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Journal of Chromatography A, 718 (1995) 429–435

JOURNAL OF CHROMATOGRAPHY A

Short communication

Improved baselines in gradient elution

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First received 8 June 1995; accepted 15 June 1995

Abstract

A technique is described to provide on-line cleanup of water-rich solvents used in HPLC gradients generated via high-pressure mixing. A short precolumn is placed between the solvent pump A and the mobile-phase mixer to remove contaminants. A six-port valve is used to enable backflushing of the collected contaminants from the precolumn between each gradient cycle. The examples given show a 5- to 100-fold reduction in baseline noise for reversed-phase gradient runs.

1. Introduction

Reproducible separations by gradient elution require that the column be equilibrated by flushing the column with the starting mobile phase after each gradient [1]. In the case of reversed-phase HPLC (RP-HPLC), this typically requires 10–20 column volumes of the initial, water-rich mobile phase [2]. Furthermore, because of the hold-up volume of the HPLC equipment (the so-called dwell volume), as much as 10 ml of additional equilibration solvent may be required before injecting the next sample [3].

The water used for RP-HPLC normally is purified to remove UV-absorbing contaminants. However, it is commonly found that residual impurities from the water collect on the column during the beginning of a gradient and later elute as interfering peaks [4]. These water-related peaks increase in size with a longer column equilibration period. This problem is commonly found when using low-UV detection (<210 nm) at the most sensitive detector settings. It has been reported that these interference peaks can be eliminated by prolonged exposure of the water to a high-intensity UV lamp [5], but this procedure is seldom used in typical HPLC laboratories.

Further purification of the water used for HPLC can be accomplished by passing the water through a reversed-phase column. However, this procedure is inconvenient–especially if large-diameter cleanup columns are not readily available. In the present study, a different approach for eliminating water-derived interference peaks in gradient elution has been developed, for application to gradient systems using high-pressure mixing. An on-line RP-HPLC precolumn is used to automatically clean the water for each gradient separation. Between gradient runs, a switching valve diverts the strong solvent to the

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SSDI 0021-9673(95)00683-4

precolumn to remove impurities that have accumulated during the previous run. In this way, the problem of interference peaks in gradient elution is greatly diminished.

2. Experimental

2.1. Materials

Reagents were obtained from different suppliers: acetonitrile (Burdick and Jackson, Muskegon, MI, USA), phosphoric acid (Baker, Phillipsburg, NJ, USA), triethylamine (TEA, Pierce, Rockford, IL, USA). Water was purified using a Milli-Q water system (Millipore, Bedford, MA, USA). The 72 m*M* TEA-phosphate (TEAP) solution was prepared by adding 10 ml of TEA to 990 ml of water. The pH of the TEAP solution was adjusted to pH 3.0 with phosphoric acid.

The analytical column was a 150 × 4.6 mm I.D. Zorbax SB-C18 (MacMod, Chadds Ford, PA, USA) with a dead volume of 1.5 ml. The 50 × 4.6 mm I.D. precolumn was packed manually using 40-μm particles from a C_{18} clean up extraction column (United Chemical Technologies, Bristol, PA, USA).

2.2. Equipment

The high-pressure mixing gradient system used was a Shimadzu LC-10A with a SCL-10A system controller, two LC-10AS solvent delivery units, and a SPD-10AV UV–VIS detector. The dwell volume for this system was determined to be 2.1 ml. ChromPerfect for Windows (Justice Innovations, Mountain View, CA, USA) was connected to the 1 V output of the detector (1 AUFS). The precolumn and analytical column were connected through a Valco 6-port valve (Valco Instruments, Houston, TX, USA) as shown in Fig. 1. During gradient elution, the cleanup configuration is used. Between gradient runs, the valve is switched to the backflush configuration to clean the precolumn.

