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Characterization of an adamantyl-modified silica used as a stationary phase in high-performance liquid chromatography

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ABSTRACT

An adamantyl-modified silica was packed and characterized by reversed-phase liquid chromatography. Initial studies involved a liquid chromatographic comparison of the adamantyl phase *versus* a conventional C_{18} stationary phase for a range of solutes including aromatic hydrocarbons, nitrobenzenes, phenols, anilines and pyridine derivatives. The coverage density of adamantyl groups on the silica surface is critical with regard to the retention behavior of these small molecules.

INTRODUCTION

This study follows up some work by Yang and Gilpin[1–3] and ourselves[4] in which adamantyl-modified silica was utilized as a reversed-phase high-performance liquid chromatographic packing. Yang and Gilpin[1] demonstrated reduced peak tailing for some basic solutes that interact strongly with residual silanols and attributed this improved peak symmetry to the bulky rigid structure of adamantane shielding the unreacted surface silanols from even small solutes. They also examined the selectivity of the adamantyl surface toward a National Institute of Standards and Technology (NITS) (formerly the National Bureau of Standards, Gaithersburg, MD, U.S.A.) test mixture of polycyclic aromatic hydrocarbons[2]. We have previously studied the effects of an adamantyl-modified silica stationary phase and other reversed-phase packings on the gradient elution of the protein ribonuclease A[4].

Similarly to Yang and Gilpin, in this study we compared the retention and selectivity characteristics of an adamantyl-modified silica packing with that of a C_{18} stationary phase for a range of solutes including an NIST test mixture of polycyclic

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aromatic hydrocarbons and an NIST test mixture for evaluating silanol activity. We also extended our earlier work[4] with adamantyl-modified silica and the gradient elution of ribonuclease A (RNase A) to more acidic conditions, to evaluate further the effect of the bulky (rigid, ball-like) structure of adamantane on the elution of proteins. The large adamantyl group should be less likely to penetrate the structure of a protein and disrupt its conformation during adsorption and elution than the thinner linear alkyl group of a conventional alkyl-bonded phase.

This work was undertaken in the hope of finding a stationary phase for reversed-phase high-performance liquid chromatography (HPLC) that would promote the irreversible unfolding of proteins to a lesser degree than the conventional *n*-alkyl bonded phases. Pearson *et al.*[5] have shown, however, that the recovery of large peptides on C_{18} phases is slightly better than on an adamantyl-bonded phase. Previously published results[4], on the contrary, have shown that ribonuclease refolds more rapidly when eluted on an adamantyl-bonded silica column than on an octadecyl-bonded column. These two sets of results are not contradictory, as Pearson *et al.*[5] analyzed denatured peptides whereas we studied the unfolding of ribonuclease[4]. This justifies the characterization of the retention properties of small molecules on the adamantyl phase.

EXPERIMENTAL

Chemicals

Bovine pancreatic ribonuclease A was purchased from Sigma (St. Louis, MO, U.S.A.) and used as received. RNase A samples were prepared as 10 and 20 mg/ml solutions in doubly distilled, deionized water.

All of the following chemicals were of analytical-reagent grade, HPLC-grade or better from J. T. Baker (Phillipsburg, NJ, U.S.A.) unless stated otherwise: acetone, acetonitrile, methanol, 2-propanol and carbon tetrachloride. Trifluoroacetic acid was purchased from Sigma and all of the solutes which were tested were obtained from Aldrich (Milwaukee, WI, U.S.A.).

Water was doubly distilled and deionized before use and all mobile phases were filtered using a vacuum filtration apparatus and 0.45-μm nylon 66 membrane filters (Schleicher & Schüll, Keene, NH, U.S.A.) before being degassed by helium sparging prior to use.

The stationary phase materials were Partisil-10 ODS-3 (C_{18}) from Alltech (Deerfield, IL, U.S.A.) and an adamantyl-bonded Partisil-10 silica. The adamantyl phase was prepared by adding 20 g of adamantylethyltrichlorosilane (Petrarch Systems, Bristol, PA, U.S.A.) to a round-bottomed flask containing 50 ml of freshly distilled toluene, 10 g of silica and 0.5 ml of pyridine and heating at 60°C for 14 h. The solid was then filtered, washed with 200 ml of methanol and finally dried at 100°C under vacuum. The particle size of both materials was 10 μm with average pore sizes of 85 Å.

