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RETENTION REPRODUCIBILITY OF BASIC DRUGS IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY ON A SILICA COLUMN WITH A METHANOL–AMMONIUM NITRATE ELUENT

INTERLABORATORY COLLABORATIVE STUDY

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SUMMARY

The reproducibility of the separation of basic drugs on silica columns has been tested in a collaborative study between nine laboratories. The results from the different laboratories were very similar. Various methods of recording the retention properties of the drugs were compared with reference to their reproducibility and ability to discriminate between different compounds. Relative retention times, relative capacity factors, and corrected capacity factors were much better than retention times and capacity factors, although all the methods suffered from large errors with weakly retained compounds. All the work was carried out using a single batch of silica to avoid variations due to the stationary phase.

INTRODUCTION

Despite the widespread use of high-performance liquid chromatography (HPLC) as a method for quantitative analysis, its application to the identification of unknown compounds has been very limited. Partly this arises from the poor reproducibility of retention properties between different laboratories, even when using the same type of column packing material and following the same eluent recipe. Small but significant differences occur when different equipment is used causing variations in the flow-rates and dead volumes, while small differences in the operating temperature and/or eluent composition can also have an effect. The method for measuring the column void volume can also be important when retention properties are recorded as capacity factors (k'), the most widely used method of reporting results.

These problems have meant that it has not been possible to collect sets of retention data, such as those available for thin-layer chromatography (TLC)[1,2] and

gas–liquid chromatography[2,3], which can be transferred between laboratories and used for the identification of an unknown sample. This led us to undertake a series of studies to investigate the significant factors which influence the reproducibility of retention in HPLC and to develop robust methods to record retention values. Work has concentrated on drug separations of forensic interest, including barbiturates[4–6], local anaesthetics[7] and thiazide diuretics[8] using reversed-phase chromatography on octadecyl-bonded silica (ODS silica). The studies on the barbiturate HPLC system concluded with an interlaboratory collaborative study, which confirmed that with care highly reproducible results could be obtained[6]. If a relative method of recording retention was used (*e.g.* relative capacity factors or retention indices) rather than capacity factors, the results were sufficiently robust for reliable interlaboratory comparisons.

These studies have recently been extended to the separation of basic drugs on silica columns using a methanol–ammonium nitrate eluent; the effect of changing the eluent composition, operating temperature and the stationary phase have been studied[9,10]. In this case the proportion of methanol in the eluent and the temperature were important but the largest effects were caused by different silica columns. Major changes occurred if different commercial brands of silica were used but significant differences were also found with different batches of the same brand.

Having identified those factors which can limit the reproducibility of this HPLC method, the present work reports a collaborative study of the reproducibility of retention values measured in different laboratories, all using the same batch of packing material. So far, very few interlaboratory studies of this type have been carried out for HPLC analyses. Apart from our earlier work with barbiturates[6], the only other detailed study was designed to investigate the robustness of retention indices as a method of recording the retentions of various drugs on ODS silicas[11].

EXPERIMENTAL

Collaborating laboratories

The analyses were carried out in seven operational forensic science laboratories at Aldermaston, Belfast, Birmingham, Chepstow, Chorley, Huntingdon and Wetherby, and at the Central Research Establishment of the Home Office Forensic Science Service and in the Chemistry Department, Loughborough University of Technology.

Test solutions

The drugs came from the reference collection of the Central Research Establishment, Home Office Forensic Science Service. Eight test solutions (A–H) were prepared, each containing a drug mixture, including protriptyline as an internal standard, dissolved in ethanol–water (90:10). A ninth solution (I) consisted of sodium nitrate in methanol–water (90:10) for void volume determination. The concentrations or the drugs were chosen so that all the compounds gave similar peak heights at a given detector sensitivity. The components in solutions A and I were declared to the collaborating laboratories whilst they were only told the total number of drugs in each of the other solutions (B–H) and that each contained protriptyline.

