*Journal of Chromatography*, 112 (1975) 171–188

CHROM. 8496

# PERFORMANCE AND SELECTED APPLICATIONS OF A NEW RANGE OF CHEMICALLY BONDED PACKING MATERIALS IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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## SUMMARY

Several modified adsorbents have been prepared by chemically bonding organic groups to form unimolecular layers on (a) a newly synthesised spherical silica gel, and (b) Spherisorb Alumina A20Y. They include three reversed-phase materials, two weak anion exchangers and a polar bonded cyano alumina. The kinetic properties of the materials have been determined by measuring their reduced plate height–velocity dependences, and their flow resistance parameters ($\phi'$). With one exception the bonded materials show performances which are as good as, or better than those of the unmodified adsorbents. The new materials have been found to be particularly useful in the separation of compounds such as: catecholamines, morphine alkaloids, tetracyclines, tricyclic antidepressants, vitamins, nucleosides, nucleotides, penicillin, barbiturates and other polar substances of complex molecular structure. They give excellent peak symmetry and high analytical speeds. The mechanisms of retention on the new materials are characterised and discussed.

## INTRODUCTION

There has been a growing appreciation over the last two or three years that, as predicted by Martin and Synge in 1941[1], highest efficiencies in liquid chromatography are to be achieved by using very small particles for the column packing[2–7]. Knox and Saleem[8] pointed out that with a pressure capability of 100–200 bar the theoretically optimum particle diameter would be around 2 $\mu$m. While practice has not yet confirmed this fully, 5-$\mu$m particles have now been sufficiently well packed that efficiencies only slightly inferior to these at the theoretical limit are now routinely obtained[9].

It is generally accepted that the best comparative measure of the intrinsic kinetic performance of a column packing material is the plot of the reduced plate height, $h$, against the reduced velocity, $\nu$, along with a measure of the column resistance parameter $\phi'$[9,10]. These parameters are defined in eqns. 1–3.

$$h = H/d_p \tag{1}$$

$$\nu = ud_p/D_m \tag{2}$$

$$\phi' = \frac{\Delta p\, d_p^2}{u\eta L} \tag{3}$$

where $H$ = plate height, $d_p$ = mean particle diameter, $u$ = eluent linear velocity, $D_m$ = diffusion coefficient of solute in eluent, $\eta$ = eluent viscosity, $L$ = column length, and $\Delta p$ = pressure drop across column.

An efficient column must have both a low reduced plate height $h$ and a low resistance factor $\phi'$. Indeed as shown by Knox and Saleem[8], the group $h^2\phi'$ is in fact the best measure of this combined requirement for good performance and is directly proportional to the time for analysis for a given pressure drop and a required number of theoretical plates.

In practice $\phi'$ is found to lie in the region 500–1000, depending upon the method of packing the column (see for example ref. 9) and the porosity of the packing material.

Generally speaking, for any type of packing material ($h$, $\nu$) curves and $\phi'$ values will be more or less independent of the size of the particles of packing. As shown previously[11,12] the ($h$, $\nu$) dependence can conveniently be expressed in the form

$$h = B/\nu + A\nu^{0.33} + C\nu$$

where the $A$, $B$ and $C$ terms have much the same significance as in the original Van Deemter equation. However, $A$, $B$ and $C$ are now dimensionless. $A$ reflects the goodness-of-packing and in the best cases is below unity. $B$ reflects axial diffusion geometry and is around 2. $C$ measures the efficiency of mass transfer and the theoretical minimum[10] for a fully porous particle and a slightly retained solute is about $10^{-2}$; experimental values are generally about $5 \times 10^{-2}$ (ref. 11) or larger.

Recently it has been shown that much improved column performance can be obtained by employing the infinite diameter effect[13–15] first described by Knox and Parcher[13] whereby in a sufficiently wide column solute injected centrally fails to reach the walls and so avoids the disturbed region of packing near the walls. For 5- and 10-$\mu$m particles packed in 5-mm-bore tubes, columns up to 500 and 250 mm in length respectively will function as if of infinite diameter[16].

A particularly useful range of dimensions for high-performance columns is: $d_p \approx$ 5–10 $\mu$m, $L$ = 100–200 mm, column diameter $\geqslant$5 mm. With such columns $h$ should be in the range 1.5–3 at $\nu \approx 5$ and between 7 and 20 at $\nu$ = 100. For the best packings $h$ will be below 10 at $\nu$ = 100 and have a minimum value of 2 or less. Values of $\phi'$ should be in the range 500–1000 (refs. 9 and 16).

While the main problems in column kinetics have now been solved, difficulties are still experienced in the area of the thermodynamics of chromatography which relates to the mechanism of retention and the symmetry of eluted bands. While substantial improvements have been made in the quality of adsorbents which now have much more uniform pore geometry and consequently show more linear adsorption isotherms and better mass transfer characteristics than previously[9], nevertheless the majority of adsorbents must still be partially deactivated before they give peaks with satisfactory elution profiles[17]. Depending upon the polarity of the solutes being separated, adsorbents may be deactivated with one of a number of polar species (*e.g.*, acetonitrile, methanol, water). It is thought that the deactivator is preferentially adsorbed onto the most active sites, which are relatively few in number, and leave the less active but more numerous sites for adsorption and retention of solutes of interest.

Unfortunately, the deactivation procedure is not always satisfactory. Indeed it

becomes highly unsatisfactory for solutes whose molecules contain a large number of polar functional groups, such as $-NH_2$, $-OH$, $-COOH$, $-SO_3H$, and ionic groups generally. Such compounds are typical in biological, clinical and pharmaceutical situations and attempts to chromatograph them often give rise to badly tailed peaks even on columns which are highly efficient when tested with simple reference compounds[18]. We suspect that this may be the result of a "template effect". Thus, while simple deactivator molecules can block individual adsorbent sites by forming say a single hydrogen bond, complex solute molecules will preferentially select from the random array of adsorbent sites specific groups of sites which have the optimum geometry for maximum interaction. Such groups will be specific to particular solute species. In this way a surface which is essentially homogeneous for adsorption of simple molecules can prove to be heterogeneous for adsorption of multifunctional molecules especially if they have a rigid molecular shape. It therefore appears that we require a more radical modification of the adsorbent surface than can be achieved by simple deactivation if we are to achieve effective chromatography of the complex molecules with highly polar functional groups. Such modifications clearly involve chemical reaction.

The idea of chemical modification of an adsorbent to improve or modify its retentive properties in chromatography is far from novel and has been pioneered by Halász and co-workers[19–22], Kirkland and De Stefano[23,24], Locke and co-workers[25,26] and others[27–35]. The field has recently been reviewed by Pryde[36]. The most satisfactory materials from the point of view of stability towards typical eluents and temperature are those where an organic group is bonded to a silica structure by $\equiv$Si–C or $\equiv$Si–O–Si–C bonds[24,29,36]. Such materials have been used in many novel applications[36]. In terms of kinetic performance the evidence suggests that silica gels having a unimolecular organic layer bonded to the support exhibit plate heights similar to or below those of the untreated silica gel[37], while materials in which a polymeric stationary phase is bonded to a support as in the DuPont Permaphases (based upon the pellicular material Zipax) give somewhat impaired plate heights and higher mass transfer coefficients ($C$ values in eqn. 4)[38,39].