Apparatus

Two chromatographic systems were employed. One consisted of a Series 400 solvent-delivery system, an LC-235 diode-array UV detector, a GP-100 graphics printer (Perkin-Elmer, Norwalk, CT, U.S.A.) and an electric six-port injector with

a 5-μl sample loop (Valco, Houston, TX, U.S.A.). The temperature difference between between the mobile phase and the column was minimized by inserting a coiled tube, with a volume of *ca.* 3 ml, between the pump outlet and the injector and immersing both the tubing and the column in a thermostated water-bath. The other system was an HP 1090 liquid chromatograph (Hewlett-Packard, Palo Alto, CA, U.S.A.). Columns were packed using a Haskel pump and a laboratory-made slurry reservoir.

Procedures

The HPLC columns were packed using 10 cm × 0.46 cm I.D. stainless-steel tubing, Parker stainless-steel unions and 0.5-μm stainless-steel frits from Alltech. Columns were packed under constant pressure (6000 p.s.i.) with the empty column filled with carbon tetrachloride and using a slurry solvent of cyclohexanol–2-propanol (50:50, v/v) with methanol as the push solvent.

RESULTS AND DISCUSSION

Initial comparisons between our adamantyl and the Partisil C_{18} stationary phases with the solute toluene indicated less carbon coverage on the former material, as suggested by the retention factors (k') for toluene in Table I. For obvious steric reasons, interactions between phenyl and adamantyl groups are also less favorable to retention than those between phenyl and long alkyl groups.

Table I also seems to indicate a stronger contribution to the retention from polar rather than hydrophobic interactions on the adamantyl phase, whereas the C_{18} phase seems to exhibit much stronger hydrophobic than polar interactions. The increasing hydrophobicities of phenol, 3-methylphenol, and 2-ethylphenol are in agreement with their increasing k' values on both phases, but the retentions are greater on the C_{18} phase. The retention factors also increase faster with increasing water content of the phase and with increasing size of the alkyl substituent(s) of the molecule.

Conversely, the much more polar 4-nitrophenol and the strongly basic pyridine and methylpyridines have smaller k' values on the C_{18} phase than on the adamantyl phase, illustrating the greater hydrophobic interactions of the C_{18} phase and the greater polar interactions of the adamantyl phase. We also observed a similar behavior of the k' values of 1,4- and 1,2-dinitrobenzene. Changing the polar characteristics of the compounds by changing the relative position of the substituents resulted in a greater change in their relative retentions on the adamantyl than on the C_{18} phase.

Evaluation of silanol activity

Both the adamantyl and the C_{18} phases were evaluated for silanol activity with an NIST recommended test mixture consisting of N,N-diethyl-*m*-toluamide (DETA) and anthracene. This mixture permits an evaluation of the relative column polarity resulting from accessible free silanols and therefore assists in predicting column suitability for the separation of polar compounds. The selectivity coefficient $\alpha_{DETA/anthracene}$ reflects the silanol activity.

The DETA–anthracene test uses acetonitrile as the mobile phase at a flow-rate of 1 ml/min and a temperature of 22°C provided by the water-bath. If the observed elution order is DETA followed by anthracene, this is an indication of a low silanol

TABLE I

RETENTIONS (k') ON ADAMANTANE AND C_{18} COLUMNS WITH WATER–METHANOL ELUENTS

Column	*Solute*	*Water in methanol (%)*			
		25	*50*	*75*	*100*
Adamantane	Phenol	0.17	0.67	1.67	3.61
	3-Methylphenol	0.26	0.97	3.35	
	2-Ethylphenol	0.4	1.58	6.39	
	4-Nitrophenol		0.81	2.61	6.98
	1,4-Dinitrobenzene	0.27	0.94	2.75	
	1,2-Dinitrobenzene	0.37	1.58	5.89	
	Aniline		0.67	2.0	5.48
	2-Ethylaniline	0.42	1.48	5.27	21.5
	Pyridine		1.48	5.12	30.42
	4-Methylpyridine	0.92	3.58	18.65	
	2-Methylpyridine	0.83	3.25	16.25	
	Toluene		0.34	1.75	
C_{18}	Phenol	0.23	0.81	3.65	19.2
	3-Methylphenol	0.27	1.57	9.5	
	2-Ethylphenol	0.5	3.38	24.65	
	4-Nitrophenol		0.35	1.54	14.61
	1,4-Dinitrobenzene	0.27	1.5	7.42	
	1,2-Dinitrobenzene	0.26	1.38	8.5	
	Aniline		0.5	2.2	12.69
	2-Ethylaniline	0.38	2.11	12.92	42.42
	Pyridine		0.44	1.69	13.54
	4-Methylpyridine	0.27	0.88	4.96	53.27
	2-Methylpyridine	0.15	0.81	4.11	48.3
	Toluene	1.04	8.0		

