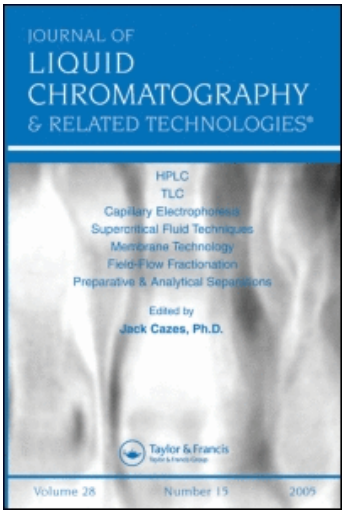

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The Effect of Chromatographic Conditions on the Retention Indices of Forensically Relevant Substances in Reversed-Phase HPLC

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THE EFFECT OF CHROMATOGRAPHIC CONDITIONS ON THE RETENTION INDICES OF FORENSICALLY RELEVANT SUBSTANCES IN REVERSED-PHASE HPLC

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ABSTRACT

This paper deals with the effect of chromatographic conditions, such as the columns with different batches and lengths, buffer concentration in eluents, gradient profiles, pH-values of buffer and flowrates of the elution, on the retention indices of forensically relevant substances in reversed phase HPLC. Our study shows that retention index is only a method of linear correction. When the retention times of the analytes change, under deviation of chromatographic conditions, proportionally to that of the scale substances, the retention indices can well balance the variation from retention times.

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INTRODUCTION

High performance liquid chromatography (HPLC) can be widely used in quantitative analysis. However, its application to systematic toxicological analysis (STA) has been limited. This may partly arise from the poor reproducibility of the retentions of analytes, which makes it difficult to collect from HPLC retention data as useful to the identification of unknown substances in different situations as those collected from thin-layer chromatography (TLC)[1] and gas chromatography (GC).[2] This led us to investigate the factors that influence the reproducibility of retentions in HPLC.

The most reproducible results were obtained when the retentions were recorded as relative values,[3,4] either as relative capacity factors, corrected capacity factors, relative retention times, or as retention indices. As capacity factors have been conventionally calculated as $k' = (t_r - t_0)/t_0$, the values of k' are susceptible to the smallest changes of the column void volume (t_0). Many methods have been proposed to determine this value,[5-7] but they often give different results with the same column and equipment. Relative retention times compared with an internal standard is a simpler method, but each laboratory may have different standard compounds, so that direct comparison of the results are impossible.

Kovats' retention indices have been widely used in GC because they are more comparable than direct retention times under different chromatographic conditions, but similar concepts have not been accepted in HPLC, so far. Since the first proposals made by Baker and Ma,[8] who suggested that the alkan-2-ones could be used as a scale for retention indices in HPLC, Smith[9] has suggested that alkyl aryl ketones would be more easily detected with UV as a retention indices scale. R. Aderjan and M. Bogusz,[10,11] have put forward 1-nitroalkane as retention indices scale both for GC and HPLC. A series of studies aimed at improving the reproducibility of retention values in HPLC have been made.[12] The influence of the eluent composition,[13] instruments setup,[14] operating temperature and the nature of the stationary phase[15] on the retention of barbiturates, local anaesthetic drugs, basic drugs and thiazide diuretics[16] with related drugs in reversed-phase HPLC has been studied. The results showed that the retention indices of neutral sample compounds were virtually independent of proportion of methanol-water in eluents over a wide range, and the retention indices of basic drugs and the references not affected in the same way by the chromatographic conditions because basic drugs have so many different chemical structures. M. Bogusz et al. used a method of correction to improve the reproducibility of retention indices in gradient elution between RP-18 columns using different groups of standards for neutral/acidic drugs or basic drugs.[17,18] M. Bogusz and M. Wu[19] used the retention indices based on 1-nitroalkane to standardize HPLC system for STA. Recently, 1-nitroalkane has been also applied[20] to the retention indices for STA in the reversed-phase HPLC.

However, most of these studies on retention indices in HPLC were done using isocratic elution and investigating neutral/acidic or basic substances with different eluents or corrected with different standards. No corresponding study has been made of the effect of changing the chromatographic conditions on the retention indices with the elution system, which is suitable for STA.

The present study is a systematic examination of the applicability of the retention indices, based on 1-nitroalkane to acetonitrile-phosphate buffer gradient elution. One aim of the study is to determine the robustness of the retention indices to small changes in chromatographic conditions and to identify the factors that must be strictly controlled in order to obtain consistent results from different laboratories.

Our work includes a detailed examination of more than 100 substances of forensic interest with various chemical structure classes in gradient elution.

EXPERIMENTAL

Instruments

Experiments were carried out with an H/P HPLC system (Hewlett-Packard, Avondale, PA, USA) equipped with a Model 1050 series pump and autosampler, HP 300 Chemstation and HP 1040 DAD detector. The DAD detector was set up at 220 nm as monitor wavelength. A LiChroCART column (125mm x 4mm ID) packed with 4 µm Supspher 100 RP-18 (Merck, FRG) was used. A guard column (4 x 4 mm), filled with the same material was installed.

The saturation column, filled with Lichrospher RP-18 was mounted between the pump and the injector to provide protection of the analytical column against the influence of amine modifier.

Chemicals

Drugs involved in this study were diluted with methanol to a concentration of 50-100 µg/mL. A series of 1-nitroalkanes—nitromethane, nitroethane, 1-nitrobutane, 1-nitropentane and 1-nitrohexane—was obtained from Fluka AG, Switzerland. 1-Nitroheptane and 1-nitrooctane were synthesized as previously[21]. Acetonitrile was analytical grade and obtained from Roth Gmbh, FRG. Triethylammoniumphosphate buffer (1 M in water) was supplied by Fluka.

Table 1

Reproducibility of Retention Times and Peak Shape of 1-Nitroalkane during Four Months

Homolog*	Rt±SD (min)	CV%	Width±SD	CV%	Symmetry±SD	CV%
C1	2.094±0.015	0.716	0.147±0.10	6.8	0.511±0.041	8.0
C2	5.168±0.047	0.909	0.150±0.014	9.3	0.533±0.033	6.2
C3	10.760±0.092	0.855	0.210±0.022	10.5	0.789±0.147	18.6
C4	17.775±0.135	0.759	0.184±0.019	10.3	0.857±0.099	11.6
C5	22.181±0.137	0.618	0.159±0.014	8.8	0.847±0.089	10.5
C6	25.479±0.134	0.526	0.150±0.011	7.3	0.883±0.084	9.5
C7	28.181±0.127	0.451	0.148±0.010	6.8	0.807±0.078	9.7
C8	30.638±0.125	0.408	0.151±0.011	7.2	0.613±0.083	10.2

* C-Atomic number of 1-nitroalkane (n = 20).

