The analysis of basic drugs by HPLC

THE ANALYSIS OF BASIC DRUGS BY HPLC

by

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Dedicated to my parents,
whose constant support and prayers
have been an invaluable source of encouragement
throughout these studies

If the Lord does not build the house,
in vain do its builders labour;
if the Lord does not watch over the city,
in vain does the watchman keep vigil.

In vain is your earlier rising,
your going later to rest,
you who toil for the bread you eat:
when he pours gifts on his beloved while they slumber.

(Psalm 126: 1-2)

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ABSTRACT

Two methods for the high performance liquid chromatographic analysis of basic drugs have been studied. In each case the methods have concentrated on separating drugs of forensic interest, based on specially designed test solutions of analytes selected to represent most of the commonly encountered classes of drug compounds.

In the first case, a HPLC method was developed on unmodified silica using an aqueous methanolic eluent of high pH. The buffer was prepared from two organic sulphonic acid amines, 3-(cyclohexylamino)-1-propanesulphonic acid (CAPS) and sodium 3-(cyclohexylamino)-2-hydroxy-1-propanesulphonate (CAPSO-Na). The method was shown to be highly reproducible within a single laboratory. The long term stability of the unmodified silica stationary phase was examined, using the newly developed method for the analysis of drugs as a monitor of column performance. Three columns from a single batch of silica were studied, and all showed pronounced changes in retention properties over the period of the study. Similar changes in retention properties were observed for silica stored dry and unused. These results led to the idea that an 'aging process' was changing the nature of the silica surface, probably by a process of hydrolysis of surface siloxanes. This aging process was also believed to be responsible for the appearance of distorted peak shapes for methylamphetamine, although no clearly defined mechanisms could be found to support this idea.

In a second study, the application of gradient HPLC methods for the screening of a wide range of analytes was examined. In this case, an Inertsil ODS-2 column was used in conjunction with an acid buffered acetonitrile / water gradient. The usefulness of the 1-nitroalkanes as a retention index series was demonstrated and a good level of retention reproducibility was achieved for most analytes studied.

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SECTION 1: BACKGROUND

CHAPTER 1: INTRODUCTION TO THE CURRENT STUDY

During the past twenty years the use of high-performance liquid chromatography (HPLC) has grown rapidly. Today it is one of the most widely used techniques in the fields of chemical toxicological analysis[1], therapeutic drug monitoring[2,3], drug purity testing[4] and forensic drug analysis[5]. HPLC has a number of major advantages which make it particularly suitable as a screening technique for multicomponent mixtures. These have been summarised by Eremin and Izotov[1], who identified simplicity of technique, specificity, precision and the non-destructive nature of HPLC to be of great importance in chemical toxicological analysis.

During this period the rapid development of powerful personal computers and expert systems for HPLC has presented the analyst with the possibility of automated method selection[6] to aid method development, or the chance of retention prediction[7] and automated solute identification[8,9]. Furthermore, the use of computer library search routines[10,11] presents the analyst with the opportunity to make a tentative identification of an analyte from a single separation. This application is most often found in the emerging area of '3-D techniques', such as HPLC-FTIR, HPLC-MS and HPLC-photodiode array detection[12]. In addition, the use of computers makes automation of HPLC methods possible; Cizmarik *et al.*[13] identified this as an advantage for applications in the Pharmacopoeial quality control of drugs.

In the analysis and identification of drug compounds and formulations by HPLC, a vast range of methodologies have been developed, based essentially on a few general techniques using different stationary phases. These methods can be identified as:

i) isocratic ion-exchange type separations on bare or RP-silica[14-40],

ii) ion-pair separations, frequently carried out on RP-silica[41-49],

iii) separations using RP-silica and amine modified eluents[48,50-63],

iv) separations on dynamically modified silica[64-67],

v) and separations on specialised stationary phases,
e.g. alumina[29,30,68,69],
chiral phases[70],
polystyrene-divinylbenzene[71,72], and
internal surface reversed-phases[73,74].

Various authors have addressed the importance of method development and selection techniques in order to reduce the number of different HPLC systems installed in a single laboratory. These have included combined extraction and HPLC selection routines for biological samples[52,53], optimisation of normal and RP separations[75,76], and method validation and selection of the best method for pharmaceutical analysis[77,78].

The vast array of stationary phases available offers the analyst the opportunity to select the most suitable material for a specific separation, although this may lead to confusion as there are many different materials available for each application. For example, the current literature suggests that new materials come onto the market at the rate of almost one per week[79,80], covering areas as diverse as reversed-phases, speciality phases (*e.g.* chiral materials) and column packings specifically for bio-molecules; hence the need for careful consideration when choosing a stationary phase. Furthermore, nominally identical stationary phases from different manufacturers often show differences in retention properties[13,81], thus making the selection of a single type of material more difficult.

Cizmarik *et al.*[13] noted that the stationary phase plays a vital role in the separation process, and that nominally identical stationary phases often differ widely in their characteristics. Whilst acknowledging that HPLC has a positive role in the future of Pharmacopoeias, they expressed concern that detailed specifications for a method could lead to the false impression that results obtained by one author could be readily reproduced elsewhere without careful control of the stationary phase used. Similar comments have been made by De Zeeuw[8] with respect to the applications of HPLC to systematic toxicological analysis. However, it has been shown by Gill *et al.*[33,34] in two inter-laboratory studies that a reasonable degree of reproducibility can be achieved provided only one batch from one brand of stationary phase is used. The problem of inter-laboratory reproducibility has itself been the subject of a recent article[82].

Problems associated with the complex retention mechanisms on ODS silicas used in the separation of basic drugs have received much attention[17,31,43,54,55,83]. Of particular concern has been the problem of poor peak shapes for basic drug compounds in RP-HPLC[18,43,81,83,84], and many efforts have been made to improve results. Frequently these involve the modification of the mobile phase in an attempt to mask the unwanted interactions from residual silanols on the silica surface[51,58]; a review of recent techniques has been published[48]. Alternative methods designed to overcome this problem have concentrated on better preparation of the stationary phase. These have included attempts to improve the homogeneity of the silica surface prior to modification[85,86], the use of highly pure silica in the preparation of RP materials[87-89] and studies aimed at trying to understand the important factors in the preparation of RP materials[90,91]. Only with the recent development of new base-deactivated RP materials has it become possible to obtain good peak shapes for basic compounds using simple, unmodified eluents[62,92,93].

Many workers have studied methods for the analysis of basic drugs in which bare silica has been used as an alternative to ODS materials. Hansen and co-workers have presented a series of papers dealing with 'dynamically modified silica' as a stationary phase for basic analytes; this work has recently been reviewed[65,66]. It was found shown that this method is relatively insensitive to the bare silica used[94] because the process of dynamic modification, in which the surface of the silica is coated with an amine modifier (typically a quaternary ammonium salt), promotes a retention mechanism which is predominantly one of hydrophobic interaction between analyte and stationary phase[65]. Other techniques use bare silica and exploit the presence of silanols on the silica surface to give ion-exchange separations. These include the use of methanolic eluents of high pH, often prepared with aqueous ammonia buffers which promote a negatively charged silica surface, thus permitting ion-exchange with cationic solutes[15,23,24,27,33,35,36,38,95]. Other systems include methanolic perchlorate eluents[25-27], methanol / methylene chloride[16] and methanol / ethylenediamine buffer[37] eluents. It is widely recognised that the retention mechanisms are more complex than a pure ion-exchange mode, and that hydrogen-bonding and dispersive forces are also important[17,18,21,31].

Previous studies in the laboratories at Loughborough led to the conclusion that ammonia based eluents were unsuitable for basic drug analysis[36] and that it would be desirable to devise an alternative mobile phase of high pH. In the current project, initial work was aimed at developing a simple, robust and reproducible high pH eluent for the analysis of basic drugs of forensic interest. It was hoped that this would also lead to a greater understanding of the role of unmodified silica stationary phases in the chromatographic separation mechanism, and the associated problem of silica stationary phase reproducibility[36].

In a second, separate study, the development of gradient screening techniques for a wider range of analytes using simple mobile phases was also addressed. This was considered possible as a result of the emergence of new 'base deactivated' reversed-phase materials. The aim of this study was to develop a single HPLC system that could be applied to a wide range of analytes of different polarity, as many of the current techniques are of limited use due to the narrow range of compounds that can be analysed in a single separation.

CHAPTER 2: LITERATURE SURVEY

2.1 INTRODUCTION

Increasingly in our Western society, governments, customs officials and police are being confronted by the problems of drug trafficking, and the medical profession by the consequences of drug abuse and overdosing. In addition to the wide range of illicit drugs encountered by police and customs, the rapid development of the pharmaceutical industry has increased the number of drug formulations legitimately available to the general public.

Consequently there is a vital need for separation and identification techniques that provide high levels of discrimination and permit rapid determination of as many components as possible in a drug mixture. This is especially important in the areas of systematic toxicological analysis, where overdosing and poisoning are rarely caused by a single compound, and of forensic drug intelligence for tracing the origins of illicit drug materials. It is the role of forensic and clinical analytical chemists to develop methods for the analysis and identification of drug compounds, either as 'pure' samples or in biological media.

Over the past 15 years, high performance liquid chromatography (HPLC) has become one of the methods of choice for the analysis of drugs. In 1975 Twitchett and Moffat[14] reported the use of an octadecylsilane (ODS) stationary phase for the analysis of a wide range of drug substances, whilst Jane[15] reported the use of unmodified silica for the analysis of drugs of abuse. Since then a great deal of interest has been shown in the use of HPLC for analysing many different classes of drug substances. Such work has included separations of drugs of forensic interest[17-19,22,41,42,96,97], along with the analysis of specific classes of compounds, for example antihistamines[22], barbiturates[98-102], benzodiazepines[103], cardioactive drugs[28], local anaesthetics[21,56,57], non-steroidal anti-inflammatory analgesics[40], thiazide diuretics[104], and tricyclic antidepressants[21,55].

2.1.1 DATA BASES FOR DRUG IDENTIFICATION

The use of HPLC for the identification of drug compounds has been hindered by the lack of standard reference data. Numerous examples of separations of drugs have been reported, using a wide variety of stationary and mobile phases. In many cases, the methods have been developed to suit the authors' specific needs, and in the cases where wide ranges of compounds have been analysed, variations between results are often quite significant. For example, Baker *et al.*[19] reported that morphine and codeine could not be resolved on a bare silica column, but Wheals[18] found that these compounds could be separated on a different type of bare silica, even when the eluent conditions were the same as those employed by Baker[19].

In addition, Wheals[18] showed that different stationary phases gave different chromatographic separations and recently it was shown that nominally identical types of silica do not give the same results for a given separation[36]. Furthermore, different batches of a single type of silica, prepared by one manufacturer, often did not give the same results[36]. These observed variations were considered significant and in some cases changes in selectivity were found between different batches of the same material[36].

The irreproducibility of results between different stationary phases has hampered progress towards standardised reference data for HPLC systems. This problem is unlike that experienced in gas-liquid chromatography (GLC) or thin-layer chromatography (TLC) where it has been possible to produce large tables of reference data[105,106]. Consequently, whilst it is possible to transfer the data from GLC and TLC experiments from one laboratory to another, it is much more difficult to do the same for HPLC systems[34,82]. Indeed, it has recently been suggested by Logan *et al.*[107] that every laboratory should prepare its own data base of retention times to avoid the problems of interlaboratory variation.

This has led to attempts to try to understand more about the nature of the silica surface and the retention mechanisms that occur during the analysis of samples by HPLC. Such knowledge could allow the analyst to develop ways of controlling experimental conditions to improve reproducibility. Ultimately the aim of such studies would be to devise a

method for the reproducible preparation of the stationary phase which would make the standardisation of methods much simpler.

2.2 HPLC SEPARATIONS OF BASIC DRUGS

In order to examine the development of current methodologies for the separation and analysis of basic drugs it is necessary to understand the importance of the role of the stationary phase in the separation process. The early methods proposed by Jane[15] and Twitchett and Moffat[14] used 'unconventional' eluent systems, {note[1]}, such that the resulting separation mechanisms were not based solely on hydrophobicity, but included ion-exchange type interactions. These were later identified and explained by Sugden *et al.*[17], and Wheals[18], who rationalised the observed similarities between retention orders on bare and modified silicas under ion-exchange conditions. Wheals[18] concluded that modified silicas had unreacted silanol groups (≡SiOH) on the surface which participated in the retention process, thus rendering such materials similar in performance to bare silica.

It was also observed[14,18] that modified silicas gave poor peak shapes for basic drugs, thus making such methods unsatisfactory for the quantitative analysis of these samples. Many efforts have since been made to overcome the problem of poor peak shape, and the most successful have employed organic modifiers in the mobile phase. For example, amines[51,58], and mixtures of amines and sulphonic acids[46,47,49] have been used to mask the presence of the interfering silanol groups, thus allowing 'conventional' RP-type separations to be achieved.

In the following discussion of the development of HPLC methods for the analysis of drugs of forensic interest, particular attention has been given to each of these methods, i.e. ion-exchange type separations, and modified-eluent separations, including the use of dynamically modified silica. The application of 'true' RP-methods in drug screening has also been reviewed, and some other methods have been briefly examined.

Note 1. A conventional normal-phase system employs a polar stationary phase and a non-polar eluent (*e.g.* silica / hexane) and a conventional reversed-phase system is based on a non-polar stationary phase and a polar eluent (*e.g.* ODS-silica / methanol). The 'unconventional' systems referred to here employ either type of stationary phase and polar eluents containing pH modifiers.

2.2.1 ION-EXCHANGE SEPARATIONS

The introduction of isocratic HPLC methods for the separation of a wide range of drugs of abuse[14,15] was an important step forward in the field of forensic drug analysis. Until that time, the few existing methods generally required gradient elution and were restricted to small numbers of drug compounds (reference 15 and references therein).

The method reported by Jane[15] used bare silica and an ammonium nitrate - methanol eluent for the separation of stimulants, alkaloids, local anaesthetics, and other drugs of forensic interest. It was reported that changes in the retention of compounds could be affected by changing the methanol - water ratio, or by changing the concentration of the buffer components. As a general rule, it was noted that less basic analytes required a less basic, more aqueous eluent for elution. The basicity and steric environment of the nitrogen atom of the analyte were also important factors in controlling retention[15].

Twitchett and Moffat[14] reported that on an ODS phase their method, in which a mobile phase comprising methanol and aqueous phosphate buffers of varying pH was used, only gave satisfactory results for the analysis of acidic and neutral species, such as acetylsalicylic acid, paracetamol and barbiturates. The efficiency for basic drugs, such as amphetamine related compounds, was poor, detracting from the overall usefulness of the method. In their study they found that retention depended on the pK_a of the analyte, the proportion of methanol in the eluent and the lipid solubility of the unionised form of the drug in the stationary phase and the mobile phase. They found a high level of correlation between *n*-octanol-water partition coefficients for the analytes and their retention behaviour, and suggested that retention could be predicted for other substances if their pK_a and partition coefficients were known. (Although not stated, the dependence of retention on pK_a was such that, for most of the analytes, maximum retention occurred when the eluent pH was similar to the pK_a of the analyte).

A detailed examination of isocratic HPLC systems for the analysis of basic drugs was reported by Sugden *et al.*[17]. They examined the retention of some local anaesthetics, including cocaine and benzocaine, and a quaternary ammonium compound, amprolium, on an acid washed bare silica,

and an ODS-modified silica. In the chromatography of the local anaesthetics, it was found that eluents of pH 7 which were devoid of salts caused the analytes to display high affinity for both stationary phases. The strong interactions were believed to involve protonated analyte species and surface silanol groups. When the experiments were repeated at pH 3.3, which is below the isoelectric point of silica (pH 4.0), similar results were obtained, which led to the conclusion that the mechanism did not involve ion-exchange. However, the existence of highly acid silanol groups on the silica surface, capable of being ionised at even lower pHs than those used in the study, was not considered. Such groups, which had previously been identified[108,109], and have since been shown to influence the chromatographic properties of silica[110-113], could have played a significant part in the retention.

Addition of salts to the mobile phase caused the retention to decrease dramatically on both stationary phases: the results on the ODS-phase indicating that retention must still be governed by residual silanols, especially for those analytes with little affinity for the ODS moieties[17]. For the more hydrophobic analyte amylocaine, ion-pair partitioning and salting-out effects were believed to be more important in influencing the retention. The observed effects of ionic strength on retention led to the belief that, even for hydrophilic analytes, such as cocaine, mixed retention mechanisms existed, where ion-pair partitioning played a part in addition to the ion-exchange type interactions. It was also found that more acidic eluents resulted in lower retention on both columns, due to enhanced ion-pair formation: the effect of pH on the ionisation of surface silanols was not considered[17].

It was also found that amprolium, the quaternary ammonium compound, had similar retention characteristics on both phases[17]. It was noted that the cation-exchange properties of bare silica, caused by the surface silanol groups, might be an important factor in the retention mechanism. They suggested that on modified phases at higher eluent pH, ion-exchange with residual silanols may be taking place; their observations led them to rule out ion-pair partition mechanisms for this compound. Later studies by Reynolds *et al.*[114], using two model quaternary ammonium compounds on a C-18 column, indicated that ion-exchange with residual silanols was the dominant mechanism under these conditions. Thus it was concluded[17] that the retention mechanisms on the two phases were similar and that mixed

mechanisms were responsible for retention, as shown by non-linear relationships between capacity ratios and the inverse of the ionic strength. Pure ion-exchange mechanisms would lead to linear plots for this function, with the line passing through the origin[17,31]. Later observations by Cox and Stout[31] support this work and it was suggested that additional mechanisms, including adsorption and salting out effects may be responsible for the non-linearity observed.

A retention mechanism was proposed by Sugden *et al.*[17] based on ion-exchange between protonated analytes and a negatively charged silica surface[17]. The mechanism (described below) was shown to be dependent on the pK_a of the analyte, as only protonated species were retained. It was shown that selectivity could be achieved by variation of the mobile phase pH and ionic strength, and this was explained by a series of equilibria as described below.

The analyte, represented as R_3N, undergoes protonation and ion-pair formation, depending on its pK_a and the eluent pH and ionic strength:

$$R_3N + H^+ \overset{K_a}{\rightleftharpoons} R_3NH^+ \qquad (1)$$

and

$$R_3NH^+ + A^- \rightleftharpoons R_3NH^+A^- \qquad (2)$$

where A^- is any anion in the eluent. The retention of the analyte takes place on ionised surface silanols, *viz.*

$$\equiv SiOH \rightleftharpoons \equiv SiO^- + H^+ \quad \text{at pH} > 4 \qquad (3)$$

$$\equiv SiO^- + R_3NH^+ \rightleftharpoons \equiv SiO^-H^+NR_3 \qquad (4)$$

Competition for the retention sites is offered by any cation, C^+, in the eluent:

$$\equiv SiO^- + C^+ \rightleftharpoons \equiv SiO^-C^+ \qquad (5)$$

The ability to exploit surface silanols is governed by the equilibrium outlined in equation (3), which shows that at pH > 4 the surface hydroxyl sites become ionised, creating negatively charged sites for which positively charged species in the eluent will compete. The effect of eluent ionic strength, described by the competing equilibria

(4), and (5), along with adsorption and partition effects were shown to alter the retention of analytes.

Similar changes in retention with pH were observed by Crommen[20], when working with aqueous eluents and a small set of drugs and related compounds. Crommen[20] suggested that the increasing ionisation of the silica surface with increasing pH was an important factor in the retention of the analytes. It was also shown that retention was dependent on the type of counter ion in the eluent, as this controlled ion-pair formation (see equation 2), and by the presence of ions of the same charge, as these could alter the retention of the analyte by a 'competition' effect (*viz.* equation 5). In this case it was found that plots of inverse capacity factor versus competing ion concentration were not linear, but showed a pronounced tendency to curve at low concentrations and only approached linearity as the concentration increased. It was suggested that this might be caused by a limited number of strong adsorption sites on the silica surface, which would be covered first by the competing ion. It is possible that these 'strong adsorption sites' were highly acidic silanols which would have been ionised under the conditions used (eluent pH 2 - 4). (This observation parallells that of Sugden *et al.*[17], see above). When the concentration of the competing ion in the eluent reached a certain level these sites would be completely covered, giving rise to a more homogeneous surface, and hence a more linear relationship between inverse capacity factor and competing ion concentration.

Wheals[18] reported similar effects with changes in pH and ionic strength and noted that the effect of pH was complex because of the simultaneous changes in the ionisation of the silica surface and protonation of the analyte. This was backed up by the observation that changes in retention with pH for a series of analytes were not systematic, some compounds showing increases in retention and others decreases as the pH was reduced from 10 to 7. Although not stated, the data suggests that retention was at a maximum when the eluent pH was close to the pK_a of the analyte, with structurally related compounds showing similar results[18], as also found in the data presented by Twitchett and Moffat[14] and Sugden *et al.*[17].

It was suggested by Wheals[18] that a set of mechanisms played a part in the separation, and that ion-exchange was dominant on both bare and

modified silicas. This was explained in terms of the procedure for the production of RP materials, in which incomplete coverage of the bare silica would leave free (residual) silanols available to participate in the retention process. The results suggested that these residual silanols were sufficient in numbers to dominate the retention process, even on bonded phase packing materials. The complexity of the retention process was further discussed, and four possible mechanisms were proposed, all of which would play a part in the separation, with one, or possibly two dominating. The mechanisms were summarised as:

i) ion-exchange of ionised molecules;
ii) liquid-liquid partition of the free base;
iii) liquid-liquid partition of the ion-pairs formed by reaction of the ionised molecules and the eluent counter-ion (*e.g.* NO_3^-);
iv) ill-defined retention mechanisms, *e.g.* dipole interactions, binding by Van der Waals forces, hydrogen-bonding etc.

In a simplified explanation of the observations, it was suggested that type (i) and (iv) interactions dominated on unbonded silica, type (i) on certain types of modified silica, and partition processes (ii) and (iii) on, for example, a mercaptopropyl ($-Si-O-(CH_2)_3-SH$) modified silica[18].

The problem of residual silanols was described by Bidlingmeyer *et al.*[21], who showed that their presence led to excessive tailing of basic amine analytes. Materials with lower amounts of surface coverage of organic phase, and thus increased quantities of free silanols, were shown to give better peak shapes than 'well covered' ODS materials, whilst the best results were obtained on bare silica. These observations led to the suggestion that the surface silanols were not, of themselves, deleterious to the retention of the amines, and that the silica gel itself, (rather than the surface modified groups, *e.g.* ODS groups), was the preferred site for retention of the analytes. Thus, the poor results on 'well covered' ODS silicas were attributed to the inaccessibility of the remaining silanols: freely accessible groups led to good peak symmetry, whilst hindered access caused poor peak symmetry.

Despite these problems, Baker *et al.*[19] successfully employed an ODS material, used with a methanol - water mobile phase buffered at pH 7, in

the separation of a wide range of drug compounds, including alkaloids, opiates, amphetamines and barbiturates. They found high levels of correlation between drug lipophilicity and retention time. Such levels of correlation were not seen on two bare silica columns, although retention did appear to be related to drug polarity. Furthermore they reported that many of the basic drugs analysed on the ODS phase gave good peak shapes, in contrast to the observations made earlier by Twitchett and Moffat[14]. However, normal phase systems were found to give much greater efficiencies for most compounds, but no explanations were offered for the differences.

2.2.1.1 Effect of the operating conditions on ion-exchange separations

A number of important experimental parameters have been identified in separations which rely primarily on an ion-exchange retention mechanism. These are eluent pH and ionic strength, the eluent composition, (*i.e.* ratio of aqueous to organic phase in the eluent), and the operating temperature. In addition, the choice of the stationary phase can often influence the separation. Each of these factors is now discussed separately.

a) Eluent pH

As noted above, Jane[15] observed variations in retention with eluent pH, which in general led to the conclusion that less basic, more aqueous, eluents were better for less basic analytes. Data presented by Twitchett and Moffat[14] and Wheals[18] appears to confirm this observation, in that maximum retention occurs when the eluent pH is close to the pK_a of the analyte. These observations were supported by experiments performed by Flanagan and Jane[25,26] when using non-aqueous ionic eluents. They observed that retention was at a maximum at intermediate pH values and suggested that at low pH the ionisation of the silica surface was suppressed, whilst at high pH the ionisation of the analyte was suppressed. These changes in retention with pH were found to alter selectivity and it was suggested that this could be used to adjust the conditions to obtain a particular separation[25]. Further work by Flanagan[26,28] led to the conclusion the pK_a of the analyte was a good marker for predicting the eluent pH of maximum retention.

These observations can be explained in terms of an ionic interaction retention mechanism in which only charged species are retained[17]. At low eluent pH, the silica surface will be largely neutral in character, with only strongly acidic silanols ionised. Under these conditions, acidic drugs, *i.e.* compounds of low pK_a, will be ionised and retained on the few ionised sites. As the pH increases, the acidic drugs become unionised and are therefore unretained[26], whilst the silica surface becomes more negative in character[115], and the basic drugs, i.e. compounds with higher pK_a values, increase in retention[24,26,32,34] due to the increased ion-exchange capacity of the silica. At very high pH levels retention is reduced as analyte ionisation is suppressed[26,32], or as analytes become negatively charged (due to ionisation of, for example, phenolic groups) and hence repelled from the negatively charged silica surface.

In a systematic study of the variation of retention with eluent pH, Law[24], derived an equation relating log (capacity factor) to eluent pH and analyte pK_a:

$$\log k' \propto \log \left[\frac{K[SiO^-M^+]}{[M^+]} \right] - \log[1 + 10^{(pH - pK_a)}]$$

where k' is the capacity factor, K is the equilibrium constant for ion-exchange interactions on the silica surface, M^+ is any protonated counter ion in the eluent, and $[SiO^-M^+]$ is the concentration of ionised silanols occupied by counter-ions reflecting the dependence on eluent ionic strength, (see (b)). Law noted that this equation indicated a linear relationship between log k' and pK_a, for $pK_a < pH$, with an inflection tending to a plateau for $pK_a > pH$. His observations tended to support this theory, particularly for structurally related compounds, where at constant eluent pH, those of higher pK_a had larger capacity factors[24]. These results suggest that pK_a is indeed a good guide to retention prediction.

It should be noted that this explanation does not account for non-ion-exchange interactions, which also contribute to retention. In a study of pH effects over a small range (pH 9 to 10), Smith *et al.*[35], found some anomalous behaviour which could not be explained solely on the basis of analyte pK_a. Dipipanone, (pK_a 8.5) did not show the expected decrease in

capacity factor with increasing pH, but instead its retention increased slightly. Wheals[18] had also found little change in retention volume for dipipanone, even when recording results over a larger pH range (pH 7 to 10). In each case no explanation was offered for these observations, but the complexity of changing interactions with changing pH and the influence of chemical structure on the basicity of the compound were considered important[18,35].

b) Eluent ionic strength

In a separation based on an ion-exchange retention mechanism, the ionic strength of the mobile phase would be expected to influence the results. Sugden *et al.*[17] showed that separations involving cation exchange were affected by the concentration of other cations in the eluent. An increase in the concentration of the competing ion in solution caused a decrease in retention of the analyte; the absence of competing ions led to very long retention times.

Crommen reported that, in addition to concentration, the nature of the competing ion and the counter ion in solution was important[20], and that this applied to cationic, anionic and neutral species. This was explained in terms of competition for retention sites from ions of the same charge, which caused the retention to decrease, whilst counter ions caused increases in retention due to the formation of ion-pairs. For neutral species, the presence of cations or anions in the mobile phase caused decreases in retention, and this was attributed to competition between ions and neutral species for adsorptions sites on the silica surface.

The dependence on ionic strength has been described in terms of a linear relationship between the capacity factor and the inverse of the ionic strength[31,32]. Cox and Stout[31] derived an equation which showed that the distribution coefficient of an analyte between the stationary phase and the mobile phase is proportional to the ion-exchange equilibrium constant and the number of accessible ionised silanol groups, and the inverse of the competing ion concentration. Thus a plot of capacity factor, which is directly related to the distribution coefficient, against inverse ionic strength, would give a straight line, with a slope proportional to the ion-exchange equilibrium constant and the number of

ionised silanol groups on the silica surface (as also shown by Law[24] in the equation quoted above; see (a)). This was found to hold in practice, with plots giving a positive intercept at "infinite" competing-ion concentration. This was taken as evidence of a mixed retention mechanism, since at very high ionic strengths, ion-exchange of the analytes would not be possible (as all retention sites would be occupied by the competing ions)[31].

Alternatively, a plot of log (capacity factor) versus log (ionic strength) has been shown to give straight lines for some structurally similar basic analytes[26]. It was shown that the retention of strong bases decreased with increasing ionic strength and that the log / log plot was linear for compounds such as tricyclic antidepressants and amphetamine[26]. In addition, Flanagan *et al.*[26] reported that weak bases, such as benzodiazepines, did not behave in a uniform manner and that changes in retention order could be achieved by changing the ionic strength. It was suggested that, as the ionic strength was dependent on the concentration of perchloric acid, the reduction in acid concentration - which led to eluents of lower ionic strength - probably caused a reduction in the protonation of the analytes, which had a significant effect on the retention.

Smith *et al.*[35] reported similar results for a wider range of drugs, but in this case the benzodiazepines behaved 'normally', showing linear decreases in retention with increasing ionic strength (when plotting the data according to the log / log method[26]). However, the plot of relative capacity factor versus relative ionic strength showed some quite different trends, with some compounds showing increases in relative k' with increasing ionic strength, although their capacity factors decreased. Structurally related compounds appeared to give similar results, but the wide range of analyte types meant that significant changes in selectivity were recorded with changes in ionic strength.

c) Eluent composition

Many workers investigating the separation of basic drugs under ion-exchange conditions have employed methanol / water mobile phases, and have investigated changes in retention caused by changing the methanol content. Jane[15] reported that less basic, more aqueous eluents were better for more

acidic analytes; this was demonstrated in the separation of a selection of ergot alkaloids, where the standard eluent (10% aqueous buffer in methanol) had to be modified to achieve a satisfactory separation (20% aqueous buffer in methanol, reduced ammonia concentration). Twitchett and Moffat[14] reported that on increasing the methanol concentration from 0 - 40%, retention volumes were dramatically reduced. Further increases in methanol content from 40 - 80% gave rise to less dramatic reductions in retention and, in the case of nicotine, the retention increased again above 60% methanol.

Similar trends in retention with increasing methanol content have since been observed by Sugden *et al.*[17], Flanagan and Jane[26], Lingeman *et al.*[29], and Cox and Stout[31]. It was suggested that the shape of the curve could be explained in terms of typical reversed-phase mechanisms at low organic content, where an increase in the amount of methanol results in a decrease in the dielectric constant of the solvent mixture which in turn enhances the electrostatic forces between a protonated amine and any counter-ion in solution. The result is ion-pair formation, which reduces retention (as only charged species are retained, in a pure ion-exchange process)[31]. It has also been suggested that hydrophobic expulsion could dominate any ion-exchange processes in water-rich eluents, which would account for the very high retentions seen in eluents with very small amounts of organic modifier[29]. In this case, the primary retention sites are believed to be the siloxane groups on the silica surface, which are considered to be hydrophobic[21]. At higher concentrations of methanol, increases in retention have been attributed to increased solvation of organic competing ions compared with solute ions[29], or to the effect of small amounts of water on the protonation of the solutes[26]. Cox and Stout[31] suggested that retention at high levels of methanol was primarily via adsorption processes rather than an ion-exchange mechanism.

One common feature of many reports has been the observation that high levels of water in the mobile phase give rise to deteriorating peak shapes and long retention times[21,26,29,32]. For example, Schmid and Wolf[32] found that on going from a 50% methanol eluent of high ionic strength, to an 80% methanol eluent of lower ionic strength, the elution order changed and the peaks became much sharper, in the case of tamoxifen the efficiency increased from 534 plates/m to 40815 plates/m. It seems to be a generally held view of these authors that eluents of higher organic

content give better chromatography, with shorter analysis times and improved peak shapes.

Another important feature of eluents rich in organic modifier has been highlighted by Atwood *et al.*[116]. They reported that eluents with a high methanol content were poor solvents of silica, and that column life was longer using this type of mobile phase. However, they suggested that the inclusion of a 'sacrificial' column, situated between the pump and the injector, would also be useful in preserving the life of the analytical column. In a recent study, Law and Chan[95] have reported that eluents of high pH, containing large fractions of methanol, do not appear to damage bare silica columns, even after long periods of use. Such observations serve to enhance the belief that predominantly organic based eluents are to be favoured for this type of analysis.

d) Operating temperature

Very few chromatographers report results obtained under controlled temperature conditions; the majority of work cited above was recorded at ambient temperature (*e.g.* see 25,26,31). Crommen[17], was one of the first to report separations carried out under temperature control; a water-bath was maintained at 25.0°C to thermostat the column, injector and mobile phase reservoir.

A series of reports by Gill and Smith *et al.*[33,34,104] noted the importance of temperature control for interlaboratory reproducibility, suggesting that temperature differences between laboratories participating in their studies could, in part, account for the observed differences in retention measurements. Kirshbaum[82] suggested that, for good transferability of HPLC methods, the use of temperature control could halve the RSD of results obtained over a 12 hour (or longer) period.

Schmid and Wolf[32] reported that an increase of temperature from 25°C to 55°C caused a general decrease in retention times for a series of related tricyclic antidepressants, but no changes in selectivity were observed. They also found that, at higher temperatures, the efficiencies for all analytes improved and this was attributed to increased rates of mass transfer in the ion-exchange mechanism as the temperature increased.

e) The stationary phase

Wheals[18] noted that the selectivity of a separation could be altered by changing the stationary phase, and that the polarity and type of organic moiety used to produce reversed-phase materials was important in determining chromatographic characteristics. Later, Law *et al.*[23] found that four different types of bare silica exhibited different retention properties, and so they decided to standardise their work on one brand of silica in order to reduce the problem of silica irreproducibility.

Since then it has been shown that, within a single brand of unmodified silica, significant differences exist between batches[36], even for batches that have passed all their manufacturing quality control criteria. A series of interlaboratory studies by Gill *et al.*[33,34] have shown that reproducibility of data is very dependent on the choice of both the brand and batch of stationary phase used. This point was also emphasised by Kirshbaum[82] as one of the major, and largely uncontrollable problems hindering the transfer of HPLC methods between laboratories.

Reversed-phase materials are also susceptible to differences between batches and brands[104], as the many different processes of modification and end-capping often lead to widely differing surface characteristics[90]. It was shown by Bidlingmeyer *et al.*[21] that variations in the carbon loading of ODS-modified phases had a significant effect on the chromatographic performance for the separation of local anaesthetics.

2.2.1.2 Examples of ion-exchange separations

Some examples of separations of basic drugs under ion-exchange conditions are shown below (Table 2.1). It is evident that many of these methods have been developed for the analysis of a wide range of analytes and that there are many common parameters. For example, most methods use methanol - water mobile phases, and either ammonium or phosphate buffers, depending on the operating pH. The list of analytes is not exhaustive, as many papers include many types of compound. In some papers, especially where many compounds have been analysed, only one or two compounds from some classes have been examined, and so not all classes of compounds have been cited in every case.

TABLE 2.1: EXAMPLES OF ION-EXCHANGE SEPARATIONS OF BASIC DRUGS

Analytes[1,2]	Stationary Phase	Eluent[3]	Reference
b,c,e,g,h,m n,t,w,x	ODS silica	Methanol / aqueous phosphate buffers (apparent pH range 3.0 - 9.0)	14
b,c,k,l,m,p,x	Silica	Methanol / aqueous ammonia, ammonium nitrate buffer	15
b,c,p,m,x	Silica	Methylene chloride / methanol / ammonium hydroxide	16
l,r,x	Bare and ODS silica	Methanol / aqueous buffers; ammonium formate, ammonium nitrate, sodium formate (apparent pH 5.5 - 9.0)	17
c,e,f,l,m,p q,w,x	Bare & various modified silicas	Methanol / aqueous ammonium hydroxide & nitrate (eluent pH 10.3)	18
b,c,g,m,n,p	ODS silica Silica Silica	Methanol / aqueous phosphate buffer, eluent pH 7.0 Methanol / aqueous ammonia, ammonium nitrate buffer Dichloromethane / concentrated ammonia (2.0 ml l^{-1})	19
a,c,d,e l,q,t,x	Silica	Various aqueous eluents (many examples), eluent pH 2 - 4: alcohol modifiers	20
a,c,e,l,x	Bare silica ODS-silica	Acetonitrile or methanol / aqueous buffers Ammonium and sodium hydroxide, HCl, H_3PO_4	21
f	Silica	Methanol / aqueous ammonium phosphate buffer	22
c,m	Silica	Methanol / aqueous ammonia, ammonium nitrate buffer	23
a,c,e	Silica	Methanol / aqueous ammonia, ammonium nitrate buffer	24
a,c,e,h,j,l m,p,s,w,x	Silica	Methanol / perchloric, sulphuric and phosphoric acid Various buffer salts (non-aqueous buffers)	25
c,e,h,r,x	Silica	Methanol / perchloric acid or sodium hydroxide (non-aqueous buffers)	26
c,e,j,r	Silica	Methanol / ammonium perchlorate, perchloric acid (non-aqueous buffers)	28
b,d,m,v	Silica (also alumina)	Methanol / water / organic modifiers & citrate (pH range 2 - 9)	29
b,c,h,m,v	Silica (also alumina)	Methanol / water / organic modifiers & citrate (pH range 5 - 8)	30
m,x (proteins)	Bare and ODS silica	Methanol / aqueous phosphate buffers (normally pH 4.6)	31
e	Silica	Methanol / aqueous buffers, e.g. sodium acetate pH 7.9 Use of H_3CCOOH or NH_4OH to adjust pH to 4.6 - 10	32

continued below....

Table 2.1: *(continued)*

Analytes[1,2]	Stationary Phase	Eluent[3]	Reference
a,b,c,e,h l,m,p,v	Silica	Methanol / aqueous ammonia, ammonium nitrate buffer (aqueous buffer pH 9.4)	33,34 35,36
a,b,c,e,h l,m,p,v	Silica	Methanol / aqueous ethylenediamine, ammonium nitrate buffers (pH 10.2)	37
e,l,t	Silica	Methanol / aqueous ammonia, ammonium nitrate buffer (pH 8)	38
a	Silica	Methanol / aqueous ammonia, ammonium nitrate buffer	39
n	Silica	Acetonitrile / aqueous phosphate buffers, pH 2.6	40

[1]Examples of major analytes examined; (not all compounds cited in each paper are listed).

[2]Code for analyte identification:

(a), adrenergics; (b), alkaloids; (c), amphetamine and related compounds; (d), anticholinergics; (e), antidepressants (including tricyclics); (f), antihistamines; (g), barbiturates; (h), benzodiazepines; (j), cardioactive drugs; (k), ergot alkaloids; (l), local anaesthetics; (m), narcotic analgesics; (n), non-steroidal anti-inflammatory analgesics; (p), other stimulants; (q), phenothiazines; (r), quaternary ammonium compounds; (s), steroids; (t), sulphonamides; (v), tetracyclines; (w), tranquillisers; (x), others (includes, for example, paraquat, quinine, and non-drug compounds).

[3]Apparent pH values are for pHs measured in the eluent (*i.e.* mixed aqueous, organic phase). Other figures quoted are pHs of aqueous buffers prior to mixing with the organic phase.

2.2.2 MODIFIED-ELUENT SEPARATIONS

One of the major problems in the reversed-phase separation of basic compounds is that they are often strongly adsorbed onto the silica surface. In a recent paper, Bayer and Paulus[117] noted that, for steric reasons, no more than 50% of the silanols on bare silica could be involved in the bonding reactions used to produce reversed-phase materials. Therefore at least 50% of the original silanols would remain on the surface of the new stationary phase. These groups would then present difficulties in the chromatography of bases, causing tailing peaks and/or irreversible adsorption of the analyte. Due to the many different methods used for the modification of bare silica, the wide range of reversed-phase materials now available have different functionalities and surface properties and so they exhibit widely differing behaviour towards basic analytes[117].

Two main methods have been developed to overcome the problems of residual silanols. These are, (i) ion-pair methods using polyalkyl ammonium compounds, sulphonates and sulphates as modifiers, and (ii) methods using alkylamines as additives to mask the presence of the silanols. Each of these is briefly reviewed below, along with the use of bare silica in conjunction with polyalkylammonium eluent modifiers, (dynamically modified silica).

2.2.2.1 Ion-pair separation techniques

The principles of ion-pairing techniques have been adequately reviewed by Bidlingmeyer[118]. Essentially ion-pair separations involve the use of counter-ions in the eluent which produce neutral ion-pairs with changed analytes enabling satisfactory retention of the sample. In addition, ion-pairing methods allow control of the surface charge of the stationary phase by careful choice of eluent modifier[118].

The most commonly used eluent modifiers in the ion-pair separation of basic drugs are polyalkylammonium compounds and alkylsulphonates. Sokolowski and Wahlund[43] demonstrated the use of tri- and polyalkyl-ammonium compounds for the reduction of peak tailing in the analysis of tricyclic antidepressants on C-8 and C-18 stationary phases. They found that ammonium compounds containing bulky side groups had little influence

on peak shape, but that N,N- and N,N,N- methyl substituted quaternary ammonium compounds were especially suitable for improving chromatographic performance. A detailed model was presented to account for the ion-pair effects observed.

Lurie and Demchuk[41,42] reported the use of alkyl sulphonates for the separation of compounds of forensic interest on modified stationary phases. They suggested that separations were dependent on the molecular surface area of the complex formed between the ion-pair and the modified stationary phase[41]. They found that the choice of column and the composition of the eluent affected the selectivity of the separations and noted that the observations could be explained on the basis of solvophobic theory (reference 42 and references therein).

A few examples of ion-pair separations of basic drugs are given in Table 2.2. Numerous others have been cited by Gilpin *et al.*[48].

TABLE 2.2: EXAMPLES OF ION-PAIR SEPARATIONS OF BASIC DRUGS

Compounds[1,2]	Stationary Phase	Method[3]	Reference
b,c,g	ODS, Cyano	Methanol / water / HAC / *n*-alkylsulphonates	41,42
a,e,g	C-8 & ODS	Methanol / phosphate buffer (pH 2 - 3) Various alkylammonium compounds	43
c	Bare & ODS	Methanol / dilute sulphuric acid Sodium dodecylsulphate / naphthalene-2-sulphonate	44
r	ODS-silica	MeOH / ACN / THF / Aqueous H_2SO_4 buffer (pH 2) Hexanesulphonic acid	45
r	ODS-silica	Methanol / aq. H_3PO_4 buffer (pH 3.0) / SOS & DMOA	46,47
q,m,p,x	C-8 & C-18	Methanol / aq. H_3PO_4 buffer (pH 3.0) / SOS & DMOA	49

[1,2,3] See Table 2.1.

Abbreviations used in Table 2.2.

ACN = Acetonitrile; DMOA = N,N-Dimethyloctylamine; HAC = Acetic acid; MeOH = Methanol; SOS = Sodium 1-octanesulphonate; THF = Tetrahydrofuran.

2.2.2.2 Amine modifiers

A study by Wehrli *et al.*[50] examined the use of RP-8 and RP-18 phases at high pH for the separation of model ergot alkaloids. They sought to use organic alkylamines to replace strong bases, such as sodium hydroxide, which were believed to be detrimental to the long term stability of silica. Triethylamine was recommended from the range of amines studied because of its low aggressivity towards the silica stationary phase and its ready availability.

The usefulness of amine modifiers for reducing peak tailing on a C-18 column has been demonstrated[51]. A series of 11 amines were examined and it was found that retention was dependent on the hydrophobicity of the modifier: the more hydrophobic the amine, the greater the reduction in retention and improvement in peak shape. In addition the molecular geometry of the amine modifier strongly influenced retention: a number of isomeric amines were compared and found to have different effects on the improvement of peak shape[51].

Kiel *et al.*[55] addressed the question of the mechanisms involved on a modified silica surface when separating positively charged analytes in the presence of alkylammonium eluent modifiers. They suggested that hydrophobic interactions were of little importance and that interactions with the residual silanols probably dominated. In this case, a combination of ion-exchange and hydrogen-bonding interactions would be possible, and these would be controlled by the ability of the amine modifier to mask the silanol sites. Secondary and tertiary amines were said to have stronger hydrogen-bonding capabilities than primary amines and would therefore be expected to show stronger interactions with the silica surface.

The main actions of the amine modifiers were identified as, a) hydrophobic interactions with the organic moieties on the silica surface, creating a charged surface layer and so repelling solute molecules; b) ion-exchange interactions with negatively charged silanols, thus blocking potential retention sites; and c) hydrogen-bonding interactions with uncharged silanols, thus blocking these retention sites. The modifier action was considered to be dependent on the length of the hydrocarbon side chain, such that small molecules would act primarily via type (b) and

(c) mechanisms, whilst larger, more hydrophobic molecules would act via type (a) mechanisms[55]. It was suggested that hydrogen-bonding interactions between the analyte and the stationary phase were the main cause of peak tailing and that this could be effectively overcome by using short-chain alkylamine modifiers, such as trimethylamine or triethylamine, which would effectively penetrate the organic layer of the stationary phase and block the unwanted sites on the silica surface. In the separation of a series of tricyclic antidepressants it was indeed found that trimethylamine and triethylamine were the most effective modifiers for improving the peak shape[55].

The usefulness of triethylamine (TEA) was further evaluated by Roos and Lau-Cam[58] for the separation of 150 drugs of pharmaceutical interest on a series of C-18 columns. They found that TEA caused an increase in efficiency of bases, and that the result was dependent on the brand of column used and on the concentration of TEA in the mobile phase. They also found that neutral and acidic compounds, such as phenol and sulphamerazine, were largely unaffected by the presence of TEA in the eluent, at an eluent pH of < 4.5, since under these conditions such compounds would remain unionised.

Bayer and Paulus[117] showed that the effect of TEA was dependent on the type of stationary phase used. In a study of the chromatographic performance of seven different ODS materials, five showed a marked dependence on the concentration of TEA in the eluent, whilst two materials showed little change with increasing TEA concentration in the eluent. All observations were found to be reversible, *i.e.* the TEA could be removed from the column, and initial behaviour restored. It was suggested that the changes in k' with TEA concentration could be attributed to silanophilic interactions (as previously noted[55]), and that TEA was effective in masking active centres on the silica surface, and that the differences between the two types of ODS-silica must be caused by differences in the original silica material used to prepare the modified materials.

Some examples of separations of basic drugs using amine-modified eluent are presented in Table 2.3. Numerous other examples have been cited by Gilpin *et al.*[48].

TABLE 2.3: EXAMPLES OF DRUG SEPARATIONS USING ALKYLAMINES

Analytes[1,2]	Stationary Phase	Eluent[3]	Reference
b	C-8 silica	Acetonitrile / water / various alkylamines	50
c	ODS silica	Methanol / aqueous buffer (pH 2.4) Various organic amine modifiers	51
b,c,e,f,l	Cyano & ODS Bare silica	Acetonitrile / water / propylamine ACN / dichloromethane / n-alkane / propylamine (n = 6 or 7)	52,53
a	Various ODS	Acetonitrile / phosphate buffer / DMOA & TMOA (mobile phase pH 2 - 3)	54
e	C-8 silica	Acetonitrile / aqueous buffers / triethylamine	55
l	ODS silica	Methanol / aqueous H_3PO_4 / n-hexylamine	56,57
b,c,f,l,q,s,t	Various ODS	Methanol / acetic acid / TEA / water	58
e	ODS silica	Acetonitrile / aqueous phosphate buffer / n-nonylamine	59
Amiodarone	Cyano-silica	MeOH / ACN / THF / water / propylamine	60
Anti-epileptics	ODS silica	ACN / phosphate buffers / n-butylamine	61
e,l,p,x	ODS silica	Methanol / phosphate buffer (pH 3.5) / TEA	62
a,x	Silica	Aqueous TEAA (pH 4) (direct serum injections)	63
a,l	ODS silica	MeOH / ACN / n-hexane / acetone / TEA (use in solid phase extraction of drugs)	119

[1,2,3] see Table 2.1.

Abbreviations used in Table 2.3.

ACN = Acetonitrile; DMOA = N,N-Dimethyloctylamine; MeOH = Methanol; TEA = Triethylamine
TEAA = Triethylammonium acetate (i.e. TEA and acetic acid); THF = Tetrahydrofuran
TMOA = N,N,N-Trimethyloctylammonium bromide.

2.2.2.3 Dynamically modified silica

a) The principles

The use of dynamically modified silica in HPLC was first reported by Ghaemi and Wall[120] and the technique has recently been reviewed by Helboe

et al.[66]. The technique employs quaternary ammonium compounds in the mobile phase which interact with ionised silanols on the surface of bare to produce a hydrophobic organic coating with retention properties similar to those of a conventional reversed-phase material. Hansen[121] found that the long-chain quaternary ammonium compound N-cetyl-N,N,N-trimethyl-ammonium bromide (CTMA-Br) was particularly suitable for this process; the cetyl chain pointed away from the silica into the mobile phase to give a hydrophobic coating.

b) The separation mechanisms

The separation mechanisms are said to be a mixture of, (a) reversed-phase partition between the hydrophobic stationary phase and the polar mobile phase, (for neutral and cationic analytes), (b) ion-exchange interactions on the silica surface, (for cationic species), and (c) reversed-phase chromatography of ion-pairs formed between anions and CTMA ions (for anionic analytes)[66,121]. In further studies it was noted that ion-exchange was only a minor factor in the retention of cationic solutes[122]. In the absence of CTMA-Br the retention of cationic species increased with increasing pH, in line with an ion-exchange separation mechanism (see 2.2.1.1 (a)). However, with CTMA-Br present, retention was found to be independent of the operating pH, indicating that no ion-exchange interactions were occurring between the analyte and ionised silanols[122].

c) The effect of the operating conditions

The effect of dynamic modification was found to be dependent on the choice of organic modifier and its concentration in the mobile phase. The maximum amount of CTMA was adsorbed at low concentration of organic modifier (*e.g.* 10% methanol), when approximately one third of the silanols were covered by CTMA ions. The presence of the organic modifier was found to be essential to avoid the build up of bi- or multimolecular layers on the silica surface[66]. In addition, if the concentration of the quaternary ammonium compound exceeded the critical micellar concentration (CMC) then secondary mobile phase characteristics began to interfere with the retention processes[123].

The pH of the buffer was also found to be important as it could

change the amount of CTMA adsorbed on the silica surface. The effect was related to the ionisation profile of the silanol groups. As the pH increased the number of 'active sites' available to the quaternary ammonium ions increased and so the amount of CTMA that could be adsorbed increased. Changes in the eluent pH were seen to alter the selectivity between neutral, cationic, and most noticeably anionic solutes[122].

In a study of 14 different columns, separations were found to be largely independent of the choice of silica packing. Only one column, packed with a silica of very high surface area, was anomalous[94]. It was concluded that the use of dynamically modified silica allowed standardisation of HPLC methods without the restrictions relating to the choice of the stationary phase that would usually apply when using alkyl bonded reversed-phase materials[94].

d) Examples of separations using dynamically modified silica

Numerous examples of the applications of dynamically modified silica have been reviewed by Helboe *et al.*[66]. A few other examples are presented in Table 2.4 below.

TABLE 2.4: EXAMPLES OF SEPARATIONS ON DYNAMICALLY MODIFIED SILICA

Analytes[1,2]	Stationary Phase	Eluent[3]	Reference
antiphologistic acids, g,t	Silica	Methanol / water / phosphate buffer (pH 7.0), CTMA-Br	64
a	Silica	Aqueous phosphate buffer (pH 2.2) / organic modifiers DMOA / TMOA / ion-pairing with DMCHS	67
a,e	Silica	Methanol / water / phosphate buffer (pH 7.0) 2.5 mM CTMA-Br (comparison with ODS materials)	124
a,n,m,x, & metabolites	Silica	Methanol / water / phosphate buffer (pH 7.0), CTMA-Br	125

[1,2,3] see Table 2.1.

Abbreviations used in Table 2.4.

CTMA-Br = Cetyltrimethylammonium bromide; DMCHS = potassium 3,5-dimethylcyclohexylsulphate; DMOA = N,N-Dimethyloctylamine; TMOA = N,N,N-Trimethyloctylammonium bromide.

2.2.3 DRUG-SCREENING TECHNIQUES

HPLC has become a popular technique for screening in drug analysis. Cizmarik *et al.*[13] have described the growth of HPLC screening methods for pharmacopoeias, Binder *et al.*[126], Watson[2] and Eremin and Izotov[1] applications in chemical toxicological analysis. Hill and Langner[11] have reviewed some of the general properties of HPLC methods for screening, and De Zeeuw[8] has discussed the role of HPLC, GC and TLC in systematic toxicological analysis.

Hill and Langner[11] highlighted some of the important features of HPLC as a screening technique. These included the use of solvent programming which permits the analysis of compounds over a wide range of polarities, the use of UV/VIS detection systems and multiple wavelength detection (diode-array detectors) to obtain spectral information about an analyte in addition to retention information, and also the application of retention indices to improve standardisation of retention data. Watson[2], in highlighting the many applications of HPLC in therapeutic drug monitoring, stressed the importance of screening for drugs by classes, thus allowing analogs and metabolites to be detected more readily. A number of these features are discussed in more detail below.

2.2.3.1 Retention indices in screening methods

One of the main problems in HPLC has been the difficulty in producing standardised sets of data for analyte retention. This can largely be attributed to the problems of small variations between HPLC systems which cause significant changes in retention times. Factors such as column dimensions, temperature, eluent flow rate and the source of the stationary phase all contribute to this phenomenon[11]. This has led to the use of retention indices as a method of reducing interlaboratory variations in retention data.

