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

## BATCH-TO-BATCH REPRODUCIBILITY OF THE STATIONARY PHASE

ROGER M. SMITH*, TONY G. HURDLEY and JAMES P. WESTLAKE
*Department of Chemistry, Loughborough University of Technology, Loughborough, Leics. LE11 3TU (U.K.)*
and
RICHARD GILL and M. DAVID OSSELTON
*Central Research Establishment, Home Office Forensic Science Service, Aldermaston, Reading, Berks. RG7 4PN (U.K.)*

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SUMMARY

The effect of changing the brand and batches of the silica stationary phase used with a methanol–aqueous ammonium nitrate eluent for the high-performance liquid chromatographic separation of basic drugs has been studied. Considerable care had to be taken to obtain a reproducible eluent and the effect of small changes in the concentration of the ammonia solution were examined closely. Large differences in both the capacity factor and relative capacity factors were found for separations on columns packed with four different brands of silica. Significant differences were also observed with columns containing eighteen different batches of Spherisorb S5W, which had been manufactured over a period of several years.

## INTRODUCTION

Although high-performance liquid chromatography (HPLC) is a very reliable technique for analytical work, intra- and interlaboratory reproducibility of separations can be very poor. With care, reproducible conditions of eluent composition and temperature can be maintained but different commercial brands of packing material or different batches of the same brand can cause variations in overall retention times and selectivities. Over the past few years manufacturers have improved batch-to-batch reproducibility but occasional changes, often unannounced, in the manufacturing processes have caused some abrupt changes to the chromatographic behaviour of packing materials[1,2]. Differences between brands of reversed-phase column packings are often large, because of differences in the chemistry of the bonding reactions and the degree of end-capping. There can also be differences in the underlying silica material.

As part of a series of studies aimed at investigating the reproducibility of retentions in the HPLC analysis of drugs, the separations of barbiturates[3], local anaesthetics[4] and thiazide diuretics[5] on ODS-Hypersil and other brands of octadecyl bonded silica have been compared. There were considerable differences between different brands but the results on different batches of ODS-Hypersil were very reproducible.

These studies have now been extended to a detailed examination of basic drug separations on silica columns using a methanol–aqueous ammonium nitrate eluent[6]. The effects on the capacity factors and relative capacity factors caused by small changes in the eluent composition and the operating conditions have been examined on a single batch of Spherisorb S5W silica. These studies showed that for consistent results it was necessary to carefully control the composition of the eluent (in particular the pH and the ionic strength) and the column temperature. The reproducibility of the retentions observed on four columns prepared from the same batch of silica suggested that, with care, reliable results could be obtained. This method was then examined in a collaborative study using the same single batch of silica[7]. The results, showed that, as expected from an earlier study on ODS-silica, relative capacity factors gave much better reproducibility than capacity factors. However, the variations were still much greater than in the study within a single laboratory and it was considered that probable causes were differences in the column temperatures and in the concentration of the ammonia solutions used to prepare the eluent. Further confirmation of the interlaboratory variation has also come from a more recent international collaborative study of the same system[8].

In the present paper, the effects of different brands of column material have been studied and differences were noted between batches of a single brand of silica. In order to test the long term reproducibility of the separation, a single column was then examined in order to assess the influence on the separation of small changes in the concentration of the ammonia solution with time due to evaporation. These results were compared with the effect of making deliberate large changes in the proportion of ammonia. These studies enabled the expected range of results for a single batch of silica under controlled conditions to be established, which was then compared with the batch-to-batch variation within a single brand of silica.

In a previous report, in which this type of HPLC system was used, it was noted that retentions on different brands of silica packing material were markedly different[9]. Similar variations between analyses on different brands of silica have also been observed with a related eluent system based on the use of methanolic perchloric acid[10] but different batches of the same brand were found to give very similar retentions for a large number of compounds[11].

## EXPERIMENTAL

### *Chemicals and standards*

Ammonium nitrate was analytical reagent-grade, methanol was HPLC-grade, and ammonia was 0.880 laboratory grade from FSA Laboratory Supplies (Loughborough, U.K.). Samples of basic drugs were from the reference collection of the Central Research Establishment, Home Office Forensic Science Service.

*HPLC equipment*

HPLC separations were carried out using a Pye Unicam Model 4020 pump and an Altex Model 153 fixed-wavelength detector (254 nm). The samples (5$\mu$l) were injected using a 7125 Rheodyne valve onto a Shandon column (25 cm × 5 mm I.D.) packed with silica. The eluent was pumped at 2 ml $min^{-1}$ and 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 consisted of methanol–aqueous ammonium nitrate buffer (9:1, v/v) and the buffer was prepared by mixing 0.880 concentrated ammonia (90 ml), ammonium nitrate (27 g) and water (900 ml). The retention times were determined using a Hewlett Packard Model 3390 integrator. The column void volume ($t_0$) was determined using an injection of sodium nitrate (30.0 mg $ml^{-1}$) 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).

*Silica column packing materials*

Spherisorb S5W, 5-$\mu$m (Numerous batches, see Table IV), was obtained from Phase Separations (Queensferry, U.K.); Hypersil, 5-$\mu$m, from Shandon Southern Products (Runcorn, U.K.); Partisil, 10-$\mu$m, from Whatman (Maidstone U.K.); LiChrosorb, 5-$\mu$m, from Merck (Darmstadt, F.R.G.).

In each case the material was slurry packed, using methanol to disperse the silica. Shandon Southern columns (250 × 5 mm I.D.) were used. Each column was equilibrated with eluent before use until consistent retention results were obtained.

*Standard test solutions of basic drugs*

Eight test solutions were made up as mixtures, each including protriptyline hydrochloride as an internal standard, in ethanol–water (90:10, v/v) with concentrations (0.02–8 mg $ml^{-1}$) chosen to give a similar detector response for each drug[6]: (A) Caffeine, imipramine hydrochloride, morphine hydrochloride, methylamphetamine hydrochloride and protriptyline hydrochloride. (B) Cocaine hydrochloride, phentermine, ephedrine and protriptyline hydrochloride. (C) Diazepam, propranolol, nortriptyline hydrochloride and protriptyline hydrochloride. (D) Amitriptyline hydrochloride, prolintane hydrochloride, phenylephrine bitartrate and protriptyline hydrochloride. (E) Nitrazepam, chlorpromazine hydrochloride, pipazethate hydrochloride and protriptyline hydrochloride. (F) Dextropropoxyphene hydrochloride, amphetamine sulphate, pholcodine and protriptyline hydrochloride. (G) Papaverine, dipipanone hydrochloride, codeine phosphate, methdilazine hydrochloride and protriptyline hydrochloride. (H) Procaine hydrochloride, promazine, ethoheptazine citrate, protriptyline hydrochloride and strychnine.