HPLC Conditions

The HPLC buffer was prepared by adding 25 mL triethylammonium phosphate buffer to 1000 mL water. The pH was about 3.1. The elution was followed by the acetonitrile buffer linear gradient: at the beginning 0% acetonitrile, after 30 min 70% acetonitrile, keeping 70% acetonitrile for 5 min, 10 min of the post time. The flow rate was 1 mL/min and the injection volume 10 µL. The above conditions were used as our standard system in this paper.

RESULTS AND DISCUSSION

Reproducibility of the System

Using the standard system, without any changes in chromatographic conditions, we have observed the retention behaviour of 1-nitroalkane and a set of test solutions for four months. The test solutions included neutral, acidic and basic substances, respectively, which, when chromatographed over a wide range, covered nearly all the important areas of the gradient elution. The results are shown in Table 1 and Table 2.

Not only the retention times but also the other chromatographic properties of 1-nitroalkane, such as the width and the symmetry factor of the peak, were reproducible. All the CV% values for retention times of 1-nitroalkane were

Table 2

Reproducibility of Retention Times and Retention Indices of Acidic, Neutral and Basic Substances in Mixed Solutions (n=20)

Substance	Rt±SD(min)	CV%	RI±SD	CV%
Paracetamol	7.05±0.14	1.99	234±1.9	0.812
Barbital	10.05±0.14	1.39	287±1.8	0.627
Brallobarbital	14.79±0.16	1.08	359±1.6	0.446
Pentobarbital	17.89±0.16	0.894	405±1.9	0.469
Secobarbital	19.15±0.15	0.783	437±1.6	0.366
Clobazam	21.31±0.20	0.939	484±2.2	0.455
Indometacine	25.64±0.25	0.975	610±3.3	0.541
Prazepam	26.79±0.12	0.448	648±3.5	0.540
Morphine	5.12±0.09	1.76	198±1.9	0.960
Chloroquine	8.80±0.07	0.795	265±0.5	0.189
Benzoylecgonine	10.52±0.06	0.570	295±0.8	0.271
Cocain	13.34±0.12	0.900	336±1.9	0.565
Diphenydramine	16.75±0.13	0.776	385±2.0	0.519
Haloperidol	18.11±0.15	0.828	409±1.7	0.416
Amitriptyline	19.69±0.03	0.152	446±2.7	0.605
Thioridazine	22.32±0.13	0.582	504±3.9	0.774
Meclozine	25.41±0.22	0.866	601±3.0	0.499
Amiodaron	29.65±0.36	1.21	762±4.4	0.577

smaller than 1.0%, 0.66% in average. The width and symmetry factor have a same level of CV% values. These width and symmetry factors demonstrate that the theoretical plate number of the column has not greatly changed after the long term run.

For the substances in test solutions, shown in Table 2, the average of CV% of retention times was 1.2%, which is greater than the average of that of retention indices, 0.66%. The reproducibility of the retention times of 1-nitroalkane was better than that of the substances in test solutions.

During the four months, we kept the instruments simply at ambient temperature (22 °C±4 °C). No serious influence of operating temperature has been found. R. M. Smith has reported that, over a small range (±5 °C), the influence is small (<10 RI units),[16] and is not likely to interfere with identification procedures. In his paper we can see that, for cyclobarbitone, for

Table 3

Characteristics of the Columns

No.	Length x i.d.	Neff./m*	Rt (C8, min)±SD**	CV%	Flowrate (mL/min)
1	125 x 4	3731	30.600±0.02	0.07	1.0
2	125 x 4	1892	30.049±0.04	0.13	1.0
3	125 x 4	4456	30.411±0.06	0.20	1.0
4	125 x 4	2441	29.920±0.12	0.40	1.0
5	50 x 4	1273	25.863±0.03	0.12	0.6

Mark: Lichrocart; Manufacturer: Merck AG; Packing material: Superspher 100 RP 18, 5 μm for columns No. 1-4, 4 μm for column No. 5
* Calculated by 1-Nitroheptane with isocratic elution, 60:40 acetonitrile:TEAP-buffer
**Calculated with n=20 for columns No. 1-4, n=16 for column No. 5
Neff./m: theoretical plate

Table 4

Linear Relationship Coefficients between Retention Time or Retention Indices on Different Lengths of Columns

Item	A	B	R
Retention time of 1-nitroalkane	0.906	-2.80	0.984
Retention indices of substances in the test solutions	0.942	62.84	0.991
Retention time of substances in the test solutions	0.902	-1.86	0.993

Linear Relationship: $Y = A*X + B$
Y: Data on the 5 cm column; X: Data on the 12.5 cm column.
R: Correlation Coefficient

instance, ΔRI was 12, 6, 10, 15 between 10 °C and 20 °C, 20 °C and 25 °C, 25 °C and 30 °C, 30 °C and 40 °C, respectively. In our work, we found temperature played an even smaller role on the retention. The reproducibility of retention times of 1-nitroalkane and the above listed analytes and of the width and symmetry factor are proof of high stability of the system, of both the equipment and the elution conditions, which is very important for gradient elution and our later investigation. In spite of this, for greater guarantee of

control we added 1-nitrooctane to each sample to monitor the reproducibility of each gradient run. And this confirmed that we could use the system to investigate the influence of small changes in operating conditions on the retention of the 1-nitroalkane scale and solutes.

Columns with Different Batches and Lengths

We know that, in interlaboratory comparisons, an important cause of irreproducibility is the differences between nominally equivalent C_{18} bonded silicas. It was reported that differences include minor, but significant, ones between batches from the same manufacturer and much bigger ones between manufacturers.

Four columns, filled with the same packing material from one manufacturer were used in our study. Their characteristics are given in Table 3. Column 3 was new and column 4 very old. Both columns 1 and 2 have been long used for our routine analysis for over a year. The batch number of the commercial columns was different in each case.

