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

THE EFFECT OF THE MOBILE PHASE AND THE OPERATING CONDITIONS

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SUMMARY

The reproducibility of capacity factors and relative capacity factors are compared as methods for recording retentions for the high-performance liquid chromatography of basic drugs on a silica column with methanol–aqueous ammonium nitrate as the eluent. The effects of changing the column temperature and the ionic strength, pH and proportion of organic modifier in the eluent on the retentions and selectivity have been studied. The results suggest that the mobile phase and operating conditions must be closely defined in order to obtain results of adequate reproducibility to develop a data base of retention values for interlaboratory comparisons and/or for the identification of basic drugs.

INTRODUCTION

Within a given laboratory most high-performance liquid chromatography (HPLC) separations will give reproducible retention values for an analyte over a short period of time. However, if the same method is carried out on different equipment, on a different day, by a different operator or in a different laboratory, the retention properties will often vary. This means that the retentions of standards in one laboratory cannot be used directly for identification purposes in another laboratory. Poor inter- and intralaboratory reproducibility arises partly from small differences in operating conditions and mobile phase compositions and partly because even nominally equivalent column materials often have different retention and selectivity properties. Consequently, it has not been possible to compile HPLC retention values in the same way that libraries or databases of retention values have been

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developed for drugs of forensic interest in thin-layer chromatography[1,2] and gas–liquid chromatography[2,3]. Instead, each laboratory has to establish its own set of retention values.

A particular problem in HPLC is that the conventional method of recording retentions using capacity factors $k' = (t_R - t_0)/t_0$, is very dependent on the reproducible measurement of the column void volume (t_0). This value is usually based on the retention of an 'unretained" polar analyte or mobile phase component. A number of different methods have been reported but these often give different values even for the same column[4,5]. So far, there is no agreed standard method of measuring t_0 or even an agreement on the rigorous definition of column void volume.

As part of a series of studies aimed at improving the reporting of retention values for qualitative analyses in HPLC, particularly for drugs of forensic interest, the use of different methods of recording retentions have been examined for various reversed-phase separations (barbiturates[6–8], local anaesthetics[9], and thiazide diuretics[10]). These studies indicated that relative measurements either based on a retention index scale such as the alkyl aryl ketones[11], or relative capacity factors compared to an appropriate internal standard gave considerably more robust results than retention times or capacity factors in both intra- and interlaboratory studies. The present paper extends this work to the separation of basic drugs on silica column materials. Although many polar compounds can be separated using reversed-phase conditions, problems have been encountered for basic compounds as these can often interact with residual silanol groups on the surface of the silica, causing peak tailing and poor efficiency. As an alternative approach, separations of basic compounds on silica columns using eluents containing high proportions of methanol have been examined[12–14]. These studies have recently led to a detailed examination of methanol–perchloric acid mixtures as eluents for the separation of drugs[15] and their use with spectrophotometric and electrochemical detectors[16]. Methanol–ammonium nitrate eluents have also been applied to the separation of basic drugs including narcotic analgesics and amphetamine-related compounds[12,13,17] and this system will be used in the present work. For both types of eluent on silica columns, the mechanism of separation is complex but both ion-exchange and ion-pairing interactions are believed to occur.

The aim of the present study was to determine the robustness of retentions with changes in the eluent composition and the separation conditions in order to identify those factors which need to be rigorously defined to obtain reproducible interlaboratory results. In related papers the measurement of interlaboratory reproducibility for the method by a collaborative study has been reported[18] and the effects of using different commercial brands and batches of silica will be described[19].

EXPERIMENTAL

Chemicals and standards

Ammonium nitrate was analytical reagent grade, methanol was HPLC grade, and ammonia was spec. grav. 0.88 laboratory grade from FSA Laboratory Supplies, Loughborough, U.K. Samples of basic drugs were taken from the reference collection of the Central Research Establishment, Home Office Forensic Science Service.

Test solutions of basic drugs

Eight test solutions were made up as mixtures, each including protriptyline as an internal standard, in ethanol–water (90:10, v/v) with concentrations (0.02–8 mg/ml) chosen to give a similar detector response for each drug.

(A) Caffeine, imipramine hydrochloride, morphine hydrochloride, methylamphetamine hydrochloride, protriptyline; (B) cocaine hydrochloride, phentermine, ephedrine, protriptyline; (C) diazepam, propranolol, nortriptyline hydrochloride, protriptyline; (D) amitriptyline hydrochloride, prolintane hydrochloride, phenylephrine bitartrate, protriptyline; (E) nitrazepam, chlorpromazine hydrochloride, pipazethate, protriptyline; (F) dextropropoxyphene, amphetamine sulphate, pholcodine, protriptyline; (G) papaverine, dipipanone hydrochloride, codeine phosphate, methdilazine hydrochloride, protriptyline; (H) procaine hydrochloride, promazine hydrochloride, ethoheptazine citrate, protriptyline, strychnine.