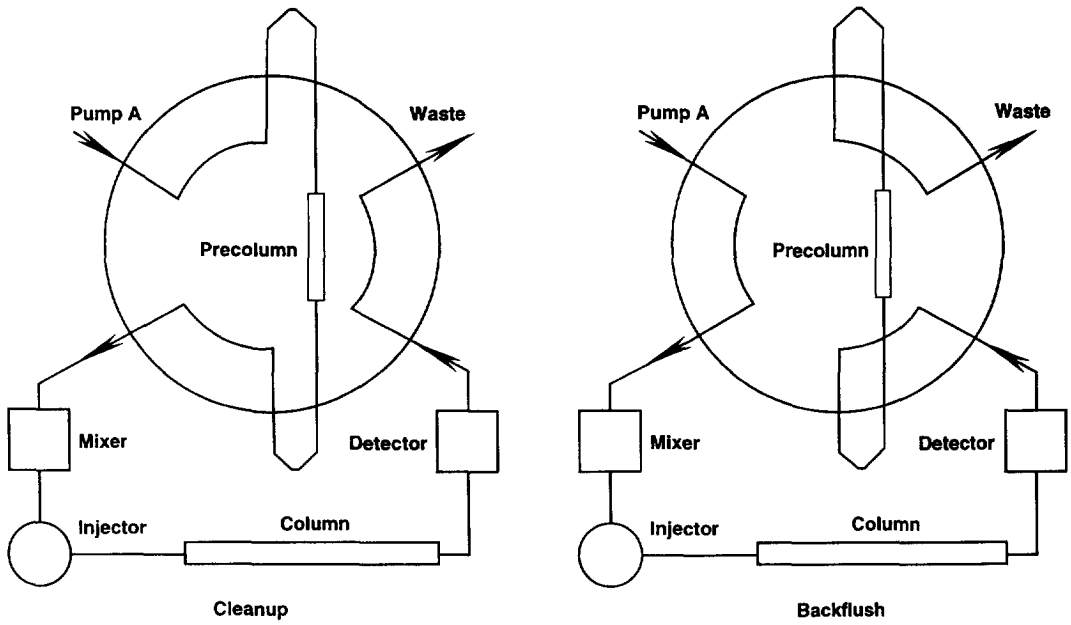

Fig. 1. Configuration of precolumn and switching valve in the HPLC system. Cleanup phase used during gradient equilibration and analysis and for initial flushing of analytical column. Backflush mode used to remove contaminants from precolumn following each gradient cycle.

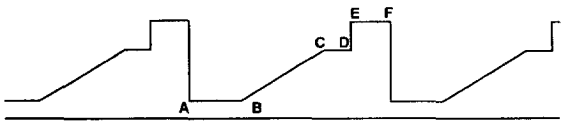

Fig. 2. Schematic of a sample gradient profile: equilibration (A–B), gradient (B–C and C–D), column and precolumn flushing (E–F). See text for details.

2.3. Procedure

Solvents A for the experiments of Figs. 3 and 4 were, respectively, acetonitrile–water (1:99) and 1% acetonitrile–72 m*M* TEAP (pH 3.0). Solvent B was 100% acetonitrile for both experiments. An example of the equilibration, gradient, and cleanup cycle is shown in Fig. 2. Equilibration: A–B, 5% B for 5 min; gradient: B–C, 5–80% B in 20 min and C–D, 80% B for 5 min; cleanup: E–F, 100% B for 10 min, including 2 min backflushing; and step from 100% B to 5% B for the next run. This corresponds to the conditions of Fig. 3. See Discussion for determination of E–F conditions. In the present studies, a flow-rate of 1.0 ml/min was used with detection at 210 nm.

3. Results and discussion

3.1. System configuration

Fig. 1 shows a diagram of the system configuration. During the cleanup phase (normal gradient operation), the precolumn is placed between the solvent A pump and the high-pressure mixer. For backflushing of the precolumn, the valve is rotated so that the precolumn is located downstream from the detector and the flow through the precolumn is reversed. In this manner, contaminants from the solvent A are adsorbed on the precolumn so that a cleaner solvent A is delivered to the analytical column (Fig. 1, cleanup). After each run, strongly retained contaminants are first washed from the analytical column using strong solvent in the normal configuration, then the valve is rotated and the clean strong solvent from the analytical column backflushes the previously adsorbed contaminants from the precolumn (Fig. 1, backflush). It should be noted that the pressure drop across the precolumn should not exceed the pressure limit of the detector cell or cell leakage could result. UV detector cells typically have pressure limits of 100–150 p.s.i., but the detector operation manual should be consulted for exact specifications.

