Journal of Chromatography, 348 (1985) 123–129
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 18 089

BRACKETING, A SIMPLE LOADING TECHNIQUE THAT INCREASES SAMPLE RECOVERY, IMPROVES RESOLUTION, AND INCREASES SENSITIVITY IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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(First received June 13th, 1985; revised manuscript received August 6th, 1985)

SUMMARY

A simple loading technique is described for high-performance liquid chromatography that permits almost quantitative sample transfer and recovery, improves resolution, and increases peak heights. A small amount of weak eluent precedes and follows the sample into the column, causing solutes to accumulate as a narrow band at the head of the column despite a relatively large injection volume. This technique differs from gradient elution in that the entire column is not equilibrated with weak eluent. Therefore, a drifting baseline is not a problem, no extra time is needed for column re-equilibration, and gradient forming equipment is not required.

INTRODUCTION

Modern high-performance liquid chromatography (HPLC) columns, especially those packed with 5- or 3-μm spherical particles, are capable of very good resolution. Their potential resolution may not be realized, however, unless the sample is introduced as a very narrow band. Therefore, samples are normally loaded in small volumes (10 μl or less).

The use of small volumes intensifies two potential problems: transfer with a syringe is not quantitative because of sample remaining in the container and in the syringe and needle, and any leak in the injector can cause partial or complete loss of sample. Recovery is important when sample size is limited (and the entire sample is to be loaded) and when two or more sequential separations, on different columns, are required for adequate purification of the component(s) of interest. This paper presents a simple loading technique for variable-volume loop injectors that permits almost quantitative sample transfer and enhances column performance.

There are two ways for loading the sample as a narrow band at the head of the column. The normal practice, mentioned above, is to inject the sample in a very small volume. This is usually accomplished by filling a small fixed-volume loop with a fraction of the sample. An alternate approach, to be discussed here, is to introduce the sample under conditions that permit virtually no movement of components of

interest on the stationary phase during column loading. Thus, the volume in which the sample is introduced becomes relatively unimportant[1–5].

If the sample is dissolved in a solvent that is much weaker than the mobile phase, a corresponding increase in sample volume then becomes possible without loss in resolution[1]. It may be difficult, however, to dissolve the sample in a very weak solvent. Another option is to use gradient elution in which the initial composition of the eluent is relatively weak in eluting power. This is a powerful technique for separating complex mixtures, but normally requires two pumps and a gradient former. A step gradient can be used in which the column is equilibrated with a weak eluent before loading the sample. The composition of the eluent is abruptly changed (stepped) sometime during the run by switching from one reservoir to another. A step gradient does not require a second pump or a gradient former. A disadvantage of gradients in general is the time required for re-equilibration of the column before the next injection. Baseline changes can also be a problem, especially at high detector sensitivity.

A technique is described that gives some of the advantages of a step gradient without the disadvantages. Rather than equilibrating the entire column with weak eluent, a relatively small volume of weak eluent is placed in the injector loop immediately before and after the sample. Thus, the weak eluent precedes and follows the sample into the column and temporarily changes the equilibrium of solute partitioning between mobile and stationary phases. This technique will henceforth be referred to a bracketing.

EXPERIMENTAL

Apparatus and chemicals

Gas-tight Hamilton* syringes (1800 Series) were used to transfer samples from 1.5-ml conical tubes into a Waters Model U6K injector equipped with a 2-ml loop. We used a 250 × 4.6 mm I.D. column packed with 5-μm spherical particles of octadecylsilane (Alltech Assoc. Adsorbosphere C_{18}). A Waters Model 440 fixed-wavelength detector, operated at 254 nm, and a Waters Model 420-AC fluorescence detector (254 nm excitation, 360 nm emission) were connected in series. Radioactive abscisic acid ([^{14}C]ABA) was purchased from Amersham (Arlington Heights, IL, U.S.A.). Indole-3-carboxylic, indole-3-acetic, indole-3-propionic, and indole-3-butyric acids, and the ABA isomers, were purchased from Sigma (St. Louis, MO, U.S.A.). OmniSolv™ methanol was obtained from Chemonics Scientific (Phoenix, AZ, U.S.A.).