HPLC eluents

The standard eluent was prepared by mixing methanol (2700 ml) with an aqueous ammonium nitrate buffer (300 ml) and had a measured pH of 9.39. The buffer (pH 10.1) was prepared by mixing 0.880 concentrated ammonia (90 ml), ammonium nitrate (27 g) and water (900 ml). For studies on the effect of pH on the separation, alternative aqueous buffer solutions were prepared: (i) ammonium nitrate (1.02 g), ammonia (9.0 ml) and water (90 ml), which gave an eluent pH of 9.95; (ii) ammonium nitrate (2.7 g), ammonia (8.0 ml) and water (91 ml), which gave an eluent pH of 9.58; (iii) ammonium nitrate (5.0 g), ammonia (8.0 ml) and water (91 ml) adjusted with concentrated hydrochloric acid to give an eluent pH of 9.03.

HPLC separations

HPLC separations were carried out using a Pye Unicam 4020 pump and an Altex 153 fixed wavelength detector (254 nm). The samples (5 μl) were injected using a 7125 Rheodyne valve onto a Shandon column (25 cm × 5 mm I.D.) packed with Spherisorb S5W (5 μm: batch 2752, Phase Separations, Queensferry, U.K.). The eluent was passed through a pre-column, which was installed between the pump and the injection valve, packed with silica. The pre-column and the analytical column were maintained at 30°C in a circulating water bath. The eluent consisting of methanol–aqueous ammonium nitrate (9:1, v/v) was pumped at 2 ml/min. The retention times were determined using a Hewlett Packard 3390 integrator. The column void volume (t_0) was determined using an injection of sodium nitrate (30.0 mg/ml) in methanol–water (9:1, v/v). At the end of each working day the column was flushed with methanol–water (9:1, v/v).

Calculations

All the retention times were measured in duplicate and the capacity factors were calculated as $k' = (t_R - t_0)/t_0$. Relative capacity factors were calculated as k'/k'_P where k'_P is the capacity factor for the protriptyline present as an internal standard in each test solution.

RESULTS AND DISCUSSION

Retention values

In previous studies on drug separations in HPLC, relative retention values, either relative capacity factors or retention indices based on the alkyl aryl ketones have been found to compensate for some of the changes in retentions caused by changing the elution conditions[13]. However, in this study the alkyl aryl ketones were virtually unretained and would therefore not span the retention times of the drugs. It was therefore planned to compare capacity factors and relative capacity factors calculated relative to protriptyline as an internal standard. This compound was selected because it has a relatively long retention time and its capacity factor should be unaffected by small inaccuracies in the determination of the column void volume.

Standardisation of the separation method

In previous HPLC methods it has been found preferable to prepare buffer solutions by weight and/or volume rather than by an adjustment with a pH meter which requires calibration[6]. In the present study the buffer was therefore prepared by adding a specified weight of ammonium nitrate (27 g) to concentrated ammonia solution (90 ml) and diluting the mixture before mixing with methanol. This method gave a very reproducible buffer pH of 10.1, which was effectively independent of small errors in the preparation. For example, changing the weight of ammonium nitrate from 10 g (pH 10.40) to 20 g (pH 10.20), 30 g (pH 10.05) to 35 g (pH 10.00) had only a small effect on the final buffer pH. Although the exact concentration of an ammonia solution is often unknown due to evaporation, changing the volume of concentrated ammonia from 80 to 100 ml only altered the pH of the buffer from 10.02 to 10.11 but would have altered the ionic strength.

A range of basic drugs of forensic interest was selected for the present study including narcotic analgesics, stimulants, sympathomimetic amines and tricyclic antidepressants (Table I). For most of the analytes, except codeine and strychnine, the peak shapes were symmetrical and the retention times were virtually independent of the volume of sample injected.

The intention of the present study was to determine the dependance of the capacity factors and relative capacity factors of the basic drugs on small changes in the experimental conditions such as might occur if the method was carried out by different operators or in different laboratories. The various experimental parameters were therefore altered in turn over a limited range.

Reproducibility of repeated separations

In order to ensure that any changes in retention are significant the chromatography of the basic drugs was repeated on four separate columns packed with the same batch of Spherisorb S5W packing material and the mean values, standard deviations (S.D.) and coefficients of variance (C.V.) for the capacity factors and relative capacity factors were calculated (Table II). The drugs are listed in order of elution. For all but the most rapidly eluted drugs, these values were very consistent and the mean values are used as the reference values in subsequent comparisons. The relative capacity factors compared to the protriptyline internal standard showed generally lower variances than the capacity factors confirming that they usually give a more

TABLE I

BASIC DRUGS USED IN THE STUDY AND THEIR IONISATION CONSTANTS

n/a = Not available.

