CHROMBIO. 5255

Evaluation of drug-free plasma profiles by high-performance liquid chromatography following on-line solid-phase extraction

MARY T. KELLY and MALCOLM R. SMYTH*

School of Chemical Sciences, Dublin City University, Glasnevin, Dublin 9 (Ireland)

and

HEATHER RUSKIN

School of Computer Applications, Dublin City University, Glasnevin, Dublin 9 (Ireland)

(First received November 16th, 1989; revised manuscript received February 1st, 1990)

SUMMARY

The effect of varying the type of column and eluent composition on drug-free plasma profiles was investigated. The study was based on a C_{18} and a CN column; methanol and acetonitrile were the organic modifiers used. The plasma profiles were evaluated quantitatively by measuring the number of interfering peaks greater than $8 \cdot 10^{-4}$ absorbance units in the area of interest along the chromatogram. Results were subjected to statistical treatment using a three-factor analysis of variance design. The three factors were the column, the type of organic modifier and either the percentage organic modifier, the pH or the ionic strength. Analysis of the data revealed that significant effects were seen with changing eluent composition, particularly with regard to the percentage of organic modifier, and that the observed effects were strongly dependent on the type of column and the type of organic modifier under consideration.

INTRODUCTION

The most widely used column packings for modern high-performance liquid chromatography (HPLC) are those with surface-reacted (chemically bonded) organic stationary phases. The most popular reversed-phase columns contain porous silica particles surface bonded with C_{18} or C_8 alkyl chains. The difference in chromatographic properties between these packings is subtle rather

than major, and probably most, if not all, separations can be achieved on either column [1]. Any apparent differences in selectivity between commercial C_{18} and C_8 phases are probably overshadowed by selectivity differences among columns from different manufacturers [2]. Selectivity differences are less likely between these two packing materials and other, more polar, bonded phases, such as cyano or phenyl columns which can also be used in the reversed-phase mode if adequate water is incorporated into the mobile phase. For example, the cyano-bonded phase provides good selectivity for separating tricyclic antidepressants [3–5].

Several workers have investigated the mechanisms of interaction involved in reversed-phase chromatography [6–8], and an exhaustive study on retentivity and selectivity involving numerous commercially available packings was undertaken by Goldberg [2]. The role of the column in reversed-phase chromatography has been extensively reviewed [9–12], and there are abundant data in the literature on the in-house preparation and evaluation of packing materials which are tailored by the experimenters to suit their individual needs. The range of packing materials which have been produced is extremely large, and it is beyond the scope of the present study to discuss them in detail.

Much data have been presented regarding the efficacy of various extraction schemes designed to effect removal of drugs from plasma, serum or urine, but this information is usually generated only in relation to the separation of the compounds of interest to the experimenter presenting the results. To date, there has not been a systematic study undertaken on the characterisation of stationary phases purely in regard to the separation of extracted plasma components. The closest approximation to a comprehensive study in this area was an investigation by Blanchard [13] into the relative merits of different methods of protein precipitation which would liberate drugs from binding sites and render the plasma suitable for direct injection onto a chromatographic column.

The objective of the present study was to determine how drug-free plasma profiles on two different analytical columns (C_{18} and CN) would be affected by varying mobile phase compositions. The variables in the eluent were the type (methanol or acetonitrile) and percentage of organic modifier, and the pH and ionic strength of the aqueous component. The plasma which was used was pooled at the outset so that this would not provide a source of variation during the course of the study, and it was extracted using the on-line column-switching technique in order to permit rapid analysis and to minimise the formation of artefacts. The plasma profiles were evaluated in terms of the number of interfering peaks in the area of analytical interest along the chromatogram, and these data were incorporated in a factorial design, the first two factors in each case being the column and the type of organic modifier with either the percentage of organic modifier, pH or the ionic strength as the third factor.

EXPERIMENTAL

Reagents and solvents

Methanol and acetonitrile (HPLC grade) were obtained from Labscan Analytical Sciences (Dublin, Ireland). AnalaR-grade sodium acetate and analytical-grade acetic acid were supplied by BDH (Poole, U.K.) and Riedel de Haen (Hannover, F.R.G.), respectively. Frozen plasma was received as a gift from the Institute of Clinical Pharmacology (Dublin, Ireland). Deionised water was obtained by passing freshly distilled water through the Millipore Milli-Q water purification system.

Plasma solutions

Small pools of mixed batches of frozen drug-free plasma were thawed at room temperature and then pooled to produce a single batch. The plasma was divided into 10-ml aliquots (in glass vials) and placed into deep freeze storage at $-20°C$. When required for use, one of these fractions was thawed at room temperature, vortexed for 30 s and diluted 1:1 with deionised water to generate 'blank' plasma solutions.