Recovery

To estimate sample recovery, 25-μl portions of [^{14}C]ABA in methanol, with an activity of about 6000 dpm per 25-μl sample, were added to 1.5-ml conical tubes and directly to scintillation vials. Solvent in the conical tubes was evaporated with a stream of nitrogen. The samples were then dissolved in 5, 10, 25, or 50 μl (25 μl plus 25 μl rinse) of methanol–0.04 *M* acetic acid (50:50), which was also used as the

* Names of products are included for the benefit of the reader and do not imply endorsement or preferential treatment by U.S.D.A.

developing eluent. The tubes were vortexed and the samples transferred to the injector with a 25-μl syringe without bracketing or with a 250-μl syringe with bracketing. In the latter case, a 250-μl portion of 0.02 *M* aqueous acetic acid was loaded into the injector loop before the sample. Another 200-μl portion of 0.02 *M* acetic acid was drawn into the syringe before the sample. Because it was drawn in last, the sample was contained in the needle end of the syringe. After loading, the sample in the injector loop was bracketed between 250-μl and 200-μl portions of 0.02 *M* aqueous acetic acid. The developing eluent, methanol–0.04 *M* acetic acid (50:50) was pumped through the column at 1 ml min^{-1}. Samples were collected in scintillation vials as they eluted from the column. A volume of 10 ml of scintillation cocktail (Beckman Ready Solv MP) was added to each vial and the samples were counted 4 min each in a Beckman LS 7500 liquid scintillation spectrometer. Scintillation quenching was automatically corrected with a DPM data reduction program. Sample recovery was estimated by comparing counts of samples collected from the HPLC with those of samples added directly to scintillation vials.

Resolution and sensitivity

Tests were conducted with unlabelled ABA and with the indole compounds to determine the effects of loading technique on resolution and sensitivity.

Volumes of 50 μl each of *cis,trans-* and *trans,trans-*ABA (about 250 ng) were placed in 1.5-ml conical tubes and evaporated to dryness. The samples were then dissolved in 10 μl of methanol–0.04 *M* acetic acid (50:50) and loaded without bracketing or were dissolved in 50 μl (25 plus 25 μl) of 5 methanol–0.04 *M* acetic acid (50:50) and loaded with bracketing between 250- and 200-μl portions of 0.02 *M* aqueous acetic acid. The column and developing eluent were the same as used for the recovery test. The recorder was operated at 5.1 cm min^{-1} to facilitate peak width measurements.

Indole-3-carboxylic, indole-3-acetic, indole-3-propionic, and indole-3-butyric acids were mixed together in methanol. Portions of 50 μl, containing about 100 ng of each indole compound, were placed in 1.5-ml conical tubes and evaporated to dryness with nitrogen. The samples were then dissolved in 50 μl of methanol–0.04 *M* acetic acid (50:50) and loaded with or without bracketing. Brackted samples were loaded between 250- and 200-μl portions of 0.02 *M* acetic acid. The column and developing eluent were the same as for the previous tests. Elution of the indole compounds was monitored with the fluorescence detector.

Another test was conducted with the isomers of ABA. Dried samples were dissolved in 50 μl of methanol–0.04 *M* acetic acid (50:50) and loaded with or without bracketing, as above. Elution was monitored at 254 nm.

RESULTS AND DISCUSSION

Recovery was poor when the sample was dissolved and transferred with 5-μl portions of eluent (Table I). Recovery was greatly increased, and variation was greatly decreased, when the sample was dissolved in a total of 50 μl of eluent and loaded with bracketing. Recovery is not a problem when large amounts of sample are available. A small fixed-volume injector loop can be filled with a fraction of the sample to give precise and reproducible volumes. In some cases, however, it is desirable to

TABLE I

RECOVERY OF [^{14}C]ABA AFTER HPLC AS INFLUENCED BY LOADING TECHNIQUE

Samples were dissolved in 5, 10, 25, or 50 μl of methanol–0.04 *M* acetic acid (50:50) and loaded into a 2-ml injector loop. The 50-μl sample was loaded between 250- and 200-μl portions of 0.02 *M* aqueous acetic acid. Eluent, methanol–0.04 *M* acetic acid (50:50); flow-rate, 1 ml/min at 18.3 MPa and 24°C through a 250 × 4.6 mm I.D. column packed with 5-μm spherical particles of octadecylsilane (Adsorbosphere C_{18}). Values are means of four replications ± SE.

