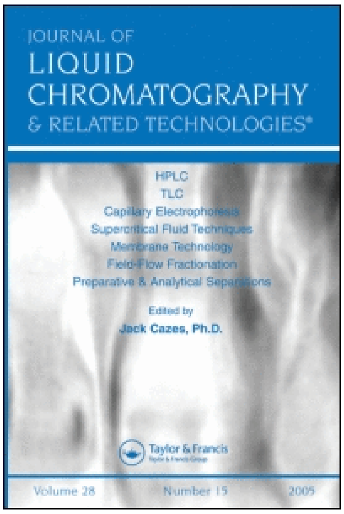

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A CONVENIENT VOID VOLUME MARKER FOR SEVERAL CHIRAL HPLC COLUMNS

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ABSTRACT

Comparison of a number of potential void volume markers under normal phase conditions shows 1,3,5-Tri-*t*-butylbenzene (TTBB) to be essentially unretained on three commercially available chiral stationary phases (CSPs). Elution times observed for TTBB agree well with void times determined by the method of minor disturbance, or with the pycnometrically determined total mobile phase volume (V_m). Therefore TTBB is recommended for use as a void volume marker for normal phase separations with these CSPs.

INTRODUCTION

Change in column temperature dissimilarly affects the elution behavior of enantiomers upon chiral stationary phases (CSPs), altering both retention and enantioselectivity (1,2). Variable temperature chromatographic studies can yield information about the enthalpy and entropy of adsorption for each enantiomer, leading to an improved understanding of the analyte-CSP interactions underlying chiral recognition. Accurate determination of these thermodynamic parameters requires careful regulation of column temperature, flow rate, and mobile phase composition. In addition, precise measurement of column void volume (V_O) is essential for accurate determination of k' and dependent parameters. Techniques for the measurement of V_O in reversed phase liquid chromatography (RPLC) have been reviewed (3) and compared (4,5). While the underlying theory for measurement of V_O during normal phase separations on CSPs is the same as that previously advanced for RPLC systems (6), we perceive a need to focus attention on the need for accurate V_0 determination during chiral separations. Often, the methods used for such determinations are either not reported or are flawed. We herein compare several reported methods for V_O measurement, and recommend one deemed suitable for CSPs similar to those reported from our laboratories (7,8).

The most commonly used method for the determination of column void volume involves measuring the retention time of an analyte which is presumed to be neither retained by nor excluded from the stationary phase (9). In the so-called "method of minor disturbance" (10), the least retained mobile phase component (9) or an isotopic analog (11) is injected and the disturbance peak obtained is regarded as indicative of V_O. There has been considerable debate about the interpretation of the complex detector signals thus obtained (12), and whether they are, in fact, a valid indicator of V_O (13). Furthermore, the difficulties involved in the detection of these peaks often leads researchers to opt for the convenience of a UV detectable void volume marker.

A recent paper (14) describes thermodynamic measurements of the normal phase separation of enantiomers upon an (*R*) 3,5-(dinitrobenzoyl) phenylglycine CSP using benzene as a void marker. Benzene, acetone and carbon tetrachloride, while convenient to use, are retained by this CSP and their use leads to erroneous V_O values. The resulting error in capacity factor (k') becomes especially significant for weakly retained analytes (k'<1).

We have investigated a number of ultraviolet absorbers as potential void markers for use with three commercially available CSPs and compared the results with those obtained with reported methods for V_O determination. We have found that the use of 1,3,5-tri-*t*-butylbenzene (TTBB) for this application gives results which compare favorably with those obtained by other techniques.

MATERIALS AND METHODS

Apparatus

Chromatographic analysis was performed using a Rainin HPX Rabbit pump, a Rheodyne model 7125 injector with a 20µl sample loop, either a Milton Roy- LDC UV absorbance monitor D (254 nm) or a Waters Associates R401 Differential Refractometer, and a Shimadzu CR1A integrating recorder.

Materials

All chemicals used were of reagent grade quality and were used without further purification. Tri-t-butylbenzene (TTBB) [1460-02-2] was obtained from Aldrich Chemical Corp., Milwaukee, WI. Solvents used were HPLC grade or distilled prior to use. Prepacked stainless steel columns of 4.6 mm ID x 25 cm length used in the study were obtained from Regis Chemical Corporation, Morton Grove, IL and are as follows: CSP 1 (*S*) DNB Leucine (Regis #731041W); CSP 2 (*R*) DNB Phenylglycine (Regis #731021W); CSP 3 (*R*) *N*-2-Naphthyl alanine (Regis #731031W).