A detailed review of the development and applications of retention index scales in RP-HPLC has been presented[127]. Baker and Ma[128] proposed the use of alkan-2-ones as the basis of a retention index scale for HPLC and Smith[129] proposed the use of the alkyl aryl ketones as an alternative, due to their greater availability and much stronger UV absorbances compared to the alkan-2-ones. More recently, Bogusz and Aderjan[130] have

suggested the use of 1-nitroalkanes, and highlighted their usefulness in covering early eluting compounds, for which reference compounds on the alkyl aryl ketone scale were not available. Smith and Finn[131] have recently presented a comparative study of these three retention index scales, which demonstrated the applicability of each scale for a range of analytes of differing polarity.

The use of retention indices has been demonstrated for the analysis of local anaesthetics[11,56,57], barbiturates[100-102], mycotoxins[11] and other drugs[130-131]. It has been shown that interlaboratory variation in retention data, for example by comparison with capacity factors, can be significantly reduced by the use of retention indices[102]. In addition, the use of the alkyl aryl ketones in the gradient HPLC analysis of *Penicillium* strains has recently been demonstrated[132], indicating that the method can be successfully transferred from isocratic systems.

Whilst it is recognised that retention indices are a more robust method of reporting retention data than either retention times or capacity factors, it has been found that they are also susceptible to changes in the properties of the stationary phase and to changes in some of the operating conditions[11]. Thus, although retention indices can be used to compensate for some changes in the operating conditions, they cannot overcome the problems of column selectivity differences.

2.2.3.2 Photodiode-array detection in drug screening

One of the major problems in toxicological screening is the selection of the most suitable wavelength for detection, as the great variety of analytes encountered have widely differing UV properties. The recent introduction of photodiode-array detectors allows the analyst to collect spectra at any number of wavelengths simultaneously during a single chromatographic run and then analyse the data at any desired wavelength in order to obtain maximum sensitivity[11].

One of the earliest applications of rapid scanning UV detection in the field of drug analysis was reported by Kabra *et al.*[133]. They presented a method for the rapid scanning of a series of barbiturates and other drugs of abuse, in which the use of both ordinary and first-derivative spectra was demonstrated for analyte identification. Later,

Overzet *et al.*[134] demonstrated the usefulness of photodiode-array detection in the analysis of a wide range of analytes (including drug metabolites), in conjunction with a gradient separation system. The ability to use spectral data, in addition to retention data, was considered particularly useful, and the use of computing techniques to subtract background noise from gradient baselines was demonstrated. This was used to good effect in the confirmation of metabolite identification from spectral information, but relied on good run-to-run reproducibility.

The general advance of computer technology has allowed the development of powerful programmes for the analysis of complex diode-array data and the production of computer library search routines for spectral identification[11,12,135-138]. These include the use of absorbance ratios for analyte identification[137] and peak purity analysis[138] and search algorithms to compare analyte spectra with library data[12,135,136]. These search and match techniques often rely on 'indices of comparison', a goodness of fit parameter designed to indicate the quality of the match of the unknown spectra with library data[11,12,135].

Photodiode-array detectors have been successfully employed in many areas of drug analysis, including the identification of heroin adulterants[12], barbiturates[139], antidepressants and neuroleptics[136], benzodiazepines[136,140], and in the analysis of multicomponent tablets[141]. Such systems have also been employed in toxicological screening[7,9,10] for automated solute identification.

2.2.3.3 Gradient separations in drug screening

Kabra *et al.*[133] reported the use of an acetonitrile / phosphate buffer gradient elution method for the analysis of a range of drugs commonly encountered in toxicological drug screening. The method was shown to provide good specificity and accuracy and enable the simultaneous analysis of a wide range of compounds. They found that gradient reproducibility was very good, but noted that there was an increase in background noise (*i.e.* baseline) when monitoring at low wavelengths (210 nm). The usefulness of the method for metabolite identification was highlighted by Overzet *et al.*[134] who used an aqueous ammonia / methanol gradient to analyse for minor components in bile samples.

The wider application of gradient methods was demonstrated by De Smet *et al.*[142] in the analysis of barbiturates, local anaesthetics, benzodiazepines, sulphonamides and other drugs. They showed that a gradient separation could be used as the first step of an optimisation procedure for the separation of multicomponent mixtures and that from the initial gradient results a suitable isocratic eluent could be devised for the analysis of each class of compound. Hill and Langner[10] separated over 300 drugs using two different gradient systems, one for acidic and neutral compounds and the other for basic and neutral compounds and Logan *et al.*[107] recently demonstrated the use of gradient separations for the rapid screening of basic drugs and metabolites in urine.

2.2.3.4 Other aspects of drug screening

It is important to note that many of the techniques discussed earlier in this review (*e.g.* ion-exchange separations, dynamically modified silica, etc.) have been applied as drug screening methods. A number of other approaches and methods for toxicological screening are discussed below.

Dong and DiCesare[143] proposed a method for screening tricyclic antidepressants, barbiturates and other drugs using short columns packed with 3-5µm ODS or bare silica, which allowed a complete scan in about 6 minutes. Gill *et al.*[103] presented a method for the analysis of benzodiazepines and some of their metabolites, again employing both bare and ODS-silica phases to achieve complete resolution of as many analytes as possible. A general method for the analysis of pharmaceutical dosage forms was produced by Sidhu *et al.*[144] and a method for the simultaneous identification and determination of non-steroidal anti-inflammatory drugs on ODS-silica was presented by Lapicque *et al.*[145].

The use of cyano phases in drug screening has been discussed by De Smet *et al.*[146] and by Badiru and Jefferies[147]. De Smet and Massart[146] concluded that the influence of organic modifier and eluent pH were the most important factors in the separation of a range of acidic and basic drugs, but that since the interactions between the various eluent parameters were small, individual optimisation of the separation parameters was possible.

2.2.4 ALTERNATIVE STATIONARY PHASES FOR DRUG ANALYSIS

Among the many examples of alternative stationary phases for the separation of basic drugs are a) alumina[29,30,68,69], b) polymer columns[71,72] and c) specialised reversed-phase columns[73,74].

2.2.4.1 Alumina

The use of alumina for the analysis of basic drugs has been investigated by Kelly *et al.*[68] and by Lingeman, Underberg and co-workers[29,30,69]. Alumina can be used in the ion-exchange mode in a similar way to silica, with the advantage that it is stable over a wider pH range (2-12)[68]. Under neutral or basic eluent conditions alumina behaves as a cation-exchanger but, due to its amphoteric nature it will behave as an anion-exchanger under acidic conditions, thus allowing the separation of both cations and anions on a single column[68].

Kelly *et al.*[68] demonstrated these properties by examining the effect of changing the eluent pH on the retention of a series of acidic, neutral and basic analytes. It was found that, over the pH range 3 - 11, the acidic drugs showed a decrease in retention with increasing pH, whilst most of the neutral compounds were virtually unretained over the whole pH range. The basic drugs behaved rather differently, in that they all showed some increase in retention with increasing pH, up to pH 5 - 7, followed by a decrease in retention at higher pH. This was explained in terms of a reduction in positive charge on the alumina surface (pH 3 - 7) which allowed an increase in retention, whilst the reduction in retention at higher pH was attributed to the reduced protonation of the bases. Lowering the ionic strength of the eluent was found to increase the retention of most of the analytes (although some of the non-retained compounds were unaffected); the observations were entirely consistent with an ion-exchange separation mechanism[68]. It was concluded that alumina was a viable alternative to silica for the analysis of drugs and plasma extracts[68].

Lingeman *et al.*[29,30,69] have shown that alumina can be used for the separation of compounds of forensic and pharmaceutical interest, including phenethylamines, opium alkaloids and benzodiazepines[30] and tetracycline derivatives[29,30,69]. In a comparison of silica and alumina it was

concluded that both materials behaved in a very similar manner, but that the retention mechanisms on alumina were more complex and in general the life time of alumina columns was shorter than silica columns[69].

2.2.4.2 Polymer columns

Lee[71] showed that poly(styrene-divinylbenzene) (PS-DVB) columns could be used over a wide pH range for the analysis of acidic and basic compounds, including sulfa and antiepileptic drugs. The columns were found to offer enhanced selectivity and better solute identification, when compared to conventional RP materials[71]. Van Liederkerke *et al.*[72] demonstrated the usefulness of polymer columns for the separation of quaternary ammonium compounds. A PS-DVB column was found to give symmetrical peaks for cationic solutes such as thiazine dyes, whose chromatography could be improved by the addition of an ion-pairing reagent to the mobile phase. The resolution and capacity factors were found to be dependent on the concentration of the ion-pairing reagent and on the eluent pH. The applicability of the column to the analysis of quaternary ammonium compounds, such as muscle relaxants in biological fluids was demonstrated.

2.2.4.3 Specialised reversed-phase columns

An important area in the analysis of basic drugs is the analysis of biological samples. The development of internal surface reversed-phase (ISRP) supports[73] has made it possible to analyse biological samples with the minimum of pretreatment without destroying the analytical column. These work on the principle of an external surface which is non-adsorptive towards the protein components of plasma, and an internal surface (*i.e.* within the pore structure), which is adsorptive towards the analyte molecules. By careful control of the pore structure, protein molecules are excluded from the matrix and eluted in the column void volume, whilst the analyte molecules are separated on the internal surface of the support[73]. A recent review of the principles, synthesis, nature and applications of ISRP materials is recommended for further information[74].

2.3 SOME IMPORTANT ASPECTS OF THE CHEMISTRY OF SILICA

A number of criteria have been put forward for the quality control of silica for HPLC stationary phases. Among these, the following properties of silica are generally considered to be the most important:

i) the specific surface area of the silica[112,148-150],
ii) the mean pore diameter and specific pore volume[148-151],
iii) the mean particle size, and the particle shape[112,150-152],
iv) the trace metal content[112,150], and
v) the surface pH[150].

In addition, the heterogeneous nature of the silica surface has been identified as a critical factor in the chromatographic performance of silica[110], and in the production of reversed phase materials[87,89,112]. In the following discussion, some of these factors are reviewed briefly.

2.3.1 THE SILICA SURFACE

The surface of silica is not simple. Detailed reviews of the properties of the silica surface have been presented[112,153-155] and these are recommended for further reading.

2.3.1.1 Silanol and siloxane groups

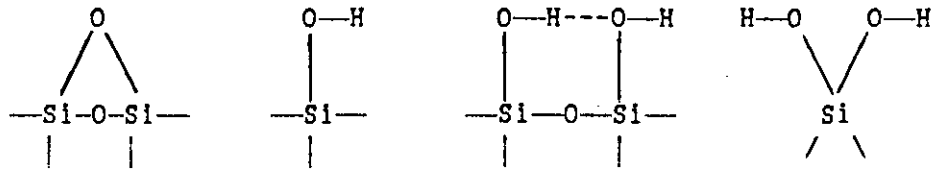
The silica surface consists of a mixture of silanol and siloxane groups and a simplified description of these is presented in Figure 2.1 (see reference 155). It is widely recognised that three different types of silanol group exist on the silica surface[112,155]; these are identified as isolated, vicinal and geminal silanols (Figure 2.1).

Vicinal silanols can interact via hydrogen-bonding, producing so-called reactive sites[112]. In general, silanols are the strong adsorption sites on the silica surface[156], and they are readily hydrated by water molecules. Siloxane groups (Si-O-Si) are usually considered to be hydrophobic[154] and are formed by the elimination of water between vicinal silanols at elevated temperature[155]. Their formation has been found to be reversible provided the heat treatment does not exceed 900K, since above

this temperature the siloxane bonds become energetically stable, and remain so after cooling[155].

Highly acidic silanol groups have been identified[108,109] and it was suggested by Kohler *et al.*[110] that these were isolated, non-hydrogen bonded silanols. These isolated silanols were believed to be the main adsorption sites[156] and responsible for the unfavourable interactions with basic analytes[110]. Mauss and Engelhardt[111] showed that the concentration of these groups increased on heating silica above 200°C, when vicinal silanols were removed. In this case the rehydration of the siloxanes was reversible if the heat treatment did not exceed 400°C[111].

Figure 2.1: The common groups on a bare silica surface



a) Siloxane Bridge b) Isolated Silanol c) Vicinal Silanols d) Geminal Silanols

2.3.1.2 Apparent surface pH and reactivity

In a detailed study by Engelhardt and Muller[152] it was found that aqueous suspensions of different brands of silica had different pHs, ranging from acidic (pH 4) to basic (pH 10). An interesting trend was revealed, in that irregularly shaped particles gave approximately neutral solutions (pH 6.5 - 8.0), whilst spherical silicas were either acidic (pH < 6.0) or basic (pH > 8.0). The differences in pH were attributed to the method of manufacture of the silica[157], and to variations in the concentration of surface silanols[151]. Kohler et al.[110] suggested that highly acidic silicas resulted from the presence of a large number of isolated silanols surrounded by siloxanes, which, being electron withdrawing, enhanced the acidity of the unbonded silanols. Silicas with fully hydroxylated surfaces, on the other hand, would have fewer siloxane groups and therefore the surface silanols would be less acidic[110]. However, data presented by Novak *et al.*[151] would appear to contradict this suggestion. They found that increasing the concentration of silanols on

the silica surface led to an increase in the acidic nature of the silica, and that the variation of apparent pH with particle shape was random. It was noted that the apparent pH of silica is dependent on the washing steps in the production process[151], suggesting that the purity of the silica must also be taken into consideration when attempting to interpret pH data.

The effect of surface pH on selectivity has been demonstrated by Muller and Engelhardt[157]. It was found that basic analytes showed severe tailing and/or irreversible adsorption on acidic silicas, but could be eluted from a silica with a surface pH of 9.0 (reference 157).

In addition to the wide variation of pH values found for different silicas, it has also been found that the pK_a value for silica seems to vary considerably. In theory, the pK_a of silica should be 7.1 ± 0.5 (references 109,158), but values from 1.5 to 10 have been reported[112]. These observations indicate that a variety of silanol groups exist with different acidities. In this case such variations could be attributed to the amorphous nature of silica, which would permit the existence of a broad range of hydrogen-bonded silanols with a variety of bond lengths, and consequently a variety of acidities and reactivities[112]. Indeed, the strength of hydrogen-bonding of the silanol group has been shown to be dependent on the acidity of the silanol group[156].

Further studies have shown that the silica surface can be quite heterogeneous[85,110] and it has been suggested that there are small groups of highly reactive, associated silanols[86] and these are believed to be responsible for many of the problems of producing homogeneous layers of organic modifier in the preparation of reversed-phases[86].

2.3.1.3 The influence of metal ion impurities on the properties of silica

The influence of trace metals on the properties of silica has recently been reviewed by Nawrocki and Buszewski[112]. It has been suggested[84,87,112] that metal ions close to the surface of the silica matrix may be responsible for the heterogeneity of the surface due to their influence on the acidity of adjacent silanols. It has been found that by acid washing the silica most metal ion impurities can be removed, resulting in a more homogeneous surface[87,89].

Studies by Sadek *et al.*[87], Ohhira *et al.*[88] and Ohtsu *et al.*[89] have shown that trace metal impurities play an important role in the production and subsequent quality of reversed-phase silicas. The use of acid washed silica for the production of reversed-phase materials has been found to give supports of much higher chromatographic quality than those prepared from silica containing much higher levels of metal ion impurities[87-89].

2.3.2 CONCLUSIONS

It is obvious from the discussion above that the nature of the silica surface is complex and not fully understood. The many reports in the literature put forward a series of explanations for the different properties of commercial silica materials, and some of these theories appear to contradict one another. At present there does not appear to be a clear consensus amongst chromatographers as to which properties are the most important for the comprehensive characterisation of silica gels for HPLC, and there are no clearly defined criteria which permit the evaluation of the retention and selectivity properties of new stationary phases[159]. Thus it would still appear to be necessary to use a series of chromatographic tests to evaluate all new silica samples to determine their individual chromatographic properties.

SECTION 2: ISOCRATIC STUDIES

CHAPTER 3: EXPERIMENTAL

3.1 CHEMICALS

2-(N-cyclohexylamino)-ethanesulphonic acid (CHES), 3-(cyclohexylamino)-2-hydroxy-1-propanesulphonic acid (CAPSO), sodium 3-(cyclohexylamino)-2-hydroxy-1-propanesulphonate (CAPSO-Na), 3-(cyclohexylamino)-1-propanesulphonic acid (CAPS) and ethanolamine hydrochloride were obtained from Sigma (Poole, Dorset, U.K.). Sodium nitrate and sodium hydroxide were AR grade and methanol was HPLC grade, from FSA Laboratory Supplies (Loughborough, Leics., U.K.). Water was either reagent grade, purified on site using a Millipore Liquipure water purification system or HPLC grade, from FSA Laboratory Supplies (Loughborough, Leics., U.K.). The drug samples were obtained from the reference collection of the Central Research and Support Establishment of the Home Office Forensic Science Service. All other chemicals were of laboratory reagent grade or better and used as purchased.

3.2 BUFFER SOLUTIONS

A variety of buffer solutions were prepared and tested, using different combinations of some of CHES, CAPSO, CAPSO-Na, CAPS and ethanolamine hydrochloride. In some cases pH adjustment of the buffer was performed using a 0.5 mol l^{-1} solution of sodium hydroxide, the pH being monitored with a pH electrode.

The selected buffer for the study was prepared by mixing CAPS (0.8852 g) and CAPSO-Na (1.0372 g) in water to give a 50 ml solution.

3.3 HPLC SEPARATIONS

The HPLC separations were performed using a Pye Unicam 4010 pump and a Pye Unicam 4020 UV detector set at 254 nm (both from Philips Scientific, Cambridge, U.K.). The eluent consisted of methanol and buffer (90:10 v/v) and was pumped at 2 ml min^{-1}. The samples (1 - 5 µl) were injected using a 7125 Rheodyne valve (Rheodyne Inc., Cotati, California, U.S.A.), fitted with a 20 µl loop, or a WISP 710B autosampler (Waters Associates, Milford, MA, U.S.A.), onto a Shandon column (25 cm x 5 mm i.d.) packed with Spherisorb S5W (5 µm, batches 2752, 5123 and 5493/1;

Phase Separations, Queensferry, U.K.). The autosamplers' auto-start function or a contact closure switch on the Rheodyne valve was used to start the integrator at the point of injection.

The temperature of the analytical column was maintained at 30°C using a water jacket and a circulating water bath. The system was fitted with a pre-column (3 cm x 5 mm i.d.) filled with open column grade silica sieved to 60 mesh, or a larger column (20 cm x 5 mm i.d.) filled with Spherisorb Prep 12W silica, (12µm, batch P6442; Phase Separations, Queensferry, U.K.). The retention times were determined using a Hewlett Packard 3390 integrator, a Shimadzu Chromatopac C-R3A integrator or a Hewlett Packard HP3396A integrator.

The HPLC columns were packed using a conventional slurry technique with methanol as the slurry and packing solvent. The packing pressure was typically 6500 - 7000 p.s.i., produced by an air-driven pressure amplification pump.

3.4 TEST SOLUTIONS OF BASIC DRUGS

Three different sets of test solutions were used during the study.

3.4.1 SIMPLIFIED TEST SET

In the initial development experiments a simplified set of test solutions[36] was used for the evaluation of new eluents. The compositions of the test solutions are given below, (compositions in mg ml^{-1} in ethanol - water (90:10 v/v)).

A. Dipipanone hydrochloride, 0.424; prolintane hydrochloride, 1.244; protriptyline hydrochloride, 0.156; strychnine, 0.08.

B. Promazine hydrochloride, 0.012; phenylephrine hydrochloride, 1.452; protriptyline hydrochloride, 0.156.

C. Codeine phosphate, 0.632; ephedrine hydrochloride, 2.02; protriptyline hydrochloride, 0.148.

D. Sodium nitrate, 30; (in methanol / water, 90:10 v/v).

3.4.2 FULL TEST SET

A set of nine solutions of basic drugs[34], was also used in the study. These solutions were used for the full evaluation of the newly developed method and their compositions are given below, (compositions in mg ml^{-1} in ethanol - water (90:10 v/v)).

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

B. Cocaine hydrochloride, 0.82; phentermine, 2.36; ephedrine hydrochloride, 3.38; protriptyline hydrochloride, 0.19.

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

D. Amitriptyline hydrochloride, 0.08; prolintane hydrochloride, 2.44; protriptyline hydrochloride, 0.24.

E. Nitrazepam, 0.04; chlorpromazine hydrochloride, 0.03; pipazethate hydrochloride, 0.22; protriptyline hydrochloride, 0.28.

F. Dextropropoxyphene hydrochloride, 1.51; amphetamine sulphate, 2.28; pholcodine, 1.61 (later reduced to 0.66); protriptyline hydrochloride, 0.2.

G. Papaverine, 0.04; dipipanone hydrochloride, 0.81; codeine phosphate, 0.80; methdilazine hydrochloride, 0.06; protriptyline hydrochloride, 0.22.

H. Procaine hydrochloride, 0.12; promazine, 0.03; ethoheptazine citrate, 3.60; protriptyline hydrochloride, 0.28; strychnine, 0.14.

I. Phenylephrine bitartrate, 1.04; protriptyline hydrochloride, 0.2.

J. Sodium nitrate, 30; (in methanol / water, 90:10 v/v).

When the new method was transferred to a second column (packed with a different batch of silica), solutions G, H, and I had to be replaced by solutions K, L, M, and N below, (compositions in mg ml^{-1} in ethanol - water (90:10 v/v)).

K. Papaverine, 0.036; dipipanone hydrochloride, 0.82; methdilazine hydrochloride, 0.07; protriptyline hydrochloride, 0.24.

L. Procaine hydrochloride, 0.044; promazine, 0.04; ethoheptazine citrate, 7.32; protriptyline hydrochloride, 0.40.

M. Codeine phosphate, 3.20; L-phenylephrine hydrochloride, 1.05; protriptyline hydrochloride, 0.22.

N. Nortriptyline hydrochloride (used as a secondary standard), 0.16; strychnine hydrochloride, 0.13.

All test solutions were stored in a fridge (4 - 7°C) when not in use, and were remade periodically. The amount of pholcodine in solution F was reduced in later test solutions because at the original concentration the pholcodine peak was off scale compared to the other components in the test solution.

3.5 CALCULATIONS

3.5.1 RETENTION DATA

The retention times(t_R) were measured in duplicate or triplicate and the mean values($t_{R'}$) were used to determine the capacity factors(k') according to the equation $k' = (t_{R'} - t_0) / t_0$, where t_0 is the retention time of methanolic sodium nitrate (solution J above). Relative capacity factors were calculated as k'/k'_p where k'_p is the capacity factor for protriptyline present as an internal standard. Solution 'N' contained nortriptyline as a secondary standard whose k' value from solution 'C' was used to determine the relative k' of strychnine according to the equation:

$$\text{Rel. } k'_{(\text{STRY.})} = \left[\frac{k'_{(\text{STRY.})}}{k'_{(\text{NORT.(N)})}} \right] \times \text{Rel. } k'_{(\text{NORT.(C)})} \qquad 3.1$$

STRY. = strychnine; NORT. = nortriptyline

3.5.2 pH AND IONIC STRENGTH OF THE BUFFER SOLUTIONS

The theoretical pH and ionic strength values were calculated for aqueous buffer solutions prepared from CAPS and CAPSO-Na. The calculations followed the principles outlined below.

The pH of the buffer solution is related to the pK_a of the buffer salts by the equations:

$$\text{CAPS-H: } pH = pK_a - \log\left[\frac{[\text{CAPS-H}]}{[\text{CAPS}^-]}\right] \qquad \{pK_a = 10.4\} \qquad 3.2$$

$$\text{CAPSO-H: } pH = pK_a - \log\left[\frac{[\text{CAPSO-H}]}{[\text{CAPSO}^-]}\right] \qquad \{pK_a = 9.6\} \qquad 3.3$$

In a mixed solution the pH will be constant and thus the two equations above can be equated as shown:

$$10.4 - \log\left[\frac{[\text{CAPS-H}]}{[\text{CAPS}^-]}\right] = 9.6 - \log\left[\frac{[\text{CAPSO-H}]}{[\text{CAPSO}^-]}\right] \qquad 3.4$$

This can be solved for the concentration of any one component in the buffer solution if the starting concentrations of the buffer salts are known. Once the concentration of the selected component has been calculated, the concentration of the other components can be determined and the pH of the solution calculated from equations 3.2 and 3.3. The ionic strength of the solution is calculated from the equation:

$$I = \tfrac{1}{2}.\Sigma(C_i z^2) \qquad 3.5$$

where C_i is the concentration of the ion 'i',
and z^2 is the charge on the ion 'i'.

It can be shown that the ionic strength of the buffer solution is controlled by the total concentration of the buffer salts, whilst the pH is controlled by the ratio of the concentrations.

N.B. Replacing CAPSO-H with CAPSO-Na does not alter the pH of the buffer solution.

3.6 ANALYSIS OF OPEN COLUMN GRADE SILICA

3.6.1 EXTRACTION OF METAL IONS FROM SILICA

A simple acid extraction procedure was devised to remove metal ions from the silica. A weighed amount of silica (ca. 2.4 g) was placed in a round bottomed flask with a few anti-bumping granules and 50 mls of AristaR grade concentrated hydrochloric acid. The flask was heated under reflux for about one hour and after cooling the yellow coloured extract was decanted into a glass container and sealed until it was analysed. The residual silica was dried overnight in an oven at 80°C and, after separation from the anti-bumping granules, the dry residue was re-weighed. The change in mass of the silica (before to after extraction) was found to be negligible, indicating that the silica itself had not dissolved to any extent during the extraction procedure.

3.6.2 ANALYSIS FOR COPPER

The analysis for copper was carried out by anodic stripping differential pulse polarography as described below:

i) A 1.5 ml aliquot of the analyte solution was diluted to 15 mls with HPLC grade water and the solution was purged for 5 minutes with nitrogen.

ii) Copper was accumulated on a static mercury drop electrode at -0.40 V for 1 minute, and the electrode was scanned from -0.40 V to 0.00 V at 10 mV sec^{-1} to record the oxidation of copper.

To determine the copper concentration the method of standard additions was employed. A 150 µl aliquot of a 1 ppm copper solution was added to the diluted silica extract and the solution was re-analysed. A second 150 µl aliquot was added and the analysis repeated. A sample blank, prepared by diluting 1.5 mls of AristaR grade hydrochloric acid to 15 mls with HPLC grade water, was analysed by the same method. In this case, however, the standard additions aliquots were reduced to 75 µl of the 1 ppm copper solution as the unspiked blank signal was very low.

3.6.3 ANALYSIS FOR IRON

3.6.3.1 Colorimetric methods

A simple colorimetric method was used for the analysis of iron in the extract. This required a portion of the extract to be boiled almost to dryness, and then taken up again in water, so that the acidity of the solution was reduced. Two 5 ml portions were boiled down, the first one to near dryness was made up to 50 mls in water, whereas the second was boiled to about 1 ml and taken back up to 5 mls with water.

The analysis was carried out by adding 1 ml of a potassium thiocyanate solution (*ca.* 2 mol l^{-1}) to 1 ml of the iron solution and recording the UV spectrum of the red iron thiocyanate complex over the range 350 nm to 650 nm, for a maximum at about 480 nm. The spectrometer was calibrated using standards of 1, 5, 10 and 20 ppm iron.

For the first extract, (remade to 50 mls), the colour was virtually undetectable due to excessive dilution. The second extract gave a detectable colour, but it did not remain stable, possibly because the extract had not been boiled to dryness and was, therefore, too acidic. (The iron thiocyanate complex is not very stable in strongly acidic solutions).

3.6.3.2 Flame atomic absorption spectroscopy

The analysis was performed on a Philips PU9100 atomic absorption spectrophotometer, (Philips Scientific, Cambridge, U.K.), using an air / acetylene flame. Detection was at 249 nm, using a lamp current of 11 mA, and a band pass of 0.5 nm. The instrument was calibrated using HPLC grade water (as a blank), and iron solutions of 1, 5 and 10 ppm, and the silica extract was analysed without further treatment. The results from three 'injections' of each sample were used to plot a calibration graph from which the iron concentration in the extract was determined.

CHAPTER 4: DEVELOPMENT OF A NEW METHOD

4.1 INTRODUCTION: THE NEED FOR A NEW ELUENT FOR BASIC DRUGS

The difficulties experienced in controlling the concentration of ammonia stock solutions used in the preparation of buffers[36] led to the conclusion that it was necessary to devise an alternative method for the analysis of basic drugs on silica. In developing the new method, the following desirable properties were identified based on experience gained from the use of the ammonia eluent. The buffer solution must be easy to prepare reproducibly and it should have a similar pH to that used in the ammonia system (pH 10.1)[35], whilst not containing any volatile components. The elution power of the eluent should be weaker than that of the ammonia eluent so that weakly retained compounds can be resolved from the solvent front. This would also extend the overall retention times and thus increase the discriminating power, *i.e.* reduce the number of compounds eluting per unit time. However, the retention of the longest retained compounds should not be excessive and unduly extend the analysis time. In addition, the eluent should not have strong UV properties in the wavelength range for detection of the analytes (254 nm), and so aliphatic compounds were considered the best candidates for new buffers.

Trials by Smith and Rabuor using buffers based on the liquid base ethylenediamine[37] gave acceptable results but failed to meet all of the criteria. It was felt that the best results would be obtained if the buffer could be prepared using solid components and so the current study was aimed at finding and testing suitable candidates.

4.2 DEVELOPMENT OF A NEW BUFFER

4.2.1 TRIAL BUFFER SOLUTIONS

The present study concentrated on potential solid organic buffer components of high pK_a, including 2-(N-cyclohexylamino)ethanesulphonic acid (CHES, pK_a = 9.3), 3-(cyclohexylamino)-2-hydroxy-1-propanesulphonic acid (CAPSO-H, pK_a = 9.6), sodium 3-(cyclohexylamino)-2-hydroxy-1-propanesulphonate, (CAPSO-Na), and 3-(cyclohexylamino)-1-propanesulphonic acid, (CAPS, pK_a = 10.4).

A range of buffers of different composition and pH were prepared and examined using a simplified set of test solutions (see 3.4.1). Buffers prepared from CHES and ethanolamine hydrochloride, (used as an ionic modifier), with pHs < 10 (*i.e.* lower than the ammonia buffer) were found to be unsuitable because they gave poor peak shapes and long retention times: the relative capacity factor for strychnine, relative to protriptyline, exceeded 2.25 for all buffers tested. Combinations of CAPSO-H and ethanolamine hydrochloride were used to prepare buffers whose pH was adjusted to *ca.* 10.1 with a sodium hydroxide solution. These were found to give lower retention times for all the analytes, the relative capacity factor for strychnine fell to 1.55, but some peak shapes remained poor, notably ephedrine and phenylephrine.

The use of CAPS and CAPSO-H in buffers with pH adjusted to 10.4 - 10.8, led to some promising results, but retention times were quite long (strychnine, 14.70 minutes). Attempts to reduce retention times by increasing the ionic strength led to complications as it was necessary to either, (a) introduce a third component into the buffer (*i.e.* ethanolamine HCl) thus making the composition more complex, or (b) to increase the concentration of the original buffer salts which led to solubility problems when the buffer was mixed with methanol.

4.2.2 CAPS / CAPSO-Na BUFFERS

Combinations of CAPS and CAPSO-Na gave buffer solutions of high pH, in the region of 9.6 - 10.4. In initial studies using the simplified test set of drug compounds (see 3.4.1), a buffer containing the two compounds in a 1:1 molar ratio, at 0.1 mol l^{-1} for each component, gave reasonable retention times (protriptyline, 10.59 minutes). These were longer than those on the ammonia system, but the efficiencies of some compounds were very low, *e.g.* phenylephrine, N = 1677 and strychnine, N = 3322. On increasing the concentration of CAPSO-Na in the aqueous buffer to 0.2 mol l^{-1}, giving a 2:1 molar ratio of CAPSO-Na / CAPS, the retention times were reduced (protriptyline, 7.15 minutes), but phenylephrine (N = 1543) and strychnine (N = 3594) still exhibited low efficiencies.

4.2.2.1 Optimisation of buffer composition

Since the results for these eluents seemed reasonably promising, a

further set of experiments was performed in which an extended test set of drug solutions (see 3.4.2) was used. Six separations were carried out using buffers varying in composition from 1:2 to 4:1 CAPSO-Na / CAPS and with different overall ionic strengths. The eluents giving the best results were those prepared from a buffer with a calculated pH about 10.0 and ionic strength 0.075 - 0.080 mol l^{-1}. If the pH was lower (1:2, pH 9.79) the later peaks were too highly retained (e.g. protriptyline, 15.63 minutes; strychnine, 16.70 minutes) whilst buffers of higher pH (4:1, pH 10.43) caused more rapid elution (protriptyline, 7.75 minutes) and thus reduced the resolution of the earlier eluting drugs.

From these studies a buffer of pH 10.0 containing the two compounds in a 1:1 molar ratio at 0.08 mol l^{-1} for each component was chosen for a more detailed study as it gave better efficiencies than a more concentrated 1:1 molar buffer (0.1 mol l^{-1} for each component) (e.g. ephedrine N = 3497 compared to N = 2573 and prolintane, 3719 compared to 2957). The eluent had a good UV range, with an absorbance < 1 at 215 nm and the retentions of the drugs ranged from 1.60 to 13.06 minutes.

Comparison of the results for this eluent and the previous ammonia system[35] (Table 4.1) showed that the present eluent gave an increase in retention time for all the drug compounds. This greater separation of the basic drugs (Figure 4.1) would improve the resolution and thus enable better discrimination between similarly retained compounds, aiding more positive identification. The capacity factors and relative retentions with the two eluents differed significantly, particularly for moderately retained compounds (relative capacity factors of 30 to 50)(Table 4.1).

The changes in relative retentions caused some compounds to be eluted relatively more rapidly in the new system, including imipramine, 26.7 (ammonia system, 31.1); promazine, 32.0 (38.5); codeine, 33.0 (46.6); morphine, 33.7 (49.7); phenylephrine 43.9 (63.8); pholcodine 44.2 (63.4); ethoheptazine, 50.0 (61.1); and strychnine, 109.3 (139.5). Other basic drugs were relatively more highly retained, including cocaine, 8.9 (6.0); dipipanone, 29.0 (22.9); prolintane, 48.6 (47.7); and pipazethate, 58.1 (54.9). These changes reflect those caused by decreasing the ionic strength of the buffer in the ammonia system[35], when the retentions of the last four compounds all increased whereas the earlier compounds decreased. There is no correlation with the pK_a' of the analytes; dipipanone and

ethoheptazine, both pK_a 8.5 behaving in a markedly different manner.

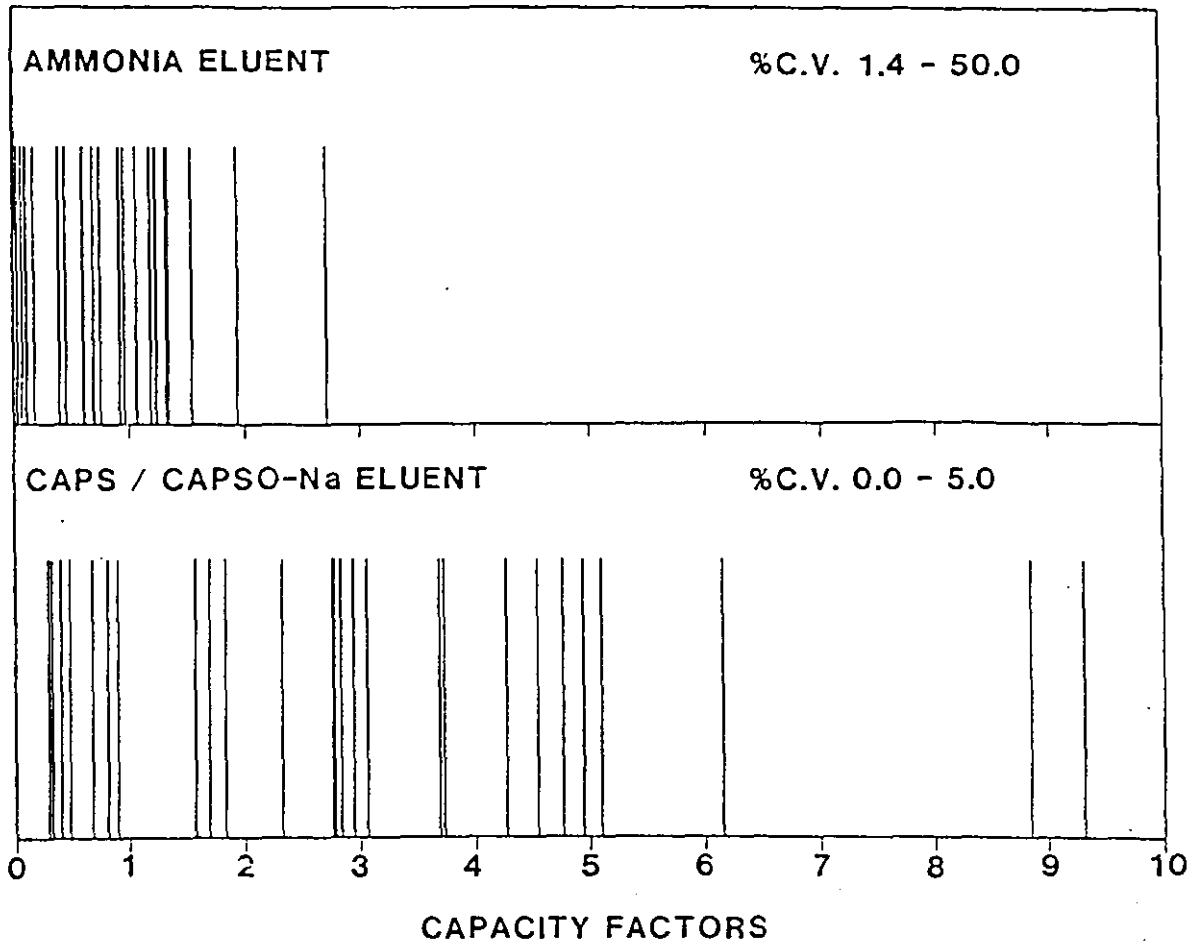
TABLE 4.1: CAPACITY FACTORS AND RELATIVE CAPACITY FACTORS USING THE CAPS / CAPSO-Na ELUENT AND THE AMMONIUM NITRATE ELUENT IN THE CHROMATOGRAPHY OF BASIC DRUGS ON A SILICA COLUMN

Conditions: column, Spherisorb S5W (batch 5123); eluent, methanol - aqueous CAPS / CAPSO-Na buffer, (each component 0.08 mol l^{-1}), 90:10 v/v; temperature, 30°C.

Compound	*Ionisation constant*[a]	*Capacity factor*		*Relative capacity factor (x100)*[b]	
		CAPS / CAPSO-Na	*Ammonia*[c]	*CAPS / CAPSO-Na*	*Ammonia*[c]
Nitrazepam	3.2,10.8	0.22	0.02	2.7	1.3
Diazepam	3.3	0.25	0.02	3.1	1.3
Papaverine	6.4	0.31	0.06	3.9	2.6
Caffeine	14.0	0.41	0.10	5.1	5.0
Dextropropoxyphene	6.3	0.56	0.09	7.0	4.5
Cocaine	8.6	0.71	0.11	8.9	6.0
Procaine	9.0	0.81	0.17	10.2	8.8
Amitriptyline	9.4	1.44	0.39	18.0	19.9
Chlorpromazine	9.3	1.53	0.44	19.9	22.4
Propranolol	9.5	1.66	0.44	20.8	22.5
Imipramine	9.5	2.13	0.60	26.7	31.1
Dipipanone	8.5	2.32	0.45	29.0	22.9
Promazine	9.4	2.56	0.75	32.0	38.5
Phentermine	10.1	2.60	0.61	32.5	31.4
Codeine	8.2	2.64	0.91	33.0	46.6
Morphine	8.0,9.9	2.69	0.96	33.7	49.7
Amphetamine	9.9	2.72	0.69	34.1	35.6
Phenylephrine	8.9,10.1	3.51	1.24	43.9	63.8
Pholcodine	8.0,9.3	3.53	1.23	44.2	63.4
Prolintane	9.7[d]	3.89	0.93	48.6	47.7
Ethoheptazine	8.5	4.03	1.19	50.0	61.1
Nortriptyline	9.7	4.32	1.19	54.1	60.9
Methdilazine	7.5	4.36	1.32	54.6	67.9
Ephedrine	9.6	4.62	1.35	57.8	69.5
Pipazethate	n/a	4.64	1.07	58.1	54.9
Methylamphetamine	10.1	5.61	1.54	70.2	79.1
Protriptyline[e]	10.0	8.00	1.94	100.0	100.0
Strychnine	2.3,8.0	8.75	2.71	109.3	139.5

[a]Data taken from reference 160 (n/a: not available).
[b]Relative capacity factors relative to protriptyline.
[c]Data taken from reference 35.
[d]pK_a unpublished value from Boehringer Ingelheim.
[e]Based on test solution H. pK_a from reference 32.

Figure 4.1: Comparison of capacity factors using the ammonia - ammonium nitrate buffer and the CAPS - CAPSO-Na buffer showing the improvement in resolution and discriminating power with the latter eluent



These results therefore contrast with studies in other laboratories[24,31,32] where, except at very low ionic strengths, there was generally no change in the relative order of retention with the strength of the buffer. However, a wider range of structural types was examined in this study.

4.2.2.2 Transfer of method to a new batch of silica

The development of the new eluent was carried out on Spherisorb S5W (batch 5123) and when the method was transferred to a new column packed with a different batch of Spherisorb S5W (batch F5493/1), significantly different results were obtained and some of the components of the test solutions were unresolved. Strychnine was now unresolved from the

internal standard protriptyline, whilst codeine and dipipanone in solution G co-eluted. Consequently the test solutions G, H and I were replaced by solutions K, L, M, and N with nortriptyline as a secondary standard for strychnine in solution N (see section 3.4.2). Good separations were now observed for all the test compounds, and examples are shown in Figure 4.2.

Having successfully set up the new method on the second batch of silica, a full evaluation of the method robustness was carried out (see Chapter 5). In addition, a study was undertaken to try to identify some quantitative structure retention relationships (QSRR) of selected test compounds and the results are presented below.

4.3 QUANTITATIVE STRUCTURE RETENTION RELATIONSHIPS

As noted above, there appeared to be no correlation between analyte pK_a and retention behaviour. Changes in elution order caused by changing the eluent could be related, in part, to differences in the ionic strengths of the two eluents, but due to the diversity of analytes used in the study, it was not possible to identify any correlation between analyte structure, pK_a and changes in retention.

Therefore, to obtain a more detailed comparison of the two eluent systems, a group of compounds related to amphetamine, along with two tricyclic antidepressants (Table 4.2) were analysed, using the full set of test solutions (see 3.4.2) and individual solutions of the additional compounds. Both the new eluent and the previous ammonia eluent[36] were used in the study and the separations were carried out on the same column (Spherisorb S5W, batch F5493/1), starting with the CAPS / CAPSO-Na eluent.

The elution order and relative position of the test compounds was largely independent of the choice of eluent (Table 4.2, for results recorded on Spherisorb S5W, batch F5493/1), indicating that the changes in selectivity observed when comparing the full set of test compounds were due to the much wider range of compounds, and hence the wider range of retention mechanisms. Significant differences in column selectivity between the reference data[24] and the current results were observed, and it was thought that these were due to the results being recorded on different batches of Spherisorb S5W.

Figure 4.2: Examples of separations of basic drugs on silica using the CAPS / CAPSO-Na eluent

Conditions: Column (25 cm x 5 mm i.d.) packed with Spherisorb S5W (batch 5493/1); eluent, methanol - aqueous CAPS / CAPSO-Na buffer 90:10 v/v; buffer composition - each component 0.08 mol l^{-1}; flow rate = 2 ml min^{-1}; temperature = 30°C; detection wavelength = 254 nm.

(a), Solution A; (b), solution C; (c), solution E; (d), solution K; (e), solution L; (f), solution N.

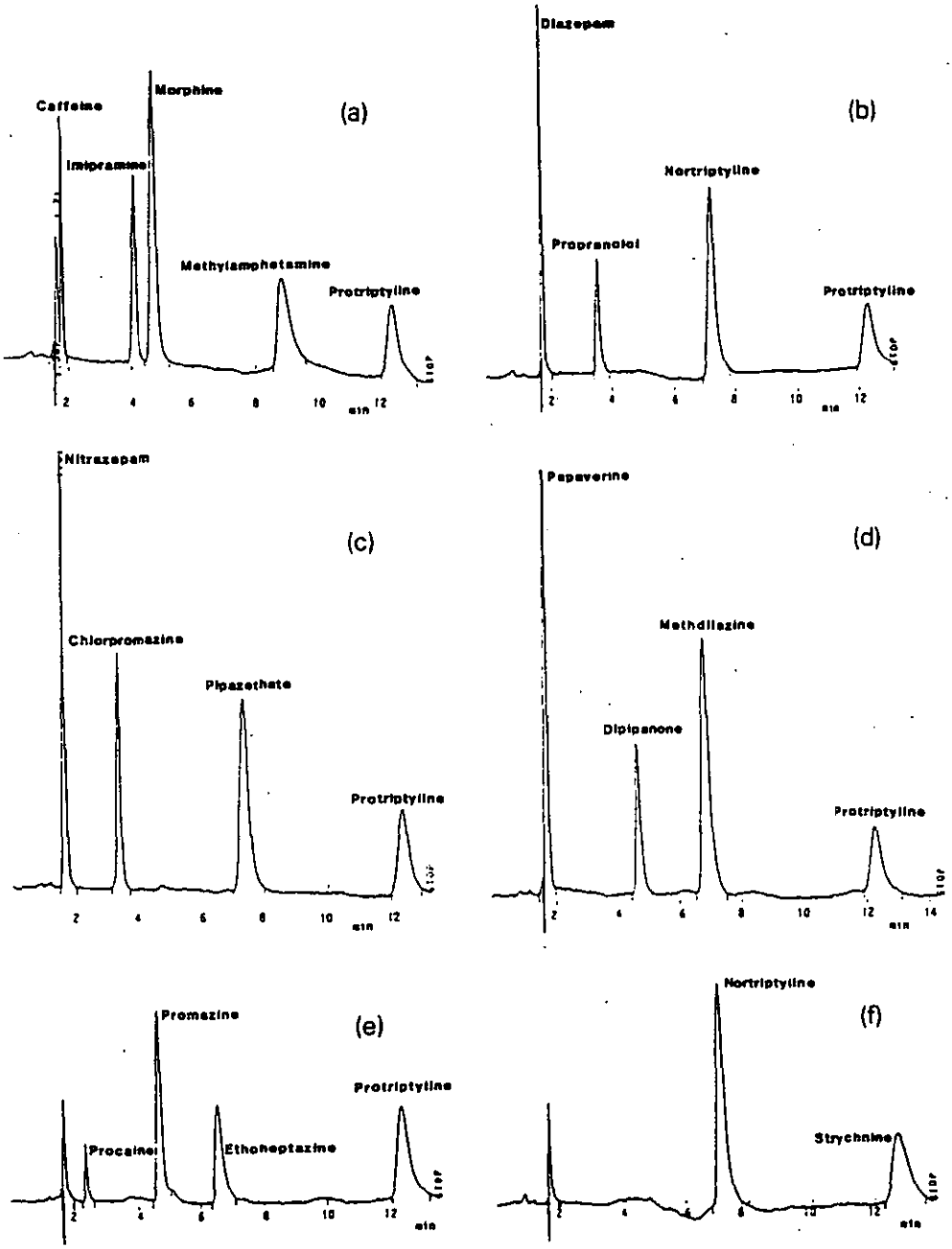


TABLE 4.2: CAPACITY FACTORS AND RELATIVE CAPACITY FACTORS FOR SELECTED TEST COMPOUNDS USING THE CAPS / CAPSO-Na AND AMMONIA ELUENTS

Compound	Capacity factor			Relative k'(x100)[a]		
	Reference data[b]	CAPS / CAPSO-Na[c]	Ammonia[c]	Reference data[b]	CAPS / CAPSO-Na	Ammonia
Amphetamine	1.46	3.29	0.85	40.6	32.8	34.5
Methylamphetamine	3.22	6.46	1.87	89.4	63.9	75.6
Ethylamphetamine	-	4.71	1.19	-	46.9	48.3
Phentermine	1.04	3.21	0.77	28.9	31.8	31.6
Mephentermine	-	9.95	2.51	-	99.1	101.7
Methoxyphenamine	-	9.47	2.24	-	94.9	90.5
N-Methylphenethylamine	3.87	-	2.22	107.5	-	89.7
Ephedrine	2.63	5.38	1.65	73.1	53.4	67.8
Amitriptyline	0.79	1.63	0.47	21.9	16.2	19.2
Nortriptyline	2.32	5.29	1.45	64.4	52.6	59.5

[a]Relative capacity factors relative to protriptyline.
[b]Data taken from reference 24 (protriptyline K' = 3.60), using Spherisorb S5W, batch 4648.
[c]Data recorded on Spherisorb S5W (batch F5493/1).

To investigate further the similarities between the two methods, group contribution analysis was performed to determine the effect of various substituents on retention. The group contribution factor, τ, was calculated according to the equation used by Law[24]:

$$\tau = \log \left[\frac{k'_s}{k'_p} \right]$$

where k'_s is the capacity factor of the substituted compound and k'_p is the capacity factor of the parent compound. A negative τ value corresponds to a decrease in retention.

A high degree of correlation was found between τ values calculated from the results obtained with the two eluents (Table 4.3). The changes were found to be of the same order of magnitude and in the same direction as those recorded by Law[24], indicating that both eluents showed the same selectivity towards these test compounds and that they behaved similarly to the eluent used by Law (which was a variant of the ammonia eluent[24]).

This would appear to confirm that the CAPS / CAPSO-Na eluent is promoting elution conditions similar to those produced using eluents based on ammonia, and that the dominant retention mechanism was almost certainly ion-exchange, as previously suggested[24].

TABLE 4.3: COMPARISON OF GROUP CONTRIBUTION FACTORS (τ) FOR THE CAPS / CAPSO-Na AND AMMONIA ELUENTS

Parent Compound	Substituted Compound	Substituent	Group Contribution Factor, τ		
			CAPS/CAPSO-Na	Ammonia	Reference[d]
Amphetamine	Methylamphetamine	N-Methyl	+0.29	+0.34	+0.34
Phentermine	Mephentermine	N-Methyl	+0.66	+0.51	-
Amphetamine	Ethylamphetamine	N-Ethyl	+0.16	+0.15	-
Amphetamine	Phentermine	1-Alkyl	-0.01	-0.04	-0.13
N-Methylphenethylamine	Methylamphetamine	1-Alkyl	-	-0.07	-0.08
Mephentermine	Methoxyphenamine	1-'O'	-0.02	-0.05	-
Methylamphetamine	Ephedrine	2-Hydroxy	-0.08	-0.05	-0.09
Nortriptyline (NHMe)	Amitriptyline (NMe_2)	N-Methyl (2nd)	-0.51	-0.49	-0.47

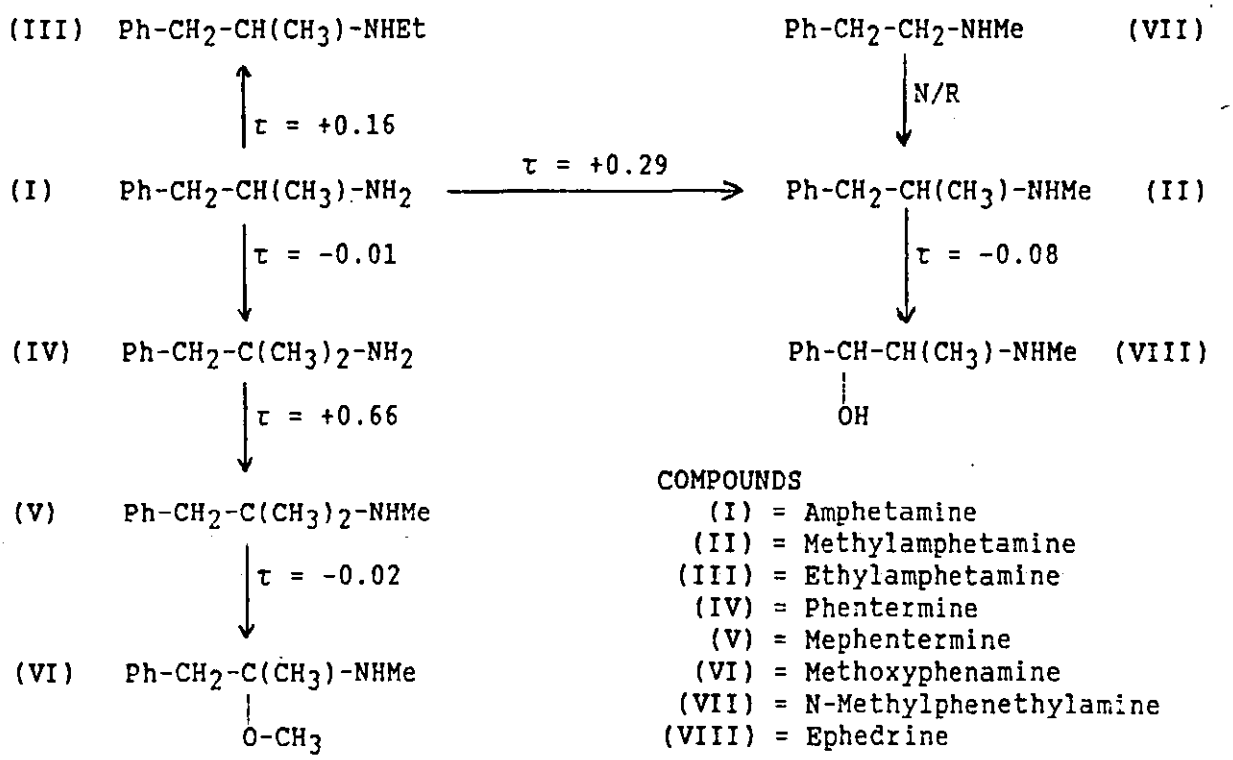
[d]Data taken from reference 24.

