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BOXCAR CHROMATOGRAPHY

A NEW APPROACH TO INCREASED ANALYSIS RATE AND VERY LARGE COLUMN PLATE NUMBERS

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SUMMARY

A new form of column-switching is described which provides significantly faster separations in routine applications of either gas or liquid chromatography. The principle of the method is the partial separation of one or a few compounds of interest on a first column, with diversion of the resulting fraction to a second column. The second column will then be filled with several samples at any given time. High-efficiency separations ($10^5 < N < 10^7$) are possible within reasonable per-sample times.

INTRODUCTION

Today the analysis of various samples by liquid chromatography (LC) is carried out mainly on small-particle columns, with increasing use of the very efficient 5-μm packings. Such columns routinely allow separations requiring 5000–10,000 plates, within a total time of 5–15 min. Column performance can be further augmented via optimization of separation selectivity, using variation of the mobile-phase composition. Especially difficult samples may require further attention, and the use of supplementary chromatographic techniques (*e.g.* ref. 1, Ch. 16 and 17), but few separation problems cannot be handled in this fashion.

The relative power of modern LC has tended to preempt interest in further extending its capabilities, particularly in the areas of higher column plate number and faster analysis rates. Also, it is apparent that we are now approaching a theoretical limit on plates per unit time in LC (*e.g.* ref. 1, pp. 238–241). As a result, although it is known that column plate numbers N of a million or more are possible, it is clear that such separations require times which are impractically long for most applications. This subject has been further detailed by Guiochon[2], and studied experimentally by Scott and Kucera[3]. Some workers have looked to coated-open-tubular (capillary) LC columns to provide a breakthrough in the area of large plate numbers. However, Knox has shown[4] that this is only possible when the inside diameter of the capillary is smaller than 10 μm. Such a system will present formidable difficulties in its design and operation, and detection sensitivity may be fundamentally

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compromised by these dimensional constraints. In any event, an advance in this area (capillary LC) beyond the present performance of packed columns cannot soon be expected.

We accept the situation as described above for the "normal" application of LC. However, there is an alternative approach to large plate numbers and faster separations, when the LC application shares the following characteristics:

(1) A need for relatively efficient columns ($N \geqslant 5000$), and the possible requirement of very large plate numbers ($10^5 < N < 10^7$);

(2) A large number of samples to be separated in the same way, either for routine analysis or for repetitive preparative separation; *i.e.*, a need to increase sample throughput per unit time;

(3) A sample where only one or a few adjacent bands are of interest, even when the sample contains many compounds.

For separation problems of the above type, "normal" chromatography is an inherently inefficient process. This is illustrated for a hypothetical separation in Fig. 1. The final chromatogram, Fig. 1a, shows resolution of the entire sample, despite the fact we are only interested in compound "X" (band 9). Fig. 1b–d portray separation of the sample within the column, at different times during the separation. It can be appreciated from this example that only a small fraction of the column is actually used at any given time during the separation of X: that portion shown in Fig. 1b, c which contains the band X and immediately overlapping compounds (labeled ΔL in Fig. 1). The remainder of the column either serves no useful function, or is separating compounds that are of no interest. As a result, the number of plates per unit time that is generated by the column is unnecessarily low.

Elsewhere[5] we describe two new techniques, primarily for application to the generation of large plate numbers in LC: boxcar chromatography (BC) and boxcar recycle chromatography (BRC)*. Here we will review the theoretical performance of these two procedures as derived in ref. 5, we will consider a number of general applications, we will examine some potential experimental problems, and we will show an example of this technique for increasing sample throughput.

EXPERIMENTAL

Equipment

The LC pumps used were all Technicon FAST-LC pumps; these are flow-controlled, single-piston reciprocating pumps capable of operating to 6000 p.s.i. with precise flow-rates and negligible pump-noise. The variable-wavelength photometric detectors used were Technicon FAST-LC spectrophotometers unless otherwise noted. These allow selection of any wavelength between 190 and 330 nm, have a noise level at any wavelength of better than 0.0001 A (peak-to-peak), and are equipped with a 10-mm optical-path-length (12-μl volume) flowcell. The automated sampling valves used were part of a Technicon Chromatographic Cartridge, and are of the standard six-port design (*e.g.* ref. 1). A digital programmable timer (Model WP6000, Minarik Electric Co., Los Angeles, CA, U.S.A.) was used in the boxcar separation described below.

* These techniques are also described in U.S. Patent 4,204,952 (5/27/80) and patents pending.