All the substances examined with the first four columns were well reprodu-cible, both in retention indices and in retention times. The greatest CV% of retention indices was 2.55% with SD = 8.18 for phenazone. This deviation is acceptable for routine analysis. It is more surprising that, with this HPLC system, no significant differences in retention times were found with different batches of the same brand, although these columns have different values of Neff/m.

The linear coefficients of the relationships between retention times or retention indices on columns 5.0 cm and 12.5 cm, shown in Fig.1, is given in Table 4. We can see from the results that there is no difference between these linear relationships when the retentions on the 5.0 cm and 12.5 cm column were expressed with retention indices and with retention times.

When the 5 cm column, which was identical with the 12.5 cm columns except for the length of the columns, was put into use with the same gradient but different flow rate (0.6 mL/min), linear consistencies were observed (Fig.1). The linear coefficient was r = 0.991 for retention indices and r=0.993 for retention times of the over 100 substances between 5cm and 12.5cm columns.

The relationship regressed with the least squares method as listed in Table 4. From Table 4, we could find that the linear relationship of the examined substances was better than that of 1-nitroalkane. For the earlier eluted substances, the reproducibility of the retention times from the 5 cm and 12.5 cm

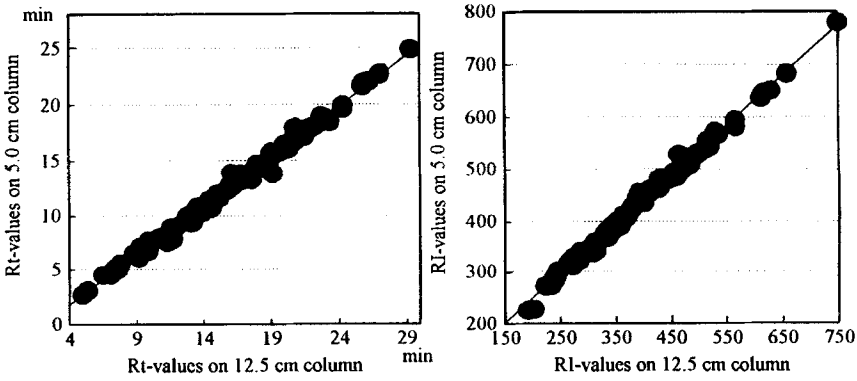


Figure 1. Linear relationships between retention times, retention indices, on 5 cm column and on 12.5 cm column.

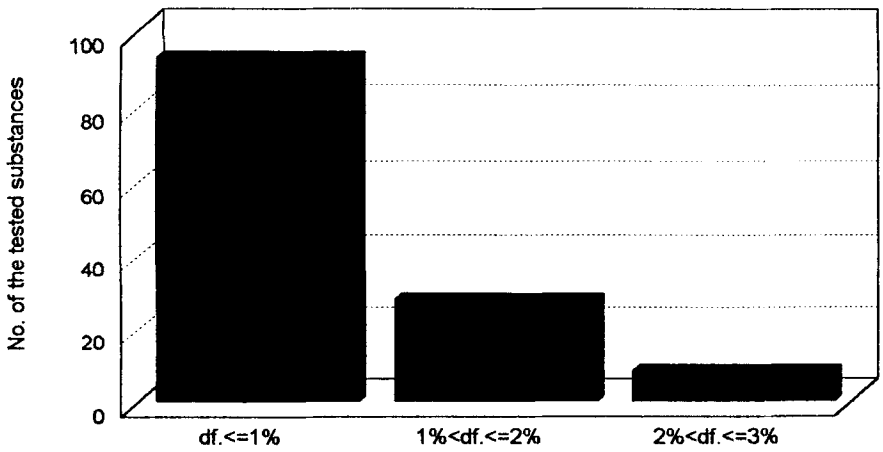


Figure 2. Distribution of the differences (df.) in retention times of the tested substances eluted with different TEAP-concentrations

Table 5

Retention Times of 1-Nitroalkane Eluted with Different TEAP-Concentrations in Eluents

Homolog*	Retention time (min)			Ev**-Rt±SD	CV%
	25 mL	20 mL	30 mL		
C 1	2.176	2.185	2.243	2.201±0.036	1.65
C 2	5.233	5.398	5.454	5.362±0.115	2.14
C 3	10.807	10.779	10.970	10.852±0.103	0.95
C 4	17.549	17.389	17.616	17.518±0.117	0.67
C 5	22.149	22.033	22.067	22.083±0.060	0.27
C 6	25.425	25.361	25.332	25.373±0.048	0.19
C 7	28.133	28.069	28.031	28.078±0.052	0.18
C 8	30.595	30.493	30.467	30.519±0.068	0.22
Ev C 8 (n=20)	30.599	30.329	30.464		
SD C 8 (n=20)	±0.015	±0.111	±0.082		
CV%	0.049	0.37	0.27		

* C-Atomic number of 1-nitroalkane.

**Average of rentention time.

columns was somewhat poorer, because nitroethane and 1-nitropropane were chromatographed later than they would be in an ideal linear relationship.

TEAP-Buffer Concentration in Eluents

It is well known that drugs with structures containing basic nitrogen atoms can show tailing peaks in reversed-phase HPLC. These problems are recognised to arise from interactions between the drugs and the adsorption sites on the silica matrix of the packing material.[22,23]

An eluent with modifier is necessary for STA in order to get sharper and more symmetrical peaks for basic substances. Triethylammonium phosphate (TEAP) has high solubility in aqueous eluents and can be used as part of the buffer system..

The effect of different TEAP concentrations in eluents on the retention times and retention indices was investigated. We changed the TEAP-concentration from 20 mL to 30 mL of 1 M TEAP in 1 L eluent, but kept all other HPLC conditions constant, then the 1-nitroalkane and over 100 forensic relevant substances were chromatographed.