4.3.1 THE EFFECT OF SUBSTITUTION ON RETENTION

N-alkyl substitution was found to increase the retention of the analytes, (Table 4.3, Figure 4.3) with N-methyl substitution having a significantly greater effect than N-ethyl. The smaller effect of N-ethyl substitution with respect to N-methyl substitution is probably caused by increased steric hindrance affecting the nitrogen - silanol interactions. Similar observations were recorded by Law[24], who ruled out the possibility that changes in retention were related to changes in pK_a at the substituted N-atom, and at present there seems to be no obvious explanation of the large increases in retention caused by N-methyl substitution. However, the addition of a second N-methyl group (nortriptyline to amitriptyline) led to a significant decrease in retention, as previously observed[24], although once again no simple explanation was offered.

Addition of 1-alkyl and hydroxy groups led to a reduction in retention. Whilst there is no obvious explanation for the effect of alkyl substitution (unless there is some remote steric effect), the influence of hydroxy groups is probably related to intramolecular interaction with the amine group, which would be expected to reduce the nitrogen - silanol interactions[24].

Figure 4.3: Group contribution factors (τ) for amphetamine and related compounds using the CAPS / CAPSO-Na eluent



CHAPTER 5: TESTING THE ROBUSTNESS OF THE NEW METHOD

The robustness and reproducibility of the new method were determined by varying the experimental parameters, temperature, flow rate, injection volume, and buffer composition. These tests included a series of runs using the selected standard conditions to monitor the reproducibility over a period of time.

5.1 REPRODUCIBILITY UNDER STANDARD CONDITIONS

Five runs were carried out using the standard conditions for the new method and the reproducibility of the results is shown in Table 5.1. These experiments included two eluents prepared from one batch of buffer solution, and three eluents prepared from a second batch of buffer.

The variation in capacity factors was about 4% RSD and, except for the rapidly eluting compounds, the variation in relative capacity factors was much lower (Table 5.1) although dipipanone stood out as being poorer that other compounds with similar retentions. In previous studies with the ammonia eluent this compound was particularly sensitive to changes in experimental conditions[35]. The variations in retention were much smaller than the difference between the results on the two columns packed from different batches of silica (section 4.2.2.2) and suggested that batch-to-batch variations in the silica had significant effects on retention in a similar manner to the differences observed with the ammonia[33,36] and diamine eluents[37].

In this series of separations the retention times and capacity factors showed a consistent downward drift with each subsequent analysis, although the relative capacity factors remained consistent. Inspection of the column at the end of the series of experiments revealed a 1 mm void at the top, indicating that the analytical column was slowly dissolving or being etched by the eluent. It is possible that the drift in retention was related to the dissolution of the silica during the study. A silica pre-column was being used between the pump and the injector to extend the lifetime of the analytical column and its presence would appear to be essential, despite the study by Law and Chan which found dissolution to be negligible[95].

TABLE 5.1: REPRODUCIBILITY OF THE CAPACITY FACTORS AND RELATIVE CAPACITY FACTORS FOR THE SEPARATION OF BASIC DRUGS ON A SILICA COLUMN

Five repeated separations; column, Spherisorb S5W (batch F5493/1); eluent, methanol - aqueous CAPS / CAPSO-Na buffer, (each component 0.08 mol l^{-1}), 90:10 v/v; flow rate = 2.0 ml min^{-1}; temperature, 30°C.

Compound	*Capacity factor*			*Relative capacity factor (x 100)*[a]		
	Mean	*S.D.*	*C.V.*	*Mean*	*S.D.*	*C.V.*
Nitrazepam	0.29	0.00	-	3.3	0.1	3.0
Diazepam	0.32	0.01	3.1	3.7	0.1	2.7
Papaverine	0.40	0.01	2.5	4.5	0.1	2.7
Caffeine	0.48	0.01	2.1	5.5	0.2	3.6
Dextropropoxyphene	0.68	0.02	3.0	7.7	0.0	-
Cocaine	0.81	0.03	3.7	9.2	0.1	1.1
Procaine	0.90	0.03	3.3	10.1	0.1	1.0
Amitriptyline	1.57	0.05	3.2	17.8	0.2	1.1
Chlorpromazine	1.67	0.05	3.0	18.9	0.2	1.1
Propranolol	1.83	0.06	3.3	20.7	0.1	0.5
Imipramine	2.32	0.07	3.0	26.3	0.2	0.8
Codeine	2.75	0.10	3.6	31.1	0.2	0.6
Promazine	2.77	0.10	3.6	31.3	0.2	0.6
Dipipanone	2.78	0.14	5.0	31.4	0.5	1.6
Morphine	2.83	0.09	3.2	32.1	0.3	0.9
Phentermine	2.94	0.11	3.8	33.3	0.2	0.6
Amphetamine	3.06	0.12	3.9	34.6	0.1	0.3
Phenylephrine	3.69	0.14	3.8	41.7	0.2	0.5
Pholcodine	3.73	0.15	4.0	42.2	0.2	0.5
Ethoheptazine	4.27	0.17	4.0	48.2	0.2	0.4
Prolintane	4.27	0.18	4.2	48.4	0.3	0.6
Methdilazine	4.54	0.17	3.7	51.3	0.3	0.6
Nortriptyline	4.77	0.17	3.6	54.0	0.3	0.6
Pipazethate	4.94	0.22	4.5	55.8	0.4	0.7
Ephedrine	5.10	0.19	3.7	57.7	0.2	0.4
Methylamphetamine	6.15	0.24	3.9	69.6	0.2	0.3
Protriptyline[b]	8.85	0.38	4.3	100.0	-	-
Strychnine	9.31	0.35	3.8	105.2	0.7	0.7

[a]Relative capacity factors relative to protriptyline.
[b]Based on test solution L.

5.2 EFFECT OF CHANGES IN THE OPERATING CONDITIONS

5.2.1 INFLUENCE OF BUFFER pH

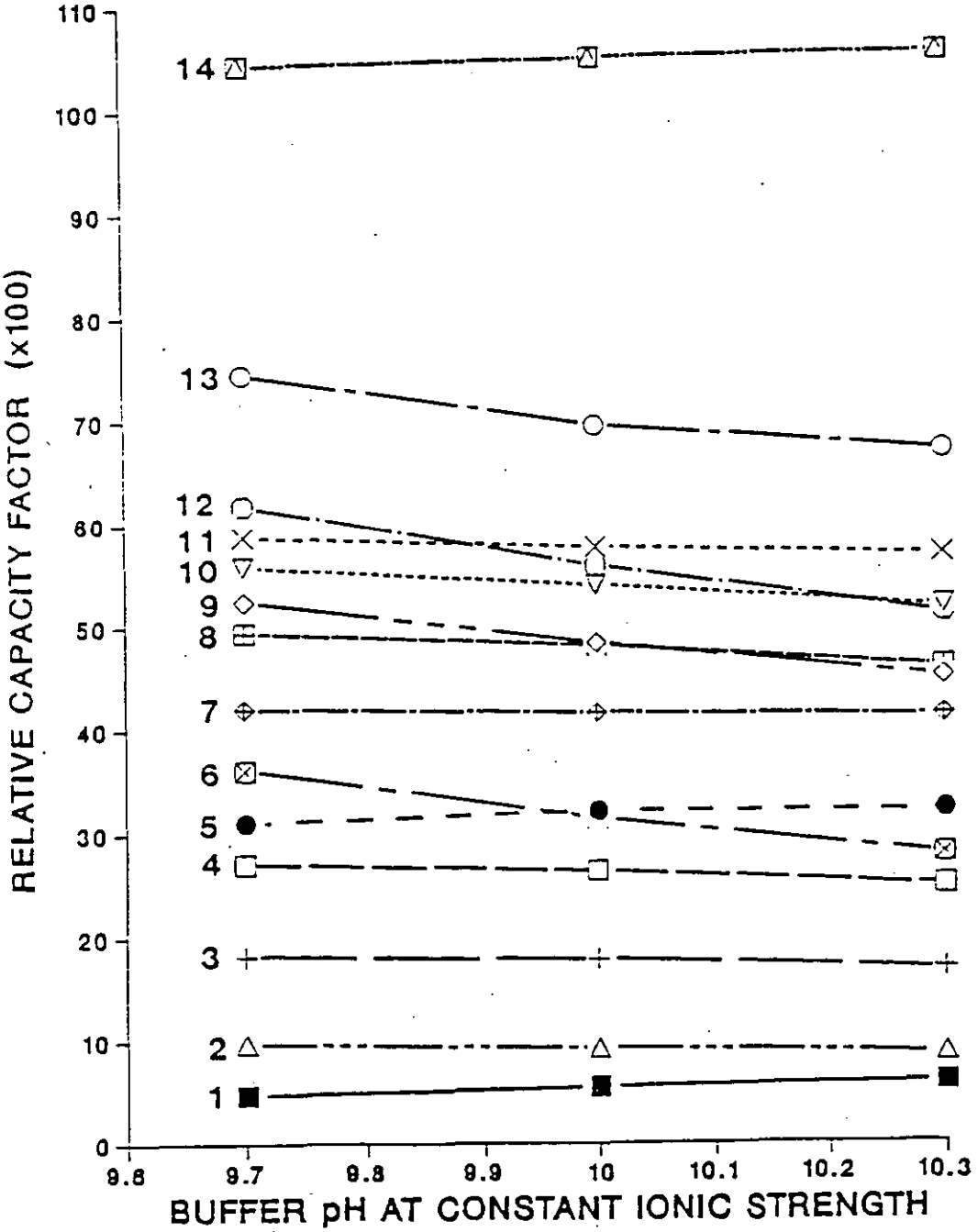
To investigate the effect of small pH changes in the buffer on the separation of the drugs, buffers of pH 9.7 and 10.3, with ionic strengths equal to that of the standard buffer, (0.080 mol l^{-1}), were tested. For

all analytes the retention times decreased on going from low to higher pH. This was probably caused by a reduction in the degree of protonation of the bases as observed earlier by Schmid and Wolf[32] (see 2.2.1.1 (a)). However, some of the bases were affected more than others, but for most of the drugs the relative capacity factors also decreased as the pH was increased (Figure 5.1). Particularly large decreases were observed for dipipanone (36.05 to 28.13) prolintane (52.41 to 45.25), pipazethate (61.64 to 51.43) and methylamphetamine (74.53 to 67.30). However, the pK_as of these compounds are similar to those of many of the other drugs (Table 4.1). The steric environment of the basic groups appears to be an important factor as the first three of these compounds all contain a cyclic tertiary amine with a substituted N-alkyl side chain. As noted earlier (see 4.2.2.1) these three compounds also showed particular sensitivity to changes in separation conditions. In contrast, tertiary amines containing only N-methyl substituents, such as methdilazine and cocaine, showed much smaller effects relative to protriptyline which is also an N-methyl compound. In his study Law[24] had found that the size of alkyl substituents had a marked effect. The introduction of N-methyl groups caused positive retention changes whereas larger alkyl substituents had a negative effect on retention (see 4.3 above). These effects suggest that the larger substituents on a cyclic amine may limit the interaction of the basic group to a particular type of silanol site on the silica surface whose ionisation changes to a different extent than the other silanol groups with changes in eluent pH.

The relative retentions increased for a few compounds, including strychnine, codeine and morphine (31.03 to 32.40; pK_a 8.0 and 9.9). In the last case this might reflect the ionisation of the phenolic group to give a doubly charged species although phenylephrine which also contains a phenolic group changed very little. These relative changes were significant as a test of the robustness of the assay and emphasise the need for a constant buffer pH to obtain reproducible results. The lower pH also caused many of the compounds to elute with a lower efficiency but the higher pH reduced the efficiency of protriptyline (pH 9.7, N = 5960, pH 10, N = 6027, pH 10.3, N = 4318). Clearly although systematic changes with pH have been observed for small sample sets, such as the tricyclic antidepressants[32] or aryl alkylamines[24] the resulting conclusions cannot be generalised to account for the relative changes observed in the present larger range of sample types.

Figure 5.1: Variation of relative capacity factors with pH

Conditions as Figure 4.2, but ratio of buffer components varied to give different buffer pHs at constant ionic strength. Compounds; (1), caffeine; (2), cocaine; (3), amitriptyline; (4), imipramine; (5), morphine; (6), dipipanone; (7), phenylephrine; (8), ethoheptazine; (9), prolintane; (10), nortriptyline; (11), ephedrine; (12), pipazethate; (13), methylamphetamine; (14), strychnine.



5.2.2 INFLUENCE OF BUFFER IONIC STRENGTH

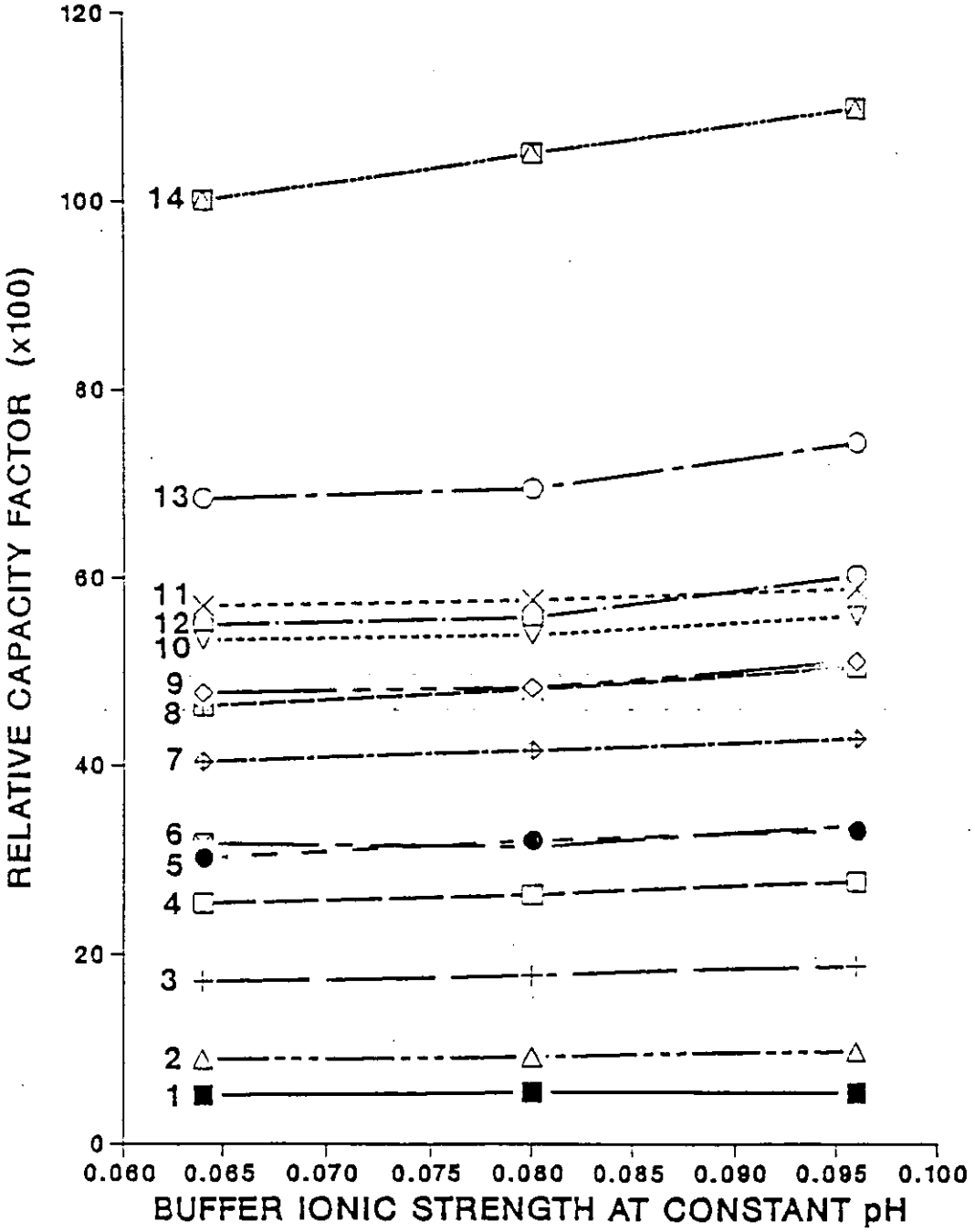
The ionic strength of the present eluent was much lower than the previous ammonia eluent and this was a major factor leading to an increase in retention times. These changes agree with the predominant mode of retention being cation exchange (see 2.2.1.1 (b)). The effects of changes in the buffer concentration by ± 20% were examined by using buffers with ionic strengths of 0.096 and 0.064 mol l^{-1} at a constant buffer pH of 10.0. Most of the compounds showed a decrease in retention as the ionic strength increased, with protriptyline showing a quite significant change (13.4 minutes decreasing to 12.6 minutes), but strychnine (13.4 minutes to 13.7 minutes) and pipazethate (7.4 to 8.2 minutes) increased slightly. For most compounds the relative K' increased slightly with some compounds showing a more marked effect (Figure 5.2). The changes in the relative capacity factors were outside the experimental range for the repeated assays and suggested that small changes in the buffer could have a significant effect on an analysis. Again the compounds most affected were those which also markedly changed with pH.

5.2.3 INFLUENCE OF ELUENT COMPOSITION

When the proportion of methanol in the eluent was changed from 90% to 88% or 92%, variations in retention times and relative capacity factors were observed. Increasing methanol content caused an increase in the retention times but a very small change in the relative capacity factors for most compounds. Decreasing the methanol content caused the retention of the compounds to decrease, as would be expected from an eluent with increased ionic strength (due to the higher % of buffer). The effect on relative capacity factors was more significant as they were between 1.8% and 12% higher than the standard results. This was due to the much larger reduction in the retention of protriptyline (relative to the other analytes) with decreasing methanol content, as had been observed previously with the ammonia eluent[35]. These observations clearly indicate that careful preparation of the eluent is essential if reproducible results are to be obtained.

Figure 5.2: Variation of relative capacity factors with ionic strength

Conditions as Figure 4.2, but ratio of buffer components varied to give different buffer ionic strengths at constant pH. Compounds as Figure 5.1.



5.2.4 INFLUENCE OF OPERATING TEMPERATURE

The retention times of the drugs decreased as the temperature increased from 20°C to 40°C, but for most of the compounds the relative capacity factors increased with increasing temperature. The increases were proportionally more significant for the weakly retained compounds (relative $K' < 10.1$, see Figure 5.3). For the rest of the compounds the changes were around ± 4%. Exceptions to this trend were strychnine and methylamphetamine which both showed decreases in relative capacity factor with increasing temperature, whilst pipazethate, methdilazine and ephedrine did not exhibit any obvious trend. Increasing the temperature also caused the efficiencies of the analytes to increase. This observation agrees with previous studies[32], where the increased efficiency was attributed to increased rates of mass transfer of the analytes (see 2.2.1.1 (d)).

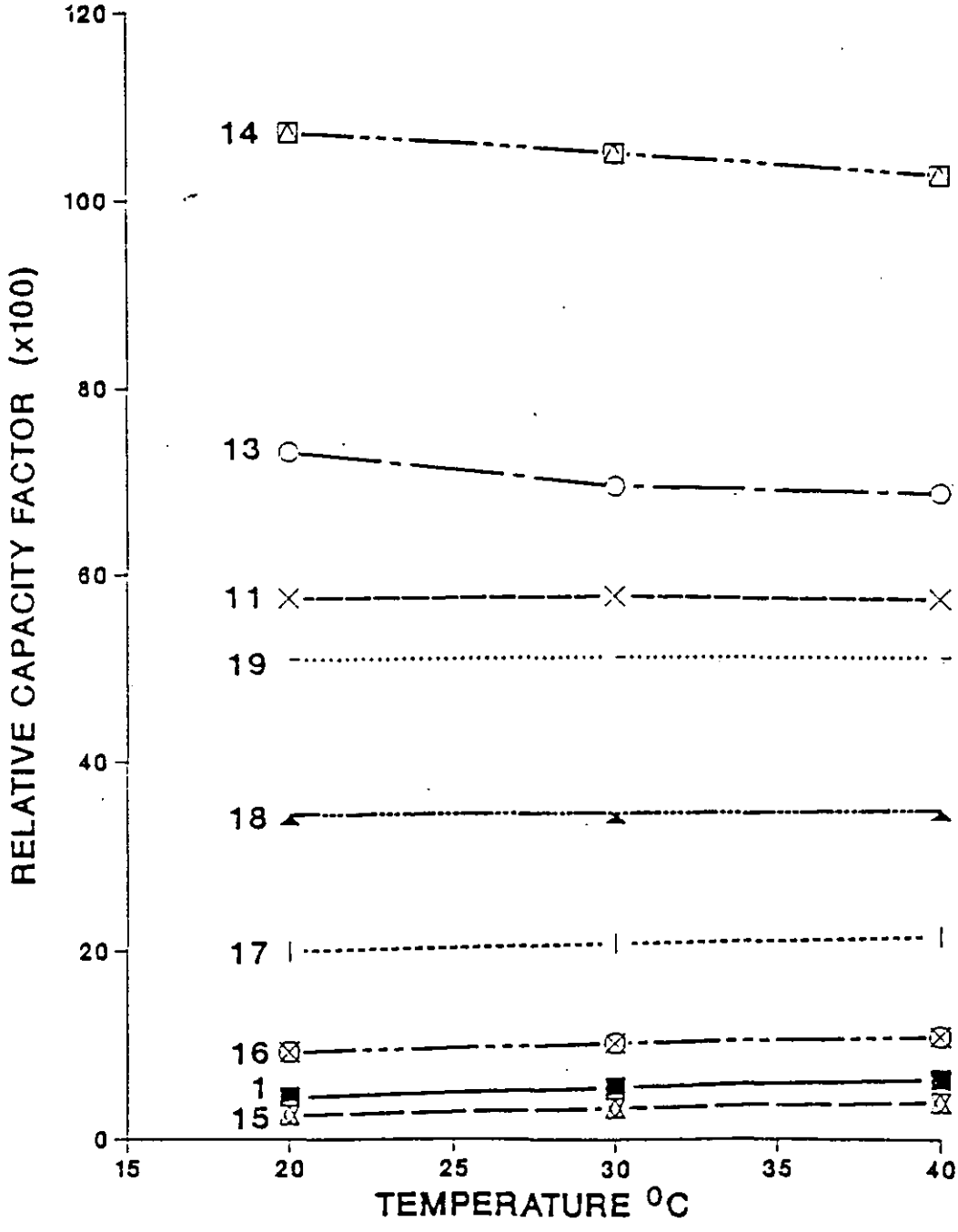
The relative capacity factors recorded at 20°C and 40°C were outside the range of experimental error determined for the five standard assays. Thus to obtain reproducible results it would be necessary to thermostat the column and to specify the temperature in any method protocol.

5.2.5 INFLUENCE OF OPERATING PRESSURE

In a recent study Tanaka et al.[161] reported large changes in retention on changing the operating pressure in the column and these were ascribed to changes in the equilibria. An ion-exchange chromatographic separation might therefore be susceptible to similar effects, particularly if the drug is partially ionised. On changing the flow rate from 2.0 ml min^{-1} to 1.0 ml min^{-1}, which changed the operating pressure from 145 - 148 bar to 76 - 78 bar, the capacity factors and relative capacity factors were unaffected, indicating that the results were independent of the operating pressure (or flow rate).

Figure 5.3: Variation of relative capacity factors with temperature

Other conditions as Figure 4.2: Compounds as Figure 5.1, plus; (15), nitrazepam; (16), procaine; (17), propranolol; (18), amphetamine; (19), methdilazine.



5.2.6 INFLUENCE OF SAMPLE SIZE

In a case of a real life sample submitted for analysis the concentration of any drug present in the solution will be unknown. Variations in relative capacity factors caused by changing the loading of the analyte on the column may therefore cause problems in identification. A sample of solution L was diluted to 20% of its original concentration and four replicate 1µl injections were examined. The retention time of protriptyline was unaltered but the retentions and relative capacity factors of the other test compounds were slightly increased. The changes were small and were less than two standard deviations (from Table 5.1) for procaine and promazine and about 3 standard deviations for ethoheptazine.

5.3 CONCLUSIONS

This study has shown that the organic buffer salts CAPS and CAPSO-Na can be used to prepare reproducible buffer solutions for use in the analysis of basic drugs on silica. The method was found to be susceptible to changes in some of the operating conditions and these parameters would need to be closely specified in the method protocol.

Increases in the operating temperature or the ionic strength of the eluent, or a decrease in the proportion of the buffer caused the relative capacity factors to increase, whilst increasing the pH of the eluent or the proportion of methanol caused the relative capacity factors to decrease. All these parameters would need to be closely controlled to obtain long term reproducibility.

CHAPTER 6: STORAGE AND LONG TERM STABILITY OF SILICA

6.1 INTRODUCTION

In previous studies by Gill and Smith *et al.*[33,34,36] using ammonia buffered eluents, it had been suggested that poor reproducibility of the eluent had hampered the detailed chromatographic comparison of silica samples. In studies within a single laboratory under carefully controlled conditions, an acceptable degree of reproducibility could be achieved on different columns from a single batch of silica[36], but interlaboratory reproducibility on a single batch had been poor[33,34], suggesting that method transfer could be difficult.

Thus it was of interest to use the newly developed method, which had been found to give highly reproducible results on a single column, (see Table 5.1), to investigate the within-batch and between-batch variations for columns packed with Spherisorb S5W. Such experiments were considered necessary to determine whether the reported variations between silica batches[36] were real, or if in part they could be accounted for by irreproducible experimental conditions.

Thus, a series of experiments were carried out over a one year period to examine the stability of three nominally identical 'storage trial' columns packed from a single bottle of silica (Spherisorb S5W, batch F5493/1). The within-batch variation and stability of the silica was further tested by comparing results obtained on a number of columns prepared from this batch of silica over a 17 month period.

The between-batch variation and stability was tested by preparing and testing a column from batch 2752, and then examining it 14 months later; these results were compared with a new column prepared from the same batch.

6.2 ONE YEAR STORAGE TRIALS

6.2.1 PREPARATION AND TESTING OF COLUMNS

Three 'storage trial' columns were prepared following the method described above (Chapter 3), using Spherisorb S5W taken from one bottle.

These columns were identified as F5493/1 - 10.1, 10.2 and 10.3.

The columns were tested immediately after packing, and then at intervals of 1, 3, 6 and 12 months, using the selected standard operating conditions (see Table 5.1). Each column was equilibrated with fresh eluent overnight prior to being tested. After testing, columns 10.1 and 10.3 were washed with methanol prior to storage whilst column 10.2 was washed with fresh eluent prior to storage, in order to determine if the storage conditions influenced the stability of the silica.

6.2.2 RESULTS OF THE STORAGE TESTS

The capacity factors for all the test compounds decreased on all three columns over the period of the study, e.g. for protriptyline on column 10.1: k' = 10.40, falling to k' = 8.04. The relative capacity factors were found to be more robust for some analytes (Tables 6.1, 6.2, 6.3), but for others significant changes (*i.e.* ± >1 RCF unit) were recorded (Table 6.4), resulting in changes in column selectivity (Figures 6.1 and 6.2). The results for methylamphetamine, where quoted, are for a 10-fold diluted sample of solution A, due to the problems experienced with the peak shape for this compound (see Chapter 7). In some cases it was not possible to obtain meaningful results even with the diluted solution; 'N.R.' indicates that no result was recorded.

The data recorded on column 10.3 at the six month interval (Table 6.3) must be treated with caution as a leak at one of the pump heads was discovered towards the end of the work, and also because it had been necessary to work at a flow rate of 1.5 ml min^{-1} due to operating difficulties with the pump. The leak may have caused variations in the retention times, as a result of variable flow rates, but the change in nominal flow rate (from the usual 2.0 ml min^{-1}) should not have had any effect on the results (see section 5.2.5).

It was necessary to repack the top of column 10.3 at the one-year interval before any meaningful chromatographic results could be recorded. The column had a void of >1 mm at the top; this was repaired with silica from the same source as that used to pack the column.

TABLE 6.1: COMPARISON OF RELATIVE CAPACITY FACTORS FOR STORAGE TRIAL COLUMN F5493/1 - 10.1

Solution	Compound	Start	1 Month	3 Months	6 Months	1 Year
A	Caffeine	3.89	3.97	4.18	4.28	4.44
	Imipramine	25.09	24.98	25.61	25.66	25.40
	Morphine	27.78	27.47	28.30	28.72	30.70
	Methylamphetamine	71.38	70.80	N.R.	71.64	N.R.
	Protriptyline	100.00	100.00	100.00	100.00	100.00
B	Cocaine	8.18	7.99	8.22	8.49	8.21
	Phentermine	32.51	31.75	31.90	32.09	31.39
	Ephedrine	54.22	53.23	53.60	54.32	55.31
	Protriptyline	100.00	100.00	100.00	100.00	100.00
C	Diazepam	2.55	2.85	2.76	2.89	2.63
	Propranolol	20.19	20.02	20.22	20.61	19.91
	Nortriptyline	53.09	52.77	52.96	53.50	53.13
	Protriptyline	100.00	100.00	100.00	100.00	100.00
D	Amitriptyline	16.30	16.38	16.99	17.11	16.87
	Prolintane	53.52	50.39	52.00	51.77	47.41
	Protriptyline	100.00	100.00	100.00	100.00	100.00
E	Nitrazepam	2.01	2.18	2.30	2.38	2.18
	Chlorpromazine	17.19	17.40	17.85	18.05	18.07
	Pipazethate	61.62	58.87	59.00	58.50	57.27
	Protriptyline	100.00	100.00	100.00	100.00	100.00
F	Dextropropoxyphene	6.89	6.85	7.02	7.20	6.65
	Amphetamine	32.77	31.88	32.48	32.69	32.94
	Pholcodine	38.44	36.85	38.02	38.93	42.21
	Protriptyline	100.00	100.00	100.00	100.00	100.00
K	Papaverine	3.28	3.51	3.91	3.74	3.50
	Dipipanone	40.67	37.55	36.68	36.28	30.91
	Methdilazine	48.50	48.57	49.67	50.57	51.41
	Protriptyline	100.00	100.00	100.00	100.00	100.00
L	Procaine	8.89	8.93	9.19	9.47	9.25
	Promazine	29.42	29.51	29.96	30.62	30.68
	Ethoheptazine	45.56	44.99	45.71	46.71	46.96
	Protriptyline	100.00	100.00	100.00	100.00	100.00
M	Codeine	27.88	27.83	28.32	28.99	30.59
	Phenylephrine	38.04	37.40	37.65	38.39	40.67
	Protriptyline	100.00	100.00	100.00	100.00	100.00
N	Nortriptyline	53.09	52.77	52.96	53.50	53.13
	Strychnine	107.00	104.44	104.36	105.99	107.63
	DATE OF RUN	24/08/88	27/09/88	29/11/88	09/03/89	29/08/89
	AGE OF COLUMN	0 DAYS	34 DAYS	97 DAYS	197 DAYS	370 DAYS

TABLE 6.2: COMPARISON OF RELATIVE CAPACITY FACTORS FOR STORAGE TRIAL COLUMN F5493/1 - 10.2

Solution	Compound	Start	1 Month	3 Months	6 Months	1 Year
A	Caffeine	3.94	4.17	4.33	4.39	4.49
	Imipramine	24.07	25.41	25.14	25.42	25.32
	Morphine	27.16	29.82	30.31	31.28	32.97
	Methylamphetamine	68.07	N.R.	70.16	70.67	N.R.
	Protriptyline	100.00	100.00	100.00	100.00	100.00
B	Cocaine	8.00	8.37	8.51	8.63	8.76
	Phentermine	32.00	32.00	31.64	31.62	31.29
	Ephedrine	53.64	55.54	55.18	55.93	57.35
	Protriptyline	100.00	100.00	100.00	100.00	100.00
C	Diazepam	2.83	2.47	2.69	2.76	2.67
	Propranolol	19.98	20.17	20.21	20.29	20.03
	Nortriptyline	52.54	53.02	53.13	53.14	53.36
	Protriptyline	100.00	100.00	100.00	100.00	100.00
D	Amitriptyline	16.14	16.39	16.80	17.08	17.00
	Prolintane	52.23	49.60	50.64	49.94	46.39
	Protriptyline	100.00	100.00	100.00	100.00	100.00
E	Nitrazepam	2.12	2.22	2.30	2.36	2.18
	Chlorpromazine	17.10	17.62	17.93	18.22	18.27
	Pipazethate	59.51	60.01	60.09	59.01	60.86
	Protriptyline	100.00	100.00	100.00	100.00	100.00
F	Dextropropoxyphene	6.83	6.76	6.79	6.80	6.20
	Amphetamine	32.14	32.61	32.69	32.68	33.58
	Pholcodine	37.69	40.19	40.62	41.65	44.61
	Protriptyline	100.00	100.00	100.00	100.00	100.00
K	Papaverine	3.40	3.46	3.52	3.85	3.48
	Dipipanone	39.07	35.21	33.48	31.98	27.66
	Methdilazine	47.95	50.03	51.09	52.11	52.75
	Protriptyline	100.00	100.00	100.00	100.00	100.00
L	Procaine	8.84	9.13	9.44	9.71	9.62
	Promazine	29.09	30.07	30.62	31.09	31.21
	Ethoheptazine	44.87	46.70	47.19	47.91	47.95
	Protriptyline	100.00	100.00	100.00	100.00	100.00
M	Codeine	27.89	29.71	30.45	31.32	32.68
	Phenylephrine	38.32	39.38	39.93	40.72	43.28
	Protriptyline	100.00	100.00	100.00	100.00	100.00
N	Nortriptyline	52.54	53.02	53.13	53.14	53.36
	Strychnine	107.09	108.74	108.56	109.71	108.70
	DATE OF RUN	26/08/88	28/09/88	30/11/88	10/03/89	30/08/89
	AGE OF COLUMN	0 DAYS	33 DAYS	96 DAYS	198 DAYS	369 DAYS

TABLE 6.3: COMPARISON OF RELATIVE CAPACITY FACTORS FOR STORAGE TRIAL COLUMN F5493/1 - 10.3

Solution	Compound	Start	1 Month	3 Months	6 Months[a]	1 Year
A	Caffeine	4.07	3.91	4.16	4.39	4.36
	Imipramine	24.99	24.89	25.16	26.38	25.23
	Morphine	27.93	27.25	28.04	30.48	31.37
	Methylamphetamine	68.26	N.R.	67.81	73.93	N.R.
	Protriptyline	100.00	100.00	100.00	100.00	100.00
B	Cocaine	8.07	7.84	8.07	8.76	8.25
	Phentermine	31.91	31.67	31.61	32.43	31.09
	Ephedrine	53.65	53.17	53.61	55.80	55.71
	Protriptyline	100.00	100.00	100.00	100.00	100.00
C	Diazepam	2.64	2.73	2.75	2.70	2.61
	Propranolol	19.96	19.83	20.02	20.85	19.67
	Nortriptyline	52.54	52.76	52.68	54.28	53.33
	Protriptyline	100.00	100.00	100.00	100.00	100.00
D	Amitriptyline	16.25	16.23	16.59	17.48	16.75
	Prolintane	51.89	50.20	51.39	52.59	44.89
	Protriptyline	100.00	100.00	100.00	100.00	100.00
E	Nitrazepam	2.19	2.18	2.25	2.37	2.18
	Chlorpromazine	17.25	17.37	17.58	18.60	18.08
	Pipazethate	59.95	58.48	58.83	61.84	55.64
	Protriptyline	100.00	100.00	100.00	100.00	100.00
F	Dextropropoxyphene	7.01	6.86	6.89	7.33	6.52
	Amphetamine	32.57	32.07	32.16	33.29	33.10
	Pholcodine	38.60	37.27	37.88	41.11	42.21
	Protriptyline	100.00	100.00	100.00	100.00	100.00
K	Papaverine	3.48	3.51	3.60	4.01	3.71
	Dipipanone	39.20	37.64	35.86	36.08	28.24
	Methdilazine	48.52	48.66	49.26	52.40	51.43
	Protriptyline	100.00	100.00	100.00	100.00	100.00
L	Procaine	8.91	8.83	9.10	9.83	9.14
	Promazine	29.26	29.18	29.78	31.54	30.88
	Ethoheptazine	45.11	44.62	45.45	48.68	46.75
	Protriptyline	100.00	100.00	100.00	100.00	100.00
M	Codeine	28.07	27.68	28.40	30.04	31.05
	Phenylephrine	38.51	37.11	37.74	39.90	41.45
	Protriptyline	100.00	100.00	100.00	100.00	100.00
N	Nortriptyline	52.54	52.76	52.68	54.28	53.33
	Strychnine	106.35	105.19	104.07	110.58	105.71
	DATE OF RUN	31/08/88	29/09/88	01/12.88	16/03/89	31/08/89
	AGE OF COLUMN	0 DAYS	29 DAYS	92 DAYS	197 DAYS	365 DAYS

[a]This data is unreliable, see text for details.

TABLE 6.4: SUMMARY OF SIGNIFICANT CHANGES IN RELATIVE CAPACITY FACTORS ON STORAGE TRIAL COLUMNS F5493/1 No. 10.1, 10.2 & 10.3

Compound	*Column*	*Relative k'(x100)*		*Total*	*% Change*
		Start	*1 Year*	*Change*	
Morphine	10.1	27.78	30.70	+2.92	+10.51
	10.2	27.16	32.97	+5.81	+21.39
	10.3	27.93	31.37	+3.44	+12.32
Ephedrine	10.1	54.22	55.31	+1.09	+2.01
	10.2	53.64	57.35	+3.71	+6.92
	10.3	53.65	55.71	+2.06	+3.84
Prolintane	10.1	53.52	47.41	-6.11	-11.42
	10.2	52.23	46.39	-5.84	-11.18
	10.3	51.89	44.89	-7.00	-13.49
Chlorpromazine	10.1	17.19	18.07	+0.88	+5.12
	10.2	17.10	18.27	+1.17	+6.84
	10.2	17.25	18.08	+0.83	+4.81
Pipazethate	10.1	61.62	57.27	-5.34	-8.67
	10.2	59.51	60.86	+1.35	+2.27
	10.3	59.95	55.64	-4.31	-7.19
Pholcodine	10.1	38.44	42.21	+3.77	+9.81
	10.2	37.69	44.61	+6.92	+18.36
	10.3	38.60	42.21	+3.61	+9.35
Dipipanone	10.1	40.67	30.91	-9.76	-24.00
	10.2	39.07	27.66	-11.41	-29.20
	10.3	39.20	28.24	-10.96	-27.96
Methdilazine	10.1	48.50	51.41	+2.91	+6.00
	10.2	47.95	52.75	+4.80	+10.01
	10.3	48.52	51.43	+2.91	+6.00
Promazine	10.1	29.42	30.62	+1.20	+4.08
	10.2	29.09	31.21	+1.12	+3.85
	10.3	29.26	30.88	+1.62	+5.54
Ethoheptazine	10.1	45.56	46.96	+1.40	+3.07
	10.2	44.87	47.95	+3.08	+6.86
	10.3	45.11	46.75	+1.64	+3.64
Codeine	10.1	27.88	30.59	+2.71	+9.72
	10.2	27.89	32.68	+4.79	+17.17
	10.3	28.07	31.05	+2.98	+10.62
Phenylephrine	10.1	38.04	40.67	+2.63	+6.91
	10.2	38.32	43.28	+4.96	+12.94
	10.3	38.51	41.45	+2.94	+7.67

Figure 6.1: Changes in Relative Capacity Factors with time for selected analytes on column F5493/1-No. 10.1

Conditions: see text. Compounds: (1), morphine; (2), pholcodine; (3), dipipanone; (4), methdilazine; (5), nortriptyline; (6), prolintane; (7), pipazethate.

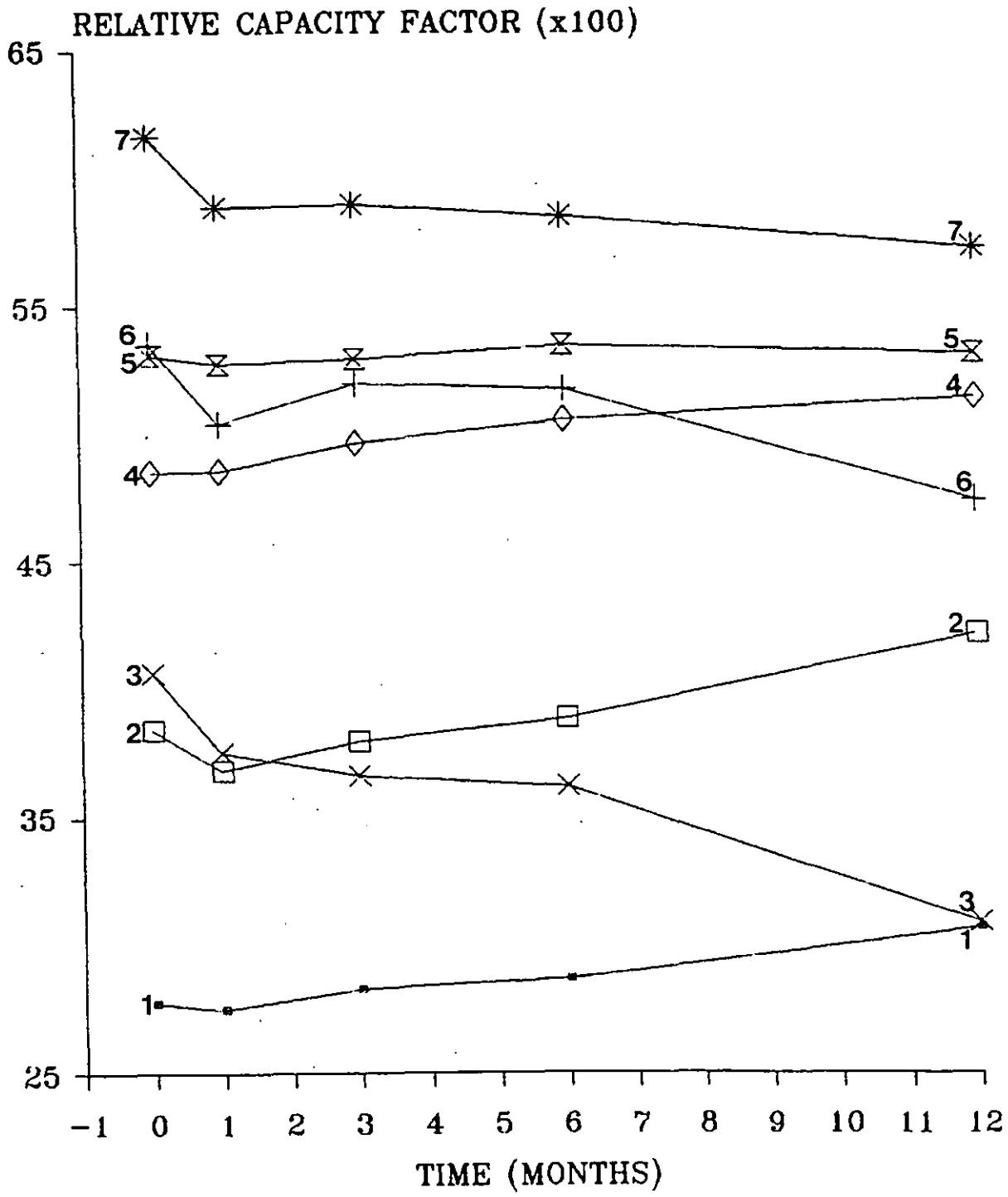
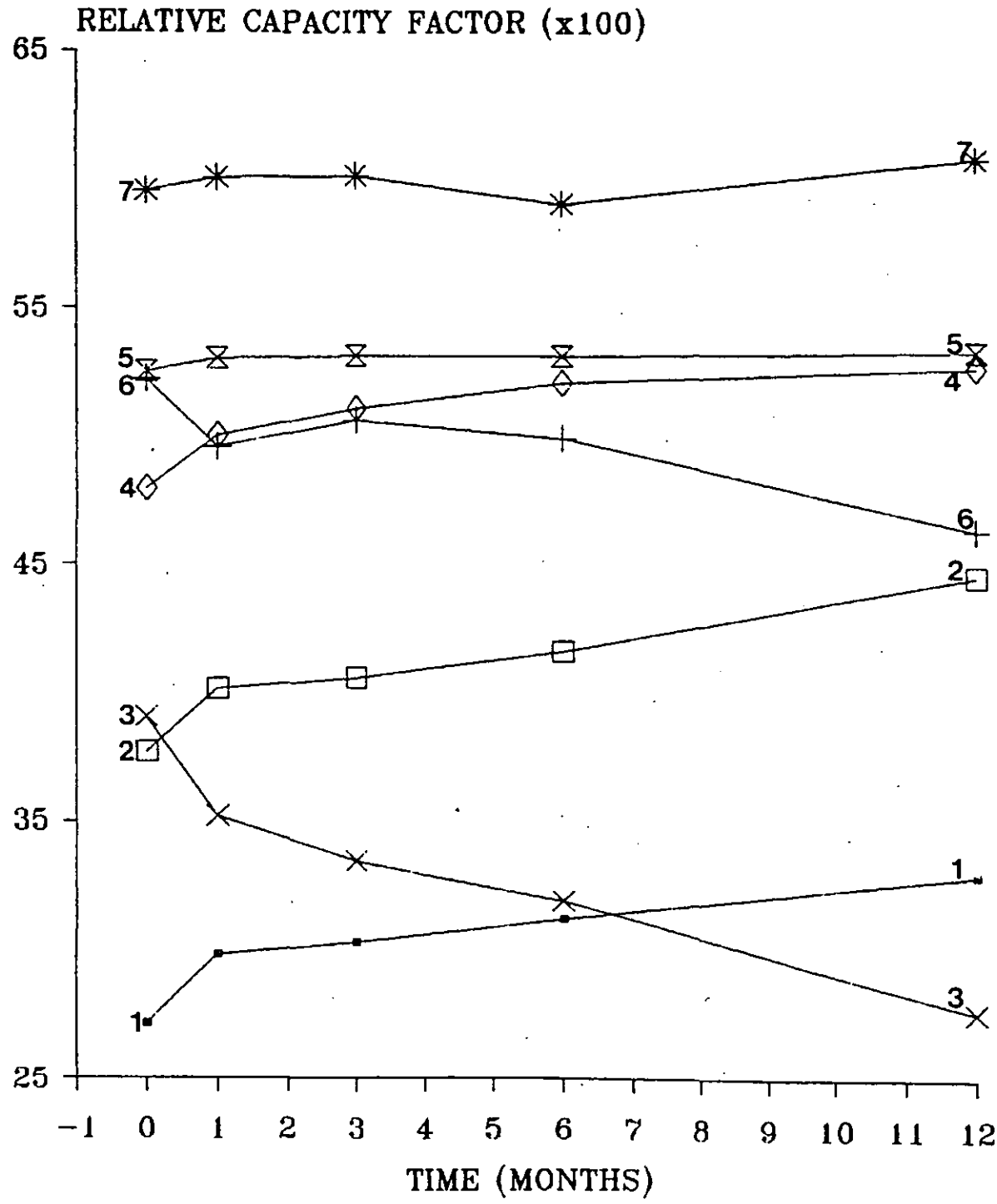


Figure 6.2: Changes in Relative Capacity Factors with time for selected analytes on column F5493/1-No. 10.2

Conditions and compounds as Figure 6.1



The performance of the column stored in eluent (10.2) changed more than the performance of either of the columns stored in methanol (Table 6.4). In most cases there were larger increases in relative capacity factors (RCFs), (*e.g.* phenylephrine) but smaller decreases (*e.g.* prolintane), although this was not true for every analyte. These results suggest that columns should be flushed with an 'inert' solvent such as methanol before storing in order to minimise changes in the retention properties.

Three compounds, dipipanone, prolintane and pipazethate, showed significant decreases in RCFs over the period of the study, with the exception of pipazethate on column 10.2, where a small increase was recorded. Both dipipanone and prolintane had been selected by Smith *et al.*[36] for use in a limited set of test compounds to test silica stability, as their retention appeared to be sensitive to the packing material. Pipazethate also stood out as a sensitive compound in robustness tests by Smith *et al.*[36], but its retention variation was less significant than either dipipanone or prolintane. These compounds are all tertiary amines and it is possible that their retention behaviour was related in some way to this common structural feature. However, strychnine, another tertiary amine which showed retention variations with the ammonia system[36], did not show any significant changes in retention during the present studies.

A number of the test compounds showed increases in RCFs during the period of the study. Within this group, sets of structurally related compounds can be identified, *e.g.* ephedrine and phenylephrine, codeine, morphine, pholcodine and ethoheptazine. Of these, phenylephrine had been identified by as a compound behaving in an 'opposite' direction to the tertiary amines[36]. The remaining three compounds in Table 6.4, chlorpromazine, promazine and methdilazine, are all phenothiazine derivatives. It should be noted that their changes in RCFs were generally smaller than for the other compounds.

Since some major changes in retention properties had been identified, it was considered necessary to investigate other samples of the same batch of silica, and samples from another batch of silica, to determine if these changes were features common to more than just this limited sample of three columns.

6.3 WITHIN AND BETWEEN BATCH STORAGE TESTS

6.3.1 OTHER F5493/1 COLUMNS

A number of columns packed with Spherisorb S5W (batch F5493/1) were studied to determine the within-batch stability of the silica. Four columns, including one of the storage trial columns, (10.1), and two others packed from a single bottle of silica, (columns 4.1 and 4.2) were packed over a 17 month period, tested within 5 days of packing, and their performances were compared (Table 6.5).

The data recorded on these columns suggested that changes in the silica had occurred during storage. Once again the capacity factors were found to decrease with increasing age of the silica (*e.g.* protriptyline *k'* decreased from 10.40 (column 10.1) to 7.84 (column 9.1)). Among the compounds which showed changes in RCFs (see Table 6.5, shown in italics), prolintane, pipazethate, dipipanone and, on this occasion strychnine, all showed decreases in RCF with increasing age of the silica. Meanwhile, morphine, ephedrine and phenylephrine all showed small increases in RCF with increasing age of the silica.

It was of interest to examine the significant changes for the two columns which had been packed from the same bottle of silica (columns 4.1 and 4.2) as this would indicate whether unused silica was susceptible to the same type of changes during storage as had been found for packed silica in the storage trial columns. In addition, the degree of any change for the important compounds might give a clue to the dependence of the results on the mode of storage of the silica.

The results indicated that silica stored dry and unpacked showed the same type of changes in chromatographic properties as silica packed in columns (Table 6.6), but that the degree of change was much lower. As before, the tertiary amines dipipanone, prolintane and pipazethate showed decreases in RCFs but, unlike the storage trial columns (10.1 - 3), strychnine also showed a decrease in RCF on the second column packed with this silica sample. Three compounds, ephedrine, pholcodine and phenylephrine, showed significant increases in RCFs; these were previously seen to behave in an opposite way to the tertiary amines.

TABLE 6.5: COMPARISON OF RELATIVE CAPACITY FACTORS SHOWING VARIATIONS BETWEEN NEW COLUMNS PACKED WITH SPHERISORB S5W (BATCH F5493/1)

Solution	Compound	Column 4.1	Column 10.1[a]	Column 4.2	Column 9.1
A	Caffeine	4.19	3.89	4.05	4.31
	Imipramine	24.68	25.09	24.47	24.71
	Morphine	27.82	27.78	28.72	29.59
	Methylamphetamine	63.92	71.38	64.10	64.48
	Protriptyline	100.00	100.00	100.00	100.00
B	Cocaine	8.00	8.18	7.78	7.93
	Phentermine	31.79	32.51	31.49	31.30
	Ephedrine	53.36	54.22	54.37	54.98
	Protriptyline	100.00	100.00	100.00	100.00
C	Diazepam	3.09	2.55	2.52	2.59
	Propranolol	19.85	20.19	19.14	19.07
	Nortriptyline	52.59	53.09	52.78	53.41
	Protriptyline	100.00	100.00	100.00	100.00
D	Amitriptyline	16.19	16.30	16.02	16.33
	Prolintane	50.00	53.52	45.18	40.83
	Protriptyline	100.00	100.00	100.00	100.00
E	Nitrazepam	2.33	2.01	2.11	2.14
	Chlorpromazine	17.19	17.19	17.11	17.47
	Pipazethate	57.33	61.62	52.92	50.19
	Protriptyline	100.00	100.00	100.00	100.00
F	Dextropropoxyphene	6.99	6.89	6.52	6.36
	Amphetamine	32.76	32.77	33.05	33.11
	Pholcodine	37.99	38.44	38.91	38.52
	Protriptyline	100.00	100.00	100.00	100.00
K	Papaverine	3.63	3.28	3.32	3.31
	Dipipanone	37.44	40.67	32.11	28.00
	Methdilazine	47.70	48.50	47.65	48.50
	Protriptyline	100.00	100.00	100.00	100.00
L	Procaine	8.80	8.89	8.44	8.48
	Promazine	29.05	29.42	29.14	29.75
	Ethoheptazine	43.95	45.56	43.95	44.10
	Protriptyline	100.00	100.00	100.00	100.00
M	Codeine	28.08	27.88	28.76	29.19
	Phenylephrine	39.40	38.04	40.51	41.19
	Protriptyline	100.00	100.00	100.00	100.00
N	Nortriptyline	52.59	53.09	52.59	53.41
	Strychnine	104.36	107.00	100.34	95.93
	DATE OF RUN	05/08/88	24/08/88	21/11/89	30/01/90
	TIME FROM COLUMN 4.1	0 DAYS	19 DAYS	473 DAYS	533 DAYS

[a]Data taken from Table 6.1.

