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Increasing data precision in reversed-phase liquid chromatography leads to new information level

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ABSTRACT

Reversed-phase liquid chromatographic systems as commonly used yield retention time data at a precision and reproducibility level of 0.05 to, at best, 0.01 standard deviation units within a set of several (typically 5–10) runs. This corresponds to a time resolution of some hundredths to some tenths of a minute. Improving the apparatus can reduce the data standard deviation to 0.001. At this precision level, several effects usually hidden in the data scatter band become clearly visible. This is demonstrated for the column dead volume determination via the $t(n+1)/t(n)$ method using *n*-alkane solutes in a octadecylsilane–methanol system. Whereas standard precision data yield erratically scattering results, improved precision data show an apparently linear decrease in the effective dead volume with increasing solute size. This observation can be explained by steric exclusion effects, which seem to have a far greater influence on retention data than is commonly assumed.

INTRODUCTION

The number of studies on the determination of kinetic and thermodynamic parameters from reversed-phase liquid chromatographic retention data is increasing continuously. The determination of partition coefficients, similar to the octanol–water partition coefficient or other solvophobicity parameters, is only one example. The desired information cannot be taken directly from the primary chromatographic results but must be calculated from the gross retention values. A prerequisite for this calculation is the column dead volume, V_m.

The determination and definition of the column dead volume are the subject of incessant discussion, manifested in more than 100 publications [1,2]. Our point of view is that V_m is the volume that is accessible to non-sorbed and partly excluded solutes during a chromatographic run [3]. The determination according to the method of Berendsen *et al.* [4] is the most effective and yields results with the highest precision if based on high-quality retention data. Insufficient comparability and reproducibility of kinetic and thermodynamic data found in the literature seem often to be based on two factors: first, the level of data precision is low, and second, the size-exclusion effect of solutes of normal size is not taken into account. The intention of this paper is to show that the first factor influences reproducibility and comparability to a high degree.

EXPERIMENTAL

The influence of the quality of the high-performance liquid chromatographic (HPLC) apparatus used on the level of data precision is shown by comparing the results from two different experimental set-ups.

Standard precision apparatus

The apparatus consisted of a Varian Model 5000 pump (flow-rate 1 ml/min), a Waters Rad PAK 10-μm C_{18} cartridge in a non-thermostated RCM 100 compression module, an air-driven Rheodyne Model 7010 six-port valve, a Melz LCD 201 refractive index detector and a Trio integrator (retention time in minutes to 2 decimals places). Methanol was used as the eluent and was not degassed. The Rheo-

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dyne valve and integrator were switched simulaneously. The extra-column dead volume, V_{extra}, of the apparatus was 150 μl.

Improved precision apparatus

The higher precision apparatus consisted of a Knauer C64 pump, an empty column (250 × 4.6 mm I.D.) connected via a T-fitting as a pulse damper, a helium-driven piloted Valco C6W valve (switching time <20 ms) [5], a Nucleosil 5-μm C_{18} column (250 × 4.6 mm I.D.) from Macherey-Nagel, which was thermostated in a water-bath (35 ± 0.03°C), a Melz LCD 201 refractive index detector and a Nelson Model 960 analogue-to-digital converter (ADC) operated with the Nelson software on a Melchers CMC 2000E computer (retention time in minutes to 3 decimal places). Methanol was used as the eluent and was degassed with helium and prewarmed in the water-bath before flowing through the column. Valve switching and the ADC start were controlled electronically. V_{extra} of the apparatus was 60 μl.

RESULTS AND DISCUSSION

The dead-volume calculation was performed according to the method of Berendsen *et al.* [4]. This method is based on the assumption that selectivity (α) between neighbouring members of a homologous series is constant (k' = capacity factor):

$$\alpha = k'(n+1)/k'(n) = \text{constant} \quad (1)$$

Transformation of eqn. 1 yields

$$V_{ms}(n+1) = \alpha V_{ms}(n) + (1-\alpha)V_m \quad (2)$$

which yields V_m from the intercept $(1-\alpha)V_m$ and slope α of a linear regression of $V_{ms}(n+1)$ against $V_{ms}(n)$.