*Simplified test mixture*

For much of the work a simplified test procedure was developed involving eight test drugs and requiring the injection of four solutions. The detailed composition of these solutions are [concentrations in mg $ml^{-1}$ in ethanol–water (90:10)]: (A) Dipipanone hydrochloride (0.40), prolintane hydrochloride (1.24), protriptyline hydrochloride (0.15) and strychnine (0.07). (B) Promazine (0.006), phenylephrine bitartrate (1.44) and protriptyline hydrochloride (0.15). (C) Codeine phosphate (1.07), ephe-

drine (2.25) and protriptyline hydrochloride (0.15). (D) Sodium nitrate [30 mg $ml^{-1}$ in methanol–water (90:10)].

*Calculations*

All the retention times were measured in duplicate and the capacity factors were calculated as $k' = (t_r - t_O)/t_O$. 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.

*Principal components analysis*

Multivariant principal components analyses on the covariance matrix were carried out using the GENSTAT programs Version 4.03 on a Honeywell Multics computer at Loughborough University.

## RESULTS AND DISCUSSION

In order to reduce the total number of packing materials in use in U.K. forensic science laboratories, thus allowing rapid transfer of methods between different laboratories, all work involving silica columns has been adapted to use Spherisorb S5W. Furthermore all laboratories were supplied from a single batch of this packing material and this material was used in the previous investigation of the effect of altering the eluent[6] and in the interlaboratory collaborative study[7].

In the present study on the separation of basic drugs it was important to determine if the results obtained with Spherisorb S5W silica would differ greatly if other brands of packing material were used and to consider whether consistent results might be obtained on subsequent batches of Spherisorb S5W once the present stocks are exhausted.

In the preceding studies on the reproducibility of retentions with the present HPLC system, it was found that relative capacity factors compared to an internal standard gave more reproducible results than capacity factors[6]. The latter are very dependent on the value determined for the column void volume whereas relative methods are virtually independent of this measurement. This study also showed that if the aqueous buffer solution was prepared by taking a specified mass of ammonium nitrate and a fixed volume of concentrated ammonia, the pH of the solution was very reproducible. Repeated analyses on four columns prepared over a short period from the same batch of Spherisorb silica gave very consistent results[6]. As no bonding reactions are involved in the production of silica packings it was expected that these materials might be more uniform in their selectivity than alkyl bonded silica columns, although the overall retention times might alter due to differences in surface area and pore size.

*Different brands of packings material*

Different brands of silica differ in particle shape (spherical or irregular), pore size, and the pH and chemistry of their surface[12,13]. In some studies, these differences appeared to have little effect on the retention properties and only minor variations between brands have been reported[14]. To determine if these factors would affect the retentions and selectivity of the basic drug separation, four different brands of silica

were tested, two with spherical (Hypersil and Spherisorb) and two with irregular particles (LiChrosorb and Partisil). The results are presented in the order of elution on Spherisorb S5W (Table I). The differences in the capacity factors of the drugs were often large and the order of elution differed between materials. The results on one column material could not therefore be easily compared directly with results on another material.

If the relative capacity factors were calculated using protriptyline as the internal standard, the results for many of the analytes were very similar on the four columns suggesting that the differences were mainly in their overall retention power (Table I). However, significant differences were still present for a number of compounds, in particular dipipanone, prolintane, codeine, pipazethate, phenylephrine and strych-

TABLE I

REPRODUCIBILITY OF CAPACITY FACTORS AND RELATIVE CAPACITY FACTORS OF BASIC DRUGS ON DIFFERENT BRANDS OF SILICA PACKING MATERIAL

Eluent, methanol–ammonium nitrate (90:10); temperature 30°C. Column packing materials: Sph, Spherisorb S5W (batch No. 2752 mean value from four columns[6]); Hyp, Hypersil, 5-μm; Part, Partisil, 10-μm; Lich, LiChrosorb, 5-μm.

