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Chemically bonded phases for the reversed-phase high-performance liquid chromatographic separation of basic substances

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ABSTRACT

    A chemically bonded phase with a peptide group (PB) for reversed-phase high-performance liquid chromatography (HPLC) is described. This packing was prepared by a two-stage modification of the surface of silica gel with mono- and trifunctional 3-aminopropylsilane and then with an appropriate derivative of a fatty acid. Packings prepared in this way were compared with standard C_{18} materials used in HPLC. Surface characteristics of the packings before and after chemical modification were determined by different physico-chemical methods, *e.g.*, porosimetry, elemental analysis, ^{13}C and ^{29}Si cross-polarization magic angle spinning NMR and HPLC. Chromatographic properties of these packings were evaluated by comparison between log k' of one phase and log k' of a second phase for substances with different chemical natures. The PB packing was found to be especially useful for the separation of basic substances.

INTRODUCTION

    The reversed-phase high-performance liquid chromatographic (RP-HPLC) separation of polar organic substances, especially those with strongly basic character, using conventional chemically bonded phases (CBP) poses many problems, connected with the strong interactions between the chromatographed substance, the mobile phase and the modified packing surface [1.2]. Hence modification of the composition of the mobile phase (careful adjustment of pH and/or addition of ionic substances) is necessary [3,4]. Improved separation selectivity can also be obtained by tailoring the properties of the column packing material [5,6].

    Hydrophobic packing materials with different lengths of the alkyl chains, *e.g.*, C_2, C_8 and C_{18}, are available for RP-HPLC [6–10]. In addition, many HPLC applications require packings containing polar functional groups (NO_2, NH_2, CN or/and OH) bonded to short alkyl chains (C_3, C_4) [7–9]. Various specific packing

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materials, *e.g.*, donor–acceptor, chiral and chelate CBPs, have also been elaborated [9,11–13].

In order to increase the separation selectivity and to widen the analytical capabilities of RP-HPLC techniques, increased attention has centred on the preparation of packing materials suitable for the selective determination of compounds belonging to a group of substances with similar physical and/or chemical properties, *e.g.*, amino acids, peptides, pharmaceuticals. In only a few papers, however, is information given concerning the preparation procedure, surface characterization and the determination of physico-chemical properties of these packing materials [14,15]. This information is very important, however, particularly when the retention mechanism during the chromatographic process is to be studied [16]. Hence for this purpose various relationships between the capacity factor (k') and mobile phase composition or/and different properties of separated compounds, *eg.*, polarity, molecular building or molecular weight, have been used [5,9,16,17]. All of these factors have an influence on the reproducibility of the retention data, particularly during routine analysis.

In this paper, a new chemically bonded phase carrying a peptide group (PB) suitable for the RP-HPLC separation of various polar substances is described. On the basis of the physico-chemical surface characteristics of the prepared phases, the chromatographic properties were compared with those of standard C_{18} packing materials. In addition, the PB phases were applied to the separation of some basic drugs ($pK_a = 9.8$–11.6) and a paracelsin–peptide mixture.

EXPERIMENTAL

Materials and reagents

LiChrospher Select B (E. Merck, Darmstadt, Germany) was used as the support for the preparation of CBPs. The physico-chemical characteristics of this unmodified silica gel are listed in Table I.

For chemical modification. 3-aminopropyldimethylmethoxysilane ($^M NH_2$), 3-aminopropyltriethoxysilane ($^T NH_2$) (Fluka, Buchs, Switzerland), octadecyldimethylchlorosilane (MC_{18}) (Wacker, Munich, Germany), stearic acid (Aldrich-Chemie,

TABLE I

PHYSICAL CHARACTERISTICS OF BARE LICHROSPHER SELECT B

Characteristic	Abbreviation	Value
Particle size (μm)	d_p	5.0
Specific surface area (m^2/g)	S_{BET}	570
Mean pore diameter (nm)	D	5.76
Pore volume (cm^3/g)	V_p	0.96
Concentration of surface accessible silanol groups[a] (μmol/m^2)	α_{SiOH}	5.18
pH, 5% aqueous suspension[b]		5.01

[a] According to ref. 18.
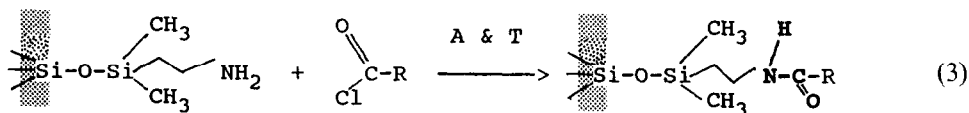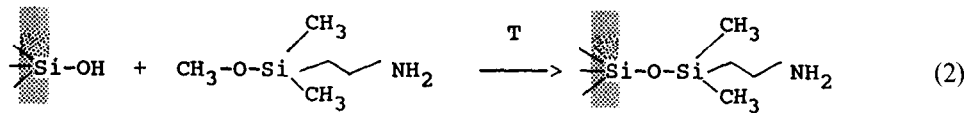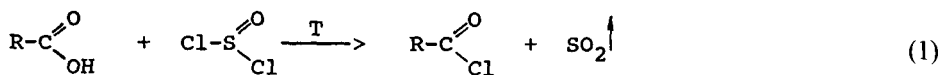[b] According to ref. 19.

