Journal of Chromatography, 455 (1988) 105–117
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 20 823

GAS PHASE SILYLATION, A RAPID METHOD FOR PREPARATION OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY SUPPORTS

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(First received October 19th, 1987; revised manuscript received May 17th, 1988)

SUMMARY

A new method for preparing chromatographic supports is described. Porous silica was treated with gaseous silanes and gaseous triethylamine at high temperature and reduced pressure. The silylation procedure was rapid and gave supports with a covalently bound monolayer of aminopropyl- or epoxysilane. Diol silica prepared by gas phase silylation was compared with diol silica prepared in the aqueous or organic phase (toluene). Seven proteins were chromatographed and the protein recovery, plate number and peak asymmetry were calculated. Silica prepared by the gas phase silylation method showed improved performance. The separation of carbohydrates on aminopropylsilica and the isolation of lactate dehydrogenase with nicotinamide–adenine dinucleotide–silica, prepared from gas phase silylated diol silica, are described.

INTRODUCTION

Chromatographic supports of inorganic origin, such as silica and controlled pore glass, are usually surface modified with various reagents, for example, alkylsilanes. The silylation procedures applied are usually carried out in organic solvents in the presence of a catalyst such as triethylamine, or in aqueous solution. The procedures often require long reaction times and laborious handling, *e.g.*, drying of solvents, sometimes taking more than 24 h per preparation. Thus, simplification of the procedures is well justified in order to facilitate production of silylated chromatographic supports especially on a larger scale.

Vapour deposition for surface coating for protection of, *e.g.*, painted surfaces, plastics and metal containers, with silane compounds has successfully been employed. Alkylsilanes have been vapour deposited on silicon wafers and on particles by using deposition from condensed silane vapour[1], in condensed thin films[2] or in the gas phase at reduced pressure[3–5]. Attractive features of vapour deposition silylation are the small amount of solvent used and the ease with which monomeric layers are obtained. It has been reported in these studies that the alkyloxysilane reaction requires a reaction time of 12–20 h at elevated temperature. This is longer compared to that in silylation

0021-9673/88/$03.50

methods performed in organic solvents or in aqueous media, and can partially be explained by the absence of the triethylamine catalyst which is used in the solvent methods.

In the present study we have employed a combination of the advantageous properties of vapour deposition (no solvents, monolayer formation) and organic phase/aqueous phase silylation (catalysed reaction) in a new method for preparation of supports for high-performance liquid chromatography (HPLC). This method involves gas phase silylation at reduced pressure and the presence of triethylamine as a catalyst. The silylation reaction is completed within 1–2 h and the total time for the preparation is less than 3 h.

MATERIALS AND METHODS

Materials

LiChrospher Si 300, Si 100, LiChroprep Si 100 and LiChrosorb 100 were obtained from E. Merck (Darmstadt, F.R.G.), 3-Glycidoxypropyltrimethoxysilane (Z-6040) from Dow Corning (Seneffe, Belgium). 3-Aminopropyltriethoxysilane, catalase (bovine liver), lysozyme (chicken egg white), haemoglobin (beef blood), ovalbumin and pepsin (porcine stomach mucosa) were obtained from Sigma (St. Louis, MO, U.S.A.), tresyl chloride (2,2,2-trifluoroethanesulphonyl chloride) from Fluka (Buchs, Switzerland). The nicotinamide–adenine dinucleotide (NAD) analogue, N^6-[N-(2-ami-noethyl)carbamoylmethyl]-NAD, was synthesized according to a previously described method[6].

Gas phase silylation (procedure 1)

Silica was evenly spread in a glass container at the bottom of a heated alumina reactor (Fig. 1). The silica was dried for 1 h at 100–160°C under reduced pressure (< 2 mmHg) using a vacuum pump. Epoxy- or aminosilane was injected with a syringe through a membrane at the top of the reactor. The silylation reaction proceeded for 1–20 h, at a constant temperature in the range 100–160°C. Any excess of silane vapour was finally evacuated from the reactor and the silica cooled to room temperature. In the case of epoxysilylation, coinjection of triethylamine (catalyst) was sometimes carried out.