| *Compound* | *Capacity factors* | | | | *Relative capacity factors (× 100)* | | | |
|---|---|---|---|---|---|---|---|---|
| | *Sph* | *Hyp* | *Part* | *Lich* | *Sph* | *Hyp* | *Part* | *Lich* |
| Diazepam | 0.02 | 0.03 | 0.03 | 0.03 | 1.3 | 2.3 | 1.7 | 1.5 |
| Nitrazepam | 0.02 | 0.03 | 0.03 | 0.03 | 1.3 | 2.3 | 1.7 | 1.5 |
| Papaverine | 0.06 | 0.04 | 0.05 | 0.04 | 2.6 | 3.1 | 2.9 | 1.9 |
| Dextropropoxyphene | 0.09 | 0.05 | 0.06 | 0.06 | 4.5 | 3.8 | 3.4 | 2.9 |
| Caffeine | 0.10 | 0.08 | 0.14 | 0.14 | 5.0 | 6.1 | 8.0 | 6.8 |
| Cocaine | 0.11 | 0.07 | 0.09 | 0.10 | 6.0 | 5.4 | 5.1 | 4.9 |
| Procaine | 0.17 | 0.11 | 0.16 | 0.20 | 8.8 | 8.5 | 9.1 | 9.7 |
| Amitriptyline | 0.39 | 0.27 | 0.37 | 0.44 | 19.9 | 20.8 | 21.1 | 21.4 |
| Chlorpromazine | 0.44 | 0.30 | 0.40 | 0.48 | 22.4 | 23.1 | 22.9 | 23.3 |
| Propranolol | 0.44 | 0.27 | 0.37 | 0.43 | 22.5 | 20.8 | 21.1 | 20.9 |
| Dipipanone | 0.45 | 0.21 | 0.19 | 0.25 | 22.9 | 16.2 | 10.9 | 12.1 |
| Imipramine | 0.60 | 0.43 | 0.57 | 0.66 | 31.1 | 33.1 | 32.6 | 32.0 |
| Phentermine | 0.61 | 0.40 | 0.55 | 0.64 | 31.4 | 30.8 | 31.4 | 31.1 |
| Amphetamine | 0.69 | 0.47 | 0.66 | 0.77 | 35.6 | 36.1 | 37.7 | 37.4 |
| Promazine | 0.75 | 0.53 | 0.70 | 0.85 | 38.5 | 40.8 | 40.0 | 41.3 |
| Codeine | 0.91 | 0.67 | 0.94 | 1.07 | 46.6 | 51.5 | 53.7 | 51.9 |
| Prolintane | 0.93 | 0.55 | 0.58 | 0.73 | 47.7 | 42.3 | 33.1 | 35.4 |
| Morphine | 0.96 | 0.71 | 1.02 | 1.15 | 49.7 | 54.6 | 58.3 | 55.8 |
| Pipazethate | 1.07 | 0.69 | 0.75 | 0.89 | 54.9 | 53.1 | 42.9 | 43.2 |
| Nortriptyline | 1.19 | 0.81 | 1.12 | 1.30 | 60.9 | 62.3 | 64.0 | 63.1 |
| Ethoheptazine | 1.19 | 0.86 | 1.16 | 1.36 | 61.1 | 66.1 | 66.3 | 66.0 |
| Pholcodine | 1.23 | 0.88 | 1.25 | 1.40 | 63.4 | 67.7 | 71.4 | 68.0 |
| Phenylephrine | 1.24 | 0.87 | 1.37 | 1.52 | 63.8 | 66.9 | 78.3 | 73.6 |
| Methdilazine | 1.32 | 0.95 | 1.25 | 1.45 | 67.9 | 73.1 | 71.4 | 70.4 |
| Ephedrine | 1.35 | 0.93 | 1.31 | 1.50 | 69.5 | 71.5 | 74.9 | 72,8 |
| Methylamphetamine | 1.54 | 1.06 | 1.47 | 1.69 | 79.1 | 81.5 | 84.0 | 82.0 |
| Protriptyline | 1.94 | 1.30 | 1.75 | 2.06 | 100.0 | 100.0 | 100.0 | 100.0 |
| Strychnine | 2.71 | 1.88 | 2.26 | 2.55 | 139.5 | 144.6 | 129.1 | 123.8 |

nine. In most of these cases, the two columns packed with irregular silica (Partisil and LiChrosorb) gave lower relative capacity factors than the spherical silica materials (Hypersil and Spherisorb). Codeine and particularly phenylephrine, both of which contain phenolic groups, which will be ionised in the eluent, showed an opposite behaviour. In their study on the characterization of silicas, Engelhardt and Müller[12] found that aqueous suspensions of the irregular materials, Partisil and LiChrosorb, had pH values of 7.2 and 7.8 while the spherical materials, Spherisorb and Hypersil, had pH values of 9.0 and 9.5. Comparable experimental values were obtained with the present packing materials on initial suspension in water; Partisil, 6.72; LiChrosorb, 7.64; Hypersil, 8.96 and Spherisorb (batch 2752), 9.59. As these values are similar to the pH of the eluent, 9.39, and to the $pK_a$ values of the compounds which appear to be sensitive to the different silicas, it suggests that small differences in the degree of ionisation of the silica surface or the degree of interaction with the analytes may cause these effects. Except for codeine, these anomalous compounds had also shown unusual behavior on changing the pH or ionic strength of the eluent with a single Spherisorb column[6].

Thus to obtain comparable results in interlaboratory studies or for the establishment of a database of retention values it will be necessary to standardise on a single brand of packing material.

*Comparison of different batches of Spherisorb S5W*

Consistent retentions were obtained in the first part of the present study with different columns packed with the same batch of Spherisorb S5W[6] and it was initially anticipated that different batches would also be very similar. However, when results on Spherisorb S5W batch No. 2752, which had been used as the standard material in U.K. forensic science laboratories and was used in the preceding paper[6], were compared with results on a column prepared from Spherisorb S5W batch No. 4488, the capacity factors (Fig. 1) and relative capacity factors of some test compounds were markedly different. The previous study had shown that no systematic changes in overall retention or selectivity occurred with the use of the HPLC columns over many weeks and thus it appears that we are dealing with permanent differences in the chromatographic properties in the silicas. Although for many compounds the relative capacity factors, which should be independent of overall changes in retention properties, on the new batch of Spherisorb were very similar, much larger values were obtained for prolintane (62.1 compared to 47.7), pipazethate (66.3 compared to 54.9) and strychnine (155.8 compared to 139.5) but a lower value was found for phenylephrine (58.5 compared to 63.4). Except for strychnine these are compounds which have previously been found to be particularily sensitive to changes in the separation conditions[6] and are also sensitive to different brands of column material (Table I).

To ensure that the observed differences between these two batches were not isolated phenomena, the variation in retention on a further eight batches of Spherisorb silica manufactured over a period of years were compared (Table II). As with earlier work large variations were observed for drugs with very short retentions ($k' < 0.4$) but these are primarily due to the errors of measuring small values. For the more strongly retained compounds ($k' > 0.4$), the coefficients of variation (C.V.) for the capacity factors on these columns were much larger (8–23%) than the C.V. values for the same compounds on columns prepared from a single batch (1–3%)[6]. The relative

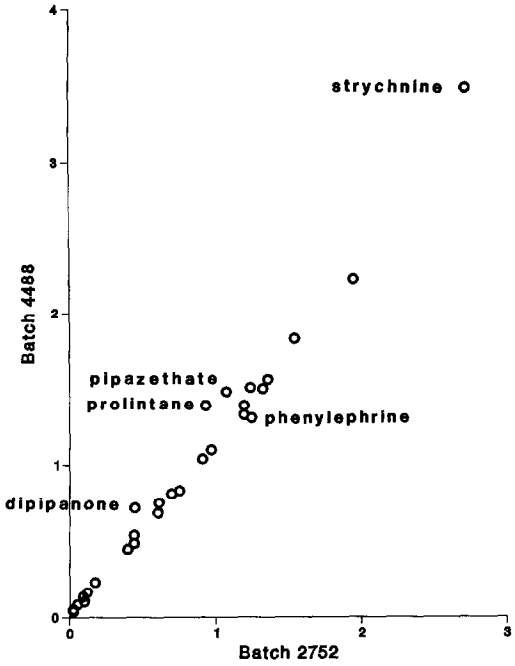


Fig. 1. Comparison of capacity factors for basic drugs on two batches of Spherisorb S5W (Nos. 2752 and 4488). Anomalous compounds labelled.

