Fast Generic-Gradient Reversed-Phase High-Performance Liquid Chromatography Using Short Narrow-Bore Columns Packed with Small Nonporous Silica Particles for the Analysis of Combinatorial Libraries

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Abstract

The extremely large number of samples generated for the quality control analysis of combinatorial libraries that are developed in the pharmaceutical research for drug discovery requires fast generic methods such as rapid-gradient reversed-phase high-performance liquid chromatography (HPLC). These methods are necessary as standard procedures to produce up to several hundreds of analytical results per day and should be optimized in order to be applied to library products of widely differing polarities. This work presents an optimized generic method using a narrow-bore column packed with 1.5-µm nonporous particles and a completely automated HPLC workstation configured for the best efficiency, throughput, and robustness with this column. A test mix of 12 compounds with a wide polarity range is separated within 1.5 min with a cycle time of 3.5 min. The throughput is further enhanced using a Gilson 233XL dual-injection sampler to feed two parallel HPLC systems in order to perform 34 analyses per hour.

Introduction

The recent trend in the areas of drug discovery and medicinal chemistry involves the high-throughput screening of molecular diversity (i.e., peptide, nonpeptide, and small-molecule libraries). Therefore, in today's pharmaceutical and biotechnology industries, large numbers of compounds resulting from combinatorial synthesis must be quickly characterized to provide structural and purity information and consequently generate a strong need for fast and reliable techniques.

Few generic methods using reversed-phase (RP) high-performance liquid chromatography (HPLC) have been reported in recent years for the analysis of drug-candidate compounds from combinatorial chemistry. They were based on short C18 columns with sharp slope gradients from acidified water to acidified organic solvents; high flow rates; and UV detection set at 220 nm or 254 nm (1–3), mass spectrometry (MS) detection (4,5), or both. These previous works emphasized the advantages of fast RP-HPLC separation methods using steep gradients; however, very little information was given on the optimization, reproducibility, and robustness of these proposed techniques.

The objective of this study was to set up a reproducible generic RP-HPLC gradient with maximum resolution and minimum run time to proceed quality control (QC) analyses of a large variety of combinatorial mixtures with wide polarity ranges. The described solution came from the selection and combination of equipment specifically adapted for advanced separation media technologies and used the advantages of a narrow-bore column filled with small nonporous particles (1.5 µm). In this work, a 12-component test mix previously described (6–8) was selected for its large log P range of particular interest to the medicinal chemists. Optimization and reliability of the method will be largely developed.

Experimental

Chemicals, reagents, and standards

All chemicals and solvents were of analytical or HPLC grade. Uracil, theophylline, acetanilide, *m*-cresol, acetophenone, benzofuran, heptanophenone, octanophenone, trifluoroacetic acid (TFA), and ammonium acetate were obtained from Sigma-Aldrich (St. Quentin Fallavier, France). Propiophenone, butyrophenone, valerophenone, and hexanophenone were purchased from Janssen Chimica (Geel, Belgium). Water and acetonitrile were from SDS (Peypin, France).

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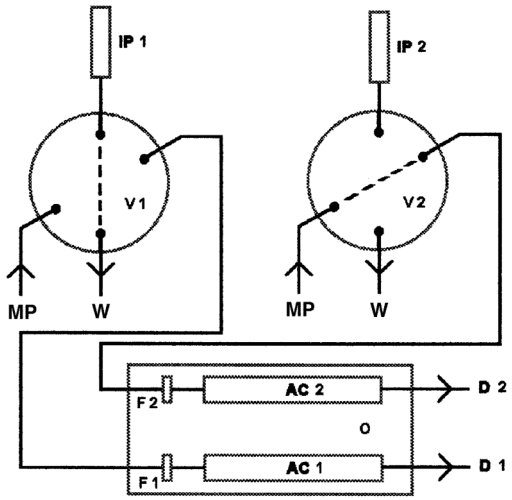

Figure 1. Valve system diagram for a dual-injection configuration using Gilson 233XL: analytical column 1, AC 1; analytical column 2, AC 2; detector 1, D 1; detector 2, D 2; precolumn filter 1, F 1; precolumn filter 2, F 2; injection port 1, IP 1; injection port 2, IP 2; mobile phase, MP; column oven, O; valve in load position for microscale injection 1, V 1; valve in inject position for microscale injection 2, V 2; and waste, W.

