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Journal of Chromatography B, 718 (1998) 187–192

JOURNAL OF CHROMATOGRAPHY B

Short communication

Use of porous pyrolytic carbon for analytical and microscale high-performance liquid chromatographic bioseparations

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Received 5 August 1997; received in revised form 30 June 1998; accepted 10 July 1998

Abstract

Porous carbonaceous adsorbent was prepared by carbonization of saccharose in silica gel pores followed by leeching out of the silica matrix. The product of pyrolysis was then deactivated by hydrogenation. The resulting adsorbent shows intermediate sorption properties between those of the highly polar pyrolytic glassy carbon and the hydrophobic graphitized carbon. The microparticulate mesoporous carbon was examined for its use in capillary HPLC separations. The separation of selected stereoisomers in a 320 μm I.D. capillary column packed with the porous carbon particles is described and discussed. Additionaly, the porous carbon filled with dextran gel was tested as a material for direct HPLC analysis of drugs in human serum.

Keywords: Bioseparations; Porous pyrolytic carbon

1. Introduction

The acceptance of separation media based on porous carbon has been growing during the last decade. The most commonly used methods of preparation of porous carbon particles that are suitable for HPLC are the controlled carbonization of the polymeric precursor particles at high temperatures [1,2] and the method in which the pores of appropriate porous matrix are filled with a carbon precursor which is subsequently pyrolysed [3,4]. The auxiliary materials such as the starting porous sorbent or porogenes are leeched out after the carbonization of the precursor. The products of carbonization usually show high chemical stability over a wide pH range, adequate mechanical stability to withstand abrasion and high pressures, as well as sufficient surface area, high pore volume and controlled pore size distribution [5–7].

Carbonization of the organic precursors below 1000°C results in an amorphous pyrolytic carbon containing micropores and mesopores. It possesses oxygenated surface bearing various functional groups such as –OH, –COOH, –C–O–C–, etc. When pyrolytic carbon is heated to about 1500°C, amorphous glassy carbon is produced. In turn, carbon

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0378-4347/98/$19.00
PII: S0378-4347(98)00341-7

heated above 2000°C has the atomic structure of two-dimensional graphite. Each of the above mentioned materials shows different adsorption properties.

Carbon prepared by pyrolysis at lower temperatures acts as a very strong polar adsorbent. However, a high sorption activity of the pyrolytic carbon often causes undesired irreversible retention [7]. The pyrolytic carbon has been successfully used for purification and preconcentration of analytical samples [8] and, after appropriate deactivation, also for HPLC separations [9]. In contrast, the porous graphitic carbon prepared at very high temperatures has a hydrophobic and chemically homogeneous surface. Its retention properties can be compared with those of commercial ODS-bonded silica phases [10–13].

A better uniformity of the surface composition of the pyrolyzed carbon can be obtained not only via the high-temperature treatment at above 2000°C, but also by a chemical modification of highly-active carbon surface. For example, hydrogenation of oxygen-containing functional groups at about 1000°C [6] or immobilization of a layer of inactive polymer on the carbon surface [14] have been used for deactivation.

Recently, we introduced a new procedure of modification, in which a polysaccharide gel was created within the pores of carbon adsorbent [15]. The polysaccharide network prevents larger solutes from contact with the carbon, while smaller molecules may penetrate the network and interact with the original carbon surface.

Both high specific surface area and a special morphology of the surface make pyrolytic or graphitic carbon adsorbents sensitive not only to the chemical nature of chromatographed compounds but also to their size, shape and position of functionalities. It is known that carbon-based adsorbents show unique selectivity towards positional isomers [16,17], and *cis–trans* isomers [9,18–20]. A separation of enantiomers using a carbon column has been illustrated [21]. The unique separation properties of carbonaceous adsorbents could be explained by a combination of various kinds of selective interactions between solutes, mobile phases and stationary phases such as electron donor–acceptor interactions, hydrogen bonding, etc. However, the largest contribution to adsorption on the carbonaceous surface seems to be associated with the dispersion forces of the sorbate molecules.

Although the carbon packing materials provide unique selectivity for numerous separations, the column efficiency for carbon-packed analytical columns usually does not match that obtained for well established silica-based materials. Therefore, a miniaturization of the column size, together with improving the quality of the packing material can increase the performance of carbon packed columns. Microcolumns and capillaries are becoming popular in both electrochromatography [22] and capillary HPLC [23].