capacity factors should compensate for any changes due to the overall retentive capacity of the columns and for most of the basic drugs the variations were much smaller (Table II). However, a number of compounds, dipipanone, pipazethate, phenylephrine, prolintane and strychnine, still showed particularly large C.V. values (4–15%). These are the same compounds which were anomalous in the initial comparison of two batches (Fig. 1). In some cases the differences between the different batches of Spherisorb S5W were sufficiently large to cause changes in elution order involving these anomalous drugs. The pH of aqueous suspensions of the Spherisorb batches were measured and compared but although the initial values varied from pH 9.19 to 9.70 no correlation was found between these results and the retention properties. Clearly, the results obtained on one batch could not be used for identification purposes on a different batch even if expressed as relative capacity factors.

It therefore appears that certain compounds and probably certain functional groups are particularly sensitive to the differences between different brands and batches of silica packing material and also to changes in the mobile phase. Thus these compounds could be potentially useful in the characterisation and testing of silica materials for reproducibility. It was desirable to identify a few compounds which show particularly characteristic retention variations and which could be used as marker compounds to simplify testing procedures. The relative factors for all 28 compounds on all ten batches were therefore examined using a multivariant principal components analysis in order to identify which compounds contributed most significantly to the overall variance between the batches.

TABLE II

REPRODUCIBILITY OF CAPACITY FACTORS AND RELATIVE CAPACITY FACTORS OF BASIC DRUGS DETERMINED ON TEN DIFFERENT BATCHES OF SPHERISORB S5W

Based on columns prepared from ten different batches of packing material. Eluent, methanol-aqueous ammonium nitrate (90:10); temperature, 30°C. S.D. = Standard deviation.

| *Compound* | *Capacity factors* | | | *Relative capacity factors (× 100)* | | |
|---|---|---|---|---|---|---|
| | *Mean* | *S.D.* | *C.V. (%)* | *Mean* | *S.D.* | *C.V. (%)* |
| Diazepam | 0.03 | 0.01 | 33.0 | 1.6 | 0.3 | 18.7 |
| Nitrazepam | 0.03 | 0.01 | 33.0 | 1.5 | 0.4 | 26.7 |
| Papaverine | 0.06 | 0.01 | 16.7 | 2.9 | 0.5 | 17.4 |
| Caffeine | 0.10 | 0.01 | 10.0 | 4.7 | 0.3 | 6.4 |
| Dextropropoxyphene | 0.11 | 0.02 | 18.2 | 5.6 | 1.0 | 17.9 |
| Cocaine | 0.14 | 0.02 | 14.3 | 6.4 | 0.6 | 9.3 |
| Procaine | 0.19 | 0.03 | 15.8 | 9.1 | 0.5 | 5.5 |
| Amitriptyline | 0.42 | 0.04 | 9.5 | 19.9 | 0.4 | 2.0 |
| Chlorpromazine | 0.46 | 0.04 | 8.7 | 22.0 | 0.5 | 2.2 |
| Propranolol | 0.49 | 0.06 | 12.2 | 23.0 | 0.8 | 3.4 |
| Dipipanone | 0.59 | 0.14 | 23.7 | 27.5 | 4.2 | 15.3 |
| Imipramine | 0.66 | 0.06 | 9.1 | 31.0 | 0.5 | 1.6 |
| Phentermine | 0.69 | 0.08 | 11.6 | 32.6 | 0.8 | 2.4 |
| Amphetamine | 0.76 | 0.08 | 10.5 | 36.1 | 0.3 | 0.8 |
| Promazine | 0.80 | 0.06 | 7.5 | 37.9 | 1.0 | 2.6 |
| Codeine | 0.98 | 0.09 | 9.2 | 46.3 | 1.1 | 2.3 |
| Morphine | 1.05 | 0.09 | 8.6 | 49.4 | 1.2 | 2.4 |
| Prolintane | 1.16 | 0.22 | 19.0 | 54.3 | 5.9 | 10.8 |
| Pipazethate | 1.26 | 0.20 | 15.9 | 59.4 | 4.5 | 7.6 |
| Nortriptyline | 1.28 | 0.11 | 8.6 | 60.5 | 0.8 | 1.3 |
| Phenylephrine | 1.29 | 0.10 | 7.7 | 61.3 | 2.6 | 4.2 |
| Ethoheptazine | 1.30 | 0.13 | 10.0 | 61.6 | 1.2 | 1.9 |
| Pholcodine | 1.37 | 0.15 | 10.9 | 64.7 | 1.8 | 2.7 |
| Methdilazine | 1.41 | 0.14 | 10.0 | 67.4 | 1.4 | 2.1 |
| Ephedrine | 1.47 | 0.14 | 9.5 | 69.6 | 0.7 | 1.0 |
| Methylamphetamine | 1.70 | 0.18 | 10.6 | 80.6 | 0.9 | 1.1 |
| Protriptyline | 2.12 | 0.20 | 9.4 | 100.0 | – | – |
| Strychnine | 3.11 | 0.42 | 13.5 | 146.5 | 7.0 | 4.8 |

Because overall differences in the retention capacity of columns contribute to the values of capacity factors, the relative capacity factors were chosen as the more usefull comparison for changes in selectivity. A plot of the loading factors from the principal components analysis showed that the loadings for most of the compounds in the first two components were very small and they were clustered near the origin. However, the five anomalous compounds (dipipanone, prolintane, pipazethate, phenylephrine and strychnine) identified earlier showed much larger loadings and could be identified as being primarily responsible for the variance between columns (Fig. 2). In the contributions to the first principal component the weighting of phenylephrine (+0.18) was of the opposite sign to the other anomalous compounds, dipipanone (−0.36), pipazethate (−0.39), prolintane (−0.52) and strychnine (−0.60). All the other test compounds had loadings in the first component of less than ± 0.12. As the