Steinheim, Germany) and specially prepared dry morpholine [19] (Riedel de Haën, Seelze, Germany) were used. Methanol, toluene and *n*-hexane (E. Merck) were used. All reagents were of analytical-reagent grade.

The prepared phases were packed into 125×4.6 mm I.D. and 60×4.6 mm I.D. stainless-steel tubes purchased from Bischoff (Leonberg, Germany).

Methanol, acetonitrile, water for HPLC (E. Merck) and phosphate buffer (pH 2.3) were used to prepare mobile phases for the chromatographic investigations.

Preparation of CBPs

The phases with a peptide group (PB) were prepared in a two-stage reaction. The silica gel was first treated with monofunctional (^{M}NH$_2$) or trifunctional (^{T}NH$_2$) aminopropylsilane modifiers, followed by reaction of the amino groups with stearyl chloride. This derivative was prepared in our laboratory according to ref. 20 (eqn. 1). In general, the reactions of PB phases can be illustrated by eqns. 1–3, where R is $C_{18}H_{37}$, T is heating and A is reaction activator (morpholine).

$$R-C\overset{O}{\underset{OH}{\diagdown}} \; + \; Cl-S\overset{O}{\underset{Cl}{\diagdown}} \; \xrightarrow{\;T\;} \; R-C\overset{O}{\underset{Cl}{\diagdown}} \; + \; SO_2\uparrow \tag{1}$$

$$\equiv Si-OH \; + \; CH_3-O-Si\overset{CH_3}{\underset{CH_3}{\diagdown}}\!\!\!\sim\!\!\!\sim\!\! NH_2 \; \xrightarrow{\;T\;} \; \equiv Si-O-Si\overset{CH_3}{\underset{CH_3}{\diagdown}}\!\!\!\sim\!\!\!\sim\!\! NH_2 \tag{2}$$

$$\equiv Si-O-Si\overset{CH_3}{\underset{CH_3}{\diagdown}}\!\!\!\sim\!\!\!\sim\!\! NH_2 \; + \; \overset{O}{\diagup}\!\!\!C-R \; \xrightarrow{\;A\;\&\;T\;} \; \equiv Si-O-Si\overset{CH_3}{\underset{CH_3}{\diagdown}}\!\!\!\sim\!\!\!\sim\!\! N\overset{H}{\underset{O}{\diagdown}}C-R \tag{3}$$

Silica gel (5 g) was dried at 180°C under vacuum (10^{-3} Pa) in a glass reactor for 12 h. Portions of 3 g of silica gel were reacted with 0.3 *M* 3-aminopropyldimethyl-methoxysilane or 0.1 *M* 3-aminopropyltriethoxysilane and heated to 110 ± 5°C for 8 h (eqn. 2). The product was filtered off and washed with dry toluene, methanol and *n*-hexane and dried at room temperature.

This intermediate product was heated at 120°C under vacuum (10^{-3} Pa) for 12 h and then allowed to react at 110 ± 5°C for 8 h with a 1:1 molar ratio of stearyl chloride in dry morpholine as an activator [19,21] (eqn. 3). The final product was filtered off, washed with dry toluene, methanol and *n*-hexane and dried at room temperature.

The packing with standard ^{M}C$_{18}$ phase was prepared using octadecyldimethyl-chlorosilane according to the procedure described previously [19,21].

The characteristics of the prepared packings are listed in Table II.

Apparatus

The porosity parameters characterizing the starting material were determined by the BET method on the basis of the low-temperature adsorption–desorption of

nitrogen using a Model 1800 automatic sorption apparatus (Carlo Erba, Milan, Italy).

The concentration of surface silanol groups (α_{SiOH}) of bare LiChrospher SB was determined using the method proposed by Nondek and Vysočil [18].

The pH of an aqueous suspension of the bare packing material was determined with a Model E-512 pH meter (Metrohm, Herisau, Switzerland).

The density of coverage (α_{RP} and/or $n\alpha_{RP}$) of the CBPs was calculated from the percentage of carbon (P_c) and nitrogen (P_N) as determined with a Model 1104 elemental analyser (Carlo Erba).