The detailed compositions are given below (concentrations mg ml^{-1} in ethanol–water, 90:10):

(A) Caffeine, 0.05; imipramine hydrochloride, 0.08; morphine hydrochloride, 1.04; methylamphetamine hydrochloride, 3.44; protriptyline, 0.26.

(B) Cocaine hydrochloride, 0.41; phentermine, 2.36; ephedrine, 3.38; protriptyline, 0.19.

(C) Diazepam, 0.04; propranolol, 0.37; nortriptyline hydrochloride, 0.15; protriptyline, 0.24.

(D) Amitriptyline hydrochloride, 0.09; prolintane hydrochloride, 2.48; phenylephrine bitartrate, 1.08; protriptyline, 0.23.

(E) Nitrazepam, 0.02; chlorpromazine hydrochloride, 0.03; pipazethate, 0.22; protriptyline, 0.28.

(F) Dextropropoxyphene, 1.51; amphetamine sulphate, 2.28; pholcodine, 1.61; protriptyline, 0.2.

(G) Papaverine, 0.04; dipipanone hydrochloride, 0.81; codeine phosphate, 3.2; methdilazine hydrochloride, 0.06; protriptyline 0.22.

(H) Procaine hydrochloride, 0.03; promazine hydrochloride, 0.04; ethoheptazine citrate, 7.34; protriptyline, 0.41; strychnine, 0.14.

(I) Sodium nitrate, 30 mg ml^{-1} in methanol–water (90:10).

The stability of the test solutions on storage was tested to ensure that no changes would occur on distribution to the collaborating laboratories. Only chlorpromazine showed any decomposition when it was exposed to light. The laboratories were therefore asked to store all solutions in the dark before analysis.

Procedure

Each laboratory was asked to use routine HPLC equipment and to work at ambient temperature or under thermostat control, in line with their normal practice. Samples were injected using a valve injector with a 5-μl loop and peaks were detected at 254 nm. In all cases a new column (25 cm × 4.5–4.9 mm I.D.) was prepared for the study using Spherisorb S5W (Batch No. 2752, Phase Separations, Queensferry, U.K.). Each laboratory used its usual slurry-packing method.

The laboratories were instructed to prepare the eluent by mixing HPLC-grade methanol (2700 ml) with an aqueous ammonium nitrate buffer (300 ml). This buffer was prepared by mixing analytical-grade ammonium nitrate (27 g), 0.880 sp. gr. concentrated ammonia (90 ml) and distilled water (900 ml). The mobile phase was pumped at 2 ml min^{-1}.

Once the HPLC system had equilibrated, 5-μl samples of the test solutions A–I were injected in turn into the column. The sequence was completed by a second injection of solution A. The retention time for all the peaks were recorded in seconds either from a chart recorder (at a chart speed of at least 40 mm min^{-1}) or electronically using an integrator. The raw data were then collected for analysis.

Calculation of retention parameters

The retention time results from each laboratory were used to calculate a series of parameters to describe the retention properties of the basic drugs on the HPLC system.

Capacity factors (k') were determined as $k' = (t_R - t_0)/t_0$, in which t_R is the retention time of the analyte and t_0 is the retention time of sodium nitrate (test solution I).

The relative retention times and relative capacity factors were calculated for each drug as t_R/t_P and k'/k'_P in which t_P and k'_P are the retention time and capacity factor for the protriptyline internal standard in the same test solution. Relative capacity factors can also be called relative adjusted retention times as $k'/k'_P = (t_R - t_0)/(t_P - t_0)$.

The corrected capacity factors were calculated by the assignment of reference capacity factors to the five components of test solution A: caffeine, 0.099; imipramine, 0.605; morphine, 0.967; methylamphetamine, 1.539; and protriptyline, 1.947. These reference values were obtained by repeated intralaboratory determinations, carried out previously on the same batch of packing material at 30°C[9,10]. For the results from each laboratory the mean experimental retention times of the components of solution A, from the two injections, were plotted against the reference capacity factors. A linear plot was obtained in each case and the best straight line was determined using a least-squares correlation. This correlation equation was then used to calculate corrected capacity factors for the compounds in solutions B–H using their experimental retention times.