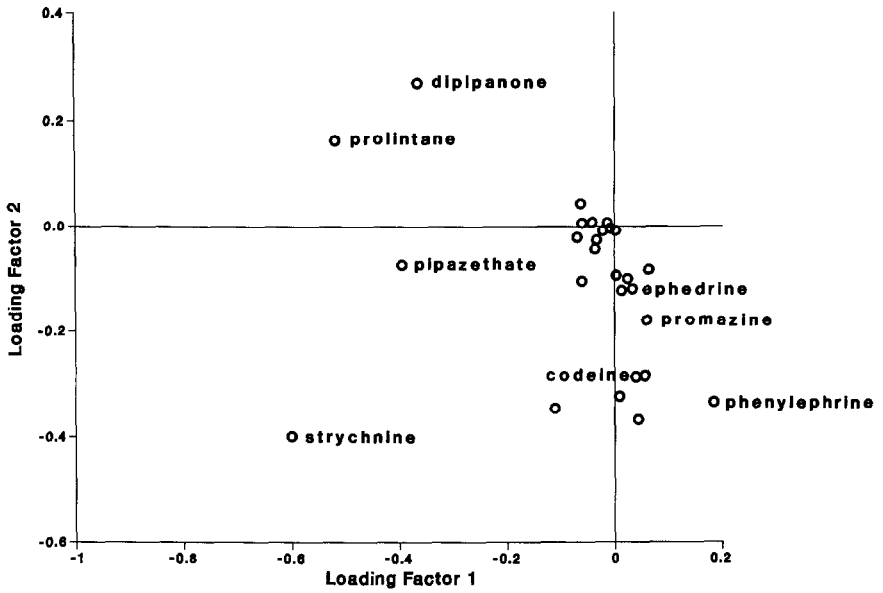


Fig. 2. Loading plot of basic drugs in principal components analysis of relative capacity factors of 27 basic drugs on ten batches on Spherisorb S5W.

first principal component accounted for almost all the variance (88.8%) in the retention of the basic drugs, it appeared that the differences between columns were primarily due to a single cause. In most cases the same anomalous compounds also contributed significantly to the second principal component.

All these columns were nominally the same material and would have satisfied the conventional quality control criteria applied by the manufacturer. It thus appears that the current HPLC system involving the separation of the basic drugs is a more severe test for silica columns than the tests which are usually used for quality control which generally involve normal phase separations. This difference probably arises because the mode of separation of the drugs involves ion-exchange and ion-pair interactions[7].

*Selection of a simplified test mixture*

In order to extend this study further and examine a wider range of different Spherisorb batches a small group of eight test drugs was selected as characteristic of the original sample set, based upon the results of the principal components analysis. Dipipanone, prolintane and strychnine were chosen as compounds whose retentions were apparently sensitive to changes in the packing material. Because the effect on phenylephrine was in the opposite direction it was also included along with codeine which had shown a large difference in the comparison of different silica brands (Table I). Two compounds, ephedrine and promazine, were included as typical compounds which were largely unaffected by differences in the batches. A set of four new simplified test mixtures was prepared and protriptyline was included in each mixture as an internal standard for the determination of relative capacity factors (see experimental). All the test compounds were well retained and thus not susceptible to small variations in the measurement of the column void volume.

*Effect of ammonia concentrations on separation and selectivity*

In order to ensure that the differences that were observed between batches were significant, it was necessary to determine the expected range of the results from a single batch over the period of the study as a consequence of small differences in the preparation of the eluent. In particular, it was desirable to estimate the possible effects that would be caused by the loss of ammonia from the stock bottle by evaporation and by deliberate changes in the ammonia concentration such as might be found between bottles from different sources. Although in the previous study it had been found the eluent pH was unaffected by small changes in the ammonia concentration[6], there was concern that the consequential changes in the ionic strength might also alter the selectivity. Also with reference to the interlaboratory study[7], it was concluded that differences in the nominal concentration of ammonia used in the different laboratories could have been a significant factor (together with differences in column temperature) in the greater variations in relative retention that were observed. As the separations observed for many of the original test mixture compounds were unaffected by changes in conditions, the simplified test mixtures were used to monitor the separation.

A single column of Spherisorb S5W (batch No. 2752) was studied over a period of four months. For each separation the eluent was prepared from the same stock bottle of 0.880 concentrated ammonia. The ammonia concentration was determined by titration and dropped from 17.1 to 16.5 *M* during the experiment. A sample of the original solution of concentrated ammonia kept in a sealed bottle showed no loss over this period. A series of nine sets of separations were carried out using the standard conditions over the study period. The relative capacity factors were very consistent and were comparable to those obtained in the repeated runs on a single column on successive days[6]. The standard deviations (Table III) were much smaller than those found in the interlaboratory study[7].

TABLE III

REPRODUCIBILITY OF CAPACITY FACTORS AND RELATIVE CAPACITY FACTORS OF BASIC DRUGS DETERMINED IN REPLICATE SEPARATIONS ON A SINGLE BATCH OF SPHERISORB S5W

Based on single column prepared from Spherisorb S5W batch No. 2752. Eluent, methanol-aqueous ammonium nitrate (90:10); temperature, 30°C. Results are based on nine separations carried over a period of four months.

| *Compound* | *Capacity factors* | | | *Relative capacity factors (× 100)* | | |
|---|---|---|---|---|---|---|
| | *Mean* | *S.D.* | *C.V. (%)* | *Mean* | *S.D.* | *C.V. (%)* |
| Dipipanone | 0.37 | 0.02 | 5.4 | 20.3 | 0.8 | 3.9 |
| Promazine | 0.71 | 0.02 | 4.2 | 38.8 | 1.3 | 3.4 |
| Codeine | 0.88 | 0.03 | 3.4 | 48.2 | 1.5 | 3.1 |
| Prolintane | 0.88 | 0.02 | 2.3 | 48.2 | 1.0 | 2.1 |
| Phenylephrine | 1.17 | 0.04 | 3.4 | 64.0 | 1.5 | 2.3 |
| Ephedrine | 1.28 | 0.04 | 3.1 | 70.1 | 1.3 | 1.9 |
| Protriptyline | 1.83 | 0.04 | 2.2 | 100.0 | – | – |
| Strychnine | 2.65 | 0.06 | 2.3 | 144.4 | 1.9 | 1.3 |

In addition runs were studied in which the ammonia concentration was deliberately increased to 160, 180 and 200% or decreased to 90, 80 and 60% of the specified value (Table IV). The 90% and 80% runs were repeated in triplicate and their variations were similar to those observed using the standard eluent. These larger changes in the eluent had marked effects on the separation and the capacity factors and relative capacity factors increased or decreased correspondingly for the sensitive test compounds. This confirmed that the separation can be very dependent on the ammonia concentration, which would be difficult to control in interlaboratory comparisons. It has lead us to undertake a subsequent examination of alternative eluent compositions with different non-volatile buffer components[15].