Solid-state NMR measurements were performed on a Bruker (Karlsruhe, Germany) MSL 200 spectrometer with 200–300-mg samples in double-bearing rotors of zirconia. Magic angle spinning (MAS) was carried out at a spinning rate of 4 kHz. ^{29}Si cross-polarization (CP) MAS NMR spectra were recorded with a pulse repetition time of 2 s. In the case of ^{13}C CP-MAS NMR spectra a contact time of 12 ms was applied. All NMR spectra were externally referenced to liquid tetramethylsilane and the chemical shifts are given in parts per million.

Chromatographic measurements were made with a Model LC-31 liquid chromatograph (Bruker) with a Model 7121 sampling valve (Rheodyne, Berkeley, CA, U.S.A.) and Model 3380A computing integrator (Hewlett-Packard, Karlsruhe, Germany). Chromatograms were recorded at 206, 220 and 254 nm using also a Model 655 A UV–VIS spectrophotometer (Hitachi-Merck, Darmstadt, Germany).

Column packing procedure

PB phases were shaken in an ultrasonic bath for 5 min with 38 ml of tetrachloroethylene–1-propanol (2:1, v/v) (for $^M C_{18}$ phase the slurry composition was 1 g of prepared packing in 35 ml of isopropanol). The slurry was then filled in the columns using 150 ml of methanol as a packing solvent. All columns were packed using a Shandon packing pump (Shandon, Frankfurt, Germany) under a pressure of 50 MPa according to procedures described previously [22].

RESULTS AND DISCUSSION

Surface characterization of CBP

Table II gives the elemental analysis data for the various packings obtained by chemical modification of bare silica gel (the most important surface characteristics of the bare support are listed in Table I). Table II also gives the coverage densities (α_{RP} and $n\alpha_{RP}$), which were calculated using equations presented previously [23]. Fig. 1 shows the ^{29}Si CP-MAS NMR spectra of unmodified and modified material packings.

From the data in Table II it can be seen that a *ca.* 20% higher density of coverage is obtained with mono functional ($^M NH_2$; packing No. 1) than trifunctional aminosilancs ($^T NH_2$; packing No. 2; eqn. 2). These observations were confirmed by ^{29}Si CP-MAS NMR measurements. On the basis of the ^{29}Si CP-MAS NMR investigations, it is possible to define the undersurface structure of chemically bonded films (Fig. 1A-b and B-b). From analysis of the spectra presented in Fig. 1A and B, it can be concluded that the first stage of chemical modification with aminosilanes reduces the silanol contribution. Almost total blocking of the geminal silanol groups takes place (Q_2; $\delta = -91$ ppm). The contribution of the free silanol groups (Q_3; $\delta = -100$ ppm) decreases and the M ($\delta = +13$ ppm), T_2 ($\delta = -56$ ppm), T_3

TABLE II

CHARACTERISTICS OF MODIFIED PACKINGS

CBP = chemically bonded phase; P_c = percentage of carbon; P_N = percentage of nitrogen; α_{RP} = concentration of chemically bonded groups (μmol/m^2); $n\alpha_{RP}$ = number of alkylsilyl ligands per unit surface area (nm^{-1}); M = monofunctional silane; T = trifunctional silane; NH$_2$ = amino phase; PB = peptide bond.

No. of packing	Type of packing	Type of CBP	Coverage			
			P_C	P_N	α_{RP}	$n\alpha_{RP}$
1	LiChrospher ^{M}NH$_2$	Monomer	6.74	1.58	2.89	1.74
2	LiChrospher ^{T}NH$_2$	Polymer	5.82	1.19	2.38	1.43
3	LiChrospher ^{M}PB	Monomer	19.75	1.64	2.33	1.33
4	LiChrospher ^{T}PB	Polymer	14.29	1.22	1.82	1.06
5	LiChrospher ^{M}C$_{18}$	Monomer	23.21	--	2.42	1.46

($\delta = -60$ ppm) and T$_4$ ($\delta = -66$ ppm) peaks appear in the spectrum. These peaks correspond to the bonded alkylsilyl ligands. Peak M corresponds to the monofunctional structure of CBP whereas peaks T$_2$, T$_3$ and T$_4$ correspond to the net polymer structure formed [23–26].

