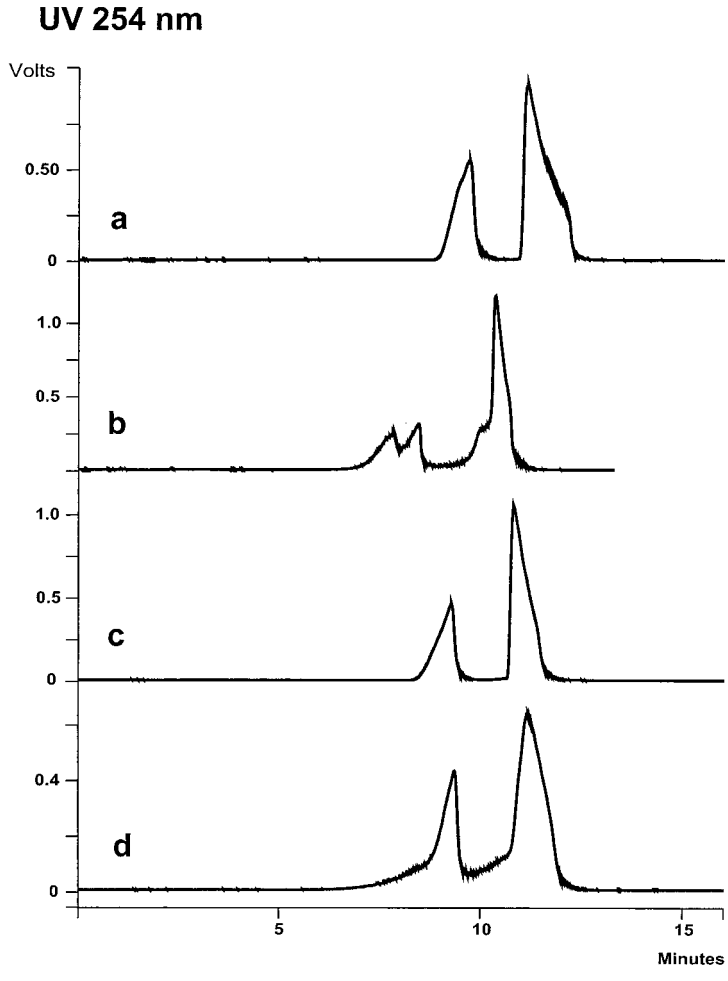

Figure 2. Preparative HPLC separation **5** and **6**. Column: Luna CN 2 × 15 cm. Flow rate: 38 m/min. Linear gradient 5–20% of MeOH in ENFB in 12 min. Detection: UV 254 nm. (a) 128 mg of the mixture (injected in 0.6 mL of CH_2Cl_2); (b) 105 mg in 1 mL of CH_2CL_2; (c) 105 mg in 0.4 mL of MeOH; and (d) 110 mg in 0.4 mL of DMSO.

~128 mg of a mixture of **5** and **6** were injected in 0.6 mL of methylene chloride to produce baseline separation of the isomers (Figure 2a). Combined fractions provided material with better than 99% purity. Overall, ~1.7 g of the mixture was processed in a similar manner with an outstanding recovery of 96%.

Effect of Injection Solvent, Sample Load, and Sample Size on the Preparative HPLC Separation of Regioisomers 5 and 6

Solubility of a sample in mobile phase and sample load are important parameters to consider for a successful preparative HPLC separation.[3,4] The gradient mode of elution often requires injection of concentrated solutions of a sample in a relatively polar solvent into the solvent stream with very different polarity and viscosity. We investigated the effect of these parameters on the outcome of preparative HPLC separation of isomers **5** and **6**.

One obvious advantage of mobile phase based on the fluorinated solvent ENFB is its miscibility with all common solvents. It allows for use of polar injection solvents (e.g., DMSO and methanol) for relatively polar compounds, something that cannot be achieved with hexane-based mobile phases.

Injection of ~128 mg of the sample in 0.6 mL of a fairly non-polar methylene chloride resulted in an excellent separation (Figure 2a). The increase of sample injection volume from ~0.6 to ~1 mL of the same solvent was clearly detrimental to the resolution observed (sample amount: ~105 mg; Figure 2b).

Surprisingly, when the mixture (~105 mg) was dissolved in 0.4 mL of very polar methanol and injected onto the column, the separation was as good as, or better than, when methylene chloride was used as an injection solvent (compare Figure 2c and 2a). When ~110 mg were injected in polar and viscous DMSO (0.4 mL), the peak shape and resolution were significantly worse, compared to methylene chloride or methanol (Figure 2d). The high viscosity of the DMSO solution seems to prevent rapid mixing of the sample components with mobile phase inside the column, leading to a significant distortion of chromatographic peaks.