Gas phase silylation with intermittent hydrolysis of remaining alkoxy groups (procedure 2; recommended procedure)

LiChrospher Si 300 (1.6 g) was placed in the same reactor as above and dried for 1 h at 160°C under reduced pressure using a vacuum pump. Epoxysilane (0.5 ml) and triethylamine (0.25 ml) were injected with a syringe and the reaction was carried out for 1 h at 160°C. Unreacted epoxysilane was removed *in vacuo*. The silica was then removed from the reactor and placed in a test-tube. Water was added and the test-tube placed in a water-bath at 90°C for 15 min (to hydrolyse the remaining methoxy groups). Using a sintered glass funnel, the silica was washed with acetone and again placed in the reactor and dried. The silylation procedure was then repeated.

Aqueous phase silylation

LiChrospher Si 300 (2.0 g) was silylated according to a previously described

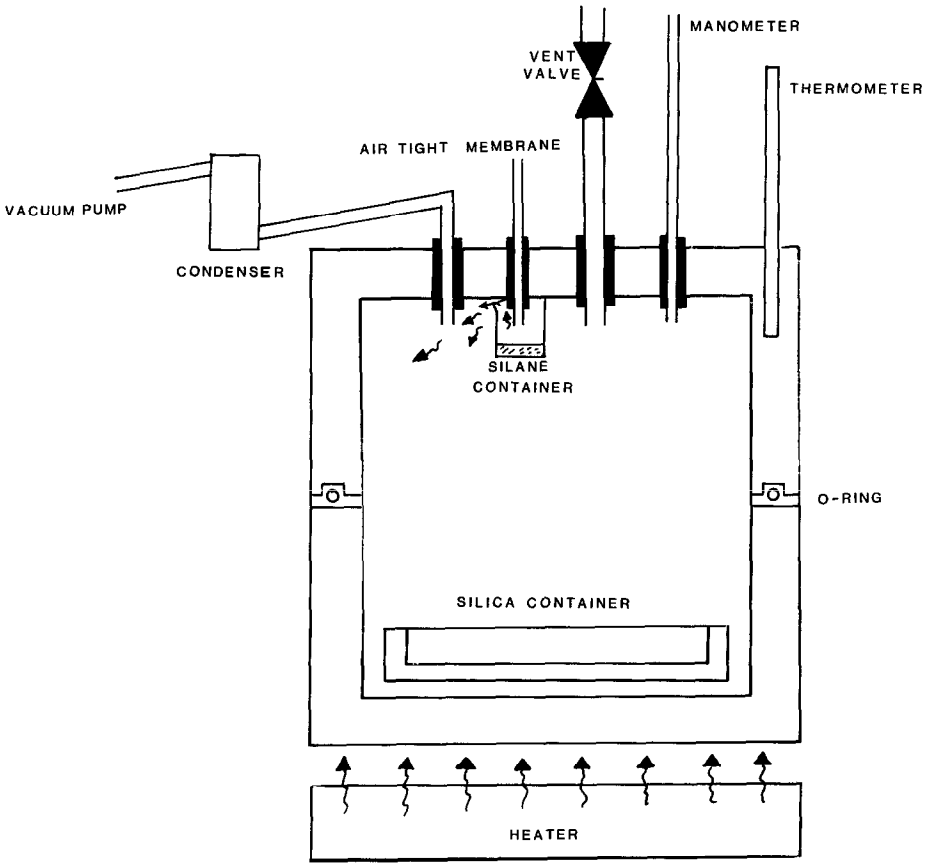

Fig. 1. A cross-section of the silylation apparatus.

method[7]. The silica was stirred for 5 h at 90°C in 10 ml 0.1 *M* sodium acetate, pH 5.5, containing 0.4 ml 3-glycidoxypropyltrimethoxysilane. The silica was subsequently washed with 0.1 *M* sodium acetate, pH 5.5 and water.