Reaction of the amino groups with chloro derivatives of a fatty acid (see eqn. 3) causes a significant increase in the surface carbon content (P_c): 65% for the monofunctional structure of the packing (packing No. 3) and 60% for the net polymer (packing No. 4, see Table II). The α_{RP} values also indicate that a higher coverage
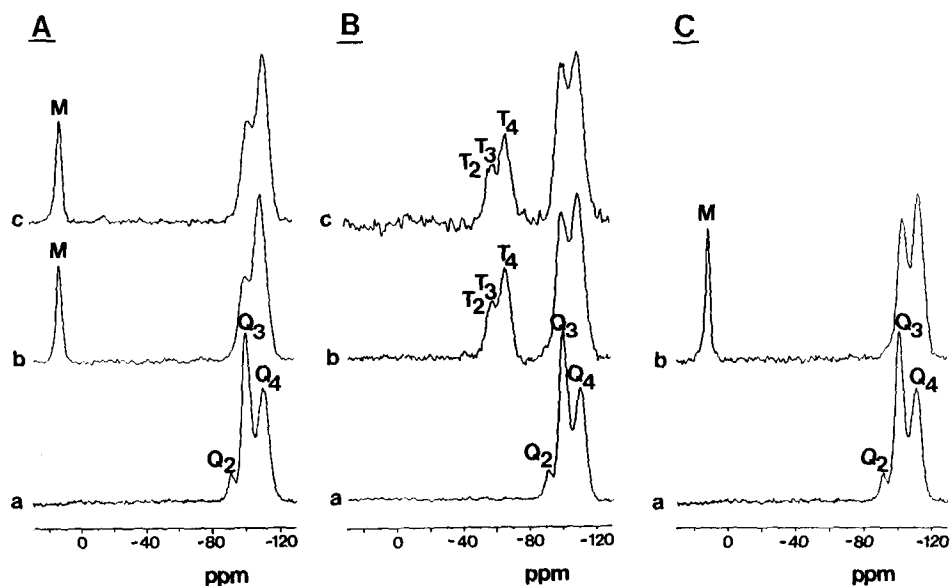

Fig. 1. ^{29}Si CP-MAS NMR spectra of bare silica gel (A, B, C-a) with (A-b) ^{M}NH$_2$ phase, (A-c) ^{M}PB phase, (B-b) ^{T}NH$_2$ phase, (B-c) ^{T}PB phase and (C-b) ^{M}C$_{18}$ phase.

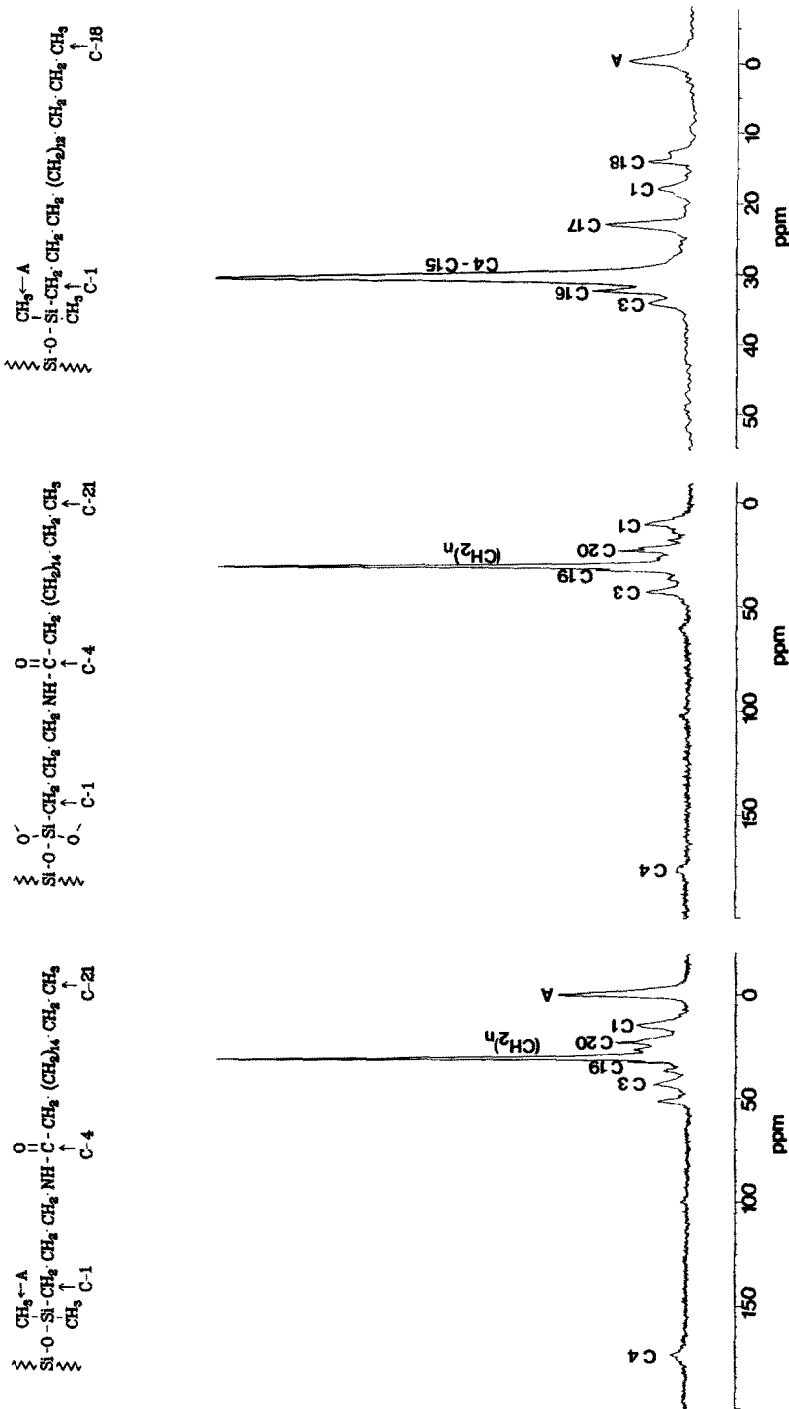Fig. 2. ^{13}C CP-MAS NMR spectra of modified silica gel with (a) ^{M}PB phase, (b) ^{T}PB phase and (c) $^{M}C_{18}$ phase.