The purpose of the present work was to explore some of these ideas and in particular to prepare chemically modified adsorbents having high kinetic and thermodynamic performance for the types of highly polar and ionic solutes of interest in biochemical and pharmaceutical applications. The thermodynamic performance is tested by examining the symmetry of elution peaks obtained with complex molecules of a high degree of functionality, while the kinetic performance is assessed by determination of the ($h$, $\nu$) curve using simple reference compounds which are not expected to show any undesirable thermodynamic effects.

## EXPERIMENTAL

### *Equipment*

The chromatographs used were a DuPont Model 830 instrument and laboratory-assembled instruments of which the principal components were either a Haskel air pump (Olin Energy Systems, Sunderland, Great Britain) or an Orlita DMP 1515 piston pump (Orlita, Giessen, G.F.R.), and a Cecil Model 212 variable-wavelength photometer (Cecil Instruments, Cambridge, Great Britain) fitted with an

8-$\mu$l flow cell. Columns 125 mm long and 5 mm in bore were of internally polished stainless steel. Injection by micro syringe through a septum was made centrally on to the top of the column packing. Columns were terminated by stainless-steel frits of 6-$\mu$m porosity (for $d_p \geqslant 10\,\mu$m) or by sandwich frits comprising a 2-$\mu$m-porosity silver disk (Selas Flotronics, Spring House, Pa., U.S.A.) held between two thin stainless-steel frits (for $d_p < 10\,\mu$m). Column fittings were made to designs previously described[40]. All columns were operated at ambient temperature in the isocratic mode.

Particles of 10 $\mu$m and above were dry packed by the rotate, bounce and tap method; particles smaller than 10 $\mu$m were packed by the balanced density method using a thick slurry in methyl iodide + carbon tetrachloride[3], or a dilute slurry in methyl alcohol followed by hexane[41]. Packings were driven home by pressures of 2000–5000 p.s.i.

*Silanes*

Octadecyltrichlorosilane and chlorotrimethylsilane were purchased from Aldrich (Milwaukee, Wisc., U.S.A.); $\gamma$-aminopropyltriethoxysilane was purchased from Pierce (Rockford, Ill., U.S.A.). Commercial silanes were used as received. In addition, 2-(4-pyridyl)-1-trichlorosilylethane[42] and 2-cyanoethyltrichlorosilane[43] were prepared by the literature methods. Their purity was checked by nuclear magnetic resonance and infrared analyses, and these silanes were freshly purified prior to use.

*Supports*

The silica used was an in-house preparation available in a variety of particle sizes down to around 6 $\mu$m, prepared according to the method of Raven and Knox, to be described elsewhere. The particles were spherical and had a surface area of approximately 200 $m^2/g$. The alumina used was 20-$\mu$m Spherisorb A20Y gifted by AERE Harwell, Materials Preparation Unit, and is available from Phase Separations (Queensferry, Flintshire, Great Britain).

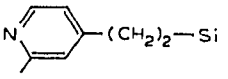
*Preparation of the bonded phases*

The silica or alumina to be reacted was dried by heating at 120° for 4 h in a vacuum oven and then immediately treated with excess of the organosilane in a dry organic solvent at reflux temperatures.

Solvent and excess organosilane were filtered off, and the support thoroughly washed with acetone (in the case of 6-$\mu$m particles, by sedimentation). Water was added to hydrolyze any residual silicon–chloride bonds, and the support was again washed with acetone before drying in a vacuum oven. Finally, the support was extracted in a Soxhlet apparatus for 24 h with benzene or chloroform to remove any higher-molecular-weight material present. In cases where the residual surface hydroxyl groups were found to confer undesirable chromatographic properties on the support, these were blocked with trimethylsilyl (TMS) groups. The bonded supports which are the subject of this work include a reversed-phase octadecyl (ODS) material bonded to silica (with and without TMS groups), denoted ODS-silica or ODS/TMS-silica, weak anion-exchange materials bonded to silica ($\gamma$-aminopropylsilyl), denoted $NH_2$-silica, [2-(4-pyridyl)-ethylsilyl)] bonded to alumina, denoted pyridyl-alumina, a polar phase (2-cyanoethylsilyl) bonded to alumina, denoted CN-alumina, and a short-chain reversed-phase material bonded to silica denoted SAS-silica[44]. A chemi-

TABLE I

CHEMICALLY BONDED SUPPORTS

| *Designation* | *Organic group* | *Support* | *Type* | *Typical analysis (%)* | | | *Total loading (%)* |
|---|---|---|---|---|---|---|---|
| | | | | *C* | *H* | *N* | |
| ODS/TMS-silica | $C_{18}H_{37}Si$ + $Me_3Si$ | 7 μm silica* | Reversed phase | 8.18 | 1.66 | — | 9.8 |
| SAS-silica | short chain | 7.5 μm silica* | Reversed phase | — | — | — | 3.6 |
| $NH_2$-silica | $NH_2(CH_2)_3Si$– | 7 μm silica* | Polar adsorbent or weak anion exchanger | 2.06 | 0.56 | 0.73 | 3.4 |
| CN-alumina | $CN(CH_2)_2Si$– | 20 μm alumina** | Polar or reversed-phase adsorbent | 1.63 | 0.36 | 0.57 | 2.6 |
| Pyridyl-alumina |  | 20 μm alumina** | Polar adsorbent or weak anion exchanger | 1.30 | 0.54 | | ≈2 |

* In house preparation of spherical silica gel.
** Spherisorb A20Y.

cally bonded γ-aminopropylsilyl alumina support has already been used for the determination of the herbicide paraquat in human urine[45]. The amount of organic group introduced by the bonding reaction was followed by the microanalysis figures which are shown in Table I.

## RESULTS AND DISCUSSION

### *Kinetic performance of adsorbents and chemically modified adsorbents*

Plots of reduced plate height, $h$, against reduced velocity, $v$, are shown in Figs. 1–3 for spherical silica gel and the three modified silica gels, SAS-silica, ODS/TMS-silica, and $NH_2$-silica. Fig. 4 compares data for cyanoethyl bonded to Spherisorb Alumina A20Y (CN-alumina). Diffusion coefficients required for calculation of reduced velocities were calculated from the Wilke–Chang equation (see ref. 10). The lines drawn are the best fits to the data given by eqn. 4, and the values of $k'$, $\varnothing'$, $A$, $B$, and $C$, for each solute are shown in Table II, along with values previously obtained for other packings. A typical test chromatogram for SAS-silica is shown in Fig. 5.