Sample volume (μl)	*Recovery (%)*
5	45.2 ± 6.0
10	76.6 ± 2.3
25	86.8 ± 1.0
50 with bracketing	95.4 ± 0.6

load the entire sample (*e.g.*, when the amount of sample is quite limited, when sequential separations on separate columns are required for adequate purification, and when radioactive internal standards are used to estimate overall recovery provided by a given purification procedure). In such cases maximum recovery is preferable to the injection of a precise fraction of the sample.

Despite the much larger volume in which the sample was loaded, bracketing increased resolution (R_s) from 2.129 with 10 μl standard loading to 2.844 when 50 μl of sample was bracketed between 250- and 200-μl portions of 0.02 *M* aqueous acetic acid. Calculated values of *N* (no. of theoretical plates) were more than twice as high with bracketing than without (Table II). Peaks were very symmetrical with very little tailing (Fig. 1). Therefore, 5σ *N* values were improved even more than

TABLE II

CALCULATED NUMBER OF THEORETICAL PLATES AND PEAK HEIGHTS AS INFLUENCED BY LOADING TECHNIQUE

Conditions as in Table I except that 5- and 25-μl loading volumes were not included. Values are means of four replications ± SE.

Loading volume and technique	*Theoretical plates (N/m)*	
	Sample	
	trans,trans-ABA	*cis,trans-ABA*
Half-peak-height method		
10 μl standard	21 357 ± 777	21 921 ± 676
50 μl with bracketing	52 089 ± 709	47 318 ± 903
5σ method		
10 μl standard	13 359 ± 387	15 596 ± 316
50 μl with bracketing	41 926 ± 834	39 314 ± 249
	Peak height (mm)	
10 μl standard	77.7 ± 3.3	62.8 ± 2.6
50 μl with bracketing	175.6 ± 3.4	131.1 ± 2.2

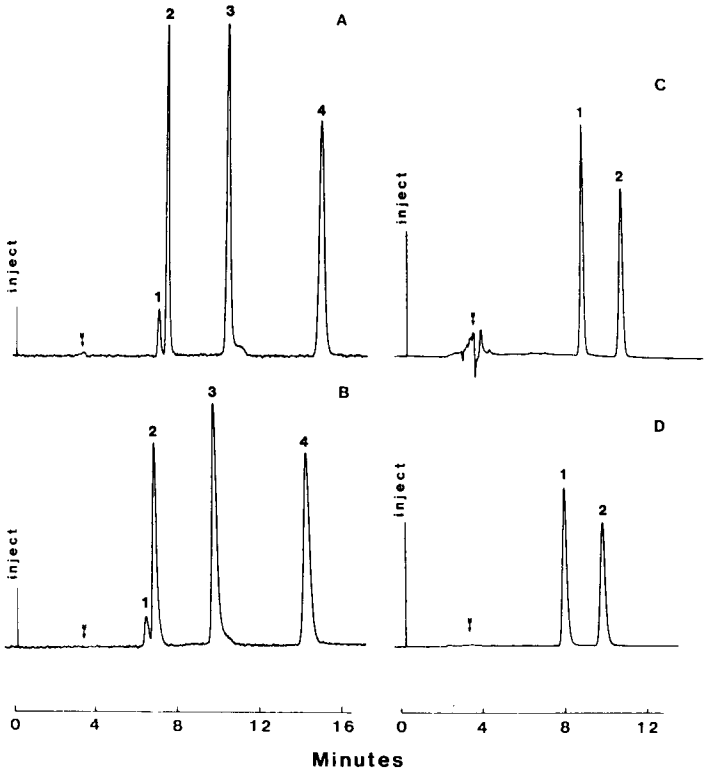

Fig. 1. Chromatograms of indole compounds (A and B) and isomers of ABA (C and D) that were loaded with (A and C) or without (B and D) bracketing between 250- and 200-μl portions of 0.02 *M* aqueous acetic acid. Peaks 1, 2, 3 and 4 of A and B were indole-3-carboxylic, indole-3-acetic, indole-3-propionic, and indole-3-butyric acids, respectively, (about 100 ng of each) monitored by fluorescence at × 16. Peaks 1 and 2 of C and D were *trans,trans*-ABA and *cis,trans*-ABA, respectively, (about 250 ng of each) monitored by absorbance at 254 nm, 0.2 a.u.f.s. Samples were eluted isocratically with methanol–0.04 *M* acetic acid (50:50) from a 250 × 4.6 mm I.D. column of 5-μm spherical particles coated with C_{18}. Approximate position of the solvent front is indicated by an arrow.