RESULTS AND DISCUSSION

All the collaborating laboratories in the study were familiar with the HPLC method for the analysis of basic drugs on silica with an ammonium nitrate eluent. As part of a rationalisation of HPLC methods within U.K. forensic science laboratories, all separations involving silica columns are carried out on Spherisorb S5W and, at the time of this study, all the laboratories were using a common batch of this packing material.

Most of the laboratories had no problems with the detection of the samples. However, two laboratories (in particular one using a diode-array spectrometer) found that the most rapidly eluted drugs, with adjusted retention times ($t_R - t_0$) less than 30 s (*e.g.* diazepam, nitrazepam, papaverine and caffeine) could not be positively distinguished from the baseline disturbance at the solvent front. The results for these compounds were omitted from the statistical analysis, even though peaks were present which corresponded to the expected components. It appeared that the geometry of the flow-cells in the detectors used by these laboratories may be responsible for causing the problem, since intense "refractive index" peaks were observed.

The pH of the prepared eluent was checked in each laboratory and varied from 9.3 to 9.5, which compared well with the standard value of 9.39 seen in the intralaboratory work[9,10]. In the preliminary studies, the preparation of the aqueous buffer from ammonium nitrate and ammonia had been found to give a very reproducible pH value which was largely independent of the volume and strength of the ammonia solution, and of the ammonium nitrate mass[9]. The column temperature used for the analyses ranged from 19 to 33°C (Table I). Some laboratories reported changes of up to 4°C during the analysis. Only two laboratories used thermostated systems (laboratories 4 and 8 in Table I).

Retention times

The retention times of the basic drugs in the present study showed coefficients of variation (C.V.) in the range 5.6–8.4% (Table I). Such relatively large variations

are not unexpected in view of the columns used by the collaborating laboratories, which had internal diameters of 4.5–4.9 mm. A similar range of internal diameters was encountered in the previous interlaboratory study, involving the barbiturates[6], but in that case much larger variations (9.1–19.5%) were observed for the retention times on a 10-cm ODS silica column. Also, in the barbiturate study it was noted that the C.V. values tended to increasse with increasing retention; no similar trend was observed with the present data.

Capacity factors

One of the supposed advantages of reporting retentions as capacity factors is that they should compensate for any differences in column internal diameters and eluent flow-rates. In order to determine the capacity factor of an analyte it is necessary to know the column void volume. There is, however, no agreed method for this determination and the various proposed methods can often give different values with the same HPLC system[12]. In the present study, aqueous sodium nitrate was used as the void volume marker. Although it has been shown that solutions with different salt concentrations may give different values for the void volume, reproducible values will be obtained if the concentration is fixed, as in the present work. The use of sodium nitrate has the advantage that is is readily detected using an ultraviolet detector, unlike some alternative markers, *e.g.* deuterated solvents.

The capacity factors for the results from each laboratory were calculated (Table II) using the retention times of sodium nitrate (test solution I) as the marker for the column void volume. The capacity factors for the five compounds in solution A (two injections from each laboratory) were then used to calculate the repeatability (r) and reproducibility (R) in accordance with the standard procedure for collaborative studies (Table III)[13]. The repeatability gives a measure of precision for intralaboratory results and the figures suggest that the within-laboratory variations were acceptable over the period of the experimental work with no significant drifts in retention. In contrast, the reproducibility data indicate far greater interlaboratory variability. The C.V. values in Table II indicate that rapidly eluted peaks ($k' < 0.5$) exhibit particularly large differences. Clearly, the identification of such early-eluted compounds from their capacity factors is highly unreliable. The capacity factors of the remaining compounds showed C.V. values ranging from 7.1 to 12.0%, and such large values indicate the difficulties of using capacity factors in retention databases. As with retention times, the C.V. values for capacity factors showed no clear trends with increasing retention.