*Spherisorb comparisons*

The retention values for components of the simplified test mixtures on nine further batches of Spherisorb were determined in duplicate. In each case the difference between the replicates was within the range which had been determined for batch No. 2752. The mean capacity factors were combined with the corresponding results from the individual batches examined previously (Table V) and the relative capacity factors were compared (Table VI). These results were then examined using principal components analysis. Almost all of the variance in the relative capacity factors on the different columns was expressed by the first and second principal components (Table VII). The loading factors of the test components were similar to those obtained for the analysis of the original ten batches. The major contributor to the variance (represented by the largest latent vector) was strychnine (0.829). As expected the contributions of ephedrine, codeine and promazine, were very small (latent vectors 0.017, 0.075 and 0.031, respectively) confirming that, relative to an internal standard, little variation had occurred between the different batches. The sensitive compounds (strychnine, prolintane and dipipanone) all showed significant contributions to the variance with the same sign of weighting suggesting a similar interaction. Phenylephrine showed a smaller contribution but with the opposite sign.

The scores for each batch (sum of latent vectors multiplied by the individual deviation for each test compound) for the first and second principal components were plotted (Fig. 3). It was noticeable that there was a progression from the older to the newer batches and the three oldest batches 876, 1290 and 1540 were markedly different from more recent batches. The standard batch (No. 2752) occupied an intermediate position quite different from batch No. 4488 discussed earlier (Fig. 1). Subsequently, the manufacturers have confirmed that this intermediate batch (No. 2752) was produced at a time when a major change occurred in the silica source used for the production of Spherisorb and some minor changes were noted in their own internal quality test[16]. The correlation between the position and the date of manufacture is not as clear with the later batches, except that the batches up to No. 4488 seemed to be bunched separately from those produced later, 5026 to 5615, with the exception of batch No. 5493. Although the majority of the later batches appeared to be grouped in one area, the differences between batches can be sufficiently large to cause inversion of the elution order for some drugs, *e.g.* prolintane and phenylephrine (Table VI).

It was of interest to try to identify common or specific features of the anomalous compounds. Phenylephrine contains a phenolic group, p$K_a$ 8.9, and would therefore be expected to be anionic under the separation conditions so its unique behav-

TABLE IV

EFFECT OF DIFFERENT AMMONIA CONCENTRATIONS ON THE RETENTION OF BASIC DRUGS ON A SINGLE BATCH OF SPHERISORB S5W

Based on single column prepared from Spherisorb S5W batch No. 2752. Standard eluent, methanol–aqueous ammonium nitrate (90:10); temperature, 30°C. Number of runs examined are indicated in parentheses.

| *Compound* | *Capacity factors* | | | | | | | *Relative capacity factors ( × 100)* | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *% ammonia relative to standard eluent* | | | | | | | *% ammonia relative to standard eluent* | | | | | | |
| | *60(1)* | *80(3)* | *90(3)* | *100*(9)* | *160(1)* | *180(1)* | *200(1)* | *60(1)* | *80(3)* | *90(3)* | *100*(9)* | *160(1)* | *180(1)* | *200(1)* |
| Dipipanone | 0.66 | 0.45 | 0.43 | 0.37 | 0.24 | 0.21 | 0.21 | 32.2 | 23.3 | 22.4 | 20.3 | 14.1 | 13.1 | 13.7 |
| Promazine | 1.01 | 0.78 | 0.77 | 0.71 | 0.53 | 0.48 | 0.63 | 49.3 | 40.4 | 40.1 | 38.8 | 31.2 | 30.0 | 41.2 |
| Codeine | 1.13 | 0.98 | 0.96 | 0.88 | 0.71 | 0.67 | 0.45 | 55.1 | 50.8 | 50.0 | 48.2 | 41.8 | 41.9 | 29.4 |
| Prolintane | 1.29 | 1.00 | 0.97 | 0.88 | 0.66 | 0.60 | 0.52 | 62.9 | 51.8 | 50.5 | 48.2 | 38.8 | 37.5 | 34.0 |
| Phenylephrine | 1.37 | 1.24 | 1.25 | 1.17 | 1.05 | 0.99 | 1.02 | 66.8 | 64.2 | 65.1 | 64.0 | 61.8 | 61.9 | 66.7 |
| Ephedrine | 1.56 | 1.39 | 1.37 | 1.28 | 1.13 | 1.07 | 0.97 | 76.1 | 72.0 | 71.4 | 70.0 | 66.5 | 66.9 | 63.4 |
| Protriptyline | 2.05 | 1.93 | 1.92 | 1.83 | 1.70 | 1.60 | 1.53 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 |
| Strychnine | 3.64 | 2.95 | 2.86 | 2.65 | 2.00 | 1.84 | 1.72 | 177.6 | 152.8 | 149.0 | 144.4 | 117.6 | 115.0 | 112.4 |

* Values from Table III.

TABLE V

CAPACITY FACTORS OF SELECTED BASIC DRUGS ON 18 DIFFERENT BATCHES OF SPHERISORB S5W

Eluent, methanol–aqueous ammonium nitrate (90:10); temperature, 30°C.