Key	*Ionisation constant*[21]
Amitriptyline	9.4
Amphetamine	9.9
Caffeine	14.0
Chlorpromazine	9.3
Cocaine	8.6
Codeine	8.2
Dextropropoxyphene	6.3
Diazepam	3.3
Dipipanone	8.5
Ephedrine	9.6
Ethoheptazine	8.5
Imipramine	9.5
Methdilazine	7.5
Methylamphetamine	10.1
Morphine	8.0, 9.9
Nitrazepam	3.2, 10.8
Nortriptyline	9.7
Papaverine	6.4
Phentermine	10.1
Phenylephrine	8.9, 10.1
Pholcodine	8.0, 9.3
Pipazethate	n/a
Procaine	9.0
Prolintane	n/a
Promazine	9.4
Propranolol	9.5
Protriptyline	n/a
Strychnine	2.3, 8.0

robust measure of retention. This was also demonstrated in a collaborative study, in which nine laboratories used the same batch and make of silica with the ammonium nitrate eluent. The capacity factors of the drugs showed large variations (*e.g.* nortriptyline C.V. = 10.0%) but the relative capacity factors were much more reproducible (nortriptyline C.V. = 1.5%)[18].

The variations were similar to those reported for separations with the methanol–perchloric acid eluent using imipramine as an internal standard, *e.g.* nortriptyline (k' = 2.0, C.V. = 6.9%; relative k' = 0.58, C.V. = 4.7% and methdilazine (k' = 6.0, C.V. = 3.9%; relative k' = 1.35, C.V. = 1.0%)[16] and compare well with earlier reproducibility studies on reversed-phase separations[6]. The large C.V. values found for rapidly eluting compounds in the present study arise from the errors in the measurement of the column void volume and the short retention times of the drugs.

The order of elution of the basic drugs corresponded to that reported previously for this system[17] but the absolute values differed significantly, (*e.g.* pholcodine k' = 1.23, reported 1.63 and morphine 0.96, reported 1.30). These differences

TABLE II

REPRODUCIBILITY OF CAPACITY FACTORS AND RELATIVE CAPACITY FACTORS ON REPEATED CHROMATOGRAPHY OF BASIC DRUGS ON A SILICA COLUMN

Repeated separation on four different columns of Spherisorb S5W (Batch 2752). Eluent, methanol–aqueous ammonium nitrate 90:10; temperature, 30°C.

Compound	*Capacity factors*			*Relative capacity factors (× 100)**		
	Mean	*S.D.*	*C.V.*	*Mean*	*S.D.*	*C.V.*
Nitrazepam	0.02	0.01	50.0	1.3	0.3	23.1
Diazepam	0.02	0.01	50.0	1.3	0.3	23.1
Papaverine	0.06	0.01	16.7	2.6	0.5	19.2
Dextropropoxyphene	0.09	0.01	11.1	4.5	0.4	8.9
Caffeine	0.10	0.01	10.0	5.0	0.2	4.0
Cocaine	0.11	0.01	9.1	6.0	0.2	3.3
Procaine	0.17	0.01	5.9	8.8	0.1	1.1
Amitriptyline	0.39	0.01	2.6	19.9	0.1	0.5
Chlorpromazine	0.44	0.01	2.2	22.4	0.5	2.2
Propranolol	0.44	0.01	2.3	22.5	0.3	1.3
Dipipanone	0.45	0.01	2.2	22.9	0.5	2.2
Imipramine	0.60	0.02	3.3	31.1	0.5	3.4
Phentermine	0.61	0.02	3.3	31.4	0.4	1.3
Amphetamine	0.69	0.01	1.4	35.6	0.5	1.4
Promazine	0.75	0.02	2.7	38.5	0.8	2.1
Codeine	0.91	0.02	2.2	46.6	1.1	2.4
Prolintane	0.93	0.03	3.2	47.7	0.8	1.7
Morphine	0.96	0.02	2.1	49.7	1.1	2.2
Pipazethate	1.07	0.03	2.8	54.9	1.0	1.8
Nortriptyline	1.19	0.02	1.7	60.9	0.4	0.7
Ethoheptazine	1.19	0.03	2.5	61.1	1.4	2.3
Pholcodine	1.23	0.03	2.4	63.4	1.5	2.4
Phenylephrine	1.24	0.02	1.6	63.8	1.9	3.0
Methdilazine	1.32	0.03	2.3	67.9	1.2	1.8
Ephedrine	1.35	0.02	1.5	69.5	0.7	1.1
Methylamphetamine	1.54	0.03	1.9	79.1	1.1	1.4
Protriptyline**	1.94	0.03	1.5	100.0	–	–
Strychnine	2.71	0.05	1.8	139.5	2.6	1.9

* Relative capacity factors relative to protriptyline.
** Based on injection of test solution H.

emphasise the problem in interlaboratory comparisons as both were carried out on the same batch of packing material, but using different HPLC equipment.