TABLE 6.6: SUMMARY OF MAJOR CHANGES IN RELATIVE CAPACITY FACTORS FOR TWO NEW COLUMNS PACKED WITH SPHERISORB S5W (BATCH F5493/1, BOTTLE 4)

Compound	*Column 4.1*	*Column 4.2*[a]	*Change*	*%Change*
Dipipanone	37.44	32.11	-5.33	-14.24
Prolintane	50.00	45.18	-4.82	-9.64
Pipazethate	57.33	52.92	-4.41	-7.69
Strychnine	104.36	100.34	-4.02	-3.85
Phentermine[b]	31.79	31.49	-0.30	-0.94
Amphetamine[b]	32.76	33.05	+0.29	+0.89
Ephedrine	53.36	54.37	+1.01	+1.89
Pholcodine	37.99	38.91	+0.92	+2.42
Phenylephrine	39.40	40.51	+1.11	+2.82

[a]This column packed and tested 473 days after column 4.1.
[b]Data included as an example of results unaffected by the age of the silica.

This data appears to confirm that the result from the storage trial columns was not an isolated phenomenon and it seems to indicate that silica is susceptible to some form of aging process which changes its chromatographic properties. This process affects both used and unused silica in a similar manner, but silica in contact with solvents (i.e. packed into columns) seems to be more susceptible to this aging process.

6.3.2 COLUMNS PACKED WITH BATCH 2752

A second batch of Spherisorb S5W (batch 2752, a previous CRSE 'standard batch'[33], was also studied in an attempt to determine if the results recorded on batch F5493/1 were unique to that batch. In this case a column was prepared, tested and then stored for 14 months before being tested a second time. At the time of the second test, another column was packed from the same sample and tested; this allowed determination of both column stability and unpacked silica stability. The largest changes in RCFs on batch 2752 (Table 6.7, shown in italics) were for the same compounds that had shown changes on batch F5493/1. Dipipanone, prolintane, pipazethate and strychnine showed decreases in RCFs with increasing age of the silica, whilst phenylephrine showed an increase in RCF with increasing age of the silica.

TABLE 6.7: COMPARISON OF RELATIVE CAPACITY FACTORS FOR COLUMNS PACKED WITH SPHERISORB S5W (BATCH 2752)

Solution	Compound	Column 2752 - No.1 Start	Column 2752 - No.1 Retested	Column 2752 - No.2 Start
A	Caffeine	4.90	4.71	4.58
	Imipramine	25.73	25.17	25.41
	Morphine	31.56	32.25	31.08
	Methylamphetamine	66.29	65.58	64.62
	Protriptyline	100.00	100.00	100.00
B	Cocaine	8.27	7.99	7.96
	Phentermine	31.17	30.35	30.38
	Ephedrine	55.89	56.16	54.90
	Protriptyline	100.00	100.00	100.00
C	Diazepam	3.02	2.71	2.82
	Propranolol	19.35	18.78	18.81
	Nortriptyline	53.85	54.05	54.01
	Protriptyline	100.00	100.00	100.00
D	Amitriptyline	17.41	17.22	17.25
	Prolintane	39.61	36.10	37.25
	Protriptyline	100.00	100.00	100.00
E	Nitrazepam	2.57	2.17	2.26
	Chlorpromazine	18.57	18.51	18.43
	Pipazethate	48.56	45.82	45.81
	Protriptyline	100.00	100.00	100.00
F	Dextropropoxyphene	6.52	5.89	6.18
	Amphetamine	33.08	33.38	33.05
	Pholcodine	39.84	41.15	39.50
	Protriptyline	100.00	100.00	100.00
K	Papaverine	3.87	3.39	3.51
	Dipipanone	24.00	20.47	22.68
	Methdilazine	51.81	52.25	51.07
	Protriptyline	100.00	100.00	100.00
L	Procaine	9.28	8.80	8.73
	Promazine	31.18	31.16	31.14
	Ethoheptazine	46.48	46.04	45.20
	Protriptyline	100.00	100.00	100.00
M	Codeine	31.26	31.80	30.51
	Phenylephrine	42.44	44.24	42.91
	Protriptyline	100.00	100.00	100.00
N	Nortriptyline	53.85	54.05	54.01
	Strychnine	95.23	92.32	91.71
	DATE OF RUN	27/07/88	09/10/89	17/10/89
	AGE OF COLUMN	0 DAYS	440 DAYS	0 DAYS

6.4 COMPARISON OF SPHERISORB S5W BATCHES 2752 AND F5493/1

As results recorded on both batches of silica suggested that some form of aging process appeared to be occurring, it was of interest to compare the performance of the two batches of silica to see if any batch-to-batch variations could be attributed to this process.

In order to put such variations into perspective, the difference in age between the two batches of silica must be taken into account. This is necessary because the aging process appears to be time dependent and so variations in retention properties between the two batches of silica might be due to the absolute age difference. The manufacturers confirmed that the difference in age between batches 2752 and F5493/1 was approximately 34 months[162] (Table 6.8). Smith *et al.*[36] reported that changes in the properties of Spherisorb S5W were noted by the manufacturer at the time of the production of batch 2752.

Examination of the data (Table 6.8) revealed that for most of the compounds used in this study the changes in RCFs with time were small (*i.e.* $< \pm 4$ RCF units) but significant. The RCF of amphetamine, one of the two robust compounds, was largely unchanged, but for phentermine (previously seen to give robust results), changes between the two batches appear to be significant (Table 6.8). On the older batch (2752) dipipanone, prolintane, pipazethate and strychnine all show lower RCFs. For those compounds showing increases in retention, structurally related groups appeared to show the same degree of change, *e.g.* the phenothiazine derivatives methdilazine, promazine and chlorpromazine all showed increases of 7 - 7.2% in RCFs.

Whilst many of the changes in RCFs were quite small, significant changes in selectivity between the two batches were observed (Figure 6.3). It was found that the differences between the batches were not systematic, and that in some cases the results were consistent on each batch, but different between batches (*e.g.* methdilazine, RCF 47.67 (F5493/1), and 51.45 (2752), see Figure 6.3). In addition, the aging process appeared to be slower on batch 2752, this being most noticeable for compounds showing the largest changes. Such variations between the batches could be related to differences reported earlier[36], thus limiting the usefulness of a direct comparison of the batches by age of the material.

TABLE 6.8: COMPARISON OF FOUR COLUMNS PACKED FROM TWO DIFFERENT BATCHES OF SPHERISORB S5W (F5493/1 AND 2752)

Relative capacity factors(x100) for test compounds (RCF > 10) recorded on each column immediately after packing. (Methylamphetamine omitted, see Chapter 7).

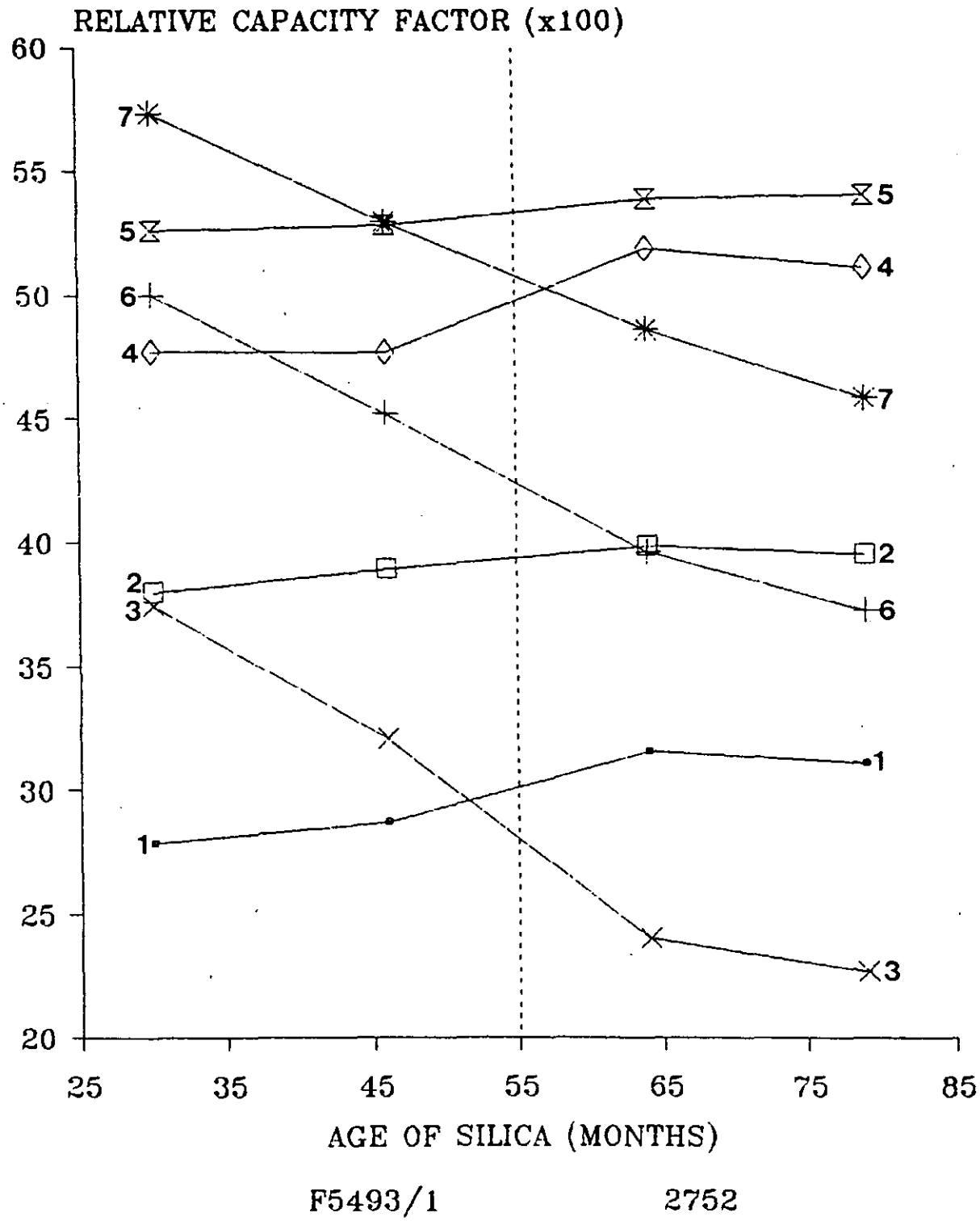
Compound	*Batch F5493/1*		*Batch 2752*		*Change*[a]	*% Change*
	No.4.1	*No.4.2*	*No.1*	*No.2*		
Dipipanone	37.44	32.11	24.00	22.68	-14.76	-39.42
Prolintane	50.00	45.18	39.61	37.25	-12.75	-25.50
Pipazethate	57.33	52.92	48.56	45.81	-11.52	-20.09
Strychnine	104.36	100.34	95.23	91.71	-12.65	-12.12
Propranolol	19.85	19.14	19.35	18.81	-1.04	-5.24
Phentermine	31.79	31.49	31.17	30.38	-1.41	-4.44
Amphetamine	32.76	33.05	33.08	33.05	+0.29	+0.89
Ethoheptazine	43.95	43.95	46.48	45.20	+1.25	+2.84
Nortriptyline	52.59	52.78	53.85	54.01	+1.42	+2.89
Ephedrine	53.36	54.37	55.89	54.90	+1.54	+2.89
Imipramine	24.68	24.47	25.73	25.41	+0.73	+2.93
Pholcodine	37.99	38.91	39.84	39.50	+1.51	+3.97
Amitriptyline	16.19	16.02	17.41	17.25	+1.06	+6.25
Methdilazine	47.70	47.65	51.81	51.07	+3.37	+7.04
Promazine	29.05	29.14	31.18	31.14	+2.09	+7.19
Chlorpromazine	17.19	17.11	18.57	18.43	+1.24	+7.21
Morphine	27.82	28.72	31.56	31.08	+2.26	+8.12
Codeine	28.08	28.76	31.26	30.51	+2.43	+8.65
Phenylephrine	39.40	40.51	42.44	42.91	+3.51	+8.91
Date of run	05/08/88	21/11/89	27/07/88	17/10/89		
Age of silica	30 months	46 months	64 months	79 months		

[a]Changes are between column F5493/1 No.4.1 and column 2752 No.2.

In comparing of the data presented by Smith *et al.*[36] on 18 batches of Spherisorb S5W (including batches 2752 and F5493/1) with the results discussed above, it was found that the current variations were similar to those found when the less robust ammonia eluent was used to examine the silica[36]. It should be stressed that differences between the batches were not as systematic as the data above might suggest, although the very old batches were different in precisely similar ways, but ephedrine was found to be unaffected by the age of the silica[36].

Figure 6.3: Changes in Relative Capacity Factors with time on two batches of Spherisorb S5W

Compounds, (1), morphine; (2), pholcodine; (3), dipipanone; (4), methdilazine; (5), nortriptyline; (6), prolintane; (7), pipazethate.



6.5 THE EFFECT OF WATER ON CHROMATOGRAPHIC PERFORMANCE

It is well known that water is readily adsorbed onto the silica surface[163] and that silica is soluble in aqueous media[164]. Since water was used in the mobile phase and would be present in trace amounts in the atmosphere above the silica in its storage container, it was thought that the action of water on the silica surface might be responsible for the observed changes in the retention properties of the silica used in this study.

To test this idea, the storage trial columns were treated with 1200 - 1300 mls of HPLC grade water in an attempt to produce 'accelerated aging' of the silica, (and also to try to restore the methylamphetamine peak, see section 7.4 below). All three columns showed improved results for methylamphetamine, but not until >1 litre of water had been used in the washing procedure; none of the columns gave a 'perfect' result, each of the methylamphetamine peaks showed some distortion.

The washing procedure also produced further changes in the retention properties for all three columns, with the capacity factors all decreasing, *e.g.* protriptyline 8.04 to 7.59 on column 10.1. Changes in relative capacity factors were also observed (Tables 6.9, 6.10 and 6.11). On all three columns the tertiary amines dipipanone, prolintane, pipazethate and strychnine all showed further reductions in RCFs as a result of washing the columns with water. The slight increase in RCF for pipazethate on column 10.2 over the one year period (59.51 to 60.86: Table 6.10) was dramatically reversed, the water wash reducing the RCF to 49.55. Phenylephrine showed further large increases in RCF on each column, but the behaviour of ephedrine was less predictable, on two of the columns its RCF increased, but on column 10.2, a small decrease was observed. In all cases the previous increases in RCF for pholcodine were reversed, the final results being only marginally different from the data recorded on the columns when new.

TABLE 6.9: COMPARISON OF RELATIVE CAPACITY FACTORS FOR STORAGE TRIAL COLUMN F5493/1 - 10.1 SHOWING THE EFFECT OF A WATER WASH ON RETENTION

Compound	*Start*	*1 Year*	*After water wash*	*Total Change*[a]	*% Change*
Dipipanone	40.67	30.91	25.62	-15.05	-37.01
Prolintane	53.52	47.41	38.74	-14.78	-27.62
Pipazethate	61.62	57.27	50.71	-10.91	-17.43
Strychnine	107.00	107.63	97.02	-9.98	-9.33
Phentermine	32.51	31.39	31.06	-1.45	-4.46
Pholcodine	38.44	42.21	37.63	-0.81	-2.11
Amphetamine	32.77	32.94	34.03	+1.26	+3.48
Ephedrine	54.22	55.31	56.31	+2.09	+3.85
Phenylephrine	38.04	40.67	47.82	+9.78	+25.71

TABLE 6.10: COMPARISON OF RELATIVE CAPACITY FACTORS FOR STORAGE TRIAL COLUMN F5493/1 - 10.2 SHOWING THE EFFECT OF A WATER WASH ON RETENTION

Compound	*Start*	*1 Year*	*After water wash*	*Total Change*[a]	*% Change*
Dipipanone	39.07	27.66	24.73	-14.34	-36.70
Prolintane	52.23	46.39	38.14	-14.04	-26.88
Pipazethate	59.51	60.86	49.55	-9.96	-16.74
Strychnine	107.09	108.70	96.43	-10.66	-9.95
Phentermine	32.00	31.29	30.88	-1.12	-3.50
Pholcodine	37.69	44.61	37.57	-0.12	-0.32
Ephedrine	53.64	57.35	55.19	+1.55	+2.89
Amphetamine	32.14	33.58	34.09	+1.95	+6.07
Phenylephrine	38.32	43.28	51.55	+13.23	+34.53

TABLE 6.11: COMPARISON OF RELATIVE CAPACITY FACTORS FOR STORAGE TRIAL COLUMN F5493/1 - 10.3 SHOWING THE EFFECT OF A WATER WASH ON RETENTION

Compound	*Start*	*1 Year*	*After water wash*	*Total Change*[a]	*% Change*
Dipipanone	39.20	28.24	25.32	-13.88	-35.41
Prolintane	51.89	44.89	39.79	-12.10	-23.32
Pipazethate	59.95	55.64	52.28	-7.67	-12.79
Strychnine	106.35	105.71	100.23	-6.12	-5.76
Phentermine	31.91	31.09	31.28	-0.63	-1.97
Pholcodine	38.60	42.21	39.55	+0.95	+2.50
Amphetamine	32.57	33.10	34.29	+1.72	+5.28
Ephedrine	53.65	55.71	57.91	+4.26	+7.94
Phenylephrine	38.51	41.45	49.67	+11.16	+28.98

[a]Changes are from 'start' to 'after water wash'.

It is clear that the effect of water on the chromatographic properties of the silica was similar to the aging process seen earlier, although the results were more erratic. It is possible that the 'accelerated aging process' led to less uniform changes in the surface structure of the silica, or that the long term aging process involved other factors besides the effect of water on the surface properties of the silica (see section 7.7.3).

6.6 CHANGES IN THE SILICA SURFACE WITH TIME

To determine whether there had been any changes in the surface characteristics of the silica during the period of study, two silica samples were submitted for surface examination by ^{29}Si Cross-Polarisation Magic-Angle-Spinning Nuclear Magnetic Resonance spectroscopy (CP-MAS-NMR). The aim was to determine if there was any significant difference in the ratio of silanols to siloxanes on the surface of the material, in the comparison of new and old silica.

CP-MAS-NMR has advantages in that it is largely a surface analysis technique, as a result of the process of Cross-Polarisation (CP), in which the energy absorbed by the silicon atoms is dissipated through the protons close to individual silicon atoms. An important factor in CP is the silicon - proton separation. Silicon atoms close to protons (e.g. HO<u>Si</u>$(OSi)_3$, "Q_3") will cross-polarise much more rapidly than those further from protons ($\underline{Si}(OSi)_4$, "Q_4"). It should be noted that Q_4 groups remote from protons will not undergo the process of CP and so they will be invisible during the experiment. This means therefore that only Q_4 groups close to the surface will be observed, along with all $\{(HO)_n\text{-}\underline{Si}\text{-}(OSi)_{4-n}\}$ groups (n = 1, "Q_3" or 2, "Q_2"), whilst the bulk matrix of the material does not contribute to the results.

Results from two samples, a reference sample of Spherisorb S5W (batch F5493/1) and the silica from column 4.2 used in this study, were obtained (Figure 6.4). The data indicated that the ratio of the Q_3:Q_4 peaks (isolated silanols:siloxanes) was:

sample F5493/1-Reference: 2:3 (Q_3:Q_4)
and, sample F5493/1-No.4.2: 1:1 (Q_3:Q_4)

Figure 6.4: ^{29}Si-CP-MAS-NMR results for two silica samples showing the Q_3 and Q_4 peaks.

Expected δSi values: Q_3, -99.8 ppm; Q_4, -109.3 ppm (see reference 165).

(I): Silica sample F5493/1-No.4.1. (II): Silica sample F5493/1-Reference.

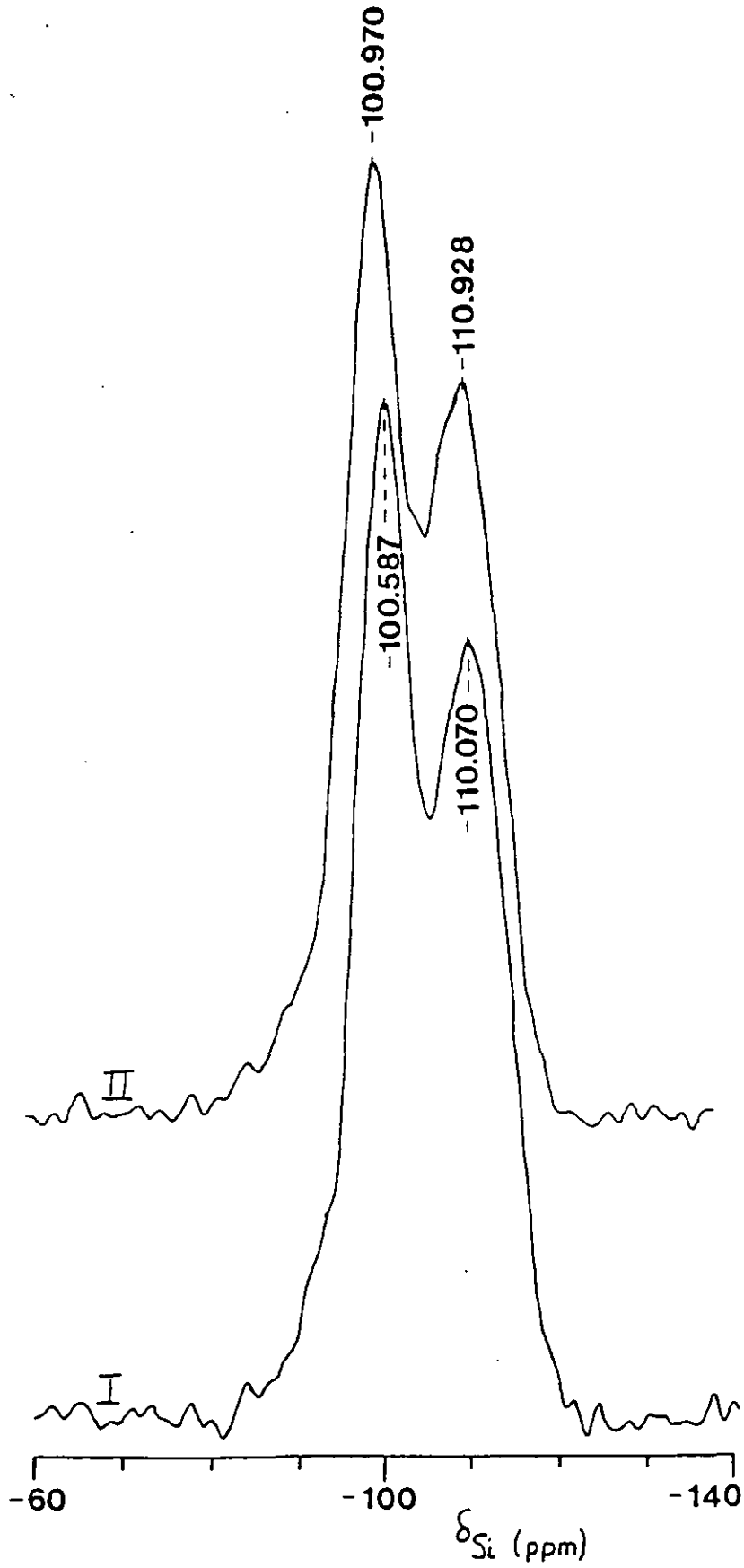
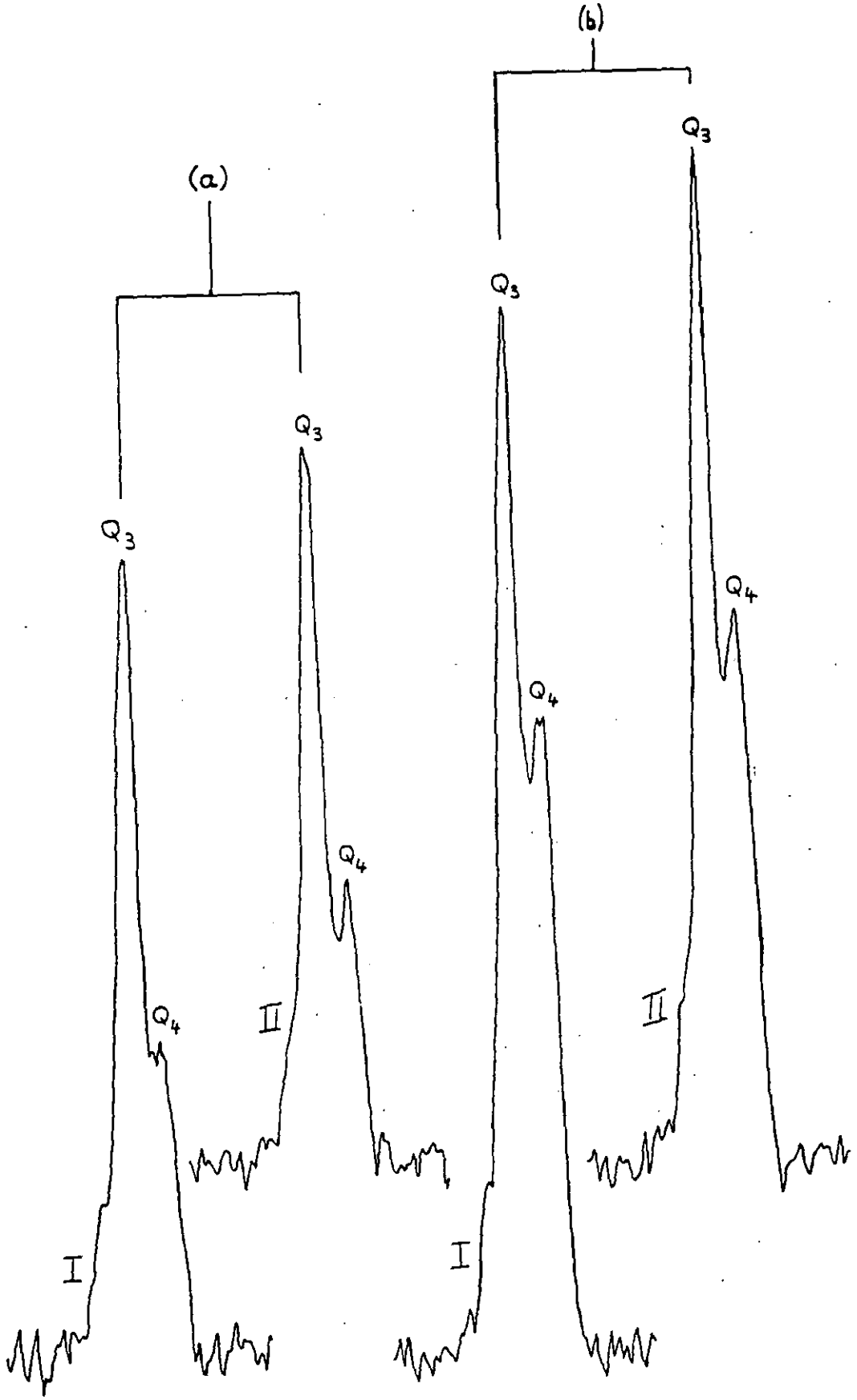


Figure 6.5: ^{29}Si-CP-MAS-NMR results for two silica samples showing differences in the Q_3 and Q_4 peaks at different contact times.

Contact times: (a) = 1 ms and (b) = 2.5 ms.

(I): Silica sample F5493/1-No.4.1. (II): Silica sample F5493/1-Reference.



The results do not, at first, suggest significant changes in the Q_3:Q_4 ratios. However, the deconvolution technique used to separate the overlapping peaks relies on data recorded from a series of experiments using different contact times[162]. Noticeable differences in results were obtained at short contact times (< 5 ms; Figure 6.5) and smaller variations were observed as the contact time increased (5 to 20 ms). These changes account for the different ratios of Q_3:Q_4 peaks in the two samples shown in Figure 6.4. However, the changes are small, and the data does not allow a calculation of absolute numbers of silanols or siloxanes on the surface of the material.

This data seems to confirm that a process of aging has occurred, involving the hydrolysis of siloxanes to silanols, as shown by the larger Q_3 peak on the used silica sample. With this result, it is possible to suggest explanations for the large changes in retention observed for some of the test compounds.

6.7 STRUCTURE RETENTION RELATIONSHIPS

During the study of the effect of age on the chromatographic performance of silica, it became clear the certain groups of compounds always behaved in the same manner and that, within these groups, the compounds usually had some structural similarity. This prompted a detailed examination of the structures of all the test compounds to determine whether the groups of compounds which were particularly sensitive to the aging of the silica were unique in their structural similarity.

6.7.1 COMPOUNDS SHOWING A DECREASE IN RETENTION WITH INCREASING AGE OF THE SILICA

The four compounds which showed decreases in RCFs with increasing silica age were found to be structurally similar (Figure 6.6). These compounds were all tertiary amines and all contained a non-aromatic ring nitrogen atom to which the rest of the molecule was linked. None of the other compounds in the test solutions contained this structural feature, although many were tertiary amines. In all these other cases, the

nitrogen atom was either aromatic (*e.g.* papaverine), double-bonded (*e.g.* diazepam and nitrazepam) or the compound contained an N-methyl group (*e.g.* cocaine and caffeine) on an aliphatic or alicyclic N-atom (Figure 6.7).

The critical difference between these groups of compounds appears to be the position of the nitrogen atom in the molecule. In each of the four compounds sensitive to the structure of the silica, the nitrogen atom is sterically crowded due to the presence of the pyrrolidine or piperidine rings and the bulky side groups. The principle mode of retention under the chosen conditions appears to be ion-exchange (see section 4.3), but in the case of these four compounds access to the nitrogen atom may be sufficiently hindered to limit interactions with ionised silanols on the silica surface. An alternative mode of retention for these compounds could be hydrophobic interactions between the aromatic groups and surface siloxane groups, as proposed by Bidlingmeyer *et al.*[21].

Figure 6.6: Structures of compounds showing large decreases in RCF with silica age.

Compounds: (1), dipipanone; (2), pipazethate; (3), prolintane; (4), strychnine.

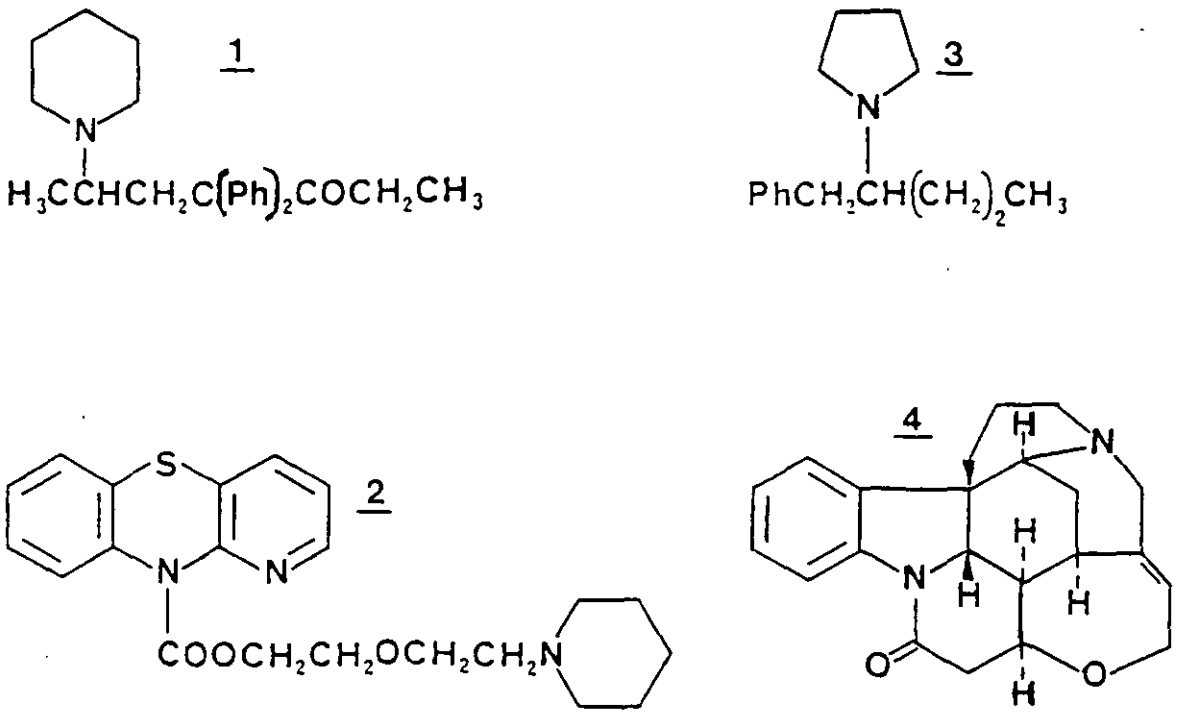
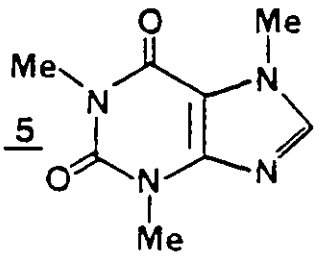
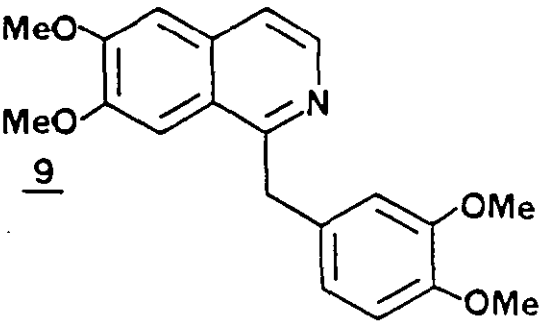
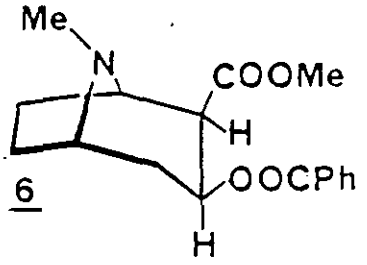


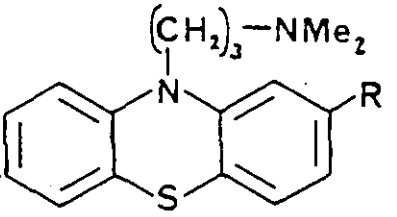
Figure 6.7: Structures of other compounds containing ring-nitrogen atoms (excluding morphine and related compounds).

Compounds: (5), caffeine; (6), cocaine; (7), diazepam; (8), nitrazepam; (9), papaverine; (10), promazine; (11), chlorpromazine; (12), methdilazine.

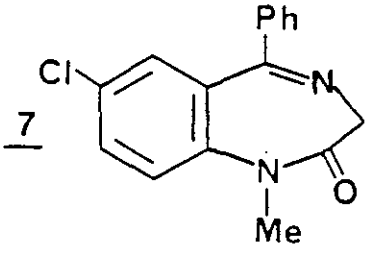


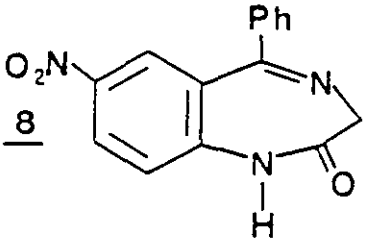


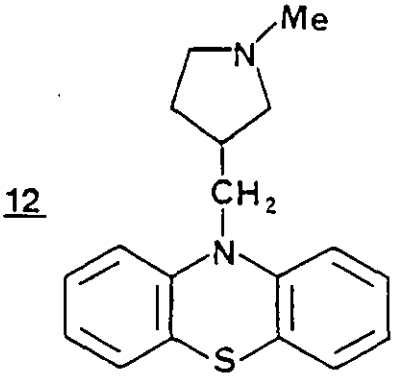




10 R = H
11 R = Cl







If this is the dominant mode of retention for these compounds, then the effect of aging on the chromatographic properties of the silica, which led to dramatic reductions in retention for these compounds, could be accounted for in terms of the hydrolysis of siloxane groups. As the silica ages, hydrolysis of the surface would lead to a reduction in the number of siloxane groups and an increase in the number of silanols, as suggested by the ^{29}Si-CP-MAS-NMR data (see section 6.6). This would result in fewer retention sites for these compounds which would explain the observed reductions in retention.

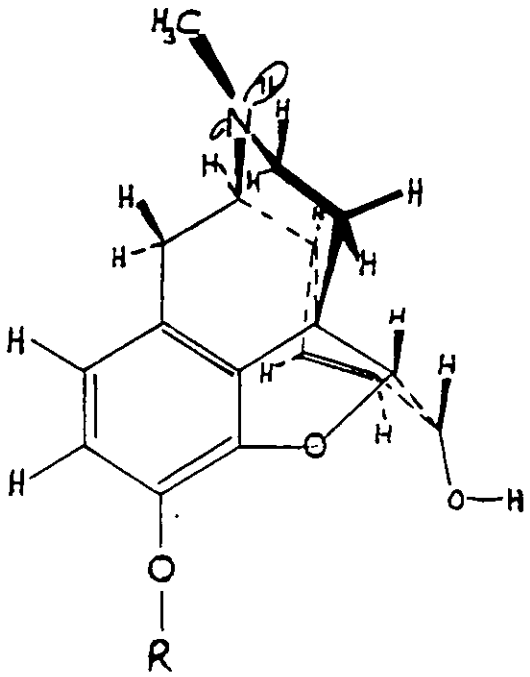
These results are in contrast to the observations by Law[24], who suggested that interactions between siloxanes and aromatic groups do not occur under ion-exchange conditions. However, the present study has examined the retentions of a wider range of analyte types, and so different mechanisms may apply in some cases (see section 4.3).

6.7.2 COMPOUNDS SHOWING AN INCREASE IN RETENTION WITH INCREASING AGE OF THE SILICA

The six compounds shown in Figure 6.8 all showed increases in retention with increasing age of the silica on the storage trial columns, the changes being most significant for morphine, codeine and phenylephrine (Table 6.4). These compounds all contain an aromatic ring and an N-methyl group which can be oriented to give similar spatial arrangements. This suggests the possibility of a two point interaction at the silica surface, involving a hydrophobic interaction between the aromatic ring and a siloxane group and an ion-exchange type interaction between the N-methyl group and an ionised silanol.

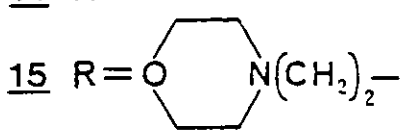
Figure 6.8: Structures of compounds showing increases in retention with increasing silica age.

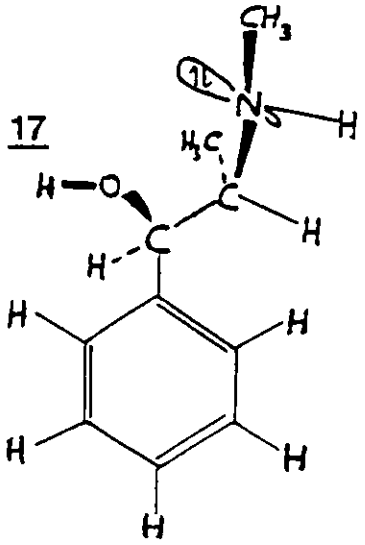
Compounds, (13) morphine; (14) codeine; (15) pholcodine; (16) ethoheptazine; (17) ephedrine; (18) phenylephrine.

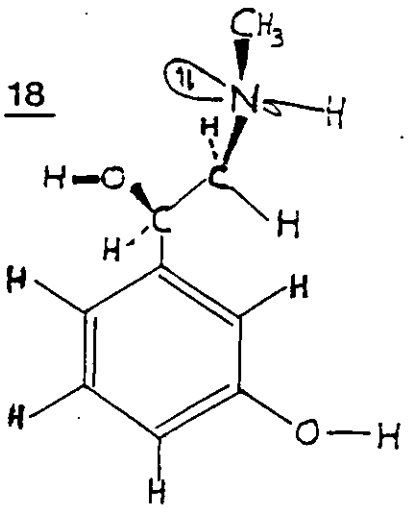


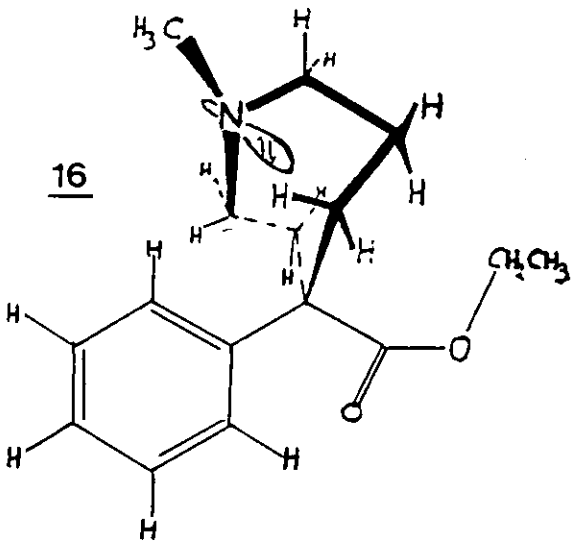
13 R = H

14 R = Me









The increases in retention for these compounds could be accounted for assuming that cation-exchange was the dominant retention mechanism, as suggested by the QSRR studies (see section 4.3). The reduction in siloxane groups, as discussed above, would lead to a drop in retention for the aromatic part of the molecule, but an increase in the number of silanols would lead to an increase in the retention of the N-methyl function via ion-exchange interactions. Assuming that an ion-exchange mechanism dominates, then the effect would be an increase in the retention of the molecule, but since two opposing changes are occurring simultaneously, the net effect would be smaller than that observed for those compounds retained by a single mechanism (see section 6.7.1).

The smaller increases in retention for many of the test compounds with increasing age of the silica batches (Table 6.8) are probably the result of similar effects, especially for structurally related compounds. It is difficult to relate the degree of change to the structure of individual analytes as a wide range of structural types was examined. It should also be noted that all the changes are recorded relative to one compound, protriptyline, whose retention properties may also have been subject to change with the age of the silica.

6.8 CONCLUSIONS

In a 12 month study of three 'storage trial' columns packed with Spherisorb S5W from a single bottle of one batch, significant changes in selectivity were observed for some compounds. Similar changes were recorded on each column for the same compounds, with some showing large decreases in retention, whilst others showed small increases. The mode of storage appeared to be significant in that columns washed with methanol seemed to be slightly less susceptible to change than a column washed with mobile phase.

Similar changes were observed on a selection of columns packed with the same batch of silica over a 17 month period. This led to the conclusion that the silica was subject to some form of aging process, both in packed columns and in dry storage. This process appeared to affect both unused silica and silica packed into columns, although unused silica appeared to be more stable. A comparison of data recorded on two

different batches of silica suggested that previously observed batch-to-batch variations could be an extension of this aging process.

Treatment of selected columns with HPLC grade water produced changes in the retention properties of the silica which appeared to be an acceleration of the aging process observed earlier. However, the changes caused by water were more erratic than those caused by the aging of the silica. Preliminary ^{29}Si-CP-MAS-NMR studies of selected silica samples indicated that changes in the ratio of silanols to siloxanes on the surface of silica were occurring, such that the older the material the greater the number of silanols. Thus it seemed reasonable to assume that the aging process was probably related to the hydrolysis of the silica surface, and that the effect of water on the silica surface was a critical factor in determining the long term stability of the material.

CHAPTER 7: INVESTIGATING DISTORTED PEAK SHAPES FOR METHYLAMPHETAMINE

7.1 INTRODUCTION

In early studies with the new eluent system (Chapters 4 & 5), some compounds, and in particular methylamphetamine, gave poor peak shapes (Figure 7.1 (a,b)). During this method development study, a pre-column (3 cm x 5 mm i.d.), filled with open-column grade silica had been used to protect the silica in the analytical column from dissolution. When a longer pre-column (20 cm x 5 mm i.d.) containing open-column grade silica was used, serious distortion of the methylamphetamine peak was observed on a number of columns and it seemed impossible to obtain meaningful results for this compound (Figure 7.1 (c-e)). Some column to column variation in retention of both methylamphetamine and protriptyline was observed (Figure 7.1), caused in part by differences in individual column history. However, when relative capacity factors were used, it was possible to identify the section of the multiple peak eluting in the expected position for methylamphetamine, based on the results obtained in earlier studies (Figure 7.1 (a)). All the other compounds in the test solutions, including those structurally similar to methylamphetamine, such as amphetamine and ephedrine, showed no changes in behaviour; only this one compound was affected.

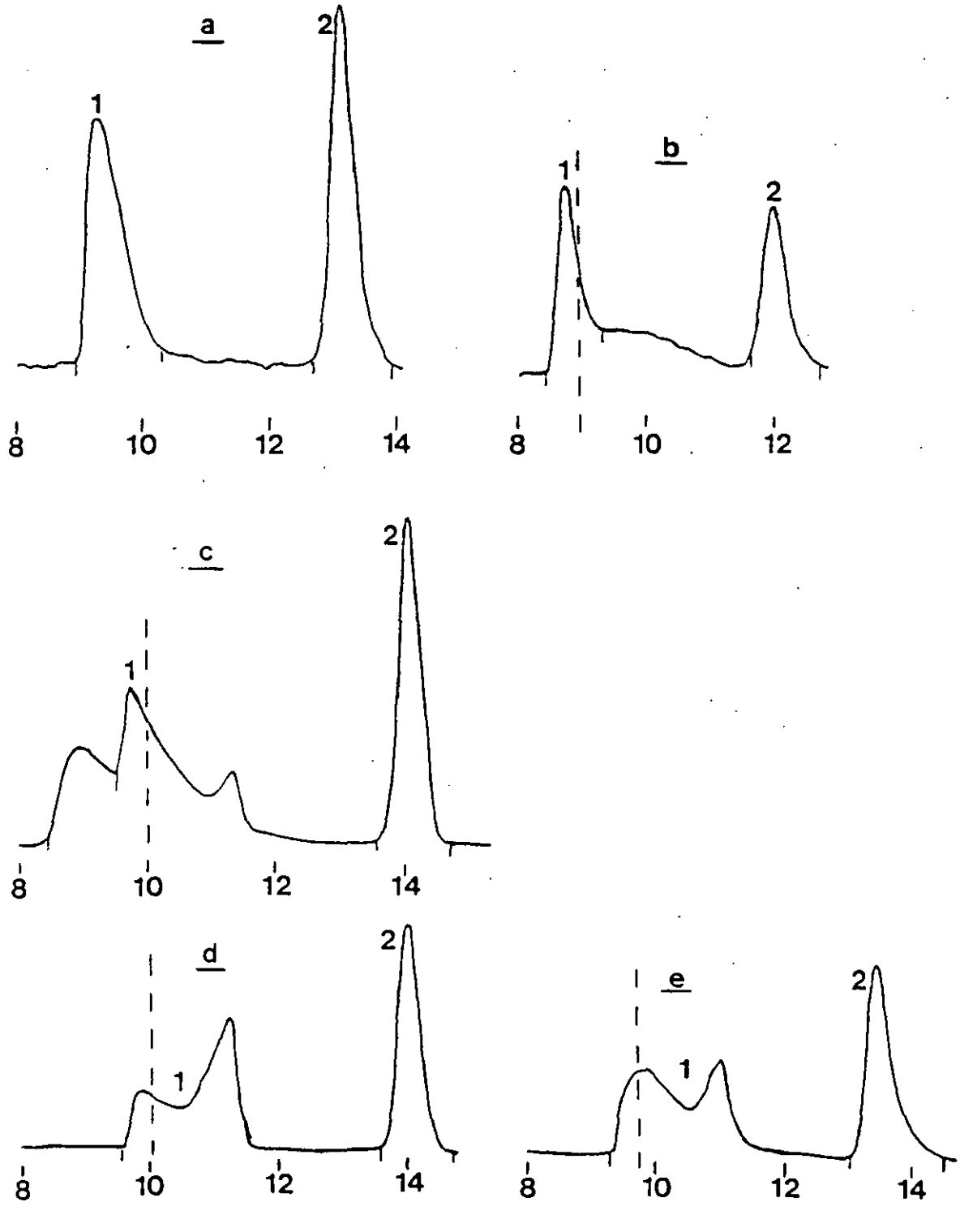
Consequently, a series of studies were carried out to investigate this problem. The main approaches were:

i) using fresh test solutions, different injection solvents and columns, cleaning the current HPLC system, and using another system,
ii) monitoring the performance of compounds structurally related to methylamphetamine,
iii) determining the influence of the pre-column silica on the chromatography of the test compounds,
iv) attempting to clean 'contaminated' columns,
v) investigating the influence of metal ions (Fe^{3+} and Cu^{2+}) on the chromatography of methylamphetamine and the other test compounds,
vi) using EDTA to remove metal ions from the HPLC columns, and
vii) assessing the influence of the age of the silica on the quality of the methylamphetamine peak.

Figure 7.1: Examples of poor peak shapes for methylamphetamine

Conditions: Eluent, methanol - aqueous CAPS / CAPSO-Na buffer 90:10 v/v; buffer composition - each component 0.08 mol l^{-1}; flow rate = 2 ml min^{-1}; temperature = 30°C; detection wavelength = 254 nm; injection volume 5µl. All columns packed with Spherisorb S5W (batch P5495/1). Compounds: (1), methylamphetamine; (2), protriptyline.

(a), A 'good' methylamphetamine peak on column No.1.1. (b), A deteriorating methylamphetamine peak on column No.1.1. (c), A badly distorted methylamphetamine peak on column No.2.1. (d), A badly distorted methylamphetamine peak using a fresh batch of test solution A on column No.1.2. (e), As (d), but on column No.1.1. In (b-e), dashed line shows expected position of methylamphetamine based on peak in (a).



7.2 INITIAL STUDIES

7.2.1 NEW COLUMNS AND TEST SOLUTIONS

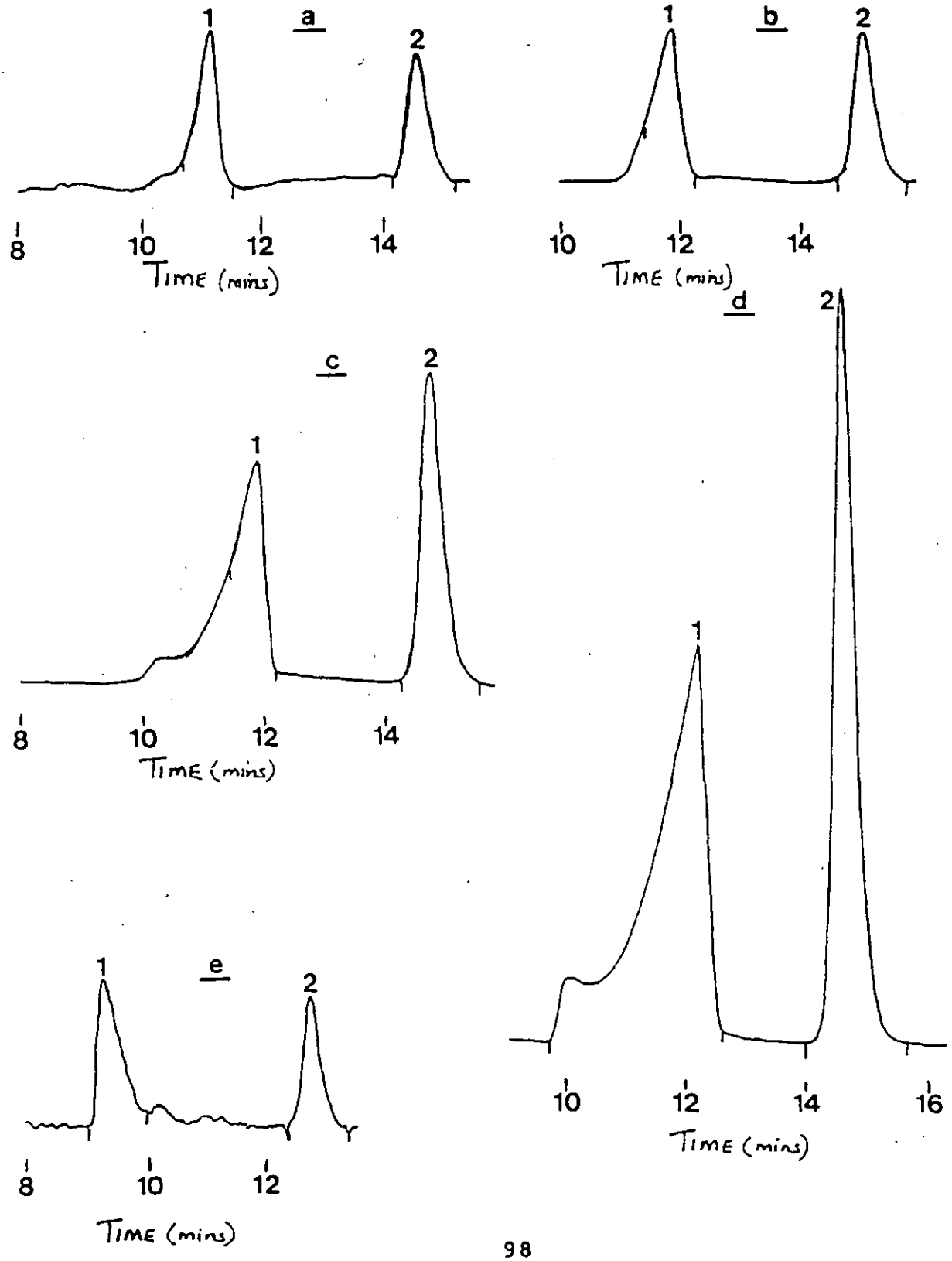
Following the observation of poor peak shapes for methylamphetamine, (Figure 7.1 (c)), a fresh batch of solution A was prepared and separated on two old columns packed with Spherisorb S5W (batch F5493/1). In each case methylamphetamine gave a distorted peak (Figure 7.1 (d,e)), indicating that the sudden change in chromatography was not caused by the test solution, and suggesting that the result was independent of the column used. Samples of methylamphetamine dissolved in methanol, methanol / water (90:10 v/v) and water all produced similar results to those recorded using fresh solution A, indicating that the distortion of the peak shape was not related to the strength of the solvent used to prepare the test solutions. In these and all the subsequent studies the other compounds in test solution A gave good peak shapes and these were used to confirm that there were no general problems with the system. Since no distortions were observed with the compounds in the other test solutions, for many of the studies only solutions A and J (the void volume marker) were used to monitor column performance.

