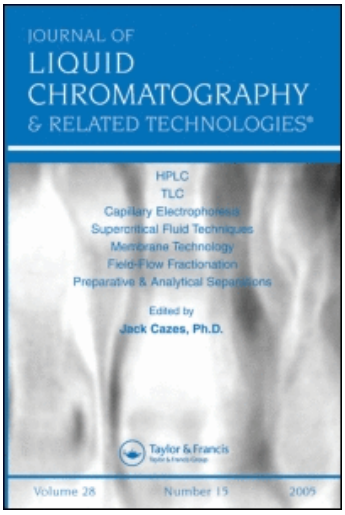

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LABORATORY TO LABORATORY REPRODUCIBILITY OF HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC RETENTION INDICES

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ABSTRACT

Using seven typical drugs, the intra- and inter-laboratory reproducibility of the measured HPLC retention indices, relative retention times, and adjusted relative retention times using the same mobile phase and various reversed-phase C-18 columns were determined. Within a given laboratory, the respective relative standard deviations were ± 0.99%, ± 1.78%, and ± 2.63%. Between laboratories, the respective relative standard deviations were found to be ± 12.6%, ± 30.2%, and ± 34.8%. These results indicated that the HPLC retention index scale may be more useful in comparing data between laboratories.

INTRODUCTION

One of the major problems in the utilization of high-performance liquid chromatographic data is the comparison of retention time data from different literature reports. It is not unknown to observe a five to ten fold variation in the capacity factor reported for the same compound even when the same mobile phase and column type were used. Recently a HPLC retention index scale very similar to the

Kovat's retention index scale for gas chromatography was introduced for use with octadecylsilyl and other reversed-phase HPLC columns (1). The HPLC retention index scale is based on the comparison of the retention time of the test compound and a series of 2-keto alkane standards. By definition, acetone is given a value of 300 and 2-butanone, 400; etc. A given column-mobile phase combination is calibrated by chromatographing the 2-keto alkane standards (C_3-C_{23}) and correlating the logarithm of the observed capacity factors in a linear manner with the defined retention indices. In previous studies, it was found that the retention index of a given compound remained nearly the same even though its retention time may vary several orders of magnitude because of changes in the composition of the mobile phase (1).

An additional advantage of the HPLC retention index scale is that the index of a given test compound (I_χ) can be estimated in advance from a knowledge of the structural properties of the compound (2,3). The retention index of the test compound (I_χ) can be calculated using the equation shown below where Π_χ is the calculated Hansch lipophilicity parameter for the test compound and $I_{ref.}$ is the experimentally observed retention index of a reference compound. The estimated and experimentally observed retention indices have been shown to be very good agreement for anthiranilic acids (2), propranolol derivatives (2), barbiturates (2), narcotics (3), steriods (4), urushiols (5), and glucuronide metabolites (6).

$$I_\chi = 200\,\Pi_\chi + I_{ref.}$$

Though it has been demonstrated that the reproducibility of the retention index values within a given laboratory is very good and the index of new compounds can be estimated easily, it is not known

if the values can be reproduced between laboratories. The major objective of present study was to determine the degree of reproducibility of the retention index values between different laboratories using seven test compounds that were representative acidic, basic, neutral, high polarity, and low polarity types of compounds.

EXPERIMENTAL

Materials

The 2-keto alkane retention index standards (C_3 to C_{23}) were obtained from Analabs (North Haven, Conn.) and the various drugs were obtained from the U.S.P. Reference standards (Rockville, Mary.) or directly from the drug manufactor. At a central laboratory, 1.0 mg/ml stock solutions of each of the drugs in methanol were prepared and distributed to each of the participating laboratories. The columns, mobile phase, and other materials were supplied by each of laboratories.

Chromatographic System

The mobile phase was prepared using 6.6 g K_2HPO_4, 8.4 g KH_2PO_4, 1.6 L CH_3OH and 2.4 L H_2O and a flow rate of 2.0 ml/min. was utilized. The C-18 reversed-phase columns utilized were supplied by each of the laboratories and each column had been in use at least two weeks before it was used for the present study. The columns shown below were used for each laboratory.

Lab A: μ Bondapak C_{18}, Waters Associates, Inc.
Lab B: μ Bondapak C_{18}, Waters Associates, Inc.
Lab C: μ Bondapak C_{18}, Waters Associates, Inc.
Lab D: μ Bondapak C_{18}, Waters Associates, Inc.
Lab E: Partisil PXS 10/25 ODS-2, Whatman Inc.
Lab F: Micropak MCH-10, Varian Instruments

Retention Index Measurement

The retention times of the 2-keto alkane standards and the test compounds (t_χ) were initially measured separately and a separate 50µl injection of methanol was used to obtain an initial estimate of the void volume. Methanol exhibited a significant retention with these columns when pure water was used as the mobile phase, but methanol gave an accurate estimate of the void volume under the conditions used in the study (7).