Relative retention times and capacity factors

In previous studies it has been found that relative methods of recording retentions are less susceptible to variation than absolute methods. For each laboratory, the relative retention times and relative capacity factors of the drugs in each injection were calculated using protriptyline as the internal standard. The means and standard deviations of the results from each method for the drugs in solutions A–H are given in Table IV. Again, the early-eluted compounds showed relatively large variations using either of the two relative parameters. This was particularly true for relative capacity factors, where the calculations involved the column void volume measurement. For later-eluted peaks, the data in Table IV indicate a general decrease in C.V.

TABLE I

RETENTION TIMES OF BASIC DRUGS MEASURED IN NINE LABORATORIES IN A COLLABORATIVE STUDY

Listed in order of injection.

Test solution	*Compound*	*Retention time (s)*											
		Laboratory									*Mean*	*S.D.*	*C.V. (%)*
		1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*			
A	Caffeine	102	–*	95	119	98	102	104	101	97	102	7	6.9
	Imipramine	147	146	135	167	144	149	159	148	144	149	9	6.0
	Morphine	185	178	169	206	178	187	201	181	179	185	12	6.5
	Methylamphetamine	241	236	213	259	230	241	272	242	234	241	17	7.1
	Protriptyline	280	278	243	290	268	269	314	294	270	278	20	7.2
B	Cocaine	103	–*	95	119	100	105	107	103	101	104	7	6.7
	Phentermine	150	149	135	167	145	150	163	151	148	151	9	6.0
	Ephedrine	224	217	199	239	215	222	247	232	219	224	14	6.3
	Protriptyline	280	277	243	290	268	270	314	295	273	279	20	7.2
C	Diazepam	94	–*	–*	110	89	96	96	94	90	96	7	7.3
	Propranolol	133	133	121	150	128	134	143	136	129	134	8	6.0
	Nortriptyline	206	202	182	220	198	203	226	214	200	206	13	6.3
	Protriptyline	280	277	243	289	266	270	314	295	271	278	20	7.2
D	Amitriptyline	128	127	118	145	124	130	135	128	124	129	8	6.6
	Prolintane	174	176	157	197	170	182	206	178	173	179	15	8.4
	Phenylephrine	214	206	194	230	208	216	231	224	207	215	12	5.6
	Protriptyline	280	277	243	289	265	271	314	295	270	278	20	7.2
E	Nitrazepam	94	–*	–*	109	89	97	96	94	92	96	6	6.3
	Chlorpromazine	131	133	122	149	127	134	140	132	129	133	8	6.0
	Pipazethate	186	186	171	208	184	192	221	192	189	192	14	7.3
	Protriptyline	280	279	243	289	264	271	314	295	276	279	20	7.2

F	Dextropropoxyphene	100	–*	92	117	97	102	105	100	97	101	7	7.0
	Amphetamine	158	158	142	174	151	158	172	161	155	159	10	6.3
	Pholcodine	209	202	191	228	197	211	235	203	205	209	13	6.2
	Protriptyline	280	279	243	289	263	270	313	295	276	279	20	7.2
G	Papaverine	96	–*	–*	112	92	98	98	97	93	98	7	7.1
	Dipipanone	129	134	116	148	128	134	147	134	130	133	10	7.5
	Codeine	178	173	163	198	170	180	195	174	174	178	11	6.2
	Methdilazine	214	202	196	236	208	220	241	219	214	216	15	6.9
	Protriptyline	280	276	243	289	264	271	313	295	278	279	20	7.2
H	Procaine	108	–*	101	124	103	110	113	107	102	109	7	6.4
	Promazine	160	158	148	179	156	163	174	162	158	162	9	5.6
	Ethoheptazine	203	198	185	223	194	208	228	203	201	205	13	6.3
	Protriptyline	280	277	244	288	263	270	314	295	278	279	20	7.2
	Strychnine	344	334	310	368	325	351	409	336	346	347	28	8.1
I	Sodium nitrate	94	94	88	108	88	95	95	91	89	94	6	6.4
A	Caffeine	102	–*	95	117	97	103	104	101	97	102	7	6.9
	Imipramine	147	147	135	166	143	150	160	149	144	149	9	6.0
	Morphine	186	180	169	205	175	187	202	182	182	185	11	6.2
	Methylamphetamine	241	236	213	258	226	240	274	244	242	241	17	7.1
	Protriptyline	281	276	243	288	262	269	315	296	279	279	21	7.5
Separation conditions													
Temperature (°C)		23	19–20	23–25	33	26–30	23–24	23	25	19–23			
Eluent pH		9.4	9.5	9.3	9.6	9.5	9.4	9.5	9.4	9.3			
Column I.D. (mm)		4.6	4.6	4.5	4.9	4.6	4.5	4.5	4.5	4.5			