The plate height curve for the new spherical silica gel is very similar to that for Spherisorb S obtained previously and lower than that for other silica gels with which Spherisorb was recently compared[9].

The overall results broadly confirm the view that the formation of a unimolecular-bonded layer on the surface of either silica or alumina has no profound

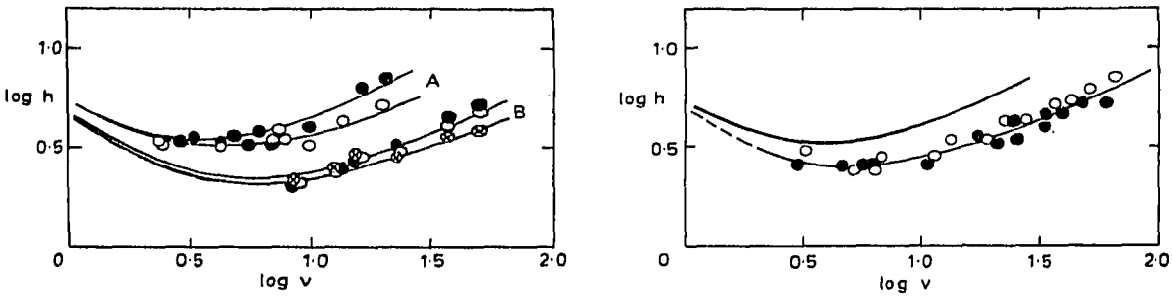



Fig. 1. Reduced plate height *vs.* velocity plots. (A) Spherical silica gel, 9 $\mu$m. Eluent, *n*-$C_6H_{14}$ + 1 % methanol; solutes, nitrobenzene (○) and *p*-aminoazobenzene (●). (B) SAS-silica, 7.5 $\mu$m. Eluent water–methanol (30:70); solutes, acetone (⊗), 2-phenylethanol (●), and 2,6-xylenol (○).

Fig. 2. Reduced plate height *vs.* velocity plot for ODS/TMS-silica, 7 $\mu$m. Eluent, water-methanol (60:40); solutes, acetone (●) and phenol (○). The solid line represents the mean data for silica gel from Fig. 1.

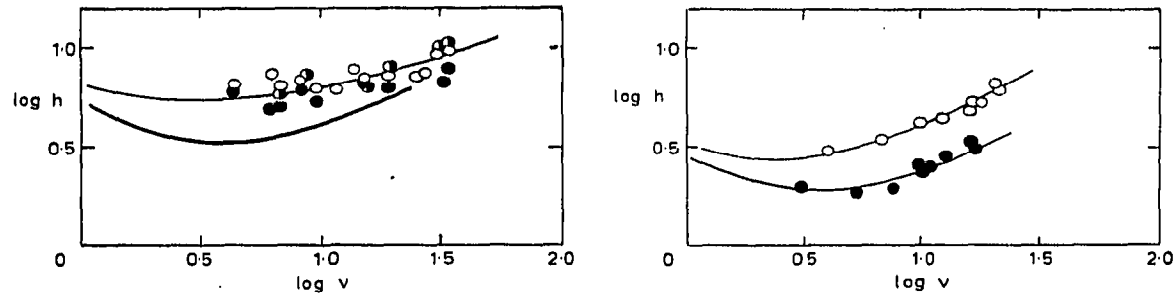



Fig. 3. Reduced plate height *vs.* velocity plot for $NH_2$-silica, 7 $\mu$m. Eluent, 0.25 *M* $Na_2HPO_4$ with $H_3PO_4$ added to give pH 2.95; solutes, ascorbic acid (●), nicotinic acid (○), and sulphanilic acid (◑). The solid line represents the mean data for silica gel from Fig. 1.

Fig. 4. Reduced plate height *vs.* velocity plots for CN-alumina (bonded to A20Y Spherisorb), 20 $\mu$m. Eluent, *n*-$C_6H_{14}$; solutes, toluene (●) and fluoranthene (○).

effect on the kinetic performance of the material. From both Figs. 1 and 2 and Table II it is clear that bonding if anything improves the performance of an adsorbent rather than the reverse in agreement with the findings of Horgan and Little[37].

The values of $\varnothing'$ fall in the lower part of the acceptable range and so indicate that the materials showed good flow properties. The parameter $B$ reflects the rate of axial diffusion. If axial diffusion occurs only in the mobile phase, then $B$ cannot exceed 2. This appears to be the best value to fit the data on the aluminas. However, the rather high value of $B = 4$ is required to fit the data on the silicas. If substantiated, this may indicate that solute molecules can move relatively freely over the surfaces of the silicas even when in the adsorbed state. Unfortunately, our data are not sufficiently extensive at low values of $v$ to pinpoint the exact value of $B$ and more data are required to confirm values higher than 2.

The values of $A$, with the exception of the high value for $NH_2$-silica, are near unity, as required for well packed columns. Difficulty was experienced in packing $NH_2$-silica, resulting in $A = 2.7$, and further work on the composition of the supporting liquid used in the slurry packing technique is required.

TABLE II

KINETIC PARAMETERS FOR ADSORBENTS, BONDED ADSORBENTS AND OTHER PACKING MATERIALS

| *Material* | *Size (μm)* | *Solute* | *k'* | *Ø'* | *A* | *B* | *C* | *Ref.* |
|---|---|---|---|---|---|---|---|---|
| Spherical silica gel | 9 | nitrobenzene | 0.3 | 540 | 1.3 | 4 | 0.05 | |
| | | *p*-aminoazobenzene | 5.1 | | 1.3 | 4 | 0.14 | |
| Spherisorb S10 | 9 | aromatics | — | | 1.0 | 4 | 0.12 | 9 |
| ODS/TMS-silica | 7 | acetone | 0.3 | 500 | 0.9 | 4 | 0.04 | |
| | | phenol | 2.3 | | | | | |
| SAS-silica | 7.5 | acetone | 0.9 | 450 | 0.7 | 4 | 0.02 | |
| | | 2,6-xylenol | 1.5 | | 0.75 | 4 | 0.04 | |
| $NH_2$-silica | 7 | ascorbic acid | 0.2 | 720 | 2.7 | 4 | 0.01–0.03 | |
| | | nicotinic acid | 0.4 | | | | | |
| | | sulphanilic acid | 0.8 | | | | | |
| Spherisorb A20Y | 20 | nitrobenzene | 6.6 | — | 0.5 | 2 | 0.08 | 9 |
| CN-alumina | 20 | toluene | 0.0 | | 0.8 | 2 | 0.05 | |
| | | fluoranthene | 2.0 | | 1.2 | 2 | 0.13 | |
| Zipax | 29 | | 8 | | 1.0 | | 0.01 | 46 |
| Permaphase ETH | 40 | | 1–3 | | 2.0 | | 0.06 | 38 |
| Corasil | 48 | | 1–5 | | 2.0 | | 0.06 | 11 |
| Porasil | 48 | | 1 | | 1.8 | | 0.05 | 11 |

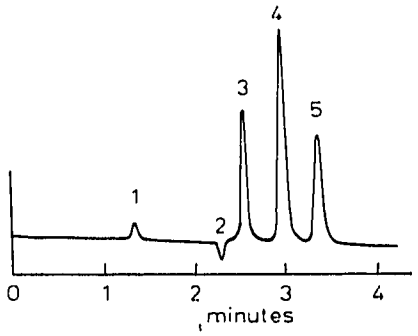



Fig. 5. Test chromatogram. Packing, SAS-silica, 7.5 μm. Eluent, water–methanol (30:70). Solutes: 1 = sulphanilic acid; 2 = water; 3 = acetone; 4 = 2-phenylethanol; 5 = 2,6-xylenol. The plate number for 2,6-xylenol is 7000, the column length is 125 mm, $H$ = 17.8 μm, and $h$ = 2.4.