In an attempt to determine the influence of the sample size on the peak shape, injections of 1, 5, 10 and 20 µl of solution A were run, along with a 5 µl injection of a 10-fold diluted sample of the same test solution. The distortion of the peak shape was found to increase as the volume of test solution increased (Figure 7.2 (a-d)), suggesting that the change in peak shape was dependent on the quantity of methylamphetamine injected onto the column. Separations of the diluted sample of solution A gave acceptable results for methylamphetamine (Figure 7.2 (e)), although some tailing of the peak was observed. This result led to the use of the 10-fold diluted sample of solution A in other studies being carried out at the same time as the present investigations into the distortion of the methylamphetamine peak.

Figure 7.2: The effect of sample load and concentration on the shape of the methylamphetamine peak

Conditions and compounds as in Figure 7.1.

(a) 1 µl injection of test solution A. (Detector range = 0.04 aufs)
(b) 5 µl injection of test solution A. (Detector range = 0.16 aufs)
(c) 10 µl injection of test solution A. (Detector range = 0.16 aufs)
(d) 20 µl injection of test solution A. (Detector range = 0.16 aufs)
(e) 5 µl injection of 10-fold diluted sample of test solution A. (Detector range = 0.02 aufs)



7.2.2 THE USE OF DIFFERENT EQUIPMENT

It was thought that one of the possible causes of the problem could be the analytical equipment itself, and so the method was transferred to a different HPLC system. In this experiment an old Spherisorb S5W column was used (F5493/1-No.1.1), but no pre-column was installed. With all other operating conditions unchanged, methylamphetamine was still found to give distorted peak shapes. Separations of the full test set of drug solutions produced acceptable results for all the other compounds, as in previous studies with the new eluent.

On returning to the original equipment, no improvement in peak shape was seen for methylamphetamine, even after extensive cleaning of the entire flow path of the eluent from the pump to the column inlet with water, methanol / water (50:50 v/v) and methanol. These results indicated that the problem of peak shape was not related to the choice of the equipment being used for the study.

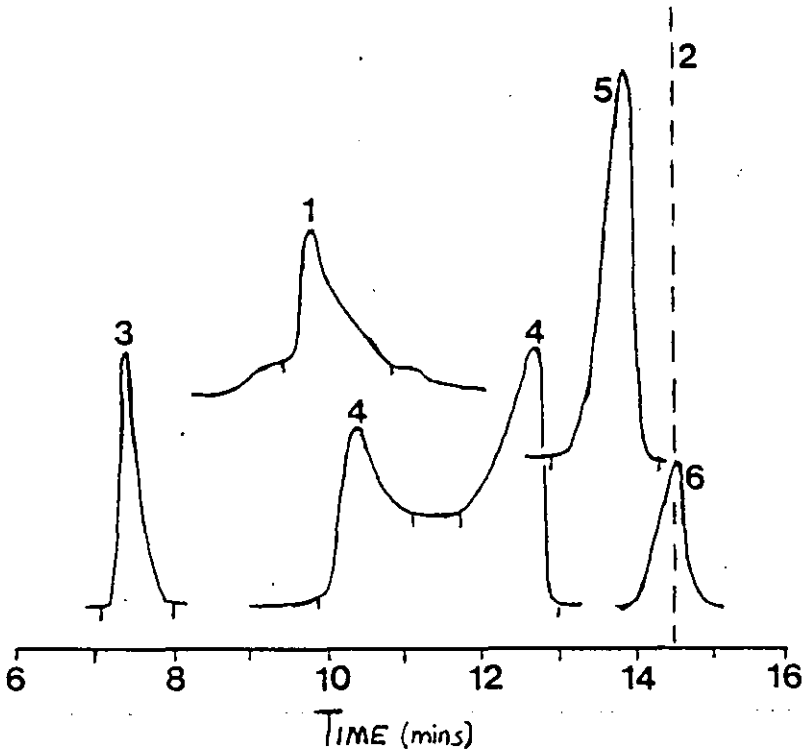
7.2.3 CHROMATOGRAPHY OF COMPOUNDS STRUCTURALLY RELATED TO METHYLAMPHETAMINE

A series of compounds structurally related to methylamphetamine (see Table 4.2), and not present in the original test solutions, were examined to determine if the peak distortion was specific to this one compound. All the compounds examined, with the exception of N-methylphenethylamine, were found to give acceptable peak shapes using this system. The difference in structure between methylamphetamine and N-methylphenethylamine is minimal (see Figure 4.3), and it was thought that the poor results for these two compounds were probably caused by the same factors. However, due to the apparent instability of N-methylphenethylamine solutions, this compound was not used in further studies.

Figure 7.3: Peak shapes for some compounds structurally related to methylamphetamine

Conditions as Figure 7.1. Column: Spherisorb S5W: F5493/1-No.4.

Compounds: (1), methylamphetamine; (2), dashed line indicates the position of protriptyline; (3), ethylamphetamine; (4), N-methylphenethylamine; (5), methoxyphenamine; (6), mephentermine.



7.3 INFLUENCE OF THE PRE-COLUMN SILICA ON THE CHROMATOGRAPHY OF METHYLAMPHETAMINE

Since the problem of the distorted peak shape for methylamphetamine had not been solved by changing the test solution, by changing the analytical column, or by cleaning the equipment, it was decided to investigate the influence of the silica pre-column on the chromatography. In all experiments where distorted peaks had been recorded, a 20 cm pre-column filled with open-column grade silica had been used, or a column which had previously been used in conjunction with the long pre-column was used. This led to the suggestion that the quality of the chromatography was being influenced by the pre-column silica.

7.3.1 OPEN-COLUMN GRADE SILICA PRE-COLUMNS

To assess the influence of the pre-column, the HPLC system was extensively flushed with fresh eluent, and a new, previously unused column (Spherisorb S5W, F5493/1-No.12.1) was fitted, and the pre-column removed. Under these conditions methylamphetamine gave good peak shapes (Figure 7.4 (a)), indicating that a new column did not produce the same distortion of the peak shape.

The long pre-column was emptied, cleaned and fitted back into the HPLC system without filling with silica. Separations of solution A carried out under these conditions produced no distortion of the methylamphetamine peak. The pre-column was then gradually filled with open-column grade silica and separations carried out at each step. With small quantities of silica in the pre-column (5 to 15 cm of the column filled) only minor distortion of the methylamphetamine peak was recorded (Figure 7.4 (b)). However, when a full pre-column was included, the distortion of the peak shape was seen to increase (Figure 7.4 (c)).

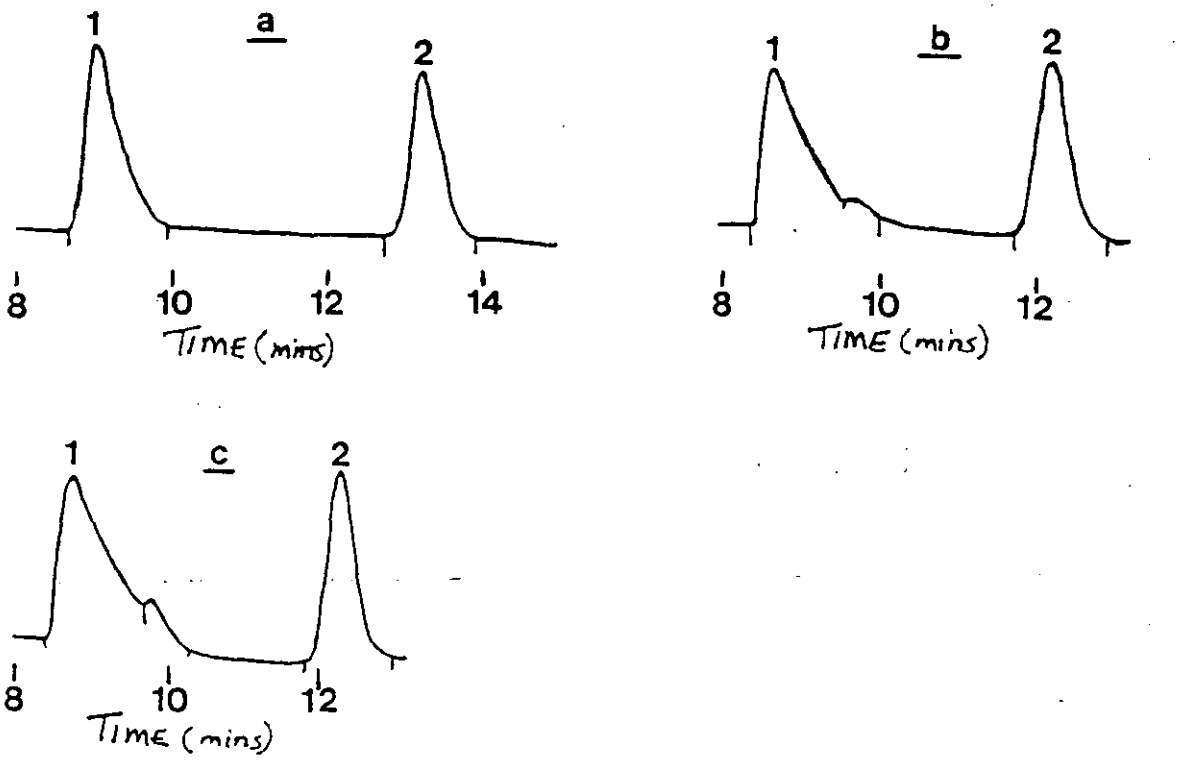
This led to the conclusion that the eluent might be transferring some sort of contaminant(s) (possibly metal ions) from the pre-column silica to the analytical column silica. These were then causing immediate and apparently irreversible contamination of the analytical column, once a sufficiently large quantity of silica was placed in the pre-column. A re-examination of the results recorded on column F5493/1-No.1.1 (Figure 7.1 (a,b,e)) suggested that deterioration of the methylamphetamine peak was occurring under conditions of prolonged exposure of the analytical column to the open-column grade silica. (This column had been used extensively with a short pre-column (3 cm) which had to be refilled at regular intervals).

Consequently it was decided to replace the open column grade silica with a higher grade silica in an attempt to avoid the possibility of contaminating the analytical column.

Figure 7.4: Influence of an open-column grade silica pre-column on the chromatography of methylamphetamine

Conditions and compounds as Figure 7.1. Column: Spherisorb S5W, F5493/1-No.12.1.

(a) A good methylamphetamine peak on a new, unused column in the absence of a pre-column.
(b) A slightly distorted methylamphetamine peak on the same column with a partially filled 20 cm pre-column containing open-column grade silica.
(c) A distorted methylamphetamine peak on the same column with a full 20 cm pre-column containing open-column grade silica.



7.3.2 HIGH GRADE SILICA PRE-COLUMNS

A high grade silica (Spherisorb Prep-W, 12 µm, batch P6442) from the same manufacturer as the analytical silica was selected to replace the open-column grade silica. The manufacturers confirmed that this silica was produced by the same process as the S5W analytical silica[162], and that it was simply a larger particle size.

Only minor deterioration in the quality of the peak shape of methylamphetamine was observed, (Figure 7.5), using a single analytical column studied over a period of 2 to 3 weeks with a 20 cm pre-column filled with the Prep-W silica. This column had previously been seen to give poor peak shapes for methylamphetamine, but had been 'cleaned up' by washing with water (see section 7.4). The gradual deterioration of the peak on this column was similar to that seen on other columns washed with water (see below), suggesting that the new, higher quality pre-column silica was probably not responsible for the return of the poor peak shapes.

Since the higher grade pre-column silica did not cause rapid deterioration of the peak shape of methylamphetamine, it was decided to use this in place of the open column grade silica whenever a pre-column was needed.

7.4 ATTEMPTS TO CLEAN CONTAMINATED COLUMNS

Since studies using different grades of pre-column silica had suggested that the analytical columns may have been contaminated by transfer of impurities from the pre-column silica, it was decided to try to clean a 'contaminated' analytical column. Washes with various solvents were carried out and it was found that washing a column with fresh CAPS / CAPSO-Na eluent or with HPLC grade methanol did not improve the results. However, washing with HPLC grade water, in the absence of an organic modifier, was found to be very successful at restoring the peak shape of methylamphetamine. After cleaning a 'contaminated' column with about 200 mls of HPLC grade water and re-equilibrating it with fresh eluent, a good peak shape was obtained for methylamphetamine (Figure 7.6). Application of this washing procedure to any column which had given poor results for methylamphetamine resulted in an improvement in column performance (see also section 6.5).

Figure 7.5: Influence of a high grade silica pre-column on the chromatography of methylamphetamine

Conditions and compounds as Figure 7.1. Column F5493/1-No.12.3.

(a) Before inclusion of pre-column; (b) 9 days after use with pre-column.

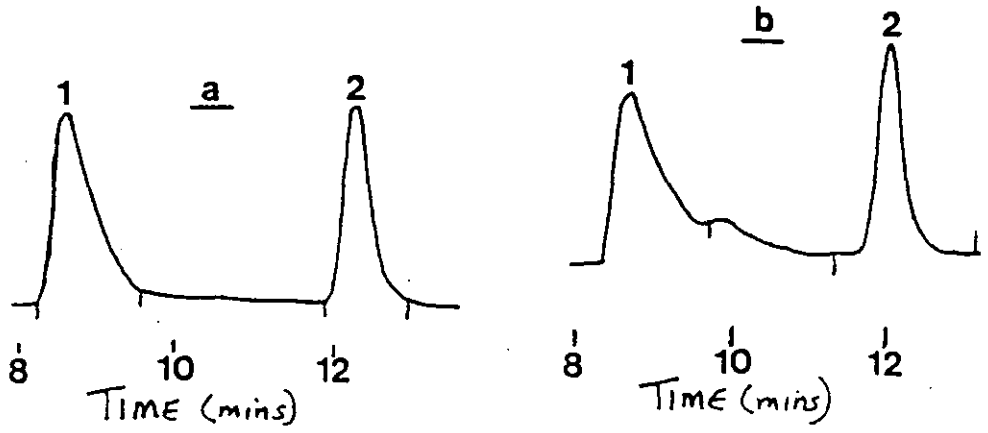
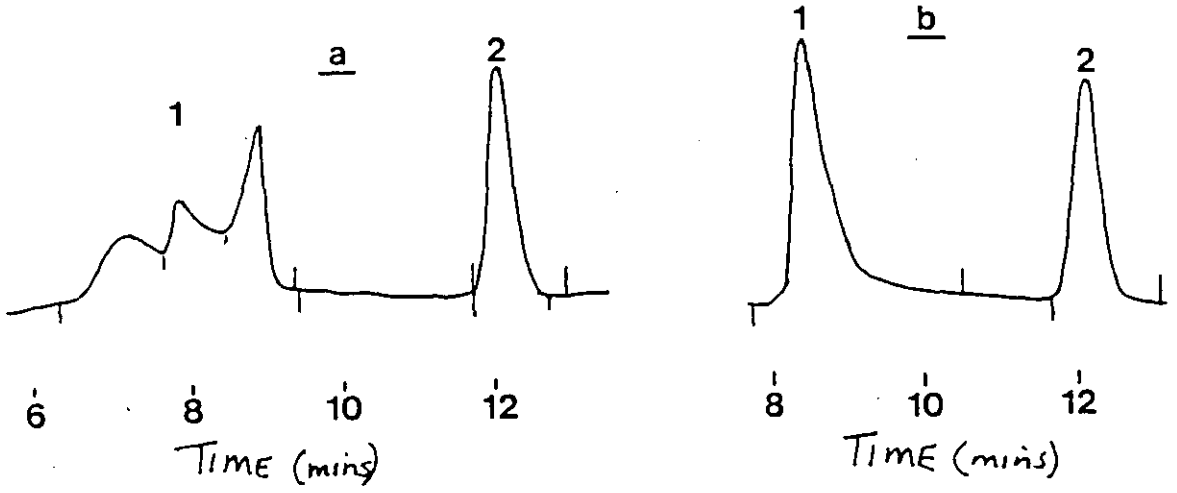


Figure 7.6: Changes in peak shapes for methylamphetamine caused by washing the analytical column with water

Conditions and compounds as Figure 7.1. Column F5493/1-No.12.3.

(a) Before washing with water; (b) After washing with water (350 mls)



The subsequent performance of columns restored by washing with water was found to deteriorate gradually, with or without a pre-column in the system. Further washing of the columns with water again restored the performance of the methylamphetamine peak, suggesting that the problem was more serious than contamination from an external source. One possible explanation for this observation was that the analytical column was contaminated fairly rapidly by impurities from the open-column grade silica, and that these were migrating into the analytical silica matrix, below the 'chromatographic surface', whilst others remained available to interfere with the chromatography. The improvement in peak shape caused by washing the columns with water could then be explained in terms of cleaning only the 'chromatographic surface' of the silica. The subsequent slow deterioration of the columns could have been caused by the 'hidden' contaminants migrating back to the 'chromatographic surface'.

As noted earlier (section 6.5), washing of the columns with water caused 'accelerated aging' of the silica, due to changes in the 'chromatographic surface'. In the studies presented here, (which were carried out during the early stages of the column storage tests), only solutions A and J were used to monitor the effect of washing the columns with water, as the primary concern was to restore column performance for methylamphetamine. Since solution A did not contain any of the compounds found to be sensitive to the age of the silica, (see Chapter 6), none of the compounds showing changes in retention reported earlier were examined during these studies (although see section 7.6.1).

7.5 ANALYSIS OF THE OPEN-COLUMN GRADE SILICA

In an attempt to understand the apparent importance of the open-column grade silica in the pre-column, it was decided to analyse the silica for two metals, copper and iron. Studies by Smith *et al.*[166] had shown that copper has a marked influence on the chromatography of 2-aminophenol and other chelating compounds, even when present at very low levels (10^{-5} mol l^{-1}). This prompted the suggestion that copper might be contributing for the poor results seen for methylamphetamine (even though this compound does not contain a chelation site). It was thought that iron, which has been identified as a major impurity even in HPLC-grade silica[89], might be a major impurity in the open-column grade silica, and that it might also be contributing to the poor results.

A simple acid extraction procedure was used to remove metal ions from the open-column grade silica, (see section 3.6.1), and the extract was then analysed for the two metals in question. Copper was determined by anodic stripping differential pulse polarography (see section 3.6.2), and iron was measured colorimetrically, as its thiocyanate complex (section 3.6.3.1), and by flame atomic absorption spectroscopy (section 3.6.3.2). The concentration of copper in the silica was determined to be about 3 ppm, and the amount of iron was found to be about 60 ppm.

7.6 THE INFLUENCE OF METAL IONS ON THE CHROMATOGRAPHY OF METHYLAMPHETAMINE

7.6.1 COPPER (Cu^{2+})

Despite the low levels of copper found in the open column grade silica, it was decided to determine the influence of copper on the chromatography of the test compounds as it had been found to affect results even at very low levels[166]. This was achieved by first testing a good analytical column under standard conditions (Table 7.1, and Figure 7.7 (a)) in order to obtain a baseline against which to compare results recorded in the presence of copper. The column was then washed with *ca.* 350 mls of an aqueous 10^{-5} mol l^{-1} copper (II) solution (as copper acetate), and solution A only was separated under standard conditions to determine the initial effect of the copper wash. The improved peak shape of methylamphetamine suggested that the aqueous copper solution had cleaned the column, as previously observed when washing columns with water (section 7.4) and that the chromatography of solution A was otherwise unaffected (Figure 7.7 (b)).

Consequently, copper acetate was added to the eluent to give a copper concentration of 0.1 mmol l^{-1}. Separation of the full test set of drug solutions was then carried out and copper was found to have had very little influence on the chromatography of methylamphetamine (Figure 7.7 (c)). However, some of the other compounds were severely affected. Most noticeable was the serious deterioration in peak shape for ephedrine and propranolol (Figure 7.8 (a,b)), and the absence of the phenylephrine peak (Figure 7.8 (c)). It was found that the chromatography of all three compounds could be restored by washing the column with a dilute aqueous EDTA solution (see Figure 7.8). A number of other compounds (Table 7.1,

shown in italics) showed large changes in relative capacity factors, of which only phentermine and amphetamine returned to 'normal' after removing the copper from the column by washing with the aqueous EDTA solution. Four compounds, prolintane, pipazethate, dipipanone and strychnine, all showed large decreases in relative capacity factors after treating the column with the aqueous copper solution and copper doped eluent. These changes were not reversed by the EDTA wash and were almost certainly not related to the presence of copper but to the effect of water on the silica. (It is highly likely that these changes were due to 'accelerated aging' of the silica, caused by the aqueous EDTA wash: see section 6.5).

The increases in retention for amphetamine and phentermine were probably caused by the complexation of copper ions with primary amine groups (i.e. $R\text{-}NH_2 : \rightarrow Cu^{2+}$). This would explain why phentermine returned to 'normal', and also why amphetamine and pholcodine were resolved once the copper was removed from the system by the EDTA wash. The disappearance of the phenylephrine peak, and the serious distortion of the ephedrine and propranolol peaks was probably also caused by the formation of copper-analyte complexes. These three test compounds all contain a ß-hydroxy amine grouping (i.e. $-CH(\underline{OH})-CH_2-\underline{N}R_2$) which may have been complexing with copper ions. Since the changes in retention for amphetamine and phentermine were of a different nature to those observed for these three compounds, some other form of complexation between copper and these analytes, such as with the ß-hydroxy group, must have been taking place. Assuming that copper was being 'deposited' on the silica surface, the disappearance of the phenylephrine peak could be explained in terms of a high stability constant for the copper-analyte complex, such that formation of the complex was favoured over solvation of the analyte by the mobile phase. For those analytes showing serious distortion of peak shape, the stability constant for complex formation would not have been large enough to prevent elution of the compound, but sufficient to cause peak tailing (in a similar manner to peak tailing caused by analyte - silanol interactions).

These observations led to the conclusion that copper was probably not responsible for the deterioration of the methylamphetamine peak, especially as the analytes which gave poor peak shapes in the presence of copper ions were unaffected when the problem was first observed.

TABLE 7.1: COMPARISON OF RELATIVE CAPACITY FACTORS ON COLUMN F5493/1 - 7 SHOWING THE EFFECT OF COPPER ON RETENTION

Solution	Compound	Before addition of copper	With copper in the eluent	After EDTA wash to remove copper
A	Caffeine	3.80	4.47	4.50
	Imipramine	24.08	22.78	22.15
	Morphine	29.23	29.53	29.47
	Methylamphetamine	63.69	62.29	61.35
	Protriptyline	100.00	100.00	100.00
B	Cocaine	7.29	7.70	7.61
	Phentermine	30.72	41.00	30.66
	Ephedrine	55.04	N.R.[a]	56.33
	Protriptyline	100.00	100.00	100.00
C	Diazepam	1.98	2.46	2.46
	Propranolol	18.31	21.87	17.75
	Nortriptyline	53.09	55.13	54.77
	Protriptyline	100.00	100.00	100.00
D	Amitriptyline	15.79	15.58	15.35
	Prolintane	39.85	31.35	29.73
	Protriptyline	100.00	100.00	100.00
E	Nitrazepam	1.56	2.39	2.05
	Chlorpromazine	17.08	16.96	16.93
	Pipazethate	50.23	44.57	43.20
	Protriptyline	100.00	100.00	100.00
F	Dextropropoxyphene	5.69	5.38	5.27
	Amphetamine	33.12	36.55[b]	34.83
	Pholcodine	38.91	36.55	36.29
	Protriptyline	100.00	100.00	100.00
K	Papaverine	2.74	3.09	2.96
	Dipipanone	26.06	19.91	18.52
	Methdilazine	48.05	45.38	44.95
	Protriptyline	100.00	100.00	100.00
L	Procaine	7.96	7.25	7.42
	Promazine	29.52	28.79	27.99
	Ethoheptazine	43.65	40.44	39.68
	Protriptyline	100.00	100.00	100.00
M	Codeine	29.15	29.25	29.41
	Phenylephrine	41.74	N.R.[a]	45.63
	Protriptyline	100.00	100.00	100.00
N	Nortriptyline	53.09	55.13	54.77
	Strychnine	96.88	88.81	89.51

[a]No peak recorded for ephedrine or phenylephrine (see Figure 7.8).

[b]Amphetamine and pholcodine co-eluted when copper was present in the eluent.

Figure 7.7: Influence of copper on the chromatography of methylamphetamine

Conditions and compounds as Figure 7.1, except eluent contained 0.1 mmol l^{-1} copper. Column: Spherisorb S5W, F5493/1-No.7.

(a) Peak shapes prior to washing the column with copper acetate solution.
(b) Peak shapes after washing column with copper acetate solution.
(c) Peak shapes with 0.1 mmol l^{-1} copper in the eluent.

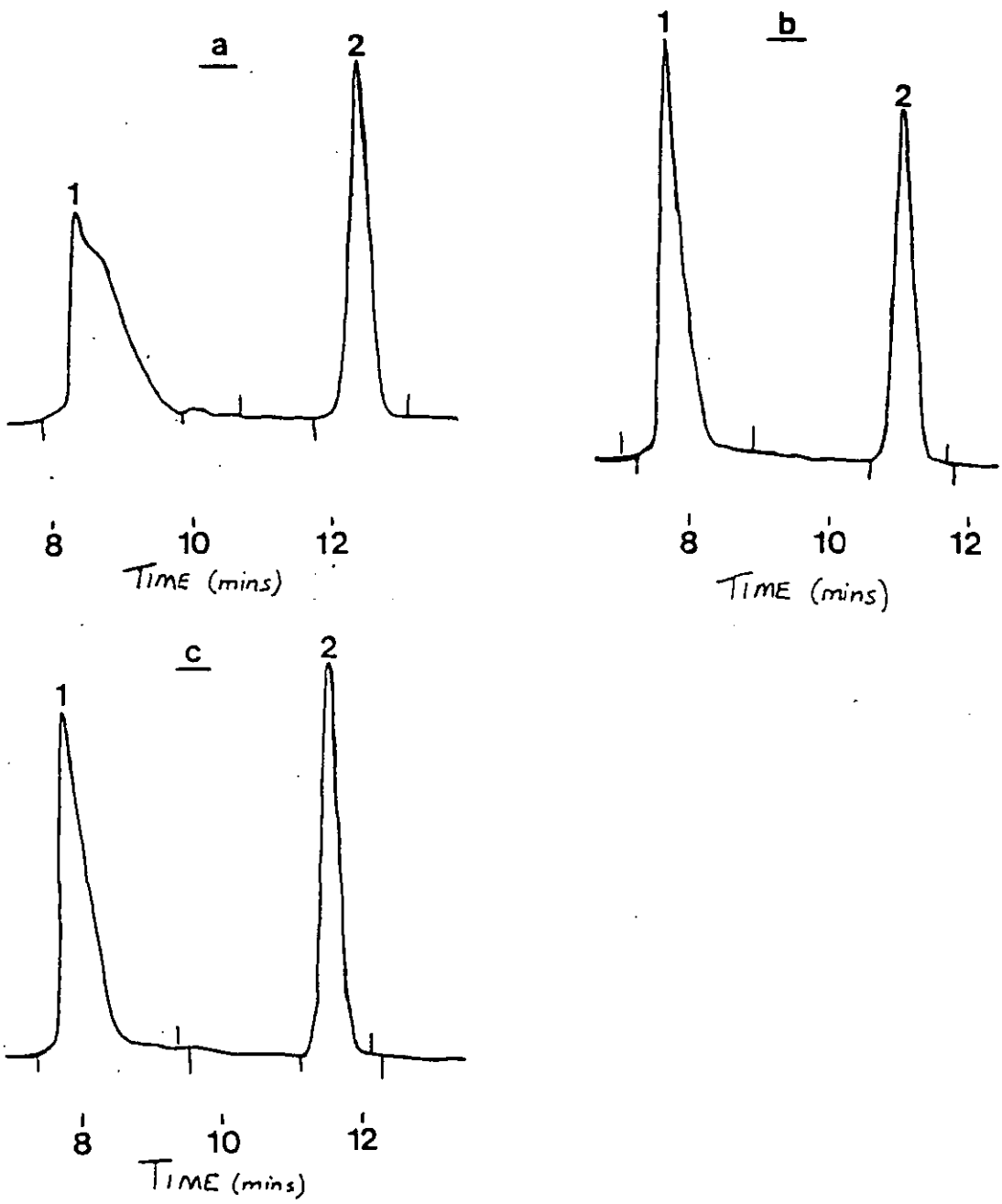
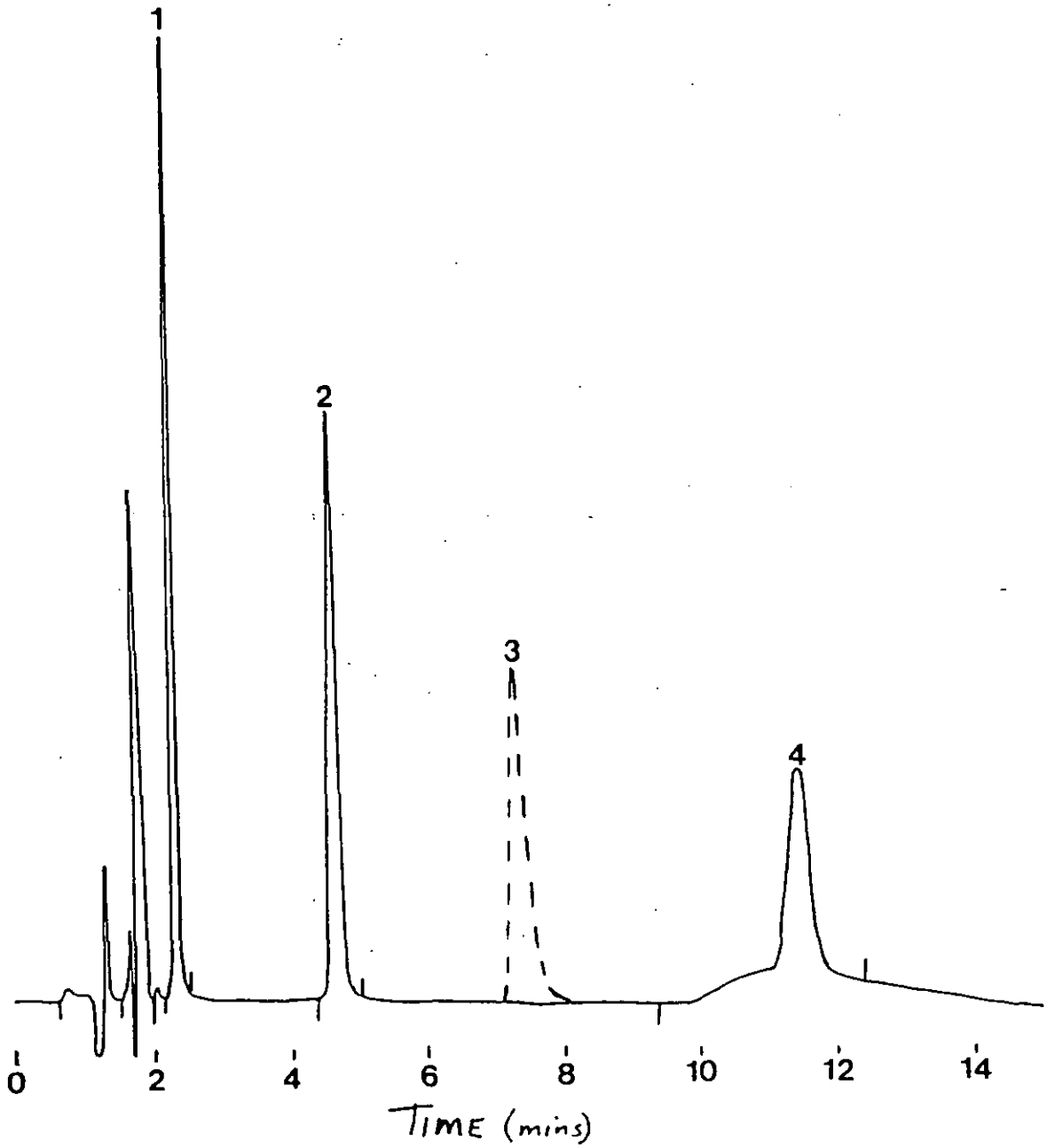


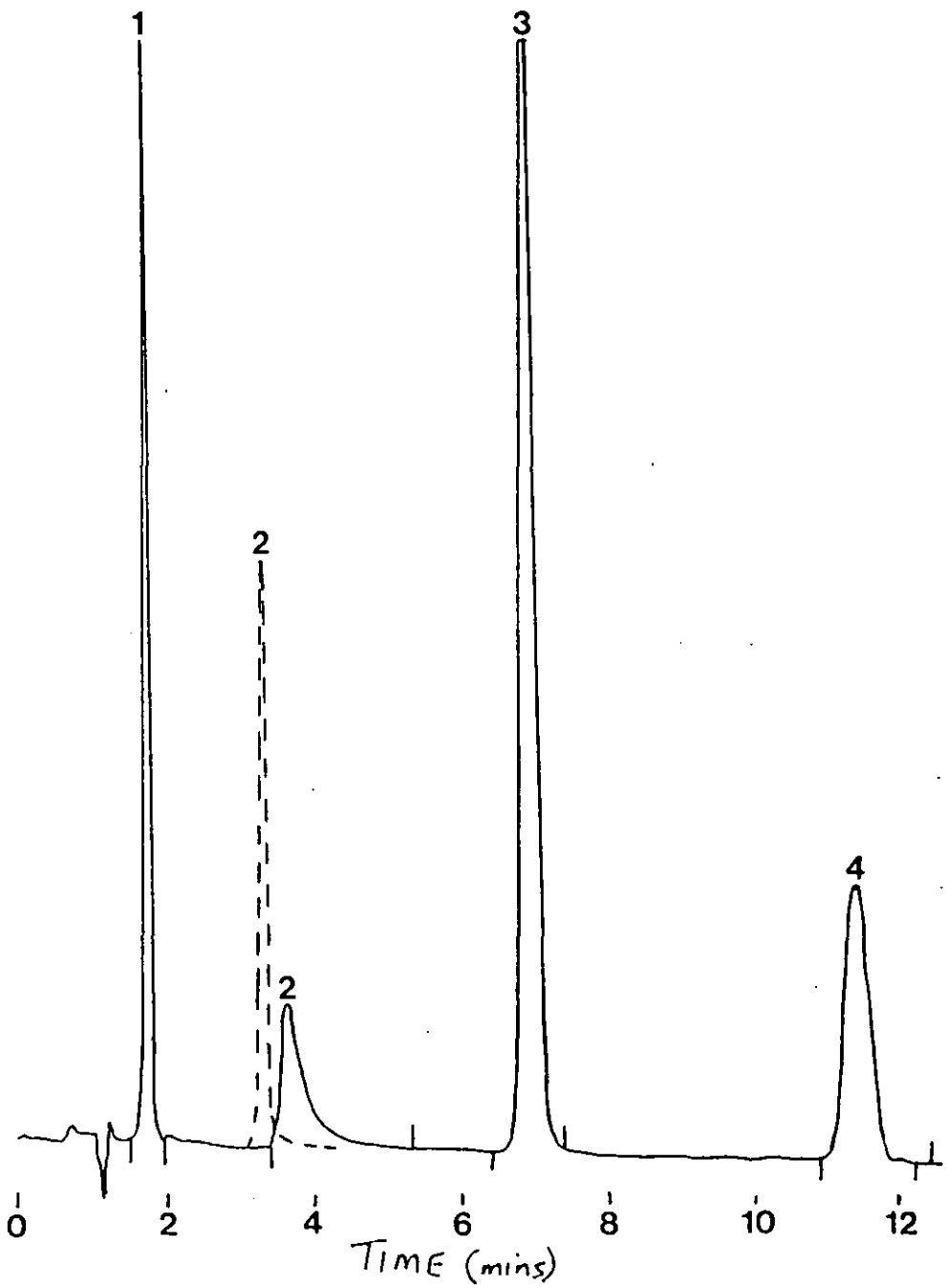
Figure 7.8: Influence of copper on the chromatography of some of the test solutions

Conditions as Figure 7.1, except eluent contained 0.1 mmol l^{-1} copper. Column: Spherisorb S5W, P5493/1-No.7. The dashed line shows the elution of the affected compound after washing the column with an aqueous EDTA solution.

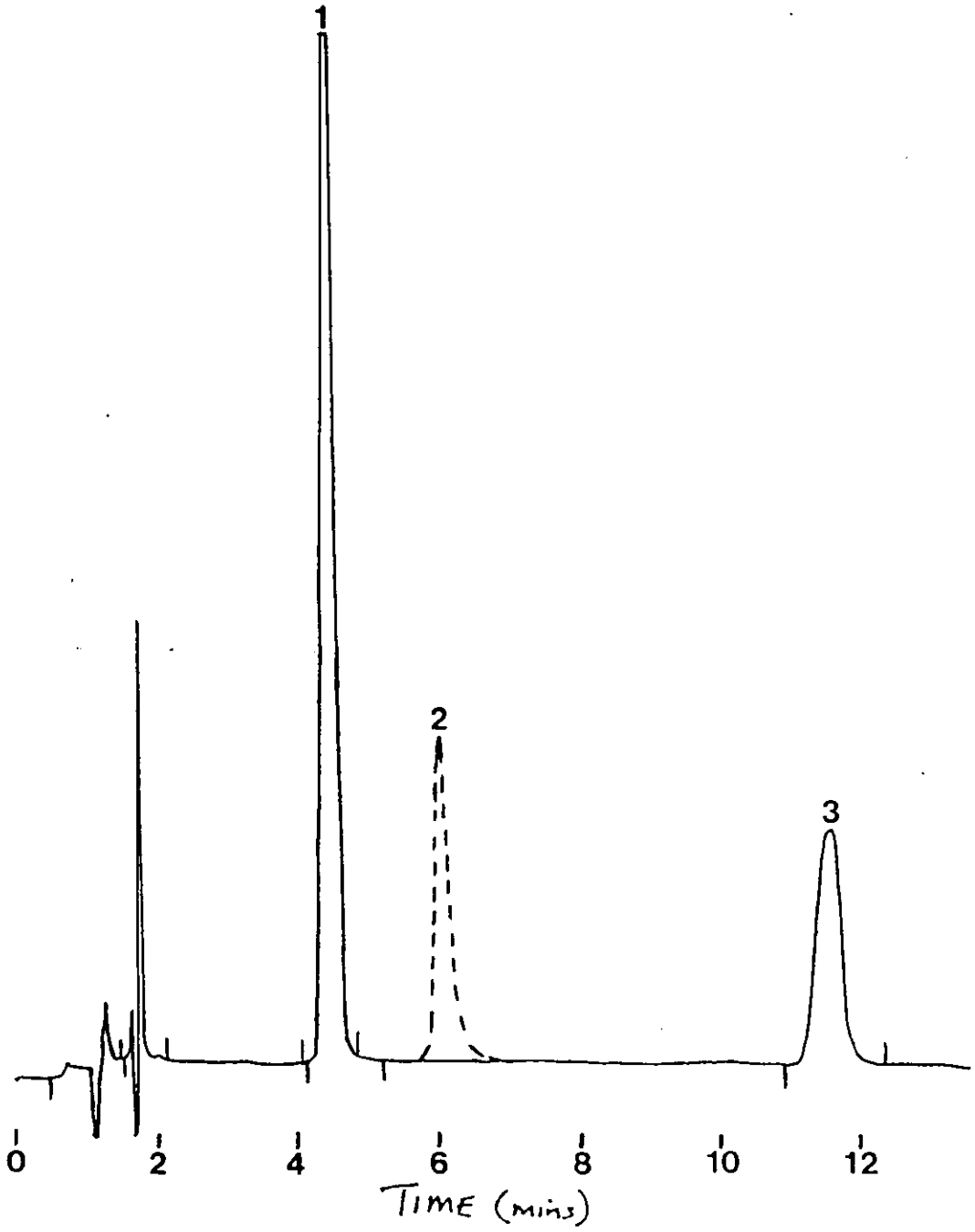
7.8: (a) Solution B; Compounds: (1), cocaine; (2), phentermine; (3), ephedrine, (note: the broad peak under the protriptyline peak could be ephedrine eluting in the presence of the copper ions); (4), protriptyline.



7.8: (b) solution C; Compounds: (1), diazepam; (2), propranolol; (3), nortriptyline; (4), protriptyline.



7.8: (c) solution M; Compounds: (1), codeine; (2), phenylephrine; (3), protriptyline.



7.6.2 IRON (Fe^{3+})

Having established that there was a significant concentration of iron in the open-column grade silica (60 ppm), it was decided to determine the influence of ferric iron on the peak shape of methylamphetamine. This would be achieved by deliberately contaminating a column with iron (III) to see if distortion of the methylamphetamine peak could be produced.

A column was tested with the standard eluent and set up to give an acceptable peak shape for methylamphetamine (Figure 7.9 (a)). A 250 ml portion of the eluent was doped with ferric iron, (concentration 1 ppm), and recycled through the column for 30 minutes. The resulting peak shape for methylamphetamine was poor (Figure 7.9 (b)), showing definite signs that it had been affected by iron in the eluent. None of the other compounds in solution A showed any sign of deteriorating peak shape although increases in relative capacity factors (RCFs) were observed (e.g. imipramine 24.9 increased to 29.3 in the presence of iron). The performance of the column was successfully recovered by washing with 200 mls of water, (the RCF of imipramine fell to 23.6). In a test on the full set of drug solutions using an eluent containing 0.1 ppm ferric iron, the methylamphetamine peak was not distorted, but phenylephrine gave a poor result, whilst amphetamine and pholcodine co-eluted. When ferric iron was added to the eluent at a concentration of 0.5 ppm, the methylamphetamine peak was distorted (Figure 7.9 (c)), prolintane and phenylephrine both gave poor results and once again amphetamine and pholcodine co-eluted (Figure 7.10).

In order to gain further evidence that ferric iron might have been responsible for the problems, it was decided to find out if it behaved in the same manner as the contaminant(s) from the pre-column. An attempt to remove ferric iron from a contaminated column using fresh eluent proved unsuccessful, and so too did washing the column with methanol. The performance of the analytical column was successfully recovered by washing it with water, suggesting that ferric iron may indeed have been contributing to the problem.

Figure 7.9: Effect of iron on the chromatography of methylamphetamine

Column: Spherisorb S5W, F5493/1-No.12.3.

Compounds: (1), methylamphetamine; (2), protriptyline.

(a) Peak shapes using the standard conditions, (see Figure 7.1).
(b) Peak shapes using the new eluent but 1.0 ppm iron added.
(c) Peak shapes using the new eluent but 0.5 ppm iron added.

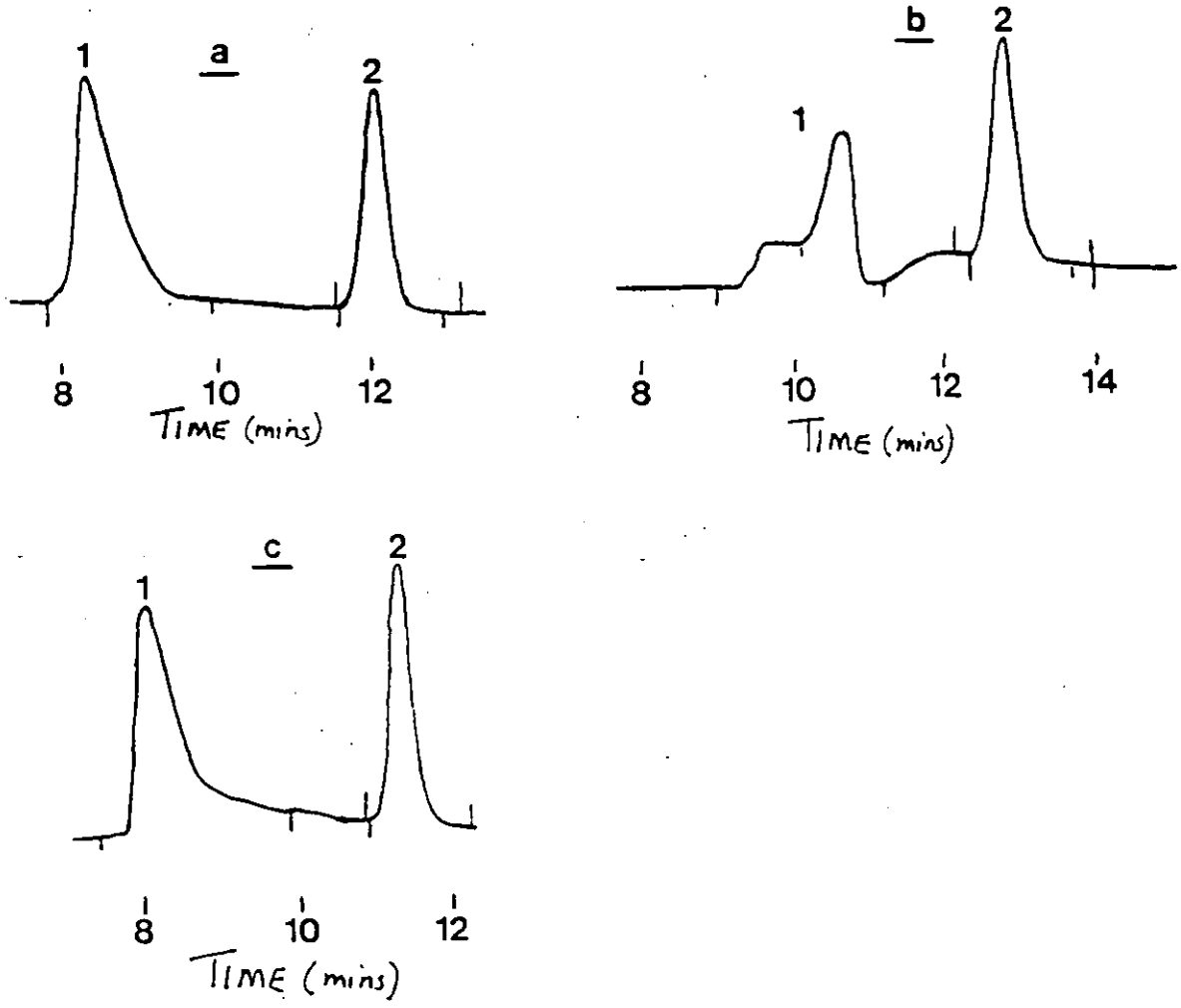
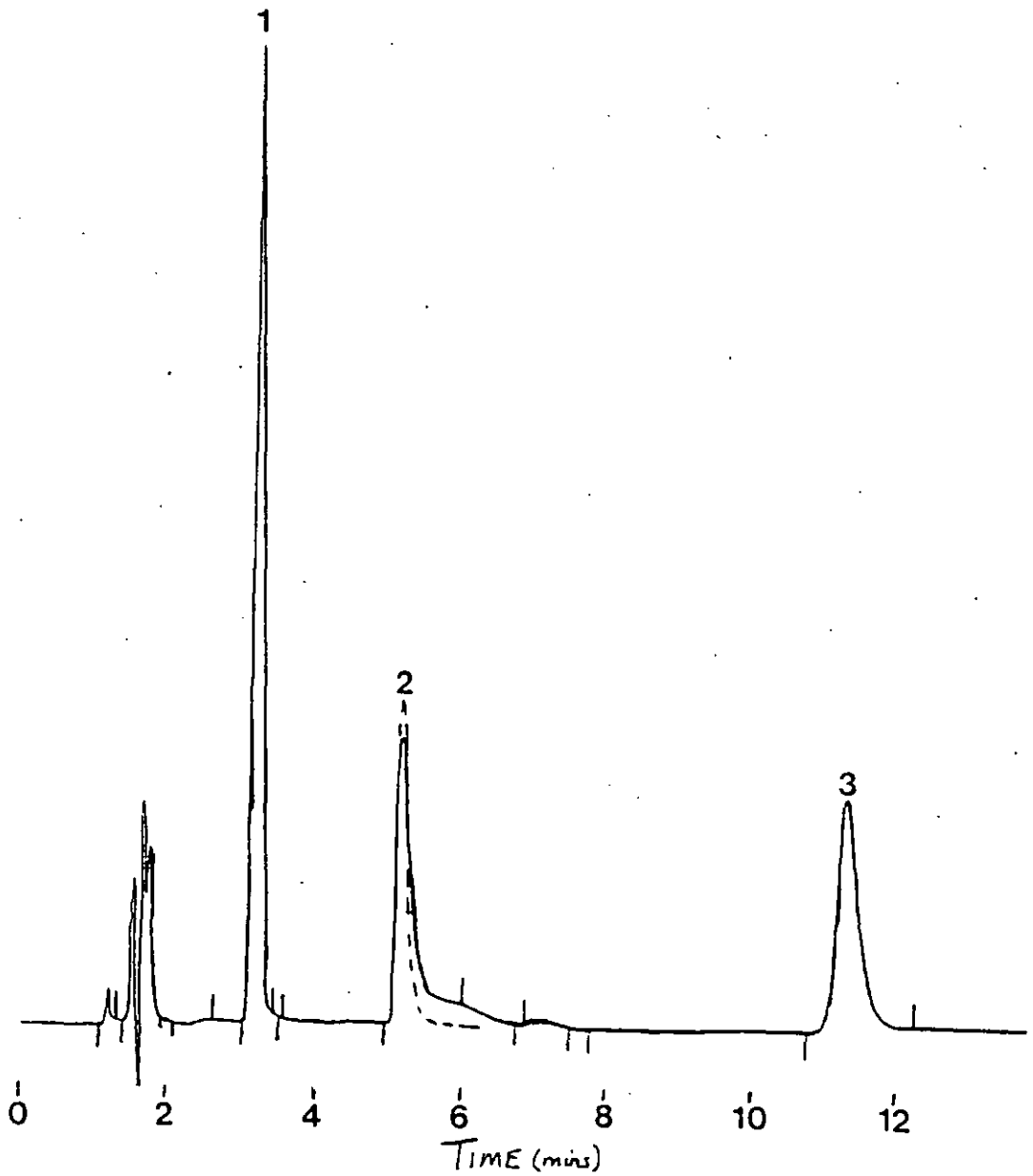


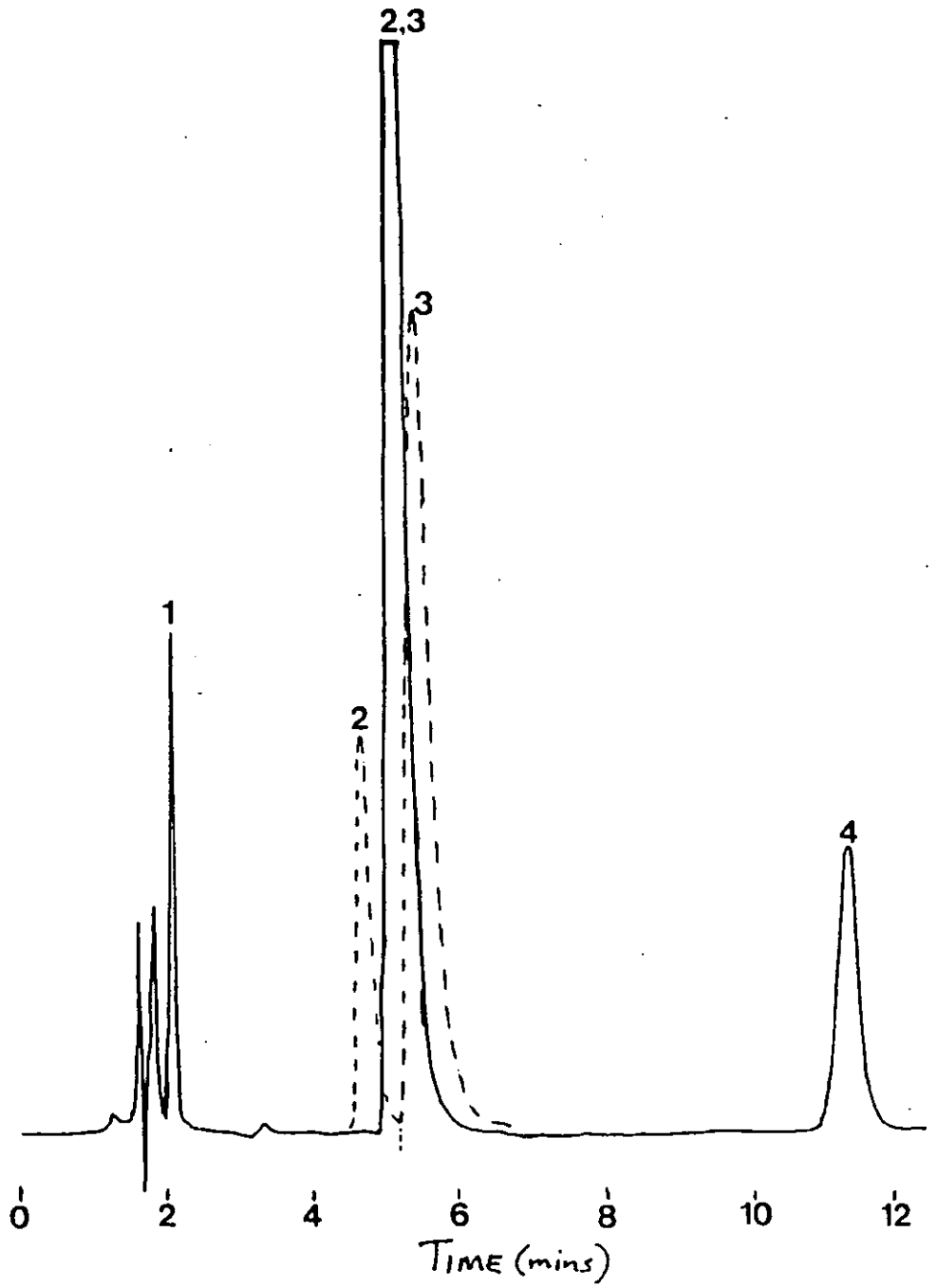
Figure 7.10: Effect of iron on the chromatography of some of the test solutions

Conditions as Figure 7.1, except eluent contained 0.5 ppm Fe^{3+}. Column: Spherisorb S5W, F5493/1-No.12.3. The dashed line shows the elution of the affected compound in the absence of iron.

