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APPLICATION OF RETENTION INDICES BASED ON THE ALKYLARYLKETONE SCALE TO THE SEPARATION OF THE LOCAL ANAESTHETIC DRUGS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The separation of the local anaesthetic drugs by reversed-phase high-performance liquid chromatography using a Hypersil ODS column has been examined in order to identify critical factors in the establishment of a retention database for interlaboratory comparisons. An eluent of methanol–aqueous orthophosphoric acid (15:85, v/v) (pH 2.5) containing 0.7% hexylamine has been used. The effects of small changes in the eluent composition, pH, temperature and the use of different column packing materials on the retentions of the analytes have been studied. The use of capacity factors, relative capacity factors and retention indices based on the alkylarylketone scale have been compared.

INTRODUCTION

One of the major problems in the use of high-performance liquid chromatography (HPLC) for the identification of drugs has been the poor reproducibility of retentions measured in different laboratories. This has meant that it has not been possible to establish databases of retention values for qualitative analysis. Partly this is because retentions are very sensitive to the small differences in the composition and pH of the eluent and the temperature of the system that inevitably occur between laboratories. Different column packing materials, even if nominally identical, can cause other significant variations in retention arising from the differences in their selectivity and retentive power.

Studies of drug analyses have therefore been carried out to determine the critical factors affecting retention and to compare different methods of recording reten-

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tions. The work has been undertaken to evaluate the most suitable approaches for establishing identification databases for forensic analyses. Conventionally in HPLC, the retentions of analytes have been recorded as capacity factors (k') calculated as $k' = (t_R - t_0)/t_0$. However, these values are very dependent on the values determined experimentally for the column void volume (t_0). It has been shown that different values can be obtained for the same column depending on the method used for the measurement[1] and despite considerable interest in this area there is no agreement on a preferred method.

In gas–liquid chromatography (GLC) a considerable degree of standardisation of retention was achieved by the use of the Kováts retention indices, which compared the retentions of analytes relative to the *n*-alkanes[2]. These standards cannot be easily used in HPLC but in 1979 Baker and Ma[3] proposed the alkan-2-ones as the basis of a retention index scale. However, these compounds have only a weak absorbance and as an alternative Smith[4] suggested that the readily available alkylarylketones could be used. It has been shown that by recording retention values as retention indices based on this alkylarylketones scale, many of the variations can be greatly reduced compared to results expressed as capacity factors[4,5]. The retention values are also largely independent of the exact value determined for the void volume[6]. Based on this technique the selectivities of eluent/column material combinations could be compared by the determination of the retention indices for a set of neutral column test compounds[6].

Initial studies on the separation of barbiturates using methanol–phosphate buffer (pH 8.5) found that within one laboratory the retention indices of both the selectivity test compounds and the barbiturates were virtually constant with significant changes in the proportion of organic modifier in the eluent and with temperature[7] and could to some extent compensate for differences in column materials[8]. However, under these conditions the barbiturates were partially ionised and their retentions were susceptible to small changes in pH. An interlaboratory collaborative study based on a single batch of column material, showed that relative capacity factors compared to a standard barbiturate gave more consistent results than retention indices. This probably arises because the close structural similarities of the barbiturates would compensate for any changes in ionisation[9].

In order to examine a more diverse set of drugs and to determine if relative capacity factors and retention indices could also be used in more complex eluent systems, a detailed examination of a HPLC system for the separation of local anaesthetics[10] has been conducted. In this system *n*-hexylamine is added to the mobile phase to control peak shapes. The effects of changes in the eluent composition and column packing materials on the retention values expressed using different techniques are reported in this paper.

EXPERIMENTAL

Chemicals

Local anaesthetics chloroprocaine, lignocaine, cocaine, amylocaine and benzocaine were reference compounds from the drug collection of the Central Research Establishment, Home Office Forensic Science Service. Methanol (HPLC grade) and phosphoric acid (analytical reagent grade) were from Fisons Scientific Apparatus

(Loughborough, U.K.). *n*-Hexylamine was laboratory grade from BDH (Poole, U.K.).