Of greatest interest are the $C$ values, which reflect the resistance to mass transfer within the particles. For the bonded materials these are either comparable with or lower than the corresponding values for the original adsorbents. Thus for the ODS/TMS-silica, for which the data are most extensive, the plate height rises very gradually with velocity above the minimum value of $h$ = 2.5, and even at $v$ = 100, $h$ is not larger than 8. The plate height curve is both lower and less steep than that for the silica gel itself and is comparable to that obtained previously for 29-μm Zipax (DuPont trade name), which until recently gave the lowest ($h$, $v$) curve of any material tested[46,47]. It is well below that for the polymer-bonded material Permaphase ETH (DuPont trade name)[38]. The excellent properties of ODS/TMS-silica are emphasised by the low $C$ value of 0.04. The new SAS-silica gives an even lower ($h$, $v$) curve and a

remarkably low $C$ value as well as low values of $A$ and $\varnothing'$. As shown below, this $C$ value of 0.02 is in the region of the theoretical minimum and makes SAS-silica one of the best materials so far devised for high-performance liquid chromatography (HPLC). The $C$ value for $NH_2$-silica cannot be determined with precision from the data obtained since the major contribution to dispersion arises from the $A$ term. We can, however, say that $C$ cannot be larger than about 0.03 and could be as low as that for SAS-silica.

The aluminas show similar features to the silicas. Thus CN-alumina shows similar values of $A$ and $C$ to Spherisorb Alumina from which it was prepared. Generally, Spherisorb Alumina has superior packing characteristics when compared with silica gels (lower $A$ values) but its mass transfer properties are less good (slightly higher $C$ values); this appears to hold for the bonded aluminas also.

The minimum $C$ value which is theoretically attainable can be evaluated from the equation given by Giddings[10] for the plate height contribution from a uniform porous sphere. If the porous sphere contains mainly eluent within its pores, the following result may be derived from Giddings' analysis:

$$C = \frac{1}{30} \cdot \frac{(1 + k' - x)}{(1 + k')^2} \cdot \frac{D_m}{D_s}$$

where $k'$ is the capacity ratio, $x$ = fraction of eluent in interparticle space (approximately 55% for a typical silica gel), and $D_s$ is the mean diffusion coefficient for molecules within the particle. Since the maximum value which $D_s$ can possess is $D_m$ (the value in bulk eluent), the theoretical minimum value for $C$ for an unretained solute ($k' = 0$) becomes $C = (1 - x)/30 = 1.5 \times 10^{-2}$, whereas for $k' = 2$ the minimum value of $C$ becomes $0.9 \times 10^{-2}$.

The reason for the improved mass transfer in bonded materials is not clear but we suspect that one effect of reaction of the hydroxyl groups of the silica or alumina surface may be to enhance the effective diffusion rate $D_s$ by eliminating layers of mobile phase held very tightly to the adsorbent surface by hydrogen bonds. Such layers would be expected to be more than usually viscous and so would restrict diffusion of foreign molecules within them. Replacement of strongly orientating Si–OH or Al–OH groups by weakly orientating O–Si–alkyl groups might therefore enhance solute diffusion and reduce $C$ values. This explanation for the superior performance of some of the bonded materials is advanced tentatively at this stage until further experimental data are available.

Taken as a whole, our results support the view that small-particle unimolecularly bonded materials perform at least as well in terms of their ($h$, $\nu$) curves as the larger pellicular materials in which a polymer is deposited within the interstices of the porous surface layer. In practice, they give very rapid separations with high resolution and there seems to be no doubt that future progress must be made in this direction. Ultimately, it seems probable that bonded materials with packing and mass transfer properties close to the theoretical limit can be produced.

*Applications and retention mechanisms for bonded materials*

As shown by the applications which follow, adsorbents to which organic groups are chemically bonded via ≡Si–O–Si–R bonds are particularly suitable for

the separation of solutes of high polarity. The molecular constitution of such solutes has a significant effect both on the degree of retention and upon the peak shape and it is therefore relevant to consider the chromatograms presented below in relation to the molecular formulae of the solutes. Representative examples of these, formulae I–XI, are accordingly given.

*ODS/TMS-silica and ODS-silica.* ODS/TMS-silica, where a very high proportion of Si–OH groups have been silanized, is particularly suitable for the separation of catecholamines and their metabolites (representative formula I) as shown in Fig. 6. This type of separation has obvious application in clinical chemistry since the important metabolites dopamine, vanilmandelic acid, and homovanillic acid are also separated.

Fig. 7 shows the separation of some common benzodiazepines, nitrazepam, diazepam (Valium) and chlordiazepoxide (Librium) (II) on ODS-silica (which had not been blocked by reaction of residual hydroxyl groups). This separation could be made the basis of a quality control method for pharmaceutical preparations.

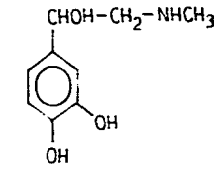
Both chromatograms show excellent peak symmetry and good efficiencies equivalent to 1500–2000 theoretical plates, indicating that the partition isotherms between the eluent and the modified silica gels are linear. The reduced plate heights are in the range 10–13.

Our results, in terms of resolution, pressure drop and speed of analysis, compare well with those of Persson and Karger for catecholamines[6], and of Rodgers[49] and Weber[50] for benzodiazepines.

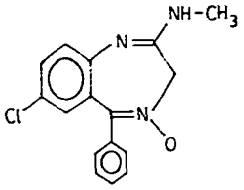
Reversed-phase partition chromatography using bonded as well as non-bonded stationary phases is, of course, well established[23,51,52] and in simple cases, for example with aromatic hydrocarbons or phenols, solutes elute in order of decreasing polarity. While this is broadly the case here for the catecholamines, the eluent used was complex. The catecholamines will be present in acid solutions as cations and in the presence of sodium lauryl sulphate, ion pairs are almost certainly the species which are sorbed into the organic stationary phase. The benzodiazepines, on the other hand, will be un-ionized in the basic eluent used, and their elution order does not seem to be readily explained in terms of the molecular structures.