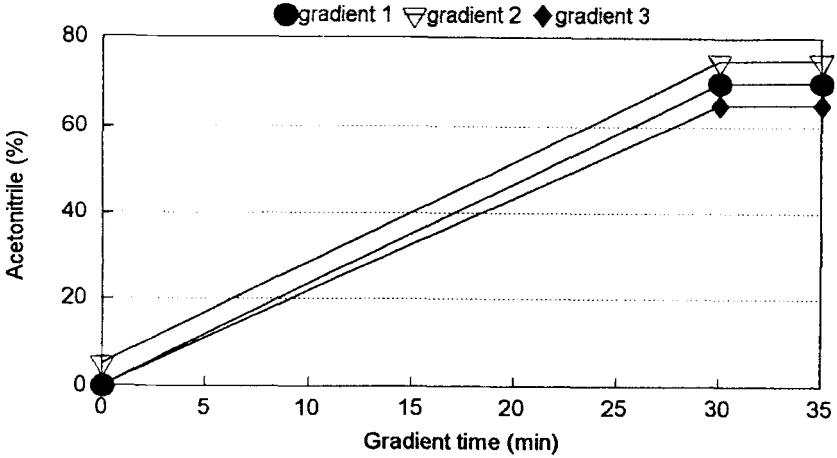


Figure 3. Three different gradient profiles used.

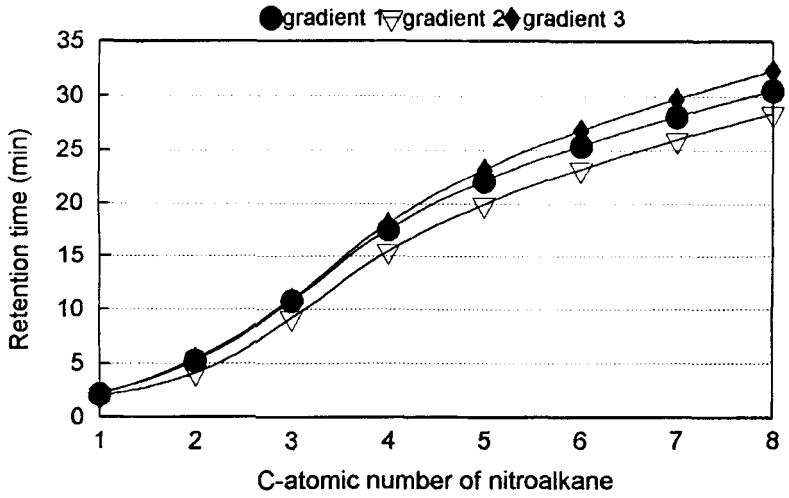


Figure 4. The retention times of 1-nitroalkane under the three different gradient profiles.

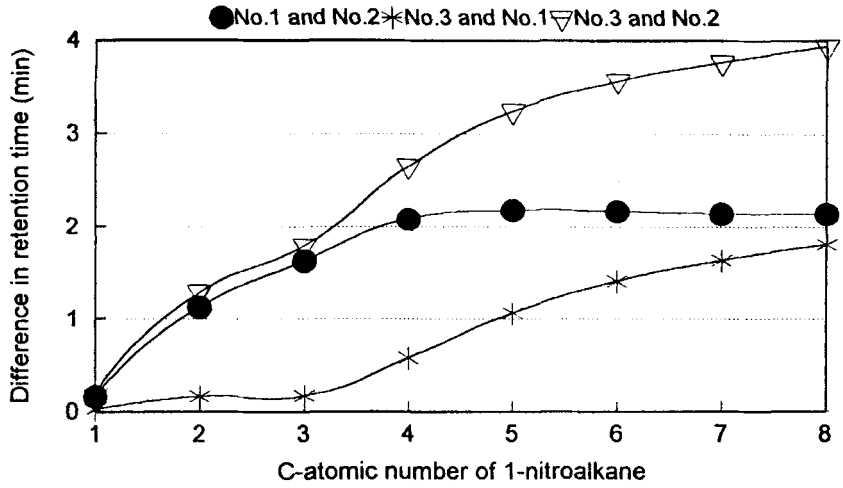


Figure 5. Differences in the retention times of 1-nitroalkane under gradient No. 1 and No. 2, that under No. 3 and No. 1 and that under No. 3 and No. 2.

The retention times of 1-nitroalkane with different concentrations of TEAP-buffer was shown in Table 5. Figure 2 showed that almost all retention times of the tested substances, eluted with different TEAP concentrations, did not change greatly. The difference of the retention times of the tested substances, eluted with three different TEAP concentrations, were mostly (72%) smaller than 1%, 22% of them between 1% and 2%, 6% of them between 2% and 3%, none of them greater than 3%. The TEAP concentrations played no significant role on the retention behaviours of 1-nitroalkane and of the tested substances.

Effect of Gradient Profiles

One of the most important causes which strongly affects the retention times in gradient elution HPLC, is the reproducibility of gradient profile. A slight change in the components of eluent on line may produce great deviations in retention times. Sometimes, in different circumstances, the HPLC systems were run under nominally identical but, in fact, under slightly varied conditions, such as, the gradient profile, the flowrate, etc.

P. Jundere and J. Churacek[24] thought that the intercept, the slope and the concentration at the beginning would be the most important factors of a linear gradient profile, which could affect the retention times in the HPLC. We used

three slightly different linear gradient profiles in order to investigate how the gradient profiles would affect retention times and retention indices. The three different gradient profiles were given in Figure 3.

The retention times of 1-nitroalkane under three different gradient profiles were given in Figure 4. As expected, there was no significant variation in retention times of nitromethane, nitroethane, 1-nitropropane and nitrobutane under gradient No. 1 and No. 3 because of their similarity during the first 15 min of gradient profiles.

Under these conditions, we would have almost the same retention times of the tested substances and, consequently, the same retention indices.

But later, the difference in retention times grew bigger with the difference in both the gradient profiles. There was a relationship between the difference in the retention times obtained under gradient No. 1 and No. 3 and the C-atomic number of 1-nitroalkane.

Over 1-nitrobutane (retention times about 17 min) the difference in retention times was constant. (See Figure 5.)

This implies that retention indices can well balance the variations in retention times which were caused by the unidentical repeat of gradient profile under different circumstances. As can be seen from the results in Table 6, a comparison of the difference in retention times and retention indices under three different gradient profiles showed that the reproducibility of retention indices was much better than that of retention times.

The average of difference in retention times of the 113 substances tested was 10.9%, but that of retention indices only 2.07%. All the substances with relatively larger variation in the retention times under gradient No.1 and No.2, for example morphine, procainamid etc., were eluted sooner under gradient No.2 than under No.1, while under the same conditions nitromethane and nitroethane were not.

In this situation, retention indices of morphine and procainamid, etc., could not balance the difference in the retention times so well as those of the other substances, such as atenolol, paracetamol, etc., which were eluted somewhat later and whose retention times changed in proportion to the changes in retention times of 1-nitroalkane.