Instrumentation and operating conditions

Plasma extracts were separated on a Spherisorb (Phase Separations, Clywd, U.K.) ODS1 Column (10 μm) and a Spherisorb CN column (10 μm) (both 250 mm $\times$ 4.6 mm I.D.). The guard column was a Chrompack (Middelburg, The Netherlands) module containing a stainless-steel column dry-packed with Corasil (Waters Assoc., Milford, MA, U.S.A.) C_{18} material (37–50 μm) or Supelco (Bellefonte, PA, U.S.A.) CN material (24–40 μm).

A stock solution of 1 M sodium acetate was prepared by dissolving the appropriate weight of substance in deionised water. The pH of this solution was then adjusted by the addition of 0.1 M acetic acid. The pH was adjusted using a standard glass electrode which was calibrated daily using aqueous standards prepared on a weekly basis. The desired ionic strength was obtained by dilution with deionised water. Mobile phases were made by mixing the aqueous component with the required volume of methanol or acetonitrile. The mobile phase was passed through a 0.45-μm filter and degassed by sonication prior to use.

The eluent was delivered by a Waters Model 501 HPLC pump (pump B) at a flow-rate of 1.0 ml/min. The plasma was introduced into the system using a Rheodyne (Cotati, CA, U.S.A.) Model 7125 six-port injection valve. For the purposes of extraction by column switching, the injector was fitted with a 500-μl loop, and a second pump (pump A) and the concentration column were connected to the analytical assembly via a Rheodyne Model 7000 six-port switching valve. All extractions were carried out on a 10 mm $\times$ 1.5 mm I.D. concentration column packed with Corasil C_{18} material. The washing eluent delivered by pump A to the concentration column was deionised water which

had been filtered through a 0.45-μm membrane and degassed by sonication. It was delivered at a flow-rate of 1.0 ml/min for 90 s. The instrument arrangement and operation of the column-switching assembly have been previously described [14].

Detection was by ultraviolet absorption at 254 nm using a Shimadzu (Kyoto, Japan) Model SPD 6A spectrophotometric detector with a sensitivity setting of 0.01 a.u.f.s. The resultant chromatograms were recorded using a Linseis (Selb, F.R.G.) recorder at a chart speed of 200 mm/min.

RESULTS AND DISCUSSION

The project was limited to the investigation of two columns. A C_{18} column was chosen because it is the most widely used stationary phase in biopharmaceutical and many other types of separations. C_8 was rejected as the second column since, as mentioned by Giese [1], the difference in chromatographic properties between C_{18}- and C_8-bonded materials is subtle rather than major. Chromatographic modes such as normal phase were not considered, since it was sought to use the same mobile phases on both columns. The cyano column was chosen since it can be used in both the normal- and reversed-phase modes and has been claimed by Massart and Buydens [15] as an almost universal stationary support. In view of the differences in columns from one manufacturer to another, both the columns were Spherisorb (10 μm), supplied by Phase Separations.

Methanol and acetonitrile were chosen as the organic solvents since they are the most commonly used organic modifiers and differ in their solvent strength and slightly in their selectivities. The chosen range was 30–80% which reflects practically the complete range which would normally be used on both columns. The aqueous phase of the eluent was buffered with sodium acetate since this buffer is widely used in biopharmaceutical applications. The pH of the aqueous component in the mobile phase was varied between 3.0 and 7.0, which represents the range where many separations are carried out. The effect of ionic strength of the aqueous component was studied since it can also affect retention, especially on C_{18} columns where separations involving basic compounds can proceed through ionic interactions with residual charged silanol groups on the column surface. The ionic strength was varied between 0.005 and 0.1 M, although the latter concentration is probably outside the upper limit of ionic strengths commonly encountered in reversed-phase applications. This broad range was selected in order to demonstrate the existence of an effect which tends to be less manifest on retention than other factors such as the type and percentage organic modifier.

As it would have been a major undertaking to study all possible combinations of pH, ionic strength and percentages of organic modifier in the ranges listed above, it was decided to set some variables as constant while examining others.

In addition, evaluation would have been difficult in a more elaborate study, as a high-order multi-factor design would have been required, and these schemes are complicated in their interpretation, especially if many-way interactions were present. The on-line column-switching technique of extraction was employed in the interests of analytical expediency and to minimise the formation of artefacts which can pose considerable problems with liquid–liquid extractions.