3.2. Timing considerations

As noted earlier, Fig. 2 shows a schematic of the mobile phase timing cycle for the runs of Fig. 3. The equilibration phase (Fig. 2, A–B) was arbitrarily chosen, and as noted in the discussion of Figs. 3 and 4, appears to be too short. The gradient conditions (Fig. 2, B–C–D) are chosen for illustrative purposes, and would be changed to match the requirements of the individual method. The timing of the E–F flush and backflush cycle needs to be determined empirically. The requirements for flushing the analytical column are determined in the normal manner by flushing with strong solvent until late-eluting peaks and contaminants are removed and the baseline stabilizes. For the present example using blank gradients, the flushing phase took 8 min for the conditions of Fig. 3 and 11 min for Fig. 4. Once the baseline stabilizes after column flushing, the switching valve is changed from the cleanup to backflush mode (see Fig. 1). Strong solvent then backflushes contaminants from the precolumn. When the baseline once again stabilizes, this phase is complete and the valve is returned to the cleanup mode and the mobile phase steps back to the initial equilibration conditions. In the present examples, the backflush phase took 2 min for the conditions of Fig. 3 and 4 min for those of Fig. 4. Once the conditions are determined for a particular analysis, they should be reproducible and can be entered into the program controlling the mobile-phase composition and switching-valve position. A wise chromatographer will add additional time to the flush and backflush phases to accommodate unexpectedly dirty samples or solvents. Because contaminant buildup on the column and

subsequent noise in the baseline is directly affected by the equilibration time between samples, optimal use of this cleanup technique will be made with fully automated analyses. The switching valve can be automated easily by using external events control from the autosampler or data system to trigger an electrically- or pneumatically-actuated switching valve.

3.3. Comparative results

Figs. 3 and 4 show baselines for acetonitrile–water and acetonitrile–TEAP gradients, respectively. The curves labeled "A" in Figs. 3 and 4 did not use a precolumn cleanup of the water, whereas the curves labeled "B" did use this cleanup procedure. The advantage of the present on-line water purification procedure is apparent. Furthermore, without on-line water purification the blank gradients in Figs. 3A and 4A were not reproducible, hence precluding baseline subtraction as a means of dealing with these interference peaks. The traces of Figs. 3 and 4 show the entire equilibration and gradient phase, starting at the moment the E–F flush cycle of Fig. 2 was changed back to the A–B equilibration conditions. The downward baseline drift and broad peaks prior to about 10 min correspond, in our experience, to equilibration of the column from a water-poor to a water-rich mobile phase. The dwell volume and column volume amount to about 3.6 ml, accounting for 3.6 min before the initial conditions reach the detector. At 10 min, only about 5 column volumes of the new mobile phase have passed though the column, so equilibration is not complete. From a practical standpoint, one should allow at least 10 column volumes (15 ml) to pass through the column before injection, and a steady baseline should be observed.

HPLC-grade water has a reputation for high purity, and as can be seen in Fig. 3A, the contribution to baseline noise is small, even at high sensitivity and low wavelength. Even so, the present cleanup technique provides a simple way to obtain even more noise-free baselines. The results of Fig. 4A confirm our practical experience that a major source of gradient baseline