For example, polymer-coated open-tubular microcolumns [24,25] or capillaries packed with silica-based particles [26,27] provide fast and low-volume analyses of isomeric compounds or complicated biological samples. Narrow-bore columns packed with a carbonaceous adsorbent were described previously [28]; however, separations on a carbonaceous adsorbent in the microcolumns with an internal diameter smaller than 1 mm have not been reported yet.

In the present paper we describe capillary HPLC separations of stereoisomers using a 320 μm I.D. fused-silica capillary column packed with the microparticulate porous carbon. Additionaly, the pyrolytic carbon modified with crosslinked dextran has been tested for its use in direct analysis of drugs in biological fluids.

2. Experimental

2.1. Materials

Mesoporous pyrolytic carbon (CF-35) was prepared according to the silica template method [6]. Saccharose was carbonized at 650°C in the pores of the silica gel matrix having an average pore diameter of 6 nm. Silica was then dissolved in aqueous sodium hydroxide. Finally, the spheroidal carbonaceous material with replicated porous structure was dried and deactivated by hydrogenation at 1000°C [6,7]. Dextran-shielded carbon adsorbent was prepared by filling the pores of original carbon CF-35 with crosslinked dextran. The crosslinking of dextran

was performed under alkaline conditions using butandiepoxide reagent as described elsewhere [15].

Samples of α-irone isomers were prepared at Faculty of Pharmacy, Turin, Italy, and ketoprofen was purchased from Sigma (St. Louis, MO, USA). The other chemicals and solvents, purchased from various commercial sources, were of HPLC or analytical reagent grade and used as received.

2.2. Packing the columns

A fused-silica capillary column Fusica (150 mm× 320 μm I.D.) was custom-packed by LC-Packings International, (Amsterdam, The Netherlands). Analytical stainless steel column (100×4 mm I.D.) was packed from a water slurry at 30 MPa using a Knauer pneumatic HPLC pump (Berlin, Germany).

2.3. LC experiments

The capillary HPLC separations were performed on a system equipped with a Kontron 322 pump (Kontron Instruments, Milan, Italy), an Acurate 70 flow-rate converter (LC-Packings), and a Rheodyne 7125 injector (Rheodyne, Berkeley, CA, USA) provided with a 20 μl polyether ether ketone (PEEK) loop. The flow-rate converter was set up at 1:70 (inlet:outlet flow) which gave a constant flow-rate of 14.5 μl/min. The whole volume of the loop was injected into the capillary column equilibrated with a water-rich starting eluent. Then, the compounds concentrated on the top of the column were eluted by a gradient of increasing concentration of acetonitrile in the mobile phase. Detection was carried out with a Kontron 433 UV capillary detector, equipped with a Z-shape flow-microcell (total volume of 90 nl, optical path length of 20 mm) or alternatively with a Kontron 440 DAD-UV unit equipped with a capillary flow-cell (total volume of 200 nl).

Conventional analytical chromatography used for direct injection analysis of drugs in serum was performed with an HPLC system consisting of Kontron 322 pump, Rheodyne 7160 injector, and Kontron 440 DAD-UV detector equipped with an analytical flow-cell.

Data from both HPLC systems were acquired and processed by a Kontron data system 450-MT2/DAD.

3. Results and discussion

3.1. Properties of carbonaceous adsorbent

HPLC packing material used in the study consists of potato-shaped particles of pyrolytic carbon with an average size between 10 and 20 μm. The pore size varies from several nm up to several hundreds nm. The sponge-like structure of carbon matrix results in a high specific surface area of 940 m^2/g (nitrogen adsorption-BET), while its good mechanical strength is preserved. The physical and chemical characteristics of the resulting material, including pore and particle size distributions, as well as results of thermal analysis and Fourier transform infrared spectroscopy, have been described more in detail previously [7].