density was obtained with the C_{18} packing (No. 5), but the alkylsilyl chains bonded with the support surface are shorter (by three methylene groups) than bonded PB ligands. However, this difference is not large (*ca.* 5% for monofunctional packings 3 and 5). Therefore, evaluation and comparison of the physico-chemical properties of both monofunctional phases were of interest. In order to obtain information on the difference between $^M C_{18}$ and ^{M}PB phases, these were investigated by ^{13}C CP-MAS NMR spectroscopy. The spectra obtained are presented in Fig. 2.

From Fig. 2a–c, it is apparent that after the reaction of amino groups with chloro derivatives of a fatty acid (packings 1 and 2; see eqn. 3), almost all accessible NH_2 groups are blocked and peptide bonds formed [27]. In Fig. 2a and b the C_4 peak ($\delta = +174$ ppm) is observed. With the monofunctional reagent, a single siloxane bond $[\equiv Si-O-Si(CH_3)_2-]$ is formed ($\delta = -2.5$ ppm) [24,25]. When the surface is modified with a trifunctional ethoxysilane, the ethanol liberated is adsorbed on the residual silanol groups via hydrogen bonding. This is reflected in a small peak ($\delta = +61.8$ ppm) in the spectrum (Fig. 2b). This effect has been described previously by Bayer *et al.* [25] for the adsorption of methanol during surface modification with methoxysilanes. The signal corresponding to the alkylsilyl part of bonded ligands for all three packings is localized between $\delta = +10$ and $+40$ ppm [24,25].

Chromatographic properties

The chromatographic behaviour of the prepared packings was evaluated with two chemically different classes of substances, alkylbenzenes and anilines. In Fig. 3, the relationship between the logarithm of the capacity factors (k') and the number of methylene groups in the alkyl chain (n_c) of alkylbenzene homologues is shown. The similarity of the plots indicates that the hydrophobic properties of the ^{M}PB and $^M C_{18}$ phases (packings 3 and 5, Table II) are almost identical (Fig. 3). However, the difference in the slopes of the lines for $^M C_{18}$ and ^{M}PB packings may be caused by stronger interactions between C_{18} chains and chains originating from alkylbenzenes.
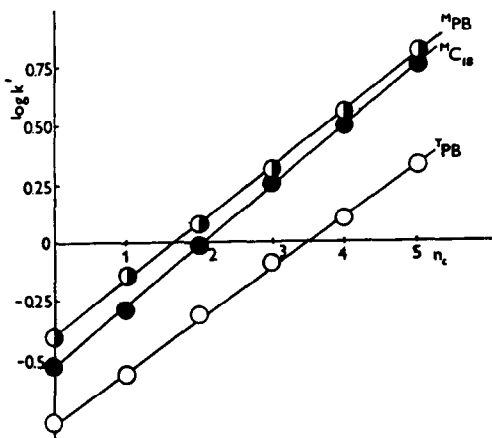