*SAS-silica.* The new SAS-silica has proved to be especially useful for the resolution of solutes of very high polarity containing a large number of functional groups. Such molecules tend to give badly asymmetric peaks when separation on currently available packing materials is attempted. As suggested in the introduction, we consider this to be attributable to what we have called the "template effect". Fig. 8 shows a separation of tetracycline (III) from its four main contaminants in 4 min[18]. While the peaks are still somewhat asymmetric, they are much less so than those in the published literature[53,54] and further improvement is almost certainly possible by adjustment of the eluent composition.

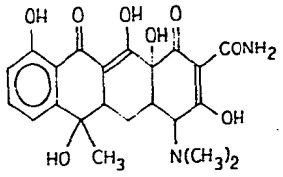
Fig. 9 shows a rapid separation of six opium alkaloids, a group which like the tetracyclines has proved somewhat intractable in the past giving tailed peaks[55–59]. It is noticeable that in Fig. 9 the peaks for papaverine (IV), narcotine, and cryptopine are narrow and symmetrical, while those for morphine (V), codeine and thebaine are somewhat asymmetric. The former group have relatively flexible molecular structures, while the molecules of the latter group all have the much more rigid morphine structure. This supports our contention that a high degree of functionality combined with



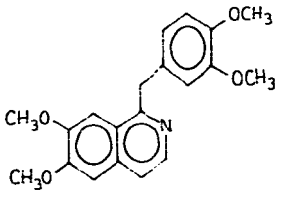
Adrenaline (I)



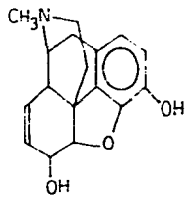
Chlordiazepoxide (Librium) (II)



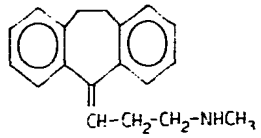
Tetracycline (III)



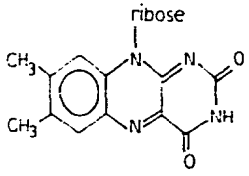
Papaverine (IV)



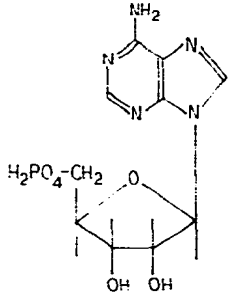
Morphine (V)



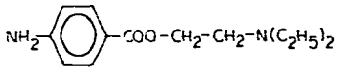
Nortriptyline (VI)



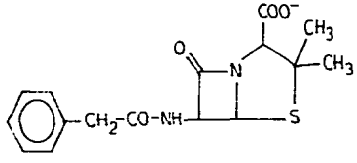
Vitamin B2 (VII)



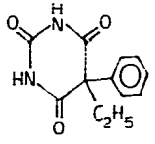
Adenosine monophosphate (VIII)



Procaine (IX)



Penicillin G (X)



Phenobarbitone (XI)

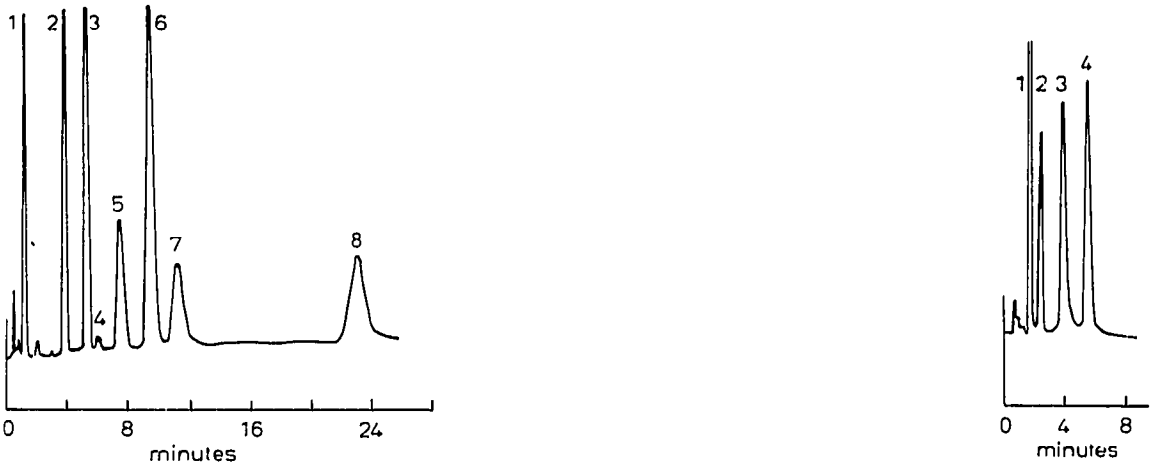



Fig. 6. Separation of catecholamines. Column packing, ODS/TMS-silica (6 μm); column dimensions, 125 mm × 5 mm I.D.; eluent, acetonitrile–water–conc. sulphuric acid [10:90:0.3 (v/v) containing 0.1 % (w/w) sodium lauryl sulphate]; pressure, 1100 p.s.i.; temperature, ambient; wavelength, 280 nm. 1 = Homovanillic acid and vanilmandelic acid; 2 = noradrenaline; 3 = adrenaline; 4 = DOPA; 5 = normetadrenaline; 6 = dopamine; 7 = metadrenaline; 8 = 3-methoxytyramine. (Reproduced by permission of J. Jurand[48].)

Fig. 7. Separation of benzodiazepines. Column packing, ODS-Silica (6 μm); column dimensions, 125 mm × 5 mm I.D.; eluent, methanol–water–ammonia (60:40:0:5, v/v); pressure, 500 p.s.i.; temperature, ambient; wavelength, 254 nm. 1 = Acetone; 2 = nitrazepam; 3 = diazepam (Valium); 4 = chlordiazepoxide (Librium). (Reproduced by permission of J. Jurand[48].)


Fig. 8. Separation of tetracyclines. Column packing, SAS-Silica (6 μm); column dimensions, 125 mm × 5 mm I.D.; eluent, 0.01 *M* $HClO_4$ in acetonitrile–water (14.5:85.5, v/v); pressure, 1000 p.s.i.; temperature, ambient; wavelength, 280 nm. 1 = 4-Epitetracycline; 2 = tetracycline; 3 = chlortetracycline; 4 = 4-epianhydrotetracycline; 5 = anhydrotetracycline. (Reproduced by permission of J. Jurand[48].)

