Properties and biomedical applications of packings with high-density coverage of C_{18} chemically bonded phase for high-performance liquid chromatography and solid-phase extraction[a]

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ABSTRACT

A series of packing materials with high-density coverage of chemically bonded C_{18} phases ($\alpha_{RP} \geqslant$ 2.8 μmol/m^2) for high-performance liquid chromatography (HPLC) and clean-up solid-phase extraction columns were prepared. The resulting packings were characterized by different physico-chemical methods including porosimetric and elemental analysis, cross-polarization magic angle spinning NMR and HPLC. The packings prepared were used for the purification by solid-phase extraction, isolation and determination of various analytes present in different biological samples.

INTRODUCTION

In spite of the elaboration and successive introduction of new types of packings for reversed-phase high-performance liquid chromatography (RP-HPLC), *e.g.*, porous graphitic carbon, carbosil and polymers [1–3], packings with chemically bonded phases (CBP) have been increasingly used. This is a result of their physico-chemical properties and relatively greater stability under the conditions of the elution process. Moreover, the possibility of preparing packings with different functional groups, *e.g.*, NH$_2$, NO$_2$ or CN [4,5], has led to an increase in separation selectivity.

In order to improve the separation selectivity and the stability of the chemically bonded film formed on the siliceous support surface, a packing with high-density coverage was developed [6,7]. Using these packing materials, an increase in hydrophobic chain–chain and chain–solute interactions has been observed [8]. On the other hand, the influence of silanol groups is limited because these active groups are

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effectively screened by long alkyl chains, so that interaction between residual silanols and polar solutes is almost impossible [8,9]. The reproducibility of the retention data and prolongation of the lifetime of these packings [10] are very important in routine clinical, biomedical, biochemical and pharmacological analysis.

Most of these applications relate to the separation of polar substances. Their separation using conventionally manufactured packing materials often gives repeatedly unsatisfactory results. Considering that clinical, biochemical and/or pharmaceutical analysis involves biological matrices, e.g. urine, blood, serum and tissue, which contain potentially interfering constituents, it is necessary to carry out an initial purification of these samples, without a quantitative or qualitative loss of the compounds to be determined. For this purpose, solid-phase extraction (SPE) on off-line clean-up columns can be ideal [11,12]. As a consequence, the purification and preconcentration of substances to be determined is possible, and a higher degree of analytical sensitivity can be obtained.

In this paper, the physico-chemical and chromatographic properties of packings with a high-density coverage of chemically bonded C_{18} phases are presented for analyses by RP-HPLC and SPE, using various biological samples as a test substances.

EXPERIMENTAL

Materials and reagents

Two types of silica gel, SG-7/G and SG-100, prepared in the Polymer Institute of Sciences (Bratislava, Czechoslovakia) [13,14], were used as the support for the preparation of chemically bonded phases (CPBs) for HPLC and clean-up SPE columns. The surface physico-chemical characteristics of the bare materials are given in Table I.

For chemical modification, momofunctional (MC_{18}), difunctional (DC_{18}) and/or trifunctional (TC_{18}) octadecylchlorosilanes (Wacker, Munich, F.R.G.) were used, and especially prepared morpholine was employed as an activator [15] (Riedel de Haën, Seelze, F.R.G.). All solvents, e.g., toluene, benzene, hexane, methanol, acetonitrile and water (Merck, Darmstadt, F.R.G.), were of analytical-reagent grade.

Stainless-steel tubes (125 × 4.6 mm I.D.) were purchased from Grom and Stagroma (Herrenberg, F.R.G.).

TABLE I

PHYSICO-CHEMICAL PROPERTIES OF BARE SILICA GELS USED AS PACKING MATERIALS IN HPLC AND SPE COLUMNS

Type of silica	Fraction d_p (μm)	Porosity[a]			α_{SiOH}[b]	Use
		S_{BET}	V_p	D		
SG-7/G	7	361	2.1	20	5.21	HPLC
SG-100	43–65	196	2.1	>23	4.93	SPE

[a] S_{BET} = specific surface area (m²/g); V_p = pore volume (cm³/g); D = mean pore diameter (nm).
[b] α_{SiOH} = concentration of silanol groups (μmol/m²).