For each column–mobile phase combination studied, analysis was carried out on three replicate injections of blank plasma. The reasons for choosing a relatively small number of replicates were that surface modification of the concentration column occurring over a large series of injections might influence findings, in addition to the fact that it was not the long-term reproducibility of the concentration column which was under consideration. A large number of replicate injections would not offer significant advantage over a smaller number in this case, since a plasma profile within a given set of experimental parameters depends on how many injections have preceded it. While it is recognised that this time effect is important, it was beyond the scope of the present study to take account of its contribution to the observed results, and measuring three replicate injections was settled upon as a suitable compromise, where any time dependence or serial correlation of responses was likely to be relatively unimportant.

From the foregoing discussion it is evident that a new concentration column would be necessary for each mobile phase on both columns. The (concentration) column-to-column variability on both a between-day and within-day basis was considered lest a large variation between columns was serving to complicate interpretation of the data. The results of this exercise are discussed later.

Having defined the variables and selected the extraction procedure, the next step was to decide how the blank plasma chromatograms would be evaluated. Obviously, the criterion was that there would be no interfering plasma peaks in the area of chromatographic interest. In regard to the latter, the area equivalent to the first 5 min on the chromatogram were eliminated since it normally contains the large early-eluting plasma peak. Measurements would be made over a time frame of 15 min (from 5 to 20 min) since the analyst would normally endeavour to execute a chromatographic run in 20 min or less.

If it is desired to quantitatively characterise a large series of chromatograms, such as was the case in the present study, some kind of response variable must be sought. If an electronic data processor (integrator) had been used, it would have been possible to measure the areas under the individual interfering peaks in the chromatogram, and to sum these if so desired. It would, however, have been a difficult task to measure the area under each peak manually using the chromatograms generated on the X–t recorder employed in this study, as the peaks were usually quite small and of indeterminate shape. Another possibility

was to measure peak heights and to use the total peak height of all interfering peaks in each chromatogram as a response factor. This approach would not, however, account for the number and location of the peaks contributing to the overall peak height. What appeared to be a practical alternative was to measure the number of interfering peaks in the area of interest and to use this as a response variable in evaluating the chromatogram.

In assessing the data, it became obvious that a large number of very small deviations around the baseline appeared in most chromatograms; though clearly they originated in the plasma, they could not be defined as 'peaks' per se, being too small and ill-defined to qualify for that definition. And since these small deviations would not, in most cases, interfere with drug quantitation, it was decided that only peaks or deviations greater than $8 \cdot 10^{-4}$ absorbance units would be counted for the purposes of chromatogram evaluation.

Since the response variable was dependent on various factors, a three-way factorial design was employed to establish whether the differences between the means (of the three replicate injections) were too great to be explained by random error. An analysis of variance (ANOVA) was carried out by computer using the Statgraphics package. A three-way design was used to assess the effects of type of organic modifier, column and either percentage of organic modifier, pH or ionic strength on the number of interfering peaks. The significance of the three possible two-factor interaction term within each experiment was also examined in order to determine how strongly the effect seen by changing one factor was dependent on the other two. The three-factor interaction terms were not computed, since the two-factor interactions are easier to examine visually, and, hence, the kinds of trends which merit more sophisticated analysis may be readily identified. A two-way ANOVA was performed on the data generated in the reproducibility study as there were, in this case, only two variables (concentration column and day).

Effect of percentage organic modifier on the number of interfering peaks

The ANOVA table for the three-factor design in which the sources of variation and their corresponding F-ratios are presented is shown in Table I. As may be seen from this table, there was, if one considers the F-ratios, a substantial difference between the various levels of organic modifier over the means for both columns and types of organic modifiers. The results also show that the observed effect with changing organic modifier concentration was strongly dependent on both the type of column and the type of organic modifier employed, indicating a strong interaction between the percentage organic modifier and these factors.

The above effects may be demonstrated graphically by constructing plots of the relevant mean values. Fig. 1 shows a plot of peak number at each level of organic modifier versus the percentage organic modifier. In this case, the means of twelve values (i.e. two columns, two types of organic modifiers, three repli-

TABLE I

EFFECT OF PERCENTAGE ORGANIC MODIFIER ON THE NUMBER OF INTERFER-
ING PEAKS

Analysis of variance for three-factor design.