Effect of the composition of the eluent

When the proportion of methanol was altered from 80 to 95%, in contrast to reversed-phase separations which are very susceptible to the proportion of organic modifier in the eluent, the capacity factors of many of the drugs varied by only a small amount (Table III) with the greatest effects occurring between 90 and 95% methanol. However, for many of the compounds the changes were not systematic

TABLE III

EFFECT OF THE PROPORTION OF METHANOL ON THE CAPACITY FACTORS AND RELATIVE CAPACITY FACTORS OF BASIC DRUGS ON A SILICA COLUMN

Column, Spherisorb S5W; temperature, 30°C; eluent, methanol–aqueous ammonium nitrate.

Compound	*Capacity factors*				*Relative capacity factors (× 100)**			
	% Methanol							
	80	*85*	*90***	*95*	*80*	*85*	*90***	*95*
Nitrazepam	0.02	0.03	0.02	0.03	1.5	1.9	1.3	1.6
Diazepam	0.01	0.04	0.02	0.03	1.0	2.3	1.3	2.0
Papaverine	0.03	0.05	0.06	0.07	1.9	3.1	2.6	4.0
Dextropropoxyphene	0.09	0.10	0.09	0.08	6.3	5.3	4.5	4.9
Caffeine	0.06	0.10	0.10	0.11	4.4	5.3	5.0	6.5
Cocaine	0.13	0.13	0.11	0.10	9.3	7.3	6.0	6.1
Procaine	0.20	0.20	0.17	0.16	14.6	11.0	8.8	9.3
Amitriptyline	0.43	0.40	0.39	0.35	31.1	22.6	19.9	21.2
Chlorpromazine	0.47	0.44	0.44	0.40	33.5	24.4	22.4	24.5
Propranolol	0.43	0.44	0.44	0.36	30.6	24.9	22.5	22.0
Dipipanone	0.53	0.49	0.45	0.30	37.9	27.2	22.9	17.7
Imipramine	0.68	0.63	0.60	0.55	49.0	35.1	31.1	33.3
Phentermine	0.56	0.62	0.61	0.47	40.5	34.9	31.4	28.5
Amphetamine	0.64	0.70	0.69	0.55	45.6	38.9	35.6	32.9
Promazine	0.80	0.76	0.75	0.70	57.3	42.0	38.5	42.3
Codeine	0.86	0.87	0.91	0.93	61.7	48.7	46.6	55.2
Prolintane	1.08	1.01	0.93	0.74	77.7	56.3	47.7	44.9
Morphine	0.95	0.94	0.96	0.97	68.4	52.3	49.7	58.5
Pipazethate	1.07	1.03	1.07	0.97	76.7	57.2	54.9	58.8
Nortriptyline	0.99	1.12	1.19	1.03	70.9	62.8	60.9	61.8
Ethoheptazine	1.30	1.24	1.19	1.11	93.7	68.6	61.1	66.7
Pholcodine	1.11	1.14	1.23	1.29	80.1	63.7	63.4	77.6
Phenylephrine	1.14	1.22	1.24	1.11	82.0	68.2	63.8	66.9
Methdilazine	1.39	1.32	1.32	1.26	100.0	73.9	67.0	75.4
Ephedrine	1.18	1.32	1.35	1.16	85.4	73.9	69.5	69.5
Methylamphetamine	1.41	1.57	1.54	1.28	101.4	87.8	79.1	77.2
Protriptyline	1.39	1.79	1.94	1.66	100.0	100.0	100.0	100.0
Strychnine	2.50	2.51	2.71	2.76	179.6	138.6	139.5	166.2

* Relative capacity factors compared to protriptyline.
** Standard conditions (values from Table II).

and some compounds even showed a maximum retention at an intermediate composition of 85 or 90% methanol (Fig. 1). These variations also caused changes in the order of elution of the drugs when compared with the standard conditions of 90% methanol, *e.g.* prolintane and morphine in 85% methanol and methdilazine and ephedrine in 80% methanol. It was noticable that groups of compounds with similar basic functional groups behaved in a similar manner *e.g.* the capacity factors of the tertiary amines, such as imipramine, amitriptyline and chlorpromazine, all decreased as the proportion of methanol increased while the fused ring cyclic amines, including codeine and morphine, showed little change or increased slightly over the same range.