TABLE II

CHARACTERISTICS OF PACKINGS WITH CHEMICALLY BONDED PHASES (CBP)

No. of packing	Type of packing	Type of CBP structure[a]	Coverage[b]		
			P_c	α_{RP}	α_{SiOH}
1	SG-7/G-^{M}C$_{18}$	M	15.23	2.20	2.97
2	SG-7/G-^{M}C$_{18}$	M	19.64	3.04	2.26
3	SG-7/G-^{M}C$_{18}$	M	25.53	4.25	0.82
4	SG-100-^{M}C$_{18}$	M	15.75	4.20	—
5	SG-100-^{M}C$_{18}$	M	8.46	2.02	—
6	SG-100-^{M}C$_{18}$	T	13.21	3.72	—

[a] M = monomeric structure of CBP; T = polymeric structure of CBP.
[b] P_c = measured carbon percentage (%); α_{RP} = degree of alkylsilyl ligand coverage (μmol/m^2); α_{SiOH} as in see Table I.

Chemical bonding procedure

The chemical surface modification was carried out in glass reactors under nitrogen. The method, mechanism and reaction conditions have been described previously [16,17]. The parameters characterizing the coverage density of prepared packings are listed in Table II.

Preparation of columns

A slurry of 2 g of the prepared HPLC phases in 35 ml of isopropanol was placed in an ultrasonic bath for 5 min and then filled into the column using 150 ml of methanol as a packing solvent. All HPLC columns were packed under a pressure of 50 MPa with a Shandon (Frankfurt, F.R.G.) packing pump according to the procedure described earlier [18].

The SPE columns were prepared by packing 2-ml plastic extraction tubes with the appropriate dry materials to a bed height of 2 cm.

Apparatus

The porosity parameters of the materials (S_{BET} = specific surface area, V_p = pore volume, D = mean pore diameter) were determined by low adsorption–desorption of liquid nitrogen using a Sorptomatic Model 1800 instrument (Carlo Erba, Milan, Italy).

The degree of alkylsilyl ligand coverage on the surface of the prepared packings was calculated from the carbon content, determined with a CHN Model 240 analyser (Perkin-Elmer, Norwalk, CT, U.S.A.).

Solid-state NMR measurements were performed on a Brucker MSL 200 spectrometer with samples of 200–300 mg in double-bearing rotors of ZrO_2. 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 length of 5 μs together with a contact time of 5 ms and a pulse repetition time of 2 s. For ^{13}C CP-MAS NMR spectra a contact time of 12 ms was used. All NMR spectra were externally referenced to liquid tetramethylsilane; chemical shifts are given in parts per million (ppm).

Fig. 1. CP-MAS NMR spectra and possible surface structure of silica gel before and after chemical modification. (a) ^{29}Si NMR spectra for (A) unmodified silica, (B) packing No. 4 modified by $^M C_{18}$ silane and (C) packing No. 6 modified by $^T C_{18}$ silane; (b) ^{13}C NMR spectrum for packing No. 3 modified by $^M C_{18}$.

RESULTS AND DISCUSSION

Characterization of CBP

In previous papers [9,10] it has been shown that by changing the preparation conditions of the chemically bonded phase (CBP) (specifically the molar ratios of individual reaction compounds), it is possible to obtain packing materials with a controlled density of coverage of CBP.

Table II presents the values characterizing the density of coverage of C_{18} CBP. From these data it appears that using silica with wide pores (Table I) as a support for CBP, packings with a high-density coverage were obtained ($\alpha_{RP} \geqslant 4.0$ $\mu mol/m^2$). Moreover, comparing α_{RP} and α_{SiOH} values, it can be seen that more than 85% of the surface-accessible silanol groups have been efficiently blocked by alkylsilyl ligands. Consequently, the packings obtained could be considered as homogeneously covered. This is confirmed by ^{29}Si and ^{13}C CP-MAS NMR spectra, as show in Fig. 1. These spectra also give information about the structure of the CBP formed during the chemical modification process [19,20].

Analysis of the example spectra shows that covalent siloxane bonds have been formed between the silica support surface and the chemical modifier (peak M in Fig. 1a, $\delta = +13$ ppm, and peak A″ in Fig. 1b, $\delta = +2.3$ ppm). Associated with the increase in α_{RP} values, there is a decrease in the individual silanol group content, *e.g.*, $Q_2, \delta = -91$ ppm; $Q_3, \delta = -100$ ppm; $Q_4, \delta = -108$ ppm [9,19,20]. Monofunctional silane gives pure monomeric structures of CBP with the creation of single $\equiv Si-O-Si \equiv$ bonds, but using trifunctional silane a polymeric network structure of CBP was obtained (peaks $T_2, \delta = -56$ ppm; $T_3, \delta = -60$ ppm; T_4 and $T_4, \delta = -66$ ppm) (Fig. 1a) [10,21]. These structures of CBP contain a relatively large population of the residual silanol groups.