Source of variation[a]	Sum of squares (SSQ)	Degrees of freedom (DOF)	Mean square (MSQ)	F-Ratio
Main effects				
A	13.8	5	2.8	3.6[b]
B	82.3	1	82.3	106.8[b]
C	0.1	1	0.1	0.2[c]
Two-factor interaction				
A–B	153.7	5	30.7	39.9[b]
A–C	35.8	5	7.2	9.3[b]
B–C	2.3	1	2 3	3.0[c]
Residual	40.9	53	0.8	—
Total	328.9	71	—	—

[a]A = Percentage organic modifier; B = column (C_{18} or CN); C = type of organic modifier (metha-
nol or acetonitrile).
[b]Significant at 5 and 1% levels.
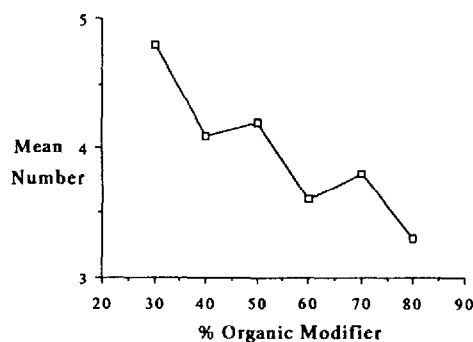[c]Not significant

Fig. 1. Plot of peak number versus percentage organic modifier: mean of types of column and
organic modifier Mobile phase: 0.025 M acetate buffer, pH 6.0–organic modifier, percentages as
per graph

cates at each level) were plotted. This plot shows that there was a decrease in
the number of interfering peaks as the amount of organic modifier in the mo-
bile phase was increased, though there were minor increases at the 50 and 70%
levels superimposed on the overall simple trend.

In order to examine this effect on the two different columns, the means of
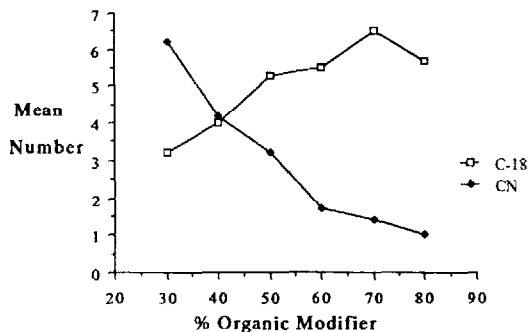the column–percentage organic modifier interaction terms were plotted as a

Fig. 2. Plot of peak number versus percentage organic modifier: breakdown on column type. Mobile phase: 0.025 M acetate buffer, pH 6.0–organic modifier, percentages as per graph.

function of the percentage organic modifier (Fig. 2). This plot shows that the effect of increasing organic modifier produced virtually the opposite effects on the C_{18} and the CN columns. These findings may be considered in terms of the elution strength of the various eluents not just on the analytical columns, but also on the concentration column following the wash cycle. Considering first the C_{18} column: using low levels of organic modifier, one would expect the plasma components swept off the concentration column to have long retention times (leading to the presence of many interfering peaks), due to the weak eluting power of the mobile phase on this highly non-polar phase. That the least number of interfering peaks was observed on this column at the lowest level of organic modifier (i.e. 30%) may be explained by the fact that the mobile phase also has weak eluting power on the concentration column and therefore only a portion of the plasma components remaining after the wash cycle were removed. Conversely, with a high proportion of organic modifier, more plasma was desorbed which produced a greater number of peaks in the chromatogram. As the level of organic modifier was increased from 70 to 80%, the number of peaks began to decrease. This is because rapid elution of the plasma components from the analytical column outweighs the adverse effects of greater plasma desorption from the concentration column with increasing eluent strength.

The opposite effect was seen with the CN column. The number of peaks decreased with increasing eluent strength in this case. As pointed out earlier, water is a stronger eluent on the more polar CN phase than the C_{18} phase, though the addition of the organic solvent enhances the solvent strength in relation to both supports. Even though more plasma was removed as more methanol or acetonitrile was added to the eluent, the overriding effect was decreased retention time for the interfering peaks which caused them not to appear in the area of interest in the chromatogram.
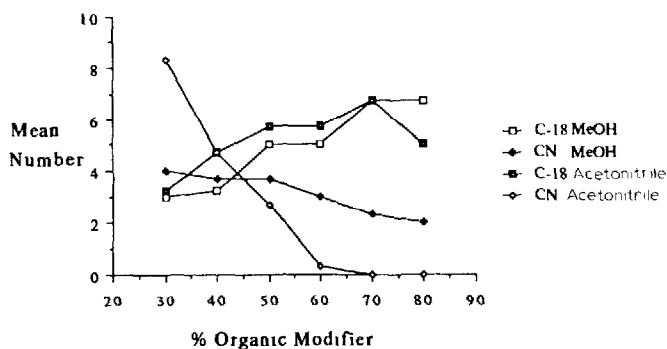
Fig. 3. Plot of peak number versus percentage organic modifier: breakdown on type of column and organic modifier Mobile phase: 0.025 M acetate buffer, pH 6.0–organic modifier.