Based on the measured capacity factors the relative capacity factors for the

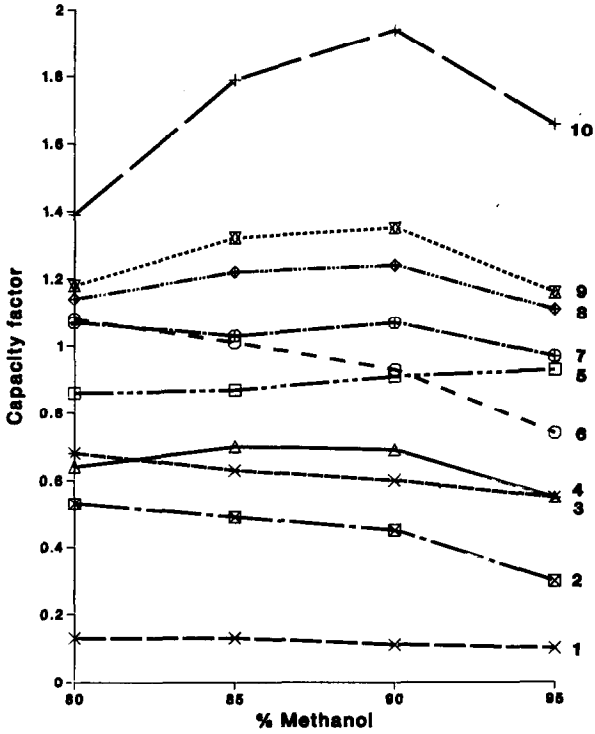

Fig. 1. Effect of proportion of methanol in a methanol–aqueous ammonium nitrate eluent on the capacity factors of basic drugs on a Spherisorb S5W column at 30°C. Drugs: 1 = cocaine; 2 = dipipanone; 3 = imipramine; 4 = amphetamine; 5 = codeine; 6 = prolintane; 7 = pipazethate; 8 = phenylephrine; 9 = ephedrine; 10 = protriptyline (internal standard).

compounds in each test solution were calculated by reference to the capacity factor of protriptyline, the internal standard (Table III). As might have been expected from the changes in elution order and the diverse behaviour of different drug groups the relative capacity factors showed considerable variation in many instances. Few compounds, even including those with basic groups similar to protriptyline, showed small variations. This suggests that in this HPLC system relative capacity measurements are unlikely to compensate for differences in the proportion of modifier in the eluent. The selectivity differences suggest that similar problems would always occur even if another drug were selected as the internal standard. Consequently it is important that the proportion of methanol used in any comparison study be carefully controlled.

Similar effects have been noted with a methanol–perchloric acid eluent on a silica column, in which the addition of increasing amounts of water to the methanolic eluent had little effect on the order of elution of related compounds such as the tricyclic antidepressants. However, if a wider range of compounds was examined many of the analytes had a minimum retention with 10% water[14,15].

Effect of eluent pH

If analytes in HPLC are partially ionised, small changes in the pH of the eluent can cause major changes in retention since the proportion of neutral and ionised forms will alter. Many of the basic drugs will therefore be particularly sensitive to the pH of the mobile phase and this may also cause changes in the selectivity of the separation. As noted earlier particular care was taken in the specification of the buffer to ensure that a reproducible eluent pH of 9.39 could be achieved which was not subject to small preparation errors. In order to examine the influence of pH on retention, the mobile phase was modified by making major changes to the buffer by the addition of acid to give an eluent pH of 9.03 or by altering the concentration of the salts and ammonia to give eluents with pH values of 9.58 and 9.95 (see Experimental). These changes are much greater than might be expected by deviations in the normal preparation procedure and would also affect the ionic strength of the eluent.

These modified eluents were then used for the separation of the basic drugs. The changes in the pH of the eluent caused marked changes in the capacity factors of the basic drugs (Table IV). Different groups of drugs showed different changes and three general types of behaviour were observed. Compounds with high pK_a values > 9.5, including the primary and secondary amines and typified by ephedrine, amphetamine, and protriptyline (Fig. 2) all showed an overall marked increase in capacity factor with increasing pH, although the pH 9.58 eluent gave a drop in capacity factor in each case. This irregularity could be caused by ionic strength differences between the eluents since these groups of bases showed a marked sensitivity to ionic strength (see later). The reduction in retention near pH 9.0 can be attributed to a reduction in the ionisation of the weakly acid silanol groups on the surface of the silica[15].

The second group of bases contained cyclic and dimethylamino-tertiary amines with pK_a values between 8.0 and 9.5. These compounds, typified by codeine and imipramine, showed small decreases in k' with increasing pH (Fig. 2). Compounds in the third group with low pK_a values less than 7.0, *e.g.* diazepam, are effectively unretained by the column and are rapidly eluted without noticable influence by pH. Because the retention properties of these drugs are so close to the column void volume, identification is difficult because of the large variations in the calculated capacity factors and this eluent system is probably unsuitable for this group of drugs.