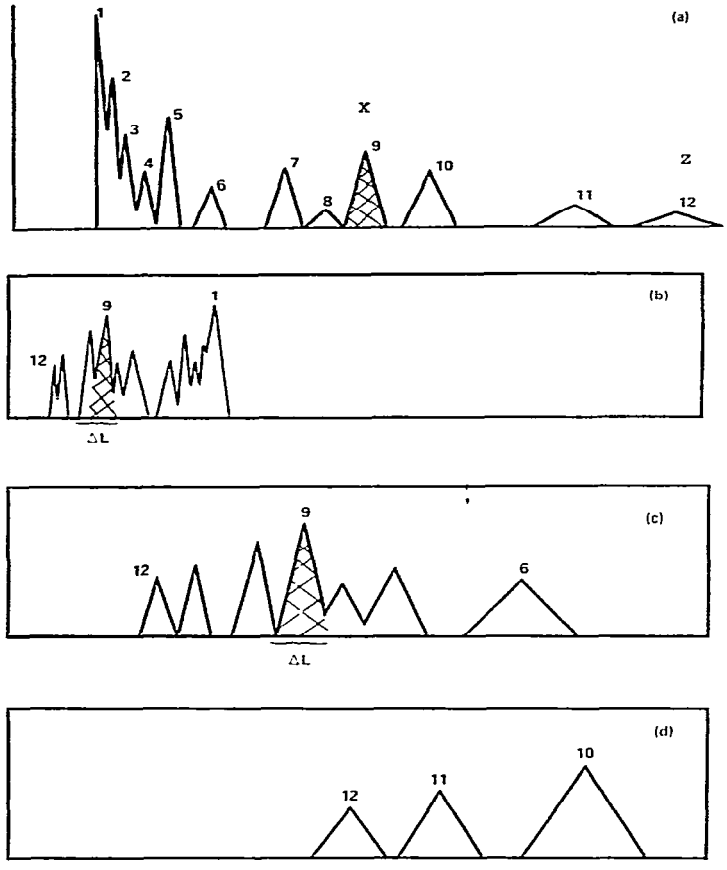

Fig. 1. Hypothetical illustration of the inefficient utilization of high-performance liquid chromatography in the separation of compound "X" (band 9) from a complex mixture. (a) Final separation chromatogram; (b–d) migration of sample through column, as function of time.

Reagents

The FAST-LC-8 column is variously a 150 × 4.6 mm I.D. or 60 × 4.6 mm I.D. column packed with 5-μm particles of C_8 bonded-phase packing material. The 6-cm columns were packed in our laboratory; the 15-cm columns are commercially available. All solvents and other reagents were of HPLC or ACS grade, as required for maximum transparency at 200 nm. Samples were mixtures of commonly available compounds.

Boxcar separation of anticonvulsants

The BC separation shown in Figs. 4 and 5 and described in a later section was carried out on a system constructed as shown in Fig. 2a. Valve V1 was an unmodified six-port valve, while valve V2 was operated functionally as a four-port valve by joining two adjacent ports with 0.25 mm I.D. stainless-steel tubing. The Technicon Chromatographic Cartridge was in each case further modified for control by the digital timer. Sample was continually perfused through valve V1 during the serial separation of

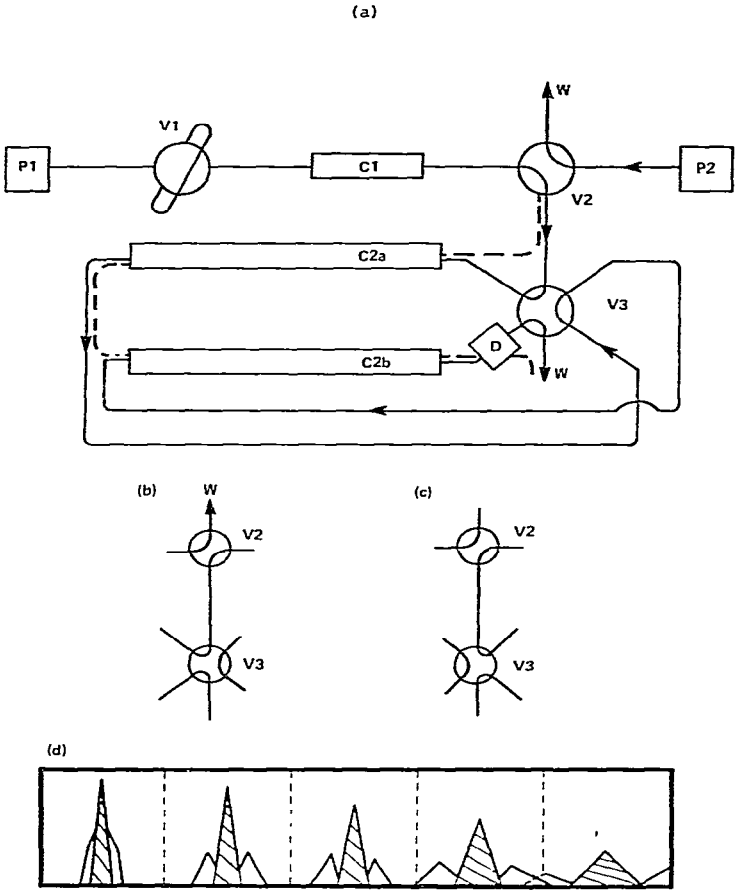

Fig. 2. System schematic for boxcar chromatography: — — —, continuous BC; ———, recycle BC.

several sample injections, and a second detector was positioned at the waste port of valve V2 to allow the effluent of column C1 to be monitored independently of the effluent from column C2. Column C1 was 6 cm in length, while column C2 (shown as C2a plus C2b in Fig. 2a) had a length of 15 cm.