half-peak-height *N* values. (The 5σ *N* values are based on peak width at 4.4% peak height and, therefore, are sensitive to tailing[6].) The combination of increased recovery and improved resolution greatly increased peak heights (Table II). Sensitivity, in terms of peak height, was more than doubled when the bracketing technique was used.

Bracketing improved resolution enough to give baseline separation of indole-3-carboxylic and indole-3-acetic acids (Fig. 1A, peaks 1 and 2) and to reveal the presence of a contaminant (Fig. 1A, peak 3). Peak sharpening was more evident for components with low *k'* values than for those that had longer retention times. For example, backeting increased peak heights 64%, 38%, and 23% for indole-3-acetic, indole-3-propionic, and indole-3-butyric acids, respectively (Fig. 1A and B). All *k'* values (retention times) were increased slightly by bracketing, probably because of temporary strong binding of all solutes to the stationary phase in the presence of

aqueous acetic acid. Bracketing caused brief perturbations of the baseline (Fig. 1C), but baseline drift was not evident (as it frequently is with gradient elution).

Solvent strength affects peak spreading[1,7]. The use of a weak solvent gives sharp peaks, but it may be difficult to dissolve the sample completely in a weak solvent. Bracketing with a weak solvent gives the peak-sharpening effect without the problem of dissolving the sample in a weak solvent. Others have used weak eluents to concentrate solutes, either in the analytical column or in a pre-column, for later elution and analysis[2–5]. Our technique differs in that the modified (weak) eluent is present only briefly as the solutes enter the column. Therefore, the time required per sample is much less because the column does not require re-equilibration for each run.

An increase in the ratio of water to organic solvent (increase in polarity) is very effective in peak sharpening with reversed-phase separations (*e.g.*, on C_{18}). A change in pH is effective with ion-exchange columns. For example, organic acids are more strongly retained during loading in an anion-exchange column if the pH is temporatily raised to increase ionization. Conversely, the binding of organic cations (*e.g.*, those with an amine group) during loading in a cation-exchange column is favored by lowering the pH to increase protonation. Care should be taken, however, that the concentration of cations in the altered eluent is not so high that competition affects binding. A change in pH can also be effective in reversed-phase separations. For example, the lower the pH the lower the polarity of an organic acid such as ABA, and the more strongly it will bind to the stationary phase. But, too low a pH may damage the column. The composition of the bracketing solvent should be chosen to maximize binding to the stationary phase, but without causing damage.

The weak eluent, drawn into the syringe before the sample, accomplishes three purposes: (a) it flushes the sample from the syringe and needle, (b) it moves the sample farther into the injector loop so that loss by leakage is less likely to occur, and (c) it precedes the sample into the column and permits temporary strong binding of solutes at the head of the column.

CONCLUSION

The bracketing technique of sample loading, when used with an appropriately altered eluent, permits almost quantitative transfer and recovery of sample, improves resolution, and increases peak height (and thus sensitivity) with isocratic development. The greatest peak sharpening occurs with components that have short retention times. A drifting baseline, sometimes a problem with gradient elution, is not a problem with the bracketing technique. Because no time is required for column re-equilibration between samples, the bracketing technique requires little more time per sample than chromatography under standard isocratic conditions. It does not require a second pump or a gradient former. Other than a large (*e.g.*, 250-μl) syringe and a large variable-volume injector loop, no special equipment is required. The technique is especially useful when all components of interest have similar retention times and the amount of sample is quite limited so that maximum resolution, recovery and sensitivity are desired.

ACKNOWLEDGEMENT

We thank D. L. Brummett for excellent technical assistance.

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