* Peak masked by solvent front disturbance.

TABLE II

CAPACITY FACTORS OF BASIC DRUGS, MEASURED IN NINE LABORATORIES IN A COLLABORATIVE STUDY

Listed in order of injection.

Test solution	*Compound*	*Capacity factors*											
		Laboratory									*Mean*	*S.D.*	*C.V. (%)*
		1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*			
A	Caffeine	0.09	–*	0.08	0.10	0.11	0.07	0.09	0.11	0.09	0.09	0.01	11.1
	Imipramine	0.56	0.56	0.53	0.55	0.63	0.57	0.67	0.63	0.62	0.59	0.05	8.5
	Morphine	0.97	0.90	0.92	0.90	1.01	0.97	1.12	0.99	1.01	0.98	0.07	7.1
	Methylamphetamine	1.56	1.52	1.42	1.40	1.61	1.54	1.86	1.65	1.63	1.58	0.14	8.9
	Protriptyline	1.98	1.97	1.76	1.69	2.04	1.83	2.30	2.22	2.03	1.98	0.20	10.1
B	Cocaine	0.10	–*	0.08	0.10	0.13	0.11	0.12	0.13	0.13	0.11	0.02	18.2
	Phentermine	0.60	0.59	0.53	0.55	0.65	0.58	0.71	0.66	0.66	0.61	0.06	9.8
	Ephedrine	1.38	1.31	1.26	1.21	1.44	1.34	1.59	1.54	1.46	1.39	0.13	9.3
	Protriptyline	1.98	1.96	1.76	1.69	2.03	1.84	2.30	2.23	2.07	1.98	0.20	10.1
C	Diazepam	0.00	–*	–*	0.01	0.02	0.01	0.01	0.03	0.01	0.01	0.01	100.0
	Propranolol	0.41	0.42	0.38	0.38	0.46	0.41	0.50	0.49	0.45	0.43	0.04	9.3
	Nortriptyline	1.19	1.15	1.07	1.04	1.24	1.14	1.38	1.35	1.24	1.20	0.12	10.0
	Protriptyline	1.98	1.96	1.76	1.68	2.02	1.84	2.30	2.23	2.04	1.98	0.20	10.1
D	Amitriptyline	0.36	0.35	0.34	0.34	0.40	0.37	0.42	0.40	0.39	0.38	0.03	7.9
	Prolintane	0.85	0.88	0.78	0.82	0.93	0.92	1.17	0.95	0.95	0.92	0.11	12.0
	Phenylephrine	1.28	1.21	1.20	1.13	1.36	1.27	1.43	1.46	1.32	1.30	0.11	8.5
	Protriptyline	1.98	1.97	1.76	1.68	2.01	1.85	2.30	2.23	2.03	1.98	0.20	10.1