| *Compound* | *Capacity factors* | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *Batch numbers* | | | | | | | | | | | | | | | | | |
| | *876* | *1290* | *1540* | *2396* | *2579* | *2683* | *2752** | *3112* | *3248* | *3882* | *4488* | *5026* | *5106* | *5115* | *5116* | *5123* | *5493* | *5615* |
| Dipipanone | 0.19 | 0.36 | 0.40 | 0.58 | 0.64 | 0.63 | 0.37 | 0.68 | 0.73 | 0.69 | 0.72 | 0.47 | 0.43 | 0.49 | 0.42 | 0.46 | 0.80 | 0.51 |
| Promazine | 0.54 | 0.71 | 0.68 | 0.85 | 0.86 | 0.79 | 0.71 | 0.85 | 0.86 | 0.81 | 0.83 | 0.79 | 0.77 | 0.80 | 0.76 | 0.81 | 0.87 | 0.77 |
| Codeine | 0.67 | 0.86 | 0.82 | 1.03 | 1.07 | 0.97 | 0.88 | 1.04 | 1.05 | 0.99 | 1.04 | 1.00 | 0.98 | 1.02 | 0.96 | 1.02 | 1.10 | 0.97 |
| Prolintane | 0.53 | 0.81 | 0.83 | 1.16 | 1.29 | 1.24 | 0.88 | 1.28 | 1.38 | 1.30 | 1.39 | 1.08 | 1.03 | 1.10 | 1.01 | 1.10 | 1.50 | 1.09 |
| Phenylephrine | 1.01 | 1.13 | 1.14 | 1.37 | 1.41 | 1.27 | 1.17 | 1.41 | 1.35 | 1.30 | 1.31 | 1.28 | 1.25 | 1.30 | 1.21 | 1.29 | 1.45 | 1.22 |
| Ephedrine | 1.04 | 1.25 | 1.24 | 1.54 | 1.55 | 1.50 | 1.28 | 1.59 | 1.61 | 1.51 | 1.56 | 1.40 | 1.36 | 1.42 | 1.34 | 1.41 | 1.67 | 1.38 |
| Protriptyline** | 1.47 | 1.79 | 1.78 | 2.19 | 2.25 | 2.17 | 1.83 | 2.28 | 2.33 | 2.18 | 2.24 | 1.94 | 1.89 | 1.96 | 1.88 | 1.96 | 2.39 | 1.95 |
| Strychnine | 1.74 | 2.45 | 2.42 | 3.19 | 3.49 | 3.21 | 2.65 | 3.36 | 3.48 | 3.28 | 3.49 | 3.10 | 3.02 | 3.21 | 2.90 | 3.14 | 3.82 | 3.12 |

* Mean values from Table III.

** Values taken from injection of test mixture H (batch Nos. 1290–2683 and 3112–4488) and mean value of the three simplified test mixtures (batch Nos. 876, 2752 and 5026–5615).

TABLE VI

RELATIVE CAPACITY FACTORS OF SELECTED BASIC DRUGS ON 18 DIFFERENT BATCHES OF SPHERISORB S5W

Eluent, methanol–aqueous ammonium nitrate (90:10); temperature, 30°C. Relative to protriptyline internal standard in each sample mixture.

| *Compound* | *Relative capacity factors ( × 100)* | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *Batch numbers* | | | | | | | | | | | | | | | | | |
| | *876* | *1290* | *1540* | *2396* | *2579* | *2683* | *2752** | *3112* | *3248* | *3882* | *4488* | *5026* | *5106* | *5115* | *5116* | *5123* | *5493* | *5615* |
| Dipipanone | 12.9 | 20.1 | 22.5 | 26.5 | 28.4 | 29.0 | 20.3 | 29.8 | 31.3 | 31.6 | 32.1 | 24.2 | 22.8 | 25.0 | 22.3 | 23.5 | 33.5 | 26.1 |
| Promazine | 36.7 | 39.7 | 38.2 | 38.7 | 38.2 | 36.4 | 38.8 | 37.3 | 36.9 | 37.2 | 37.1 | 40.7 | 40.7 | 40.8 | 40.4 | 41.3 | 36.4 | 39.5 |
| Codeine | 45.6 | 48.0 | 46.1 | 47.0 | 47.6 | 44.7 | 48.2 | 45.6 | 45.1 | 45.4 | 46.4 | 51.5 | 51.9 | 52.0 | 51.1 | 52.0 | 46.0 | 49.7 |
| Prolintane | 36.0 | 45.3 | 46.6 | 53.0 | 57.3 | 57.1 | 48.2 | 56.1 | 59.2 | 59.6 | 62.1 | 55.7 | 54.5 | 56.1 | 53.7 | 56.1 | 62.8 | 55.9 |
| Phenylephrine | 68.7 | 63.1 | 64.0 | 62.6 | 62.7 | 58.5 | 64.0 | 61.8 | 57.9 | 59.6 | 58.5 | 66.0 | 66.1 | 66.3 | 64.4 | 65.8 | 60.7 | 62.6 |
| Ephedrine | 70.7 | 69.8 | 69.7 | 70.3 | 68.9 | 69.1 | 70.0 | 69.7 | 69.1 | 69.3 | 69.6 | 72.2 | 72.0 | 72.4 | 71.3 | 71.9 | 69.9 | 70.8 |
| Strychnine | 119.0 | 136.9 | 136.0 | 145.7 | 155.1 | 147.9 | 144.4 | 147.4 | 149.4 | 150.5 | 155.8 | 159.8 | 159.8 | 163.8 | 154.3 | 160.2 | 159.8 | 160.0 |

* Mean values from Table III.

TABLE VII

WEIGHTING OF LATENT VECTORS IN PRINCIPAL COMPONENTS ANALYSIS OF RELATIVE CAPACITY FACTORS OF EIGHT TEST DRUG COMPOUNDS ON 18 DIFFERENT BATCHES OF SPHERISORB S5W

Eluent, methanol–aqueous ammonium nitrate (90:10). All remaining vectors had weightings of less than 0.7%.

| *Compound* | *Vector 1 weighting 80.2%* | *Vector 2 weighting 18.6%* |
|---|---|---|
| Dipipanone | 0.290 | 0.553 |
| Promazine | 0.031 | −0.238 |
| Codeine | 0.075 | −0.385 |
| Prolintane | 0.462 | 0.364 |
| Phenylephrine | −0.084 | −0.420 |
| Ephedrine | 0.017 | −0.160 |
| Strychnine | 0.829 | −0.393 |

iour is not unexpected. The three tertiary amines strychnine ($pK_a$ = 8.0), dipipanone ($pK_a$ = 8.5) and prolintane ($pK_a$ unreported but estimated about 8.5) have relatively low $pK_a$ values compared to the pH of the mobile phase (9.39) and would be only slightly ionised compared to many of the more basic amines such as methylamphetamine ($pK_a$ = 10.1). The effects appear therefore to be complex and possibly due to