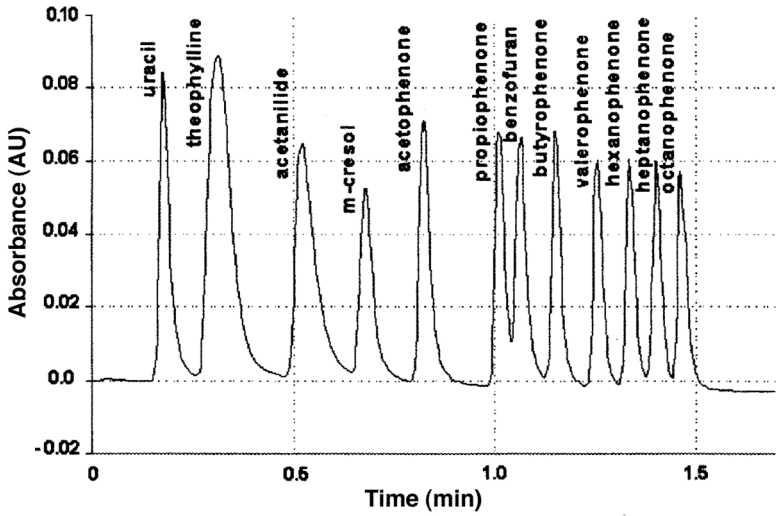

Figure 2. Chromatogram of the 12-component test mix.

Table I. Optimization of Flow Rate and Gradient Time

Flow rate (mL/min) pressure (mPa)	Gradient time* (s)	Elution time (min)	Resolution factor† p1–p2	Resolution factor† p6–p7	Resolution factor† p7–p8	Resolution factor† p9–p10	Resolution factor† p10–p11
0.5–27	40	1.48	0.86	0.75	0.90	1.02	0.85
	60	1.61	0.66	0.68	0.95	1.05	0.99
	80	1.82	0.50	0.60	0.99	1.09	1.04
0.6–31.5	40	1.28	0.95	0.70	0.94	1.00	0.99
	60	1.45	0.90	0.66	1.00	1.08	1.02
	80	1.67	0.65	0.55	1.00	1.09	1.05
0.7–36	40	1.17	1.06	0.67	0.96	1.05	1.00

* From 0% B to 80% B.
† Between peak *n* and peak *n* + 1.

Individual stock solutions of uracil, theophylline, acetanilide, *m*-cresol, acetophenone, propiophenone, benzofuran, butyrophenone, valerophenone, hexanophenone, heptanophenone, and octanophenone were prepared at a concentration of 5–10 mg/mL each in 50mM ammonium acetate–acetonitrile (1:1, v/v) and stored at 4°C for up to one month. The test mix was extemporaneously prepared by combining 2 to 10 mL of each stock solution to finally obtain similar UV detector responses for all analytes.

Instrumentation

Injection was carried out using a Gilson (Villiers le Bel, France) 233XL dual-valve sampling injector consisting of a large-capacity XYZ sampling injector equipped with two automated 4-port Model 7413 valves (Rheodyne, Berkeley, CA) fitted with 0.5-µL internal injection loops and a Model 402 syringe pump equipped with a 500-µL syringe. The solvent of the syringe pump was acetonitrile, and the sampling injector was configured for dual-system injection (Figure 1).

The 233XL sampling injector was interfaced to two HPLC systems (Gilson). Each system consisted of a Model 321 pump fitted with two H1 pump heads and a Model 151 UV–vis detector equipped with a 2-mm path-length microbore flow cell.