Methods

All chromatographic experiments were carried out at a nominal flow rate of 1.00 mL/min. The recorder was triggered as an injection was made. Retention times are reproducible to ± 0.01 min, the smallest interval of time registered by the recorder.

Variable temperature data were collected with the mobile phase reservoir and pump at ambient temperature, and with the column immersed in a large constant temperature bath. About two feet of 0.009 in ID stainless steel tubing was used to connect the column to the injector and was wrapped around the inverted column as a heat exchanger to thermally equilibrate the mobile phase prior to column entry.

The extracolumn volume (V_{con}) was determined by measuring the elution time of TTBB (or any other UV adsorber) with the column replaced by a low dead volume union. All elution times reported herein are corrected values in which the extracolumn elution time has been subtracted from the measured elution time. Cyclohexane and carbon tetrachloride were used to gravimetrically determine the solvent accessible column volume (V_m) using a modified literature procedure (6).

TABLE 1

Elution Times for Candidate Void Volume Markers

	CSP 1	CSP 2	CSP 3
V_m (mL)	2.75	2.71	2.74
Compound		Elution Time (min)*	
benzene	3.03	3.11	3.02
toluene	3.02	3.10	2.98
t-butylbenzene	2.90	2.94	2.86
pentamethylbenzene	3.30	3.51	2.93
p-cymene	2.89	2.93	2.86
1,3,5-tri-*t*-butylbenzene	2.72	2.71	2.68
1,2,4,5-tetra-*i*-propylbenzene	2.73	2.72	2.71
carbon tetrachloride	2.89	2.90	2.91
dodecane†	2.68	2.65	2.65
dodecane®	2.69	2.65	2.65
decane®	2.70	2.67	2.67
cyclohexane®	2.78	2.74	2.74
pentane®	2.76	2.72	2.72

Conditions: 1.0 mL/min.; 5% 2-propanol/hexane; Room Temp.
* The reported elution times are corrected for extracolumn void volume (V_{con}).
† The first minimum of the complex UV trace afforded by dodecane is reported.
® Data collected with refractive index detector

RESULTS AND DISCUSSION

Retention times for several analytes on the three columns are summarized in Table 1 along with values obtained for the solvent accessible column volume (V_m), which provides an upper limit on the actual column void volume (V_o). Since elution volumes greater than V_m must represent retention, it can be seen that benzene, toluene and other simple monosubstituted benzenes are retained on all three CSPs. Pentamethylbenzene is quite strongly retained, especially on the π-acidic CSPs. This presumably reflects an increase in π basicity with increasing alkyl substitution. Substitution with sterically bulky alkyl groups appears to shield the aromatic systems from interaction with the stationary phase. Indeed, 1,3,5-tri-*t*-butylbenzene (TTBB) and 1,2,4,5-tetra-*i*-propylbenzene (TIPB) are not retained by the CSP relative to V_m. TTBB has been shown to be essentially unretained in a

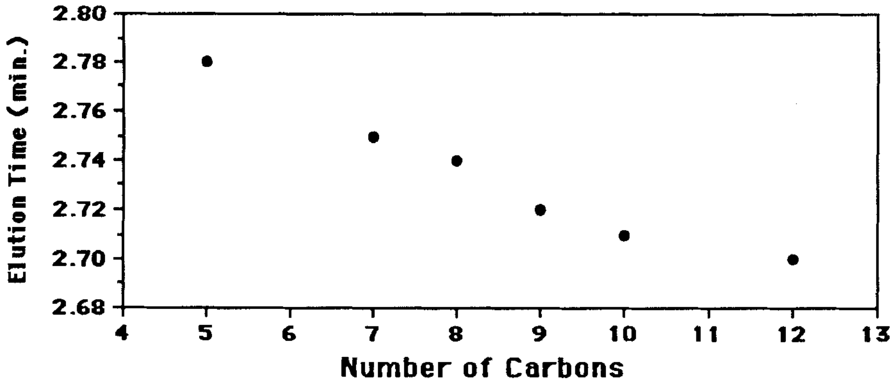

Figure 1: Elution times for the normal alkane series as measured by refractive index.
Column: Regis (*S*) DNB Leucine (CSP 1); Flow Rate: 1.0 mL/min; Mobile Phase: 5% 2-propanol in hexane; Injection Size: 0.5 µl neat liquid.

study of the retention of a number of substituted benzenes on both silica and alumina stationary phases (15). In addition, TTBB has been suggested as an void volume marker for triacetylcellulose-derived CSPs using water-ethanol mobile phases (16). However TTBB is retained by the three CSPs used in this study when reversed phase eluents are employed.