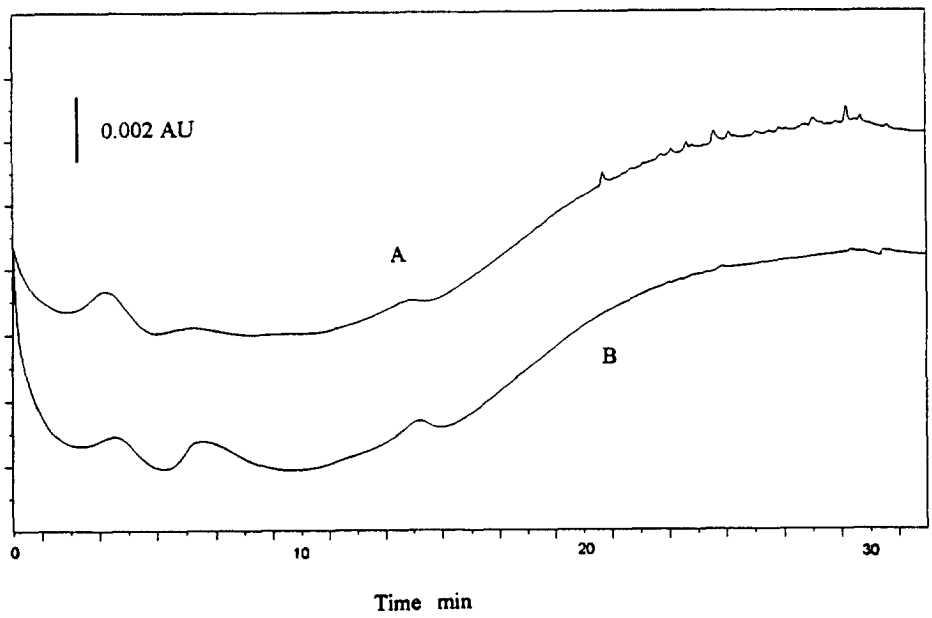

Fig. 3. Comparison of baselines obtained (A) without and (B) with precolumn cleanup of solvent A. Zorbax SB-C18, 150 × 4.6 mm I.D. column operated at 1 ml/min. Solvent A: acetonitrile–water (1:99); solvent B: acetonitrile. Gradient, 5/5/80/80% B at 0/5/25/30 min. Flushing cycle, 8-min column flush at 100% B followed by 2-min precolumn backflush. Detection, UV 210 nm; ambient temperature.

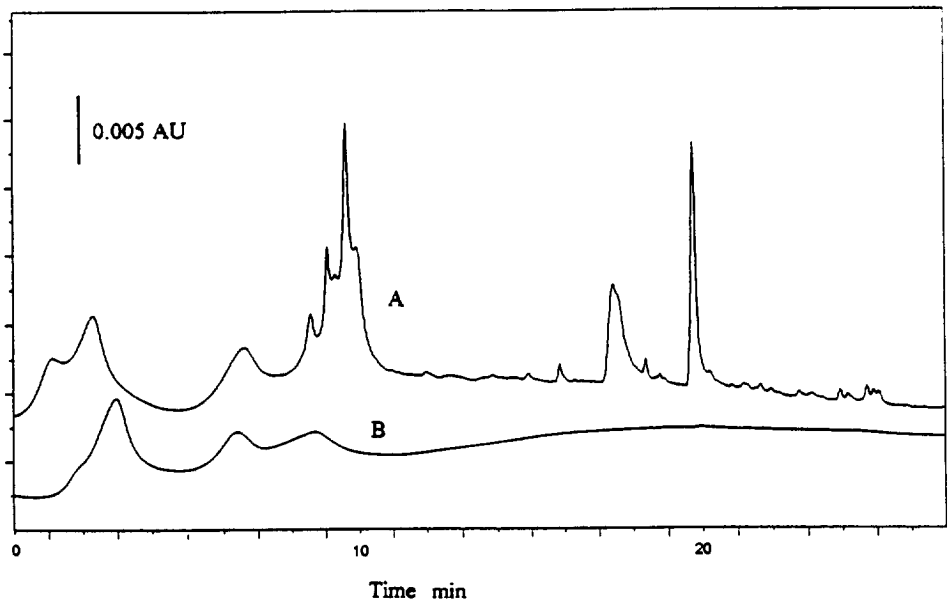

Fig. 4. Same conditions as Fig. 3, except solvent A is 1% acetonitrile–72 m*M* TEAP. Flushing cycle, 11-min column flush and 4-min precolumn backflush.

noise comes from mobile phase additives. In the present example of TEAP additives, a 50-fold increase in baseline noise is observed over the additive-free mobile phase (compare Figs. 3A and 4A). With the on-line cleanup technique, however, the baseline is comparable with or without mobile phase additives (the difference in baseline offset is due to the scale differences).

3.4. Practical considerations

In addition to the discussion above regarding valve timing and detector pressure limit precautions, several other items of practical consideration are important. Fig. 5 shows five consecutive gradients run under conditions similar to those of Fig. 4. This illustrates that the cleanup procedure is sufficiently reproducible to consistently remove contaminants from the aqueous portion of the mobile phase.

The use of the present cleanup technique reduces baseline noise, and thus allows detector operation at lower wavelengths and higher sensitivities. As these more demanding conditions are used, baseline drift becomes increasingly important, as is illustrated by the drift of Fig. 3 for the upward drift of the baseline between 10 and 30 min. With the conditions of Figs. 3 and 4, the baseline drift indicates that solvent B absorbs UV light more strongly at 210 nm. Baseline drift in cases such as these often can be corrected by adding an unretained, UV-absorbing compound to solvent A. Compounds such as nitrate or thiourea have been reported as effective in correcting absorbance mismatch in cases like this [6]. With the addition of absorbance-matching additives comes the risk of further inadvertent contamination of the mobile phase by UV-absorbing impurities (as in Fig. 4A), but the present technique should remove such contaminants. The correction of absorbance mismatch would complement the present cleanup technique in improving signal-to-noise ratios through the reduction of baseline noise and drift with low-wavelength gradient applications.