Organic phase silylation (epoxysilane)

LiChrospher Si 300 (1.8 g) was dried *in vacuo* for 4 h at 160°C. Toluene (300 ml, sodium dried) was added to the silica followed by 1.1 ml epoxysilane and 100 μl of the catalyst, triethylamine. The slurry was stirred and refluxed under dry nitrogen for 12 h. When the silylation reaction was complete, the amino silica was filtered off and washed extensively with dry toluene, and acetone[8].

Organic phase silylation (aminosilane)

LiChrosorb Si 100 (1.3 g) was dried overnight at 160°C. Toluene (300 ml, sodium dried) was added to the silica followed by 3 ml 3-aminopropyltriethoxysilane. The slurry was stirred and refluxed for 12 h. When the silylation reaction was complete, the slurry was filtered off and washed extensively with dry toluene and acetone[8].

Preparation of diol silica

Epoxysilica was converted into diol silica by suspending the silica in water acidified to pH 2.3 with sulphuric acid and stirred at 90°C for 3 h.

Preparation of NAD-bound silica

The diol silica (LiChrospher Si 300, 1 g) was activated with tresyl chloride, according to a published procedure[9]. For 1 g of dry diol silica, 8 ml of dry acetone, 0.2 ml of pyridine and 0.1 ml of tresyl chloride were used. The slurry was thoroughly mixed and allowed to react on a rocking table for 15 min. The activated silica was washed with 100 ml of acetone, acetone–5 m*M* hydrochloric acid (70:30, 50:50 and 30:70), 5 m*M* hydrochloric acid and 0.2 *M* sodium bicarbonate buffer pH 7.5. The silica gel was then suspended in 8 ml of the same buffer containing 20 mg of N^6-[N-(2-amino-ethyl)carbamoylmethyl]-NAD. The slurry was placed in a sealed test-tube and allowed to react at room temperature for 1 h. Unreacted tresyl groups were then blocked with 0.2 *M* 2-mercaptoethanol pH 7.5, overnight.

Functional group analysis of modified silica

The epoxy group content was determined by reaction with sodium thiosulphate and titration of released hydroxide ions[10]. The amino group content was determined from the picric acid ion-pairing capacity according to Alpert and Regnier[11]. Methyl red dye adsorption was used to give a qualitative measure of the remaining exposed silanol groups on the silica surface[12].

Enzyme assay

Lactate dehydrogenase activity was monitored by the oxidation of NADH at 340 nm and 21°C using puruvate as the substrate. The enzyme was added to an end volume of 3.1 ml, containing 0.21 m*M* NADH and 1.0 m*M* sodium pyruvate in 0.05 *M* sodium phosphate buffer, pH 7.0.

Packing of columns

All columns used were standard HPLC columns with compression fittings. They were packed using the slurry technique[13].

Chromatographic conditions

HPLC equipment from LKB (Bromma, Sweden) was used. Samples (20 μl) were injected in triplicate and emerging proteins were monitored at 280 nm. The data scatter (retention times, peak heights, peak widths) was in all cases within 5%.

RESULTS AND DISCUSSION

Gas phase silylation procedure

The general outline of the gas phase silylation procedure developed here was straightforward: porous silica was placed in a vacuum chamber (Fig. 1) and heated for 1 h to remove any adsorbed water. While maintaining the vacuum, the silane was injected into the chamber, where it vaporized and reacted with the silica. The procedure normally involved coinjection of triethylamine, which catalyzed the silylation reaction, as will be discussed further. Silylation was complete after 1 h at 160°C.

TABLE I

EPOXY- AND AMINOSILANE ADSORPTION ON LICHROSPHER Si 100

Gas phase silylation was carried out as described in Materials and Methods (Procedure 1). Epoxy groups were determined via reaction with sodium thiosulphate and the amino groups were detected by the picric acid ion-pairing capacity method.