It is also known from that study, that a large amount of aromatic and oxygen-containing groups is situated on the pyrolythic carbon surface. Therefore, a mixed behaviour owing to the presence of both polar and nonpolar sites is typical for such a material. Large and very active surface bearing a variety of functionalities supposedly causes strong retention of chromatographed compounds with different chemical structures. In the system studied by us, the elution strength of a mobile phase for solutes of medium polarity increased in the sequence from water, methanol, acetonitrile to tetrahydrofuran. This makes the situation similar to typical reversed-phase chromatography. However, separation on the pyrolytic carbon studied did not obey the theory of conventional retention developed for reversed-phase chromatography, e.g. a logarithm of retention factor was not a linear function of eluent composition. To completely elute some hydrophobic compounds, especially those with condensed aromatic rings, an extremely nonpolar eluent such as hexane had to be used. On the other hand, highly polar compounds such as phenols, interacting strongly with polar groups of carbon surface via hydrogen bonding or dipole–dipole interactions, were retained by column using nonpolar eluents and could be eluted from the column with more polar mobile phases, thus resembling the normal-phase HPLC. The mixed character of the carbon surface can cause peak tailing and complicate the optimization of separation. However, in certain cases, it may help to discriminate the

compounds which are otherwise very difficult to separate.

3.2. Separation of cis–trans isomers

It has been previously found that the pyrolytic carbon adsorbent shows high selectivity for separation of *cis–trans* (*E*/*Z*) isomers [9]. Various *E*/*Z* isomers have been separated using commercially available or laboratory made columns based on graphitized carbon [18–20]. We used our carbon-packed capillary for the separation of *E*/*Z* isomers of an α-irone. An extract containing α-irone is used for preparation of perfumes. The separation may help to determine the quality of a perfume, since particular isomers differ significantly in their scent.

Fig. 1 shows that the isomers can be easily separated using the capillary-HPLC column packed with the micrometer-sized pyrolytic carbon. The separation with a selectivity of 6.63 was achieved under simple isocratic conditions in about 15 min. Since only a very diluted sample was available, 20 μl of the sample was let to concentrate on the top of the capillary column and then eluted with acetonitrile–water (80:20, v/v). The sensitivity of the method can be increased significantly using on-line sample preconcentration without sacrificing the selectvity of separation. The retention depends on the difference in the geometric structure of the molecules. In the case of α-irone, the *E*-isomer molecule is more planar and therefore provides a larger contact area with the carbonaceous adsorbent. As a result, the *E*-isomer is more strongly retained than the *Z*-isomer.

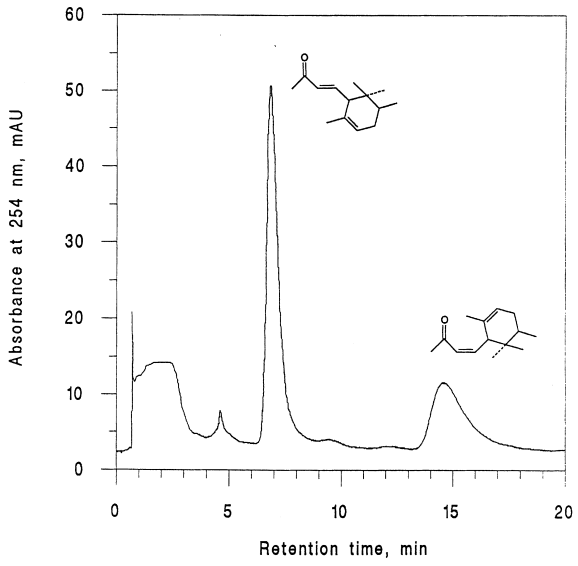

Fig. 1. Capillary HPLC of *cis* and *trans* isomers of α-irone. Conditions: column, fused-silica capillary, 150 mm×320 μm I.D.; packing material, pyrolytic carbon CF-35; mobile phase, acetonitrile–water (80:20, v/v); flow-rate, 14.5 μl/min; detection, absorbance at 254 nm; injection, 20 μl of sample dissolved in acetonitrile–water (30:70, v/v).

3.3. Direct serum injection

Proteins are irreversibly adsorbed on the pyrolytic carbon surface. Therefore, protein-containing samples such as serum cannot be chromatographed directly, but only after an expensive and time consuming preseparation step. To prevent proteins from irreversible adsorption on a carbonaceous adsorbent it must be chemically deactivated, e.g. by a layer of hydrophilic polymer [14]. However, the carbon coated with an inert layer often loses its sorption activity and separation selectivity also for small analytes. In order to obtain a sorbent which would selectively adsorb only the compounds of interest from a multicomponent sample, we filled the pyrolytic carbon with a dextran gel of a specific pore size [15]. The modification allows small molecules to interact with the carbon surface after penetration the hydrophilic dextran layer, while large molecules are prevented from contact with the active surface because of their steric exclusion from the dextran gel pores. As a result, protein molecules should pass through the column unretained. The mechanism is typical for ‘restricted access media’ [29].