The mobile phase used was methanol–water (44:56, v/v), with added buffer and amine-modifier to minimize band-tailing and maintain a constant solution pH. Samples consisted of 10 μl of a mixture of the six drugs shown in Fig. 4, with concentrations chosen to give suitable peak heights. Valve sequencing for the chromatogram shown in Fig. 5 involved: (1) initial injection of sample; (2) switching of valve V2 to allow elution of sample onto column C2, 23 sec after injection; (3) switching of valve V2 back to waste, 47 sec after injection; (4) injection of the next sample, 65 sec after the previous injection.

Testing of columns connected in series

In the column-testing experiments of Table II, Technicon FAST-LC-8 columns (150 × 4.6 mm I.D.) were connected by *ca.* 40 mm of 0.25 mm I.D. stainless-steel

tubing. A controlled-temperature water bath (Technicon) equilibrated the mobile phase temperature via capillary tubing of *ca.* 2 ml volume before the sampling valve, and also maintained the immersed columns at 50°C. The column eluate was monitored at 200 nm with a Waters 450 variable-wavelength detector (Waters Assoc., Milford, MA, U.S.A.) from which the heat exchanger was removed to minimize extra-column band-broadening. The plate number N was calculated in the usual fashion, using the peak width at half-height[1].

BOXCAR CHROMATOGRAPHY — THEORY

Continuous mode

A general system for carrying out both BC and BRC is outlined in Fig. 2. For the continuous (BC) mode, the valve V3 is positioned as shown in Fig. 2a, or is eliminated altogether; in the latter case, column C2a is connected directly to C2ab, which is in turn connected to the detector D as shown (dashed flow-path, Fig. 2a). The length of column C1 is given as L_1, and the combined length of C2a plus C2b is L_2 (the length of an equivalent single column C2); a single column can be substituted for C2a plus C2b. The application of BC, using the system of Fig. 2, can be illustrated by reference to the sample of Fig. 1. Here we have designated the band of interest (band 9) as "X", and the last band in the chromatogram (band 12) as "Z". It is assumed that the separation of Fig. 1a, where X is just resolved from surrounding bands, can be achieved on a column of length $L_1 + L_2 = L$. It is further assumed that the retention times of bands X and Z on a column of length L are t_x and t_z, respectively. Thus, the normal time required for the separation of the compound X in each sample will be equal to t_z.

In BC, samples are continuously injected onto column C1 at intervals $t_z q$. The quantity q ($q < 1$) is determined as described below. At the time each sample is injected onto column C1, the valve V2 is positioned as shown in Fig. 2b, which allows the effluent from C1 to vent to waste. When compound X begins to elute from C1, the valve V2 is reset to the position shown in Fig. 2a, allowing X to be directed to column C2. When all of band X has been transferred to C2, valve V2 is repositioned as in Fig. 2b. In this way, only band X (and associated overlapping bands) is diverted to column C2. The remainder of the sample goes to waste. Because $L_2 \gg L_1$, the entire sample is eluted from C_1 before band X has moved very far through column C2. Consequently, a second sample will be separated on column C1, and a second band X from this sample will be transferred to C2, while band X from the first sample is still moving through C2. In fact, with proper adjustment of the ratio $L_1/L = q$, several different samples (containing band X and adjacent peaks) can be separated simultaneously on column C2 (see Fig. 2d). Thus with continued injection of samples onto C1 (one sample every $t_z q$ sec), samples containing separated band X eventually emerge from C2 at the same rate ($t_z q$ sec per sample). This process continues until all samples have been injected onto C1 and have cleared C2. The net effect is full utilization of columns C1 and C2 throughout the serial separation of a set of samples where only band X is of interest.

During that part of BC where samples are entering column C1 and leaving column C2, it is apparent that the separation time per sample is reduced by the factor q. For separation of a sample as in Fig. 1, q has been derived as a function of

separation conditions in ref. 5. The solution to the resulting cubic equation can be approximated (±0.5%) by

$$q = \left[\frac{4C + 3\,(5C)^{2/3}}{3\,(5C)^{1/3}}\right]^2 \tag{1}$$

where $C = N^{-1/2}(t_x/t_z)$. However, for some number n of samples which is not very large, the *average* reduction factor in separation time is equal to q' ($q' > q$), where

$$q' = [1 + (n - 1)q]/n \tag{1a}$$

The separation example shown in Fig. 1 is referred to in ref. 5 as Case-IV. Other separation possibilities (*e.g.*, two adjacent bands of interest) are described in ref. 5, with derived values of q for each case. These q values are generally somewhat smaller for these other cases, meaning that BC is then even more advantageous in terms of reducing separation time per sample.

The theory of ref. 5 allows us to maximize sample throughput rate (maximum q') for various values of N, (t_z/t_x) and n, for different separation situations. The use of eqns. 1 and 1a in this connection is illustrated here for the situation of Fig. 1; Table I summarizes representative values of the effective increase in sample separation rate $r' = 1/q'$ for various conditions. It is seen that under appropriate circumstances, sample-throughput rates can be greatly increased using the technique of continuous BC. For example, if 100,000 plates are required for the separation, t_z/t_x is equal to 2, and there are 25 samples to separate, the value of $1/q'$ in Table I is equal to 12. That is, sample throughput is 12 times greater compared to normal LC separation. Large values of $1/q'$ are favored by large values of N, n and/or t_z/t_x.