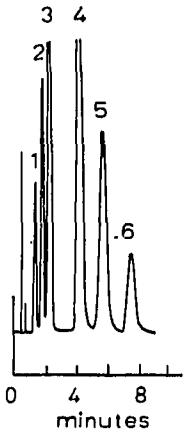
Fig. 9. Separation of opium alkaloids. Column packing, SAS-Silica (6 μm); column dimensions, 125 mm × 5 mm I.D.; eluent, 0.025 *M* $NH_3$ in methanol–water (1:1); pressure, 800 p.s.i.; temperature, ambient; wavelength, 240 nm. 1 = Acetone; 2 = morphine; 3 = papaverine; 4 = narcotine; 5 = codeine; 6 = cryptopine; 7 = thebaine. (Reproduced by permission of J. Jurand[48].)

rigidity of the molecular structure leads to bad peak shape probably because there always exist a small number of surface sites which have the correct geometry for strong adsorption of these complex rigid molecules.

Tricyclic tranquillizers (VI) (Fig. 10) and a selection of the B group of vitamins (VII) (Fig. 11) are also well resolved on SAS-silica. These species are somewhat less polar than the tetracyclines and present less of a problem in regard to peak symmetry. In both Figs. 10 and 11, the efficiency is equivalent to up to 2000 theoretical plates, and the analytical speed is a considerable improvement on what has previously been attained both for the water-soluble vitamins[30,60,61] and for the tricyclic tranquillizers[62].

Unlike the partitioning of non-polar compounds into a long-chain hydrocarbon stationary phase such as ODS, partitioning of such highly polar compounds as tetracyclines into a short-chain hydrocarbon phase is quite unexpected since these compounds are almost insoluble in hydrocarbon solvents. Yet SAS-silica is clearly both retentive and of high chromatographic efficiency. It is also selective and its selectivity can be altered over wide limits by change in the composition of the eluent.

We believe that SAS-silica partitions because it extracts the less polar component(s) from the eluent and so provides what amounts to an adsorbed stationary phase layer whose composition is substantially different from and much less polar than that of the mobile phase. Thus in the case of the tetracyclines, which are eluted by aqueous perchloric acid containing acetonitrile[18], it seems most likely that we are partitioning tetracycline–perchlorate ion pairs between a predominantly aqueous mobile phase and a relatively, but not completely, non-polar adsorbed stationary phase which is rich in acetonitrile.



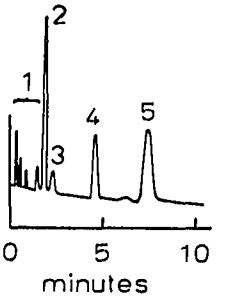




Fig. 10. Separation of tricyclic tranquillizers. Column packing, SAS-silica (6 μm); column dimensions, 125 mm × 5 mm I.D.; eluent, methanol–water–ammonia [75:25:0.5 (v/v) containing 0.1% (w/w) sodium lauryl sulphate]; pressure, 1300 p.s.i.; temperature, ambient; wavelength, 240 nm. 1 = Trimipramine; 2 = amitriptyline; 3 = imipramine; 4 = nortriptyline; 5 = desipramine; 6 = protriptyline. (Reproduced by permission of J. Jurand[48].)

Fig. 11. Separation of B vitamins. Column packing, SAS-silica (6 μm); column dimensions, 125 mm × 5 mm I.D.; eluent, water–methanol–conc. sulphuric acid [85:15:0.25 (v/v), containing 0.1% (w/w) sodium lauryl sulphate]; pressure, 2000 p.s.i.; temperature, ambient; wavelength, 270 nm. 1 = Impurities; 2 = caffeine; 3 = vitamin B2; 4 = vitamin B3; 5 = vitamin B6. (Reproduced by permission of J. Jurand[48].)

In the separation of the tricyclic tranquillizers in basic solution, and of the vitamins in acid solution, the addition of a small concentration of sodium lauryl sulphate had a profound effect as it did on the elution of the catecholamines from ODS/TMS-silica. It is probable that here again the detergent is strongly adsorbed on to the non-polar surface and that the formation of ion pairs and probably also of micelles play a part in the partitioning process. Further work on this form of chromatography will be published in due course[63]. Recent papers by Kraak and Huber[64] and Persson and Karger[6] also deal with ion pair partition chromatography.

The short-chain alkyl-bonded silica is evidently much more versatile than would at first sight be anticipated. It appears to act very much like an adsorbent which partitions mainly by virtue of the influence it has on the composition of the liquid in the immediate vicinity of its surface (say within 1–5 nm). But unlike conventional adsorbents such as silica or alumina, the SAS-silica preferentially extracts the non-polar component(s) rather than the polar components of an eluent. It is thus the true reversed-phase analog of a normal adsorbent such as silica gel or alumina.

*$NH_2$-silica.* Anion-exchange materials have long been used for the analysis of nucleic acid materials but generally chromatographic performance measured as the rate of generation of plates for a given pressure drop has been relatively low[65]. The bonded pellicular support AAX Permaphase (DuPont trade name) showed a considerable improvement on homogeneous ion-exchange resins and on previous pellicular materials for nucleotide analysis[66] but was still not as efficient as the equivalent unbonded material Zipax. Fig. 12 shows that by using a unimolecular-bonded material the four DNA nucleotide monophosphates (VIII) can be separated very rapidly under quite gentle pressure conditions and with excellent plate efficiency. Likewise, AMP and cyclic AMP can be resolved in 2 min, as shown in Fig. 13.

Fig. 14 shows an interesting separation where procaine penicillin G gives two peaks, one for the base cation of procaine (IX) and the second for the penicillin anion (X). The penicillin peak is wide and tailed presumably because of the highly functionalized structure of the anion which is adsorbed onto rare but specific sites on the silica underlying the organic phase. Cephalosporins may also be separated on this material under similar conditions.

The retention of a base cation (such as that of procaine) by an anion exchanger is unexpected but has previously been observed with the dicationic herbicides paraquat and diquat[45] using aminopropylsilyl bonded to alumina and an aqueous–methanolic buffer as eluent. It is believed that the retention mechanism in such cases is essentially ion-pair partition onto a highly polar surface rather than ion exchange. It was therefore of interest to examine whether or not $NH_2$-silica was behaving as a genuine ion exchanger when retaining simple acids.