E	Nitrazepam	0.00	−*	−*	0.01	0.01	0.02	0.01	0.03	0.03	0.02	0.01	50.0
	Chlorpromazine	0.39	0.42	0.39	0.38	0.44	0.41	0.47	0.45	0.45	0.42	0.03	7.1
	Pipazethate	0.98	0.99	0.94	1.93	1.08	1.02	1.32	1.11	1.12	1.05	0.12	11.4
	Protriptyline	1.98	1.98	1.76	1.67	1.99	1.85	2.30	2.23	2.10	1.99	0.21	10.6
F	Dextropropoxyphene	0.06	−*	0.05	0.08	0.10	0.07	0.10	0.09	0.09	0.08	0.02	25.0
	Amphetamine	0.68	0.69	0.61	0.61	0.71	0.66	0.81	0.76	0.74	0.70	0.07	10.0
	Pholcodine	1.22	1.15	1.17	1.11	1.24	1.22	1.47	1.22	1.30	1.23	0.10	8.1
	Protriptyline	1.98	1.98	1.76	1.68	1.99	1.84	2.29	2.23	2.10	1.98	0.20	10.1
G	Papaverine	0.02	−*	−*	0.04	0.05	0.03	0.03	0.06	0.05	0.04	0.01	25.0
	Dipipanone	0.37	0.43	0.32	0.37	0.45	0.41	0.55	0.47	0.46	0.43	0.07	16.3
	Codeine	0.89	0.85	0.85	0.83	0.93	0.89	1.05	0.91	0.96	0.91	0.07	7.7
	Methdilazine	1.28	1.15	1.23	1.18	1.35	1.32	1.54	1.40	1.40	1.32	0.12	9.1
	Protriptyline	1.98	1.95	1.76	1.67	1.99	1.85	2.29	2.23	2.11	1.98	0.21	10.6
H	Procaine	0.15	−*	0.15	0.15	0.17	0.16	0.19	0.17	0.15	0.16	0.02	12.5
	Promazine	0.70	0.69	0.68	0.66	0.77	0.72	0.83	0.78	0.77	0.73	0.06	8.2
	Ethoheptazine	1.16	1.12	1.10	1.06	1.20	1.19	1.39	1.22	1.26	1.19	0.10	8.4
	Protriptyline	1.98	1.96	1.77	1.67	1.98	1.84	2.30	2.24	2.11	1.98	0.21	10.6
	Strychnine	2.66	2.57	2.52	2.41	2.69	2.69	3.30	2.69	2.88	2.71	0.26	9.6
A	Caffeine	0.09	−*	0.08	0.08	0.10	0.08	0.09	0.11	0.09	0.09	0.01	11.1
	Imipramine	0.56	0.57	0.53	0.53	0.63	0.58	0.68	0.64	0.62	0.59	0.05	8.5
	Morphine	0.98	0.92	0.92	0.89	0.99	0.97	1.12	0.99	1.04	0.98	0.07	7.1
	Methylamphetamine	1.56	1.53	1.42	1.38	1.56	1.53	1.88	1.67	1.72	1.58	0.15	9.5
	Protriptyline	1.99	1.95	1.76	1.67	1.97	1.83	2.32	2.25	2.13	1.99	0.22	11.0

* Peak masked by solvent front disturbance.

TABLE III

REPRODUCIBILITY AND REPEATABILITY OF CAPACITY FACTORS FOR FIVE REFERENCE COMPOUNDS IN TEST SOLUTION A

Calculated as described in ref. 13, based on nine laboratories and two replicate injections of each test solution.

Compound	*Repeatability (r)*	*Reproducibility (R)*
Caffeine	0.017	0.030
Imipramine	0.019	0.149
Morphine	0.029	0.196
Methylamphetamine	0.073	0.412
Protriptyline	0.087	0.592

with increasing retention when using relative retention times; a similar trend with relative capacity factors is less clear.

Corrected capacity factors

When using TLC for the tentative identification of drugs, reference compounds are usually run on the plate alongside the unknown compounds[1]. These standards are assigned reference R_F values, enabling the experimental R_F values of the unknown to be "corrected" in order to improve the reproducibility of the measurement for comparison with R_F databases. In our previous collaborative study with barbiturates[6], an analogous approach was successfully adopted to correct capacity factors in HPLC, and this method has been applied to the present data.