Alkylarylketones (acetophenone, propiophenone and butyrophenone) and column test compounds (toluene, *p*-cresol, nitrobenzene and 2-phenylethanol) were laboratory reagent grade from a number of manufacturers.

HPLC separation

HPLC separations were carried out using a Pye-Unicam PU4010 pump and Altex 153 fixed-wavelength detector set at 254 nm. Samples (10 μl) were injected using a Rheodyne 7125 valve onto a Hypersil ODS column (100 × 5 mm I.D.), which was thermostated in a water jacket at 30°C. The eluent was prepared from methanol (300 ml), water (700 ml), 1.0% (v/v) aqueous orthophosphoric acid (1000 ml) and *n*-hexylamine (14 ml, 10.7 g) and had a pH 2.5. The column void volume was determined using an injection of methanol and retention times were recorded using a Hewlett Packard 3390 integrator.

RESULTS AND DISCUSSION

The conditions chosen for the analysis of the local anaesthetics [methanol–water–1% (v/v) aqueous phosphoric acid–*n*-hexylamine (30:70:100:1.4, v/v) at pH 2.5] enabled most of the principal local anaesthetic compounds of interest to be eluted within a reasonable time with symmetrical peak shapes (k' less than 21)[10]. Under these conditions, most of the analytes will be fully ionised but the alkylarylketone retention standards and column test compounds will be neutral.

The potential advantage of relative retention methods such as retention indices is that they are much less susceptible than direct retention measurements to small changes in elution conditions, such as might occur between different laboratories, particularily those which alter overall elution times. It was therefore of interest to test the effect of changing the composition, pH and temperature of the eluent on the retention values of the local anaesthetics. In order to be able to monitor changes in the selectivity of the system the column test compounds, toluene, 2-phenylethanol, nitrobenzene and *p*-cresol were also examined[6]. The same conditions have also been used in a parallel study to test the effects of these changes on the separation of a set of N-alkylanilines as typical weak bases[11]. Despite earlier reports that aniline showed marked differences in its silanophilic interactions with different column materials the results from these controlled changes, if expressed as retention indices, were highly reproducible.

One major problem in the comparison of results from literature reports is that the value of the void volume, and hence the capacity factors, is dependent on the technique used for its measurement[1]. The methods frequently differ between laboratories and are often not stated. In this study this has been standardised by using a sample of methanol. Although previous studies based on retention indices have used a solution of sodium nitrate, the elution volume of this salt in the present system was found to be larger than that of some samples. Further independence has been achieved in the present study by expressing the retentions as relative values which have been shown earlier to be less susceptible to small differences in the value used for the void volume[6].

The reproducibility of the method was tested using the standard analytical conditions at 30°C by determining the capacity factors and retention indices of the local anaesthetics and test compounds for three replicate analyses carried out on different days (Table I). The results showed only a small variation and the intra-laboratory repeatability was comparable to that obtained previously for the barbiturate analyses[7]. However, the capacity factors of the local anaesthetics were significantly different from those reported previously using the same column material and eluent system at ambient temperature (*i.e.* chloroprocaine, $k' = 0.24$; lignocaine, $k' = 0.79$; cocaine, $k' = 2.68$; amylocaine, $k' = 7.19$; benzocaine, $k' = 20.06$)[10]. This emphasises the difficulty of using the direct measurement of capacity factors as the possible basis of a database for identification purposes.

TABLE I

REPEATABILITY OF CAPACITY FACTORS AND RETENTION INDICES OF LOCAL ANAESTHETICS AND COLUMN TEST COMPOUNDS

Based on three assays carried out on different days. Eluent methanol–water–1% (v/v) aqueous orthophosphoric acid–*n*-hexylamine (30:70:100:1.4, v/v).