activity. The relative retention of DETA to anthracene increases with increasing silanol activity. DETA elutes last for the most active columns.

In our tests, the relative retention of DETA to anthracene was 5.08 on the adamantyl phase and 1.0 on the C_{18} phase. This illustrates the much stronger silanol activity and therefore the greater polarity of the adamantyl phase compared with those of the C_{18} phase. This greater polarity may be explained, at least in part, by the difference in the chemical nature of the bonding, and hence in the chemical nature of the surface prepared. The C_{18} phase is a monomeric alkyl phase, prepared with a monochlorodimethylsilane whereas the adamantyl phase is prepared with a trichlorosilane. Although it is improbable that the adamantyl phase could rightly be called a polymeric phase, the steric hindrance of the bulky adamantyl groups preventing any extent of polycondensation with water close to the silica surface, the density of residual silanol groups is probably more important with the adamantyl than with the C_{18} phase. The adamantyl groups, although large, seem to be unable to shield sufficiently these silanol groups and to prevent them from interacting with small-molecule probe solutes.

Evaluation of selectivity for polynuclear aromatic hydrocarbons (PAHs)

As in an earlier study[2], we examined the adamantyl phase with respect to PAH selectivity by using a test mixture of PAHs provided by the NIST. Sander and Wise[6–8] proposed and demonstrated that selected PAH solutes (*e.g.*, benzo[*a*]pyrene, BaP; phenanthro[3,4-*c*]phenanthrene, PhPh; and 1,2:3,4:5,6:7,8-tetrabenzonaphthalene, TBN) can be used to classify phases in terms of their selectivity.

Both the adamantyl and the C_{18} phases were evaluated using acetonitrile–water

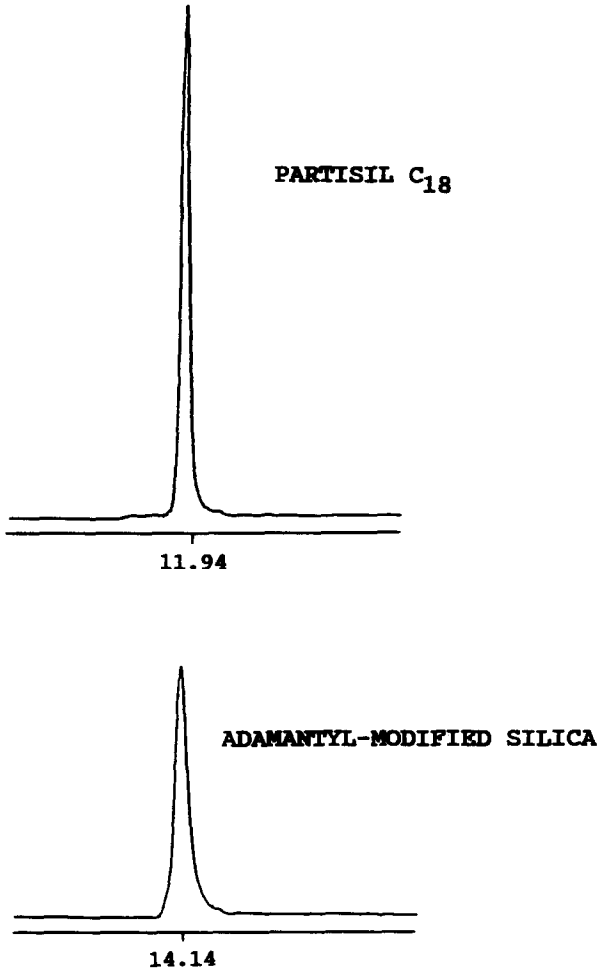

Fig. 1. Behavior of ribonuclease A on Partisil C_{18} and adamantyl-modified silica stationary phases. Eluent: solvent A, 0.1% TFA in water; solvent B, 0.1% TFA in acetonitrile; gradient from 0 to 85% B in 25 min, linear. Flow-rate, 1 ml/min; Sample size, 100 μg RNase A; Temperature, 20°C; Detection, 280 nm (0.2 a.u.f.s.).