Some substances, such as trifluoperazine and parathion, etc., eluted under gradient No.1 and No.3, had relatively larger differences in

Table 6

Retention Times and Retention Indices of 113 Substances under the Three Different Gradient Profiles, No. 1, No. 2, and No. 3

Substance		Retentions[a] (1)	(2)	(3)	M±SD	CV, %
Acebutolol	RT:	11.58	9.41	12.08	11.02±1.42	12.9
	RI:	311	304	316	310±6.0	1.94
Acetanilide	RT:	11.68	9.67	12.25	11.20±1.35	12.1
	RI:	313	308	318	313±5.0	1.60
Alimenazin	RT:	13.41	11.40	14.27	13.03±1.47	11.3
	RI:	338	335	346	340±5.7	1.67
Allobarbital	RT:	13.14	10.84	13.55	12.51±1.46	11.7
	RI:	334	326	336	332±5.3	1.59
Alprazolam	RT:	19.97	18.09	21.09	19.72±1.52	7.69
	RI:	452	458	458	456±3.5	0.76
Alprenolol	RT:	16.09	14.14	17.08	15.77±1.50	9.49
	RI:	378	379	385	381±3.8	0.99
Aminophenazon	RT:	7.57	5.44	7.74	6.92±1.28	18.5
	RI:	241	226	242	236±9.0	3.79
Amitritylin	RT:	20.21	18.46	21.47	20.05±1.51	7.50
	RI:	458	466	464	463±4.2	0.90
Amobarbital	RT:	17.94	15.76	18.93	17.54±1.62	9.24
	RI:	409	406	416	410±5.1	1.25
Aprobarbital	RT:	13.93	11.88	14.62	13.48±1.43	10.6
	RI:	346	342	350	346±4.0	1.16
Aspirin	RT:	13.06	10.91	13.85	12.61±1.52	12.1
	RI:	333	327	340	333±6.5	1.95
Atenolol	RT:	6.49	4.16	6.79	5.89±1.44	24.8
	RI:	223	201	225	216±13	6.16
Azinphos-Methyl	RT:	24.28	22.26	25.50	24.01±1.64	6.81
	RI:	565	569	563	566±3.1	0.54
Barbital	RT:	9.92	7.20	10.39	9.17±1.72	18.8
	RI:	284	261	289	278±15	5.38
Benzoylecgonine	RT:	10.60	8.48	11.16	10.08±1.41	14.0
	RI:	296	286	303	295±8.5	2.90
Brallobarbital	RT:	14.64	12.74	15.27	14.22±1.32	9.26
	RI:	357	357	360	358±1.7	0.48
Bromazepam	RT:	16.22	14.12	17.11	15.82±1.54	9.72
	RI:	380	378	385	381±3.6	0.95

(continued)

Table 6 (continued)

Substance		Retentions[a] (1)	(2)	(3)	M±SD	CV, %
Butabital	RT:	16.09	14.06	16.91	15.59±1.35	8.64
	RI:	378	378	383	380±2.9	0.76
Camazepam	RT:	23.95	21.92	24.81	23.56±1.48	6.30
	RI:	554	559	544	552±7.6	1.38
Carbamazepin	RT:	16.91	15.94	19.03	17.29±1.58	9.14
	RI:	391	410	418	406±14	3.42
Chlordiazepoxide	RT:	14.71	12.70	15.48	14.29±1.44	10.0
	RI:	358	356	363	359±3.6	1.00
Chloroquin	RT:	8.83	6.61	9.16	8.20±1.39	16.9
	RI:	265	249	267	260±9.9	3.69
Chlorprothixen	RT:	21.74	20.01	23.08	21.61±1.54	7.12
	RI:	491	501	498	497±5.1	1.03
Clobazam	RT:	21.77	19.26	23.23	21.42±2.01	9.37
	RI:	492	484	503	493±9.5	1.93
Clomipramin	RT:	21.38	17.86	22.51	20.58±2.43	11.8
	RI:	483	497	488	489±7.1	1.45
Clonazepat	RT:	19.61	17.54	20.66	19.27±1.59	8.24
	RI:	445	446	450	447±2.6	0.59
Clopamid	RT:	14.76	12.57	15.41	14.25±1.49	10.4
	RI:	357	354	361	357±3.5	0.98
Cocain	RT:	13.45	11.56	14.35	13.12±1.42	10.9
	RI:	339	338	347	341±4.9	1.45
Caffein	RT:	9.37	7.20	9.92	8.83±1.44	16.3
	RI:	274	261	281	272±10	3.73
Codein	RT:	7.7	5.61	8.09	7.14±1.34	18.7
	RI:	244	230	248	241±9.5	3.93
Cyclopentabarbitol	RT:	15.91	13.90	16.78	15.53±1.48	9.5
	RI:	376	375	381	377±3.2	0.85
Diazepam	RT:	23.27	21.21	24.33	22.94±1.59	6.92
	RI:	534	538	531	534±3.5	6.57
Diazoxid	RT:	13.88	11.62	14.55	13.35±1.54	11.5
	RI:	346	339	350	345±5.6	1.61
Dibenzepin	RT:	14.38	12.46	15.29	14.04±1.44	10.3
	RI:	353	352	360	355±4.4	1.23
Diclofenac	RT:	25.72	23.72	27.15	25.53±1.72	6.75
	RI:	611	616	611	613±2.9	0.47
Dimethoat	RT:	13.19	10.99	13.90	12.69±1.52	12.0
	RI:	335	329	341	335±6.0	1.79

Table 6 (continued)