TABLE I

SAMPLE THROUGHPUT ADVANTAGE OF CONTINUOUS BOXCAR CHROMATOGRAPHY FOR VARIOUS CONDITIONS

Plate number, N	*Throughput advantage, r'*					
	$t_z/t_x = 1$		$t_z/t_x = 2$		$t_z/t_x = 3$	
	$n = 5$	$n = 25$	$n = 5$	$n = 25$	$n = 5$	$n = 25$
3000	2.4	3.4	3.0	5	3.4	6
10,000	2.9	4.8	3.5	7	3.8	9
30,000	3.4	7	3.9	9	4.1	11
100,000	3.8	9	4.2	12	4.4	14
300,000	4.2	11	4.4	14	4.6	16

Recycle mode

Recycle chromatography is a general technique for obtaining large plate numbers in shorter overall separation time[5]. The technique of BRC is essentially similar to continuous BC as described in the preceding section, and makes use of the apparatus shown in Fig. 2. The initial operation of BRC is the same as for BC.

Fractions containing band X are serially loaded onto column C2a of Fig. 2, using valve settings (V2) as shown in Figs. 2a and b. When column C2a is loaded with sample fractions, the valve settings are then varied between Figs. 2b and c to allow continuous recycle of the samples initially loaded onto C2a. In this mode —the alternate pumping scheme of ref. 6— samples are alternately transferred between columns C2a and C2b without passing through the pump, and without undergoing significant extra-column band-broadening (see general discussion of recycle in LC given in ref. 7). The further description and theory of separation for BRC is given in ref. 5. The potential performance of BRC under optimized conditions is illustrated in Fig. 3. Here, column plate number is plotted against the separation time per sample, which is equal to the total recycle separation time divided by the number of samples loaded onto column C2a. It is seen that very large N values can be achieved via this technique, although separation times (per sample) will normally exceed 1 h. Thus, for a separation time of 1 day per sample, 4,000,000 plates are possible (with $k' = 3$). This would require *ca.* 100 passes (recycles) through each column (C2a and C2b), and the loading of two samples initially (*i.e.*, total separation time of 2 days).

It can also be seen in Fig. 3 that continuous BC is less efficient than BRC when the required plate number exceeds a value of *ca.* 200,000. Thus BRC is the preferred approach for separations that demand very large N values, while continuous BC is

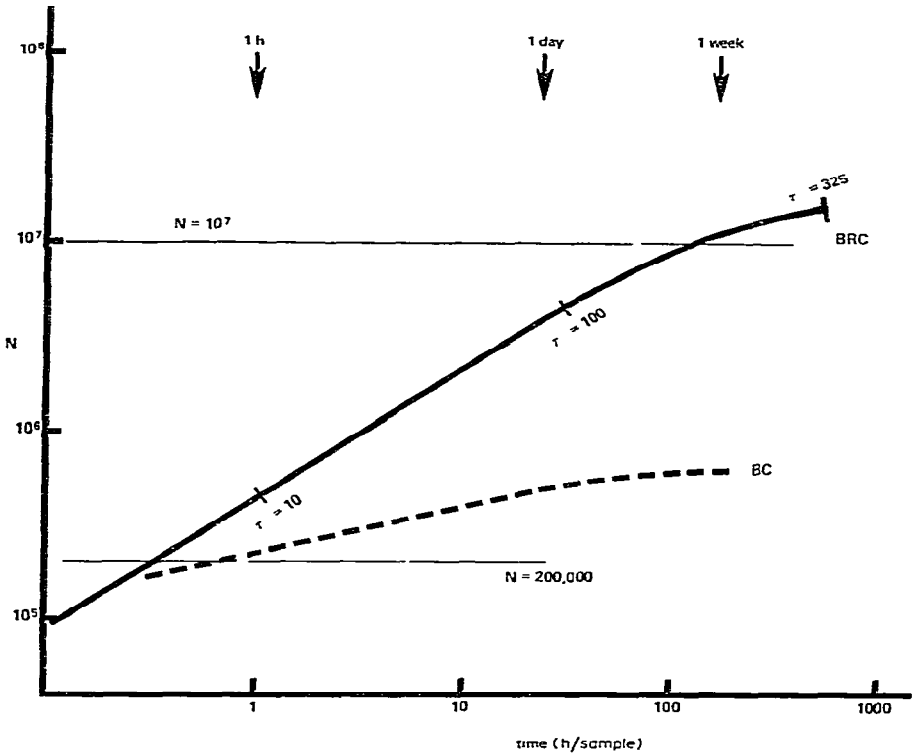

Fig. 3. Calculated plate numbers for boxcar recycle chromatography *vs.* separation time per sample. Typical conditions as in ref. 5: ———, recycle; - - -, continuous. Two 60-cm columns of 5-μm particles; reversed-phase separation at 25°C; $k' = 3$.

the better technique for lower N values because of its greater convenience. BRC seems practical for separations requiring as much as 5,000,000–10,000,000 plates, inasmuch as it can be operated in an automated, unattended mode for extended periods.

BOXCAR CHROMATOGRAPHY — EXPERIMENTAL CONSIDERATIONS

In this section we will consider some possible experimental difficulties in the application of BC and BRC, and we will show a preliminary application of the technique of BC.