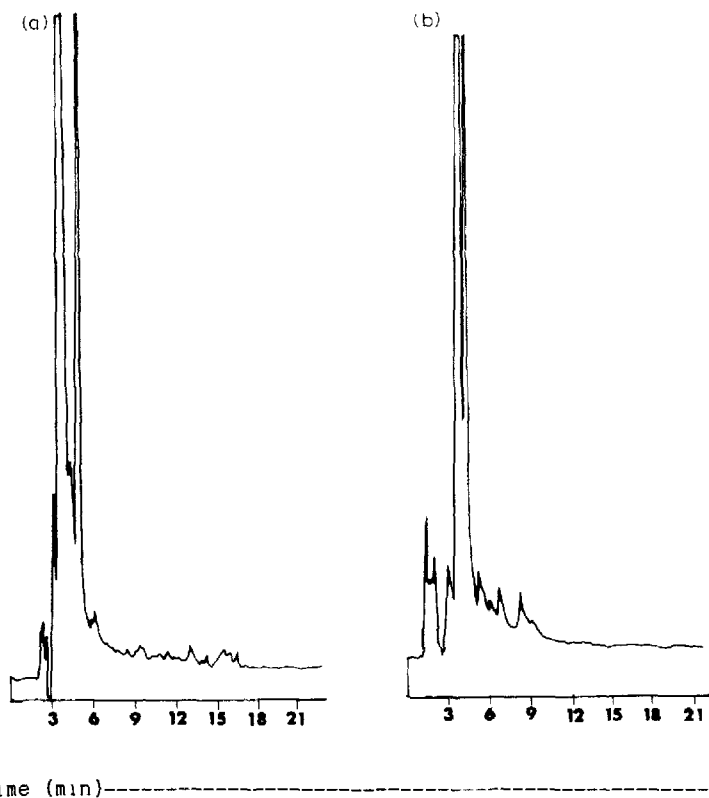Time (min)——>

Fig. 4. Chromatograms showing effect of organic modifier. Mobile phase: 0 025 M acetate buffer, pH 6.0 plus (a) 70% methanol or (b) 30% acetonitrile on the C_{18} column.

If it is desired to separate the effect of percentage organic modifier between the two columns and the two organic modifiers, the means of each cell (i.e., the three replicate analyses) may be plotted as a function of percentage organic

modifier (Fig. 3). This graph reflects the increase in the number of peaks on the C_{18} column and the corresponding decrease on the CN column. However, it further depicts how the decrease in peak number in going from 70 to 80% organic modifier on the C_{18} column was due entirely to the contribution from acetonitrile since the number of peaks remain constant between 70 and 80% methanol on this column. In addition, the decrease in peak number on the CN column is much more profound with increasing concentrations of acetonitrile rather than methanol.

The above points are illustrated chromatographically in Fig. 4. The chromatograms also serve to demonstrate that one set of parameters might be superior to another in one respect, but inferior in another respect. For example, in terms of peak number, 70% methanol on the C_{18} column offers a better proposition than 30% acetonitrile on the C_{18} column. However, by studying the chromatograms themselves, it can be seen that although the peaks are more numerous in the former case, they are larger in the latter case and more likely to operate to the detriment of a quantitative determination. This is an example of the limitation of the chosen response variable: the size of the peaks are not accounted for in evaluating the various mobile phases, and this can sometimes create ambiguity as to what constitutes an inferior or a superior chromatogram.

Effect of pH on the number of interfering peaks

The pH of the aqueous component was varied in 1-unit increments over the range 3.0–7.0. The ionic strength was set at 0.025 M, and a constant organic-to-buffer ratio of 1:1 (with both methanol and acetonitrile) was employed. This yielded twenty cells of three replicate injections. The three-way ANOVA table for the determination of the significance of the results is shown in Table II. The results show that over the means of both columns and types of organic modifiers, there was a systematic effect observed with changing pH. Similarly, there was a systematic effect seen between columns and between types of organic modifiers over the means of the remaining two factors. As evidenced by the very large F-ratio, there was a strong interaction between the pH and organic modifier, and an even larger interaction between the column and type of organic modifier; the pH–column interaction was marginally below the 5% level of significance, though again, it was non-negligible.