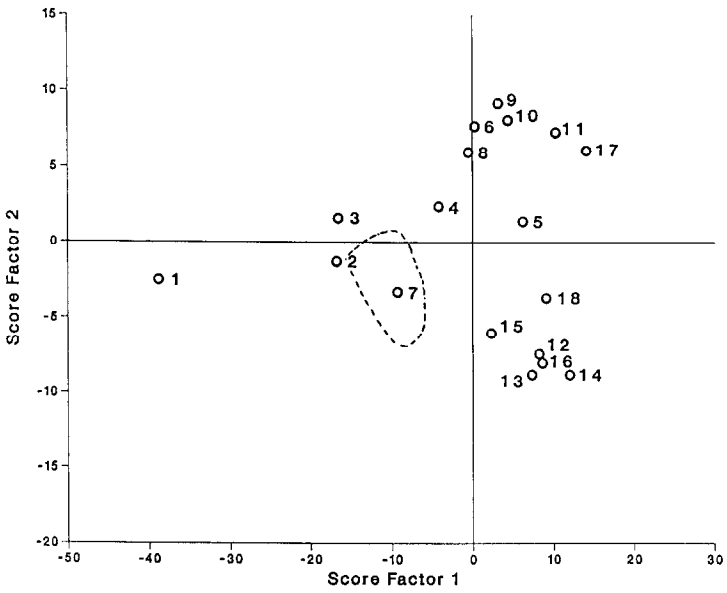


Fig. 3. Comparison of first two principal component scores for relative capacity factors of eight test drugs (dipipanone, promazine, codeine, prolintane, phenylephrine, ephedrine, protriptyline and strychnine) on 18 different batches of Spherisorb S5W: 1, No. 876; 2, No. 1290; 3, No. 1540; 4, No. 2396; 5, No. 2579; 6, No. 2683; 7, No. 2752; 8, No. 3112; 9, No. 3248; 10, No. 3882; 11, No. 4488; 12, No. 5026; 13, No. 5106; 14, No. 5115; 15, No. 5116; 16, No. 5123; 17, No. 5493; 18, No. 5615.

small changes in the steric environment of the basic groups and their interaction with the reactive sites on the silica surface. An attempt had been made previously to examine the separation of similar drugs on silica using a methanol–perchloric eluent but no relationship could be identified between the structures and the changes with eluent composition[9].

In order to compare the reproducibility of the retentions on a single batch with those on different batches the loading factors and mean values from the principal components analysis can be used to calculate the principal component scores for the replicate runs on batch No. 2752 over the four month period and the effects of deliberately changing the ammonia concentration. If these results are plotted on the same axes as the results for the different batches it can be seen that the interbatch results (Fig. 4 and indicated by the dotted line on Fig. 3) confirm that the differences between batches are greater than the variation expected within one batch. Thus, if the conditions are closely controlled real differences in batches can be identified. These results contrast with those obtained in an international collaborative trial in which the variations between columns packed from one batch of silica were as great as the variations between batches[8] although, as already indicated above, the variations in this case may arise from different temperatures and ammonia concentrations.

Similarly if the results from the separations conducted with different ammonia concentrations are plotted the significant effects of the large changes in the ammonia concentration can be seen. The positions on the plot of the three other brands of

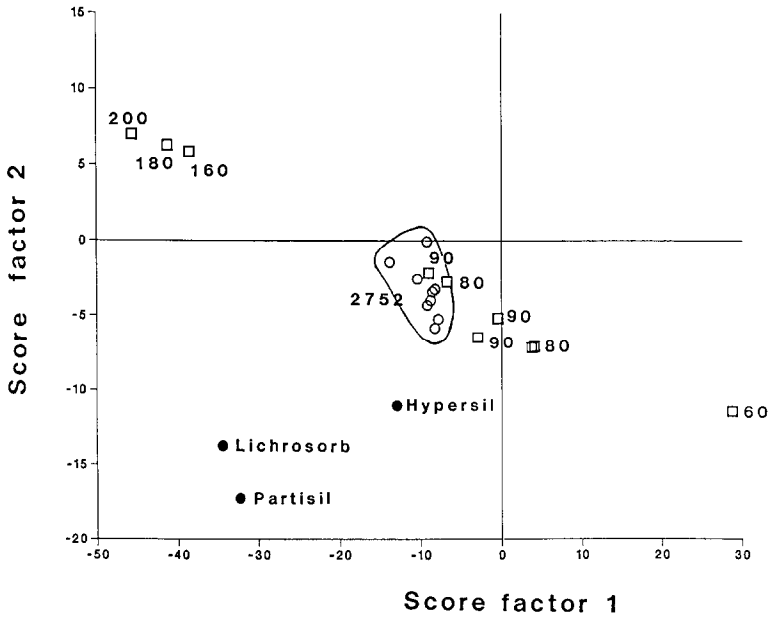


Fig. 4. Calculated first two principal component scores for changes in the conditions of the separation of the simplified test mixtures based on relative capacity factors for seven test drugs (dipipanone, promazine, codeine, prolintane, phenylephrine, ephedrine and strychnine) compared to protriptyline: ○, replicate separations on a single batch of Spherisorb S5W (batch No. 2752); □, different ammonia concentrations as indicated; other brands of silica (●); H, Hypersil; L, LiChrosorb; P, Partisil.

stationary phase can be calculated and, as expected, these are markedly different (Fig. 4).

## CONCLUSIONS

With the present HPLC system for basic drugs the large differences in the retention properties of different commercial brands of silica mean that for reproducible results to be obtained in interlaboratory studies each laboratory must standardise on the same brand of packing material. However, the retention properties of several basic drugs have been shown to vary on different batches of a single brand of silica. Consequently standardisation should involve ideally a single batch of packing material. This may be possible within one organisation, as has been achieved in U.K. forensic science laboratories, but poses severe limitations on the generation of retention databases for more general use. The results have shown that batches manufactured over a short period of time can show significant variations and the matching of batches as old materials are used up and replaced by new ones can only be accomplished through trial and error. As yet no simple method can be used to predict which column materials will give similar results.

Subsequently a number of these batches were used in an international collaborative study but the interlaboratory variation obscured any differences between batches confirming that although the ammonia concentration and temperature can be controlled within one laboratory, when different labororatories are involved, the variations can be too large. Work is therefore in progress to devise an alternative mobile phase to increase the robustness of the assay and temperature control would appear to be essential.

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