The adjustable mixer volume of the pump was set at 0.25 mL to reduce gradient delay, and the tubing was stainless steal and exactly calibrated (0.005-inch i.d.). This was carried out to minimize extra-column band broadening and optimize efficiency and reproducibility from one HPLC system to another. The HPLC column was a Micra RP-18 nonporous silica column (3 × 33 mm, 1.5-µm particles) from Bischoff Chromatography (Leonberg, Germany). The column was preceded by a low dead-volume precolumn filter containing a 0.5-µm-pore frit from Supelco (St. Quentin Fallavier, France). Both systems used a single Model 864 4-channel degasser from Gilson and a single Croco-CIL column oven from Cluzeau Info Labo (Ste. Foy La Grande, France). Simultaneous control of both HPLC systems and data handling was provided by UniPoint (V2.0) system software.

Methodology

HPLC mobile phases A and B were respectively composed of 0.1% TFA in water and 0.065% TFA in acetonitrile and were online degassed. A simple binary gradient elution at 0.6 mL/min was performed from 0% to 80% B in 1.0 min and then maintained at 80% B during 0.2 min and returned to 0% B in 0.1 min. The column was thermostatted at 35°C. Analytes were detected at 220 nm with 0.1 AUFS using a raw detector signal mode to allow for the detection of rapidly eluting and narrow peaks. The total cycle time from one injection to another was 3.5 min.

Results and Discussion

Optimization of HPLC conditions

A narrow-bore column filled with 1.5-µm nonporous silica particles was selected for the following reasons.

The sample quantities and solvent volumes were smaller than the ones generally used in ordinary analytical HPLC and low flow rates give a better compatibility with MS detectors, avoiding therefore a high percentage split to waste. Moreover, sensibilities increase when internal column diameter decreases.

Small nonporous particles provide high efficiencies and strong resistance to high pressure. Also, with very small particles (1.5 µm) Van Deemter's curves show that after the optimum the H.E.P.T. value does not significantly change if the flow rate increases (9).

The chromatographic equipment was completely setup and optimized according to the selected column–small injection loop valves, optimized calibrated tubing, the mixing chamber of the pump adjusted at 0.25 mL to get the best compromise between gradient delay and composition pulsation amplitude, and a microbore flow cell (1.6-µL internal volume). Using this configuration, the system volumes were determined and respectively provided a total delay volume of 0.17 mL and an effective mixing volume of 0.28 mL.

The main chromatographic parameters requiring optimization were mobile phase composition, flow rate, gradient speed, and temperature. In each case, compromises had to be established between resolution, cycle time, and system pressure. The best conditions led to a chromatographic separation of the 12 compounds within 1.5 min (Figure 2).

For the mobile phase composition, using 0.1% TFA in acetonitrile produced an important baseline drift at the end of the gradient and therefore generated erroneous integration of the last peaks. Several percentages were tried and finally 0.065% TFA in acetonitrile produced the best results regarding baseline drift and retention times of the last peaks.

The parameters of flow rate and gradient speed were selected after comparing several critical pairs of peaks. The resolution factors of the critical pairs were calculated from the retention times of the corresponding peaks (9) (i.e., uracil (p1)–theophylline (p2), propiophenone (p6)–benzofuran (p7), benzofuran (p7)–butyrophenone (p8), valerophenone (p9)–hexanophenone (p10), and hexanophenone (p10)–heptanophenone (p11)) and are reported according to several flow rates (i.e., 0.5, 0.6, and 0.7 mL/min) and gradient speeds (i.e., 0% B to 80% B within 40, 60, and 80 s) in Table I. The best results were obtained at 0.6 mL/min and with a gradient time of 1 min.

Three different temperatures were tested (30°C, 35°C, and 40°C), and three critical pairs of peaks were significant for this test, which were p1–p2, p7–p8, and p11–p12. Table II shows that increasing the temperature of the oven from 35°C to 40°C only

Table II. Optimization of Temperature

Temperature (°C)	Pressure (MPa)	Elution time (min)	Resolution factor* p1–p2	Resolution factor* p7–p8	Resolution factor* p11–p12
30	34	1.48	0.94	0.93	0.50
35	31.5	1.45	0.90	1.00	0.70
40	30.5	1.44	0.88	1.00	0.70

* Between peak *n* and peak *n* + 1.