Fig. 3. Dependence of log k' on the number of methylene groups (n_c) in the homologous series of alkylbenzenes (benzene, toluene, ethylbenzene, propylbenzene and butylbenzene) for packings ^{M}PB, ^{T}PB and $^M C_{18}$ (see Table II).
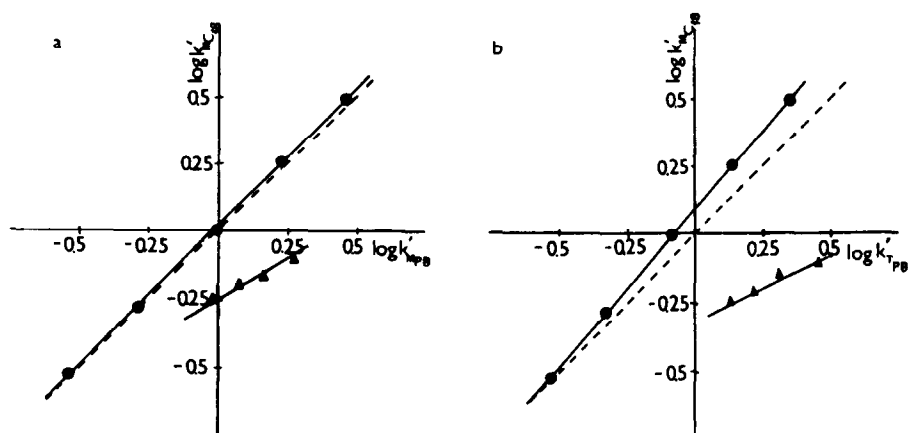
Fig. 4. Relationships between (a) $\log k'$ for $^M C_{18}$ and $\log k'$ for $^M PB$ and (b) $\log k'$ for $^M C_{18}$ and $\log k'$ for $^T PB$ phases for (●) alkylbenzenes (see Fig. 3) and (▲) anilines (aniline, methylaniline, dimethylaniline and diethylaniline.

This effect appears particularly with an increase in the alkyl chain length in the solute molecule (n_c values). The weaker hydrophobic interactions observed with the $^M PB$ phase are probably caused by a strong charge localized in the peptide group. This conclusion was also confirmed by the plot obtained for the $^T PB$ phase (packing No. 4; Fig. 3), which was also a straight line with a slope similar to that of the $^M PB$ packing. The vertical displacement of the plot of the $^T PB$ phase in relation to the $^M PB$ phase can be attributed to the lower coverage density of the net polymer structure of CBP on the silica gel surface. This influences the degree of shielding of the residual silanol groups, which also participate in the RP-HPLC elution process. This effect is clearly seen in Fig. 4, where $\log k'$ of the test substances on one phase is plotted against the corresponding $\log k'$ values on a reference phase ($^M C_{18}$).

According to hydrophobic theory [1–5,16,17], when using the $\log k'$–$\log k'$ relationship for comparison of two packings with similar surface properties compensation of the interactions between the separated solutes the mobile phase and the surface of the material packing should be expected. In this connection, the slopes of the straight line plotted for both packings should be equal to unity (dashed lines in Fig. 4a and b). Different effects taking place during RP-HPLC elution in chromatographic columns cause the deviations of real straight lines from the theoretical lines.

Considering the $\log k'$ *vs.* $\log k'$ relationship for both monofunctional phases (packings 3 and 5; Fig. 4a), it can be stated that the experimental result of this relationship for alkylbenzenes is virtually identical with the theoretical assumption. Small differences in the slopes of straight lines may result from different α_{RP} values (Table II). In both instances the hydrophobic character of the packings can be observed (chain–chain interactions). An effect of the peptide group on the elution of aniline is also observed. However, the residual silanol groups can also participate in the elution mechanism. This effect manifests itself in the correlation of $\log k'$ *vs.* $\log k'$ for $^T PB$ and C_{18} phases (Fig. 4b). In this instance the slopes of the straight lines for both test mixtures (alkylbenzenes and anilines) were significantly greater, as shown by the

deviations from unity. The differences in α_{RP} values were also higher (Table II). Moreover, the silanolophobic interactions also influence this effect [1–6,9,16,17]. As a consequence, for the ^{T}PB packing, greater mobility of the bonded chains can be expected owing to the nitrogen atom (in the peptide group) which has a strong tendency for interaction [26–28]. Hence the influence of these effects is better represented as in Fig. 5.

On the basis of Fig. 5, a comparison of both phases containing the peptide group and a different undersurface structure of CBP was made. The linear dependences of alkylbenzenes and anilines indicate that (i) the hydrophobic interactions are preferentionally found for the packings with a monofunctional structure of PB, (ii) the influence of silanols is more significant for packings obtained using a trifunctional silane, (iii) the straight line shift for alkylbenzenes is due to differences in α_{RP} values and (iv) the peptide group does not play an important role in the retention mechanism of non-polar solutes as is the case with polar solutes.

The comparion of the separation of the mixture of basic substances (pK_a = 8.98–11.74) presented in Fig. 6 is interesting especially from the viewpoint of selectivity. Daldrup and Kardel [29] proposed three of these four substances (peaks 1, 3 and 4) as sensitive indicators of the quality of test packings. We added melperone [4'-fluoro-4-(4-methylpiperidino)butyrophenone, pK_a = 9.2] to the test mixture. In Table III the most important retention parameters (k' and $\alpha_{i,j}$) and asymmetry factors (f_{As}) are listed.