The five drugs in test solution A (caffeine, imipramine, morphine, methylamphetamine and protriptyline) were designated as the reference compounds with capacity factors 0.099, 0.605, 0.967, 1.539 and 1.947, respectively, based on the earlier intralaboratory studies[9,10]. For each laboratory, a graphical approach was adopted to relate the experimental retention times and reference capacity factors for these standard drugs, thus allowing the corrected capacity factors of all the other drugs to be determined. The means and standard deviations of the corrected capacity factors for the drugs in solutions B–H are given in Table IV. Because they are used as the reference compounds, no values are included in this table for the compounds in solution A and for protriptyline in the other solutions.

As with all the other methods examined, compounds showing low retentions gave high C.V. values. Nevertheless later-eluted compounds show relatively good reproducibility. The major disadvantage of the corrected capacity factor method is that it requires the injection of a standard mixture as well as the unknown sample and that the data handling and calculation is more time-consuming.

Comparison of methods for reporting retention

In the previous collaborative study on retention measurement in HPLC the need to compare the reproducibility of widely different methods of reporting retention led to the development of discrimination numbers (DN)[6]. This concept considers the number of compounds which can be discriminated when using particular methods

TABLE IV

MEANS, STANDARD DEVIATIONS AND COEFFICIENTS OF VARIATION OF RELATIVE RETENTION TIMES, RELATIVE CAPACITY FACTORS AND CORRECTED CAPACITY FACTORS OF BASIC DRUGS, DETERMINED IN THE COLLABORATIVE STUDY

Test solution	*Compound*	*Relative retention time* (× 100)*			*Relative capacity factor* (× 100)*			*Corrected capacity factor*		
		Mean	*S.D.*	*C.V. (%)*	*Mean*	*S.D.*	*C.V. (%)*	*Mean*	*S.D.*	*C.V. (%)*
A	Caffeine	36.8	2.5	6.8	4.6	0.6	13.0			
	Imipramine	53.5	2.3	4.3	29.9	1.5	5.0			
	Morphine	66.5	3.0	4.5	49.5	3.1	6.3			
	Methylamphetamine	86.6	2.2	2.6	79.8	2.9	3.6			
B	Cocaine	37.4	2.3	6.1	5.6	0.7	12.5	0.121	0.025	20.7
	Phentermine	54.2	1.9	3.5	30.9	1.0	3.2	0.614	0.017	2.8
	Ephedrine	80.3	1.6	2.0	70.4	1.7	2.4	1.371	0.020	1.5
C	Diazepam	33.8	2.4	7.1	0.6	0.5	83.3	0.027	0.027	100
	Propranolol	48.3	1.9	3.9	22.0	0.6	2.7	0.441	0.025	5.7
	Nortriptyline	74.0	1.3	1.8	60.7	0.9	1.5	1.183	0.021	1.8
D	Amitriptyline	46.3	2.3	5.0	19.0	1.0	5.3	0.384	0.014	3.6
	Prolintane	64.4	2.4	3.8	46.3	2.9	6.3	0.907	0.045	5.0
	Phenylephrine	77.2	2.4	3.1	65.6	2.7	4.1	1.277	0.044	3.4
E	Nitrazepam	33.8	2.4	7.1	0.8	0.5	62.5	0.030	0.029	96.7
	Chlorpromazine	47.8	2.4	5.0	21.3	1.0	4.7	0.430	0.020	4.6
	Pipazethate	68.9	2.3	3.3	53.1	2.9	5.5	1.040	0.038	3.7
F	Dextropropoxyphene	36.3	2.3	6.3	4.0	0.7	17.5	0.090	0.025	2.8
	Amphetamine	57.0	1.8	3.2	35.2	0.8	2.3	0.696	0.019	2.7
	Pholcodine	75.3	3.1	4.1	62.8	3.6	5.7	1.226	0.048	3.9
G	Papaverine	34.6	2.4	6.9	1.9	0.6	31.6	0.052	0.027	51.9
	Dipipanone	47.9	1.8	3.8	21.4	1.8	8.4	0.430	0.045	10.5
	Codeine	64.1	2.9	4.5	45.9	2.8	6.1	0.901	0.028	3.2
	Methdilazine	77.8	3.0	3.9	66.5	3.9	5.9	1.297	0.053	4.1
H	Procaine	39.0	2.6	6.7	8.1	0.6	7.4	0.169	0.017	10.1
	Promazine	58.2	2.5	4.3	37.1	1.8	4.9	0.731	0.012	1.7
	Ethoheptazine	73.5	2.8	3.8	60.1	3.1	5.2	1.174	0.034	2.9
	Strychnine	124.5	5.2	4.2	137.1	8.2	6.0	2.651	0.127	4.8