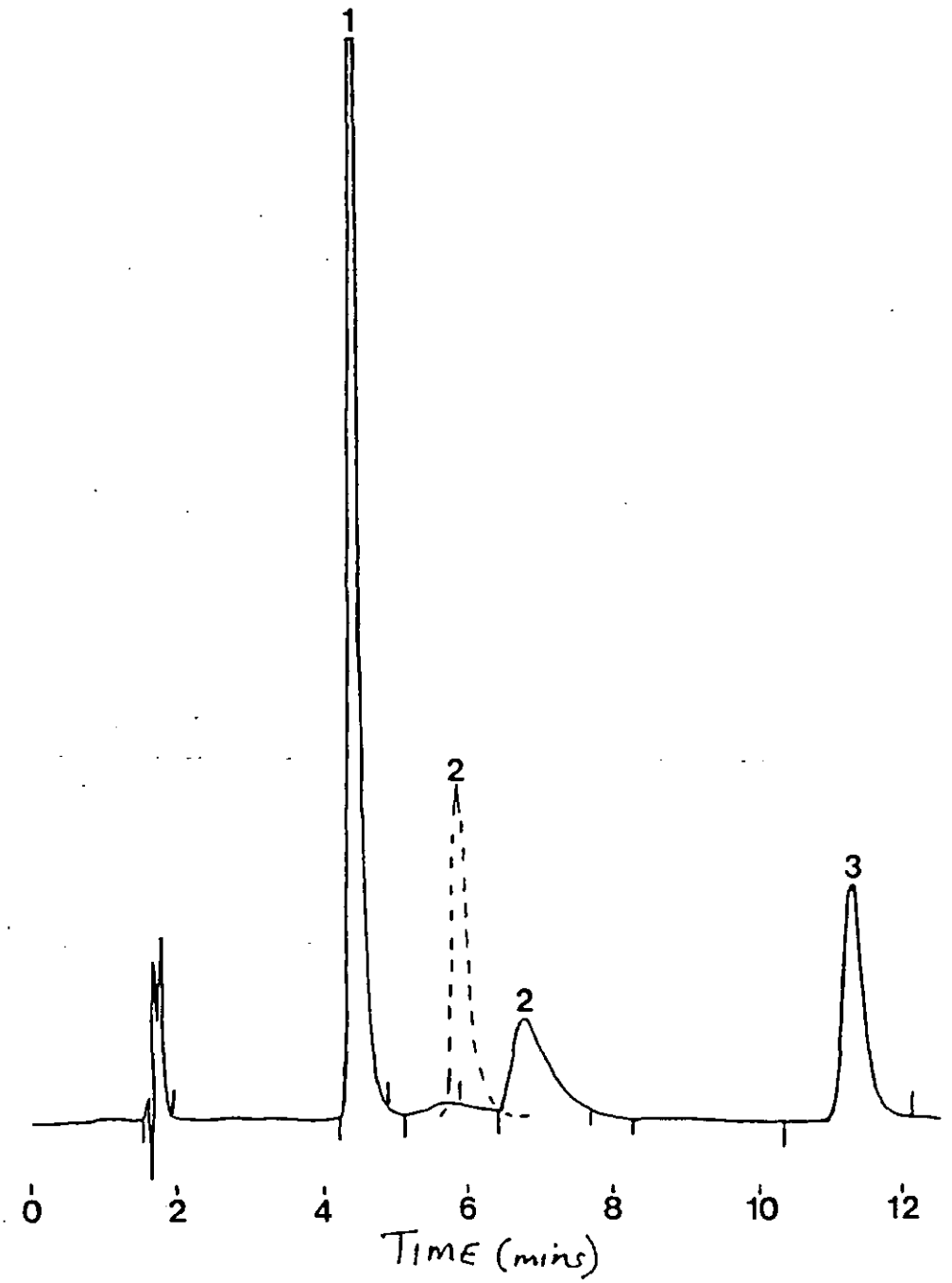
7.10 (a) Solution D; Compounds: (1), amitriptyline; (2), prolintane; (3), protriptyline.



7.10 (b) solution F; Compounds: (1), dextropropoxyphene; (2), amphetamine; (3), pholcodine, (co-eluted with amphetamine in the presence of iron); (4), protriptyline.



7.10 (c) solution M; Compounds: (1), codeine; (2), phenylephrine; (3), protriptyline. (Compare with Figure 7.8 (c)).



7.6.3 CONCLUSIONS FROM METAL ION STUDIES

These studies revealed that copper was almost certainly not responsible for the deterioration of the methylamphetamine peak. The results from the studies using iron (III) revealed that when it was present at sufficiently high concentrations (> 0.5 ppm in the eluent) poor results were obtained for methylamphetamine. However, when iron (III) was used at a concentration of only 0.5 ppm in the eluent, a separation of the full test set revealed problems for a number of the test compounds. Some of the peak shapes were seriously distorted, suggesting that the effect of iron was different from the effect of the contaminant(s) believed to be present in the system. In addition, at lower concentrations in the eluent (0.1 ppm) iron (III) had a much less dramatic effect than the contaminants on the chromatography of methylamphetamine, suggesting that it was not solely responsible for the deterioration of the methylamphetamine peak.

7.7 FURTHER STUDIES

7.7.1 DISTORTED METHYLAMPHETAMINE PEAKS FROM NEW COLUMNS

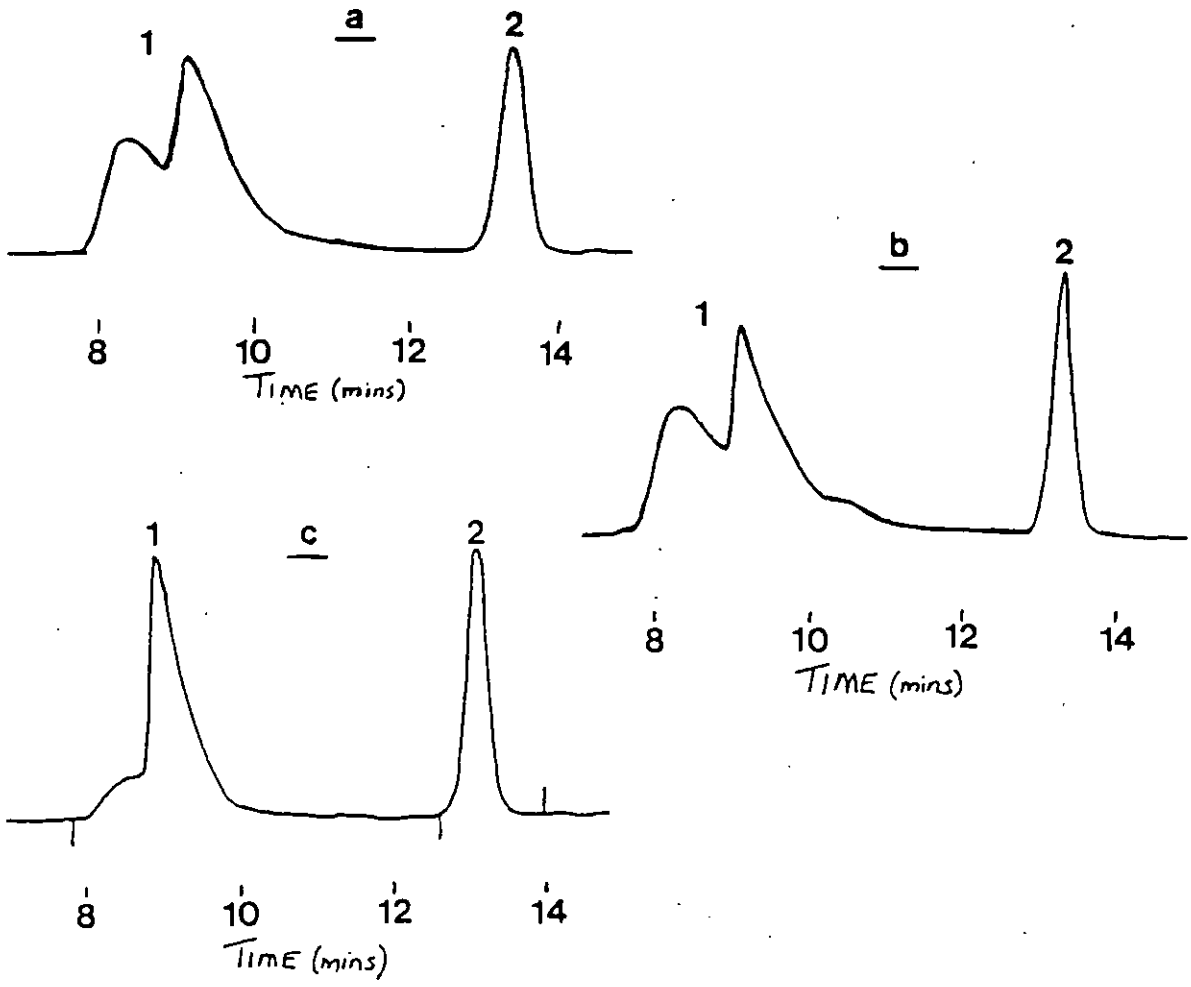
As none of the experiments aimed at investigating the problem of methylamphetamine had been successful in identifying the cause of the poor peak shapes, new columns were packed with Spherisorb S5W, (batch F5493/1), in preparation for new experiments. When these columns were tested immediately after packing, using the standard conditions described earlier (see Figure 7.1), but in the absence of a pre-column, poor results were recorded for methylamphetamine (Figure 7.11). All other compounds in the test solutions gave acceptable results.

This observation led to the conclusion that the problem had to be independent of the nature of the pre-column silica, as deterioration of the methylamphetamine peak on these columns was recorded before they were subjected to use in conjunction with a pre-column. However, it was found that these columns could all be cleaned by washing with water, suggesting that the cause of the problem on this occasion was similar in nature to whatever had produced poor results in previous experiments.

Figure 7.11: Poor methylamphetamine peaks on new, unused columns

Conditions and compounds as Figure 7.1. All columns packed with Spherisorb S5W (batch F5493/1).

(a), column No.2.2; (b), column No.7; (c), column No.9.3.



7.7.2 THE SIGNIFICANCE OF THE WATER WASH

The observation that water could be successfully employed to clean any column producing poor results, irrespective of the column history, was thought to be of considerable significance. The effect of water washes on analytical columns was found to be two-fold. Firstly, when used in sufficient amounts, water was able to clean even the most severely contaminated columns (see section 6.5), and secondly, the effect of water was found to extend beyond improving the peak shape of methylamphetamine as considerable changes in retention were observed for certain compounds,

(Table 7.1, shown in italics, and also Tables 6.9, 6.10 and 6.11).

As noted earlier (Chapter 6) the chromatographic properties of the silica were found to be changing with time. These changes were attributed to alterations in the silica surface (section 6.6), where the effect of water on silica was explained in terms of the hydrolysis of siloxane groups to silanols. In addition, changes in properties were found between columns packed with silica from a single source at different times (see Table 6.6), indicating that the same processes of aging were occurring on dry silica and on silica packed in columns.

7.7.3 INFLUENCE OF THE AGE OF THE SILICA ON THE PEAK SHAPE OF METHYLAMPHETAMINE

These observations led to the idea that the problem of poor peak shapes for methylamphetamine might be related to the absolute age of the silica. In the initial stages of the study, when good peak shapes could be obtained, the silica was relatively new (ca. 1 year old[162]). The first observation of poor peak shapes for methylamphetamine (Figure 6.1 (c)) was recorded on a column which had been packed 6 months earlier, and at a time when the silica was about 17 months old. By the time poor results were being recorded on new columns packed from the same bottles of silica as those used in the early studies (batch F5493/1), the silica was 2½ - 3 years old. From this observation, along with the results from the storage studies presented in Chapter 6, one possible explanation for the erratic behaviour of methylamphetamine can be proposed.

In the early stages of the study it would appear that the new silica had not aged to any significant extent, and so a clean 'chromatographic surface' existed, on which it was possible to obtain acceptable results for methylamphetamine. As packed columns were used, and in some cases stored for long periods (> 6 months) between experiments, the silica was undergoing an aging process which caused major changes in chromatographic properties. Most noticeable was the dramatic reduction in RCFs for some compounds and smaller, but significant increases in retention for others (Chapter 6). It would appear from the observations presented in this chapter that the deterioration of the methylamphetamine peak may have been caused by the same aging process. As the silica aged, almost certainly by hydrolysis of siloxane groups, changes in the nature and purity of the

'chromatographic surface' would be expected. Breakdown of the siloxane groups on the surface might lead to the exposure of any impurities that exist in the atomic layers close to the silica surface. These impurities, almost certainly metal ions (*e.g.* Na, Al, Ti and Fe (reference 89)), could have been responsible for the distortion of the methylamphetamine peak, although there are no obvious mechanisms to support this theory.

Application of water or aqueous EDTA washes was found to clean any 'contaminated' column, but further deterioration of column performance was recorded, although in most cases the changes were slow and less dramatic than the original loss of column performance. It could be argued that the application of a substantial water (or EDTA) wash removed the majority of exposed contaminants as soluble metal hydroxides, whilst simultaneously producing an artificial aging of the silica, breaking down more siloxane bridges and removing any rogue metal ions exposed in the process. This would have been possible because the pH of the water was not controlled, and so the aqueous wash conditions were probably slightly acidic.

Upon return to the standard chromatographic conditions, the aging of the silica during use would then have been much slower, as the more strained siloxane sites would have been hydrolysed by the water wash, and only the more stable siloxanes would have been available for subsequent hydrolysis by the mobile phase. Most of the metal ions exposed during aging of the silica by the eluent would not have been removed from the silica matrix under normal chromatographic conditions due to the high operating pH, (at which most heavy metal hydroxides are insoluble).

This speculation would also account for the observation of poor results on new columns packed with older silica from the same batch. The natural aging of this material led to the same type of deterioration of the 'chromatographic surface' as the aging process occurring in packed columns, or during a water wash (see Chapter 6). Also, the considerable variation in peak shapes for methylamphetamine could be accounted for by variations in the distribution and accessibility of contaminants on the chromatographic surface, along with blocking of these 'active' sites by other analyte molecules or components of the eluent.

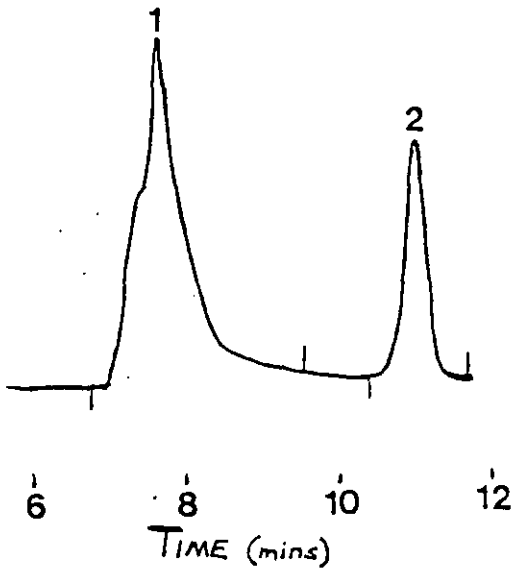
Another interesting observation was recorded which appears to support this speculation. When the test compounds were analysed on

'contaminated' columns using the ammonia eluent, no deterioration of the methylamphetamine peak was recorded. The eluting power of the ammonia eluent was much greater than that of the new eluent (see Table 4.2), due to its much higher ionic strength. This factor may have been critical in masking the effect of changes in the chromatographic properties of the silica when using the ammonia eluent. The ionic strength of the ammonia eluent might have been sufficient to mask all the 'active sites' on the aged silica surface, possibly by the formation of ammine complexes, so preventing peak distortion. Since poor results for methylamphetamine were obtained on subsequent use of the columns with the new eluent, it would appear that the ammonia eluent was not removing any exposed metal ions. This is hardly surprising as the ammine complexes of many of the likely metal ion contaminants as insoluble at the high pH of the ammonia eluent.

However, there remains one interesting observation that has not yet been explained. In all the studies discussed so far, the poor results were recorded on Spherisorb S5W, batch F5493/1. When columns prepared from batch 2752 were examined, the extent of peak deterioration was much less than might have been expected (Figure 7.12). This observation appears, at first sight, to be contrary to the proposal presented above, especially as the other aging effects (related to changes in retention of certain analytes caused by changes in the siloxanes and silanols) were observed on this batch (see section 6.4 and Figure 6.3). However, another factor must be taken into consideration when comparing columns from different batches, namely the individual levels of impurities in each batch. Smith *et al.*[36] reported that the manufacturers had found changes in chromatographic properties of Spherisorb S5W at the time of the production of batch 2752, and it is possible that such changes, although not explained by the manufacturers, may have been related to the purity of the silica. Such changes could be responsible for the differences in performance of this batch (with respect to F5493/1), both in terms of its retention properties for methylamphetamine, and in terms of its changes in behaviour during storage (see section 6.4).

Figure 7.12: A distorted methylamphetamine peak on a second batch of Spherisorb S5W

Conditions and compounds as Figure 7.1. Column: Spherisorb S5W, batch 2752, column No.2.



7.8 CONCLUSIONS

These studies have shown that distorted peak shapes for methylamphetamine recorded on silica columns could not be accounted for in conventional chromatographic terms. Factors such as sample integrity, solvent strength and the cleanliness of the HPLC system were all ruled out. Detailed examination of the influence of the pre-column silica on the chromatography suggested that the quality of the silica might be a contributory factor in the process leading to the deterioration of the peak shape for this compound. However, the most likely cause, namely, the direct transfer of metal ions from the pre-column silica to the analytical column, could not be conclusively demonstrated. Studies involving two metal ions, iron (III) and copper (II), suggested that other compounds in the test solutions should be affected, either in preference to or in addition to methylamphetamine, if these metal ions were indeed involved in the processes leading to poor peak shapes.

Continuing studies into the problem led to the conclusion that the phenomenon was related to the age of the silica. This idea was backed up by a series of observations of changes in chromatographic properties for the silica with time, and that the changes in properties appeared to happen in a systematic, (but unquantifiable) manner. The results suggested that, as the silica aged, the nature of its surface changed, and that this resulted in the production of 'active sites' which selectively affected the chromatography of methylamphetamine.

At present there is insufficient data to confirm whether this theory is right or wrong, and evidently these observations merit further detailed examination. It would appear that such experiments would require the use of a series of compounds closely related to methylamphetamine, to determine if other compounds are affected in the same manner. However, throughout these studies amphetamine, phentermine and ephedrine, compounds closely related to methylamphetamine, showed no signs of anomalous behaviour.

CHAPTER 8: CONCLUSIONS FROM THE ISOCRATIC STUDIES

These studies have shown that it is possible to produce an eluent of high pH using only solid components for the preparation of the aqueous buffer. The two components that were found to give the best results, within guidelines laid down at the start of the study (section 4.1), were sodium 3-(cyclohexylamino)-2-hydroxy-1-propanesulphonate (CAPSO-Na) and 3-(cyclohexylamino)-1-propanesulphonic acid (CAPS). These were used at a concentration of 0.08 mol l^{-1} each in the aqueous buffer to produce an eluent (90:10 methanol:buffer, v/v) which was simple to prepare, which had a UV absorbance < 1 at 215 nm, and which, in a typical assay, eluted the longest retained analyte, strychnine, in about 13 minutes.

The new mobile phase was found to have a higher discriminating power than the ammonia eluents used previously, and the reproducibility of separations over a one month period on a single column was found to be very good. Variations in the elution order of the test compounds were observed when comparing the performance of the new eluent with the previous ammonia system, and these were attributed, in part, to differences in the ionic strengths of the two eluents.

In studies carried out over a one year period on three columns packed from a single bottle of silica and using the newly devised eluent, significant changes in relative capacity factors (relative to protriptyline, the internal standard) were observed for some of the analytes. Similar changes in chromatographic performance were found for silica stored dry and unused, both in columns packed from the same batch of silica as the original three columns, and for columns packed from a second batch of the same brand of material. These differences were found to be enhanced by washing the columns with water, suggesting that some form of 'aging process' was occurring, and that it was related to the action of water on the silica surface. Preliminary ^{29}Si-CP-MAS-NMR results suggested that 'used' silica had a higher ratio of silanols to siloxanes, (when compared to a 'reference' sample of the same material). This led to the idea that the changes in chromatographic properties of the silica were caused by hydrolysis of surface siloxane groups to silanols.

The sudden appearance of distorted peak shapes for methylamphetamine could not be accounted for by conventional chromatographic phenomena. Columns producing poor results for methylamphetamine could be 'cleaned up' by washing with water, and from this observation it was concluded that the occurrence of poor peak shapes was related to the aging process which had been discovered in separate experiments. It was thought that the aging process, which involved the hydrolysis of surface siloxane groups, may have led to the exposure of impurities (probably metal ions) in the silica matrix, creating 'active sites' on the silica surface. These sites may have been responsible for the distortion of the methylamphetamine peak, although no straightforward mechanisms could be proposed to account for the observations. It was concluded that washing the columns with water removed any exposed impurities on the silica surface, as well as causing further hydrolysis of the siloxane groups.

SECTION 3: GRADIENT STUDIES

CHAPTER 9: EXPERIMENTAL

9.1 GRADIENT SYSTEM 1

9.1.1 CHEMICALS

Water was HPLC grade and acetonitrile far-UV HPLC grade, both from FSA Laboratory Supplies, (Loughborough, U.K.). Trifluoroacetic acid (TFA) was AnalaR grade and sulphuric acid an AnalaR grade volumetric solution (2.5 mol l^{-1}), both from BDH Ltd., (Poole, U.K.). All drug samples were obtained from the reference collection of the Central Research and Support Establishment (CRSE) of the Home Office Forensic Science Service.

9.1.2 THE ELUENTS

Two different eluent systems were examined under both isocratic ("dial-a-mix") and gradient conditions. In the first system, eluent A was prepared by adding 1 ml of TFA to 1 litre of HPLC grade water, and eluent B by adding 1 ml of TFA to 1 litre of HPLC grade acetonitrile. In the second system, the TFA was replaced by an identical quantity of 2.5 mol l^{-1} sulphuric acid in both eluents A and B.

9.1.3 HPLC SEPARATIONS

9.1.3.1 Instrumentation

The two eluents, A and B, were passed through an ERMA on-line degasser, (Model ERC 3311, supplied by Applied Chromatography Systems, Macclesfield, U.K.) before reaching the pumps. Each eluent was pumped using a Waters 590 programmable HPLC pump, (Waters Associates, Milford, MA, U.S.A.); the two pumps being controlled by a Waters automated gradient controller (Model 680), set to deliver a total flow of 1.5 ml min^{-1}. The two eluents passed from the pumps into a 1.5 ml mixing chamber on a Gilson dynamic mixer (Model 811; supplied by Anachem, Luton, U.K.). The output of the mixing chamber was connected to a Rheodyne 7125 injection valve (Rheodyne Inc., Cotati, California, U.S.A.), fitted with a 20 µl loop. The valve was connected to the analytical column (150 mm x 4.6 mm i.d.) packed with Inertsil ODS 2 (5 µm: Lot No. SQ5-737: Gasukuro Kogyo, Japan: supplied by Jones Chromatography Ltd., Hengoed, U.K.). The samples were

detected using a Waters 990 diode-array detector. The detector was controlled by a Waters 990 computer control system, which was also used for data analysis. The wavelength chosen for on-line monitoring of the chromatographic separations was 215 nm, from a diode-array scan of 200 to 310.6 nm.

9.1.3.2 The Gradient Profile

The standard gradient profile is described below:

0 - 3 mins 2% B
3 - 23 mins 2% B to 98% B Linear Gradient (4.8% B min^{-1})
23 - 26 mins 98% B (End of run)

The return to initial conditions, i.e. 98%B - 2%B, was by direct changeover, and the reequilibration time prior to commencing a new run was typically about 10 minutes.

9.1.4 TEST SOLUTIONS

Most of the test solutions were prepared in water to give a peak on-scale using the diode-array detection system (which allowed rescaling of the peaks after the separation, to a maximum of 2AU). Where compounds were found to be insoluble in water, mixtures of acetonitrile and water were used as solvents, *e.g.* a mixture of acetonitrile / water (80:20 v/v) was used as a solvent for cannabinol (the most hydrophobic analyte). In all cases full loop injections of the test samples were performed by injecting ≥ 30 µl of sample through a 20 µl loop.

Two test solutions were prepared in detail. Their compositions are given below.

9.1.4.1 Aqueous test solution

The following samples were dissolved in 5 mls of HPLC grade water to produce the aqueous test solution, (quantities in mg).

Paraquat, 6.85; morphine-3ß-glucuronide, 3.75; morphine hydrochloride, 2.01; ephedrine hydrochloride, 4.58; paracetamol, 3.26; amphetamine

sulphate, 5.71; bretylium tosylate, 5.78.

9.1.4.2 Organic test solution

The following samples were dissolved in a mixture of 5 mls of HPLC grade acetonitrile and 0.5 mls HPLC grade water to produce the organic test solution, (quantities in mg).

Tubocurarine chloride, 1.70; diamorphine hydrochloride, 4.20; cocaine hydrochloride, 8.19; dibenzepin hydrochloride, 2.04; propranolol hydrochloride, 1.04; phenobarbitone, 2.11; promazine hydrochloride, 2.70; dextropropoxyphene hydrochloride, 2.97; dimethisoquin hydrochloride, 1.43; methaqualone hydrochloride, 2.09; triazolam, 1.69; diazepam, 1.73; nandralone, 4.81; cyclopenthiazide, 1.56; cannabinol, 1.43.

9.1.5 CALCULATIONS

Capacity factors were calculated relative to the solvent front(t_0) from each individual chromatogram, according to the equation:

$$k' = \left[\frac{(t_R - t_0)}{t_0} \right] \qquad 9.1$$

where t_R is the retention time of the analyte.

Standard deviations for small data sets were calculated using σ_{n-1} and coefficients of variance (%C.V.) as ($100\sigma_{n-1}/\bar{x}$), where $\bar{x}$ is the mean.

9.2 GRADIENT SYSTEM 2

9.2.1 CHEMICALS

Acetonitrile was far-UV HPLC grade (FSA Laboratory Supplies, Loughborough, U.K.). Sulphuric acid was an AnalaR grade volumetric solution (2.5 mol l^{-1}; BDH Ltd., Poole, U.K.). Various water supplies were tested (see section 9.2.6), and for most of the chromatography, Rathburn HPLC grade water (Rathburn Chemicals Ltd., Walkerburn, Scotland) was used. All drug samples and 1-nitroalkane standards were obtained from the reference collection of the Central Research and Support Establishment

(CRSE) of the Home Office Forensic Science Service. (C_1 to C_6 1-nitroalkanes were obtained from commercial sources whilst C_7 to C_{16} 1-nitroalkanes were custom synthesised for CRSE (by Cookson Chemicals Ltd., Southampton, U.K.). All the compounds were chromatographed separately and found to give only one detectable peak using HPLC system 2).

9.2.2 THE ELUENTS

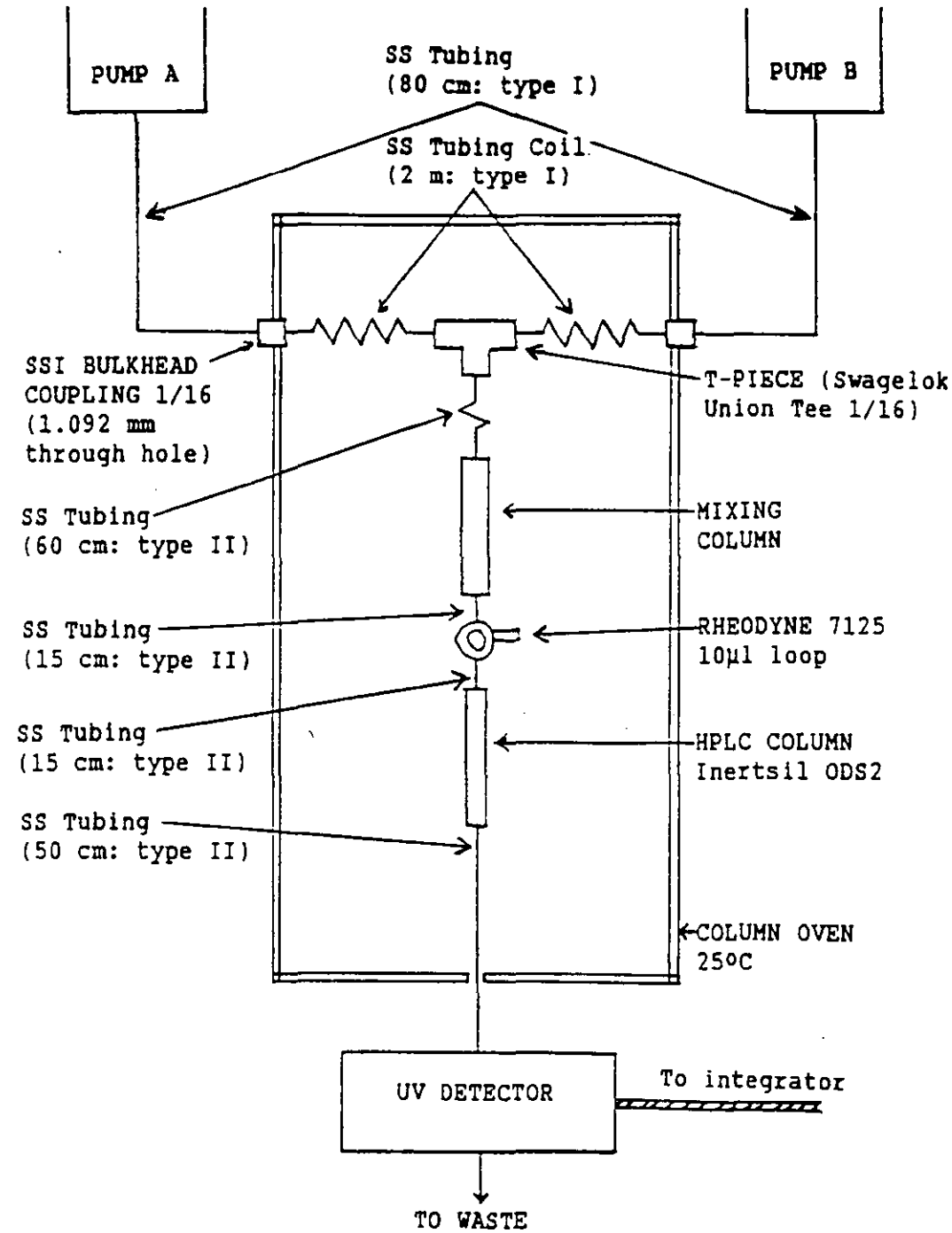
Eluent A was prepared by adding 1 ml of sulphuric acid (2.5 mol l^{-1} volumetric solution) to water (1 litre) and eluent B by adding 1 ml of sulphuric acid (as in eluent A) to acetonitrile (1 litre). Both eluents were degassed continually during use by sparging with a slow stream of helium.

9.2.3 HPLC SEPARATIONS

9.2.3.1 Instrumentation

The two eluents, A and B, were delivered using Waters 6000A pumps, (Waters Associates, Milford, MA, U.S.A.), which were controlled by a Waters 660 Solvent Programmer, set to give a total flow rate of 1.5 ml min^{-1}. Each pump was connected to a bulk head fitting (SSI bulkhead coupling, part no. 01-0156) on the door of a column oven and from the bulk heads to a low dead volume T-piece (Swagelok Union Tee, part no. SS-1FO-3GC). The outlet of the T-piece was connected through a specially designed mixing column (see below) to the inlet of a Rheodyne 7125 injection valve, (Rheodyne Inc., Cotati, California, U.S.A.), fitted with a 10 µl loop. The outlet of the injection valve connected to the analytical column (15 cm x 4.6 mm i.d.), which was packed with Inertsil ODS2 (5 µm: Lot No. SQ5-790: Gasukuro Kogyo, Japan: supplied by Jones Chromatography Ltd., Hengoed, Wales). Detection of the analytes was by UV at 215 nm (0.16 aufs) using a Pye Unicam PU-4020 variable wavelength UV detector (Philips Scientific, Cambridge, U.K.). Chromatograms were recorded using a Shimadzu C-R3A integrator (supplied by Dyson Instruments, Houghton-le-Spring, Tyne and Wear, U.K.), which was connected to the Rheodyne valve by a contact closure switch for remote start at the time of injection. All tubing from the bulkhead couplings to the analytical column outlet was enclosed in a Liquid Chromatography Advanced Air Oven (Kariba Instruments, Cardiff, U.K.) set at 25°C (see Figure 9.1).

Figure 9.1: Schematic diagram of HPLC system 2 showing the arrangement of the components in the column oven.



Stainless Steel Tubing type I: (1.588 mm o.d. x 1.092 mm i.d.)
Stainless Steel Tubing type II: (1.588 mm o.d. x 0.152 mm i.d.)

9.2.3.2 The mixing column

The mixing column was constructed by filling a 15 cm length of stainless steel tubing (8 mm i.d.) with 20 PTFE balls (7.938 mm diameter) and compressing these into the tube using low dead volume end fittings. The dead volume of the column was calculated to be 2.30 ml, which at a flow rate of 1.5 ml min^{-1}, corresponded to a delay time of 1.53 minutes.

9.2.3.3 The gradient profile

The standard gradient profile is described below (with a definition of the terms used to identify each part of the cycle):

0 - 3 mins 2% B (= *Gradient delay time*: t_d)
3 - 23 mins 2% B to 98% B Linear gradient (4.8% B min^{-1})
23 - end 98% B (= *Gradient hold time* ≥3 mins.)

The return to initial conditions, *i.e.* 98%B - 2%B, was by direct changeover. The time from the switch back to the initial conditions to the next injection was identified as the *Gradient reequilibration time*.

9.2.4 GRADIENT TEST SOLUTIONS

The following test solutions were provided by CRSE, except aqueous test solutions 2 and 3 and the glycine solution. In most cases full loop injections of the test solutions were performed by injecting ≥ 20 µl of sample through a 10 µl loop. Occasionally individual injections of 5 µl were performed (usually for the organic test solution).

9.2.4.1 1-Nitroalkanes test solutions

a) Short test solution

A 'short' test solution containing 6 1-nitroalkanes was prepared as shown, (volumes in µl of 1-nitroalkanes in 25 mls HPLC grade acetonitrile):

Nitromethane, 2; nitroethane, 3; nitropropane, 5; nitrobutane, 5;

nitropentane, 5; nitrohexane, 5.

b) Full test solution

A 'full' test solution containing 16 1-nitroalkanes was prepared as shown, (volumes in µl of 1-nitroalkanes in 10 mls HPLC grade acetonitrile):

Nitromethane, 1.0; nitroethane, 1.5; nitropropane, 1.5; nitrobutane, 2.0; nitropentane, 2.0; nitrohexane, 2.0; nitroheptane, 2.0; nitrooctane, 2.5; nitrononane, 3.0; nitrodecane, 3.0; nitroundecane, 3.0; nitrododecane 3.5; nitrotridecane, 3.5; nitrotetradecane 4.0; nitropentadecane, 4.0; nitrohexadecane, 4.0.

N.B. C_{15} and C_{16} 1-nitroalkanes are solids at room temperature and so they needed to be melted prior to pipetting.

9.2.4.2 Aqueous test solutions

a) Test solution 1

For most of the gradient studies an aqueous test solution containing five compounds was used. It was prepared as shown below, (quantities in mg in 15 mls HPLC grade water).

Morphine-3ß-glucuronide, 2.00; morphine hydrochloride, 1.47; ephedrine hydrochloride, 2.07; paracetamol, 1.40; bretylium tosylate, 3.16.

b) Test solution 2

For some of the gradient studies a second aqueous test solution was used. It was prepared as shown below, (quantities in mg in 15 mls HPLC grade water).

Morphine-3ß-glucuronide, 2.6; morphine hydrochloride, 1.5; ephedrine hydrochloride, 1.8; paracetamol, 1.1.

c) Test solution 3

For some isocratic runs a test solution was prepared with the following composition (quantities in mg in 15 mls HPLC grade water):

Morphine-3ß-glucuronide, 2.5; morphine hydrochloride, 1.3.

9.2.4.3 Organic test solution

The stock solution was prepared by dissolving the components in a mixture of 0.5 mls water and 4.5 mls acetonitrile (quantities in mg as shown below). The solution for chromatography was prepared by diluting a fraction of the stock solution with 40% acetonitrile (in water) to 1/10 of the original concentration.

Tubocurarine chloride, 2.06; diamorphine hydrochloride, 4.15; dibenzepin hydrochloride, 1.91; propranolol hydrochloride, 1.05; phenobarbitone, 1.80; dextropropoxyphene hydrochloride, 3.02; diazepam, 2.19; cannabinol, 1.49.

9.2.4.4 Void volume marker

A solution of glycine, 22.36 mg ml^{-1} in eluent A (section 9.2.2), was used to measure the column void volume.

9.2.5 CALCULATIONS

9.2.5.1 The 'Nitro-Index' scale

An injection of the 1-nitroalkanes was used to calibrate the gradient on a retention time basis. The 1-nitroalkanes were assigned index values of *100 x Carbon Number* and nitro-index values were calculated for the test compounds by linear interpolation between the two nearest 1-nitroalkane standards. For a compound with retention time t_R, eluting between 1-nitroalkanes C_n, retention time t_n, and C_{n+1}, retention time t_{n+1}, the nitro-index was calculated according to:

$$\text{Nitro-index} = 100 \times \left[n + \frac{(t_r - t_n)}{(t_{n+1} - t_n)} \right] \qquad 9.2$$

9.2.5.2 Statistical analysis of the retention data

Standard deviations for the data sets were calculated using σ_{n-1} and coefficients of variance (%C.V.) as $\{(100\sigma_{n-1})/\bar{x}\}$, where $\bar{x}$ is the mean of the data set.

To test for systematic error between certain HPLC separations, the T-test was applied to the mean retention times recorded under different conditions. In addition, a one-tailed F-test was applied to determine whether one method was more precise than the other[167]. In all calculations the confidence limit was 95%.

9.2.6 SELECTION OF A SUITABLE WATER SUPPLY

One of the most important criteria in gradient HPLC is a clean baseline over the entire range of eluent composition to be used. As a part of the set up procedure for gradient system 2, a series of baselines were recorded using different water samples, to determine which would be most suitable for the study. In all these studies, only one source of HPLC grade acetonitrile was used, as a recent report had indicated that variations in chromatographic results could be caused by using different batches / brands of acetonitrile[168].

Initial blank gradient runs, using in-house deionised and scrubbed water, gave poor results with many peaks in the baseline (see Figure 9.2). One source of contamination was found to be the tubing used for the helium supply for eluent degassing. Lengths of blue plastic tubing had been used for the helium line, and evaporation of acetonitrile into the tubing (when the helium supply was off) caused leaching of the blue dye, which was then blown back into the acetonitrile reservoir when the helium was used. It was thought that this dye then accumulated on the column and eluted during the gradient run, giving a number of peaks. By replacing all the blue tubing with clear Teflon tubing many of the peaks were eliminated, apparently confirming that the dye was the source of much of the contamination.

However, peaks still remained in the gradient baseline (see Figure 9.3), even after careful cleaning of both solvent reservoirs. These were traced to organic impurities in the water supply, which were found to be present as a result of operating problems with the scrubber units in the water purification system[169].

Therefore, a number of alternative water supplies were tested, and all gave better results than the original deionised water used for eluent A. Fisons HPLC grade water produced an acceptable baseline (Figure 9.4), but Rathburn HPLC grade water gave even better results (Figure 9.4), with only a few small peaks in the baseline. It was decided that, in order to keep the blank gradient baseline as clean as possible Rathburn HPLC grade water would be used throughout the study for the preparation of eluent A.

Figure 9.2: A typical poor gradient baseline recorded in the early stages of the method development.

Gradient conditions: 0 - 20 minutes 2 - 98%B (linear gradient), then 98%B constant.
Water: in-house deionised and scrubbed. System contaminated with dye components from plastic tubing used in helium degassing lines.

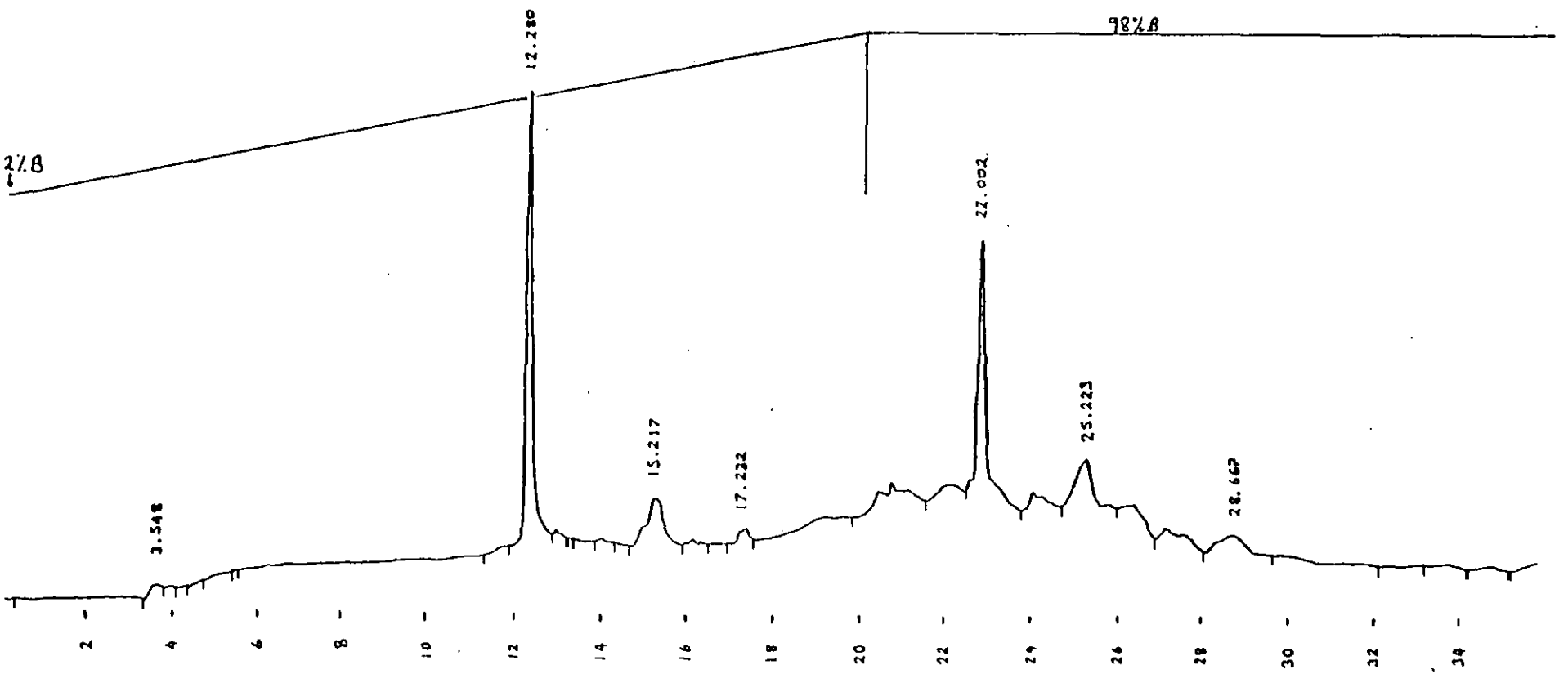


Figure 9.3: A typical gradient baseline using a clean system and in-house de-ionised water for eluent A.

Gradient conditions: 0 - 3 minutes 98%B, 3 - 12 minutes 2%B, 12 - 32 minutes 2 - 98%B (linear gradient), 32 - 40 minutes 98%B. Water: in-house deionised and scrubbed.

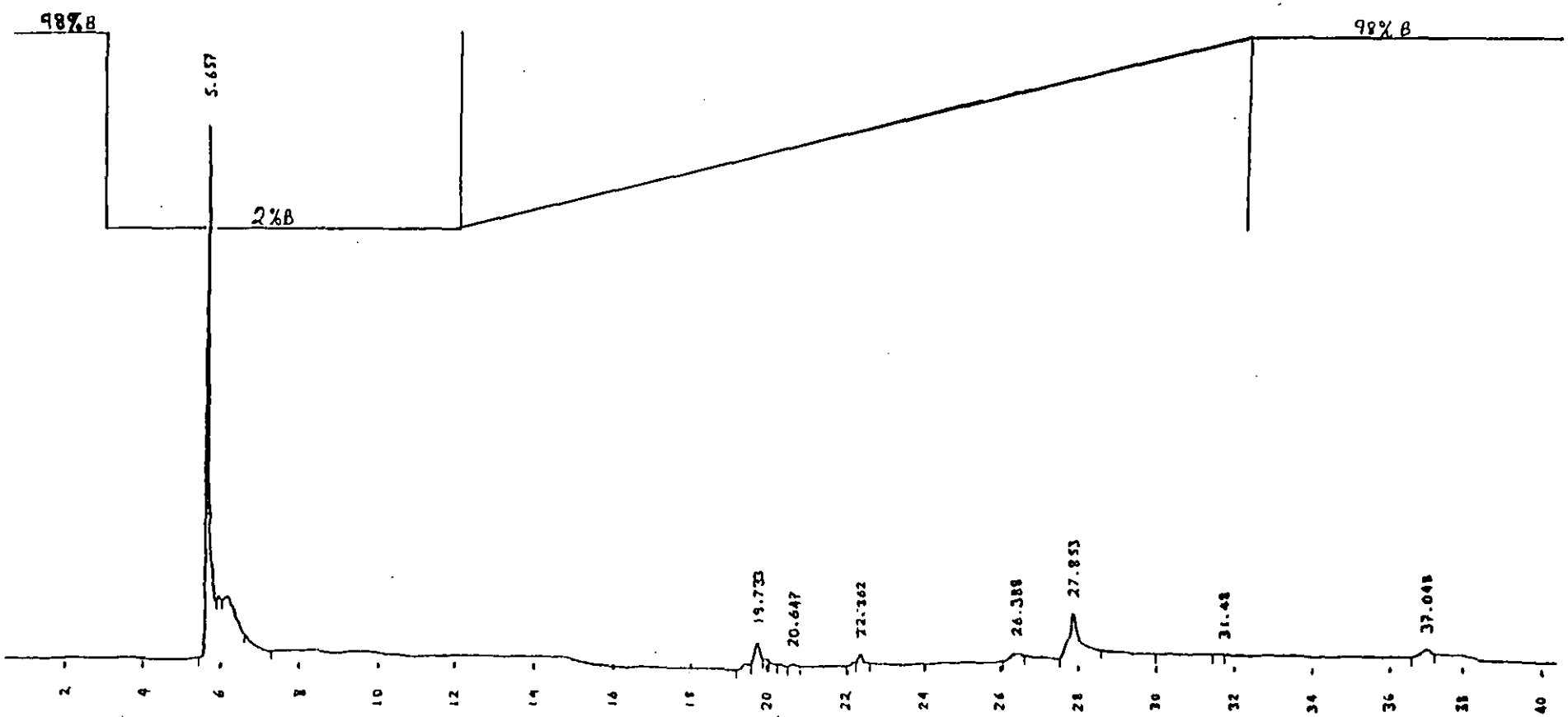
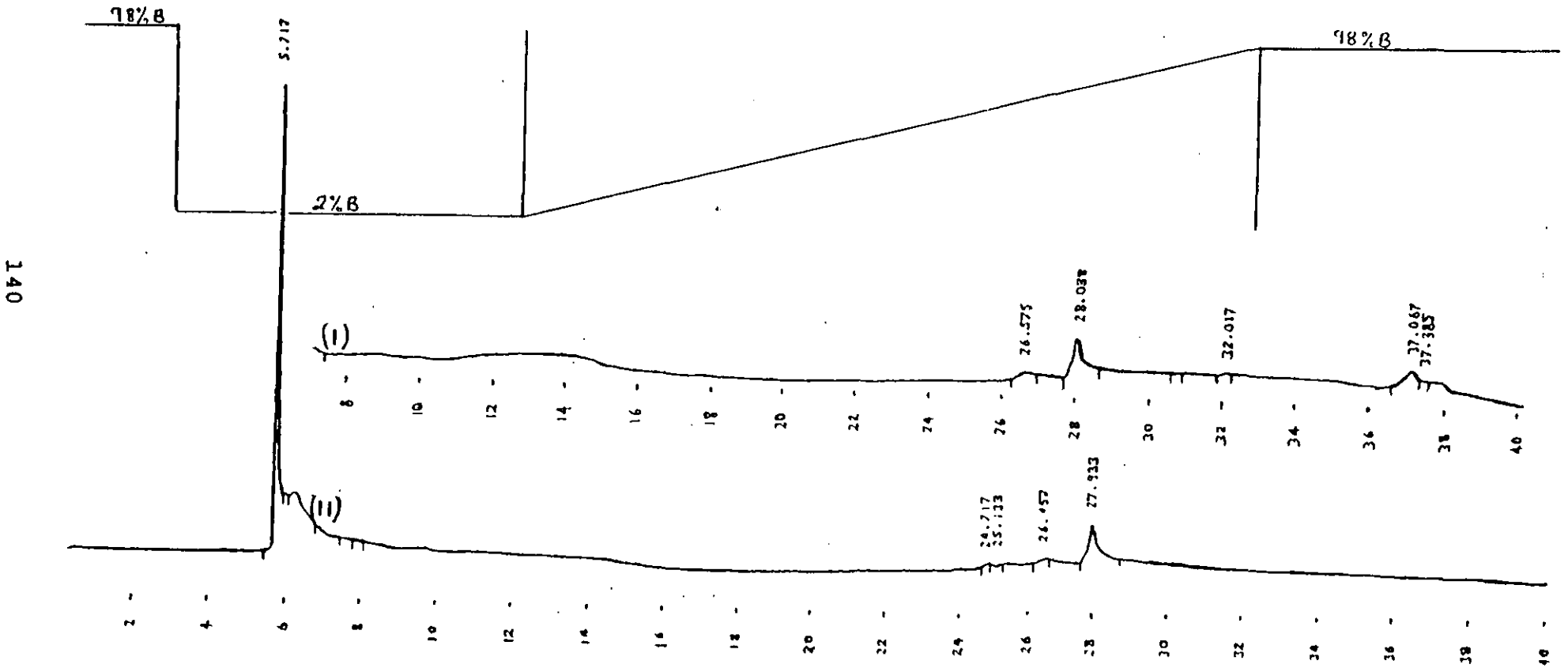


Figure 9.4: A typical gradient baseline using a clean system and Rathburn HPLC grade water for eluent A.

Gradient conditions as Figure 9.3. Water samples: (I), Fisons HPLC grade; (II), Rathburn HPLC grade. The peaks at 28.038 minutes (sample I), and 27.933 minutes (sample II), were caused by impurities in the acetonitrile.



CHAPTER 10: GRADIENT STUDIES

10.1 STUDIES USING GRADIENT SYSTEM 1

The work presented below using this gradient system was carried out at CRSE as part of a development project examining the use of gradient HPLC systems for simultaneous screening of a wide range of drugs. The aim of the project was to design a single chromatographic system that could be equally well applied to acidic, neutral and basic drugs along with quaternary ammonium compounds. This was to overcome the major drawback of ion-exchange separations and other common HPLC methods used for drug analysis, namely their limited applicability to a wide range of compounds. The current work was carried out on an Inertsil ODS-2 column, as this had been identified as the best 'base-deactivated' stationary phase available at the time[170].

10.1.1 TRIFLUOROACETIC ACID ELUENTS

Trifluoroacetic acid (TFA) was selected for use in the initial development of the gradient system because:

i) it was found to be necessary to maintain an acidic environment in the column to suppress activity from residual silanols[170],
ii) it is a liquid acid, (solid buffer components were not considered desirable because of the possibility of precipitation of the buffer salts, particularly in the pump heads, causing unnecessary wear and tear of the pump seals)[170], and
iii) it had been identified as a good acid modifier for working at low detection wavelengths (215 nm)[171].

10.1.1.1 Isocratic studies

A large selection of compounds were chromatographed at different mobile phase compositions to identify the conditions giving a capacity factor in the range 1 to 2 for each analyte. This was achieved by using the solvent programmer to select different percentages of eluents A and B (0.1% v/v TFA in water and acetonitrile respectively) to make up each

mobile phase. The aim of the study was to determine the relative elution order of the analytes in order to predict their likely elution positions in a gradient run. From this data it would then be possible to prepare gradient test solutions with compounds reasonably well spaced. The results are presented in Table 10.1, in order of increasing mobile phase strength necessary to achieve a satisfactory analysis, where the solvent front from each run was taken as a measure of the void volume.