The graph in Fig. 5 was generated by plotting the mean peak number at each pH level versus pH. This graph shows that there was a general decrease in the number of interfering peaks with increasing pH. This effect may be explained by the fact that many endogenous plasma components are acidic in nature and, therefore, in more basic eluents they would become more ionised, tend less to partition into the stationary phase and would elute rapidly off the analytical column. A breakdown on the types of organic modifiers and columns (Fig. 6) reveals that with acetonitrile on both columns there is a marked decrease in the number of peaks with increasing pH. That at most pH levels there was a greater number of peaks seen with acetonitrile may be accounted for by the

TABLE II

EFFECT OF pH ON THE NUMBER OF INTERFERING PEAKS

Analysis of variance for three-factor design.

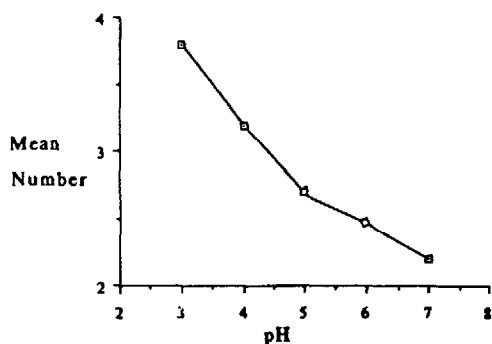| Source of variation[a]	Sum of squares (SSQ)	Degrees of freedom (DOF)	Mean square (MSQ)	F-Ratio
Main effects				
A	16.4	4	4.1	8.4[b]
B	11.3	1	11.3	23.1[b]
C	29.4	1	29.4	60.0[b]
Two-factor interaction				
A–B	4.9	4	1.2	2.4[c]
A–C	16.4	4	4.1	8.4[b]
B–C	56.1	1	56.1	114.5[b]
Residual	21.4	44	0.5	–
Total	155.9	59	–	–

[a] A = pH, B = column (C_{18} or CN); C = type of organic modifier (methanol or acetonitrile).
[b] Significant at 5 and 1% levels.
[c] Not significant.

Fig. 5. Plot of peak number versus pH: mean of columns and types of organic modifier. Mobile phase: 0.025 M acetate buffer–organic modifier (1:1), pH as per graph.

fact that it removed more plasma from the concentration column than methanol, and thus more peaks appeared on the chromatogram at the 1:1 buffer-to-organic ratio employed in this experiment. The situation is less clear with methanol. On the CN column, the general tendency was for a decrease in peak number to occur, with a small increase between pH 5.0 and 6.0. On the C_{18} column, there was a greater number of peaks in the chromatogram at higher pH values. This was, however, a small increase in peak number and, from a
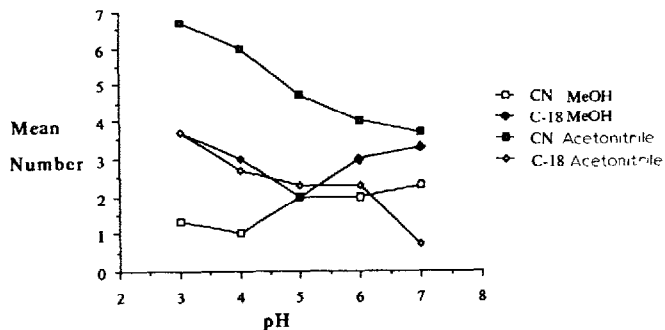
Fig. 6. Plot of peak number versus pH: breakdown on type of column and organic modifier. Mobile phase: 0.025 M acetate buffer–organic modifier (1:1), pH as per graph.

chromatographic point of view, would not represent a serious deterioration in chromatogram quality.

Effect of ionic strength on the number of interfering peaks

The ionic strength of the aqueous component was varied between 0.005 and 0.1 M acetate buffer at a constant pH of 6.0. Mobile phases containing either methanol–buffer (1:1) of acetonitrile–buffer (1:1) were prepared. The three-

TABLE III

EFFECT OF IONIC STRENGTH ON THE NUMBER OF INTERFERING PEAKS

Analysis of variance for three-factor design.