The retention index of the test compound (I_χ) was then determined by chromatographying a mixture of the test compound, a 2-keto alkane eluting just before the test compound (carbon no. = N), and a 2-keto alkane standard eluting after the test compound (carbon no. = N+1) which allowed the calculation of the corresponding capacity factor (k'_χ) and retention index as shown below.

$$k'_\chi = \frac{t_\chi - t_o}{t_o}$$

$$I_\chi = 100\left(\frac{\log k'_\chi - \log k'_N}{\log k'_{N+1} - \log k'_N}\right) + 100N$$

Adjusted Relative Retention Time Measurements

The C_6 2-keto alkane standard was selected as a single reference standard because its retention time was the nearest the average of the retention time of the various test drugs. The relative retention time of the test drug (t_R) and the adjusted relative retention time of the test drug (t'_R) were calculated from the retention time of the test compound (t_χ) and the retention time of the C_6 standard (t_{C6}) using the equations shown below.

$$t_R = \frac{t_\chi}{t_{C6}}$$

$$t'_R = \frac{t_\chi - t_o}{t_{C6} - t_o}$$

TABLE 1

Variation of the Capacity Factor for the C_6 2-Keto Alkane Standard

Laboratory	k'
A	3.4
B	2.6
C	4.0
D	6.0
E	14.3
F	5.3

RESULTS AND DISCUSSION

When the data was collected from the various laboratories, it was found that there was an extremely wide variation in the retention times that were observed for the drugs and the 2-keto alkane standards. For example, the retention time of androsterone was found to vary from 34 to 258 minutes (a 758% variation) between laboratories. The retention of the 2-keto alkane standards (Tab. 1) were also found to vary considerably between laboratories.

The carbon load on the columns used by laboratories A-D would typically be 10%, while the carbon load on column E would be 15% and column F, 12% (8). The correlation coefficient observed for the relationship of the C_6 capacity factor and the carbon load ($r=0.93$) indicated that major part of the variation was probably due to variations in the carbon load of the columns.

Within a given laboratory, it was found that the relative retention time and retention index measurements were very reproducible (Tab. 2). The average of the relative standard deviation (i.e. avg. coefficient

TABLE 2

Chromatographic Data Obtained by Laboratory B

	t_R	t'_R	Retention Index
Aspirin	0.3264 ± 0.0020	0.0705 ± 0.0023	178.6 ± 9.4
Caffeine	0.5343 ± 0.0006	0.3547 ± 0.0015	456.7 ± 1.2
Phenobarbital	0.7308 ± 0.0022	0.6270 ± 0.0058	538.3 ± 1.3
Phenacetin	0.9640 ± 0.0028	0.9500 ± 0.0040	593.0 ± 0.7
Methaqualone	3.099 ± 0.017	3.877 ± 0.042	759.7 ± 0.6
Chlordiazepoxide	5.19 ± 0.17	6.67 ± 0.29	825.0 ± 1.4
Androsterone	7.90 ± 0.58	10.38 ± 0.83	876.0 ± 7.2
avg. std. dev.	± 0.22	± 0.31	± 3.1
avg. relative std. dev.	± 1.78%	± 2.63%	± 0.99%

of variance) of the t_R values for the drugs was found to be ± 1.79% and the average for the t'_R measurements was found to be ± 2.63%. The reproducibility of the retention index measurements (± 0.99%) was found to be slightly better, but the superiority was small enough to be of little practical advantage.

The reproducibility of the data between laboratories (Tab. 3) was found to be much worse. For androsterone for example, the overall

TABLE 3

Laboratory to Laboratory Reproducibility Among All of the Columns

	t_R	t'_R	Retention Index
Aspirin	0.224 ± 0.086	0.060 ± 0.038	197 ± 60
Caffeine	0.507 ± 0.073	0.38 ± 0.13	471 ± 45
Phenobarbital	0.66 ± 0.13	0.59 ± 0.13	534 ± 24
Phenacetin	1.02 ± 0.17	1.02 ± 0.20	612 ± 50
Methaqualone	4.4 ± 1.5	5.1 ± 1.5	793 ± 54
Chlordiazepoxide	8.1 ± 3.7	9.5 ± 3.9	893 ± 131
Androsterone	12.3 ± 5.1	14.7 ± 5.3	952 ± 133
average std. dev.	± 1.53	± 1.59	± 71
avg. relative std. dev.	±30.2%	±34.8%	±12.6%

laboratory average of t_R was found to be 12.3 with a standard deviation of $\pm$ 5.1 ($\pm$ 41%). Though a variation of $\pm$ 41% seemed large; it was smaller than the 758% variation if the simple retention time measurement were used. The average relative standard deviation of the t_R measurement for all of the drugs was found to be $\pm$ 30.2%. If these results were then generalized, one could only expect a $\pm$ 30.2% reproducibility of retention data even where exactly the same mobile phase, exactly the same internal standard, and the same column type were used.