Problems

In ref. 5 it was noted that maximum column N values will normally involve the use of 5-μm or smaller columns. Several workers (*e.g.* ref. 8) have noted that large N values cannot be achieved when the usual short lengths (*e.g.* 15 cm) of these small-particle columns are connected in series. However, Kirkland *et al.*[9,10] have found that this effect —the non-additivity of N when combining 5-μm columns— is due to the use of columns which show significant band-tailing or peak asymmetry. Such columns, with peak asymmetry values[7] greater than 1.25, actually have smaller N values than are calculated in the usual manner (peak half-height or tangents-to-baseline[1]). As a result, the expected additivity of band variances and column N values is then not found in practice. However, for "good" columns with peak asymmetry factors less than 1.2, and similar column-to-column H values, it is found that small-particle columns can be connected in series to give overall column N values that are the sum of N for each column in the set[11]. We have also observed this in the present study, for the 5-μm RP C_8 columns described in the Experimental section. These data are summarized in Table II. Eighteen columns were selected from our inhouse supply, all having similar plate numbers (10,300 plates for conditions of Table II). Nine columns having lower asymmetry factors (average 1.06) were grouped into set B of Table II, and the remaining nine columns (set A) had an average asymmetry factor of 1.15. Three, six and nine columns were next connected in series from each of sets A and B, and plate numbers and band asymmetries were determined as shown in

TABLE II

CONNECTING SMALL-PARTICLE COLUMNS IN SERIES. ADDITIVITY OF N VALUES

Conditions: acetonitrile–water (70:30, v/v) at 50°C; 1 ml/min; average of data for pentyl-, hexyl- and heptylbenzene solutes ($2.2 \leq k' \leq 3.9$); each FAST-LC-8 column as described in Experimental section.

Number of columns	*Column set A**		*Column set B**	
	*expt. %N***	*As****	*expt. %N***	*As****
3	96	1.10	98	1.16
6	90	1.28	91	1.15
9	82	1.39	91	1.35
Average	89	1.26	93	1.22

* Average N value for each column in set A or B equal to 10,300 plates; average band asymmetry value for set A equal to 1.15, for set B, 1.06.

** Experimental N value divided by summed N values of columns in set.

*** Band asymmetry value[1].

Table II. For set A, the experimental plate number *vs.* theoretical (sum of individual-column N values) decreased with increasing column length: 96% for three columns in series, 90% for six columns, and 82% for nine columns. Similar results were obtained for the columns from set B, although experimental values were closer to theoretical. Band asymmetry increased with increasing column length, as summarized in Table II.

The major conclusion to draw from Table II is that near-theoretical plate numbers result in each case (set A or B) as we connect our columns together in series. Similarly, large overall N values can be achieved by combining up to nine columns in series (76,000–85,000 plates). The increase in band asymmetry with increasing column length is bothersome, but the average band asymmetries of 1.2–1.3 for these experiments of Table II are not a practical problem. The use of larger column sets might result in asymmetry factors greater than 1.4, which begins to be significant; we are studying this effect further.

Pre-selection of columns with lower individual asymmetry factors (set B) is seen to give somewhat better results in terms of combined plate numbers and band asymmetries, which was expected. Possibly the further reduction of band asymmetry will be beneficial, but we are already approaching the limiting value of 1.00.

Finally, if cumulative column efficiency is less than expected when columns are coupled together, one would expect a similar reduction in overall column efficiency in any recycling technique. Therefore, solution of the problem for columns in series should avoid a similar problem in BRC.

Another question which can be raised concerning these BC procedures is that of detection sensitivity. On the one hand, Done has shown[12] that very efficient columns —those with small H values— are more quickly overloaded by sample, so that column N values begin to decrease significantly after injection of more than a few micrograms of sample per gram of column packing. On the other hand, we have seen[5] that very large N values will require long lengths of 5-μm-particle columns, and band-broading (with sample dilution) increases with increase in column length. The general issue of column loading and detection sensitivity for small-particle, long-length columns represents an important area which needs to be further studied. However, three points can be made concerning BC *per se*:

(1) Very efficient 5-μm columns are being used for practical separations at the present time, and the associated problems of low-loading-capacity have not proven insuperable;

(2) An increase in column length by 100-fold for 5-μm columns will lead to an increase in band width (and increase in band dilution) of no more than four-fold, when operating at constant pressure[5]; this is due to the corresponding decrease in H as mobile-phase velocity decreases;

(3) The total mass of column packing increases with increase in column length, and the amount of sample that can be injected before overloading becomes apparent should also increase—thus at least partly nullifying the effect of increased band-broadening.

It therefore appears that decreased detection sensitivity in BC with large N values is less serious than might have been assumed. Furthermore, if band-broadening and sample dilution become significant in a particular application, the resulting loss in sensitivity could be overcome by the use of longer-light-path (*e.g.* 25–50 mm)

flowcells. While the volume of such cells would be greater (20–40 μl), the extra-column effect would not be serious, because we are now dealing with sample bands of increased volume.