* Measurements relative to protriptyline.

for reporting retentions. The discrimination number is calculated as the number of time windows, representing the uncertainties in recording the retention values, each two standard deviations wide, which can be fitted into a defined chromatographic range. It therefore represents, in each case, the theoretical maximum number of compounds that could be positively identified within the elution range.

In the present work, the DN values were calculated over the range $k' = 0.5$–5.0, corresponding to the useful chromatographic region for the present HPLC system (Table V). The retention properties of compounds eluted with $k' < 0.5$ are highly

TABLE V

DISCRIMINATION NUMBERS (DN) FOR DIFFERENT METHODS OF RECORDING THE RETENTIONS OF BASIC DRUGS

Method	*Range**	*DN***
Retention times	141 –564 s	11
Capacity factors	0.5 – 5.0	15
Relative retention times	0.5 – 2.02	22
Relative capacity factors	0.25– 2.51	27
Corrected capacity factors	0.5 – 5.0	41

* An arbitrary range of $k' = 0.5$–5.0 or its equivalent when considering alternative methods for recording retention.

** Calculated by the method described in ref. 6.

irreproducible, irrespective of the method of recording, and thus were excluded from the DN calculations. Equivalent ranges, corresponding to $k' = 0.5$–5.0, were used for the other methods of recording retentions (see Table V). The data used for the calculations were taken from Tables I, II and IV, and ignore those drugs with retentions outside the defined ranges.

As expected, the experimental retention times (DN = 11) and capacity factors (DN = 15) both showed fairly poor discrimination. The relative retention times (DN = 22) and relative capacity factors (DN = 27) gave much better results, indicating that almost twice as many drugs could be distinguished. The best discriminations were obtained for the corrected capacity factors (DN = 41), but, as noted earlier, their determination is more laborious than the other methods.

CONCLUSIONS

With the present system, in which a silica HPLC column and an aqueous ammonium nitrate eluent were used, the most reproducible results were obtained when retentions were recorded as corrected capacity factors, involving a comparison with five reference compounds. Relative retention times and relative capacity factors were less effective for reducing interlaboratory variability. In these cases, the retention of the unknown compound was compared with a single reference compound. Capacity factors were worse still, involving a comparison with an unretained compound whose retention is notoriously unreliable. The results indicate that such a comparison is scarcely better than no comparison at all, *i.e.* the use of retention times. All the methods applied were unable to achieve reproducible results for compounds weakly retained on the HPLC column.

Overall, the results demonstrate that good interlaboratory reproducibility can be achieved for retained compounds when common batches of packing material are used and the eluent recipe is carefully specified. Nevertheless, it is clear that the method used for recording chromatographic retention in HPLC can have a significant effect on the interlaboratory reproducibility, and this point must be fully appreciated when setting up databases for identification purposes. The general principle emerging from the results is that the reproducibility of a retention measurement

method increases in line with the number of reference compounds used. Similar observations were made with the previous collaborative study involving a reversed-phase HPLC system for barbiturates[6]. In that work, all the drugs examined were of a similar chemical class with both the reference compounds and the "unknowns" often belonging to a homologous series. This contrasts with the present study, where the chemical structures of the basic drugs were very different. In such circumstances variations in the chromatographic conditions may not influence all compounds in the same way. Thus with this HPLC system it is perhaps more surprising that the use of several chemically dissimilar reference compounds is more effective than a single reference compound.

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