The present example uses a hand-packed precolumn to remove mobile phase contaminants. From a practical standpoint, it may be more convenient to use a cartridge-type guard column instead. Ideally the bonded-phase type in the precolumn should match the phase in the ana-

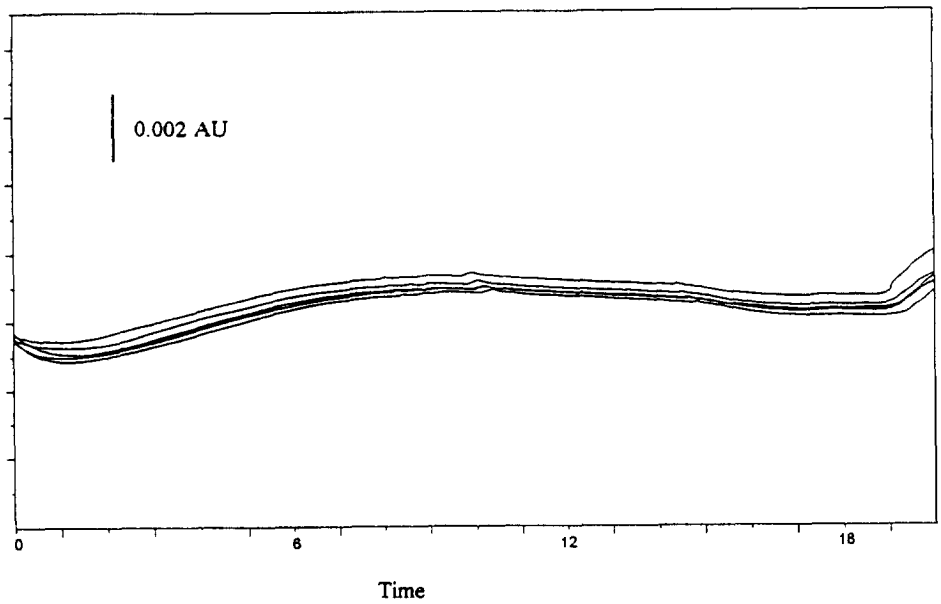

Fig. 5. Reproducibility of baselines for five successive gradients. Same conditions as Fig. 4, except detector scale as noted.

lytical column, but as the current example illustrates, an exact match is not essential. Furthermore, the precolumn stationary phase should be no weaker than the analytical column so that it will readily trap contaminants. For example, a C_{18} phase could be used with a C_{18} or C_8 analytical column, but a C_8 precolumn would not be expected to work as well with a C_{18} analytical column.

The lifetime of the precolumn was not determined in the present study; in 12 days of use, no deterioration was noticed. Because a relatively small amount of contaminant is trapped on the precolumn and it is flushed between each use, it is expected that the life of such a cleanup column would be adequate. We suggest that a blank gradient be run periodically as a standard practice for routine methods, just to be sure extraneous peaks are not present; this blank gradient could also be used to monitor the effectiveness of the precolumn. When the quality of the blank gradient deteriorates, the cleanup column should be replaced.

We are aware of two limitations of this technique. First, it is applicable only to high-pressure mixing systems. The precolumn must be configured such that it can be backflushed to remove contaminants, and this configuration is not possible with low-pressure mixing systems. Second, the on-line cleanup technique can be used only for the weak (A) solvent; the strong (B) solvent must be pure organic. If an additive, such as TEAP in the present example, were added to the B solvent, its contaminants would be trapped on the analytical column under weak-solvent conditions and then eluted later in the gradient under strong-solvent conditions. A precolumn placed between the pump for solvent B and the mixer would not trap these contaminants because solvent B would wash them off immediately (this is the same as the backflush condition used for the precolumn).

Except for the two limitations noted above, the use of a precolumn configured as in Fig. 1 will enable the practical removal of contaminants from water-rich solvents A in binary gradients generated by high-pressure mixing HPLC systems.

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