Based on our observations, two factors—total volume of the sample and viscosity of the sample's solution, and not the polarity of an injection solvent—appear to play major roles in the successful outcome of a particular preparative HPLC separation. Consequently, we could double, and even triple, the sample load (up to ~300 mg per injection) for this separation without noticeable change in its quality by keeping the sample injection volume low (less than 1 mL) and using a mixture of low viscosity solvents, e.g., methylene chloride and methanol, to dissolve the sample (Figure 3a and 3b).

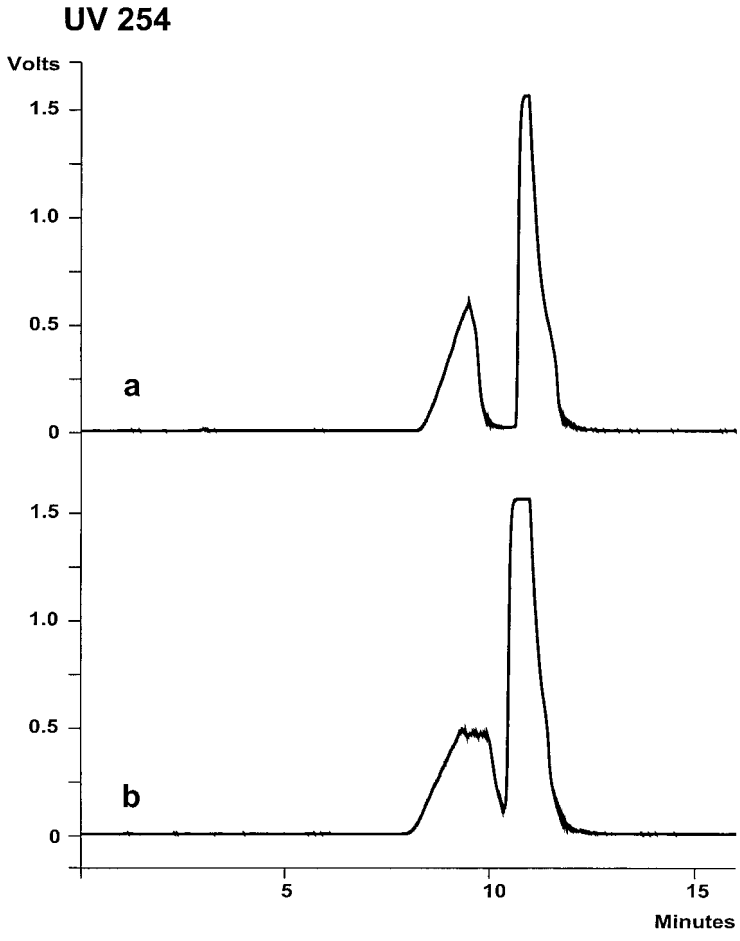

Figure 3. Preparative HPLC separation of regioisomers **5** and **6**. Chromatographic conditions as in Figure 2; (a) 210 mg of the mixture (injected in 0.5 mL of CH_2Cl_2–MeOH, 1 : 1); (b) 300 mg (1 mL of CH_2Cl_2–MeOH, 1 : 1).

Relatively high flow rate gradients described above, and used successfully for the separation of regioisomers, appear to be of certain practical importance. The use of mobile phases based on normal-phase organic solvents generally leads to much lower column back pressures (compared to conventional RP methods) and allows employing an HPLC column of sufficient length and, therefore, with adequate separation power to resolve even difficult-to-separate mixtures. The loss of chromatographic resolution associated with increased flow rates may be less pronounced when very efficient columns are used, which, in turn, leads to the increased productivity of a preparative HPLC purification. Using this technique, we were able to

separate multi-gram quantities of a mixture of regioisomers **5** and **6** in a very short period of time.

CONCLUSIONS

High speed methanol gradients in hexane, e.g., ethoxynonafluorobutane on cyano columns, were used to separate preparative amounts of t-butyl esters 3-(4-benzenesulfonylamino-pyrimidin-2-yloxy)-pyrrolidine-1-carboxylic acid and its 2-benzenesulfonylamino-pyrimidin-4-yloxy-analog. Such gradients were unique for this particular mixture for the production of significant amounts of needed isomers. Performance of a preparative separation seemed to be affected by the sample solvent viscosity rather than by its polarity. High viscosity solvents, e.g., DMSO, produced distorted chromatographic peaks due to what appears to be a much slower process of mixing with mobile phase at the top of the preparative column. Sample volume, and not necessarily sample load, seemed to play a major role in the peak distortion process. The optimal sample volume did not exceed $\sim$1 mL for the experimental setup (2×15 cm column, flow rate 38 mL/min, sample loop 2 mL) described in this report. Ethoxynonafluorobutane miscibility with all common solvents allowed for injection of polar samples in polar solvents under normal-phase conditions. The overall productivity of HPLC purification was aided by a 2-fold increase (compared to conventional) in the flow rate of the mobile phase.

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