Table III. Repeatability of Retention Times

	System 1		System 2	
Peak name	Retention time (min)	%RSD*	Retention time (min)	%RSD*
Uracil	0.15	5.8	0.14	9.5
Theophylline	0.31	2.7	0.30	4.3
Acetanilide	0.50	2.0	0.50	2.2
m-Cresol	0.63	1.3	0.63	1.3
Acetophenone	0.79	0.9	0.81	0.7
Propiophenone	1.00	0.6	1.02	0.6
Benzofuran	1.05	0.5	1.07	0.5
Butyrophenone	1.14	0.3	1.17	0.6
Valerophenone	1.25	0.4	1.28	0.5
Hexanophenone	1.33	0.4	1.36	0.4
Heptanophenone	1.39	0.4	1.43	0.4
Octanophenone	1.45	0.3	1.50	0.4

* *n* = 10.

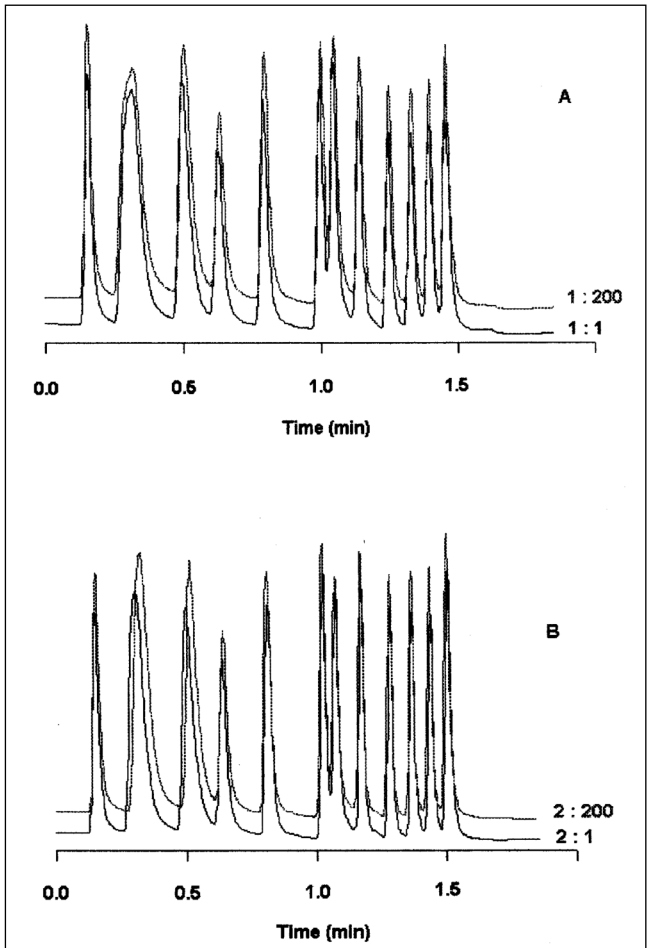

Figure 3. Comparison of the chromatograms of sample 1 and sample 200 from system one (A) and system two (B).

decreased the pressure from 31.5 to 30.5 MPa and the retention time of the last peak from 1.45 to 1.44 min; therefore, the temperature was set at 35°C.

Reliability and ruggedness

This study was completed by operating several injections in both chromatographic systems (Gilson 233XL alternatively feeding system one and system two), which provided an overall throughput of 34 analyses per hour. Table III shows the reliability of the method. The relative standard deviation (RSD) (n = 10) of the retention times in both systems were less than 1% for the 8 latter compounds. The RSDs of uracil were particularly high, which was principally because of the calculation obtained with the very low retention time of uracil (0.14 min).

Figure 3 shows the ruggedness of the method. It represents a perfect superposition of the chromatograms obtained from sample 1 and sample 200 with system one and sample 1 and sample 200 with system two.

Conclusion

This work demonstrates that with appropriately adjusted commercially available equipment and short HPLC narrow-bore columns packed with 1.5-µm nonporous silica particles, a 1.5-min separation can be obtained as a rapid generic method for the QC analysis of combinatorial libraries. This fully automated method has been proven reliable and rugged for hundreds of samples. Moreover, lower flow rates used with narrow bore columns presented a better compatibility for MS detectors, a lower cost with solvents, and a better response to environmental considerations compared with traditional analytical HPLC methods.

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