A third question concerns the operational approach to these boxcar separation techniques. How is the separation set up and monitored during its operation? Our approach is illustrated in a following section and in the Experimental section. Basically, in continuous BC a sample is first separated on a total column set ($L_1 + L_2$) that is just long enough to provide the required resolution. The relative lengths L_1 and L_2 of the two-column set will have been determined by application of eqn. 1 or similar relationship from ref. 5 for other separation cases. Assuming that the retention characteristics of columns C1 and C2 are identical, the valve-switching times are determined so as to fractionate the band of interest (X) on C1 and divert that band to column C2. The center (retention time) of band X as it leaves column C1 is calculable as $(L_1/L)t_x$, and the width of band X will be $(L_1/L)^{\frac{1}{2}}$ times the width of band X as it leaves column C2. Alternatively, as in the present study, a second detector can be plumbed into the system so as to monitor directly the effluent from column C1 of Fig. 2a.

Boxcar operation is now begun (continuous mode), with sampling rate determined as $t_z q$. Alternate samples in the latter sequence can be omitted initially, to confirm that there is no overlap of one sample onto the next in the final chromatogram. The calculated trapping-interval for band X as it leaves C1 can also be increased (*e.g.* by 25%) initially, to provide a safety margin for the resulting operation. This margin can then be reduced during running, provided that the result for a reference sample is monitored so as to establish that overtightening of the trapping-window has not occurred.

A final problem that can be visualized in the operation of continuous BC concerns column stability and the possible failure of the boxcar conditions initially established as above. Since several lengths of column (*e.g.* 15 cm) will normally be combined in the overall BC system, the probability that each column will work satisfactorily over some period of time is required to be greater than when only a single column is used. This requires that each column maintains its initial plate count and retention characteristics over long periods of time. Other studies carried out on FAST-LC columns as described here indicate that they are capable of this performance, and experimental details concerning this question will be published elsewhere. Two kinds of failure of the BC separation can be visualized if retention and/or plate number characteristics of the system change during use. First, incomplete transfer of the compound "X" from column C1 to C2 might result, leading to variable recovery of that compound from column C2. Second, incomplete separation of compound X from surrounding interferences might occur on column C1 and/or C2. This could result in overlap of such interferences onto the band of compound X, either within the same sample, or across adjacent samples. Either type of failure can be detected by suitable in-process experiments. For example, injection of control samples can be used to monitor the relative overall recovery of compound X. When the recovery of X drops by some critical fraction, the system can be rephased for more complete transfer of band X to column C2. Similarly, the occasional, deliberate omission of an injection provides an opportunity to evaluate potential overlap of interfering bands from one sample onto adjacent samples (carryover).

An experimental example of continuous BC

We have carried out preliminary experimental work on the application of BC to various problems, using off-the-shelf equipment as described in the Experimental section. Our initial results have shown that the technique is practical and free from unexpected problems. One such result will both illustrate the experimental approach used in continuous BC, and show the potential of the technique.

We have described earlier[13] the automated determination of various anticonvulsant drugs and their metabolites in serum, using LC with sample pretreatment. It is possible to carry out the separation of a six-component drug mixture in *ca.* 6 min, using a 6-cm plus 15-cm column in series, as shown in Fig. 4a. Since these drugs are often coadministered, and the metabolites phenylethylmalonamide (PEMA) and carbamazepine epoxide (CE) will accompany the respective parent drugs primidone (Pr) and carbamazepine (Cb), it is useful to assay simultaneously all six compounds. However, in some cases there will be an interest in only one or two of these compounds. Then the analysis for all six compounds is not needed, and continuous BC can shorten the per-sample analysis time.

The compounds in the separation of Fig. 4a can be grouped for BC separation

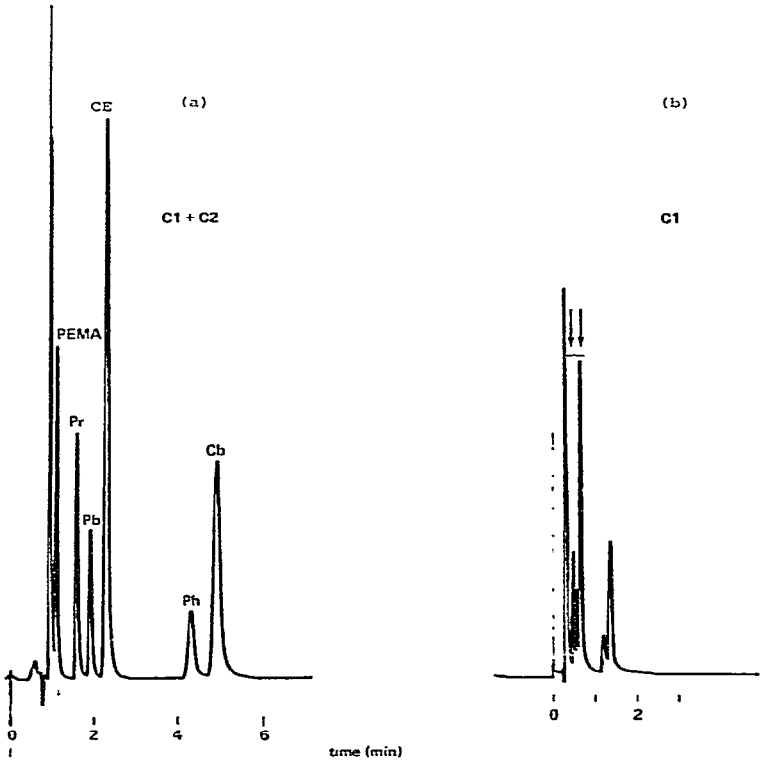

Fig. 4. Continuous boxcar separation of anticonvulsant drug mixture as described in Experimental section. C1 is 6-cm column, C2 is 15-cm column. (a) Separation on both columns; (b) separation on first column (C1). PEMA = phenylethylmalonamide; Pr = primidone; Pb = phenobarbital; CE = carbamazepine epoxide; Ph = phenytoin; Cb = carbamazepine.