HPLC applications

It is known that unblocked, residual silanol groups can play an important role in the elution process of solutes. This has been demonstrated by considerable differences in the capacity factor (k') values, low resolution (R_s) values and peak tailing (*i.e.*, higher asymmetry factor, f_{As}). The last effect is especially important during the routine analysis of polar substances, where the interactions between the analyte substances and the residual silanol groups is more apparent. Fig. 2 shows example chromatograms of five purines separated from blood serum, run on two columns filled with packings with different values of α_{RP}. The retention data (k') for all three columns are listed in Table III.

Comparing the k' values, it can be seen that changing the coverage density leads to a change in the separation selectivity, and that better separations are obtained using column No. 3 with $\alpha_{RP} = 4.25$ $\mu mol/m^2$ (Tables II and III). It can be seen that the residual silanol groups are well shielded by the dense film of CBP formed and therefore silanolophobic interactions are minimized. Moreover, when these packings are used for the determination of substances of biological origin, this leads to an increase in detection sensitivity (of about two orders of magnitude) and prolongation of the column lifetime. These are very important factors in routine analysis.

Fig. 3 presents the test proposed by Daldrup and Kardel [22] for substances characterized by their organic base nature, which were obtained on the packing with
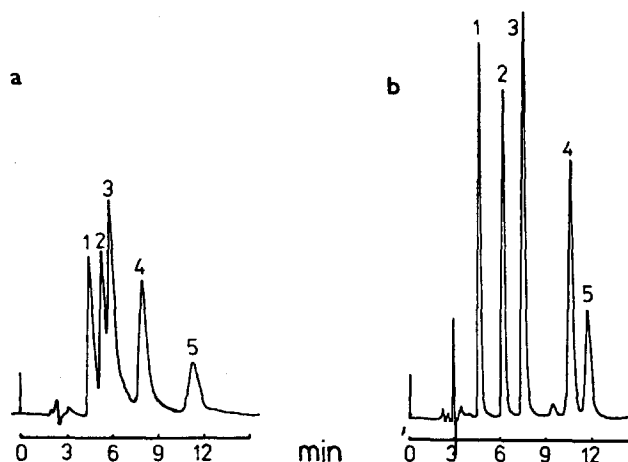
Fig. 2. Separation of purines (6.2 μg/μl) (theophylline and its metabolites) on columns packed with C_{18} chemically bonded phases. (a) α_{RP} = 2.20 μmol/m^2; (b) α_{RP} = 4.25 μmol/m^2. Peaks numbers as in Table III. Chromatographic conditions: column, 125 × 4.6 mm I.D.; mobile phase, acetonitrile–water (10:90) containing 0.1 M acetic acid; flow-rate, 0.9 ml/min; detection, UV (273 nm).

a high-density coverage (packing No. 3). The chromatogram shows that the peaks of individual substances are symmetrical and that the separation is characterized by a large peak capacity [23]. This gives a further important reason for using these packings in routine analysis, considering the higher sorption capacity and better mass transfer obtained.

Fig. 4 shows the separation with electrochemical detection of catecholamines isolated from human urine by means of the SPE clean-up column. The separation selectivity is high and the resolution of individual substances was obtained in a relatively short time (*ca.* 32 min).

SPE applications

Many factors determine the efficiency and effective application of SPE for the isolation and/or clean-up of substances in biological samples. The type of packing used, structure of CBP, the height of the packing bed and the appropiate choice of the