The reproducibility of the retention index measurements between laboratories (Tab. 3) was found to be considerably better. The average of the relative standard deviation for all of the drugs was found to be $\pm$ 12.6%. The best laboratory to laboratory reproducibility of the retention index measurement was observed for phenobarbital ($\pm$ 4.5%) and the worst reproducibility was observed for aspirin ($\pm$ 30%). The large error for aspirin was primarily the result of it falling outside of the retention index scale (300-2,300) which necessitated an extrapolation using the C_3 and C_4 2-keto alkane standards.

The primary reason that the retention index scale gave better reproducibility than the simple relative retention time scale was most likely the result of an "automatic" compensation that occurs when the retention index scale is calibrated for a specific chromatographic system. The plot of k' vs retention index produced a linear calibration curve which has a very high slope when a low percent of methanol is used in the mobile phase and a very low slope when a high percent of methanol is used in the mobile phase (1). Because of the change in the slope of the curve, the retention time of the C_3, C_4 or C_5 2-keto alkane standards relative to a single standard (e.g. C_6) will always decrease when there is

an increase in the methanol content of the mobile phase. Because of the same mechanism, the retention time of any given test compound relative to the single C_6 standard will almost always show a decrease as the methanol content is increased. Since the slope of the k' vs retention index curve changes nearly to the same extent, the retention index of the test compound will be nearly independent of the mobile phase composition (1).

When the laboratory to laboratory comparison was limited to these laboratories using only one type of column (ie μ-Bondapak C-18), the reproducibility of the retention index measurements were found considerably better (Tab. 4). Within this group, the average relative standard deviation was found to be $\pm$ 3.6% for the seven drugs. Within this group the measurements using the relative retention time scale or adjusted relative retention time scale were also found to have lower standard deviations ($\pm$ 8.8% and $\pm$ 12.9%), but they were not as good as the retention index measurement.

As indicated previously, the capacity factors for the C_6 standard (Tab. 1) served as a measure of the carbon load of the columns. In general,

TABLE 4

Laboratory to Laboratory Reproducibility Using Only μ-Bondapak C-18 Columns

	t_R	t'_R	Retention Index
Aspirin	0.276 $\pm$ 0.033	0.077 $\pm$ 0.035	231 $\pm$ 37
Caffeine	0.471 $\pm$ 0.053	0.303 $\pm$ 0.047	443 $\pm$ 10
Phenobarbital	0.740 $\pm$ 0.050	0.670 $\pm$ 0.064	548 $\pm$ 13
Phenacetin	0.938 $\pm$ 0.032	0.927 $\pm$ 0.035	590.3 $\pm$ 4.8
Methaqualone	3.44 $\pm$ 0.26	4.15 $\pm$ 0.21	756.6 $\pm$ 4.7
Chlordiazepoxide	5.68 $\pm$ 0.39	7.04 $\pm$ 0.26	828.7 $\pm$ 7.9
Androsterone	7.00 $\pm$ 1.07	11.29 $\pm$ 0.75	891 $\pm$ 22
average std. dev.	$\pm$ 0.27	$\pm$ 0.20	$\pm$ 12.9
avg. relative std. dev.	$\pm$ 8.8%	$\pm$12.9%	$\pm$ 3.6%

TABLE 5

Correlation of the Observed Retention Index with the Capacity Factor[a] (Carbon Load) of the Column

compound	correlation coefficient
aspirin	- 0.42[b]
caffeine	+ 0.65
phenobarbital	- 0.65
phenacetin	- 0.001
methaqualone	+ 0.17
chordiazepoxide	+ 0.10
androsterone	+ 0.06

a: capacity factor of the C_6 2-keto alkane standard
b: correlation between retention index and k'_{C6}

as the carbon load of the columns increased, the retention of both the drugs and the retention index standards increased markedly. If the increase were exactly the same, the retention index of the drugs would be independent of the carbon load. In a attempt to determine if the small variations in the retention index of the drugs ($\pm$ 12.6%, Tab. 3) was related to the column carbon load, the correlation coefficients of the retention index and k'_{C6} of each drug-column pair was determined. The result of the statistical analysis (Tab. 5) indicated that in general, there was no correlation between the change in the retention index and the carbon load. Thus it appeared that carbon load was not a major factor in determining the selectivity of the column. However, it was noted that the retention index of the two acidic drugs aspirin (r= -0.42) and phenobarbital (r= -0.65) did show a slight tendency to decrease with an increase in carbon load. The index for caffeine (r = +0.65) showed a slight tendency to increase with an increase in carbon load.