There also appeared to be two anomalous compounds, dipipanone (pK_a 8.5), and prolintane, which might both be expected to be in the second group because of their tertiary amine structures. However, these drugs show changes which are more typical of the first group with small increases in capacity factors with increasing pH (Fig. 2). Unlike the other cyclic tertiary amines these compounds contain unsubstituted piperidine or pyrrolidine ring systems. As will be seen later these compounds, along with the structurally related tertiary amine pipazethate, behave anomalously during other changes in conditions. Unfortunately, pK_a values have not been reported for prolintane and pipazethate and the reported data for related model compounds with similar basic groups suggest a wide range of possible values.

The complex changes observed with pH suggest that, as with the methanol–perchloric acid eluent system, the separation mechanism is complex and depends on both the degree of ionisation of the analytes and of the silanol groups on the silica packing material[14,15]. Because of the complex changes in interaction which can occur

TABLE IV

EFFECT OF ELUENT pH AND IONIC STRENGTH ON THE CAPACITY FACTORS OF BASIC DRUGS ON A SILICA COLUMN

Column, Spherisorb S5W; temperature, 30°C; eluent, methanol–aqueous ammonium nitrate (90:10).

Compound	*Capacity factors*				*Capacity factors*		
	pH of eluent				*Relative ionic strength*		
	9.03	*9.39**	*9.58*	*9.95*	*0.5*	*1.0**	*2.0*
Nitrazepam	0.02	0.02	0.01	0.04	0.06	0.02	0.01
Diazepam	0.02	0.02	0.01	0.05	0.07	0.02	0.02
Papaverine	0.05	0.06	0.04	0.08	0.10	0.06	0.03
Dextropropoxyphene	0.10	0.09	0.08	0.10	0.20	0.09	0.03
Caffeine	0.09	0.10	0.09	0.13	0.15	0.10	0.06
Cocaine	0.14	0.11	0.11	0.13	0.25	0.11	0.03
Procaine	0.20	0.17	0.18	0.19	0.33	0.17	0.04
Amitriptyline	0.44	0.39	0.37	0.38	0.67	0.39	0.19
Chlorpromazine	0.50	0.44	0.41	0.43	0.73	0.44	0.23
Propranolol	0.34	0.44	0.40	0.46	0.75	0.44	0.22
Dipipanone	0.33	0.45	0.37	0.41	0.90	0.45	0.13
Imipramine	0.67	0.60	0.59	0.59	1.01	0.60	0.33
Phentermine	0.32	0.61	0.56	0.71	1.08	0.61	0.29
Amphetamine	0.40	0.69	0.64	0.78	1.17	0.69	0.35
Promazine	0.85	0.75	0.74	0.73	1.24	0.75	0.42
Codeine	1.04	0.91	0.90	0.91	1.35	0.91	0.61
Prolintane	0.77	0.93	0.86	0.92	1.65	0.93	0.45
Morphine	1.10	0.96	0.97	0.96	1.39	0.96	0.69
Pipazethate	1.15	1.07	1.02	1.00	1.94	1.07	0.53
Nortriptyline	0.74	1.19	1.10	1.29	1.94	1.19	0.64
Ethoheptazine	1.30	1.19	1.19	1.18	1.96	1.19	0.71
Pholcodine	1.42	1.23	1.22	1.21	1.87	1.23	0.81
Phenylephrine	0.89	1.24	1.20	1.37	1.82	1.24	0.84
Methdilazine	1.42	1.32	1.31	1.29	2.13	1.32	0.78
Ephedrine	0.84	1.35	1.28	1.52	2.15	1.35	0.80
Methylamphetamine	0.89	1.54	1.47	1.68	2.51	1.54	0.91
Protriptyline	0.91	1.94	1.77	2.35	3.20	1.94	1.03
Strychnine	3.05	2.71	2.63	2.55	4.24	2.71	1.64

* Standard conditions (values from Table II).

with pH, relative capacity factors compared to protriptyline can only be expected to compensate for changes in the retentions for drugs having primary or secondary amino groups and to show marked changes with other basic compounds.

Effect of the ionic strength of the eluent

Because it appears that the major mechanism causing retention is a cation-exchange interaction with the silanol groups on the surface of the silica, the ionic strength of the eluent is expected to have a major effect. In the separations with methanol–perchloric acid it was found that increasing the ionic strength of the eluent caused a decrease in retention times and that for structurally related compounds