Temperature (°C)	*Epoxysilane (μmol/g silica)*	*Aminosilane (μmol/g silica)*
100	530	425
120	640	560
135	550	590
150	490	580

In gas phase silylation, silane is initially deposited on the silica surface and later covalently bound[4], The deposition of epoxy- and aminosilane on LiChrospher Si 100 at temperatures ranging from 100 to 150°C is detailed in Table I. Although the data show some scatter, no temperature effect is seen. The deposition of silane is thus considered to be independent of temperature in the range investigated. Assuming a surface concentration of available silane binding sites of 2 μmol/m^2 silica would result in 500 μmol bound silane per gram for a LiChrospher Si 100 preparation[14,15]. Thus the data in Table I are consistent with the formation of a monolayer at all temperatures investigated, meaning that the transport rate of silane to the surface is sufficient. However, investigation of the stability of the silica indicated insufficient anchoring of the monolayer to the silica surface. For example, washing of the treated beads for 30 min at pH 2 and 70°C resulted in a significant loss of silanes according to the methyl red test. The release of silanes generates free silanol groups, which adsorb methyl red, leading to a coloured silica preparation. Clearly, the deposited silane monolayer must be firmly bound to its support if the gas phase silylation is to be considered a viable procedure.

In previous works concerning gas phase silylation, covalent binding was

TABLE II

EFFECT OF TRIETHYLAMINE (TEA) AND TEMPERATURE ON COVALENT ATTACHMENT OF EPOXYSILANE

Gas phase silylation was carried out as described in Materials and Methods (procedure 1). The silylated silica was treated with acidified water pH 2 for 30 min at 70°C, washed, dried and finally subjected to the methyl red test.

Silica	*Amount (mg)*	*Silane (μl)*	*TEA (μl)*	*Temp. (°C)*	*Time (h)*	*Methyl red colouring*
LiChroprep 100	200	200	–	160	1	Strong
LiChroprep 100	200	200	50	100	1	Strong
LiChroprep 100	200	200	50	100	5	Weak
LiChrosorb 100	200	200	50	100	20	Weak
LiChrosorb 100	200	200	50	160	1	None
LiChrospher 300	1200	1000	500	160	1	None

achieved by increasing the reaction time to 12–24 h[2–4]. We suggest an alternative procedure based on silylation in the presence of the catalyst triethylamine for 1–3 h.

Table II shows the catalytic effect of triethylamine on the silylation reaction. In the presence of triethylamine, silylation occurs at 100°C although the reaction is not complete after 20 h. However, as indicated by Table II, increasing the temperature increases the reaction rate such that silylation of silica of pore size 100 Å and surface area of 250 m^2 at 160°C is complete within 1 h. If the catalyst triethylamine is excluded, insufficient reaction takes place even at 160°C.

Gas phase silylation in the presence of triethylamine has been carried out many times according to the protocols given in Materials and Methods. We have found the protocols reliable, and to give very consistent results.

Chromatographic performance of gas phase silylated silica

In order to characterize the performance of gas phase silylated silica and its general usefulness in comparison to silica produced with other methods, a number of chromatographic tests were carried out. Wide pore (300 Å) silica supports, primarily related to chromatography of proteins which should neither be excluded nor retained by an ideally coated support, were employed. The tests involved recovery studies, plate number calculations and peak asymmetry measurements.

Protein recovery. Protein recovery was chosen as a sensitive measure of anomalies on the silica surface, especially residual unsilylated areas. Seven well characterized proteins with widely different isoelectric points and molecular weights were selected (Table III) as a representative group. To enhance the sensitivity of the test, the amount of proteins injected on the columns was small, 5 μg. Four columns (50 mm × 4.6 mm) were used, packed with diol silica prepared by aqueous silylation, organic phase silylation (toluene), gas phase silylation or by a modified gas phase silylation which involved a second silylation after removal of the methoxy groups introduced in the first silylation step (Procedure 2).

TABLE III

RECOVERY OF PROTEINS FROM DIOL SILICA PREPARED BY DIFFERENT METHODS

The proteins were chromatographed on the diol silica columns and the recoveries calculated from the peaks obtained. Column: 50 mm × 4.6 mm I.D. Temperature: 20°C. Detection: UV, 280 nm. Sample: 0.25 mg/ml injected with a 20-μl loop (5 μg protein). Buffer: 0.05 *M* phosphate buffer pH 7.0 containing 0.1 *M* sodium chloride; flow-rate, 1 ml/min.