According to the simple theory of ion exchange[39], the $k'$ values of an acid held by an anion exchanger should be inversely proportional to the ionic strength of the eluent, $\mu$, and for weak acids should increase with increase in pH as the degree of ionization of the acid is increased. For degrees of ionization below say 10% $k'$ should be proportional to $1/[H^+]$. Although for a weak ion exchanger the degree of ionization of the base and hence its retentive power will eventually decrease as the pH is raised, this is not expected to occur for aminopropyl for pH $< 7$ since the $pK_a$ value of the base cation is around 10 or 11. Accordingly, $k'$ should increase with pH until the acid solute is completely dissociated; thereafter $k'$ should remain independent of pH. In

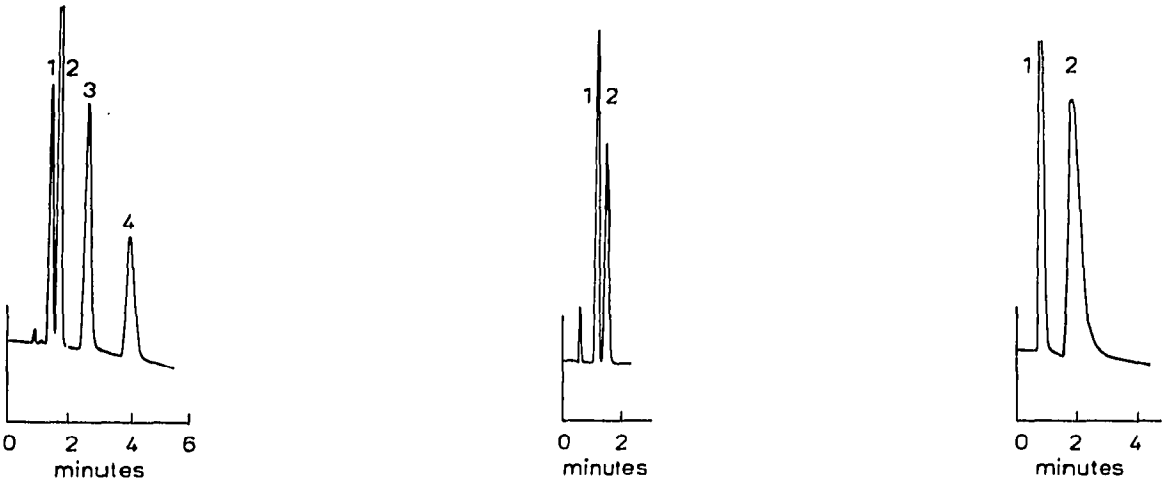



Fig. 12. Separation of DNA nucleotides. Column packing, $NH_2$-silica (6 $\mu$m); column dimensions, 125 mm × 5 mm I.D.; eluent, 0.01 *M* $Na_2HPO_4$ + $H_3PO_4$ to pH 2.46; pressure, 420 p.s.i.; temperature, ambient; wavelength, 254 nm. 1 = 2'-Deoxycytidine-5'-monophosphoric acid; 2 = 2'-deoxyadenosine-5'-monophosphoric acid; 3 = 2'-deoxyguanosine-5'-monophosphoric acid; 4 = thymidine-5'-monophosphoric acid.

Fig. 13. Separation of AMP and cyclic AMP. Column packing, $NH_2$-silica (6 $\mu$m); column dimensions, 125 mm × 5 mm I.D.; eluent, 0.005 *M* $Na_2HPO_4$ + $H_3PO_4$ to pH 2.46; pressure, 550 p.s.i.; temperature, ambient; wavelength, 258 nm. 1 = AMP; 2 = cyclic AMP.

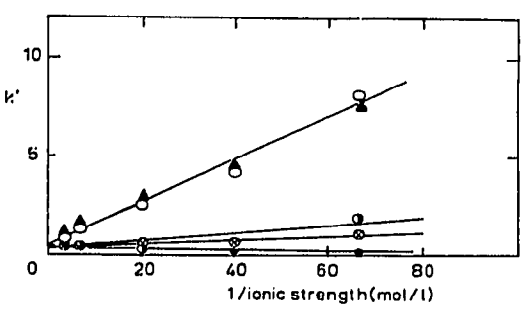
Fig. 14. Chromatogram of procaine penicillin G. Column packing, $NH_2$-silica (6 $\mu$m); column dimensions, 125 mm × 5 mm I.D.; eluent, 0.01 *M* $Na_2HPO_4$ + $H_3PO_4$ to pH 3.25; pressure, 625 p.s.i.; temperature, ambient; wavelength, 230 nm. 1 = Procaine; 2 = penicillin G.

practice, these simple predictions will be more qualitative than quantitative since retention by simple adsorption and partition will generally occur and since activities (which should strictly be used in the above arguments) only roughly follow concentrations in ionic solutions.

Nevertheless, the effects of variation of ionic strength and pH still provide the best tests for the mechanism of retention on a supposed ion exchanger.

Fig. 15 shows that at pH 3.0 there is indeed a roughly linear relationship between $k'$ and $1/\mu$. Over a tenfold range of $\mu$, $k'$ changes about tenfold for *o*-phthalic acid ($pK_a = 2.59$; ≈30% anion) and *p*-toluenesulphonic acid (≈99% anion), whereas for the weakly ionized benzoic acid ($pK_a = 4.19$; <10% anion) there is a lesser increase in $k'$ and for 2,6-xylenol, which is un-ionized, $k'$ is small (<0.45) and independent of ionic strength. These trends are thus good evidence that with moderately and strongly ionized acids the $NH_2$-silica acts as a true ion exchanger while for the un-ionized 2,6-xylenol retention is probably by adsorption. For the intermediate case of a weakly ionized acid both ion exchange and adsorption contribute to retention.

The effect of variation of pH is shown in Fig. 16 and a maximum is clearly shown at a pH of around 4.5 in those cases where partial ionization occurs, *viz.* *o*-phthalic acid, benzoic acid and sulphanilic acid ($pK_a = 3.23$; 35% zwitterion and 65% cation at pH 4.5). For fully ionized *p*-toluenesulphonic acid ($pK_a < 1$) pH has no effect at values below 4, while for the un-ionized 2,6-xylenol very little effect is



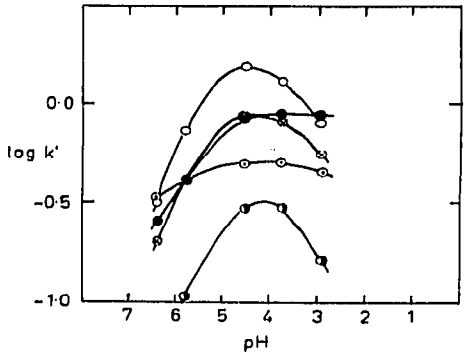




Fig. 15. Plot of capacity ratio ($k'$) *vs.* 1/ionic strength ($1/\mu$) for $NH_2$-silica. Eluent, $Na_2HPO_4$ + $H_3PO_4$ to pH 3.0. Solutes: *o*-phthalic acid ($pK_a$ 2.59: 30% anion) (○); *p*-toluenesulphonic acid ($pK_a < 1$: 99% anion) (▲); benzoic acid ($pK_a$ 4.19: ≈10% anion) (⊘); 2,6-xylenol (un-ionized) (●); sulphanilic acid ($pK_a$ 3.23: cation/zwitterion) (◑).