From the data collected, two solutions were prepared for gradient tests. An aqueous solution, designed to test the polar region of the gradient, contained morphine-3β-glucuronide, chlorothiazide, amphetamine sulphate, cocaine hydrochloride, diazepam, triazolam and cyclopenthiazide, whilst an acetonitrile solution, designed to test the non-polar region of the gradient, contained cannabinol only.

10.1.1.2 Gradient studies

a) Blank gradients

A number of gradient baselines were recorded using water / acetonitrile eluents which did not contain TFA. The aim was to determine the shape of the gradient baseline and to identify any peaks caused by impurities in the solvents. In most cases the gradient shape was linear from 2%B to 98%B in 20 minutes. Different delay times and intervals between gradients were used to determine the source of the contamination peaks.

It was found that there was a very small increase in the baseline during each gradient run, with a major impurity peak eluting at 16.1 - 16.2 minutes after starting the gradient (Figure 10.1). The size of the peak was found to be independent of the equilibration time at 2%B prior to the start of the gradient (Figure 10.1), suggesting that the impurity was contained in the acetonitrile rather than the water (the peak size would have increased after a longer equilibration time if the impurity had been present in the water). The retention time of the peak was found to be dependent on the gradient profile, indicating that the impurity peak was caused by one or more hydrophobic compounds which were eluted only when the acetonitrile concentration reached a certain level.

TABLE 10.1: COMPARISON OF %B FOR ISOCRATIC SEPARATIONS WITH TFA MOBILE PHASES

%B	Analyte	k'	%B	Analyte	k'
0	Paraquat[a]	-	40	Benzoic acid	0.91
5	Morphine-3B-glucuronide	1.29	40	Stanozolol	1.02
10	Paracetamol	1.31	40	Diazepam	1.37
12	Pheniramine maleate[b]	1.43	42	Phenytoin	1.37
15	Chlorothiazide	1.32	43	Promazine HCl	0.82
15	Ephedrine	1.58	44	Cyclizine HCl[d]	0.33
16	Hordenine hemisulphate	1.49	44	Dipipanone HCl[d]	2.56
17	Quinine	1.66	45	Methaqualone HCl	0.99
18	Bretylium tosylate[c]	1.52	45	Dextropropoxyphene HCl	1.19
20	Amphetamine sulphate	1.17	45	Amitriptyline HCl	1.82
20	Strychnine	1.19	47	Triazolam	1.06
20	Pseudoephedrine	1.55	47	Benorylate	1.40
21	Atropine	1.10	48	Chlorpromazine HCl	1.02
22	Triprolidine HCl	0.89	48	Dimethisoquin HCl	1.27
22	Barbitone	1.50	50	Methadone	1.27
23	Methylamphetamine HCl	1.17	50	Dipipanone HCl	1.33
23	Tubocurarine chloride	1.73	50	Mandralone	1.62
30	Phensuximide	1.85	50	Benzilic acid	1.90
30	Dibenzepin HCl	2.00	50	Iprindole HCl	2.00
31	Cocaine HCl	0.71	55	Cyclopenthiazide	1.06
34	Sulphamethoxazole	1.05	90	Δ8-THC-acid[e]	0.44
34	Hydrocortisone	1.51	90	Digitoxin	0.98
35	Lysergide (LSD)	0.56	95	Cannabinol	1.00
35	Propranolol HCl	1.06			

[a]Paraquat was unretained (eluted ahead of solvent front).

[b]Pheniramine maleate gave 2 peaks. The maleate ion eluted at the solvent front and was identified by comparison with maleic acid: the result in the Table is for the pheniramine ion.

[c]Bretylium tosylate gave 2 peaks. The tosylate ion had a k' of 0.38 and was identified by comparison with p-toluenesulphonic acid: the result in the Table is for the bretylium ion.

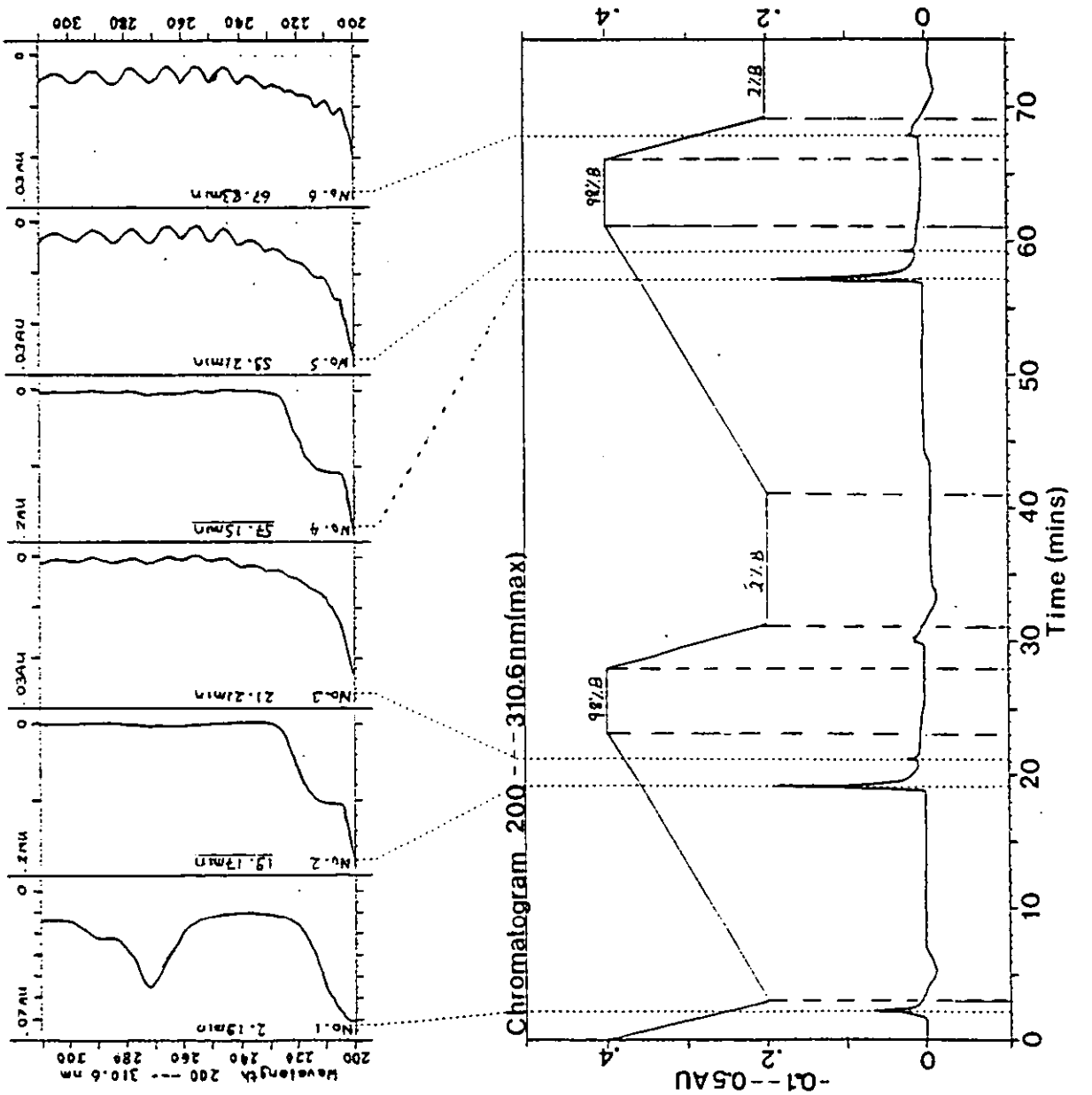
[d]Cyclizine hydrochloride and dipipanone hydrochloride (components of the tablet "Diconal") were separated in a single analysis. (The conditions were not optimised separately for cyclizine HCl).

[e]Δ8-THC-acid = 11-nor-9-carboxy-Δ8-tetrahydrocannabinolic acid.

To determine whether the 'gradient peak' interfered with the chromatography, the two test solutions were analysed using the linear gradient described above, with no TFA present in the mobile phase. The results revealed that the gradient peak (16.2 minutes) eluted between cyclopenthiazide (13.5 minutes) and cannabinol (19.8 minutes), in a region where none of the other test compounds had eluted (Table 10.1). With these results in mind, it was decided to determine the effect of TFA on the gradient baselines.

Figure 10.1: A typical blank gradient baseline using water / acetonitrile eluents

Conditions: Eluent A = water, eluent B = acetonitrile. The chromatogram is a spectrum index maximum plot (i.e. plot of maximum absorbance at any wavelength (200 - 310.6 nm) versus time).



b) TFA gradients

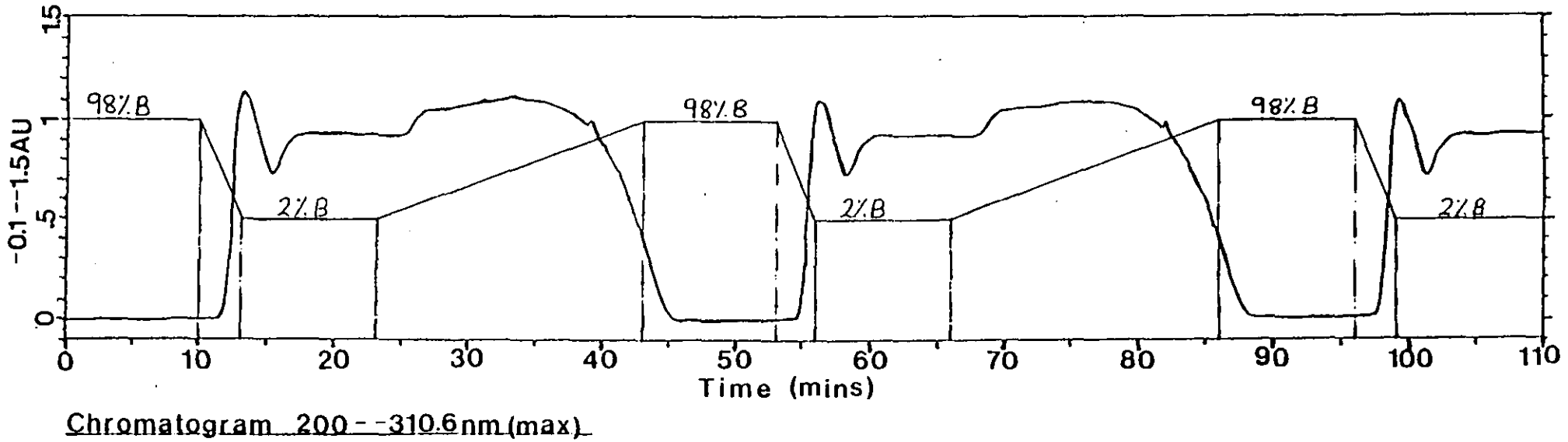
Severe problems were encountered when TFA was added to the eluents. The generally good baselines recorded earlier could not be reproduced with TFA present. Very large changes in background absorbance were recorded on the spectrum index maximum plots (Figure 10.2), making it impossible to analyse the test compounds.

A detailed examination of the results revealed that the TFA eluent had a strong UV absorbance in the wavelength range 225 - 235 nm (see below and Figure 10.3) and that this resulted from the presence of TFA. Any variation in the concentration of TFA between the two eluents would result in a TFA gradient during the change from 2%B to 98%B. This would alter the UV properties of the mobile phase during a gradient run, which could, in part, account for the poor baselines observed. Indeed, absorbance mismatches of this nature have recently been blamed for large baseline shifts when only one eluent of a gradient system contains TFA[172].

A second problem was observed which suggested that TFA was being adsorbed onto the ODS-silica surface. As noted earlier, in the absence of TFA, observation of the gradient baseline had led to the conclusion that impurities in the acetonitrile accumulated on the column during the early part of the gradient and were eluted later in the run. The 'gradient peak' was followed by a stable baseline, with little overall change in background absorbance between initial and final conditions. When TFA was added to the eluents, the 'gradient peak' was followed by a large negative baseline shift (see Figure 10.2) which was attributed to the loss of TFA from the mobile phase after the elution of the acetonitrile impurities. This can be explained by assuming that the impurities in the acetonitrile occupied some of the adsorption sites on the stationary phase surface, and once they (the impurities) had been eluted, TFA was adsorbed onto these sites. This would lower the concentration of TFA reaching the detector, which would result in a reduction in UV absorbance, producing a negative baseline shift. The large difference in peak size between the 'gradient peak' and the negative shift might have been caused by differences in UV properties between the impurities and the TFA.

Figure 10.2: A typical gradient baseline using water / acetonitrile / TFA eluents.

Conditions: Eluent A = water + 1 ml l^{-1} TFA, eluent B = acetonitrile + 1 ml l^{-1} TFA. The chromatogram is a spectrum index maximum plot (i.e. plot of maximum absorbance at any wavelength (200 - 310.6 nm) versus time).



In an attempt to understand the reason for the large baseline shifts during TFA gradient runs, an experiment was carried out to determine the absorbance of isocratic eluent mixtures in the range from (98%A : 2%B) to (2%A : 98%B). This was achieved by controlling the pumps to mix the eluents in a series of steps. It was hoped that the results would indicate whether the observed baseline disturbances were caused by absolute changes of absorbance with eluent composition, or whether dynamic phenomena also contributed, possibly through refractive index effects at the detector flow cell, or as a result of changes in the concentration of TFA in the eluent caused by variations in the amount of TFA adsorbed by the ODS-surface of the stationary phase.

The results revealed that the changes in apparent absorbance were probably caused by a combination of the factors suggested above. Significant absolute changes in absorbance were recorded over the whole composition range (Figure 10.3), with particularly large shifts at 225 and 235 nm. Dynamic positive shifts in absorbance were observed between eluent compositions for all steps up to 50%B (Figure 10.4). The size of the shift decreased as the concentration of 'B' increased. At the 60%B elution step, the profile was further disturbed by the elution of impurities corresponding to the gradient peak. Above 70%B, negative dynamic shifts were recorded, and (at 215 nm) the absolute baseline absorbance decreased (Figure 10.4). These observations were largely in agreement with those of Winkler[171], who showed that the optimum monitoring wavelength for acetonitrile / water / TFA eluents was 215 nm, which had been determined to be the isosbestic point, (i.e. the wavelength at which the absorbance does not change with composition), of this ternary mixture[171]. It was also found that at higher wavelengths there was a large increase in the background absorbance as the amount of acetonitrile increased (as shown in Figure 10.3), which was why monitoring at a lower wavelength was recommended[171].

In an attempt to identify the cause of the dynamic changes in absorbance between stepped eluent compositions, similar experiments were carried out in the absence of TFA. Under these conditions no significant dynamic changes were observed, and the overall shift in baseline was less than 0.04 AU. Thus it was concluded that the dynamic phenomenon observed earlier (and also the absolute changes in absorbance), (Figure 10.4) must have been caused by the presence of TFA in the eluents. However, whilst

the absolute changes in absorbance could be accounted for[171] it was unclear why the addition of TFA to the eluents caused dynamic changes in absorbance to occur. Since dynamic shifts were not seen with eluents that did not contain TFA, it would appear that refractive index effects at the detector cell can be ruled out. (If these had been responsible, similar dynamic changes should have occurred in the absence of TFA, unless TFA contributed significantly to the RI properties of the eluents). One possible explanation is that the effects were related to changes in the concentration of TFA in the eluent caused by adsorption / desorption of the TFA on the hydrophobic surface of the stationary phase, as discussed above. However, there was insufficient data to conclusively demonstrate this to be the cause.

Thus it appears that the poor gradient baselines recorded with TFA eluents (especially when using the spectrum index maximum plot), can be attributed to a significant change in absorbance between the initial and final conditions at certain wavelengths. The (unidentified) phenomenon causing the large dynamic changes in absorbance between stepped compositions (Figure 10.4) would probably also occur during a gradient run, thus accentuating the positive shift in baseline in the early stages of the gradient, and the negative shift seen after the elution of the 'gradient peak'.

Figure 10.3: Graph showing changes in absorbance with eluent composition for water / acetonitrile / TFA eluents.

Conditions: Eluent A = water + 1 ml l^{-1} TFA, eluent B = acetonitrile + 1 ml l^{-1} TFA. (N.B. No result was recorded at 60%B / 215 nm due to baseline instability: this problem was not seen at higher wavelengths).

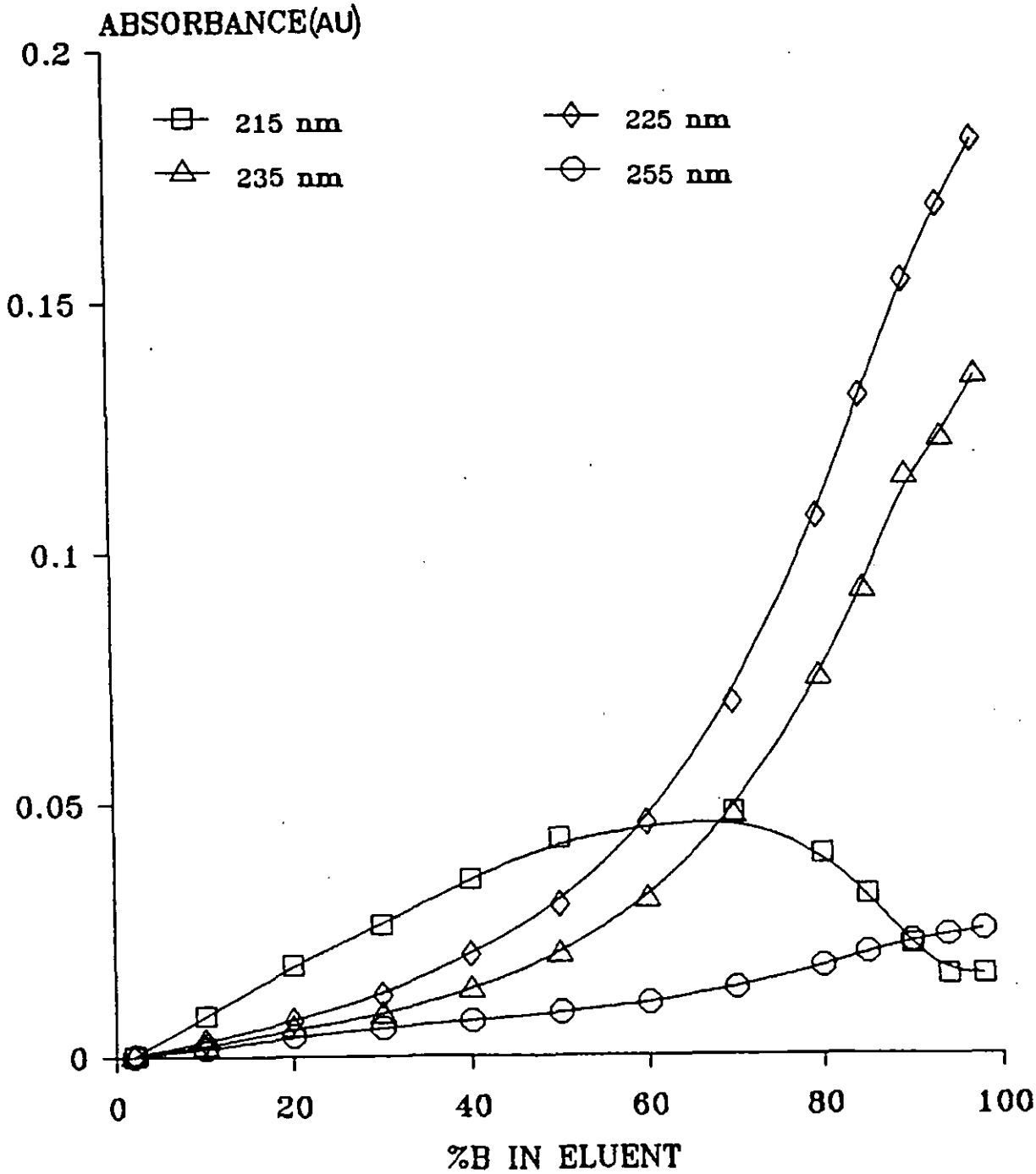
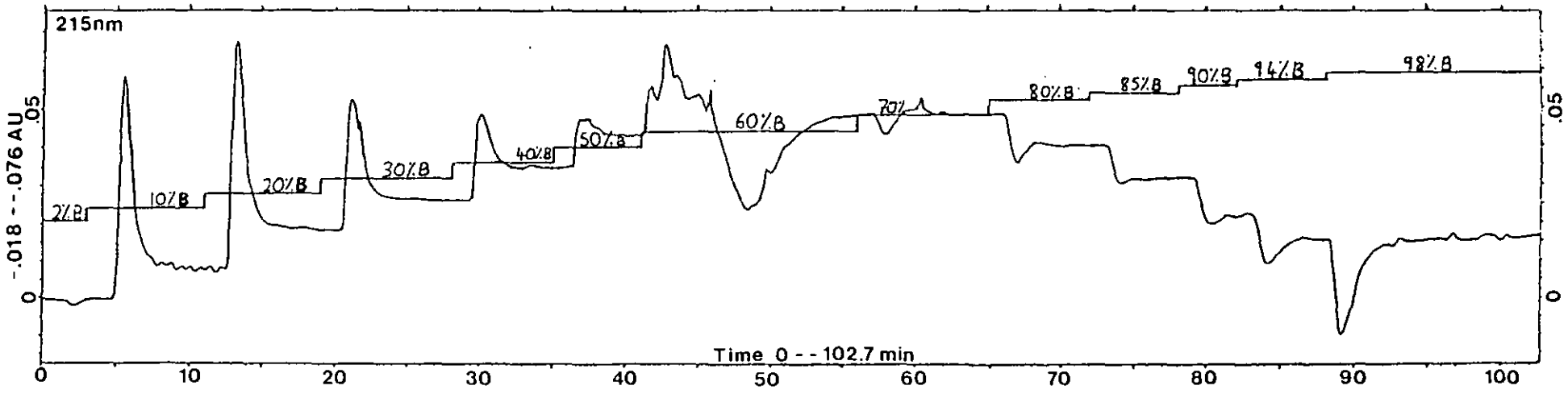


Figure 10.4: Chromatogram showing absolute shifts in absorbance with eluent composition and dynamic shifts in absorbance between different compositions.

Conditions: Eluent A = water + 1 ml l^{-1} TFA, eluent B = acetonitrile + 1 ml l^{-1} TFA. Monitor at 215 nm. Eluent compositions as shown.



10.1.2 SELECTION OF AN ALTERNATIVE ACID

As a result of these observations it was decided to replace TFA with a different acid. The alternative would have to exhibit little or no UV activity at low wavelengths (< 220 nm) and be unlikely to adsorb on the ODS-silica surface, but be able to produce eluents of low pH (*ca.* 2 - 3 in the aqueous fraction was considered desirable).

The most suitable candidate, fulfilling all these requirements, seemed to be sulphuric acid. The preparation of aqueous eluents containing small quantities of sulphuric acid indicated that 1 ml l^{-1} of a 2.5 mol l^{-1} volumetric solution of the acid would be appropriate. The resulting solution had a pH of 2.44, was UV transparent over the whole of the wavelength range of interest and was easy to prepare. Thus the new eluent system was prepared by mixing 1 ml l^{-1} of the sulphuric acid solution with both water and acetonitrile.

10.1.3 SULPHURIC ACID ELUENTS

10.1.3.1 Isocratic studies

A few compounds from the wide range studied earlier were selected for analysis under isocratic conditions. These separations were performed to evaluate the new mobile phase, and to compare its performance with the TFA system. Results are presented in Table 10.2 for the analysis of 14 acidic, neutral and basic compounds.

It was found that the acidic and neutral analytes were largely unaffected by the change to sulphuric acid, but that the basic analytes all required less acetonitrile in the mobile phase with the new system. The most likely reason for this is that sulphuric acid (*i.e.* the HSO_4^- ion) is a weaker ion-pairing reagent than trifluoroacetic acid (*i.e.* the CF_3-COO^- ion). Using the TFA eluent, the basic analytes would form ion-pairs with the counter-ions in the mobile phase, and these neutral species would be 'highly' retained by the ODS-silica. The reduction in ion-pair formation caused by changing to sulphuric acid would therefore lead to a much lower retention for the basic analytes at a given %B in the mobile phase. Thus, to increase the retention of these analytes (to suit the

original conditions of k' = 1 to 2), a reduction in the acetonitrile content of the mobile phase would be necessary.

All the compounds analysed were found to have good peak shapes using the new eluent, and it was concluded that this system was suitable for detailed examination under gradient conditions.

TABLE 10.2: COMPARISON OF THE TWO MOBILE PHASES

Compound	Sulphuric acid eluent		Trifluoroacetic acid eluent	
	%B	k'	%B	k'
Paraquat[a]	0	-	0	-
Morphine-3B-glucuronide	1	2.24		
" "	3	0.47	5	1.29
Ephedrine	10	0.81	15	1.58
Paracetamol	10	0.96	10	1.31
Amphetamine sulphate	15	0.53	20	1.17
Strychnine	15	1.00	20	1.19
Bretylium tosylate[b]	18	1.09	18	1.52
Amitriptyline hydrochloride	35	1.79	45	1.82
Cyclizine hydrochloride[c]	35	0.46	44	0.33
Dipipanone hydrochloride[c]	35	3.91	44	2.56
Dipipanone hydrochloride	40	1.80	50	1.33
Methadone hydrochloride	40	0.95	50	1.27
Benzoic acid	40	0.94	40	0.91
Phenytoin	42	1.50	42	1.37
Cannabinol	95	0.78	95	1.00

[a]Paraquat was unretained, (retention time 0.91 minutes; solvent front 1.50 minutes).

[b]Bretylium tosylate gave 2 peaks: the tosylate ion had a capacity factor of 0.30 at 18%B; the result presented in the Table is for the bretylium ion.

[c]Cyclizine HCl and dipipanone HCl, (components of the tablet "Diconal"), separated in a single assay.

10.1.3.2 Gradient studies

The gradient baselines recorded using sulphuric acid eluents were found to be very clean. The 'gradient peak' eluted about 16.0 minutes after the start of the gradient with a maximum absorbance of *ca.* 0.13 - 0.14 AU at 215 nm and the baseline difference between initial and final conditions was typically about 0.1 AU (Figure 10.5).

a) Method protocol

A method protocol was prepared for the analysis of a range of test compounds under gradient conditions (see 9.1.3.2 above). Using this protocol the gradient baselines were found to be highly reproducible, (*e.g.* the gradient peak retention time recorded over 27 runs was 16.02 ± 0.03(4) minutes, %CV = 0.21), indicating that it would be valid to compare data from different gradient runs, and it was concluded that retention times could be used for comparison of results. (Capacity factors were not used because it was considered unadvisable to use paraquat (a potent human toxin) as the void volume marker in each gradient run. If the solvent front in each run had been used then paraquat would have had a negative capacity factor! (see Table 10.2, note (a))).

A number of problems were encountered in the choice of injection solvents for the analytes examined. Polar compounds with very low retention times gave poor, and in some cases totally split peaks, in solvents containing a significant percentage of acetonitrile (> 20%, see Figure 10.5 and Table 10.4). Thus it was felt necessary to specify closely the choice of solvent for all analytes and this was achieved using 'predicted retention times' for the compounds (based on the TFA eluent). All compounds with short retention times (say, less than *ca.* 8 minutes) were dissolved in either HPLC grade water or eluent A. The remaining compounds were dissolved in either eluent A or B, or a mixture of both, depending on the polarity of the compound. In all cases, the minimum quantity of eluent B necessary to achieve complete dissolution of the sample was used. This was to avoid any possibility of poor peak shapes due to the solvent strength exceeding the eluent strength at the point of injection.

Figure 10.5: Typical blank gradient baseline, recorded at 215 nm.

Conditions: Column, Inertsil ODS2 (5 µm: 15 cm x 4.6 mm i.d.); Eluent A = water + 1 ml l^{-1} sulphuric acid (2.5 mol l^{-1}); eluent B = acetonitrile + 1 ml l^{-1} sulphuric acid (2.5 mol l^{-1}); total flow rate = 1.5 ml min^{-1}; UV detection at 215 nm. Standard gradient programme (see 9.1.3.2).

Sample = 5 µl injection of water. Baseline rescaled to the same vertical axis as Figure 10.7 (see below), to allow direct comparison.

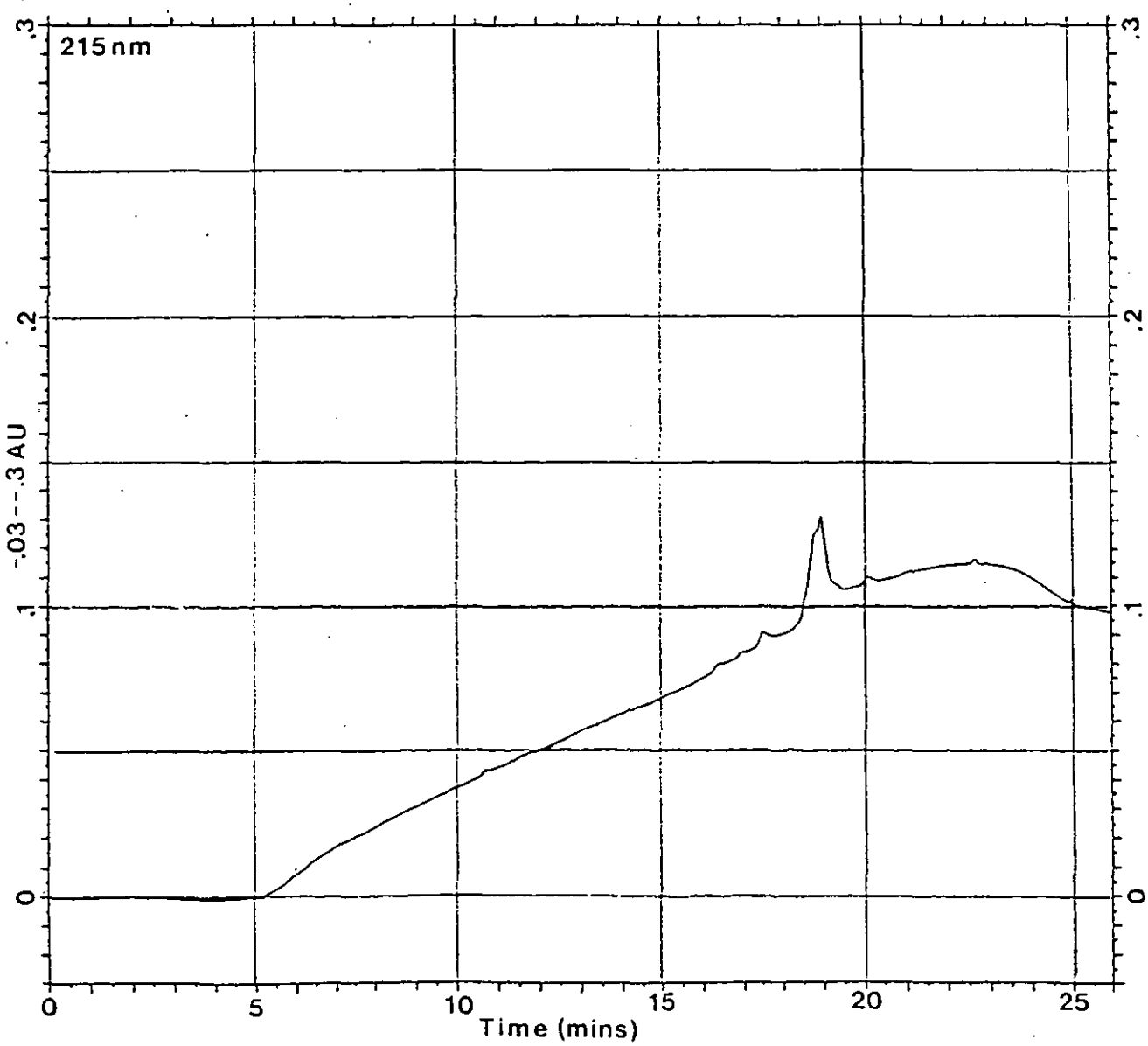
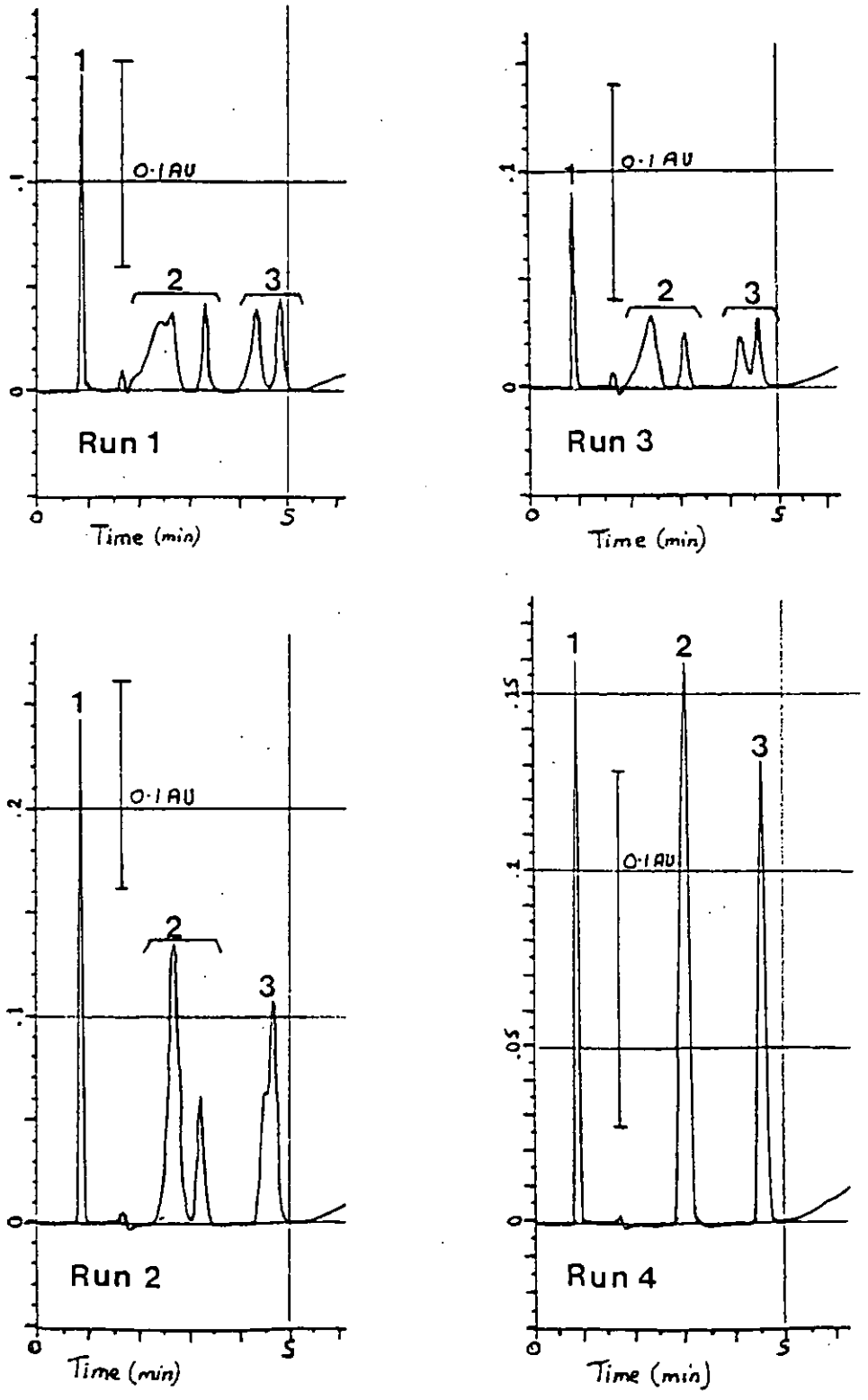


Figure 10.6: Examples of poor peak shapes for morphine-3β-glucuronide and morphine hydrochloride caused by different solvent strengths at the point of injection.

Conditions as Figure 10.5. Compounds: (1), paraquat; (2), morphine-3β-glucuronide; (3), morphine hydrochloride. The rising baseline at 5 minutes corresponds to the beginning of the gradient (see Figure 10.7). Run numbers correspond to details presented in Table 10.4.



b) Analysis of the test compounds

A large number of drug compounds were analysed using the sulphuric acid gradient system. The samples were chosen to represent a wide cross section of polarities and drug classes. The results are shown in Table 10.3.

TABLE 10.3: COMPARISON OF RETENTION TIMES FOR GRADIENT SEPARATIONS USING THE SULPHURIC ACID MOBILE PHASE

Compound	RT (mins)	Compound	RT (mins)
Paraquat[a]	0.89	Propranolol hydrochloride	11.40
Maleate ion	2.83	Sulphamethoxazole	11.48
Hordenine hemisulphate	3.39	Cyclizine hydrochloride	11.60
Morphine-3ß-glucuronide	3.63	Benzoic acid	11.65
Morphine hydrochloride	4.66	Phenobarbitone	12.06
p-hydroxyamphetamine	5.24	Promazine hydrochloride	12.44
Ephedrine hydrochloride	6.93	Phensuximide	12.51
Pseudoephedrine	7.11	Hydrocortisone	12.68
Pheniramine ion	7.20	Dextropropoxyphene hydrochloride	12.95
Paracetamol	7.29	Methadone	12.98
Amphetamine sulphate	7.62	Amitriptyline hydrochloride	13.01
Methylamphetamine hydrochloride	8.04	Chlorpromazine	13.39
Quinine	8.09	Dimethisoquin hydrochloride	13.53
Tosylate ion	8.18	Iprindole hydrochloride	13.70
6-O-Monoacetylmorphine	8.24	Stanozolol	13.70
Chlorothiazide	8.28	Phenytoin	13.74
Caffeine	8.71	Dipipanone hydrochloride	13.92
Strychnine	8.72	Methaqualone hydrochloride	14.15
Tubocurarine chloride	8.86	Benzilic acid	14.26
Atropine	8.93	Phenolphthalein	14.31
Bretylium ion	9.21	Triazolam	14.44
Barbitone	9.45	Benorylate	14.49
Triprolidine hydrochloride	9.54	Diazepam	14.70
Diamorphine hydrochloride	9.97	Nandralone	15.11
Cocaine hydrochloride	10.13	Cyclopenthiazide	15.43
Lysergide (LSD)	10.54	Cannabinol	22.79
Dibenzepin hydrochloride	10.60		

[a]Paraquat was unretained.

From these results, two main test solutions, (see 9.1.4.1 and 9.1.4.2 above), were designed for calibration of the gradient system. Four test runs were carried out using different combinations of these solutions in a single injection and the results are given in Table 10.4. The inter-run reproducibility was found to be extremely good. An example of the chromatography of the two solutions is shown in Figure 10.7.

TABLE 10.4: REPRODUCIBILITY OF GRADIENT SEPARATIONS

Compound	Retention time (mins)[a] Run 1	Run 2	Run 3	Run 4	Mean RT	SD	%CV
Paraquat[b]	0.89	0.89	0.88	0.88	0.89	0.01	1.1
Morphine-3β-glucuronide[c]	-	-	-	3.04	3.04	-	-
Morphine hydrochloride[c]	-	-	-	4.58	4.58	-	-
Ephedrine hydrochloride	7.07	7.04	7.05	7.03	7.05	0.02	0.3
Paracetamol	7.42	7.39	7.38	7.36	7.39	0.03	0.4
Amphetamine sulphate	7.72	7.69	7.71	7.68	7.70	0.02	0.3
Tosylate ion	8.21	8.19	8.19	8.19	8.20	0.01	0.1
Tubocurarine chloride	8.92	8.92	8.91	8.89	8.91	0.01	0.1
Bretylium ion	9.30	9.27	9.29	9.30	9.28	0.02	0.2
Diamorphine hydrochloride	10.00	10.00	9.99	9.99	10.00	0.01	0.1
Cocaine hydrochloride	10.20	10.20	10.19	10.20	10.20	0.01	0.1
Dibenzepin hydrochloride	10.68	10.68	10.67	10.67	10.68	0.01	0.1
Propranolol hydrochloride	11.39	11.39	11.38	11.38	11.39	0.01	0.1
Phenobarbitone	12.09	12.08	12.06	12.06	12.07	0.02	0.1
Promazine hydrochloride	12.54	12.54	12.53	12.53	12.54	0.01	0.1
Dextropropoxyphene HCl	13.02	13.02	13.01	13.03	13.02	0.01	0.1
Dimethisoquin hydrochloride	13.64	13.64	13.63	13.64	13.64	0.01	0.1
Methaqualone hydrochloride	14.22	14.22	14.21	14.22	14.22	0.01	0.1
Triazolam	14.47	14.48	14.47	14.47	14.47	0.01	0.1
Diazepam	14.83	14.83	14.84	14.84	14.84	0.01	0.1
Nandralone	15.13	15.13	15.14	15.14	15.14	0.01	0.1
Cyclopenthiazide	15.45	15.43	15.42	15.43	15.43	0.01	0.1
Cannabinol	22.77	22.77	22.76	22.74	22.76	0.01	0.0

[a]Preparation of sample for injection was as follows, (volumes of each test solution used, in order of 'take up' into the syringe):

Run 1: 2 µl organic test solution; 1 µl water / acetonitrile (50:50 v/v); 2 µl aqueous test solution.
Run 2: 2 µl aqueous test solution; 1 µl water; 2 µl organic test solution.
Run 3: 2 µl organic test solution; 1 µl water; 2 µl aqueous test solution.
Run 4: 1 µl aqueous test solution; 3 µl water; 1 µl organic test solution (see Figure 10.7).

Injection solvents: Run 1, 46.4% ACN; runs 2 and 3, 36.4% ACN: run 4, 18.2% ACN.

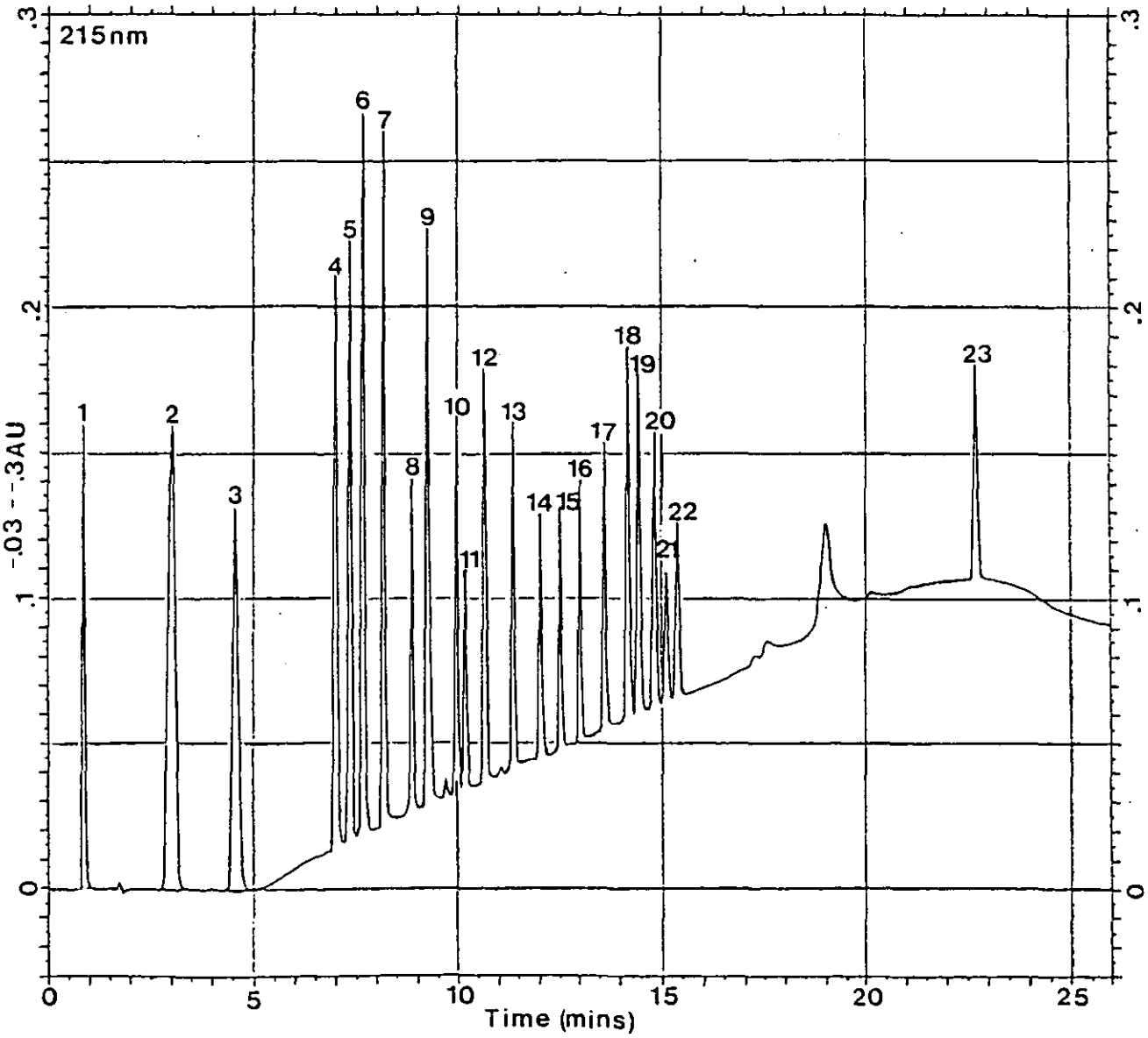
[b]Paraquat was unretained.

[c]In runs 1,2 and 3 no meaningful results were obtained for these two compounds. The peak shapes were "distorted" by the excess acetonitrile present in the sample solution at the time of injection (see Figure 10.6). Acceptable peak shapes were only obtained in the 4th run (see Figure 10.6). (N.B. None of the other peaks were affected by the acetonitrile).

Figure 10.7: Separation of the test compounds selected to calibrate the gradient

Conditions as Figure 10.5. Sample: See note 'a' and run 4 in Table 10.4.

Compounds: (1), Paraquat; (2), morphine-3β-glucuronide; (3), morphine HCl; (4), ephedrine HCl; (5), paracetamol; (6), amphetamine sulphate; (7), tosylate ion; (8), tubocurarine chloride; (9), bretylium ion; (10), diamorphine HCl; (11), cocaine HCl; (12), dibenzepin HCl; (13), propranolol HCl; (14), phenobarbitone; (15), promazine HCl; (16), dextropropoxyphene HCl; (17), dimethisoquin HCl; (18), methaqualone HCl; (19), triazolam; (20), diazepam; (21), nandralone; (22), cyclopenthiazide; (23), cannabinol.



10.1.4 CONTINUATION OF METHOD DEVELOPMENT

These results were considered to be very good, indicating that the new method was suitable for development into a full drug screening system. Since the work reported here was completed, further studies have been carried out by staff at CRSE[170] to produce a detailed HPLC system and a rigorously defined method protocol, along with detailed system performance tests. The new HPLC system and method protocol are outlined in section 9.2 above, and the system performance tests are described in Appendix A.

Following the completion of the method development at CRSE, a 'duplicate' system was set up at Loughborough, taking care to use the same lengths and types of tubing, and the same design of mixing chamber[170]. The following section describes work carried out using this system.

10.2 STUDIES USING GRADIENT SYSTEM 2

10.2.1 'MECHANICAL SET-UP' OF THE EQUIPMENT

The system performance tests, as described in Appendix A, were used to set up the new system. The important results are presented below.

10.2.1.1 Calibration of pumps

Each pump was calibrated separately at a solvent programmer flow rate setting of 1.5 ml min^{-1}, and 100% flow for the pump being tested. The flow rates were determined by measuring the time required for the pump to fill a specially calibrated volumetric flask. The calibration results (for flow through the entire HPLC system) were:

Pump A: flow rate 1.509 ml min^{-1}. Pump B: flow rate 1.506 ml min^{-1}.

10.2.1.2 Calibration of solvent programmer

In an attempt to determine the accuracy of the eluent composition profile, three fractions of eluent were collected at solvent programmer settings of 0%B, 10%B, 25%B, 50%B, 75%B and 100%B and their refractive indices were measured. An equilibration time of at least 10 minutes was

allowed between changing the eluent composition and collecting the eluent fractions. Refractive indices (two measurements per sample) were recorded on a refractometer thermostated at 25°C. The results (means of six measurements at each %B), and reference data, are shown in Table 10.5.

TABLE 10.5: REFRACTIVE INDICES OF ELUENT SAMPLES

%B in eluent	Mean R.I.	S.D.	Reference R.I.[a]
0	1.3330	$1x10^{-4}$	1.3321
10	1.3368	$1x10^{-4}$	1.3363
25	1.3416	$3x10^{-4}$	1.3411
50	1.3454	$1x10^{-4}$	1.3446
75	1.3459	$1x10^{-4}$	1.3449
100	1.3421	$1x10^{-4}$	1.3415

[a]Reference data supplied by CRSE[170].

The results suggested that there was a systematic difference between the two data sets, the results recorded at Loughborough always being greater than the reference data provided by CRSE. It was concluded that, for accurate checks on eluent compositions, it would be necessary for individual laboratories to prepare their own calibration graphs of refractive indices at different eluent compositions; it has since been confirmed that this would be specified in a detailed method protocol[170]. Since the aim of the current test was to determine the usefulness of the method for checking the accuracy of the eluent profile, the shape of the refractive index 'curve' for the eluents measured at LUT was compared with the reference data. The two curves were found to be very similar (Table 10.5) and it was concluded that the eluent profile produced by the solvent programmer was correct, but that the results could not be used to determine individual eluent compositions.

10.2.3 1-NITROALKANE CALIBRATION OF THE GRADIENT

10.2.2.1 The short test solution

Initially, calibrations of the gradient were carried out using the short test solution of C_1-C_6 1-nitroalkanes. These were used to test the

reproducibility of the gradient between runs and between days. A downward drift in retention times was observed, which was found to be due to changes in flow rates for both pumps. All other conditions in the set-up routine (Appendix A) were virtually unchanged. No adjustments were made as the full test solution of C_1-C_{16} 1-nitroalkanes had become available to calibrate the gradient more rigorously.

10.2.2.2 The full test solution

The reproducibility of the gradient system was tested over a one month period using the test solution of 16 1-nitroalkanes. Some of the results are shown in Table 10.6.

TABLE 10.6: GRADIENT CALIBRATIONS USING THE FULL TEST SOLUTION OF 1-NITROALKANES

Date of run	27/3/90	28/3/90	30/3/90	02/4/90	03/4/90	23/4/90	24/4/90	25/4/90	27/4/90
Compound	Retention times (mins)								
Nitromethane	2.013	2.072	2.042	2.048	2.012	2.052	2.047	2.050	2.020
Nitroethane	4.392	4.485	4.435	4.557	4.398	4.438	4.520	4.550	4.438
Nitropropane	10.408	10.502	10.478	10.448	10.460	10.408	10.448	10.425	10.413
Nitrobutane	14.187	14.248	14.225	14.262	14.227	14.172	14.207	14.192	14.180
Nitropentane	16.245	16.285	16.265	16.308	16.275	16.222	16.253	16.240	16.228
Nitrohexane	17.730	17.755	17.740	17.792	17.757	17.703	17.735	17.725	17.705
Nitroheptane	18.983	19.003	18.990	19.048	19.007	18.950	18.985	18.973	18.985
Nitrooctane	20.133	20.145	20.130	20.192	20.148	20.092	20.128	20.115	20.100
Nitrononane	21.212	21.233	21.208	21.275	21.225	21.170	21.207	21.192	21.178
Nitrodecane	22.220	22.230	22.217	22.283	22.233	22.175	22.215	22.200	22.183
Nitroundecane	23.133	23.142	23.128	23.197	23.150	23.088	23.117	23.115	23.098
Nitrododecane	23.963	23.957	23.942	24.017	23.972	23.908	23.927	23.922	23.923
Nitrotridecane	24.758	24.745	24.723	24.803	24.758	24.697	24.707	24.705	24.712
Nitrotetradecane	25.753	25.578	25.555	25.637	25.575	25.522	25.537	25.535	25.535
Nitropentadecane	26.418	26.463	26.433	26.518	26.423	26.398	26.413	26.413	26.408
Nitrohexadecane	27.325	27.397	27.360	27.452	27.328	27.313	27.338	27.342	27.318
Glycine	-	1.083	1.070	1.087	1.072	1.085	1.087	1.085	1.080

The results revealed a high level of reproducibility for most of the 1-nitroalkanes. Nitroethane stood out as the poorest result; this could have been because it gave a slightly fronting peak, quite close to the point when the gradient profile first reached the detector. The quality of the results indicated that the system was now very robust and suggested that long term reproducibility would be good.

10.2.3 PROBLEMS WITH THE DRUG TEST SOLUTIONS

10.2.3.1 The aqueous test solution

Results for three separations of the aqueous test solution are presented in Table 10.7. The data suggested that the reproducibility of retention times for the first three compounds in this test solution was poor. However, the nitro-indices showed much better reproducibility for ephedrine and, to a lesser extent, morphine. The results for morphine-3β-glucuronide were not improved by using the nitro-indices. The problem of irreproducible retention of early eluting compounds was investigated in more detail and results are presented later (see section 10.2.6).