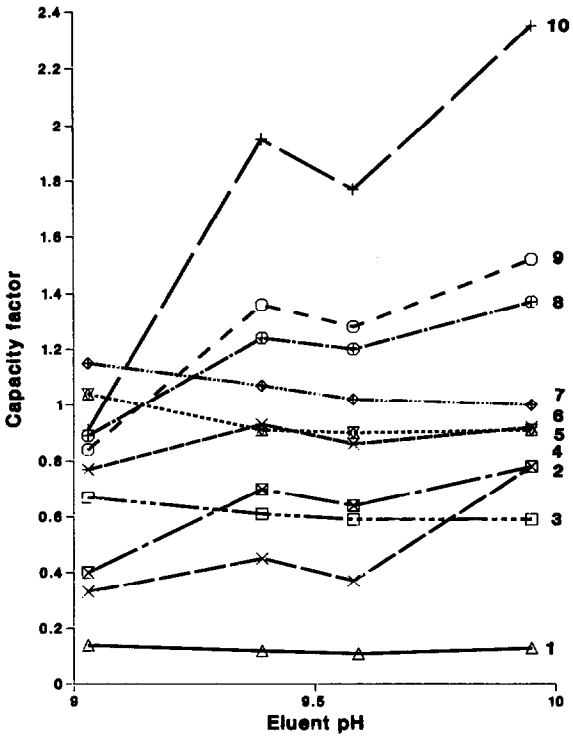

Fig. 2. Effect of eluent pH on the capacity factors of basic drugs. Conditions and drugs as in Fig. 1.

there was a linear relationship between the log k' and the log(ionic strength)[15,20]. Nevertheless, compounds with different pK_a values (and hence showing different degrees of ionisation) were susceptible to different extents.

In the present study the ionic strength of the eluent was altered by doubling and halving the concentration of the aqueous ammonium nitrate solution. These changes had a negligible effect on the pH of the eluent. With increasing ionic strength the capacity factors of all the analytes decreased markedly (Table IV) and the order of elution changed (*e.g.* morphine and prolintane). However, it is difficult to compare the changes directly using the capacity factor data. The changes in the relative capacity factors compared to protriptyline are more easily observed (Fig. 3). The more basic primary, secondary and tertiary amines behaved very much as protriptyline and showed very small relative changes with the ionic strength. The relative capacity factors of some of the amines such as strychnine, ephedrine, phenylephrine, and codeine, increased markedly with ionic strength while retentions of the anomalous compounds, dipipanone, pipazethate, and prolintane, decreased with increasing ionic strength. These studies of the pH and the ionic strength emphasise the need to keep both constant for good retention reproducibility.

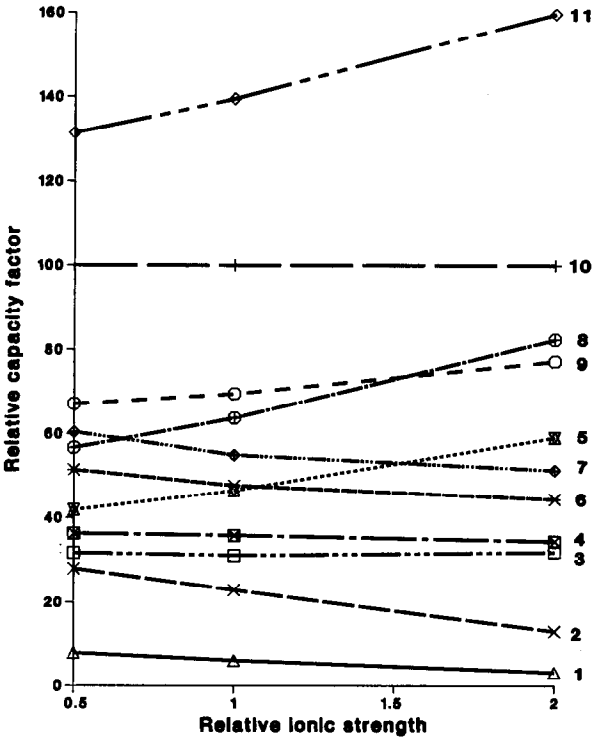

Fig. 3. Effect of ionic strength of the aqueous component of the mobile phase on the relative capacity factors of selected basic drugs. Conditions and drugs as in Fig. 1; 11 = strychnine.

The effect of the temperature of the column

Very often in HPLC the effect of temperature is ignored and separations are carried out under ambient conditions although an increase in temperature usually causes retention times to decrease and column efficiency to increase. In the earlier studies on the separation of barbiturates, the temperature was found to be an important factor whose effect was apparently enhanced because it also altered the degree of ionisation of the partially ionised samples[6]. A similar situation is likely to be present in the current study and the separations were therefore repeated with the column temperature over the range 10–40°C. As expected, the capacity factors decreased with increasing temperature (Table V). Normally in partition HPLC there is a linear relationship between log k' and the reciprocal of the absolute temperature. In ion-exchange chromatography the effects can be more variable and changes with temperature can be an important method of altering selectivity[22]. A plot of selected drugs showed that although the linear relationship was approximately correct the slope of the lines for the different drugs were very different and marked changes in selectivity occurred (*e.g.* codeine and prolintane, Fig. 4). Thus, again relative capacity factors will only provide compensation for compounds with similar basic groups and marked changes can be expected with some amines. Clearly to attain maximum reproducibility the temperature of the column should be specified and carefully controlled.