	Capacity factor		*Retention index*	
	Mean	*S.D.*	*Mean*	*S.D.*
Chloroprocaine	0.55	0.06	461	8.62
Lignocaine	1.15	0.07	530	4.51
Cocaine	3.48	0.27	630	6.11
Amylocaine	8.68	0.72	714	6.03
Benzocaine	25.39	0.77	812	3.51
Acetophenone	22.44	0.96	800	–
Propiophenone	66.70	2.57	900	–
Butyrophenone	201.11	8.36	1000	–
2-Phenylethanol	15.84	0.65	769	3.21
Nitrobenzene	20.93	0.59	794	3.06
p-Cresol	21.31	0.17	796	2.52
Toluene	84.76	1.36	921	4.16
Void volume (min)	0.59	0.01		

Effect of eluent composition

On changing methanol composition, temperature, or pH of the eluent there were large changes in the capacity factors of all the local anaesthetics but with the exception of benzocaine the retention indices were virtually unaffected (Tables II–IV). The consistency of the results is particularly good when it is realised that the values of many of the drugs represent a considerable extrapolation from the most rapidly eluted retention index standard (acetophenone, index value 800). Unlike the other local anaesthetic drugs, which have pK_a values in the range 7.70–9.0 (ref. 12), and are therefore fully protonated under the chromatographic conditions, benzocaine has a pK_a of 2.5 and would be expected to be only partially ionised in the eluent. The degree of ionisation and hence its retention would thus be expected to be susceptible to small changes in the pH of the eluent and column temperature. The retention indices of the column test compounds were very constant over these changes

TABLE II

EFFECT OF METHANOL CONCENTRATION ON THE CAPACITY FACTORS AND RETENTION INDICES OF LOCAL ANAESTHETIC DRUGS AND COLUMN TEST COMPOUNDS

Eluent: methanol–water–1.0% (v/v) aqueous orthophosphoric acid–*n*-hexylamine in the ratios 20:80:100:1.4 (v/v) ("10% methanol"), 30:70:100:1.4 (v/v) ("15% methanol") or 40:60:100:1.4 (v/v) ("20% methanol") adjusted to pH 2.50 in each case.

Compound	*Capacity factor*			*Retention index*		
	Methanol (%)			*Methanol (%)*		
	10	*15*	*20*	*10*	*15*	*20*
Chloroprocaine	1.07	0.55	0.38	496	461	443
Lignocaine	1.64	1.15	0.94	534	530	530
Cocaine	6.20	3.48	2.48	651	630	623
Amylocaine	15.04	8.68	6.07	730	714	710
Benzocaine	39.50	25.39	15.66	815	812	801
2-Phenylethanol	21.61	15.84	11.09	762	769	767
Nitrobenzene	27.84	20.93	15.85	784	794	802
p-Cresol	29.13	21.31	14.99	788	796	796
Toluene	103.54	84.76	64.95	900	921	938
Void volume (min)	0.56	0.58	0.54			

confirming that the selectivity of the separation system for neutral compounds was effectively unchanged.

Over the limited range of separation conditions used in the study the capacity factors showed the expected relationship of log k' with the proportion of methanol

TABLE III

EFFECT OF ELUENT pH ON THE CAPACITY FACTORS AND RETENTION INDICES OF LOCAL ANAESTHETIC DRUGS AND COLUMN TEST COMPOUNDS

Eluent: methanol–water–1.0% (v/v) aqueous orthophosphoric acid–*n*-hexylamine (30:70:100:1.4, v/v).