TABLE III

CAPACITY FACTORS OF PURINE BASES ISOLATED FROM HUMAN BLOOD SERUM

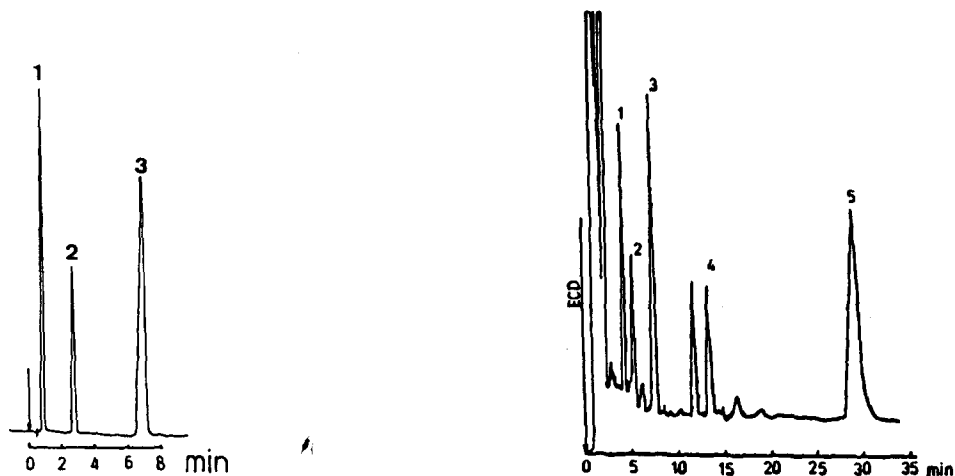| No. of peak	Substance	Capacity factor (k')		
		Column 1[a]	Column 2[a]	Column 3[a]
1	Theophylline	1.16	0.90	0.57
2	Theobromine	2.80	1.54	1.21
3	8-Methoxymethyltheophylline	3.02	1.79	1.59
4	7-(2-Hydroxymethyl)theophylline	3.88	2.47	2.63
5	Caffeine	5.07	3.87	2.98

[a] See Table II.

Fig. 3. Test mixture according to ref. 22 where 1 = diphenylhydramine chloride (0.5 μg/μl), 2 = 5-(p-methylphenyl)-5-phenylhydantoin (0.25 μg/μl) and 3 = diazepam (0.25 μg/μl). Chromatographic conditions: column, 60 × 4.6 mm I.D. with packing No. 3; mobile phase, 156 ml of acetonitrile + 340 ml of phosphate buffer (pH 2.3); flow-rate, 0.5 ml/min; detection, UV (220 nm).

Fig. 4. Separation of catecholamines isolated from human plasma, where 1 = noradrenaline (3.5 mmol/l), 2 = adrenaline (3.0 mmol/l), 3 = dihydroxybenzylamine (4.0 mmol/l), 4 = dopamine (4.2 mmol/l) and 5 = serotonin (3.5 mmol/l). Chromatographic conditions: column, 125 × 4.6 mm I.D. with packing No. 3; mobile phase, 50 mM sodium acetate + 20 mM acetic acid + 3.75 mM sodium octanesulphonate + 1 mM dibutanolamine + 0.134 mM ETDA + 950 ml of water + 50 ml of methanol; detection, electrochemical (working potential + 0.6 V, sensitivity 0.2 mA); flow-rate, 0.8 ml/min.

eluent are the main parameters [11,12,23]. However, these factors are closely connected with the chemical character of the analytes separated.

Fig. 5 shows example control chromatograms, demonstrating the efficiency of the SPE technique during isolation of 5-hydroxyindolacetic acid (5-HIAA) from human urine using an SPE column packed with material No. 4 (α_{RP} = 4.20 μmol/m^2; Table II). These results indicate that the determination of 5-HIAA without prior purification of the biological material is impossible when using electrochemical detection. Based on the adsorption effect, 5-HIAA was sorbed in the first step on the packing bed and then the 5-HIAA was displaced using an eluent with a higher elution strength. A recovery of 96 ± 2% was obtained.

Table IV lists the recoveries for two drugs, Melperone (M) and Paracetamol (P) isolated from individual standard solutions (SE) (6.5 · 10^{-6} g/ml) and also from various biological samples (RR) (M from rat brain, P from human urine) using the SPE clean-up columns packed with materials Nos. 4–6 (Table II).

On comparing the recoveries (in Table IV, it can be seen that for substances of basic character (M, pK_a = 9.6) a better recovery was obtained with the packing with a low concentration of alkylsilyl ligands on the support surface (material No. 4). This confirms that the recoveries of both SE and RR are due to small differences between them. Using the packing with a polymeric network structure of CBP in both instances did not guarantee reproducible recoveries. This is due to irreversible sorption of these substances, resulting from different interactions between the isolated solutes and the modified surface of the packings [8,9,21].
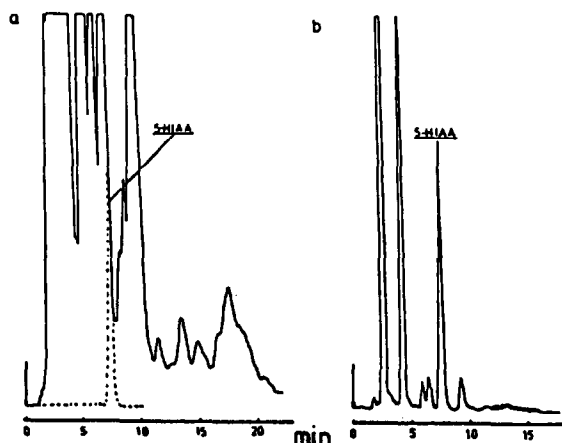
Fig. 5. Chromatograms illustrating the determination of 5-HIAA (167.3 μmol/l) in urine samples by HPLC with electrochemical detection. (a) Direct injection of an untreated urine sample; (b) injection of a purified urine sample using the SPE column with packing No. 4. Chromatographic conditions: column, 125 × 4.6 mm I.D. with packing No. 3; mobile phase, 0.08 M ammonium acetate–methanol (85:15%, v/v) (pH 4.5); flow-rate, 0.8 ml/min; detection, electrochemical (+0.55 V).