TABLE 10.7: GRADIENT SEPARATIONS OF THE AQUEOUS TEST SOLUTION

Compound	Retention times (mins)			Nitro-indices		
Morphine-3β-glucuronide	3.867	3.528	3.617	170.6	159.9	161.6
Morphine HCl	5.065	4.550	5.013	207.6	200.4	207.1
Ephedrine HCl	7.548	7.460	7.538	249.7	249.4	250.0
Paracetamol	8.247	8.198	8.220	261.6	261.8	261.6
Tosylate ion	8.653	8.627	8.648	268.5	269.1	268.9
Bretylium ion	10.688	10.660	10.608	304.8	305.2	303.3

10.2.3.2 The organic test solution

Results for three separations of the organic test solution are presented in Table 10.8. It was found that a 10 µl injection of this test solution caused severe fronting, and occasionally splitting, of the tubocurarine and diamorphine peaks. This was thought to have been caused by the injection solvent (90% acetonitrile) being too strong for these compounds, when injected into the 98% aqueous starting eluent. However, by lowering the injection volume to 5 µl, the peak shapes were improved, suggesting that column loading may also have been important.

As with the aqueous test solution, nitro-indices were found to be much more reproducible than retention times, and so it was decided to quote all subsequent retention data in this form.

TABLE 10.8: GRADIENT SEPARATIONS OF THE ORGANIC TEST SOLUTION

Compound	Retention times (mins)			Nitro-indices		
Tubocurarine Cl	9.477[a]	9.397[a]	9.437[c]	282.5	282.0	282.4
Diamorphine HCl	10.325[b]	10.307[b]	10.313[a]	296.9	297.3	297.1
Dibenzepin HCl	10.863	10.852	10.860	309.5	310.3	310.0
Propranolol HCl	11.510	11.507	11.508	326.8	327.6	327.3
Phenobarbitone	12.532	12.525	12.515	354.2	354.6	354.2
Dextropropoxyphene HCl	12.890	12.883	12.900	363.8	364.1	364.5
Diazepam	14.780	14.732	14.770	426.5	424.3	426.2
Cannabinol	22.612	22.617	22.625	1042.4	1045.9	1041.3

[a]Peaks show serious fronting / splitting.

[b]Peak shows slight fronting.

[c]Peak split, the front shoulder had a retention time of 9.180 minutes (Nitro-index = 277.9).

10.2.4 COMPARISON OF TWO GRADIENT SYSTEMS

A careful comparison of data obtained on the system described here with data provided by CRSE[170] (recorded on an almost identical system) revealed a change in selectivity between the two Inertsil columns used in the study. The change was specifically for the bretylium ion, which was more highly retained on the column used at Loughborough. The nitro-index for the bretylium ion increased from 291 on the column used at CRSE, to 308 on the column used at Loughborough; all the other nitro-indices were remarkably similar on both columns (Table 10.9). The columns were prepared from different batches of silica, and differences between the batches were probably responsible for these changes in selectivity towards the bretylium ion (a quaternary ammonium ion), which may have been very sensitive to variations in the stationary phase as a result of its permanent positive charge.

The results indicated that the interlaboratory reproducibility was generally very good, and that the use of retention indices, rather than retention times, for comparing the results was to be preferred, as has been noted by Gill *et al.*[102]. However, as observed by Hill and Langner[11], the use of nitro-indices could not eliminate the variations in column selectivity.

A recent report by Snyder and Dolan[173] suggested that one of the

main reasons for irreproducibility of gradient separations when a method is transferred between laboratories is differences in the HPLC equipment used. In the studies presented here, the use of standardised HPLC systems at both CRSE and Loughborough was almost certainly the main reason for the high level of interlaboratory reproducibility for most of the results, (although see 10.2.6 for problems with early eluting compounds).

TABLE 10.9: COMPARISON OF RETENTION CHARACTERISTICS FOR TWO INERTSIL COLUMNS PREPARED FROM DIFFERENT BATCHES OF SILICA

Compound	Loughborough data[a]		CRSE data[b]	
	RT (mins)	Nitro index	RT (mins)	Nitro index
Morphine-3B-glucuronide	Split peak[c]	-	3.25	145.9
Morphine hydrochloride	4.330	197.4	4.68	200.9
Ephedrine hydrochloride	7.447	250.8	7.40	247.8
Paracetamol	8.167	262.8	7.96	257.5
Tosylate ion	8.617	270.2	8.61	268.7
Tubocurarine Chloride	9.560	285.8	9.27	280.1
Diamorphine hydrochloride	10.317	298.5	10.29	297.8
Bretylium ion	10.723[d]	308.3	9.87	290.5
Dibenzepin hydrochloride	10.848[d]	311.6	10.92	313.0
Propranolol hydrochloride	11.495	328.8	11.63	331.5
Phenobarbitone	12.515	355.8	12.43	352.1
Dextropropoxyphene hydrochloride	12.887	365.6	13.19	371.8
Diazepam	14.772	428.4	14.90	429.0
Cannabinol	22.633	1045.2	23.26	1042.6

[a]Data obtained from single injection of both test mixtures. The syringe was filled (in order) with 2 µl organic test mixture, 1 µl water and 2 µl aqueous test solution. Column: Inertsil ODS-2, 5 µm, (15 cm x 4.6 mm i.d.), column number OAI 10066, batch number SQ5 790.

[b]Data provided by CRSE, derived from separate injections of the two test solutions. Column: Inertsil ODS-2, 5 µm, (15 cm x 4.6 mm i.d.), column number 9KI 2392, batch number SQ5 779.

[c]Morphine-3B-glucuronide peak split due to strength of sample solvents compared to the eluent at time of injection. Retention times of the two parts of the peak were 3.018 minutes (nitro-index = 142.2) and 3.187 minutes (nitro-index = 149.4).

[d]These two peaks were unresolved.

10.2.5: EFFECT OF GRADIENT DELAY TIME ON RETENTION

A study was undertaken to determine the effect of changing the gradient delay time on the retention times of the 1-nitroalkanes and the

test compounds. The aim of the study was to determine the importance of the gradient delay time as a part of the overall gradient profile, and to find out whether the selectivity of the separations was affected by changes in the conditions. This would be shown by changes in the nitro-indices of the analytes as the delay time was varied.

10.2.5.1 The 1-nitroalkanes

The effect of changes in the gradient delay time, from 0 to 6 minutes, on the retention of the 1-nitroalkanes is shown in Table 10.10 and Figure 10.8. The data recorded for the 1-nitroalkanes was used to calculate the nitro-indices for the compounds in the two test solutions, according to the delay time for each injection.

TABLE 10.10: EFFECT OF GRADIENT DELAY TIME ON THE RETENTION OF THE 1-NITROALKANES

Delay time (mins)	0	1	2	3	4	5	6
Compound	Retention times (mins)						
Nitromethane	2.077	2.067	2.057	2.050	2.058	2.053	2.052
Nitroethane	4.402	4.480	4.613	4.570	4.567	4.455	4.532
Nitropropane	8.362	9.063	9.832	10.486	11.155	11.743	12.342
Nitrobutane	11.407	12.342	13.325	14.248	15.188	16.120	17.063
Nitropentane	13.322	14.305	15.323	16.294	17.277	18.258	19.243
Nitrohexane	14.777	15.770	16.795	17.780	18.767	19.758	20.750
Nitroheptane	16.022	17.015	18.043	19.031	20.020	21.013	22.007
Nitrooctane	17.165	18.158	19.187	20.176	21.165	22.157	23.152
Nitrononane	18.245	19.240	20.265	21.254	22.243	23.233	24.230
Nitrodecane	19.255	20.252	21.277	22.265	23.248	24.243	25.242
Nitroundecane	20.163	21.158	22.187	23.180	24.163	25.158	26.157
Nitrododecane	20.973	21.965	22.995	23.994	24.980	25.962	26.960
Nitrotridecane	21.765	22.757	23.787	24.780	25.775	26.750	27.757
Nitrotetradecane	22.607	23.600	24.633	25.616	26.622	27.598	28.598
Nitropentadecane	23.497	24.492	25.527	26.500	27.518	28.495	29.488
Nitrohexadecane	24.435	25.432	26.468	27.435	28.462	29.437	30.425

The effect of the gradient delay time on the retention of the analytes can be understood in terms of the following equation:

$$(RT_{td} - RT_0) = \delta t \qquad \text{Equ. 10.1}$$

where RT_{td} is the retention time of the analyte at delay time t_d minutes and RT_0 is the retention time of the analyte at delay time = 0 minutes. If $\delta t = t_d$, then the analyte is eluted only under gradient conditions, (*i.e.* it will never be eluted under the starting conditions). If $\delta t = 0$, then the analyte is eluted under initial isocratic conditions (*i.e.* its retention is not influenced by the gradient). In cases where $0 < \delta t < t_d$, the analyte is eluted partially by the isocratic conditions at the start of the run, and partially by the gradient conditions that follow. The degree of elution under each of the conditions can be determined qualitatively by the value of δt: the closer it is to t_d the less the influence of the isocratic section of the gradient profile.

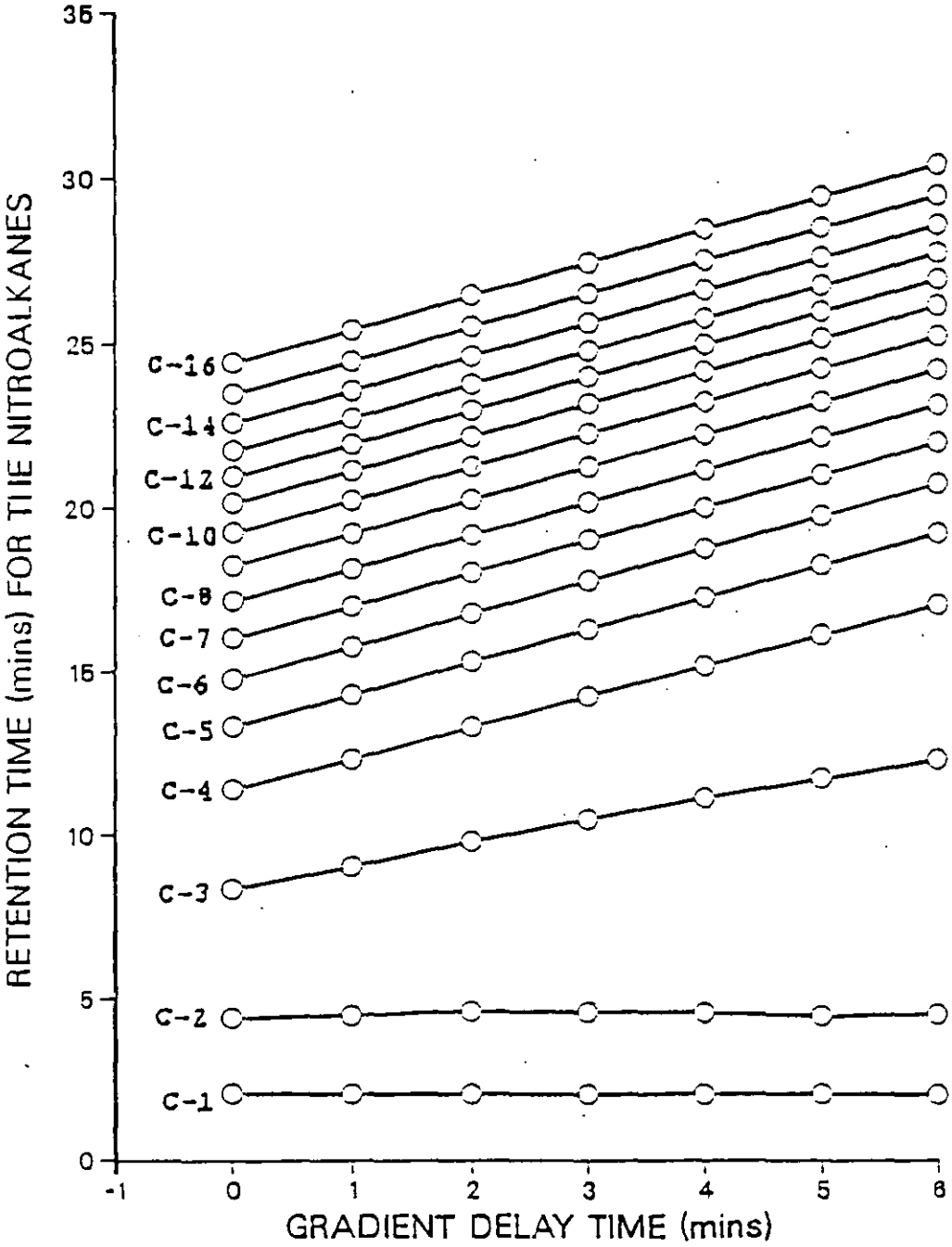
As shown in Figure 10.8, there was a change in the retention behaviour of the 1-nitroalkanes between nitroethane (unaffected by the delay time, $\delta t \approx 0$ minutes) and nitrobutane (whose retention was almost totally dependent on the gradient, $\delta t = 5.65$ minutes, when $t_d = 6$ minutes). The retention of nitropropane was dependent on both the isocratic and gradient sections of the run ($\delta t = 3.98$ minutes, when $t_d = 6$ minutes). This suggests that the smaller 1-nitroalkanes are eluted rapidly due to their small size, whilst the larger compounds (C-*n* > 4) are separated by a hydrophobic mechanism. The intermediate behaviour of nitropropane can thus be explained: as the delay time increases nitropropane is partially eluted in the starting conditions, *i.e.* 2%B, (because of its small size), but the eluting power of the eluent is insufficient to cause complete elution before the gradient conditions reach the column and speed up the elution.

It is important to note that if the initial conditions are changed from 2%B to, say 10%B, the transition point between the two mechanisms would change to, say nitrobutane. This would occur because of the increased 'hydrophobic elution power' of the mobile phase, which would allow elution of the next higher homologue(s) to begin before the gradient reached the column.

The change in behaviour in this region of the gradient was found to have a marked effect on the nitro-indices on the test compounds (see 10.2.5.4 below).

Figure 10.8: Variation of retention time with gradient delay time for the 1-nitroalkanes.

Conditions: eluents as in section 9.2.2; gradient profile as in 9.2.3.3, but variations in t_d as shown.
Compounds: C-*n* = carbon number of 1-nitroalkane.



10.2.5.2 The aqueous test solution

Data is presented in Table 10.11 for runs at delay times from 0 to 6 minutes. Considerable irreproducibility in retention was again seen for morphine-3ß-glucuronide and morphine hydrochloride, and this is clearly shown in Figure 10.9. It was thought that these variations may have been caused by different reequilibration times between the gradients. This factor may have been critical because differing reequilibration times may have caused variations in the eluent composition on the silica surface at the time of injection[174]. This was investigated by repeating the experiment with a fixed reequilibration time of six minutes. The results are summarised in Figure 10.10.

TABLE 10.11: EFFECT OF GRADIENT DELAY TIME ON RETENTION TIMES AND NITRO -INDICES FOR THE AQUEOUS TEST SOLUTION

Gradient delay time (mins)	0	1	2	3	4	5	6
Compound	Retention times (mins)						
Morphine-3ß-glucuronide	3.492	3.243	2.890	3.617	3.028	3.708	3.620
Morphine hydrochloride	4.037	4.350	4.528	5.013	4.275	4.863	4.742
Ephedrine hydrochloride	4.965	5.083	6.710	7.538	8.230	8.950	9.217
Paracetamol	5.492	6.355	7.313	8.220	9.067	9.962	10.822
Tosylate ion	5.747	6.695	7.695	8.648	9.605	10.563	11.558
Bretylium ion	7.815	8.643	9.630	10.608	11.482	12.430	13.385
	Nitro indices						
Morphine-3ß-glucuronide	160.9	148.7	132.6	162.2	138.7	168.9	163.2
Morphine hydrochloride	184.3	194.6	196.7	207.5	188.4	205.6	202.7
Ephedrine hydrochloride	214.2	213.2	240.2	250.2	255.6	261.7	260.0
Paracetamol	227.5	240.9	251.7	261.7	268.3	275.6	280.5
Tosylate ion	234.0	248.3	259.0	268.9	276.5	283.1	290.0
Bretylium ion	286.2	290.8	296.1	303.4	308.1	315.7	322.1

An improvement in the results for morphine-3ß-glucuronide, morphine hydrochloride and ephedrine hydrochloride was observed (Figure 10.10). For the first two compounds the irreproducibility of retention times and nitro-indices was reduced but not eliminated. The problems of irreproducibility with these rapidly eluted compounds were subsequently studied in more detail (see 10.2.6).

Figure 10.9: Variation of retention indices with gradient delay time for the main aqueous test solution (using uncontrolled reequilibration times between gradients).

Conditions as in Figure 10.8. Compounds: (1), morphine-3β-glucuronide; (2), morphine HCl; (3), ephedrine HCl; (4), paracetamol; (5), tosylate ion; (6), bretylium ion.

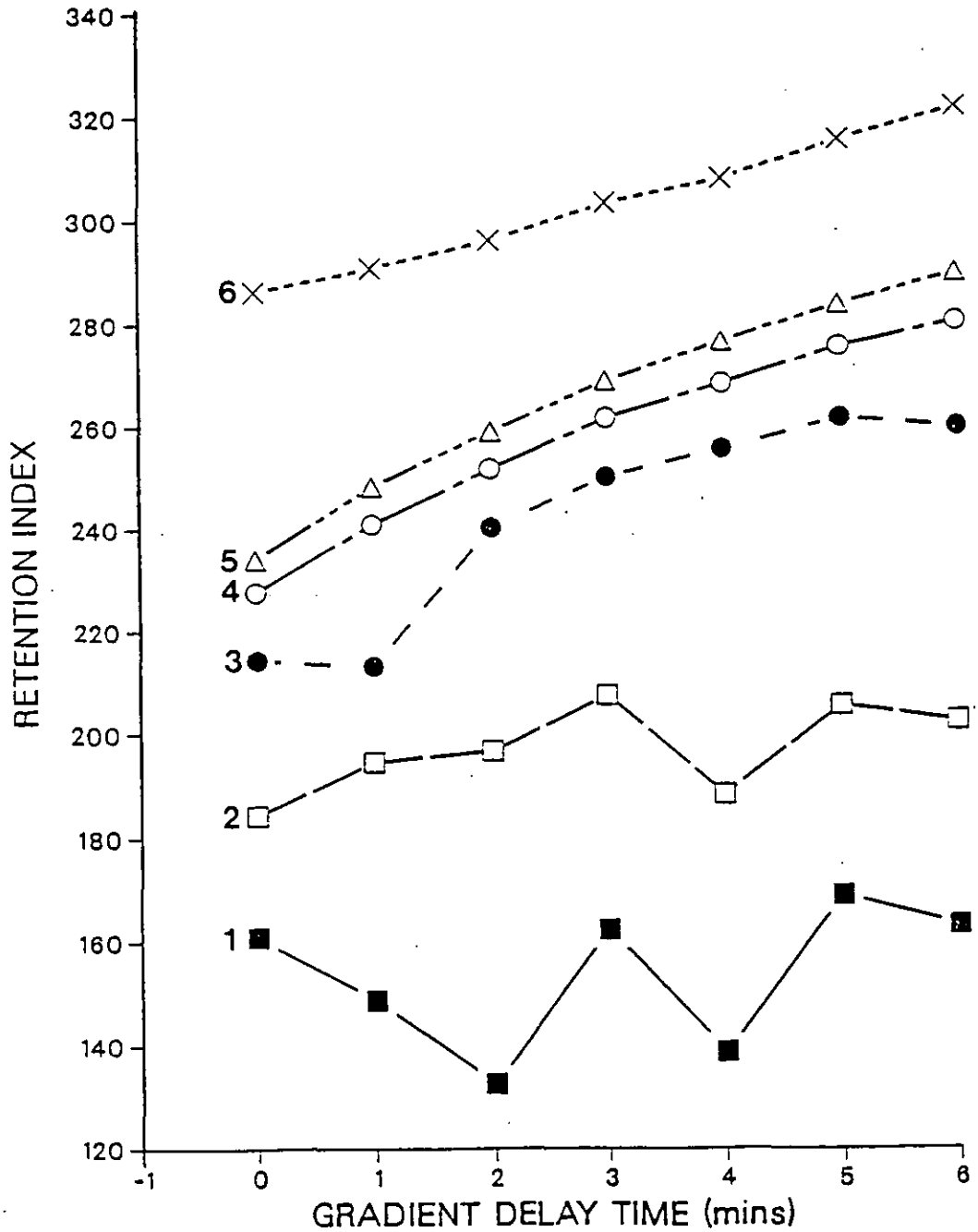
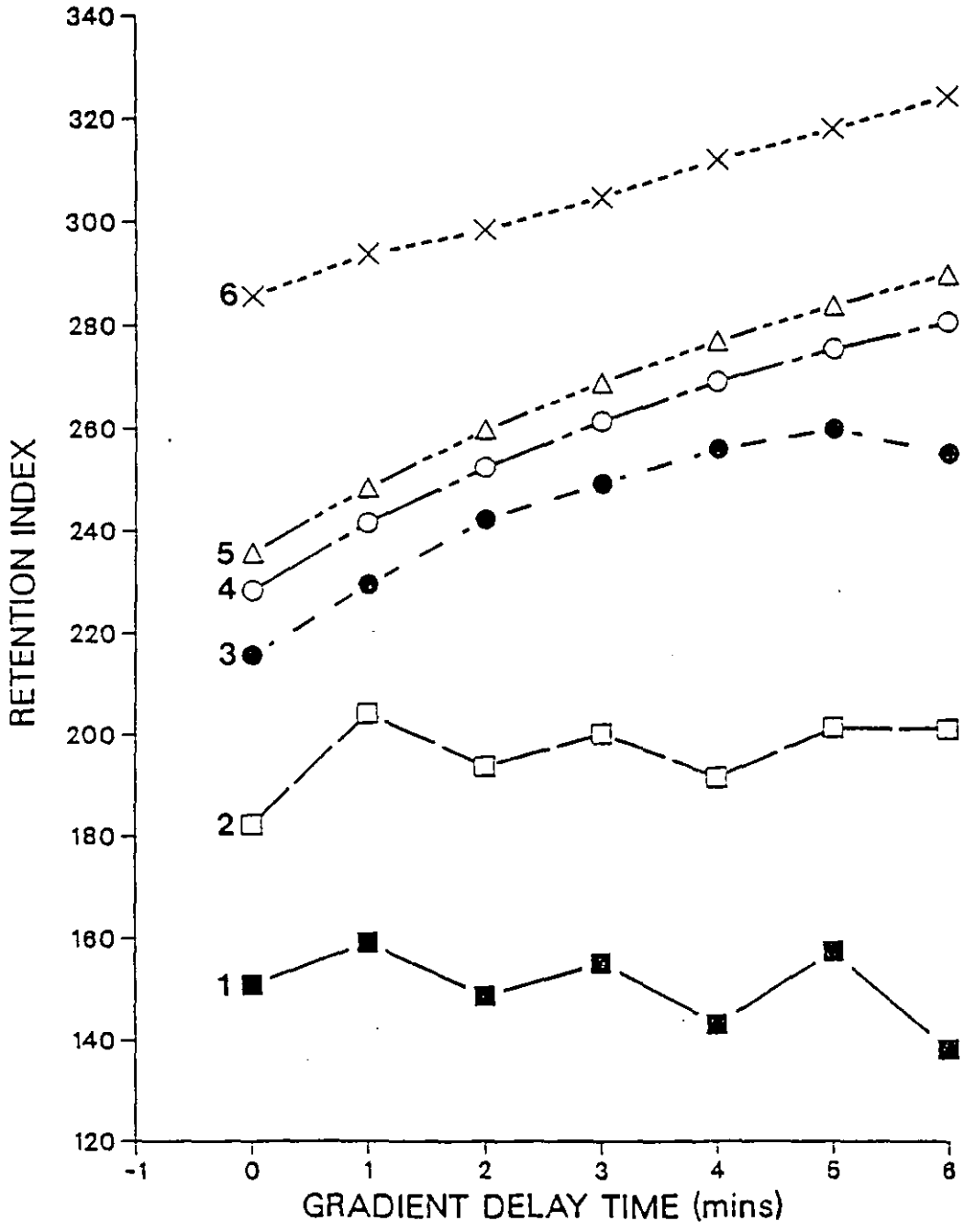


Figure 10.10: Variation of retention indices with gradient delay time for the main aqueous test solution (using controlled reequilibration times of 6 minutes between gradients).

Conditions and compounds as in Figure 10.9.



The results for ephedrine revealed that at delay times ≥4 minutes there was a dramatic reduction in efficiency (N = 21425 at gradient delay time of 4 minutes falling to N = 3935 at 6 minutes). The reduction in efficiency suggested that ephedrine was almost completely eluted by the isocratic section of the run at t_d = 6 minutes (where δt = 3.82 minutes), especially as the onset of the gradient ought to sharpen broad analyte bands on the column, given sufficient time. The two morphine compounds also showed reductions in efficiency at longer delay times, indicating that the more polar analytes were all susceptible to band broadening on this 'inert' column. Paracetamol, on the other hand, showed a large increase in efficiency as the delay time increased (N = 23539 at delay time = 0 minutes to N = 54480 at 6 minutes).

For all the analytes in the aqueous test solution the value of δt (Equ. 10.1) was less than t_d, (*e.g.* for paracetamol, δt = 5.33 minutes when t_d = 6 minutes) indicating that they are all eluted, either totally or in part, by the isocratic section of the gradient profile. (For further discussion in the importance of this result, see 10.2.5.4).

10.2.5.3 The organic test solution

Results are presented for separations of the components in the organic test solution at gradient delay times from 0 to 6 minutes (Table 10.12). Figure 10.11 shows the variation in nitro-index with gradient delay time (cannabinol has been omitted to allow clear presentation of the data). For all the compounds in this test solution, $\delta t \approx t_d$ (Equ. 10.1), indicating that they were only eluted under gradient conditions.

10.2.5.4 Changes in selectivity with delay time

The changes in nitro-indices for the test compounds must be examined with reference to the absolute behaviour of the nitroalkanes themselves. For compounds eluting before nitroethane, (*i.e.* with indices below 200), the results should be independent of the gradient delay time as these compounds were eluted under isocratic conditions. However, considerable irreproducibility was found and the problems encountered for compounds eluting in this part of the gradient are discussed in detail below (see 10.2.6).

TABLE 10.12: EFFECT OF GRADIENT DELAY TIME ON RETENTION TIMES AND NITRO -INDICES FOR THE ORGANIC TEST SOLUTION

Gradient delay time (mins)	0	1	2	3	4	5	6
Compound	Retention times (mins)						
Tubocurarine Cl	6.387	7.423	8.393	9.423	10.402	11.397	12.437
Diamorphine HCl	7.303	8.338	9.310	10.333	11.305	12.308	13.330
Dibenzepin HCl	7.855	8.895	9.858	10.880	11.853	12.863	13.877
Propranolol HCl	8.513	9.545	10.513	11.530	12.512	13.513	14.525
Phenobarbitone	9.507	10.538	11.527	12.522	13.517	14.510	15.513
Dextropropoxyphene HCl	9.903	10.945	11.902	12.922	13.895	14.908	15.923
Diazepam	11.718	12.760	13.748	14.748	15.732	16.757	17.758
Cannabinol	19.598	20.642	21.627	22.620	23.608	24.625	25.627
	Nitro indices						
Tubocurarine Cl	250.1	264.2	272.4	282.0	288.6	295.3	302.0
Diamorphine HCl	273.3	284.2	290.0	297.4	303.7	312.9	320.9
Dibenzepin HCl	287.2	296.3	300.7	310.5	317.3	325.6	332.5
Propranolol HCl	305.0	314.7	319.5	327.8	333.7	340.4	346.7
Phenobarbitone	337.6	345.0	348.5	354.1	358.6	363.2	367.2
Dextropropoxyphene HCl	350.6	357.4	359.3	364.8	367.4	372.3	375.9
Diazepam	416.2	421.3	421.2	424.4	426.0	429.8	431.9
Cannabinol	1037.8	1043.0	1038.5	1038.9	1039.3	1041.8	1042.0

10.2.5.4 continued...

For compounds eluting between nitroethane and nitrobutane, (i.e. with nitro-indices in the range 200 to 400), changes in nitro-indices with gradient delay time were observed. The increases in nitro-indices for these compounds were caused by the difference in behaviour of nitropropane with respect to the test compounds, as shown in Figure 10.12. Most of the test compounds behaved in a similar manner to nitrobutane (i.e. δt values in the range 5.3 to 5.8 minutes, compare with nitrobutane, δt = 5.65 minutes, for t_d = 6 minutes), whilst nitropropane showed a slower rate of increase of retention with increasing delay time (δt = 3.98 minutes when t_d = 6 minutes). This shows up in Figure 10.12 as a difference in slope for the nitropropane line compared with the test compounds and its higher homologues.

Figure 10.11: Variation of retention indices with gradient delay time for the organic test solution (cannabinol omitted from the graph).

Conditions as Figure 10.8. Compounds: (7), Tubocurarine Cl; (8), diamorphine HCl; (9), dibenzepin; (10), propranolol HCl; (11), phenobarbitone; (12), dextropropoxyphene; (13), diazepam.

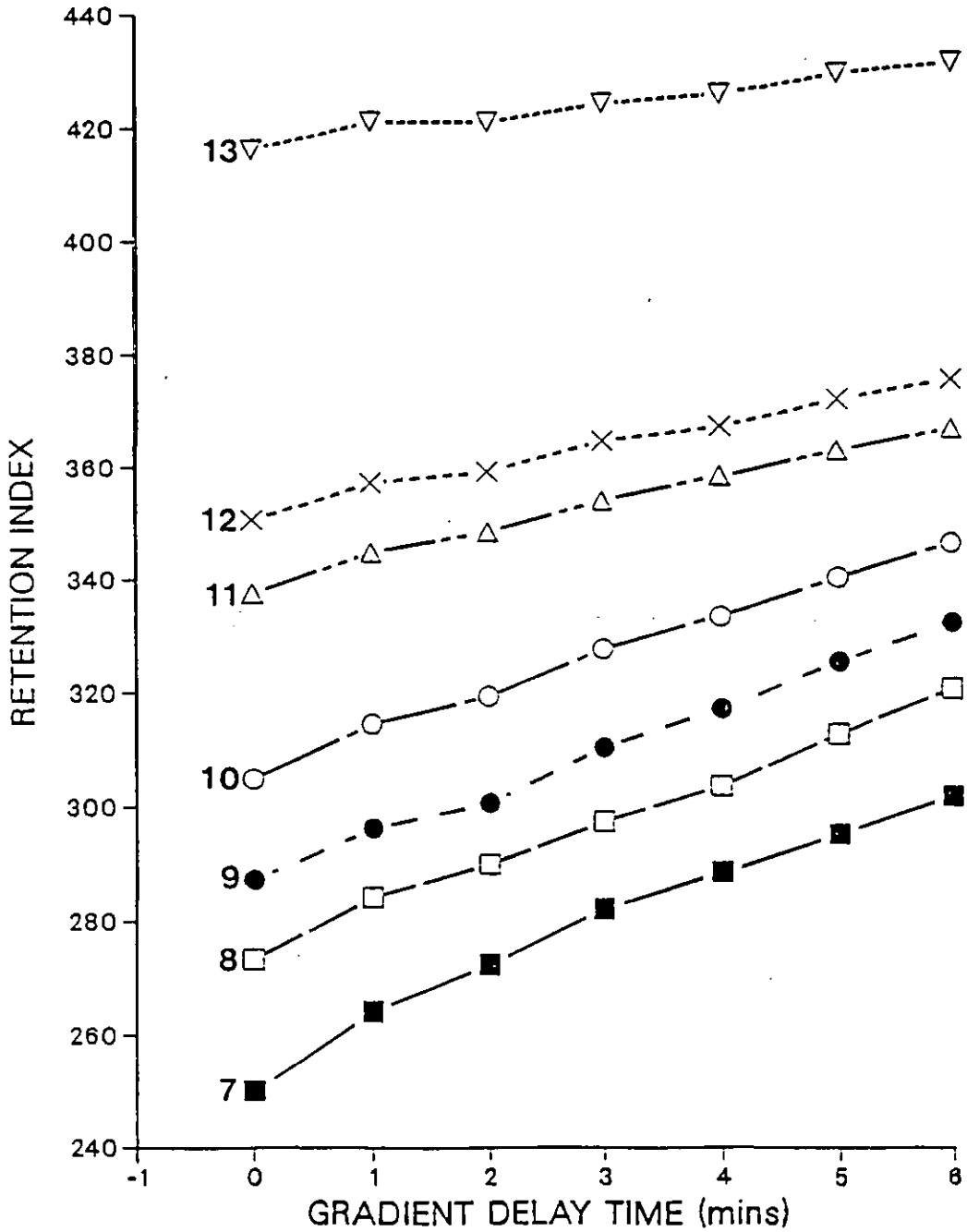
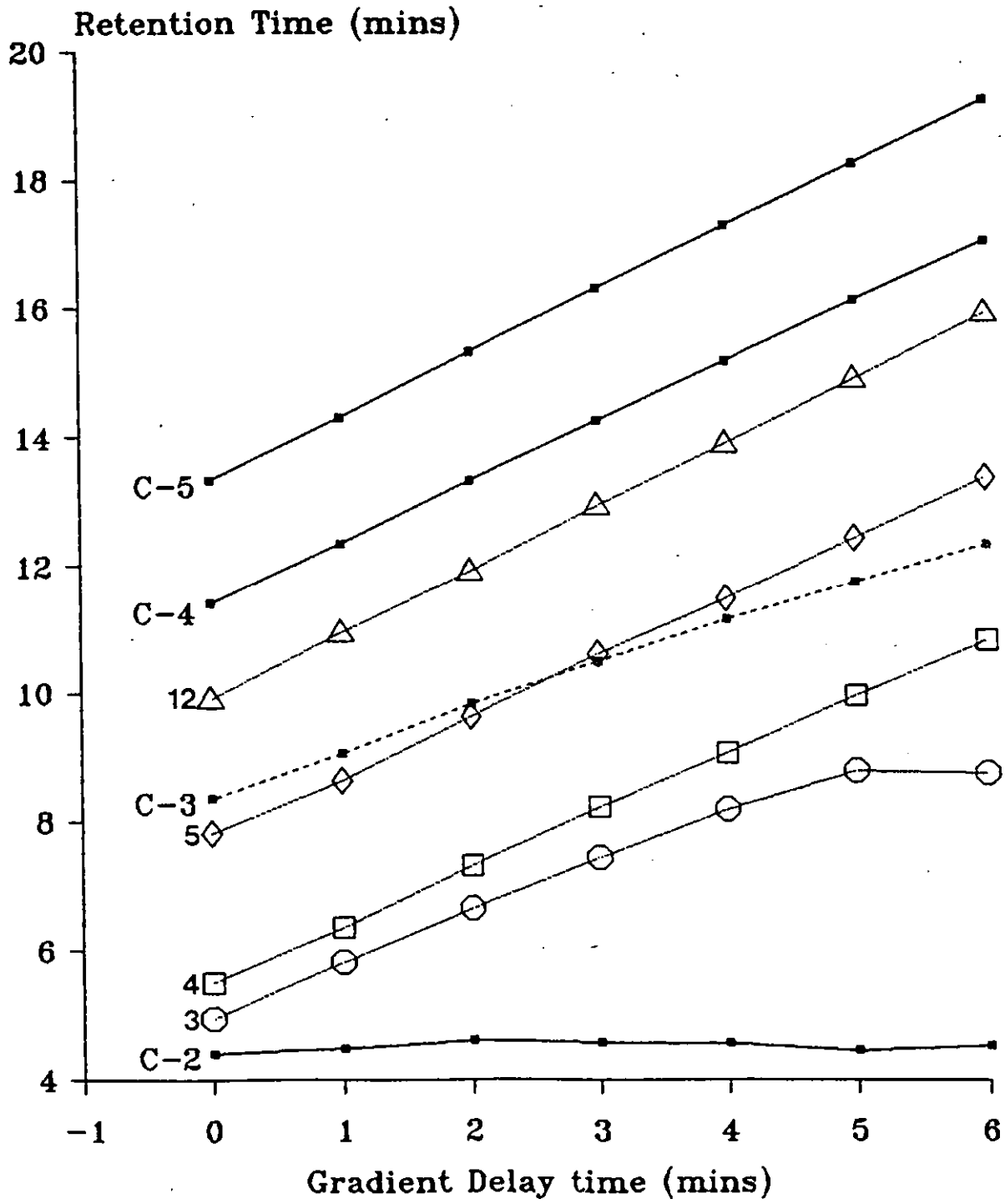


Figure 10.12: Variation of retention times with gradient delay time for some early eluting compounds.

Conditions as Figure 10.8. Compounds: C-*n* = carbon number of 1-nitroalkane; (3), ephedrine; (4), paracetamol; (5), bretylium ion; (12), dextropropoxyphene.



In an ideal situation the transition from a polar retention mechanism ($\delta t = 0$, elution at 2%B), to a hydrophobic retention mechanism ($\delta t = t_d$, gradient elution) would be gradual, such that, as the retention of the analyte increased (*e.g.* from ephedrine to the bretylium ion), the value of δt (Equ. 10.1) would increase from 0 to t_d. To maintain constant selectivity, compounds with short retention times would have low δt values, whilst longer retained compounds would have larger δt values.

The data recorded here clearly shows that the transition from one mechanism to the other does not follow this pattern, as most of the test compounds behaved in a similar manner to nitrobutane (Figure 10.12). Changes in selectivity and nitro-indices for the rapidly eluting compounds can be accounted for in terms of different retention mechanisms. The effect of changing the gradient delay time on the retention of the test compounds which were rapidly eluted due to their high polarity (*e.g.* ephedrine) was different to that found for the C_1 to C_3 1-nitroalkanes, as these were eluted rapidly due to their small size. These results are in agreement with recent data presented by Smith and Finn[131], who reported changes in column selectivity with increasing proportions of methanol in the mobile phase. Also, Bogusz and Aderjan[130] have reported that the nitro-indices of short retained compounds (*e.g.* paracetamol), were found to decrease as the amount of acetonitrile in the eluent increased, showing that most selectivity changes would occur at the beginning of a gradient run.

Cannabinol and diazepam showed near constant nitro-indices. Being more hydrophobic than the other analytes they should have retention mechanisms similar to the larger 1-nitroalkanes and so their selectivity should remain constant. Again these observations are in agreement with those of Bogusz and Aderjan[130], who found that the nitro-index of diazepam was independent of the %B in the mobile phase.

These results indicate the need to define the gradient protocol with care in order to maintain constant selectivity. This would be especially necessary when comparing results from different HPLC systems, where variations in the delay time between changing in the gradient at the pumps and on the column could occur due to different arrangements of the plumbing[173].

10.2.6 VARIABLE RETENTION OF EARLY ELUTING ANALYTES

It seemed reasonable to assume that morphine-3β-glucuronide and morphine hydrochloride ought to be unaffected by the gradient delay time as they both eluted before the gradient profile reached the detector, *i.e.* they were eluted isocratically in the starting eluent of 2%B ($\delta t \approx 0$). The irreproducible nature of the results recorded under these conditions was therefore of considerable concern, and so further studies were carried out to investigate this problem.

10.2.6.1 Tests using both gradient and isocratic conditions

To investigate the problem of variable retention for morphine-3β-glucuronide and morphine hydrochloride, experiments were carried out under carefully controlled conditions to test the effect of different reequilibration times between gradient runs and the reproducibility of retention under isocratic conditions. In each case, six injections of the test solutions were run.

The first two experiments, using aqueous test solution 2, involved running gradients using the standard profile, *i.e.* with a delay time of 3 minutes, and fixed reequilibration times of 6 and 12 minutes between consecutive injections. The reason for controlling the reequilibration time was to determine whether the irreproducible retention was caused by starting some of the runs too early, *i.e.* before the column was at equilibrium. The results for these experiments are shown in Table 10.13.

The other two experiments, carried out under 'identical' conditions, involved running repeat injections of aqueous test solution 3 under isocratic conditions at 2%B, set by the gradient programmer. These were run to determine the day to day reproducibility of the retentions under isocratic conditions. This would indicate whether the problem was caused by the gradients or was simply one of poor reproducibility for these compounds. The results for these experiments are shown in Table 10.14.

TABLE 10.13: RETENTION VARIATION FOR AQUEOUS TEST SOLUTION 2 UNDER CONTROLLED GRADIENT REEQUILIBRATION TIMES

Reequilibration: 6 mins Compound	Retention times (mins)						Mean R.T.	S.D.	%C.V.
Morphine-3B-glucuronide	3.463	3.475	3.215	2.953	3.467	2.973	3.258	0.249	7.63
Morphine hydrochloride	4.592	4.625	4.365	4.297	4.563	4.563	4.501	0.135	3.00
Ephedrine hydrochloride	7.423	7.447	7.465	7.467	7.465	7.532	7.467	0.036	0.49
Paracetamol	8.168	8.162	8.180	8.178	8.168	8.255	8.185	0.035	0.43

Reequilibration: 12 mins Compound	Retention times (mins)						Mean R.T.	S.D.	%C.V.
Morphine-3B-glucuronide	2.967	3.253	3.302	3.267	3.478	3.247	3.252	0.164	5.05
Morphine hydrochloride	4.182	4.338	4.405	4.708	4.627	4.343	4.434	0.197	4.44
Ephedrine hydrochloride	7.427	7.413	7.428	7.438	7.425	7.428	7.427	0.008	0.11
Paracetamol	8.150	8.143	8.150	8.148	8.145	8.160	8.149	0.006	0.07

TABLE 10.14: RETENTION VARIATION FOR AQUEOUS TEST SOLUTION 3 UNDER ISOCRATIC CONDITIONS

1st Isocratic set Compound	Retention times (mins)						Mean R.T.	S.D.	%C.V.
Morphine-3B-glucuronide	2.958	2.902	3.103	3.073	3.225	3.263	3.087	0.142	4.61
Morphine hydrochloride	4.185	4.117	4.232	4.208	4.312	4.345	4.233	0.084	1.98

2nd Isocratic set Compound	Retention times (mins)						Mean R.T.	S.D.	%C.V.
Morphine-3B-glucuronide	3.157	3.103	3.132	2.998	3.067	3.037	3.082	0.060	1.94
Morphine hydrochloride	4.278	4.203	4.240	4.150	4.173	4.157	4.200	0.051	1.20

10.2.6.2 Statistical analysis of the retention data

To determine if the apparent differences in the mean retention times (MRTs) and %CVs between the various runs were significant, statistical analysis of the results was carried out using F- and T- tests (see 9.2.5.2).

a) Morphine-3β-glucuronide

To test for systematic error between the different methods, the results with the largest and smallest MRTs were compared. The calculated value of T_{exp} was 1.667; the critical value, $T_{5,5}$ is 2.23 at the 95% confidence level[175]. Thus it was assumed that there was no systematic error between the methods, i.e. the choice of gradient or isocratic conditions did not significantly alter the results.

Application of the one-tailed F-test to the %CVs revealed that the second isocratic run was more precise than either of the gradient runs. In all other cases the variation in precision was not significant.

b) Morphine hydrochloride

The T-test showed that the MRTs recorded under gradient conditions did not differ significantly from each other, and that the MRTs recorded under isocratic conditions did not differ significantly from each other. However, the MRTs recorded under gradient conditions differed significantly from the MRTs recorded under isocratic conditions, i.e. the choice of method appeared to be significant, although there was no obvious explanation for this observation.

A one-tailed F-test was applied to the %CVs to determine if the isocratic runs were more precise than the gradient runs. The calculations revealed that both isocratic runs were more precise that the 12 minute reequilibration run, and that the second isocratic run was more precise than the 6 minute reequilibration run.

Thus it seems that the choice of method for morphine affects both the precision and the accuracy of the results, although it was unclear which of the methods gave the correct result for this compound.

10.2.6.3 ADDITIONAL STUDIES

In a continuation of these studies, work carried out by Ertas[176] at Loughborough University showed that the use of pre-prepared eluents of 2%B / 98%A gave much more robust results than eluents produced using the solvent programmer to control the mobile phase composition. This led to the conclusion that a likely cause of the reproducibility problem was that the HPLC pumps were unable to produce an eluent of constant composition when one of the components was being pumped at very low flow rates (*e.g.* 2%B at 1.5 ml min^{-1} total flow requires 0.03 ml min^{-1} from pump B).

The general conclusion appeared to be that this type of system was not suitable for the separation of early eluting compounds unless a pre-prepared eluent A, containing 2%B, was used, as the instrumentation could not meet the required level of eluent composition reproducibility at the ends of the gradient[176].

10.2.7 CONCLUSIONS

These studies have shown that sulphuric acid is more suitable than trifluoroacetic acid for use as an eluent modifier in the gradient separation of a wide range of drugs on an ODS-silica stationary phase. At the chosen wavelength for monitoring the separations (215 nm) clean baselines were achieved, and the 'gradient peak' (from impurities in the acetonitrile) did not interfere with the separation of the test compounds. Successful separation of a broad range of drugs, from highly polar compounds such as paraquat to very non polar ones such as cannabinol, was achieved using a gradient from 2%B to 98%B in 20 minutes.

The use of C_1 to C_{16} 1-nitroalkanes for calibration of the gradient was found to be successful, with the most polar and non-polar compounds studied (i.e. morphine-3ß-glucuronide and cannabinol), both eluting within the calibration range. Repeated separations of the 1-nitroalkanes were found to be very reproducible, and the use of nitro-indices, along with highly standardised equipment, resulted in a very good level of inter-laboratory reproducibility for all but one of the test compounds. Variations in nitro-index were recorded for the bretylium ion (a quaternary ammonium ion), which may have been susceptible to stationary

phase variations due to its permanent positive charge.

Alterations to the gradient profile were found to cause changes in selectivity for compounds with low retention indices (< 400), indicating that a number of retention mechanisms were involved in the separation of the analytes. However, when a single gradient profile was used, the separations were reproducible for most of the analytes, provided nitro-indices were used to report the results. For the two polar compounds eluting in the early part of the gradient, retention was found to be irreproducible due to difficulties in producing eluents of constant composition when one of the components was being pumped at very low flow rates.

SECTION 4: CONCLUSIONS, REFERENCES AND APPENDICES

CHAPTER 11: OVERALL CONCLUSIONS AND AREAS FOR FUTURE STUDY

In the isocratic studies it was found that a simple mobile phase, suitable for the analysis of basic drugs on bare silica, could be prepared by mixing an aqueous buffer, containing the two organic buffer compounds 3-(cyclohexylamino)-1-propanesulphonic acid (CAPS) and sodium 3-(cyclohexylamino)-2-hydroxy-1-propanesulphonate, with methanol. The eluent was found to give highly reproducible results on a single column over a one month period.

Studies carried out on two batches of silica revealed that the material was undergoing an 'aging process' which caused changes in its chromatographic properties. This process resulted in significant variations in retention for some test compounds, leading to changes in column selectivity over the period of the study. The process was also thought to be responsible for the appearance of distorted peak shapes for methylamphetamine, although no straightforward mechanism could be put forward to account for this observation.

Clearly such changes in the chromatographic properties of the silica are worrying, as they suggest that it would not be possible to maintain constant selectivity on a single column over a long period of use. These observations clearly merit further detailed study, with the particular aim of attempting to identify what is happening to the silica during storage. The observations reported here give a clear indication as to the types of compounds that would be suitable for use as sensitive probes of column performance for use in such studies.

A separate study, to assess the applicability of the new method for a wider range of test compounds, for 'real forensic samples' and for the analysis of samples from biological extracts, could also prove to be useful as this would give an idea of the range of samples that could be effectively analysed by this new method, (assuming that column stability could be maintained).

The gradient studies revealed that the first choice of eluent modifier, namely trifluoroacetic acid, was unsuitable for use at the wavelength chosen for detection (215 nm), as there was a very high

background absorbance change during the gradient run. Changing the modifier to sulphuric acid was found to give a system which produced clean gradient baselines with very little background absorbance. The selected eluent system was found to be applicable to a wide range of drugs, varying in size and polarity from paraquat and morphine-3β-glucuronide to cannabinol.

The use of 1-nitroalkanes as retention index standards was found to be very successful for the calibration of the gradient, and for the interlaboratory comparison of HPLC systems. The retention indices of most of the test compounds were found to be reproducible, but problems were experienced with rapidly eluted, polar analytes. It was concluded that the irreproducible retention of these compounds was probably caused by a variation in the eluent composition with time when one pump was required to work at very low flow rates.

The problem of irreproducible retention of early eluting analytes is clearly one requiring further attention. Ideally, the development of better technology (which was outside the scope of these studies!), should lead to improvements in the performance of the pumping systems, but a chromatographic solution to the problem is still needed. The development of a different gradient profile, or variations in the initial conditions of the gradient could be investigated, with a view to obtaining reproducible elution of all early eluting compounds without loss of resolution. Further studies could also involve the comparison of the 1-nitroalkanes (used here) with the alkan-2-ones to determine the most suitable retention index scale for use with this system.

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APPENDIX A

GRADIENT SYSTEM PERFORMANCE TESTS

The following performance tests were designed at CRSE[170] to measure the accuracy of flow rates and eluent composition, and to check for system errors such as leaks in the plumbing and faulty check valves. (Certain aspects of the tests were modified to suit equipment used at Loughborough. This was necessary because different pumps were used and the original tests could not be applied without modification).

A.1 LEAK TESTING

Using the solvent programmer, set the pumps to deliver 50% A, 50% B at 1.5 ml min^{-1}, and allow the system to equilibrate until a constant operating pressure is obtained. Using the torn edges of filter paper, the base and top of <u>every</u> joint throughout the system must be examined for leaks. These tests must include all fittings from the pump heads to the detector inlet, including fittings on the HPLC column and the Rheodyne valve. All leaks should be eliminated by careful tightening of the fittings, or by the use of PTFE tape.

A.2 CHECKING THE OVEN TEMPERATURE

The oven temperature should be set at 25°C, in the recycle mode (for the Kariba Advanced Air Oven, see 9.2.3.1), and the oven left for 30 minutes after switching on, or until a stable temperature is reached. The exact oven temperature must then be recorded using a NAMAS calibrated thermometer, inserted into the oven through a 3½ mm hole just below the Rheodyne valve.

For the system set up at Loughborough, a calibrated thermometer was unavailable for regular use, but the oven temperature was checked at the time the system was tested (using a calibrated thermometer, (NAMAS certificate of calibration 03069T), provided by CRSE). The temperature of the oven, when set at 25°C, was found to be 25.33°C.

A.3 TESTING PUMP CHECK VALVES

Using the solvent programmer, set the pumps to deliver 50% A, 50% B at 1.5 ml min^{-1}, and allow the system to equilibrate until a constant operating pressure is obtained. Then turn the Rheodyne valve into the 'half-way' position, and stop the pumps. Allow the pressures to stabilise, and record the pressure on each pump. Wait for 5 minutes, record the pressures again and calculate any changes in pressure. If significant changes are recorded, re-check the pump fittings for leakage and test the check valves to ensure that there is no 'back-flow' of eluent through the pump.

A.4 TESTING PUMP FLOW RATES

Switch off the electrical power to pump B, and set the gradient controller to deliver 100% A at 1.5 ml min^{-1}. Allow the system to settle at constant pressure and then record the flow rate at the detector outlet. This can be done either by use of a flow meter (recommended in CRSE set-up procedure), or by recording the time taken to collect a known volume of eluent in a calibrated volumetric flask (method employed at Loughborough). If the flow rate differs significantly from 1.5 ml min^{-1}, adjust the compressibility and/or flow rate settings on the pump until the correct flow rate is obtained.

The procedure must be repeated for pump B until the correct flow rate is obtained. (In the case of the pumps used at Loughborough, flow rates of 1.5 ml min^{-1} $\pm$ 0.01 ml min^{-1} were considered acceptable in the initial set-up).

A.5 TESTING THE GRADIENT COMPOSITION AND RUN TIME

To test the gradient run time, turn off the pumps and set the gradient controller to run a standard gradient programme, *i.e.* 2% B to 98% B in 20 minutes. Set the gradient to run and record the time taken for the programme to run. If the recorded run time differs significantly from the preset run time, adjust the gradient controller until the recorded run time corresponds to the preset run time. (N.B. This test is an addition

to those designed at CRSE, to account for the use of an older gradient controller, which did not contain modern microchip circuitry. However, it should be noted that microchips are precisely inaccurate!).

To test the accuracy of the gradient composition, set the pumps to deliver 0%, 10%, 25%, 50%, 75% and 100% B, at a flow rate of 1.5 ml min^{-1}. At each composition allow the system to equilibrate to constant pressure and effluent composition (*ca.* 10 minutes), and then collect three separate samples of column effluent in vials. Measure the refractive indices of each effluent sample using a refractometer, operating at the sodium D line and thermostated at 25°C. Allow each sample to reach 25°C in the refractometer before recording the RI. Take two separate measurements on each effluent sample (making six measurements at each eluent composition). Then compare the results with data recorded by manual mixing of the two eluents at 25°C.

N.B. Since the original test was designed, it has become clear that it is necessary for individual laboratories to calibrate their own refractometer to allow better determination of the accuracy of the eluent profile. The test is not designed to determine exact eluent compositions, but as a check on the eluent profile as the programmed composition changes.

APPENDIX B

PRESENTED AND PUBLISHED WORK BASED ON THESE STUDIES

1. Retention reproducibility of basic drugs in high-performance liquid chromatography on a silica column with a methanol-high pH buffer eluent.
   Effect of operating conditions on separations using an organic buffer.

   Roger M. Smith and James P. Westlake, Richard Gill and M. David Osselton.

   J. Chromatogr., 514 (1990) 97-109.

2. Lecture at *SAC '89*, Cambridge, (U.K.), 1989, entitled:

   "Improved reproducibility for the separation of basic drugs on a silica column."
   (Lecture abstract A16, see *Anal. Proc.*, 26 (1989) 204.)

3. Poster and 'Blitz presentation' at 2^{nd} *International Symposium on Pharmaceutical and Biomedical Analysis*, York, (U.K.), 1990, entitled:

   "Separation of basic drugs by HPLC on a silica column."
   (Poster abstract F-P-F1).