Elution volumes less than V_m may reflect either an actual reduction in void volume owing to the presence of a solvation layer on the stationary phase (16) or may represent exclusion of the marker from the pores of the silica (17). The elution times (RI detection) of several members of the normal alkane series on CSP 1 are illustrated below in Figure 1. The decrease in elution time with increasing alkyl chain length may indicate a degree of exclusion of the higher homologs. Hexane, which would be the analyte of choice for measuring V_0 by the method of minor disturbance, failed to give an adequate detector response in the 2-propanol-hexane mobile phases employed. However, an interpolated value of 2.76 min is in good agreement with the pycnometrically-obtained V_m (2.75 mL), and the elution time of TTBB (2.74 min) obtained with UV detection.

The observed elution times of all candidate markers are dependent upon temperature and mobile phase composition as illustrated in Figure 2a for TTBB.

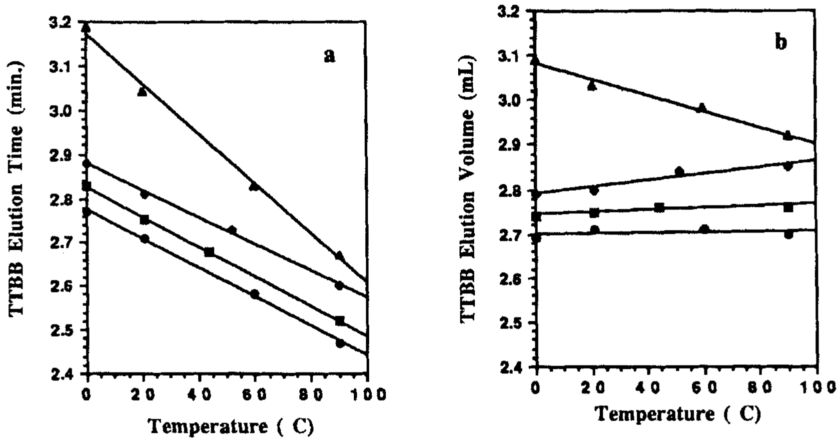

Figure 2: a) Effect of temperature and mobile phase composition on the elution time of TTBB. b) The same data corrected for temperature induced changes in flow rate. Column: Regis (*S*) DNB Leucine (CSP 1); Flow Rate: 1.0 mL/min.
Mobile Phase Composition (% 2-propanol in hexane) ● 0; ● 0.25; ■ 5; ● 20

All other candidate markers, including the above mentioned hydrocarbons, display similar temperature and mobile phase dependence. The bulk of the temperature dependence stems from the change in flow rate within the column brought about by thermal expansion (or contraction) of the mobile phase as it enters a column maintained at a temperature different than that of the pump and mobile phase reservoir (5,19). Elution volumes, corrected for thermal expansion (or contraction) of the mobile phase, (Fig. 2b) are nearly independent of temperature. Thermal expansion of the stainless steel column and the silica-based stationary phase have a negligable effect on V_0 Note that the corrected elution time of TTBB in pure hexane displays a temperature dependence which indicates some retention. Thus some caution should be exercised in using TTBB as a void marker with eluents of very low polarity. The horizontal displacement in the corrected TTBB elution volume curves for different 2-propanol concentrations may reflect an actual difference in column void volume owing to adsorption of 2-propanol by the stationary phase. The sense of the slight slope of these curves may indicate a shift of this adsorption equilibrium with temperature, the effect being greatest for 0.25%

2-propanol/hexane. The effect of mobile phase composition upon compressibility and hence flow rate was considered, however, no detectable elution time variation occurred when the compressibility setting of the pump was altered.

In summary, while static measurements provide a value for V_m which may be a good estimate of V_0 at ambient temperature (20), it is desirable to include a void marker in each chromatographic run. This not only affords information on changes in void times with changes in temperature or mobile phase composition, but is also useful in detecting leaks or unexpected changes in flow rate delivered by the pump. Tri-*t*-butylbenzene (TTBB) is recommended as a convenient and commercially available void marker for use with CSPs 1-3 under normal phase conditions. Furthermore, as a presumed unretained solute, TTBB may be of additional use in column characterization by bandshape analysis (6,16).

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