as follows: (1) PEMA, (2) primidone, phenobarbital and carbamazepine epoxide, (3) phenytoin and carbamazepine. It is possible to determine any of these three compound-groups by BC, and we chose the second group for the following illustration. Therefore, the latter group of three compounds will be separated from the remainder of the sample on the 6-cm C1 column, and diverted to column C2 (15 cm) for BC separation. Fig. 4b shows the partial separation of the sample of Fig. 4a on the C1 column. The arrows in Fig. 4b indicate the valve-switching times for diversion of the compounds of interest to column C2. It is seen that the resolution of these three compounds on column C1 alone is inadequate for quantitative analysis.

A series of eighteen identical samples were next charged for BC separation as described above. The output from column C2 of the resulting samples is shown in Fig. 5. It is seen that each sample is eluted over an interval of 1.1 min, *vs.* 6 min in Fig. 4a. Therefore, there is a roughly five-fold increase in analysis rate *vs.* normal separation as in Fig. 4a. The precision of the resulting assays (peak-height quantitation) was observed to be better than 1% coefficient of variation for all three compounds. The combination of fast assay rate plus high assay precision means that external standards can be run frequently, thereby insuring both accuracy and precision in final reported data. Further improvements in assay technique, including the possible use of internal standardization and multiple assays of the same sample (with averaging) could probably reduce assay imprecision to the level of 0.1–0.2%. The technique of continuous BC creates many options that were previously impractical in the high-precision analysis of biological samples by LC.

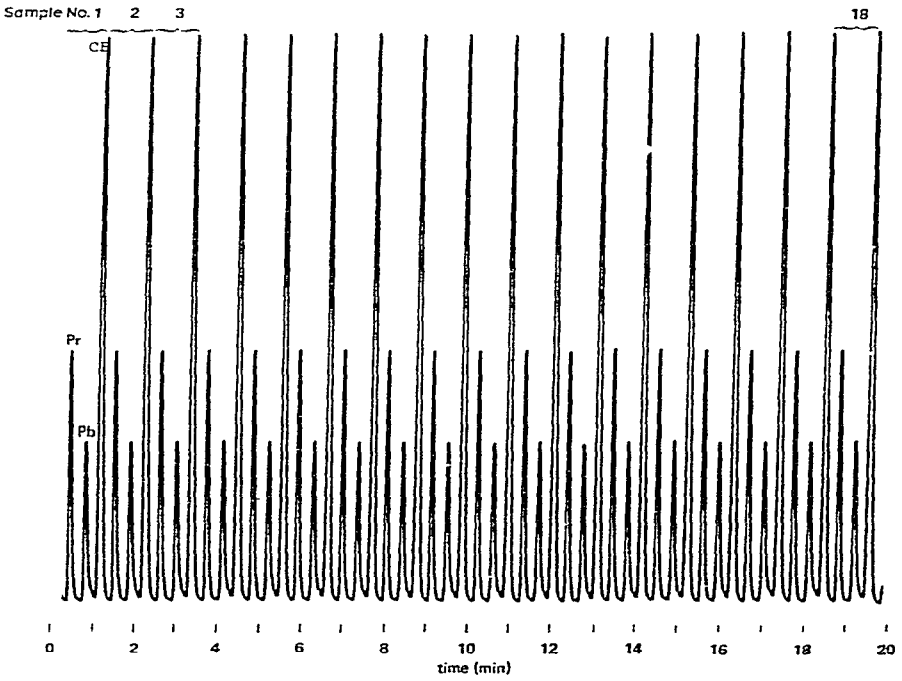

Fig. 5. Boxcar separation as in Fig. 4; output from detector during BC operation.

CONCLUSIONS

We have described a new approach to the goal of increased separation rate in LC applications, particularly where larger column plate numbers are required. We have also summarized the theory of such separations as derived in ref. 5, we have examined various hypothetical problems that might restrict the use of BC, and we have reduced the technique to practice. On balance, BC has proven simple to use and free from any major problems.

A complete boxcar system will include the elements of Fig. 2a, plus an automatic sampler for continuous feeding of samples to column C1, and a timer or microprocessor for coordinating sample pickup, sample injection and the switching of the various valves. With such a system it is possible to easily and routinely perform separations using either continuous or recycle boxcar operation, on either an analytical or preparative scale. A few general applications that we have so far conceived are discussed below.