Compound	*Capacity factor*			*Retention index*		
	Eluent pH			*Eluent pH*		
	2.0	*2.5*	*3.0*	*2.0*	*2.5*	*3.0*
Chloroprocaine	0.57	0.55	0.69	463	461	484
Lignocaine	1.23	1.15	1.38	533	530	548
Cocaine	3.85	3.48	4.05	638	630	646
Amylocaine	9.39	8.68	10.21	720	714	730
Benzocaine	11.81	25.39	29.94	741	812	829
2-Phenylethanol	15.54	15.84	14.96	766	769	765
Nitrobenzene	20.95	20.93	20.43	793	794	794
p-Cresol	20.19	21.31	20.37	790	796	793
Toluene	82.67	84.76	82.46	919	921	921
Void volume (min)	0.56	0.58	0.56			

TABLE IV

EFFECT OF COLUMN TEMPERATURE ON THE CAPACITY FACTORS AND RETENTION INDICES OF LOCAL ANAESTHETIC DRUGS AND COLUMN TEST COMPOUNDS

Eluent as in Table III.

Compound	*Capacity factor*			*Retention index*		
	Temperature (°C)			*Temperature (°C)*		
	10	*30*	*40*	*10*	*30*	*40*
Chloroprocaine	1.03	0.55	0.42	486	461	449
Lignocaine	1.45	1.15	1.01	517	530	531
Cocaine	5.67	3.48	2.75	640	630	625
Amylocaine	13.88	8.68	6.70	720	714	708
Benzocaine	34.21	25.39	21.07	801	812	814
2-Phenylethanol	21.03	15.84	13.11	758	769	770
Nitrobenzene	28.72	20.93	17.74	786	794	798
p-Cresol	32.97	21.31	17.28	798	796	796
Toluene	106.81	84.76	72.21	904	921	929
Void volume (min)	0.58	0.58	0.57			

or with the reciprocal absolute temperature. Increasing the temperature also increased the column efficiency.

In the study on the barbiturate analysis, relative capacity factors compared to a standard barbiturate were even more reproducible than retention indices. Therefore the relative capacity factors compared to amylocaine (rel. $k' = 100$) for the local anaesthetics were calculated (Tables V–VII). As expected, the value for benzocaine showed a marked dependance on pH. The relative capacity factors of the other local anaesthetics, particularily of lignocaine, markedly changed with both temperature and percentage of methanol in the eluent. When the retentions reported in the previous study[10] were used to calculate relative capacity factors the values were signifi-

TABLE V

EFFECT OF METHANOL CONCENTRATION ON THE RELATIVE CAPACITY FACTORS OF LOCAL ANAESTHETIC DRUGS

Eluents as in Table II.

Compound	*Relative capacity factor*		
	Methanol (%)		
	10	*15*	*20*
Chloroprocaine	7.1	6.3	6.3
Lignocaine	10.9	13.2	15.5
Cocaine	41.2	40.1	40.9
Amylocaine	100	100	100
Benzocaine	263	293	258

TABLE VI

EFFECT OF ELUENT pH ON THE RELATIVE CAPACITY FACTORS OF LOCAL ANAESTHETIC DRUGS

Eluent as in Table III.

Compound	*Relative capacity factor*		
	Eluent pH		
	2.0	*2.5*	*3.0*
Chloroprocaine	6.1	6.3	6.7
Lignocaine	13.1	13.2	13.5
Cocaine	41.0	40.1	39.7
Amylocaine	100	100	100
Benzocaine	126	292	293

cantly different from the present separations particularily for the earlier eluted samples (*i.e.* relative capacity factors chloroprocaine, 3.4; lignocaine, 11.0; cocaine, 32.3, benzocaine, 280.4). In both cases the void volume was measured using an injection of methanol. However, the previous data was recorded at ambient temperature. From the present results if therefore appears that running the analyses at a specified temperature will be an important requirement to obtain reproducible interlaboratory results for this separation method.

Concentration of n-hexylamine

The absence of *n*-hexylamine from the mobile phase lead to an increase in the capacity factors of both the local anaesthetics and test compounds. Even though the drugs would be fully protonated they were strongly retained presumably by an ion-exchange interaction with the acidic silanol groups on the surface of the Hypersil ODS. The peak shapes of the bases without the amine modifier were also very poor

TABLE VII

EFFECT OF COLUMN TEMPERATURE ON THE RELATIVE CAPACITY FACTORS OF LOCAL ANAESTHETIC DRUGS

Eluent as in Table III.