Source of variation[a]	Sum of squares (SSQ)	Degrees of freedom (DOF)	Mean square (MSQ)	F-Ratio
Main effects				
A	13.4	3	4.5	11.2[b]
B	14.1	1	14.1	35.2[b]
C	30.1	1	30.1	75.2[b]
Two-factor interaction				
A–B	5.1	3	1.7	4.2[c]
A–C	6.4	3	2.1	5.2[b]
B–C	36.7	1	36.7	91.8[b]
Residual	14.1	35	0.4	–
Total	119 9	47	–	–

[a]A = Ionic strength; B = column (C_{18} or CN); C = type of organic modifier (methanol or acetonitrile).
[b]Significant at 5 and 1% levels.
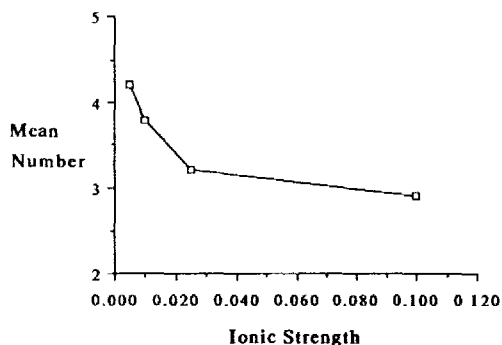[c]Significant at 5% level only.

Fig. 7. Plot of peak number versus ionic strength: mean of columns and organic modifiers. Mobile phase: acetate buffer, pH 6.0-organic modifier (1:1), ionic strength as per graph.
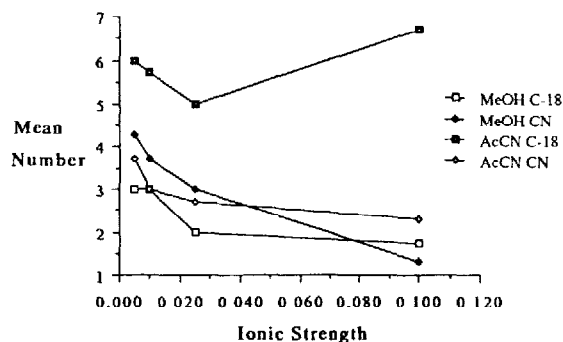

Fig 8. Plot of peak number versus ionic strength: breakdown on types of column and organic modifier. Mobile phase: acetate buffer, pH 6.0-organic modifier (1:1), ionic strength as per graph.

way ANOVA table used to determine the significance of the observed results is presented in Table III. From these data it may be seen that there was a significant difference between the columns and between the organic modifiers as indicated by the large F-values. There were also significant interactions between the ionic strength and the columns and the ionic strength and the types of organic modifier. By plotting the number of peaks (averaged over both columns and organic modifiers) as a function of ionic strength (Fig. 7), it may be seen that there was a general decrease in peak number with increasing ionic strength. By plotting the mean number of peaks in each cell (Fig. 8), it becomes apparent that, except for acetonitrile on the C_{18} column, the changing trend was for the number of peaks to decrease with increasing ionic strength. The effect appeared to be most pronounced for methanol on the CN column.

The effect of ionic strength in reversed-phase chromatography is more difficult to define than in ion-exchange chromatography. In the latter case the effect of ionic strength is readily explained in terms of the number of ions available to compete with solute molecules for sites on the column surface.

With reversed-phase chromatography, the same argument has been put forward to explain the behaviour of strongly basic amines in reversed-phase systems, i.e. that ionised bases interact with oppositely charged residual silanol groups on the reversed-phase column, and therefore their retention times are strongly affected by ionic strength [16,17]. It is known that species which are uncharged under the employed experimental conditions are also affected by ionic strength [16–18], though the nature of these effects have not been fully clarified. Snyder and Kirkland [18] proposed that when the addition of a salt to the mobile phase increases the solubility of a solute in the mobile phase, retention decreases, and that when it decreases the solubility of the solute in the mobile phase, retention is increased. Hence, the results in the present study might possibly be explained by a 'salting-in' effect which lead to decreased retention of the peaks, thus causing them to elute near the solvent front and be undetectable in the area of interest along the chromatogram. This is, however, a tentative speculation, since the natures of the interferences have not been identified, and it is also possible, if they possessed a basic function, that decreased retention is due to reduced competition at the charged silanol sites.

Concentration column reproducibility

In order to establish that the differences between concentration columns themselves were not contributing significantly to the trends observed in the foregoing experiments, reproducibility among different concentration columns was measured both on a within-day basis and a between-day basis. Three replicate injections were analysed on each of three Corasil C_{18} concentration columns on each of three consecutive days (9 columns, 27 injections). The analytical conditions used were a mobile phase of 0.025 M sodium acetate, pH 6.0–acetonitrile (1:1) on the C_{18} analytical column. Having counted the number of peaks greater than $8 \cdot 10^{-4}$ absorbance units, the results were analysed by carrying out a two-way factorial analysis in order to identify significant differences among the columns within days and between days.