Compound	*pI*	*MW (kilodaltons)*	*Recovery (%) after silylation in*			
			Aqueous phase	*Organic phase*	*Gas phase procedure 1*	*Gas phase procedure 2*
Pepsin	2.9	33.0	76	59	75	79
Ovalbumin	4.7	45.0	94	86	94	95
Albumin	5.1	67.0	91	59	70	83
Haemoglobin	7.0	16.0	91	2	45	67
Catalase	8.0	240.0	68	46	54	62
Trypsinogen	9,3	24.0	71	42	59	67
Lysozyme	10.5	14.3	94	6	72	97
Mean value			84	42	67	79

TABLE IV

SILANE SURFACE CONCENTRATION

The silane surface concentration was determined by elemental analysis of carbon.

Method	*Surface concentration (μmol of silane per m²)*
Organic phase	2.8
Aqueous phase	5.8
Gas phase (Procedure 1)	3.2
Gas phase (Procedure 2)	3.7

The recovery varied considerably depending on the protein as well as the silylation method used (Table III). Water-free toluene silylation gave, for all proteins investigated, lower recovery than the other methods. In the case of, for example, lysozyme a particularly low recovery (0.6%) was obtained for the organic phase silylated material. This may be at least partially explained by a strong interaction between the highly positively charged protein and the negative charges of the remaining silanol groups on the silica surface. Other types of interactions, *e.g.*, hydrogen bonds, have also been suggested to take part in irreversible protein adsorption on silica[16].

On the other hand, aqueous phase silylated silica gave a very good recovery of lysozyme (94%), indicating a more complete coating and shielding of any remaining silanol groups. The good recovery accords with the carbon content data in Table IV, where aqueous phase silylated silica is shown to have about twice the carbon content compared to organic phase silylated material. The silane concentration of 5.8 μmol/m^2 obtained for aqueous phase silylation indicates a silica surface covered by more than a monolayer. Clearly, such a multilayer cover would provide a more efficient shielding of silanol groups.

As is also shown in Table III, gas phase silylation gives a fair recovery of 72% for lysozyme, suggesting only a limited amount of non-specific adsorption sites on the silica surface. An even better recovery (97%) was achieved using a silica prepared by the modified gas phase silylation procedure (Procedure 2). The initial gas phase silylation product was treated with water (to hydrolyze unreacted methoxy groups), dried at 160°C and subjected to a second silylation. Additional silanes were thereby bound as indicated in Table IV. The recovery properties of the silica were thereby improved for lysozyme as well as for other proteins to the level of recovery obtained using silica silylated with the aqueous phase method. There may be several explanations for the beneficial effects of a second silylation. The process may result in "end capping" of remaining silanol groups. Another possible explanation would be a more complete engagement of silane-derived silanol groups in the second silylation step.

Table V also gives the k' values obtained, calculated from $k' = (V_e - V_0)/V$ where V_e is the elution volume for the substance under study and V_0 the elution volume for a non-retarded, non-excluded substance (potassium nitrate). Ideally, the k' values for larger molecules like proteins should all be zero or slightly negative for an inert support based on medium pore (300 Å) silica, indicating the absence of attractive

TABLE V

k' VALUES ON DIOL SILICA PREPARED BY DIFFERENT METHODS

k' was calculated by $k' = (V_e - V_0)/V_0$, where V_0 is the elution volume for a non-retarded non-excluded substance (potassium nitrate). Chromatographic conditions as in Table III.