Substance		Retentions[a] (1)	(2)	(3)	M±SD	CV, %
Diphenhydramin	RT:	17.19	15.19	18.25	16.88±1.55	9.21
	RI:	395	396	402	398±3.8	0.95
Dipyridamol	RT:	16.66	14.61	17.48	16.25±1.48	9.10
	RI:	387	386	391	388±2.6	0.68
Doxepin	RT:	17.82	15.95	18.95	17.57±1.52	8.62
	RI:	406	410	416	411±5.0	1.23
Dosulepin	RT:	19.07	17.13	20.36	18.85±1.63	8.62
	RI:	433	436	444	438±5.7	1.30
Ethenzamid	RT:	14.56	12.59	15.41	14.19±1.45	10.2
	RI:	356	354	362	357±4.2	1.17
Fenbufen	RT:	22.67	20.55	24.00	22.41±1.74	7.77
	RI:	516	518	521	518±2.5	0.49
Flecainid	RT:	18.03	16.12	19.06	17.74±1.49	8.41
	RI:	410	414	418	414±4.0	0.97
Flunitrazepam	RT:	20.72	18.72	21.74	20.39±1.54	7.53
	RI:	468	472	471	470±2.1	0.44
Fluphenazin	RT:	23.14	21.18	24.19	22.84±1.53	6.69
	RI:	530	537	527	531±5.1	0.97
Flurazepam	RT:	16.96	14.95	18.00	16.63±1.55	9.31
	RI:	391	392	398	394±3.8	0.96
Furosemid	RT:	17.96	16.07	19.09	17.71±1.53	8.62
	RI:	409	413	419	414±1.22	1.22
Glibenclamid	RT:	25.81	23.84	27.14	25.60±1.66	6.49
	RI:	615	621	611	616±5.0	0.82
Glipizid	RT:	20.39	18.22	21.57	20.06±1.70	8.47
	RI:	462	461	469	464±4.4	0.94
Gliquidon	RT:	28.81	26.64	30.43	28.63±1.40	6.64
	RI:	726	726	731	728±2.9	0.40
Heptabarbital	RT:	17.62	20.01	18.50	18.71±1.21	6.46
	RI:	401	402	407	403±3.2	0.80
Hydrochlorothiazid	RT:	9.18	7.10	9.80	8.69±1.41	16.3
	RI:	271	259	279	270±10.1	3.73
Ibuprofen	RT:	26.20	24.07	27.71	26.00±1.83	7.05
	RI:	628	629	630	629±1.0	0.16
Idobutal	RT:	17.08	14.87	17.95	16.63V1.59	9.55
	RI:	393	391	398	394±3.6	0.92

(continued)

Table 6 (continued)

Substance		Retentions[a] (1)	(2)	(3)	M±SD	CV, %
Imipramin	RT:	19.46	17.56	20.82	19.28±1.64	8.49
	RI:	442	446	453	447±5.6	1.25
Ketotifen	RT:	15.10	13.00	16.03	14.71±1.55	10.6
	RI:	363	361	370	365±4.7	1.30
Linuron	RT:	24.26	22.23	25.49	23.99±1.65	6.86
	RI:	565	570	563	566±3.6	0.64
Lorazepam	RT:	19.11	17.06	20.22	18.80±1.60	8.53
	RI:	434	435	441	437±3.8	0.87
Lormethazepam	RT:	21.46	19.29	22.66	21.14±1.71	8.08
	RI:	485	485	491	487±3.5	0.71
Meclozine	RT:	26.66	24.94	28.31	26.64±1.69	6.33
	RI:	645	662	650	652±8.7	1.34
Medazepam	RT:	17.57	15.73	18.76	13.35±1.53	8.80
	RI:	400	405	412	406±6.0	1.49
Mescalin	RT:	19.14	17.21	2-.31	18.89±1.57	8.29
	RI:	435	439	443	439±4.0	0.91
Metamizol	RT:	9.95	7.71	10.37	9.34±1.43	15.3
	RI:	285	271	289	282±9.5	3.36
Metoclopramid	RT:	11.38	9.47	12.15	11.00±1.38	12.5
	RI:	309	305	316	310±5.6	1.80
Metronidazol	RT:	7.30	5.14	7.66	6.70±1.36	20.3
	RI:	237	220	241	233±11.2	4.79
Mianserin	RT:	17.28	15.35	18.50	17.04±1.59	9.32
	RI:	396	398	405	400±4.7	1.18
Midazolam	RT:	16.85	14.88	17.94	16.56±1.55	9.37
	RI:	389	390	398	392±4.9	1.26
Morphin	RT:	4.95	2.68	5.04	4.22±1.34	31.7
	RI:	191	132	189	171±33.5	19.6
Nadolol	RT:	9.28	7.12	9.73	8.71±1.40	16.0
	RI:	273	259	278	270±9.8	3.65
Nafopam	RT:	14.82	12.82	15.83	14.49±1.53	10.6
	RI:	360	358	368	362±5.3	1.46
Nalorphin	RT:	7.49	5.32	7.89	6.90±1.38	20.0
	RI:	240	224	244	236±10.6	4.48
Naproxen	RT:	20.72	19.81	22.84	21.12±1.55	7.36
	RI:	493	495	493	494±1.2	0.23

Table 6 (continued)

Substance		Retentions[a] (1)	(2)	(3)	M±SD	CV, %
Nifedipin	RT:	22.13	20.09	23.43	21.88±1.68	7.69
	RI:	500	503	506	503±3.0	0.60
Nitrazepam	RT:	18.86	16.75	19.94	18.52±1.62	8.76
	RT:	427	428	436	430±4.9	1.15
Nordiazepam	RT:	20.51	18.49	21.73	20.24±1.64	8.08
	RI:	464	467	471	464±3.5	0.75
Noscapin	RT:	14.70	12.68	15.63	14.34±1.51	10.5
	RI:	358	356	365	360±4.7	1.31
Opipramol	RT:	16.52	14.50	17.43	16.16±1.50	9.27
	RI:	3385	385	390	387±2.9	0.75
Orphenadrin	RT:	18.83	16.89	20.00	18.57±1.57	8.45
	RI:	427	431	437	432±5.0	1.17
Oxazepam	RT:	18.64	16.54	19.57	18.25±1.55	8.51
	RI:	423	424	428	425±2.6	0.62
Oxyphenbutazon	RT:	21.92	19.77	23.27	21.65±1.77	8.15
	RI	495	496	502	498±3.8	0.76
Papaverin	RT:	14.27	12.32	15.19	13.93±1.47	10.5
	RI:	351	350	359	353±4.9	1.40
Paracetamol	RT:	7.06	4.76	7.17	6.33±1.36	21.5
	RI:	233	213	232	226±11.3	4.99
Paraoxon	RT:	21.39	19.42	22.51	21.11±1.56	7.41
	RI:	483	488	486	486±2.5	0.52
Parathion	RT:	29.29	27.32	30.06	28.56±1.08	3.77
	RI:	747	754	711	759±15.7	2.07
Pemolin	RT:	9.88	7.65	10.12	9.22±1.36	14.8
	RI:	283	270	284	279±7.8	2.80
Pentazocin	RT:	15.03	12.94	15.86	14.61±1.50	10.3
	RI:	363	360	369	364±4.0	1.11
Pentobarbital	RT:	17.77	15.92	18.65	17.45±1.39	7.99
	RI:	405	405	411	407±3.5	0.85
Perphenazin	RT:	20.00	18.09	21.21	19.77±1.57	7.96
	RI:	453	458	460	457±3.6	0.79
Phenacetin	RT:	14.78	12.75	15.55	14.36±1.45	10.1
	RI:	358	357	364	360±3.8	1.05
Phenazon	RT:	11.69	9.64	12.33	11.22±1.41	12.5
	RI:	313	307	319	313±6.0	1.92