(85:15, v/v) as the mobile phase at flow-rates of 1 and 2 ml/min and a temperature of 22°C. The relative retentions TBN/BaP (2.22) and PhPh/BaP (1.41) for four adamantyl phase compared favorably with those for the adamantyl phase (2.10 and 1.47, respectively) of Yang and Gilpin[2]. This, and the low surface coverage of our adamantyl phase, suggest a degree of independence of the relative retention for the PAHs studied on the surface covered by adamantyl groups.

The $\alpha_{TBN/BaP}$ value of 1.93 for our Partisil-10 ODS-3 (C_{18}) phase was identical with that found by Sander and Wise[7] for a Partisil-5 ODS-3 (C_{18}) phase. Sander and Wise classified the probable synthesis chemistry of the phases based on their experience and have found that $\alpha_{TBN/BaP}$ values greater than 1.7 and the elution order (BaP < PhPh < TBN, as observed with the adamantyl phase) characterize monomeric phases. We also found that the k' values on our adamantyl phase were about 5–6 times less than those on our Partisil C_{18} and 3–4 times less than those reported by Yang and Gilpin[2] for their adamantyl phase. These low k' values again seem to illustrate the possibility of a lower coverage of adamantane on our silica than that achieved by Yang and Gilpin, as did the fairly high polar interactions mentioned earlier.

Gradient elution of ribonuclease A

In a brief extension of an earlier study[4] we examined the effect of protein adsorption and elution on both the adamantyl and the C_{18} phases in a more acidic medium. This solvent system consisted of 0.1% trifluoroacetic acid (TFA) in water as one solvent (A) and 0.1% TFA in acetonitrile as the second solvent (B), with a gradient from 100% A to 85% B in 25 min at 25°C. This gradient was utilized because of its ability to denature completely the RNase A at 25°C, unlike the gradient system used in the previous study[4]. Fig. 1 shows the chromatograms obtained with the adamantyl and C_{18} phases for a sample size of 100 μl. It is obvious that the protein seems to be completely denatured on both columns from the sharp peak shapes in comparison with those in the previous study[4]. In agreement with what was reported previously[4], the retention time of RNase A was longer on the adamantyl than on the C_{18} phase.

CONCLUSION

The results indicate that whereas hydrophobic interactions contribute much more than polar interactions to retention on a Partisil C_{18} column, the converse is true on adamantyl phases at low surface coverage. As the earlier work of Yang and Gilpin[1] demonstrated an inhibition of polar interactions as a result of the bulky structure of adamantane blocking the underlying free silanols, it seems reasonable to assume that the polar effects and the silanol activity observed on our adamantyl phase are due to a low coverage density of adamantane on the surface of the silica. It is remarkable, however, that the presence of these polar effects does not seem to affect the PAH selectivity of our adamantyl phase compared with that of Yang and Gilpin[2].

Unlike the results of previous work[4] where we found differences between the gradient elution profile of RNase A on our adamantyl phase and other reversed-phase columns, we did not find any significant differences between the RNase A profiles on our adamantyl phase and the Partisil C_{18} phase when eluted under more acidic conditions.

Based on these results, the importance of a good coverage of adamantane on the

surface of the silica is critical with regard to the adsorption and elution of small basic molecules and the reduction of free silanol accessibility. Because of the bulkiness of the adamantyl group, a high coverage density is difficult to achieve, in agreement with the low reaction rate observed. The use of a monochlorodimethylsilane in the synthesis and the inclusion of an end-capping step in the preparation of the stationary phase would probably give a less polar surface. However, the amount of adamantane coverage did not seem to play a dominant role in the adsorption and elution RNase A. The difficulty in achieving a low density of residual silanols could explain also the previous results of Pearson *et al.*[5] and the slight loss that they observed in their study of protein recovery.

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