Compound	*Relative capacity factor*		
	Temperature (°C)		
	10	*30*	*40*
Chloroprocaine	7.4	6.3	6.3
Lignocaine	10.4	13.2	15.1
Cocaine	40.8	40.1	41.0
Amylocaine	100	100	100
Benzocaine	246	292	314

TABLE VIII

EFFECT OF THE ADDITION OF *n*-HEXYLAMINE TO THE ELUENT ON CAPACITY FACTORS AND RETENTION INDICES OF LOCAL ANAESTHETIC DRUGS AND COLUMN TEST COMPOUNDS

Eluents: methanol–water–1.0% (v/v) aqueous orthophosphoric acid (30:70:100, v/v) and methanol–water–1.0% (v/v) aqueous orthophosphoric acid–*n*-hexylamine in the ratios 30:70:100:0.7 (v/v) ("0.35% *n*-hexylamine"), 30:70:100:1.4 (v/v) ("0.7% *n*-hexylamine"), or 30:70:100:2.8 (v/v) ("1.4% *n*-hexylamine") adjusted in each case to pH 2.50.

Compound	*Capacity factor*				*Retention index*			
	n-Hexylamine (%)				*n-Hexylamine (%)*			
	0.0	*0.35*	*0.7*	*1.4*	*0.0*	*0.35*	*0.7*	*1.4*
Chloroprocaine	9.02	0.89	0.55	0.35	684	508	461	437
Lignocaine	11.36	1.56	1.15	0.88	704	559	530	523
Cocaine	39.25	5.19	3.48	2.15	815	668	630	605
Amylocaine	69.62	11.91	8.68	5.94	866	744	714	700
Benzocaine	34.94	21.70	25.39	16.76	804	799	812	796
2-Phenylethanol	21.74	14.98	15.84	12.90	762	765	769	772
Nitrobenzene	26.94	20.75	20.93	18.96	781	795	794	807
p-Cresol	24.04	19.37	21.31	19.66	771	789	796	811
Toluene	102.38	83.26	84.76	82.06	900	921	921	944
Void volume (min)	0.52	0.57	0.58	0.54				

with extensive tailing (asymmetry ratios of 8–15 at 10% of peak height), whereas the neutral column test compounds gave symmetrical peaks.

On the addition of small amounts of *n*-hexylamine the peak shapes of the drugs rapidly improved (asymmetry ratios 1–4) and the retention times of all the analytes decreased. For the neutral compounds, whose peak shapes were unaltered, the shortening of the retentions probably arose from the increasing polarity of the mobile phase. The bases showed larger changes as can be seen by the decrease in their retention index values (Table VIII). The active sites on the silica surface are

TABLE IX

EFFECT OF *n*-HEXYLAMINE CONCENTRATION ON THE RELATIVE CAPACITY FACTORS OF LOCAL ANAESTHETIC DRUGS

Eluents as in Table VIII.

Compound	*Relative capacity factor*			
	n-Hexylamine (%)			
	0.0	*0.35*	*0.7*	*1.4*
Chloroprocaine	13.0	7.4	6.3	5.9
Lignocaine	16.3	13.1	13.2	14.8
Cocaine	56.8	43.6	40.1	36.2
Amylocaine	100	100	100	100
Benzocaine	57.4	182.2	292	282

presumably being neutralised by interaction with the *n*-alkylamine. Further addition of *n*-hexylamine above a minimum value had only a limited effect; both the capacity factors and retention indices seemed to stablise. Benzocaine behaved in a very similar manner to the neutral test compounds and its retention indices were virtually independent of the *n*-hexylamine concentration suggesting that at pH 2.5 it was almost completely unprotonated. The relative capacity factors of the local anaesthetics were also very variable (Table IX). Throughout this study large changes in the phosphoric acid concentration were needed to maintain the pH so some changes might have been caused by the changes in ionic strength. Thus as long as a minimum percentage of *n*-hexylamine is present small changes in the level would have little effect on the retention indices and this is not likely to be an important factor in their use for interlaboratory reproducibility comparisons.