Compound	k'			
	Aqueous phase	*Organic phase*	*Gas phase procedure 1*	*Gas phase procedure 2*
Pepsin	−0.02	0.01	−0.02	−0.04
Ovalbumin	−0.12	−0.10	−0.11	−0.13
Albumin	−0.13	0.45	−0.07	−0.10
Haemoglobin	−0.05		0.11	−0.05
Catalase	−0.08	−0.01	−0.04	−0.08
Trypsinogen	−0.07	0.15	0.03	−0.06
Lysozyme	0.13		2.4	0.14

forces and the presence of a slight size-exclusion effect. However, as is clear from the recovery discussion above, several support–protein combinations behaved far from ideally. This is also reflected in the k' values that in several cases are well above zero. As expected, low recovery often co-varies with high k' values, with some exceptions. For example, lysozyme is fairly strongly retained on gas phase (Procedure 1) silica ($k' = 2.4$) but the recovery is still high (72%). Trypsinogen on the other hand is hardly retained ($k' = 0.03$) but the recovery is lower (59%). The discrepancy probably reflects that several types of binding sites are present on the silica.

Plate number calculations. The number of theoretical plates was calculated for

TABLE VI

PLATE NUMBER FOR DIOL SILICA PREPARED BY DIFFERENT METHODS

Chromatographic conditions as in Table III. The plate number was calculated as described by Kirkland and Snyder[17].

Compound	*Silylation in*			
	Aqueous phase	*Organic phase*	*Gas phase procedure 1*	*Gas phase procedure 2*
Pepsin	3400	2800	4000	4200
Ovalbumin	2300	4200	6900	5700
Albumin	2400	310	2700	2600
Haemoglobin	2600	–	1000	4000
Catalase	1400	2200	2600	1700
Trypsinogen	7500	800	3000	8500
Lysozyme	4200	–	600	4100
Mean value	3400	2100	3000	4400
Nitrate	8600	9500	8700	7500

seven proteins chromatographed on four types of diol silica columns (Table VI). Significant differences between the different types of silica were seen.

The aqueous phase silylation gave high plate numbers for most of the proteins, the average for the seven proteins being 3400 plates/m. A low plate number was observed only for catalase. This very large protein may require a lower flow-rate for satisfactory performance.

The organic phase silylated silica gave somewhat heterogeneous results. Due to heavy adsorption, thus low recovery, the values for lysozyme and haemoglobin could not be calculated. The remaining proteins had plate numbers ranging from 310 (serum albumin) to about 4200 (ovalbumin).

Diol silica prepared by the standard gas phase silylation procedure showed intermediate plate numbers with the exception of lysozyme and haemoglobin. However, the use of twice silylated material improved the plate number considerably, for lysozyme ten times and four times for haemoglobin. This material also showed the highest average plate number of all the preparations, 4400.

Peak asymmetry. Protein peaks are often not "well behaved", *i.e.*, they show considerable tailing and other deviations from the ideal Gaussian distribution. For high resolution work, narrow (high plate number) and symmetric peak (asymmetry factor close to unity) are required. A closer examination of the peak shapes obtained by the gas phase silylated material was therefore considered appropriate. A comparison of asymmetry factors is given in Table VII[17]. The same trend occurs as for the plate numbers described in Table VI, *i.e.*, the best results are obtained with gas phase silylated silica and aqueous phase silylated silica.

The peaks of lysozyme and trypsinogen generated on the four silica preparations are shown in Fig. 2a and b. The amount of protein injected was the same for all columns. Trypsinogen (Fig. 2b) behaves fairly well on all columns. Fig. 2b shows the appearance of lysozyme peaks. Note that the very small peak obtained on silica silylated in the organic phase is due to heavy adsorption onto the silica. Also note the

TABLE VII

PEAK ASYMMETRY FACTORS ON DIOL SILICA PREPARED BY DIFFERENT METHODS

Chromatographic conditions as in Table III. Peak asymmetry factors were calculated as described by Kirkland and Snyder[17].