TABLE IV

COMPARISON OF RECOVERIES (%) AND RELATIVE STANDARD DEVIATIONS [R.S.D. (%)] FOR MELPERON AND PARACETAMOL ISOLATED FROM VALUES STANDARD SOLUTION (SE) AND BIOLOGICAL SAMPLES (RR)

No. of packing[a]	Melperone				Paracetamol			
	SE	R.S.D.	RR	R.S.D.	SE	R.S.D.	RR	R.S.D.
4	67.2	4.3	52.5	5.2	100.0	1.2	98.2	2.1
5	92.1	2.8	89.4	3.4	37.3	3.9	15.7	5.3
6	27.8	7.6	18.3	9.8	46.1	5.7	31.8	7.3

[a] See Table II.

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REFERENCES

1 J. H. Knox and B. Kaur, in P. R. Brown and R. A. Hartwick (Editor), *High Performance Liquid Chromatography*, Wiley, New York, 1989, Ch. 4, p. 189.
2 D. J. Pietrzyk, in P. R. Brown and R. A. Hartwick (Editors), *High Performance Liquid Chromatography*, Wiley, New York, 1989, Ch. 5, p. 223.
3 B. Buszewski and R. Leboda, *Chem. Stosow.*, 34 (1990) 191.
4 J. Nawrocki and B. Buszewski, *J. Chromatogr.*, 449 (1988) 1.
5 L. R. Sander and S. A. Wise, *CRC Crit. Rev. Anal. Chem.*, 18 (1987) 299.
6 K. Szabó, N. Le Ha, Ph. Schneider, P. Zeltner and E. sz. Kováts, *Helv. Chim. Acta*, 67 (1984) 2128.
7 B. Buszewski, A. Jurášek, J. Garaj, L. Nondek, I. Novák and D. Berek, *J. Liq. Chromatogr.*, 10 (1987) 2325.

8 R. K. Gilpin, *J. Chromatogr. Sci.*, 22 (1984) 371.
9 B. Buszewski, Z. Suprynowicz, P. Staszczuk, K. Albert, B. Pfleiderer and E. Bayer, *J. Chromatogr.*, 499 (1990) 305.
10 B. Buszewski and Z. Suprynowicz, *Anal. Chim. Acta*, 208 (1988) 199.
11 C. K. Lim, F. Li and T. J. Peters, *Int. Lab.*, 16 (1986) 60.
12 B. Tippins, *Int. Lab.*, 17 (1987) 28.
13 D. Berek and I. Novák, *U.S. Pat.*, 4 255 286 (1981) and 4 382 070 (1983).
14 L. Šoltes, I. Novák and D. Berek, *Czech. Pat.*, 234 100 (1983).
15 B. Buszewski, R. Lodkowski and J. Trocewicz, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 10 (1987) 527.
16 B. Buszewski and Z. Suprynowicz, *Chromatographia*, 22 (1987) 573.
17 B. Buszewski, L. Nondek, A. Jurášek and D. Berek, *Chromatographia*, 23 (1987) 442.
18 B. Buszewski, D. Berek, I. Novák and J. Garaj, *Chem. Listy*, 81 (1987) 552.
19 E. Bayer, K. Albert, J. Reiners, M. Nieder and M. Müller, *J. Chromatogr.*, 264 (1983) 197.
20 K. Albert, B. Evers and E. Bayer, *J. Magn. Reson.*, 62 (1985) 428.
21 B. Buszewski, *Chromatographia*, 29 (1990) 233.
22 T. Daldrup and B. Kardel, *Chromatographia*, 18 (1984) 81.
23 B. Buszewski, *J. Pharm. Biomed. Anal.*, 8 (1990) 645.