It appeared that the major factor relating to the small changes in selectivity of the columns was the amount of end-capping used in preparation of the column. Columns A-D were extensively end-capped with approximately 95% coverage (8). Column E had approximately 75% surface

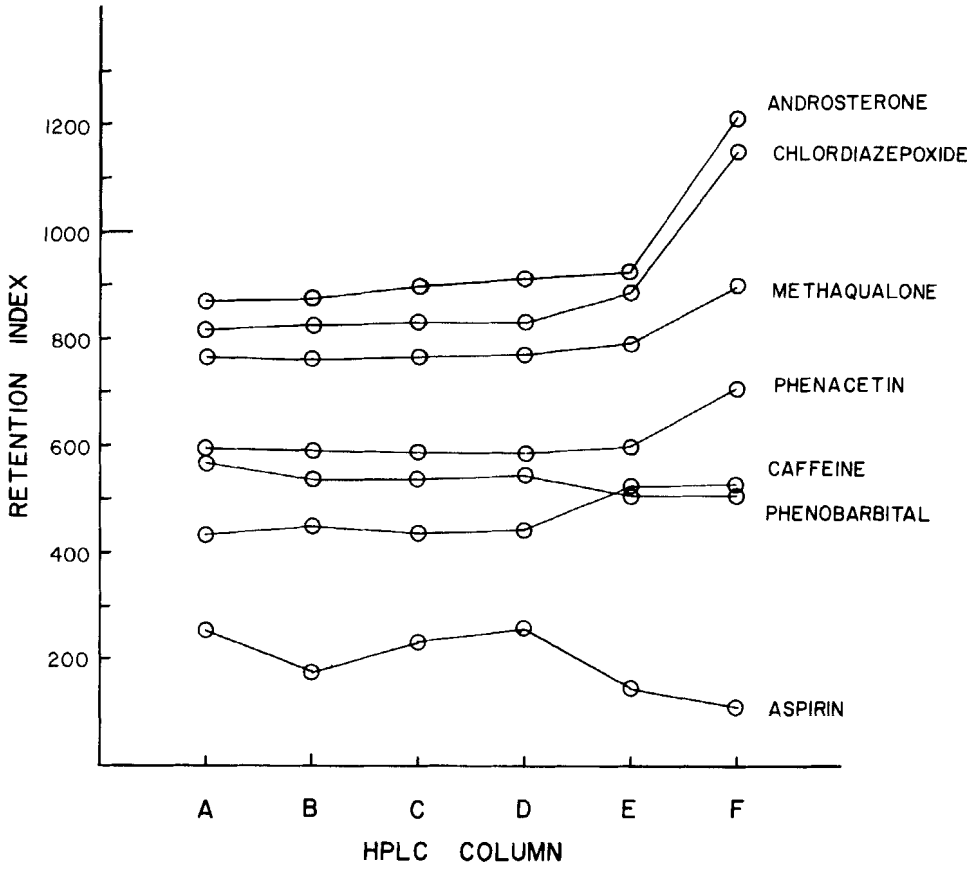

FIGURE 1

Variation in column selectivity from laboratory to laboratory

coverage while no end-capping was used for column F. It was noted (Fig. 1) that as the number of free silanol sites increased most of the drugs showed an increase in the observed retention index. The two acidic drugs, phenobarbital and aspirin, were found to have lower retention indices as the number of free silanol sites increased.

CONCLUSIONS

It was found that the laboratory to laboratory reproducibility of the retention index measurement was better than the relative retention time or adjusted relative retention time measurements. If the columns of only one manufactor were used, one could anticipate a relative standard deviation of approximately $\pm$ 3.6% for the retention index measurement. If reversed-phase columns from different manufactors were used, one could anticipate a $\pm$ 12.6% variation. Though the retention index measurements were remarkably constant, the small variations that were noted arose from variations in the selectivity of the columns. The major factor relating to the change in selectivity appeared to be the extent of surface coverage of each column.

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