The dead-volume calculation was performed in two ways. First, it was done for the whole set of C_5 to C_{17}. The mean column dead volume found for the Rad PAK cartridge is $V_{m,av.}$ = 2.487 ml and for the Nucleosil C_{18} column $V_{m,av.}$ = 2.700 ml. Second, sub-sets of six members, *e.g.*, C_5–C_{10}, C_6–C_{11}, ..., C_{12}–C_{17}, were subjected to the same calculation [6]. With the standard precision apparatus three runs were performed and with the improved apparatus fifteen runs, as the probability of finding "mavericks" increases with the number of experiments performed. In Table I, standard precision data are shown. The data quality would normally be regarded as good. Table II gives the results obtained with the improved apparatus. It is seen that the data scatter is unusually low.

The sub-set dead-volume calculation with the standard precision data is shown in Fig. 1. C_{start} is

TABLE I

RETENTION VOLUMES OF *n*-ALKANES ON THE RAD PAK C_{18} CARTRIDGE

n_C, Number of carbon atoms; $V_{msr,run\ x}$, gross retention volume (including V_{extra}) of three runs; $V_{msr,av.}$, average of the gross retention volumes; $V_{ms,av.}$, mean net retention volume (excluding V_{extra}); S.D., standard deviation; R.S.D. relative standard deviation referred to $V_{msr,\ av.}$

n_C	$V_{msr,run\ 1}$ (min)	$V_{msr,\ run\ 2}$ (min)	$V_{msr,\ run\ 3}$ (min)	$V_{msr,\ av.}$ (min)	$V_{ms,av.}$ (min)	S.D. (min)	R.S.D. (%)
5	4.40	4.40	4.40	4.40	4.25	0	0
6	4.77	4.78	4.77	4.77	4.62	0.01	0.23
7	5.25	5.25	5.25	5.25	5.10	0	0
8	5.83	5.83	5.83	5.83	5.68	0	0
9	6.57	6.57	6.57	6.57	6.52	0	0
10	7.43	7.47	7.45	7.45	7.30	0.02	0.27
11	8.57	8.57	8.57	8.57	8.42	0	0
12	9.90	9.92	9.92	9.91	9.77	0.01	0.1
13	11.57	11.58	11.57	11.57	11.42	0.01	0.09
14	13.60	13.60	13.60	13.60	13.45	0	0
15	16.12	16.10	16.10	16.11	15.96	0.01	0.06
16	19.13	19.18	19.18	19.16	19.01	0.03	0.16
17	22.88	22.87	22.88	22.88	22.73	0.01	0.04

TABLE II

RETENTION VOLUMES OF *n*-ALKANES ON THE NUCLEOSIL C_{18} COLUMN

n_C, Number of carbon atoms; $V_{msr,max}$ and $V_{msr,min}$, maximum and minimum gross retention volumes (including V_{extra}) of 15 runs; $V_{msr,av.}$ average of the gross retention volumes (including V_{extra}); $V_{ms,av.}$, average of the net retention volumes (excluding V_{extra}); S.D., standard deviation; R.S.D. relative standard deviation referred to $V_{msr,av.}$.

n_C	$V_{msr,max}$ (min)	$V_{msr,min}$ (min)	$V_{msr,av.}$ (min)	$V_{msr,av.}$ (min)	S.D. (min)	R.S.D. (%)
5	4.160	4.158	4.159	4.099	0.001	0.025
6	4.390	4.387	4.388	4.328	0.001	0.022
7	4.663	4.658	4.660	4.600	0.001	0.021
8	4.985	4.982	4.983	4.923	0.001	0.016
9	5.365	5.362	5.363	5.303	0.001	0.018
10	5.812	5.808	5.810	5.750	0.002	0.034
11	6.337	6.333	6.335	6.275	0.001	0.016
12	6.952	6.948	6.950	6.890	0.001	0.014
13	7.673	7.667	7.671	7.611	0.002	0.026
14	8.515	8.508	8.512	8.452	0.002	0.023
15	9.498	9.490	9.493	9.433	0.002	0.021
16	10.647	10.637	10.641	10.581	0.003	0.028
17	11.983	11.975	11.980	11.920	0.003	0.025

the carbon number of the first member of a sub-set. V_m found for the various sub-sets show a strong scatter around a linear regression line of V_m against C_{start}:

$$V_m = 2.819 - 3.9 \cdot 10^{-2} C_{start} \quad (r = 0.769) \quad (3)$$

The correlation coefficient is so poor that a linear dependence is not really justified.

The results obtained with the improved precision data are shown in Fig. 2. The scatter of data points around the regression

$$V_m = 2.990 - 3.4 \cdot 10^{-2} C_{start} \quad (r = 0.987) \quad (4)$$

is greatly reduced, and the correlation coefficient is acceptable, although nopt entirely satisfactory.