Fig. 16. Plot of log $k'$ *vs.* pH for the $NH_2$-silica packing. Eluent, 0.1 *M* $Na_2HPO_4$ + $H_3PO_4$. Solutes: *o*-phthalic acid (○); *p*-toluenesulphonic acid (●); benzoic acid (⊘); 2,6-xylenol (○); sulphanilic acid (◑).

seen over a still wider pH range. As pH is increased above 4.5, $k'$ falls for all the acids. The position of the maximum is, however, far below the pH at which propylamine would itself be un-ionized. The fall in $k'$ as pH is increased above 4.5 cannot therefore be due to a fall in the degree of ionization of the base. We believe quite a different explanation applies. The isoelectric point of silica gel occurs at a pH in the range of 4–5, silica being negatively charged at higher pH and neutral or positively charged at lower pH[67]. We believe that when the silica becomes negatively charged the ion-exchange capacity of the aminopropyl cations can be neutralized by the negative charges on the silica. Thus the effect is somewhat analogous to an increase of counter-ion concentration in the eluent in that the availability of bonded cations for ion exchange is reduced. We are dealing not with failure of the base to ionize but with saturation of the ion-exchange capacity by fixed charges on the silica gel surface.

*Pyridyl-alumina.* The material formed by bonding 2-(4-pyridyl)trichlorosilylethane to the surface of 20-$\mu$m alumina is a useful chemically bonded weak anion-exchange material in which the 4-alkylpyridine group is separated from the alumina by a two-carbon spacer unit. Majors[29] has recently prepared a similar support by bonding a 2-ethylpyridyl group to silica. The bonded pyridyl and cyanoethyl groups and the previously reported bonded aminopropylsilyl group[45] are, we believe, the first examples of stationary phases chemically bonded to alumina for use in HPLC. These materials have been used with a range of acid buffers and in the case of the aminopropyl material for the repeated injection of neat urine samples, without deterioration in chromatographic performance[45]. The pyridyl material can be used either as a modified polar adsorbent, as shown in the separation of some aromatic hydrocarbons (Fig. 17), or as a weak anion exchanger, as demonstrated by the separation of some barbiturates (XI) (Fig. 18) and nucleoside components (VIII) (Fig. 19). Again the

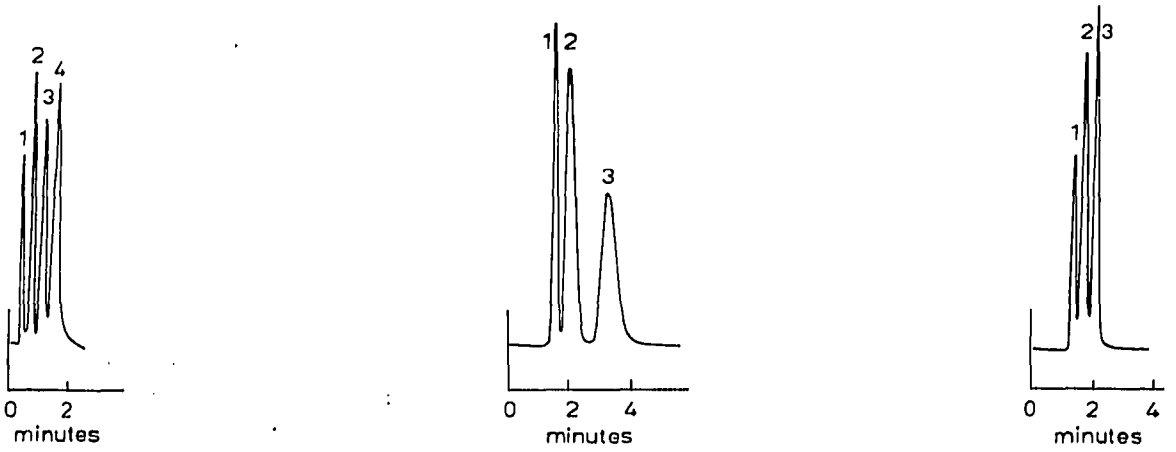



Fig. 17. Separation of aromatic hydrocarbons. Column packing, pyridyl-alumina (20 $\mu$m); column dimensions, 125 mm × 5 mm I.D.; eluent, hexane (water saturated)–dioxan (95:5, v/v); pressure, 120 p.s.i.; temperature, ambient; wavelength, 254 nm. 1 = Toluene; 2 = pyrene; 3 = 9,10-benzophenanthrene; 4 = 1,2,5,6-dibenzofluorene.

Fig. 18. Separation of barbiturates. Column packing, pyridyl-alumina (20 $\mu$m); column dimensions, 125 mm × 5 mm I.D.; eluent, 0.04 *M* sodium hydroxide + boric acid to pH 7.1; pressure, 350 p.s.i.; temperature, ambient; wavelength, 254 nm. 1 = Barbituric acid; 2 = hexobarbitone; 3 = phenobarbitone.

Fig. 19. Separation of nucleoside components. Column packing, pyridyl-alumina (20 $\mu$m); column dimensions, 125 mm × 5 mm I.D.; eluent, 0.01 *M* $KH_2PO_4$ at pH 4.75; pressure, 210 p.s.i.; temperature, ambient; wavelength, 254 nm. 1 = Uracil; 2 = adenine; 3 = adenosine. (Reproduced by courtesy of *Laboratory Practice*[71].)

separations obtained compare well with those reported in the literature for barbiturates[68–70] and for nucleosides and nucleic acid bases[65] in terms of speed and chromatographic efficiency.

## CONCLUSIONS

The main conclusions of this work may be summarised as follows:

(1) Both silica gel and alumina may be chemically modified by reaction with silanes to give materials having a unimolecular surface layer of organic material. These materials are stable over a wide range of solvent composition and pH. They exhibit partitioning properties which are radically different from those of the unmodified adsorbents.

(2) Chemical modification of an adsorbent can improve the plate height obtained with the material. Thus the ODS/TMS- and SAS-silicas showed significantly lower plate heights than the spherical silica gel from which they were prepared (and other silica gels for HPLC). Their mass transfer coefficients closely approached the theoretical minimum.

(3) Chemically modified adsorbents, especially when fully reacted, are particularly suitable for the separation of highly polar substances of complex molecular structure which give severely tailed peaks with normal adsorbents. Typical examples

of this type are tetracyclines, morphine alkaloids, and tricyclic antidepressants. In many instances ion pairs appear to be involved in partitioning into the organic phase.

(4) Aminopropyl silica or pyridyl alumina can act both as polar adsorbents or as weak anion exchangers. In the latter capacity, retention of acids on $NH_2$-silica showed the expected response to increase of ionic strength and decrease in pH below 4.5 (namely a decrease), but at pH above 4.5 retention again decreased unexpectedly. This is thought to be due to saturation of the ion-exchange capacity of the amine by negative surface charges on the silica.

(5) The development of new chemically modified adsorbents is likely to have a profound effect on the future usefulness of HPLC.

## ACKNOWLEDGEMENTS

The authors wish to thank Mrs. B. Trafford for skilled experimental assistance, Dr. J. Jurand for permission to reproduce several original chromatograms, and the Materials Preparation Unit, AERE, Harwell, Great Britain, for the gift of samples of Spherisorb Alumina.

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