(continued)

Table 6 (continued)

Substance		Retentions[a] (1)	(2)	(3)	M±SD	CV, %
Phenylbutazon	RT:	26.96	24.86	28.67	26.83±1.91	7.11
	RI:	657	658	662	659±2.6	0.49
Pindolol	RT:	9.53	7.49	10.06	9.03±1.36	15.0
	RI:	277	267	283	276±9.1	2.93
Prazepam	RT:	27.09	25.04	28.68	26.94±1.82	6.77
	RI:	661	665	668	665±3.5	0.53
Procain	RT:	7.39	5.22	7.71	6.77±1.35	20.0
	RI:	239	221	241	234±11.0	4.71
Procainamid	RT:	5.34	2.92	5.41	4.56±1.42	31.1
	RI:	202	143	200	182±33.5	18.4
Propranolol	RT:	15.90	13.93	16.83	15.55±1.48	9.52
	RI:	376	376	381	378±2.9	0.76
Protriptylin	RT:	19.07	17.15	20.25	18.82±1.56	8.31
	RI:	432	437	441	437±4.5	1.03
Quinidin	RT:	11.70	8.74	12.26	10.90±1.89	17.4
	RI:	313	291	318	307±14.4	4.67
Quinin	RT:	11.12	9.08	11.66	10.62±1.36	12.8
	RI:	306	298	309	304±5.7	1.87
Reserpin	RT:	31.43	19.50	23.00	21.31±1.75	8.23
	RI:	484	489	496	490±6.0	1.23
Salicylamid	RT:	11.31	9.05	11.58	10.65±1.39	13.0
	RI:	307	297	309	304±6.5	2.11
Secbutabarbital	RT:	15.14	12.94	15.93	14.67±1.55	10.6
	RI:	367	360	369	365±4.7	1.29
Sulpirid	RT:	7.29	4.99	7.54	6.61±1.41	21.3
	RI:	237	217	238	231±11.8	5.14
Temazepam	RT:	20.68	18.61	21.82	20.37±1.63	7.99
	RI:	468	470	473	470±2.5	0.54
Theophyllin	RT:	7.77	5.61	8.19	7.19±1.38	19.3
	RI:	245	230	250	242±10.4	4.30
Thioridazin	RT:	23.27	21.56	24.67	23.17±1.56	6.72
	RI:	543	548	540	541±7.0	1.30
Tolbutamid	RT:	20.73	18.66	21.89	20.43±1.64	8.01
	RI:	469	471	474	471±2.5	0.53
Triazolam	RT:	20.27	18.38	21.39	20.01±1.52	7.60
	RI:	459	465	464	463±3.2	0.69

Table 6 (continued)

Substance		Retentions[a] (1)	(2)	(3)	M±SD	CV, %
Trichlormethiazin	RT:	15.80	13.97	16.99	15.59±1.52	9.76
	RI:	374	376	384	378±5.3	1.40
Trifluoperazin	RT:	22.77	21.02	24.30	22.78±1.52	6.67
	RI:	519	532	530	527±7.0	1.33
Triflupromazin	RT:	22.62	20.46	23.93	22.34±1.75	7.84
	RI:	514	515	520	516±3.2	0.62
Viloxazin	RT:	11.97	10.01	12.70	11.56±1.39	12.0
	RI:	317	313	324	318±5.6	1.75
Vinylbital	RT:	17.87	15.75	18.80	17.47±1.56	8.95
	RI:	406	406	413	408±4.0	0.99

[a] RT = Retention Times.
RI = Retention Indices.

retention indices, while others, such as ibuprofen, prazepam, gliquidon and fenbufen, etc., did not.

The retention times of gliquidon changed from 30.43 min under gradient No.3 to 28.81min under gradient No.1 and that of the corresponding 1-nitrohepthane from 29.76 min to 28.13 min, so the retention indices of gliquidon stayed almost the same, with values of 731 and 726.

On the other hand, under the same situation, the retention times of parathion varied from 30.06 min to 29.29 min, so the retention indices of parathion, under gradient No.3 and No.1, which were 711 and 747 respectively, could not balance the difference in their retention times.

Effect of the pH Values in Eluent

The effect of the pH-values in the TEAP-buffer on the retention times and retention indices was tested by changing the pH value in the eluent and keeping all other conditions mentioned in the experimental section constant. The pH values were changed by adding 1 M NaOH to the eluent, up to pH 4 or pH 5.

During gradient elution, the pH values should be increased with the increase of percentage of acetonitrile in the eluent on line. The changes of pH values during gradient elution are linear as shown in Figure 6.

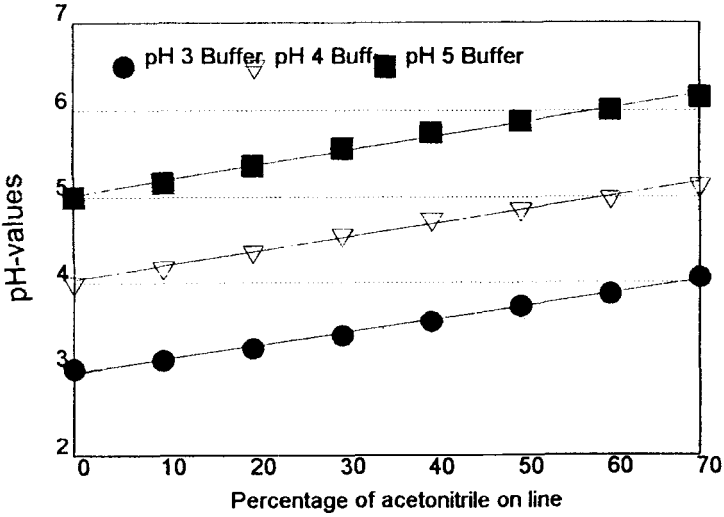


Figure 6. pH values of eluent during the gradient elution.