Compound	*Silylation in*			
	Aqueous phase	*Organic phase*	*Gas phase procedure 1*	*Gas phase procedure 2*
Pepsin	2.2	3.2	3.0	2.6
Ovalbumin	2.2	2.7	1.4	2.4
Albumin	1.4	3.5	2.3	2.2
Haemoglobin	2.7	–	4.1	2.7
Catalase	1.8	2.5	1.8	1.5
Trypsinogen	2.2	5.2	3.1	2.2
Lysozyme	5.5	–	5.0	3.4
Mean value	2.6	3.4	3.0	2.4

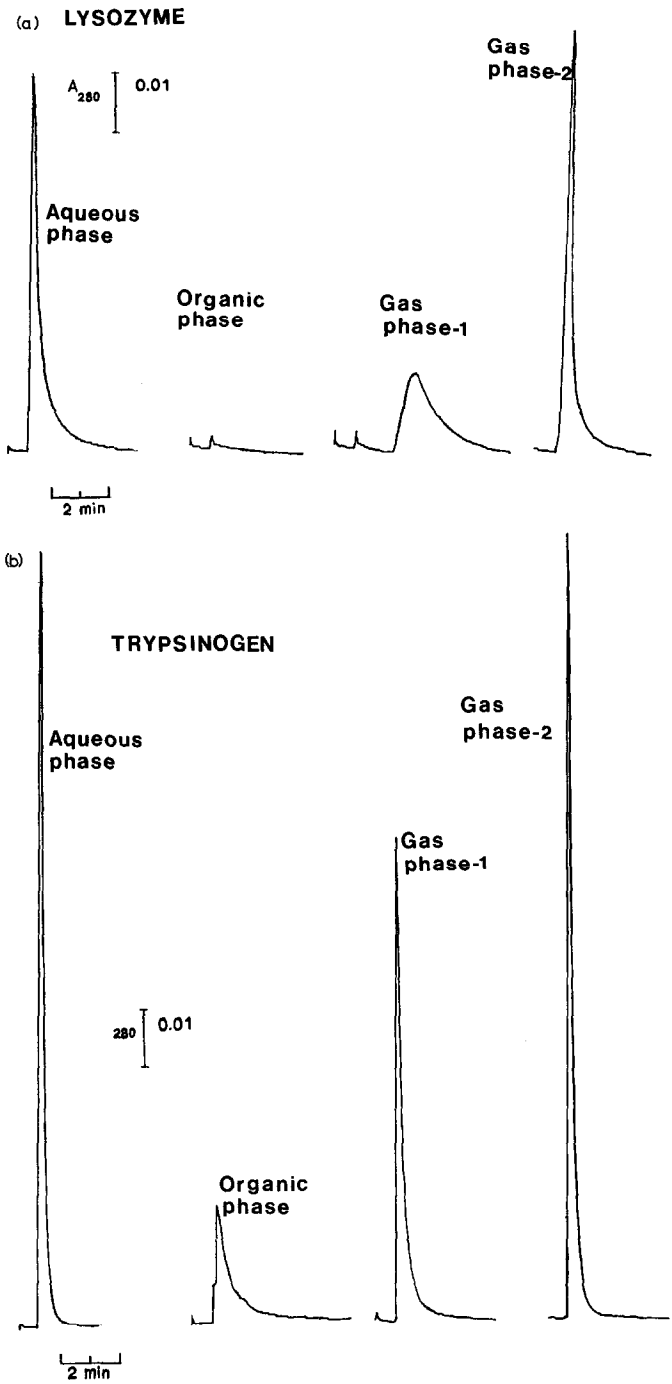

Fig. 2. A comparison of the chromatograms obtained for lysozyme (a) and trypsinogen (b) using silica prepared by the four silylation methods. The buffer used was 0.05 *M* sodium phosphate, 0.1 *M* sodium chloride, pH 7.0. The protein concentration was 5 μg per 20-μl sample and the flow-rate was 1.0 ml/min.