On comparing the chromatograms in Fig. 6a–c, it can be seen that the separation of all four substances was only been obtained on the packings containing the peptide group. In both instances the peaks are symmetrical and sharp and the retention time is relatively short (Table III). However, improved resolution and separation selectivities between peaks 1 and 2 and between 3 and 4 were obtained on the packing with a monofunctional structure of the PB phase. Although the retention time was about half of that for a conventional C_{18} HPLC packing (Fig. 6c), peaks 1 and 2 could not be resolved (Table III). The C_{18} phase also has a tendency to produce tailing, particularly
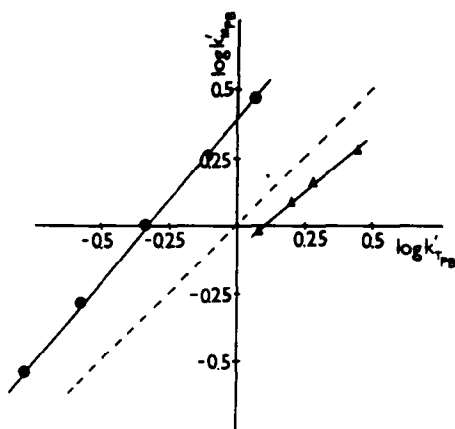

Fig. 5. Relationship between log k' for ^{M}PB and log k' for ^{T}PB phases obtained by injection of alkylbenzenes and anilines (abbreviations as in Fig. 4).
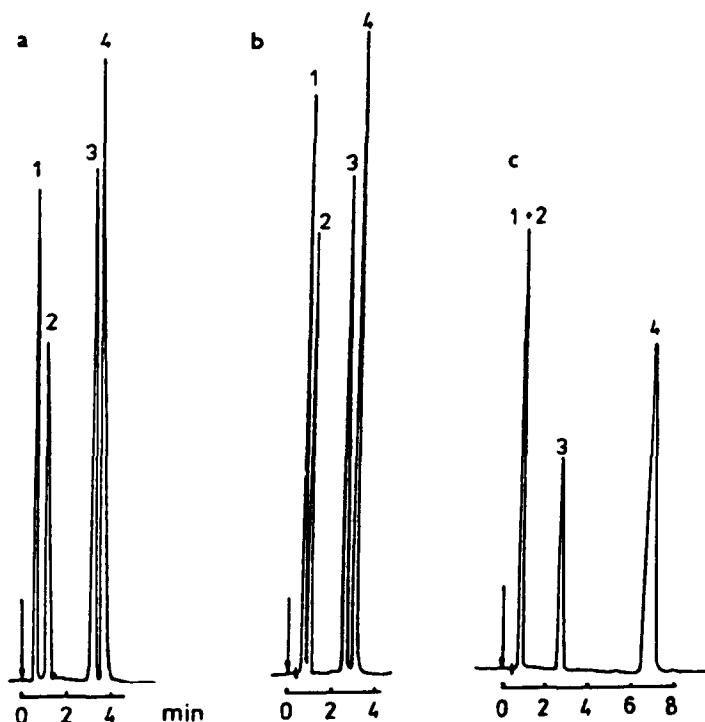
Fig. 6. Chromatograms of the test mixture obtained on columns packed with (a) ^{M}PB phase, (b) ^{T}PB phase and (c) ^{M}C$_{18}$ phase. Peaks: 1 = DPHA; 2 = MELP; 3 = MPPH; 4 = DZPM. For abbreviations and separation conditions, see Table III.

TABLE III

COMPARISON OF k', α_{ij} AND f_{As} VALUES FOR TEST MIXTURE OF SUBSTANCES WITH BASIC CHARACTER, OBTAINED ON COLUMNS 1, 4 AND 5

Mobile phase: 156 ml acetonitrile + 340 ml phosphate buffer, pH 2.3 (6.66 g of KH$_2$PO$_4$ + 4.8 g of 85% H$_3$PO$_4$ in 1 l of water for HPLC). DPHA = diphenylhydramine hydrochloride; MLPN = melperone; MPPH = 5-(p-methylphenyl)-5-phenylhydantoin; DZPM = diazepam.