To test the dextran-modified carbon for its use in direct serum analysis, we determined recoveries of various proteins injected. Recoveries between about 80 and 100% were found for proteins of both acidic and alkaline nature, such as chymotrypsinogen A and ovalbumin, and of different molecular mass ranging from 14 500 (ribonuclease A) to 675 000 (thyroglobulin). Pure human serum, ketoprofen and the serum spiked with the various amounts of ketoprofen were injected repeatedly onto analytical HPLC column packed with the dextran-modified carbon to test its ability to separate the drug from the serum. Fig. 2

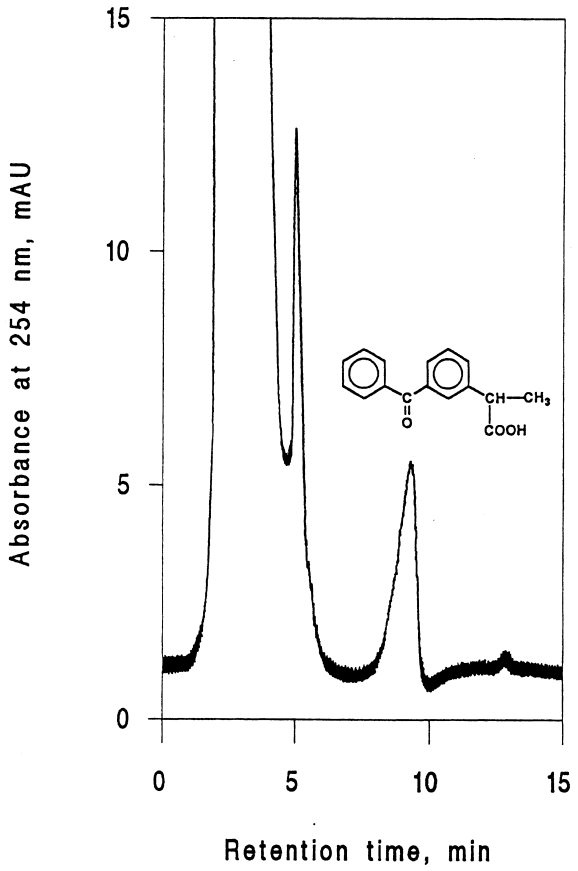

Fig. 2. HPLC of ketoprofen in serum on dextran-shielded carbon column. Conditions: column stainless steel, 100×4 mm I.D.; packing material, pyrolytic carbon CF-35 with pores filled with dextran gel; mobile phase, acetonitrile–water (10:90, v/v), followed by a step gradient to acetonitrile–water (70:30, v/v), in the third minute from injection; flow-rate, 1.0 ml/min; detection, absorbance at 254 nm; sample, human serum spiked with 2 μg/ml of ketoprofen; injection volume, 20 μl.

shows a typical chromatogram of the mixture. All proteins were eluted from the column using acetonitrile–water (10:90, v/v) as the starting mobile phase, followed by elution of the adsorbed drug by a step gradient to acetonitrile–water (70:30, v/v), in the third minute from injection. A drawback of this method is that many drugs are retained so strongly that only organic solvent (pure acetonitrile, tetrahydrofuran, or even mixtures with hexane) can elute them from the column, while the dextran gel incorporated in the carbon pores shrinks under these conditions. After several tens of cycles of adsorption and elution using repeatedly aqueous and organic eluents, the back pressure of the column began to increase probably due to partial degradation of the incorporated dextran gel. Even if this limits the stability and the application area of the dextran filled carbon column, the results show that the approach might be used for preparation of novel 'restricted access' separation media.

4. Conclusions

The carbon adsorbent prepared by low temperature pyrolysis of various organic precursors is widely used as an adsorbent in gas chromatography or for bulk adsorption from liquid phase, but its use in HPLC is limited, mainly because of inhomogeneity of its surface. Therefore, almost all HPLC separations on carbonaceous adsorbents described in the literature were performed with graphitized carbon columns. This paper shows that nongraphitized carbonaceous adsorbents can be used after suitable deactivation for highly selective separations. The capillary columns packed with pyrolytic carbon combine high resolving power of the capillary device with unique selectivity of the separation medium.

Advantageous properties of the pyrolytic carbon were demonstrated on selected micro HPLC separations of *cis–trans* isomers and enantiomers.

Additionally, we showed that carbon with pores filled with croslinked dextran can be used as an alternative material for direct HPLC analysis of drugs in serum.

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