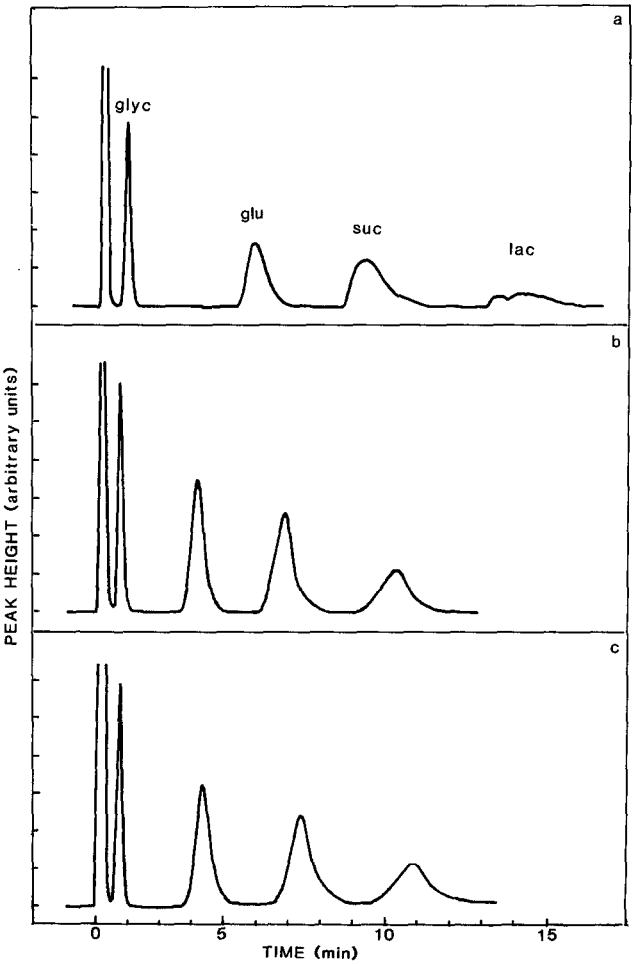

Fig. 3. A comparison of the chromatograms obtained for glycerol (8 mg/ml) glucose, sucrose and lactose (each 25 mg/ml) dissolved in water–acetonitrile (20:80). The injection volume was 20 μl and the flow-rate was 1.0 ml/min.

improvement in the peak shape when going from standard gas phase silylated material (Procedure 1) to twice silylated gas phase material (Procedure 2).

Applications

Fig. 3 shows the separation profiles for a test mixture of glucose, sucrose, lactose and glycerol on aminopropylsilica prepared by three different methods. The three columns readily separate the mixture into its components. However, a closer look reveals that the gas phase silylated silica (Procedure 1) gave the best result, with regard to peak height and peak shape. The relative plate numbers are given in Table VIII.

In another application, gas phase silylated silica was used for chromatography of lactate dehydrogenase using an affinity chromatography column. The affinity column

TABLE VIII

RELATIVE PLATE NUMBERS FOR AMINOPROPYLSILICA

Chromatographic conditions as in Fig. 3. The plate numbers were calculated as described by Kirkland and Snyder[17].

Compound	*Silylation in*		
	Undried organic phase	*Dried organic phase*	*Gas phase procedure 1*
Glycerol	1.11	1.02	1.0
Glucose	0.82	0.92	1.0
Sucrose	0.57	0.88	1.0
Lactose	0.38	0.86	1.0

was prepared by coupling an NAD analogue to tresyl chloride activated diol silica[18]. Lactate dehydrogenase was eluted using 5 m*M* NADH and the enzyme activity of the eluate was monitored. On loading 0.20 mg of enzyme, a recovery of 99% was obtained using the above procedure.

CONCLUSION

This work has demonstrated (1) that silanes can be covalently bound to silica in a much shorter reaction time when the silylation is done in the gas phase in the presence of a catalyst, and (2) that the silica material obtained shows a significantly improved chromatographic performance compared with materials derived from previously described methods.

An additional advantage of special relevance for large scale preparation of derivatized silica is the fact that the gas phase procedure will give a defined product even when the reagents are of varying quality. For instance, the gas phase procedure will allow only unpolymerized silanes to react with the silica support. Also the absence of solvents should be of importance when preparing derivatized supports in large amounts.

ACKNOWLEDGEMENT

The authors thank Perstorp Biolytica AB (Lund, Sweden) for valuable support.

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