Consider first the analysis of extremely complex mixtures, which contain hundreds or thousands of constituents; *e.g.*, urine, petroleum, etc. Here there might exist particular interest in one or more compounds which are present at the trace level. Typically the interference-free separation of such compounds in sample matrices of this type would require tens or hundreds of thousands of theoretical plates (although this requirement can be somewhat reduced via additional work aimed at increasing separation and detection selectivity). With continuous BC operation, the per-sample time required to separate and measure or collect the desired compound can be reduced by a factor of 10 or more, when several samples are to be processed.

A second application is that of separating simpler mixtures, when two or more compounds have k' values that are extremely close; *e.g.*, α values of 1.01 or less. If separation selectivity cannot be increased, use of columns with large plate numbers (10^5–10^6) cannot be avoided, which means long separation times will be required in conventional LC. Here the use of continuous or recycle BC can reduce per-sample separation time by a factor of as much as 100. The recycle mode of operation will be preferred when the required N value is greater than 200,000; continuous operation is better when N is smaller. Examples of samples of the present type would include diastereomers, certain positional isomers, and compounds differing in isotopic substituents (*e.g.* C_6H_6 *vs.* C_6H_5D). While separation selectivity is the first variable that should be optimized in such separations (*e.g.* ref. 1), sometimes the best possible α values will be small for a given pair of compounds.

An offshoot of the latter case is represented by preparative separations where α cannot be made larger than *ca.* 1.05. Here, the required plate number for the separation will be 20,000 or more, suggesting the use of small-particle columns. But such columns cannot be loaded with more than 1–100 μg of sample without excessive loss in efficiency (*e.g.* ref. 12), and furthermore the maximum column dimensions are restricted by pressure-drop considerations. In this situation, continuous BC operation allows the steady sampling of the mixture to be separated. As the resolved bands leave the detector, they can be diverted to different collection vessels via a switching valve under the control of the timer or microprocessor. The resulting application is then essentially continuous in operation, provides maximum sample throughput, and can

be run unattended for long periods of time. The separation would continue as long as sample remained for injection.

A third general application is in the area of ultra-purification and the use of chromatography to certify "chromatographically pure" materials. We know that "chromatographically pure" at present provides limited assurance of sample identification and purity; many compounds can typically elute at essentially the same place in the chromatogram for present LC separations. But suppose that in the typical case there is a 10% likelihood of chromatographic overlap, using a defined LC system, with 5000 theoretical plates. Increase of the N value would then lower the possibility of co-eluting bands; for N equal to 500,000, the probability of overlap is reduced to 1%; for N equal to 5,000,000, 0.3%. Now we can begin to assign some significance to the statement "chromatographically pure", or "chromatographically characterized". For purposes of purification, the degree of purity achievable increases in like proportion. And we can use the continuous separation scheme described above for practical throughput rates.

A fourth possibility for the effective use of these boxcar techniques is as an alternative to extensive method development, where both separation and detection selectivity are to be optimized, and where additional sample handling may be required, as in multi-dimensional separation involving two or more successive separation steps. This traditional approach to LC separation (*e.g.* ref. 1) can be carried to considerable limits so as to expand enormously the separation power of LC as normally carried out; and it is likely that few separation problems cannot be ultimately handled in this fashion. However, it should be kept in mind that this traditional approach often requires a considerable effort on the part of a near-expert practitioner.

The alternative based on the boxcar techniques could be called a "brute force" approach, where one simply increases column efficiency to a level where additional method development becomes unnecessary. For example, if a separation is obviously going to be difficult using an initial column with N equal to 5000, why not simply increase the N value to 50,000 . . . or 5,000,000? Depending on the number of samples for analysis, and other factors, a boxcar separation at this point may be more cost-effective than the usual approach.

Other possible uses of these new LC techniques will suggest themselves to workers facing separation problems of unusual difficulty. We are presently working with several groups outside the Technicon organization in reducing these possibilities to practice.

LIST OF SYMBOLS AND ABBREVIATIONS

BC	boxcar chromatography; refers to continuous mode unless otherwise stated
BRC	boxcar recycle chromatography
C	constant in eqn. 1; equal to $N^{-1/2}(t_x/t_z)$
Cb	carbamazepine (Fig. 4)
CE	carbamazepine epoxide (Fig. 4)
C1, C2a, C2b	columns in Fig. 2a
H	height equivalent of a theoretical plate (cm)
L	column length (cm); equal to $L_1 + L_2$

L_1, L_2	lengths of columns C1 and (C2a + C2b), respectively
n	number of samples assayed during a single BC operation
N	column theoretical plate number
p	equal to L_1/L
PEMA	phenylethylmalonamide (Fig. 4)
Pb	phenobarbital (Fig. 4)
Pr	primidone (Fig. 4)
P1, P2	pumps in Fig. 2a
q	fractional time required per assay in BC, *vs.* normal separation; assumes n is large
q'	"true" fractional time required per assay in BC for any value of n (see eqn. 1a)
t_x,t_z	retention time for compounds X and Z (Z is last band in chromatogram) for elution through columns C1, C2a and C2b of boxcar system
V1, V2, V3	valves in Fig. 2a
W	waste line (Fig. 2a)
X, Z	bands in Fig. 1a; X is a band of interest, Z is the last band in the chromatogram
τ	number of passes (recycles) of sample through both column C2a and C2b in BRC

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