It can be seen that the dead volume is not constant, but decreases almost linearly from V_m = 2.825 ml for the C_5–C_{10} subset to V_m = 2.593 ml for the C_{12}–C_{17} subset. The moderate scatter around the Nucleosil C_{18} curve indicates that the data precision achieved with the improved apparatus is still not high enough to allow a decision about

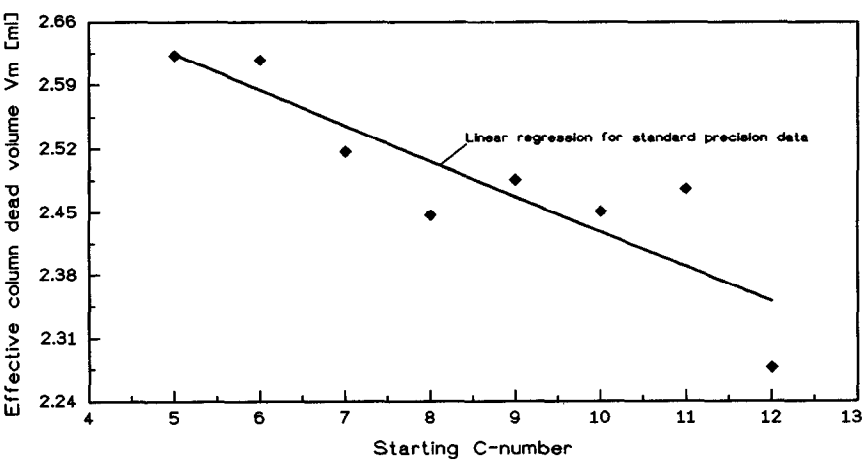

Fig. 1. Effective column dead volume as a function of the starting carbon number of the sub-set with standard precision apparatus.

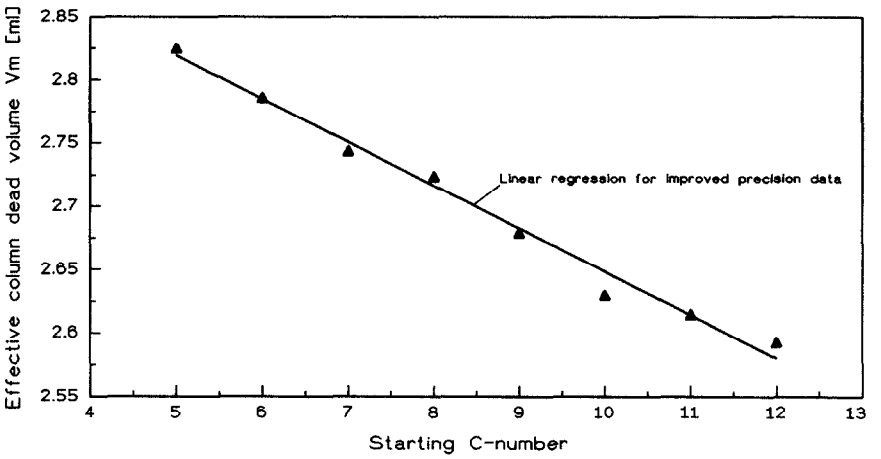

Fig. 2. Effective column dead volume as a function of the starting carbon number of the sub-set with improved precision apparatus.

an actually linear dependence of V_m on carbon number to be made.

In order to differentiate between influences of columns and apparatus, the Nucleosil C_{18} column and several others (Intersil ODS, LiChrospher ODS) were run under the same conditions as the standard precision apparatus. In all instances the same large data scatter as with the Rad PAK column was found.

CONCLUSIONS

It has been shown that the "effective" dead volume decreases with increasing solute size. This decrease is probably caused by a partial size exclusion of solutes. Solute size and ability to penetrate into the pores are directly related. To a first approximation, only those solutes whose effective diameter is smaller than the pore diameter can enter. Among the solutes fulfilling this condition, smaller ones have a higher probability per unit time than larger ones of actually finding the pore opening. These effects have to be evaluated over the pore-size distribution of the column used. A detailed report on this subject will be published elsewhere.

From the results presented, it is obvious that the data precision commonly attained in HPLC is not sufficient for a reliable dead-volume determination [7]. Our attempt to improve the apparatus led to the conclusion that commercially available HPLC pumps do not reach the required level of flow constancy. In order to solve this problem, we are working on a high-precision flow meter which monitors the actual flow at the detector exit continously on a ± 0.001 ml level. The flow-rate–time function allows the measured retention times to be converted into *true* retention volumes. We shall report on this principle later.

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