The results of the ANOVA analysis are presented in Table IV. These findings show that the differences among the columns, on either a within- or between-day basis, were not statistically significant, though, as indicated by the higher F-ratio, the between-day variation was, as expected, greater than the between-column variation. The results further demonstrate that the day–column interaction was not significant indicating that the variation in peak number between columns is not dependent on the day the analysis was carried out. Based on these results, it can be concluded that variation among concentration columns did not contribute significantly to the systematic effects seen in previous experiments.

TABLE IV

REPRODUCIBILITY STUDY OF CONCENTRATION COLUMNS

Analysis of variance for two-factor design.

Source of variation[a]	Sum of squares (SSQ)	Degrees of freedom (DOF)	Mean square (MSQ)	F-ratio
Main effects				
A	0.5	2	0.3	1.0[b]
B	0.1	2	0.1	0.1[b]
Two-factor interaction				
A–B	0.8	4	0.2	0.8[b]
Residual	4.7	18	0.3	–
Total	6.1	26	–	–

[a]A = Day; B = concentration column number.
[b]Not significant.

CONCLUSION

The difference in selectivity between a C_{18} and a CN column for extracted plasma components has been demonstrated. It has been shown that the percentage of organic modifier exerts a profound effect on blank plasma profiles and that this effect is strongly dependent on the column and on the type of organic modifier employed. Based on the chosen response variable, best results were obtained on the cyano column at high percentages of acetonitrile and on the C_{18} column with low percentages of methanol. The number of interfering peaks was also affected by eluent pH and ionic strength, though not to the same extent as by percentage organic modifier. The observed effects were also dependent on the type of column and organic modifier. Using the number of peaks criterion, the differences with changing pH and ionic strength seemed greater than was shown by the general appearance of the chromatogram, which suggests that an alternative response variable may be more appropriate when evaluating the influence of these factors.

The three-factor design using the peak number response variable served to demonstrate the significance of the effects resulting from changing mobile phase composition, particularly in relation to percentage organic modifier. It also highlighted some very significant two-factor interactions within each set of experiments, and the significance of more than two of the three possible interaction terms in each case is strong presumptive evidence that a significant higher-order interaction is present. The significance of this three-factor interaction was not tested as the two-factor interactions are easier to visualise and interpret. It is, however, recognised that since this term was not extracted from

the residual term, the results for both main effects and interactions were conditional, and that this interaction may account for some findings, particularly in relation to the pH and ionic strength experiments.

The on-line extraction technique proved useful for the evaluation of plasma extracts in this study. Because of the speed and convenience of the method, a large number of samples could be analysed; because of the inherent reproducibility of the method, effects resulting from changing mobile phase composition were easy to identify. However, as the amount of plasma extracted is related to the analytical mobile phase, an interesting exercise would be to perform a similar investigation based on off-line solid-phase extraction or the more traditional liquid–liquid extraction. Such an approach would substantially increase the number of extraction schemes possible, thus adding considerably to the potential for further research in this area of biopharmaceutical analysis.

ACKNOWLEDGEMENT

REFERENCES

1 R.W. Giese, Clin. Chem., 29 (1983) 1331.
2 A.P. Goldberg, Anal. Chem., 54 (1982) 342.
3 The Supelco Reporter, 1 (1982) 8.
4 A. Power and D. Dadgar, Anal. Proc., 23 (1986) 416.
5 D Dadgar and A. Power, J. Chromatogr., 416 (1987) 99.
6 C. Horvath and W.J. Melander, J. Chromatogr. Sci., 15 (1977) 393.
7 W. Melander, D.E. Campbell and C. Horvath, J. Chromatogr., 158 (1976) 215
8 R.P.W Scott and C.F. Simpson, J. Chromatogr., 197 (1980) 11.
9 I. Halasz, Anal. Chem., 52 (1980) 1393A.
10 H F. Walton, Anal. Chem., 50 (1978) 36 (R).
11 B.L Karger and R.W. Giese, Anal. Chem., 50 (1978) 1048A
12 H.F. Walton, Anal. Chem., 52 (1980) 15 (R).
13 J. Blanchard, J. Chromatogr., 226 (1981) 455.
14 M.T. Kelly, M.R. Smyth and D Dadgar, J. Chromatogr., 473 (1989) 53.
15 D.L. Massart and L. Buydens, J. Pharm. Biomed. Anal., 6 (1988) 535.
16 E. Papp and Gy. Vigh, J. Chromatogr., 282 (1983) 59.
17 E. Papp and Gy. Vigh, J. Chromatogr., 259 (1983) 49.
18 L.R. Snyder and J.J. Kirkland, Introduction to Modern Liquid Chromatography, Wiley Interscience, New York, 1979.