TABLE V

EFFECT OF TEMPERATURE ON THE CAPACITY FACTORS OF BASIC DRUGS ON A SILICA COLUMN

Column, Spherisorb S5W; eluent, methanol–aqueous ammonium nitrate (90:10).

Compound	*Capacity factors*				
	Temperature (°C)				
	10	*20*	*25*	*30**	*40*
Nitrazepam	0.03	0.03	0.03	0.02	0.03
Diazepam	0.03	0.03	0.03	0.02	0.03
Papaverine	0.05	0.06	0.06	0.06	0.05
Dextropropoxyphene	0.07	0.09	0.10	0.09	0.08
Caffeine	0.10	0.10	0.10	0.10	0.08
Cocaine	0.11	0.11	0.12	0.11	0.10
Procaine	0.17	0.18	0.18	0.17	0.16
Amitriptyline	0.37	0.39	0.40	0.39	0.36
Chlorpromazine	0.43	0.44	0.45	0.44	0.40
Propranolol	0.49	0.47	0.46	0.44	0.37
Dipipanone	0.38	0.41	0.46	0.45	0.38
Imipramine	0.62	0.61	0.62	0.60	0.55
Phentermine	0.71	0.66	0.65	0.61	0.52
Amphetamine	0.82	0.74	0.74	0.69	0.58
Promazine	0.76	0.76	0.77	0.75	0.69
Codeine	1.03	0.98	0.95	0.91	0.83
Prolintane	0.93	0.94	0.96	0.93	0.82
Morphine	1.13	1.05	1.02	0.96	0.87
Pipazethate	1.07	1.07	1.09	1.07	0.95
Nortriptyline	1.41	1.28	1.26	1.19	0.99
Ethoheptazine	1.35	1.28	1.25	1.19	1.06
Pholcodine	1.41	1.33	1.30	1.23	1.12
Phenylephrine	1.67	1.41	1.36	1.24	1.03
Methdilazine	1.44	1.40	1.37	1.32	1.19
Ephedrine	1.75	1.53	1.45	1.35	1.12
Methylamphetamine	1.93	1.72	1.65	1.54	1.30
Protriptyline	2.43	2.14	2.09	1.94	1.60
Strychnine	2.90	2.84	2.84	2.71	2.44

* Standard conditions (values from Table II).

CONCLUSIONS

The initial study on retention reproducibility for a single batch of Spherisorb S5W suggested that relative capacity factors would be more robust than capacity factors for this HPLC system for basic drugs. However, further studies, involving various changes to the separation, have shown large changes in the selectivity of the separation and suggest that the usefulness of the internal standard may be limited to compounds with related structures and with similar pK_a values. Particular problems were found for the proportion of methanol, pH of the eluent, and the temperature of the column as all caused the selectivity of the separation to alter markedly. Relative

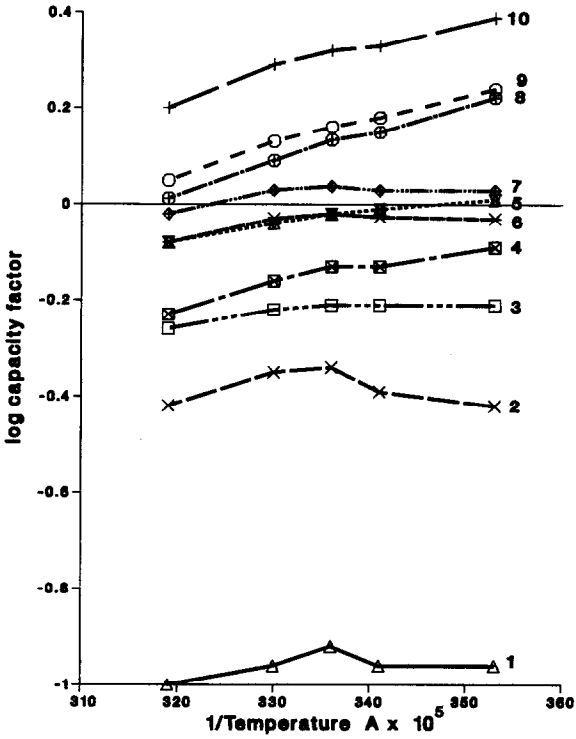

Fig. 4. Effect of temperature on the capacity factors of selected basic drugs. Conditions and drugs as in Fig. 1.

capacity factors may help compensate for variations due to ionic strength because of the very large changes observed with all the drugs. However, because of the selectivity changes which can occur it is clearly important to specify carefully all the operating conditions if reproducible results are to be obtained. A robust method has been used to achieve reproducible results for the preparation of the mobile phase. Retention values obtained from this HPLC system under such controlled conditions can be highly reproducible and could form the basis for a database used for identification purposes.

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