No. of peak	Compound	No. of column								
		3			4			5		
		k'	α_{ij}	f_{As}	k'	α_{ij}	f_{As}	k'	α_{ij}	f_{As}
1	DPHA	0.61		1.09	0.74		1.25	0.78		1.20
			2.00			1.55			—	
2	MLPN	1.22		1.10	1.15		1.25	—		—
			6.05			2.84			6.14	
3	MPPH	7.20		1.12	3.27		1.30	4.73		1.35
			1.16			1.24			2.91	
4	DZPM	8.60		1.13	4.05		1.50	13.75		1.50

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 1  2  3  4  5  6  7  8  9  10  11  12  13  14  15  16  17  18  19  20
Ac-Aib-Ala-Aib-Ala-Aib-Ala-Gln-Aib-Val-Aib-Gly-Aib-Aib-Pro-Val-Aib-Aib-Gln-Gln-Pheol A
Ac-Aib-Ala-Aib-Ala-Aib-Ala-Gln-Aib-Leu-Aib-Gly-Aib-Aib-Pro-Val-Aib-Aib-Gln-Gln-Pheol B
Ac-Aib-Ala-Aib-Ala-Aib-Aib-Gln-Aib-Val-Aib-Gly-Aib-Aib-Pro-Val-Aib-Aib-Gln-Gln-Pheol C
Ac-Aib-Ala-Aib-Ala-Aib-Aib-Gln-Aib-Leu-Aib-Gly-Aib-Aib-Pro-Val-Aib-Aib-Gln-Gln-Pheol D
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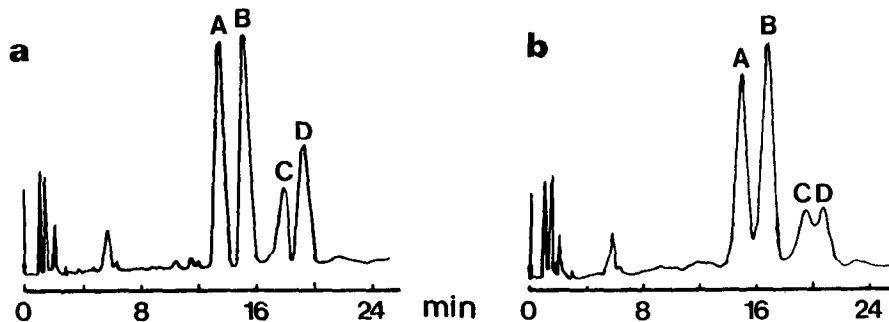
Fig. 7. Amino acid sequence of four peptides of paracelsine (A–D) and RP-HPLC separations of this protein obtained on columns packed with (a) ^{M}PB and (b) ^{M}C$_{18}$ phases. Separation conditions: column, 125 × 4.6 mm I.D.; mobile phase, acetonitrile–methanol–water (34:33:33, v/v/v); flow-rate, 1 ml/min; detection, UV at 206 nm.

for peak 4 (diazepam, Fig. 6c). This can be seen in Table III, where the capacity factors increase with increasing pK_a values. The smallest f_{As} values (1.09–1.13) are found on the packing with a monofunctional PB phase.

HPLC applications

The hydrophobic properties and the specific selectivity of the packings with ^{M}PB phase toward basic substances may be utilized to advantage in the separation of four peptides contained in the protein mixture paracelsine [30]. This natural product, which is particularly important for haemolysis of erythrocytes, contains four peptides with slightly different primary structures of the amino acids in positions 6 and 9 (see Fig. 7). All chiral components of paracelsine (A–D) are of L configuration. In Fig. 7 the chromatograms obtained from the RP-HPLC separation of this substance with (a) ^{M}PB and (b) C$_{18}$ phases (packings 3 and 5. Table II) are shown.

This improved selectivity of the ^{M}PB phase toward this class of substances is apparent, full resolution of peptide pairs A, B, C and D being obtained. In spite of the higher α_{RP} values, the C$_{18}$ phase (Table II) does not allow optimum resolution of the peptides, although the retention times are similar for both phases. This is probably due to specific interactions between the separated proteins and hydrophobic alkylsilyl ligands contaning peptide groups.

CONCLUSIONS

The chemical modification of the LiChrospher Select B surface using ^{M}NH$_2$ silane yielded a higher coverage density (about 20%) than modifying it with ^{T}NH$_2$ silane. This difference in the coverage density was found to be independent of the PB

structures and of the structure of CBP resulting from the first modifying stage with 3-aminopropyl silanes.

CP-MAS NMR investigations not only confirmed the elemental analysis but also gave information about the structure of the CBP formed and detailed insight into the contributions of the different silanol groups (Q_2, Q_3 and Q_4) before and after chemical modification.

RP-HPLC measurements indicated that the hydrophobic interactions between the packing surfaces, solute and mobile phase are preferentially found for packings with a dense film of the monomeric structure of CBP and therefore the mass transfer of the separated substances is better.

For the separation of polar substances (particularly with a basic character), better selectivity and resolution were found for packings involving the peptide group than for conventional C_{18} packings. In addition, the material containing a PB group was successfully applied to the separation of peptides and proteins.

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