Table 7

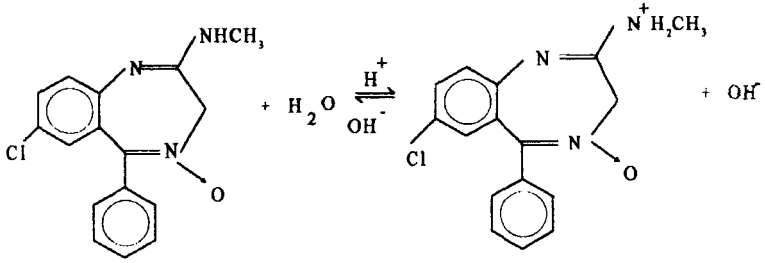
The Retention Times of 1-Nitroalkane under Three Different Elution Flow Rates

Homolog	Flowrate mL/min 1.0	1.1	0.9	Average Rt (min)	CV%
C 1	2.176	2.005	2.459	2.214±0.239	10.4
C 2	5.233	4.936	5.844	5.338±0.463	8.71
C 3	10.807	10.175	11.657	10.880±0.744	6.83
C 4	17.549	16.926	18.306	17.594±0.691	3.93
C 5	22.149	21.684	22.873	22.235±0.599	2.69
C 6	25.425	25.028	26.167	25.540±0.578	2.26
C 7	28.133	27.729	28.883	28.248±0.586	2.07
C 8	30.595	30.151	31.344	30.685±0.585	1.91
MW C 8 (n=20)	30.599	30.135	31.310		
SD C 8 (n=20)	0.015	0.057	0.044		
CV%	0.049	0.189	0.141		

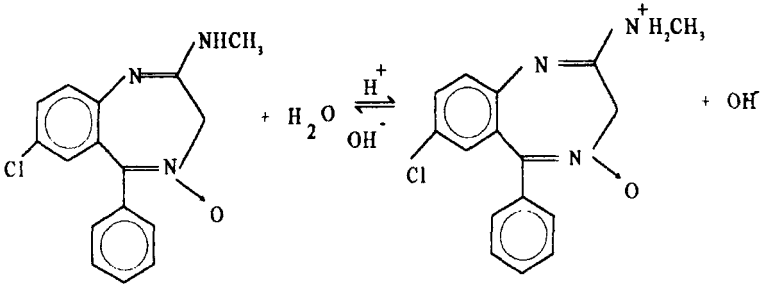
The retention times of the substances whose pK_a or pK_b values were in the range of the changes in pH values during the gradient elution, such as aspirin, chlordiazepoxide, quinine, etc., changed greatly when eluents with different pH values as in our test with pH 3, pH 4 or pH 5 were used. Under such conditions,

the retention indices of these substances could not stay the same because the retention times of the retention index scale used, 1-nitroalkane, changed only slightly.

The changes of the retention times of the substances mentioned above are related to their acid-base equilibria. For example, aspirin, with a pK_a value of 3.25, has the following acid-base equilibrium:



With the increase in the pH-values of the buffer used, the equilibria should move to the right in ion form. This may result in the decrease of the retention times of aspirin, because the ion form of aspirin is eluted more quickly than aspirin itself. On the other hand chlordiazepoxide has a different acid-base equilibrium:



and a pK_b value of 4.6. The ion form of chlordiazepoxide may be chromatographed more quickly too. With an increase in the pH of the buffer, the equilibrium of chlordiazepoxide should move to the neutral form, so the retention times of chlordiazepoxide should then increase.

Most retention times of the other substances tested have not been seriously affected by the changes in pH of the eluent. The retention times of over 100 selected substances, whose pK_a or pK_b values are not in the range between 3 and 6, had good reproducibility with an average CV% value of 2.09. But, the retention indices of the substances mentioned above had better reproduciblity with the CV% value of 1.57. The largest CV% value (22.5) expressed as retention times under the three pH values, decreased to 13.0% when the retentions under the same conditions were described as retention indices.

Effect of the Flow Rates

The aim of our test being to investigate the effect of the flowrate on the retention index, we chromatographed all the substances with flowrates of 0.9, 1.0 and 1.1 mL/min. Other conditions remained the same.

The retention times of 1-nitroalkane changed regularly and greatly under the three different flowrates. The results are given in Table 7. The retention times of the 115 tested substances also changed greatly under the same conditions. The average of the CV% values of the tested substances in retention times was 3.015, with a standard deviation of 0.817. Meanwhile, the average of the CV% values of the same substances is 1.143 with a standard deviation of 0.763.

It is well known that the flowrate of the eluent affects the retention times in HPLC. The greater the flowrate, the more quickly are the substances eluted. There is a simple relationship between the capacity factor k' and other chromatographic parameters:[25]

$k' = (\text{constant}) t_G * F/(\%B * Vm)$, where

%B = (%Acetonitrile at the beginning) – (%Acetonitrile at the end of the gradient elution);

F = Flowrate, in mL/min;

Vm = Volume of the column used;

t_G = gradient time.

There is a linear proportionality between flow rate and the capacity factor. Flow rate affects the retention times of 1-nitroalkane and the tested substances in a similar manner. That is why retention indices counteract the effect of flow rate on the reproducibility of the retention expression.

CONCLUSION

When the operating system, including pump, gradient profile, buffer, etc., is stable, the retentions, expressed both in terms of retention times and retention indices, in gradient HPLC, are well reproducible.

Retention index is only a method of linear correction. When the retention times of the analytes change proportional to that of the scale substances, the retention indices can well balance the variation from retention times. Some chromatographic conditions, such as column length, flow rate, gradient profile, etc., affect retention times greatly, meanwhile retention indices can decrease the effect and improve the reproducibility of the retention expression.

By contrast, some chromatographic conditions, for example, pH values of the buffer, etc., affect both retention times and retention indices of some substances because the retention times of the analytes and that of the retention index scale – 1-nitroalkane – do not change in the same way. However, the retention index method can somewhat improve the reproducibility of HPLC retention data.

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