Different column materials

A major problem in HPLC is the poor reproducibility of the retentive power and selectivity of column materials even if nominally identical. To test the consistency of the column packing material three different batches of Hypersil ODS were compared. The capacity factors were virtually identical and the range was comparable to the repeatability study on a single column. The relative standard deviations (R.S.D.) of the capacity factors ranged from 2–5% except for chloroprocaine (R.S.D. = 11.79%) whose retentions are so short that measurement errors are significant and toluene (R.S.D. = 7.1%) which has previously been found to be very sensitive to intercolumn differences[6]. The retention indices were similarily reproducible with standard deviations of 1–5 units (chloroprocaine 10 units).

However, if three different column packing materials were compared, Hypersil ODS, Partisil ODS, and Zorbax ODS the capacity factors showed very large varia-

TABLE X

EFFECT OF DIFFERENT STATIONARY PHASES ON THE CAPACITY FACTORS AND RETENTION INDICES OF LOCAL ANAESTHETICS AND COLUMN TEST COMPOUNDS

Eluent as in Table III. Abbreviations: H-ODS = Hypersil ODS (5 μm); P-ODS = Partisil ODS (10 μm); Z-ODS = Zorbax ODS (5 μm).

Compound	*Capacity factor*			*Retention index*		
	Column			*Column*		
	H-ODS	*P-ODS*	*Z-ODS*	*H-ODS*	*P-ODS*	*Z-ODS*
Chloroprocaine	0.55	2.13	2.59	461	654	487
Lignocaine	1.15	1.50	2.62	530	609	488
Cocaine	3.48	6.41	13.20	630	795	643
Amylcocaine	8.68	5.36	18.48	714	772	675
Benzocaine	25.39	7.10	53.00	812	808	776
2-Phenylethanol	15.84	3.46	19.12	769	716	678
Nitrobenzene	20.93	5.34	42.06	794	771	754
p-Cresol	21.31	3.46	22.06	796	716	692
Toluene	84.76	9.81	159.76	921	850	881
Void volume (min)	0.58	0.68	0.52			

TABLE XI

EFFECT OF DIFFERENT STATIONARY PHASES ON THE RELATIVE CAPACITY FACTORS OF LOCAL ANAESTHETIC DRUGS

Eluent as in Table III. Abbreviations as in Table X.

Compound	*Relative capacity factor*		
	H-ODS	*P-ODS*	*Z-ODS*
Chloroprocaine	6.3	39.7	14.0
Lignocaine	13.2	28.0	14.2
Cocaine	40.1	119.6	71.4
Amylocaine	100	100	100
Benzocaine	292	132	287

tions (Tables X and XI) with nearly a five-fold difference between some of the values. If the results were expressed as retention indices these changes were much smaller but still significant. The selectivities of the columns judged by the column test compounds were also very different and even with the limited number of local anaesthetics examined the elution order was different on the three column materials. The fully protonated analytes were particularly affected on the Partisil ODS column whereas the values on the other columns were similar. Benzocaine was more independent of the column material and behaved much more like the neutral test compounds presumably again reflecting its smaller pK_a and suggesting that under the assay conditions it is only partially, if at all, protonated. For any identification system it is therefore essential that the column packing material be specified and if possible the same batch be used in each laboratory.

CONCLUSIONS

Retention indices would be a better basis for the establishment of a database for the identification of the local anaesthetic drugs than capacity factors or relative capacity factors as they are apparently less sensitive to small changes in conditions which might be expected in setting up systems in different laboratories or on different occasions. Within the limited range of compounds studied only benzocaine could cause problems but other related compounds with similar pK_a values close to the pH of the eluent might also be less reproducible.

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